SCREENING, CHARACTERIZATION, AND IN VITRO EVALUATION OF PROBIOTIC PROPERTIES OF LACTOBACILLUS STRAINS

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ABSTRACT

Objective: The aim of the present investigation was to isolate and identify Lactobacillus strains from dairy and cattle dung samples. Potent isolates were selected for screening by antimicrobial activity; selected lactobacilli were further tested for probiotic properties and adhesive attributes.

Methods: Lactobacilli were isolated aseptically on specific de man, rogosa and sharpe medium from dairy and cattle dung samples. Isolates were identified by Gram-staining, motility, catalase, endospore, and carbohydrate fermentation tests. Further, the isolates were screened for antimicrobial activity by disk diffusion assay, and potent lactobacilli were observed for probiotic properties: Acid and bile salt tolerance, gelatinase activity, and autolytic activity. For analyzing the adhesive attributes, isolates were observed for autoaggregation, coaggregation and microbial adhesion to solvents assay.

Results: About 12 Lactobacillus strains among 98 isolates exhibited maximum antimicrobial activity were further selected for identifying their probiotic and adhesive attributes. Among 12 selected isolates, cell-free supernatant (CFS) of buffalo milk BM10 and goat milk GM10 showed excellent antimicrobial activity 20.34±0.02 mm against Staphylococcus aureus and 18.65±0.11 mm against Escherichia coli. Isolates showed survival at pH 2 and 3 and can tolerate 0.2-0.3% bile salt concentrations. The GM5 showed maximum autoaggregation (67.04±0.61%) and minimum coaggregation (11.51±0.50%) showed by GM3. The BM10 exhibited maximum adherent value 64.84±1.41% for n-hexadecane.

Conclusion: The two lactobacilli, BM10 and GM10 identified as Lactobacillus fermentum and Lactobacillus pentosus on the basis of phenotypic and sugar utilization tests. The CFS of both lactobacilli can be used as antimicrobial agent. Both isolates showed significant results of probiotic and adhesive attributes, therefore, can be evaluated for clinical and therapeutic applications.

Keywords: Lactobacillus, Antimicrobial, Probiotic, Adhesive properties.
Antimicrobial activity

Antimicrobial activity of the lactobacilli isolates was checked by disk diffusion method. Isolates were screened against Bacillus subtilis MTCC 1143, Escherichia coli MTCC 433, Enterococcus faecalis MTCC 439, Pseudomonas aeruginosa MTCC 6642, and Staphylococcus aureus MTCC 9886, as the indicator microorganisms. Isolates were subcultured in sterile test tubes containing MRS broth at 37°C for 24 hrs and transferred into a sterile flask containing 150 ml MRS broth and the broth culture were incubated at 37°C for 3 days in thermostat water bath. The cell-free supernatant (CFS) was prepared by centrifuging the 3 days old cultures at 10000 rpm for 20 minutes [18]. Test (indicator) microorganisms were grown in a nutrient broth at 37°C for 24 hrs. Streptomyces and Gentamyces were used as standard. Experiments were conducted in triplicates.

Acid and bile salt tolerance

Acid tolerances of selected lactobacilli were determined by the method described by Skladal et al. [19]. Lactobacilli isolates were cultured for 6 hrs in MRS broth at 37°C. 100 ml fresh MRS broth was prepared, and pH had been adjusted to 2, 3 or 7 using 1N HCl or NaOH. Add 1 ml of the 6 hrs old culture in flasks. Optical density was recorded at 620 nm after 6 and 24 hrs incubation period at 37°C. Surviving (%) can be calculated by following formula:

\[
\text{Surviving} = \frac{\text{OD}_{620} - \text{OD}_{620} \text{H}2 \text{O} \text{r} \text{or} \text{3}}{\text{OD}_{620} \text{H}2 \text{O} \text{r}} \times 100
\]

A modified method given by Dora and Glenn [20] is used for estimation of bile salt tolerance in a similar method of acid tolerance. MRS broth supplemented with different concentration 0.2 and 0.4% of bile salts were used for the experiment. Surviving (%) in bile salt can be calculated by following formula:

\[
\text{Surviving} = \frac{\text{OD}_{0} \% \text{BS} - \text{OD}_{0} \% \text{BS} \times 0.4 \% \text{BS}}{\text{OD}_{0} \% \text{BS}} \times 100
\]

Gelatinase activity

Gelatinase activity of lactobacilli isolates was determined by Harrigan and McCance [21] method. 2 μl of a 6 hrs old culture was spot-inoculated into the nutrient gelatin agar (Himedia, India). The plates were incubated anaerobically for 48 hrs at 37°C after which plates were submerged with saturated ammonium sulfate solution and observed for clear zones surrounding colonies (positive reaction for gelatin hydrolysis). A strain of S. aureus MTCC 9886 was used as positive control.

Autoaggregation and coaggregation assay

Autoaggregation was measured according to the method given by Basson et al. [22]. The lactobacilli strains were inoculated in MRS broth at 37°C for 24 hrs. The cells were then harvested (7000 g, 10 minutes, 20°C), washed, resuspended in sterile physiological water and diluted to optical densities (OD) at 660 nm = 0.3. 1 ml of the cell suspension was transferred to a 2 ml sterile plastic cuvette and the OD at 660 nm recorded over 60 minutes using a microplate reader (Cyber Elisa R01, Cyberlab USA). Autoaggregation was determined using the given equation:

\[
\text{Auto-aggregation} = \frac{\text{OD}(0) - \text{OD}(60)}{\text{OD}(0)} \times 100
\]

Where OD(0) and OD(60) are the initial and final OD recorded at 0 and after 60 minutes of incubation, respectively.

Coaggregation (%) was determined similar autoaggregation method. Isolate culture and coaggregation partners were inoculated in 10 ml MRS and MHA, respectively. Coaggregation (%) is determined using the given equation:

\[
\text{Coaggregation} = \frac{\text{OD}(0) - \text{OD}(60)}{\text{OD}(0)} \times 100
\]

Where OD(0) is the initial OD, taken immediately after the relevant strains were paired. OD(60) is the OD of the supernatant after 60 minutes. Experiments were conducted in triplicate.

Microbial adhesion to solvents (MATS)

MATS was measured according to the method given by Kos et al. [24]. The sample was prepared by centrifuging the bacterial culture at 5000 rpm for 15 minutes, washed twice in PBS and resuspend in 0.1 mol KNO₃ (pH 6.2). The absorbance of the cell suspension was recorded at 600 nm (A₅ₐ). 1 ml solvent (xylene, toluene, and n-hexadecane) was added into 3 ml cell suspension. After pre-incubation at room temperature, the two-phase system was developed, mixed it by vortexing for 2 minutes. The aqueous phase was removed after 20 minutes of incubation at room temperature and its absorbance at 600 nm (A₄) was measured. The percentage of bacterial adhesion to solvent was calculated as (1-A₄/A₅). 100. Experiments were carried out in triplicates.

Results and Discussion

Antimicrobial activity

In the present study, out of 141 lactobacilli isolates, 94 isolated strains were nonmotile, Gram-positive, catalase-negative, rod-shaped; some were rods in chains and nonspore forming. These were coded according to advanced bacterial software which suggested that 12 isolates were identified using biochemical tests and identified by Jain et al. [27]. The isolates were predominated lactobacilli (B. subtilis, E. coli, E. faecalis, P. aeruginosa, and S. aureus). The CFS of BM and goat milk GM10: S. aureus MTCC 439, P. aeruginosa MTCC 9886, as the indicator microorganisms. Isolates were subcultured for clear zones surrounding colonies (positive reaction for gelatin hydrolysis). A strain of S. aureus MTCC 9886 was used as positive control.
Table 1: Carbohydrate fermentation results of lactobacilli isolates

| Isolates | BM2 | BM5 | BM10 | CH4 | CH9 | CH12 | CD1 | CM3 | CS6 | GM3 | GM5 | GM10 |
|----------|-----|-----|------|-----|-----|------|-----|-----|-----|-----|-----|------|
| Origin   | BM  | BM  | BM   | CH  | CH  | CH   | CD  | CM  | CS  | GM  | GM  | GM   |
| Lactose  | +   | +   | +    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| Maltose  | -   | -   | +    | -   | -   | +    | -   | +   | +   | +   | +    | +    |
| Fructose | +   | +   | +    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| Dextrose | +   | -   | +    | +   | -   | -    | -   | -   | +   | -   | +    | +    |
| Galactose| +   | -   | +    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| Raffinose| -   | -   | +    | +   | -   | +    | -   | -   | +   | -   | +    | +    |
| Trehalose| +   | -   | -    | +   | -   | -    | +   | -   | +   | -   | +    | +    |
| Melibiose| ±   | +   | -    | +   | +   | -    | +   | +   | -   | +   | ±    | +    |
| Sucrose  | +   | +   | +    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| L-arabinose| -    | -   | -    | -   | -   | -    | +   | -   | -   | -   | -    | -    |
| Mannose  | +   | +   | -    | +   | +   | -    | -   | +   | +   | +   | +    | +    |
| Inulin   | -   | -   | -    | -   | -   | -    | -   | -   | -   | -   | -    | -    |
| Sodium   | +   | +   | +    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| Glucuronate| Glycerol| Glycerol| Glycerol| Glycerol| Glycerol| Glycerol| Glycerol| Glycerol| Glycerol| Glycerol| Glycerol| Glycerol|
|          | +   | +   | +    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| Salicin  | +   | +   | +    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| Dukitol  | +   | +   | +    | ±   | ±   | ±    | -   | -   | ±   | -   | -    | -    |
| Inositol | +   | +   | +    | -   | -   | -    | +   | -   | -   | -   | -    | -    |
| Sorbitol | +   | -   | -    | +   | V   | -    | -   | V   | +   | -   | -    | -    |
| Mannitol | +   | -   | -    | +   | -   | -    | +   | +   | +   | -   | -    | -    |
| Adonitol | ±   | +   | -    | -   | -   | -    | -   | -   | -   | -   | -    | -    |
| Arabitol | +   | -   | -    | +   | -   | -    | +   | -   | -   | -   | -    | -    |
| Erythritol| -    | -   | -    | -   | -   | -    | -   | -   | -   | -   | -    | -    |
| α-methyl | -   | +   | ±    | ±   | ±   | ±    | ±   | ±   | -   | -   | -    | -    |
| D-glucose| -    | -   | -    | -   | -   | -    | -   | -   | -   | -   | -    | -    |
| Rhamnose | -    | -   | -    | -   | -   | -    | -   | -   | -   | -   | -    | -    |
| Gentiobiose| +    | +   | +    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| Melezitose| +    | -   | +    | +   | -   | -    | +   | +   | -   | -   | -    | -    |
| α-Methyl | +    | +   | -    | -   | -   | -    | -   | -   | -   | -   | -    | -    |
| D-mannoside| Xyitol| Xyitol| Xyitol| Xyitol| Xyitol| Xyitol| Xyitol| Xyitol| Xyitol| Xyitol| Xyitol| Xyitol|
|          | +   | +   | -    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| Esculin  | +   | +   | -    | -   | +   | +    | +   | +   | +   | +   | +    | +    |
| Hydrolysis| D-arabinose| D-arabinose| D-arabinose| D-arabinose| D-arabinose| D-arabinose| D-arabinose| D-arabinose| D-arabinose| D-arabinose| D-arabinose| D-arabinose|
|          | -   | +   | -    | -   | +   | +    | -   | -   | -   | -   | -    | -    |
| Sorbose  | -   | -   | +    | +   | -   | -    | -   | -   | -   | -   | -    | -    |
| Glucose  | +   | +   | +    | +   | +   | +    | +   | +   | +   | +   | +    | +    |
| Designated species* | Lactobacillus casei | Lactobacillus acidophilus | Lactobacillus plantarum | Lactobacillus casei | Lactobacillus acidophilus | Lactobacillus plantarum | Lactobacillus amylovorus | Lactobacillus casei | Lactobacillus pentosus | Lactobacillus acidophilus |
| % of similarity** | 99.99 | 98.73 | 78.67 | 99.97 | 99.89 | 78.67 | 98.99 | 99.99 | 79.99 | 78.99 | 89.99 | 99.99 |

*(+): Positive reaction, (-): Negative reaction, (±): Weak reaction and (V): Variable reaction, *BM: Buffalo milk, CD: Cow dung, CS: Curd sample, GM: Goat milk, **percentage of similarity of identified isolates were checked by advanced bacterial software
results suggested that the CFS of isolates can be used as antimicrobial agents.

Acid tolerance
To investigate the survival of lactobacilli isolates in the presence of acid and bile salt, their growth was observed at low pH (2 and 3) and at bile salt concentrations (0.2% and 0.3%). Since, from entry into the mouth, to establish in the gut and colon, the LAB have to survive in acidic environment and bile salt presence which secrets in the liver. The stomach has low pH ranged from 1.5 to 3.5, due to secretion of gastric juice and intestine is moderately alkaline, pH ranged from 8 to 8.5. In the investigated study, results indicated that all 12 isolates exhibited a survival <50, ranged from 56.93% to 80.88% at pH 2 and 61.44% to 61.25% at pH 3. According to the classification criteria, the survival percentage of isolates in acidic condition has been divided into four categories: Excellent if the isolate survived at pH 2 after 24 hrs; very good if the isolate survived at pH 2 after 6 hrs but not after 24 hrs; good if the isolate survived at pH 3 after 24 hrs but not at pH 2; poor if the isolate did not survive in any experimental condition. The results (Fig. 2) showed that out of 12 isolates, 3 isolates (BM10, curd sample (CS)5 and GM10) exhibited excellent growth in pH 2 and 3, 4 isolates (BM5, CH12, GM3 and GM5) exhibited very good growth, 3 isolates (cow dung [CD]1, CH4 and CM3) exhibited good growth while 2 isolates (BM2 and CH9) exhibited poor survival at low pH 2. Singh et al. [26] reported the maximum acid tolerance of nine lactobacilli isolates ranged from 46.47% to 79.74% at pH 2 and 46.70% to 102.48% at pH 3.

Bile salt tolerance
Bacterial cell wall contains lipids and fatty acids, which disrupt in duodenal part of the gut by bile salts, as it has a detergent like nature. Hence, survival in bile salt than acidic environment is an important property of LAB, which facilitates it to efficiently perform their action in gut [27]. Isolates showed good survival in the presence of 0.2% and 0.4% bile salt varies from 36.65% to 79.91% in 0.2% bile salt concentration, while survival percentages on increasing bile salt concentration 0.4% decreased up to 30.41% and GM10 showed maximum (61.59%) value of bile salt tolerance. Classification criteria of bile salt tolerance also have been divided into: Excellent if the isolate survived at 0.4% bile salt after 24 hrs; very good if the isolate survived at 0.4% bile salt after 6 hr but not after 24 Hr; good if the isolate survived at 0.2% bile salt after 24 hrs but not at 0.4% bile salt; poor if the isolate did not survive in any experimental condition. Among 12 isolates, two isolates (GM3 and GM10) exhibited an excellent survival, three isolates (BM10, CH4, and CS6) exhibited a very good survival of bile salt tolerance, and five isolates (BM2, BM5, CD1, CM3, and GM5) showed good survival while two isolates (CH9 and CH12) showed poor bile salt tolerance (Fig. 3). The previous studies showed that Lactobacillus strains showed tolerance in 0.05–2.5% bile salt concentrations [28,29].

Autolytic activity
In this study, the autolysis rate of isolates measured in MRS medium after 3 and 5 hrs. GM3 showed the lowest value of autolytic activity 8.32±0.16% after 3 hrs and 15.85±0.13% after 5 hrs. Saran et al. [23] evaluated the autolytic activity of Lactobacillus acidophilus 291 in the presence of honey and inulin; their observation suggests that the autolytic activity in MRS was approximately 9% after 3 hrs and further reached to 12% after 5 hrs. However, in the presence of inulin the autolytic activity was reduced to 5.6–6.09%. The results (Table 2) obtained in the present study are closely comparable to previous results reported in literature and in the presence of prebiotics like honey and inulin; autolytic activity of these isolates can also be improved. Autolysis is a spontaneous degeneration of bacterial cell due to age or unfavorable conditions. During autolysis intracellular enzymes release out of the cell, this property of lactobacilli is useful for cheese ripening as these enzymes help in texture and flavor improvement. An enzyme autolysin during autolysis activates which disrupt peptidoglycan subunits of the cell wall by hydrolyzing covalent bonds. It reduces the number of probiotic bacteria [30] and decreases adhesiveness.

Gelatinase, autoaggregation and coaggregation activities
Among isolated 12 lactobacilli, 8 isolates (BM2, CD1, CH4, CH9,CH12, CS6, BM10 and GM5) exhibited no positive gelatinase activity compared to positive control S. aureus, while 4 isolates (BM5, CM3, GM3 and GM10) exhibited positive gelatinase activity. Adhesive properties are essential for probiotic bacteria, as these provide protection of host mucosal surfaces against entry of pathogens. Aggregation inhibits adherence of pathogen via forming a barrier, which prevents colonization of pathogen thereby limiting their infection [31]. It can also increase the concentration of excreted inhibitory substances [32]. Coaggregation involves the interaction between a Lactobacillus and a pathogenic strain (between genetically different strains) which facilitate direct or indirect clearance of pathogens. Adherence depends on cell surface characteristics. Results (Table 2) exhibited that the GM5 showed maximum and GM3 showed minimum autoaggregation phenotype. The coaggregation of lactobacilli isolates with E. coli differs in values ranged from 13.04±0.21% to 34.97±0.37%. The BM5 showed minimum coaggregation ability while it was significantly higher in GM5 (Table 2). Kos et al. [24] reported the relationship between
autoggregation and the adhesiveness ability of \textit{L. acidophilus} M92, which mediated by proteaceous components on the cell surface. The selection rate of lactobacilli isolates was ranged from 3.65±1.34% up to 67.04±0.61% over a period of 60 minutes. Recently, Gudina et al.\cite{33} reported a similar and comparative study on \textit{Lactobacillus paracasei} performed with washed cells suspended in PBS and their own culture supernatant fluid, the autoggregation observed in both the conditions were similar 51.1 and 49.4\%, respectively, after 2 hrs of incubation. In this study, xylene, toluene and n-hexadecane were used, as these solvents are non-polar in nature, their hydrophobic nature help to interact with hydrophobic surfaces of microbes. Lactobacilli with hydrophobic cell surfaces can easily adhere to host epithelium and enhances competition and colonization in the gastrointestinal tract against pathogens.

**Microbial adhesion to solvents (MATS)**

Microbial adhesion to n-hexadecane is considered as a marker for evaluating adhesiveness of microbial cells\cite{34} and minimum 40% hydrophobicity is required for a probiotic strain for adhesiveness\cite{35}. In our study, eight out of 12 isolates showed MATS <40% and BM10 showed maximum hydrophobicity (50.10±2.06%, 53.70±0.86%, and 64.84±1.41\%) in xylene, toluene and n-hexadecane, respectively (Table 2). Nikolovic et al.\cite{36} reported that ten lactobacilli and one Leuconostoc strains showed high adhesion activity to n-hexadecane. \textit{L. paracasei} subsp. \textit{paracasei} BG52-8, BGDP1-84 and BGNJ1-61 showed high percentage of adhesion to chloroform and ethyl acetate. Similarly, \textit{L. fermentum} strains CFR5, CFR1, CFR2 and CFR4 also showed a higher hydrophobicity than the \textit{Lactobacillus delbrueckii} CFR6, with a maximum value of 53.6±8.3\%\cite{37}.

**CONCLUSION**

Probiotic bacteria are beneficial for human health. These can be a perfect replacement of antibiotics. Antibiotics have broad spectrum growth inhibition activity, therefore kill useful flora in gut with the target pathogen while probiotics contain narrow antimicrobial activity. Antibiotics also generate resistance in pathogenic microorganisms. Researches must be proceed to identify and exploitation of new lactobacilli isolates, which fulfill the requirement as probiotic. Keeping ahead a step in this direction, to get potent lactobacilli isolates, in this present study, 141 isolates were isolated from 150 dairy and cattle dung samples. All strains were identified by Gram-staining, catalase test, motility test, and endospore test. 94 isolates were Gram-positive, catalase negative, non-motile and non-sporing in nature. After screening of these lactobacilli isolates, 12 potent probiotic strains with maximum antimicrobial activity were chosen for further study. Out of 12, two isolates (BM10 and GM10) showed significant results of antimicrobial, probiotic properties and as well as adhesive attributes. The results concluded that these isolates fulfill the requirements of probiotic strains and can be further analyzed for clinical and biotherapeutic applications.

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