Screening of Lactic Acid Bacteria of Different Origin for their Probiotic Potential

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Abstract

This study was to isolate lactic acid bacteria (LAB) from different dairy and non-dairy sources and to evaluate the probiotic potential of the isolated microorganism(s). Sixteen Lactobacillus isolates viz. seven human (LHI), six dairy (LMI) and three from kimchi (LKI) were subjected to screening for probiotic attributes: simulated stomach duodenum passage, bile salt hydrolase; cell surface hydrophobicity, adhesion, auto-aggregation, antimicrobial activity, antibiotic susceptibility and antioxidative potential, following standard protocols. The isolates exhibited resistance to stomach pH (pH=3.0) and tolerance against gastric juice and bile. The isolates also displayed antimicrobial activity against Escherichia coli, Klebsiella sp., S. typhimurium and were uniformly sensitive towards amikacin, amoxycillin, ampicillin, chloramphenicol, clindamycin, erythromycin, oxetetracyclin and cefuroxime. Highest hydrophobicity of up to 86 and 44% against both n-hexadecane and xylene were recorded with LHI7 followed by LHI6. Moreover, LMI5 and LKI2 showed high anti-oxidative and auto-aggregation properties, respectively. Bacterial adhesion increased with a decrease in pH with highest affinity for Caco-2 cells in LHI7, showing 68.7 ± 0.53% adhesion, followed by LHI6 with 65.4 ± 0.15%. Overall, these selective strains may possibly be utilized as probiotics after further validation through in vivo studies and/or human clinical trials.

Keywords: Probiotics; Lactic acid bacteria; Lactobacilli; Acid tolerance; Bile tolerance; Hydrophobicity

Abbreviations: LAB: Lactic Acid Bacteria; CSH: Cell Surface Hydrophobicity; SSDP: Simulated Stomach Duodenum Passage

Introduction

Lactic acid bacteria (LAB) having GRAS status, consists of genetically and physiologically diverse group of Gram positive, catalase negative, non-spore forming, non-pathogen [1,2], known for their health benefits. Members of this family, Lactobacillus and Bifidobacterium are recognized as probiotics; live bacteria, which when administered in adequate amount confer some health benefits to the host [3]; and have wider applications in food, feed, dairy and fermentation industry, as non-pharmacological approaches for health management [2]. The concept that food-borne bacteria can have health promoting effects is credited to Russian Nobel laureate, Elie Metchnikoff, who hypothesized that consuming large amounts of fermented milk products could prolong the life of Bulgarians [4].

Newer applications of probiotics are studied for their potential in lactose intolerance [5,6], natural resistance to infections of GIT [7], improved digestion [8], cancer suppression [9], cholesterol lowering [10], anti-obesity [11,12], anti-diabetic [13], anti-allergic [14], anti-hypertensive [15], anti-inflammatory [16], enzyme inhibitions [17,18], mood changer [19] etc. Thus, the concept of using beneficial microbes, as bio-therapeutics and for improved health has become an area of immense interest [20].

Although, several strains of LAB viz. L. acidophilus [21,22], L. brevis [23], L. casei [22,24], L. fermentum [25], L. gasseri [26], L. helveticus [22], L. plantarum [27], L. rhamnous [28] etc. have been isolated and validated as probiotics; studies are still focusing on potential strains of LAB. It is a fact that probiotic attributes and health benefits are strain specific [29], yet it is of high significance to screen for new strains with more potent health benefits.

In order to survive and colonize the GIT, probiotics should have resistance to acid and bile of GIT, followed by adhesion to the host gut, which is prerequisite for sufficient interaction with host [30]. Good adherence to GIT enables the probiotics to persist enhancing the host-bacteria interactions [31]. Adherence of probiotics also helps to overcome peristalsis of stomach [32]. Other essential characteristics include cell surface hydrophobicity (CSH), auto-aggregation, antimicrobial properties and safety. The CSH of bacterial surfaces is a determinant of adhesion and biofilms formation on animate and inanimate surfaces [33]. It is likely due to a complex interplay between negatively-charged, positively charged, hydrophobic and hydrophilic components on bacterial cell surface. Display of antimicrobial activity against pathogens is also preferred [34-38]. In addition, these genes coding for resistance in probiotics should be natural and located over chromosomal DNA i.e. non-transferable to other fellow microbes [40].

The identification of unique probiotics for newer applications is a very complex process that takes substantial research effort. Thus the aim of this study was to identify LAB isolates as probiotics, based on assessment of attributes necessary to qualify them as potential probiotic agent.

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Materials and Methods
Isolation of LAB

Samples of dahi, lassi and raw milk (i.e. cow, goat and buffalo) were collected from the experimental dairy plant and cattle yard of ICAR-NDRI, Karnal, as well from the local market and rural areas of Karnal and Chandigarh. Commercially available samples from organized sectors were also collected from local market. Faecal samples from healthy human adults and infants, with no recent history of antibiotic treatment (≥ 6 months) or infection, were also collected from Karnal with consent from parents. Also, Kimchi, a fermented Korean product was procured from ‘microbial metabolite lab’ for isolation. Following sampling, one gram/ml of food/faecal samples were suspended in 9.0 ml of peptone water, homogenized exhaustively, serially diluted (10⁻⁵-10⁻⁸), poured plated in BCP-MRS agar and incubated an-aerobically at 37°C for 24-48h for the development of typical yellow coloured rice shape colonies.

Identification of LAB

Individual colonies were picked and transferred to MRS broth for further examination and selection at morphological, bio-chemical and molecular levels. Isolates were screened for catalase activity by plating a loop of 16-18h grown cultures on slide followed by the addition of a drop of H₂O₂ (3%) and effervescence was noted as positive. Isolates were further characterized at molecular level by genus specific PCR. Genomic DNA was extracted by Neumann and Pospiech [41] method following quantification using Nanodrop (Shimadzu Bio Spec-nano). PCR reactions were performed using oligo-nucleotide primer sets (LbLMA-1, CTCAAAAATACAAAAAGTTC and R-161, CTTGTACACCGGCGCGTCA) described by Dubernet et al., [42]. PCR reaction was carried out in Eppendorf thermalcycler including initial denaturation at 95°C/5 min, followed by 35 cycles each of denaturation, 95°C/30 sec; annealing, 55°C/30 sec and extension, 72°C/30 sec; and final extension at 72°C for 7 min and final holding at 4°C. PCR products were electrophoreses in 1.5% agarose gel (Mini submarine, Hoeffer, USA) following the standard protocol of Sambrook et al. [43]. A product size of ~250 bp confirmed the presence of Lactobacillus isolates.

All identified Lactobacillus isolates were subjected to series in vitro tests for confirming their probiotic attributes as recommended by FAO/WHO [5]. All experiments were conducted in vitro, anticipating that the qualified isolates may also mimic their activities under in vivo conditions.

Simulated Stomach Duodenum Passage (SSDP) assay

SSDP assay is designed to represent a simplified and standardized test giving predictable values for survival of test strain in human stomach and duodenum under normal gut conditions [44]. The principle involves an initial simulation of stomach containing ingested LAB after a meal. After 1h, bile and artificial duodenum secretions were added to simulate the further gastric passage. Counts refer to the log cfu/ml at 0h. Synthetic duodenum juice was prepared by dissolving NaHCO₃ (6.4 g/l), KCl (0.239 g/l), NaCl (1.28 g/l) in distilled water. The pH was adjusted to 7.5 using 5M HCl. The oxgal solution was prepared by reconstituting 10 g of oxgal in 100ml distilled water. All the solutions were sterilized by autoclaving at 121°C/15 min. The required volumes of the overnight grown cultures and MRS broth adjusted at pH 3.0 were aseptically mixed in sterile flask to give a final concentration of 10⁶ cfu/ml. After mixing initial counts were determined by spread plating. Samples were drawn after 1h of incubation at 37°C and viable counts determined. Four milliliters of oxgal solutions were added to the culture in flasks, followed by 17 ml of duodenum juice. After mixing, the flasks were further incubated at 37°C. Samples were withdrawn after 2 and 3h and counts were taken. Experiment was carried out in duplicate and repeated three times.

Cell surface hydrophobicity

Ability of the organisms to adhere to hydrocarbons is a measure of their adherence to the epithelial cells in the gut i.e. CSH. The CSH was determined according to the method described by Rosenberg et al. [45] with slight modification using n-hexadecane and xylene. Overnight grown cultures were centrifuged at 5,000 g and the cell pellets were washed twice with phosphate urea magnesium (PUM) buffer. The washed pellets were re-suspended in PUM buffer and the absorbance (Aₖ₃₂₀) was adjusted to 0.7-0.9. The cell suspension (3.0ml) and n-hexadecane (1.0ml) were vortex and incubated at 37°C for 10 min. The mixture was again vortex briefly and incubated at 37°C for 1h for phase separations and the hydrocarbon layer was allowed to rise completely. After 1h, aqueous phase was removed carefully with a Pasteur pipette and absorbance (Aₖ₃₂₀) was measured using Spectrophotometer (Jenway Genova, Jenway Ltd. UK). The decrease in absorbance was taken as a measure of the CSH (H%) calculated as:

\[
H(\%) = \frac{OD_{\text{initial}} - OD_{\text{final}}}{OD_{\text{initial}}} \times 100
\]

Where, \( OD_{\text{initial}} \) = initial absorbance and \( OD_{\text{final}} \) = final absorbance; with the two hydrocarbons at 610 nm.

Cell auto-aggregation

Cell auto-aggregation was determined using the method of Tomas et al. [46]. Overnight grown Lactobacillus cultures were harvested by centrifugation and the cell pellets washed twice with Phosphate Buffer Saline (PBS) and re-suspended again in PBS to an absorbance of ~0.5 at 600 nm (Abs₆₀₀). The suspension was centrifuged and the pellets were re-suspended in equal volume of broth removed at step 1. The mixture was allowed to stand at 37°C for 2 h. Thereafter, 1.0 ml of the upper suspension was taken to measure the absorbance (Abs₆₀₀) using broth as reference. The percent difference between the initial and final absorbance gives an index of cellular auto-aggregation as follows.

\[
\text{Aggregation (\%) = } \frac{(Abs_{\text{initial}} - Abs_{\text{final}})}{Abs_{\text{initial}}} \times 100
\]

Adhesion of lactobacilli isolates to Caco-2 cell lines

The quantitative binding of Lactobacillus cultures was also investigated on Caco-2 cell line by two independent methods i.e. direct microscopic examination after Giemsa staining and enumeration by plating on MRS. The human adenocarcinoma cell line, Caco-2 (nonsense secreting) for adhesion assay were procured from National Center of Cell Sciences, Pune, India. The cell lines were cultured in Dulbecco’s Modified Eagle’s Minimal Essential Medium (DMEM; Sigma, USA) supplemented with 10 % (v/v) heat-inactivated (30 min, 56°C) fetal bovine serum, 25 mM HEPES (Sigma, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO₂.

Adhesion assay

Adhesion of the Lactobacillus cultures was measured as per the method of Jacobsen et al. [47]. The cell suspension with 1 x 10⁵ cells prepared in 4ml complete DMEM was transferred to each well of six-well tissue culture plates. The medium was changed on alternate day. When cells reached 80% confluency, the medium was replenished each day for 20 days. The spent medium was completely removed 24h
before adhesion assay and cells were fed with antibiotic free DMEM. Cells were then washed twice with 3ml PBS (pH 7.4). An aliquot of 2 ml of DMEM (without serum and antibiotics) was added to each well and incubated at 37°C for 30 min. Different Lactobacillus cultures (1 x 10^6cfu) suspended in 1ml DMEM (without serum and antibiotics) were added to different wells. The plates were incubated at 37°C in 5% CO₂ for 2 h. The monolayers were washed 5 times with sterile PBS (pH 7.4). Percent adhesion was determined by plating method.

Cells from monolayers were detached by trypsinization. The detached cells were repeatedly but gently aspirated to make homogenous suspension. The cell suspension was then serially diluted with saline solution, plated on MRS agar, incubated for 24-48 h at 37°C and colonies were counted (B1,cfu/ml). Bacterial cells initially added to each well of six-well plates were also counted (B0,cfu/ml). The adhesion percent was calculated as:

\[
\text{Adhesion} \% = \left( \frac{B1}{B0} \right) \times 100
\]

Where \( B1 \) = Number of bacterial colonies after incubation and \( B0 \) = no of bacterial cells added initially.

**Antimicrobial activity**

Antimicrobial activities of probiotics are thought to be a significant functional criterion for competitively excluding or inhibiting the activities of pathogenic intestinal microflora via production of antimicrobial compounds like organic acids (e.g. acetic and lactic acids), hydrogen peroxide, bacteriocins etc. Antimicrobial activity of Lactobacillus isolates were recorded against Klebsiella sp., S. typhimurium and E. coli following agar spot test by Schillinger andLucke [48]. Lactobacillus cultures for spot inoculation of agar surfaces were grown in MRS broth at 37°C by three consecutive transfers at 24 h intervals. Indicant strains for seeding the soft agar for overlay were also grown in BHI broths by three successive transfers at 24h intervals at their optimum growth temperatures. The surface of the solidified and dried (overnight at 37°C) tryptone glycocholate yeast extract (TGE) agar plates were spot inoculated with 5 mL of Lactobacillus cultures. The inoculated agar plates were incubated at 37°C for 18-24 h and then overlaid with 7.0 mL of TGE soft agar (0.7% agar) seeded with 30 µL of the indicator culture. The diameter of zone of inhibition was measured and a clear zone of 1 mm or more was considered positive inhibition.

**Antibiotic susceptibility**

Antibiotic resistance/susceptibility of lactobacilli isolates were studied against large spectra of clinically important antibiotics by disc diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI; Wayne, USA) [49]. A total number of 12 antibiotics (HiMedia Ltd, Mumbai, India) viz. Amikacin (AK, 30 µg), Amoxyccillin (AM, 10 µg), Ampicillin (AMP, 10 µg), Ciprofloxacain (CIP, 10 µg), Cephalothin (CET, 30 µg), Chloramphenicol (C, 10 µg), Clindamycin (CD, 2 µg), Erythromycin (E, 10 µg), Novobiocin (NV, 30 µg), Kenamycin (K, 30 µg), Oxytetracyclin (OXT, 30 µg) and Cefuroxime (CXM, 30 µg) were applied. Fifteen milliliters of MRS agar was poured in petriplate and allowed to solidify. These were subsequently over laid with 4ml of soft agar tempered at 45°C and seeded with 200 µL of active cultures. Petriplates were allowed to stand at room temperature for 15 min and then the antibiotic discs were dispensed onto agar using disc dispenser under aseptic conditions. The agar plates were incubated at 37°C aerobically for 24 h. Diameter (mm) of zone of inhibition was measured using antibiotic zone scale and results were expressed in terms of resistant, moderately susceptible or susceptible by comparing with the interpretative zone diameters given by ‘Performance Standards for Antimicrobial Disk Susceptibility’ tests [49] for disc diffusion antibiotic susceptibility test.

**Antioxidative potential**

Measurement of antioxidative capacity of the selected Lactobacillus cultures was performed according to Re et al. [50] based on the principle of scavenging ABTS⁺ radicals by test cultures. Overnight grown culture supernatant was collected by centrifugation at 5400 × g for 15 min at 4°C. The ABTS working solution was prepared by mixing 88 µL of 140 mM potassium persulphate with 5mL of 7 mM ABTS stock solution and incubating overnight in dark bottles for generation of radicals. An aliquot of 200 µl of this solution was added to 15 ml PBS to adjust the absorbance at 734 nm to 0.7 ± 0.02. An aliquot of 10 µl of cell supernatant collected in step 1 was added to 1.0 mL ABTS in PBS solution in an optical cuvette and the contents were mixed for 10 sec and decrease in absorbance at 734 nm was recorded over a period of 10 min at 10 sec intervals using SPECORD-200 double beam spectrophotometer (Analytical zena).

**Bile salt hydrolase (BSH) assay**

Direct plate assay method of Singh et al. [52] was employed for detection of BSH activity. BSH activity was examined by streaking the fully activated culture on MRS agar containing 0.5% (w/v) bile salt viz. sodium taurocholate, sodium taurodeoxycholate and sodium tauroglycocholate and 0.37 g/l of CaCl₂. Petriplates were then incubated at 37°C anaerobically for three days. BSH activity was indicated, when the hydrolyzed products of salts, viz. cholic acid or deoxycholic acid, precipitated in agar medium in and around the spots.

**Results and Discussion**

**Isolation of LAB**

A total of 125 colonies were selected from raw milk (cow, 20; goat, 5; buffalo, 25); Dai (10); lassi (5); faeces (adult, 25; infant, 30) and Kimchi (5) on basis of variations in colonial morphology (i.e. size: small, medium and large i.e. 0.7 mm to 2.1 mm; margins: entire and undulate; shape and colour: circular, rough, smooth and compact, irregular, white, greyish-white, dark-centered, cream) clearly indicating a wider diversity. Isolates were identified as member of Lactobacillaceae by negative and Gram’s staining. Figures 1a and 1b are representative for negative and Gram’s staining, respectively. Based on morphological screening, out of the total 125 isolates, a total of 101, were identified as Gram positive rods. After subjecting to genus specific PCR, as described in materials and methods, a total of 16 isolates were confirmed as Lactobacillus isolates, on basis of observed band size of 250 bp (Figure 2).

![Figure 1: (a) Representative smears for Negative staining and (b) Gram’s staining performed with bacterial isolates randomly collected from BCP-MRS agar plates.](image-url)
Response to simulated stomach duodenum passage (SSDP)

As the two stresses of stomach and small intestinal transit might interact and thereby, affect the viability of strains in a synergistic fashion, therefore, it is important to evaluate low pH, bile salts and duodenum juice in one system, rather than evaluating the effect of each separately [44]. In this study, combined effect of gastric and intestinal fluids in simulated GIT transit resistance was tested. Ten strains showed higher survival and tolerance to acid and bile environments after 2 h incubation presented as log cfu/ml (Table 1 and Figure 3). However, 13 isolates maintained viability after 3 h of stress. Survival of bacterial strains in human gastric juice is a more accurate indication of the ability of strains to survive passage through the stomach. Resistance to bile salts is considered as an important property in strains envisaged the ability of strains to survive passage through the intestine. Kociubinsky et al. [44] reported that all the Lactobacillus strains showed higher survival and tolerance to acid and bile environments. As already reported [57,58], in vitro studies can partially mimic the gut ecosystem.

Cell surface hydrophobicity

CSH of Lactobacillus isolates was measured using the microbial partition to xylene and n-hexadecane. Results indicated wide variations in CSH ranging from 11.88 to 86.79% between different isolates (Table 2 and Figure 4). Highest hydrophobicity of 86.79 ± 2.03 and 44.82 ± 0.63% against both n-hexadecane and xylene were recorded with LHI17 followed by LHI6, having 72.22 ± 0.28 and 42.85 ± 0.94% adhesion against both the hydrocarbons. As depicted in Table 2, other isolates showed moderate to low affinity against both the hydrocarbons. The variations in hydrophobicity can be correlated with the earlier reports [59-62]. This variable affinity of different isolates may be attributed to their net negative surface charge. The hydrophobic potential varies between different organisms, strains and is also influenced by age and surface chemistry of strains along with the medium constituents [63]. Among several factors responsible for adhesion of bacterial cells to host tissues, CSH plays a key role [64] and might also play an important role in biofilm formation [63]. The significant hydrophobicity observed with selected isolates could indicate their upper hand in establishing firmly in GIT [65]. However, few studies contradict the correlation usually established between hydrophobicity and adhesion. Schillinger et al. (2005) [65] showed that L. acidophilus BFE 719, having poor hydrophobicity of only 2%, showed good adhesion to HT29 cells. This study suggests that the strains depicting low CSH values may have higher adhesion capabilities under in vitro and in vivo.

Cell auto-aggregation

Sedimentation rate of all isolates was determined for a period

<table>
<thead>
<tr>
<th>Isolate</th>
<th>0h</th>
<th>2h</th>
<th>3h</th>
<th>Isolate</th>
<th>0h</th>
<th>2h</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHI1</td>
<td>9.3</td>
<td>8.14</td>
<td>6.5</td>
<td>LMI2</td>
<td>9 ± 0.33</td>
<td>8.59 ± 0.18</td>
<td>-</td>
</tr>
<tr>
<td>LHI2</td>
<td>10.2</td>
<td>8.17</td>
<td>6.16</td>
<td>LMI3</td>
<td>8.5 ± 0.76</td>
<td>8.38 ± 0.11</td>
<td>-</td>
</tr>
<tr>
<td>LHI3</td>
<td>7.8</td>
<td>6.41</td>
<td>0.35</td>
<td>LMI4</td>
<td>9 ± 0.66</td>
<td>8.54 ± 0.15</td>
<td>7.54 ± 0.22</td>
</tr>
<tr>
<td>LHI4</td>
<td>8.8</td>
<td>8.23</td>
<td>0.09</td>
<td>LMI5</td>
<td>8.7 ± 0.29</td>
<td>8.34 ± 0.11</td>
<td>7.07 ± 0.17</td>
</tr>
<tr>
<td>LHI5</td>
<td>9 ± 0.57</td>
<td>8.3 ± 0.20</td>
<td>6.77 ± 0.22</td>
<td>LMI6</td>
<td>11 ± 0.57</td>
<td>8.65 ± 0.19</td>
<td>7.65 ± 0.28</td>
</tr>
<tr>
<td>LHI6</td>
<td>8.9</td>
<td>8.74</td>
<td>0.44</td>
<td>LK11</td>
<td>10 ± 0.57</td>
<td>8.41 ± 0.12</td>
<td>7.38 ± 0.41</td>
</tr>
<tr>
<td>LHI7</td>
<td>8.8</td>
<td>8.54</td>
<td>0.15</td>
<td>LK12</td>
<td>9 ± 0.55</td>
<td>8.67 ± 0.22</td>
<td>7.71 ± 0.27</td>
</tr>
<tr>
<td>LMI1</td>
<td>10 ± 0.33</td>
<td>8.54 ± 0.15</td>
<td>7.68 ± 0.20</td>
<td>LMI3</td>
<td>8.4 ± 0.78</td>
<td>8.23 ± 0.14</td>
<td>7.00 ± 0.26</td>
</tr>
</tbody>
</table>

Values are presented in triplicate ± SEM

Table 1: Response to SSDP (log cfu/ml).
Values are presented in triplicate, ± SEM

<table>
<thead>
<tr>
<th>Isolate</th>
<th>n-Hexadecane</th>
<th>Xylene</th>
<th>Isolate</th>
<th>n-Hexadecane</th>
<th>Xylene</th>
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</thead>
<tbody>
<tr>
<td>LHI1</td>
<td>51.61 ± 0.46</td>
<td>16.86 ± 0.63</td>
<td>LMI2</td>
<td>43.75 ± 1.28</td>
<td>38.48 ± 0.66</td>
</tr>
<tr>
<td>LHI2</td>
<td>14.99 ± 0.36</td>
<td>13.88 ± 0.35</td>
<td>LM13</td>
<td>19.32 ± 1.34</td>
<td>19.32 ± 0.59</td>
</tr>
<tr>
<td>LHI3</td>
<td>11.88 ± 0.60</td>
<td>12.81 ± 0.77</td>
<td>LM14</td>
<td>62.35 ± 2.14</td>
<td>29.67 ± 1.58</td>
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<tr>
<td>LHI4</td>
<td>15.83 ± 0.37</td>
<td>21.23 ± 0.87</td>
<td>LM15</td>
<td>69.35 ± 0.58</td>
<td>12.96 ± 0.75</td>
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<tr>
<td>LHI5</td>
<td>31.09 ± 1.01</td>
<td>18.50 ± 1.59</td>
<td>LM16</td>
<td>34.63 ± 1.78</td>
<td>17.18 ± 0.33</td>
</tr>
<tr>
<td>LHI6</td>
<td>72.22 ± 0.28</td>
<td>42.85 ± 0.94</td>
<td>LKI1</td>
<td>36.90 ± 2.16</td>
<td>17.88 ± 0.71</td>
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<tr>
<td>LHI7</td>
<td>86.79 ± 2.03</td>
<td>44.82 ± 0.63</td>
<td>LKI2</td>
<td>14.42 ± 1.77</td>
<td>13.79 ± 0.43</td>
</tr>
<tr>
<td>LMI1</td>
<td>18.75 ± 15.85</td>
<td>15.85 ± 1.77</td>
<td>LKI3</td>
<td>17.10 ± 1.01</td>
<td>12.26 ± 0.65</td>
</tr>
</tbody>
</table>

Values are presented in triplicate, ± SEM

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Aggregation</th>
<th>Isolate</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHI1</td>
<td>58.4 ± 0.92</td>
<td>LMI2</td>
<td>49.5 ± 2.68</td>
</tr>
<tr>
<td>LHI2</td>
<td>70.8 ± 1.79</td>
<td>LM13</td>
<td>48 ± 0.75</td>
</tr>
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<td>LHI3</td>
<td>68.7 ± 0.49</td>
<td>LM14</td>
<td>59 ± 0.87</td>
</tr>
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<td>LHI4</td>
<td>73.0 ± 0.2</td>
<td>LM15</td>
<td>67.7 ± 2.24</td>
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<td>57.8 ± 0.76</td>
<td>LM16</td>
<td>56.1 ± 1.87</td>
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<tr>
<td>LHI6</td>
<td>62.4 ± 0.81</td>
<td>LKI1</td>
<td>50 ± 2.09</td>
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<td>LHI7</td>
<td>72.4 ± 1.04</td>
<td>LKI2</td>
<td>72 ± 0.45</td>
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<tr>
<td>LMI1</td>
<td>69.0 ± 1.70</td>
<td>LKI3</td>
<td>65.4 ± 0.61</td>
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</tbody>
</table>

Values are presented in triplicate, ± SEM

Adhesion of Lactobacilli on Caco-2 cell lines

Adhesion of LAB isolates viz. LHI6, LHI7, LMI5 and LKI1; showing promising CSH, were assessed for their adhesion potential over Caco-2 cells (Table 4 and Figure 6). Highest affinity for Caco-2 cells was with LHI7, showing 68.7 ± 0.53% adhesion, followed by LHI6 with 65.4 ± 0.15%. Interestingly, the results were in accordance with CSH. LMI5 showed moderate adhesion of 30.2 ± 0.30%. Studies support varied Caco-2 adhesion potential of Lactobacillus [69-71]. However, in contrast to earlier reports, our study reported high adhesion around 30-68%, in comparison to those in range of 3-15% [69-71]. Out of the four strains screened, only LKI1 showed very low i.e. 12 ± 1.18% adhesion.

Antimicrobial activity

Antagonistic activity exhibited by different lactobacilli isolates was determined against enteric pathogens viz. Klebsiella sp., S. typhimurium and E. coli ATCC. All the isolates tested were inhibitory against the pathogens employed (Table 5). Among all, LHI4 and LHI7, LMI5 and LKI2 showed highest zone of inhibition against all the tested pathogens. Highest potency against tested pathogens was exhibited by LHI4 and LHI7. This indicates that the Lactobacillus strains exhibited inhibitory activity against enteric pathogens and may have similar effect over other pathogens. Klebsiella sp. and S. typhimurium were inhibited by all the test isolates at high or moderate level. However, E. coli showed resistance against three isolates viz. LKI1, LKI2, LKI3.

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of 2 h. The aggregation of the isolates ranged between 48-73%. Four isolates viz. LHI4, LHI7, LKI2 and LHI2 exhibited auto-aggregation over 70% with values of 73 ± 0.2, 72.4 ± 1.04, 72 ± 0.45 and 70.8 ± 1.79, respectively (Table 3 and Figure 5). Auto-aggregation potential of cells plays an important role in adhesion to intestinal cells [58] and prevention of pathogens colonization [66]. Auto-aggregation is the ability of bacteria to interact themselves in a nonspecific way, a prerequisite for colonization of the GIT [67]. As reported by del Re et al. (2000) [67], probiotics should have auto-aggregation potential over 40%. All the isolates evaluated showed affinity over 40% and have moderate to high degree of aggregation, which can be correlated to earlier findings [68]. Our results are in line with Kassaa et al. [61], who reported auto-aggregation values 30-76% of different Lactobacillus strains. Among other strains, showing good aggregation, LHI7, also showed highest affinity to hydrocarbons. This confirms the ability of LHI7 to adhere, persist and divide in GIT, qualifying it as potential probiotic [61].

Adhesion and auto-aggregation of LAB isolates. Error bars represents ± SEM.

Adhesion of LAB isolates viz. LHI6, LHI7, LMI5 and LKI1; showing promising CSH, were assessed for their adhesion potential over Caco-2 cells (Table 4 and Figure 6). Highest affinity for Caco-2 cells was with LHI7, showing 68.7 ± 0.53% adhesion, followed by LHI6 with 65.4 ± 0.15%. Interestingly, the results were in accordance with CSH. LMI5 showed moderate adhesion of 30.2 ± 0.30%. Studies support varied Caco-2 adhesion potential of Lactobacillus [69-71]. However, in contrast to earlier reports, our study reported high adhesion around 30-68%, in comparison to those in range of 3-15% [69-71]. Out of the four strains screened, only LKI1 showed very low i.e. 12 ± 1.18% adhesion. Schillinger and co-workers [65], reported that it is not mandatory to have direct correlation between CSH and adhesion. Adhesion of lactobacilli to Caco-2 cells, representing intestine, increases their possibility of adherence and survival in GIT. Also, this activity varies from strain to strain that support our results [72]. Adhesion to the gastrointestinal cells offers an additional advantage to cells, protecting them from frequent removal by gut contractions and peristaltic flow [71]. The present study documents adhesion of Lactobacillus strains to in vitro cell line model. However, studies in animal models are required to further support their candidature as probiotic.

Antimicrobial activity

Antagonistic activity exhibited by different lactobacilli isolates was determined against enteric pathogens viz. Klebsiella sp., S. typhimurium and E. coli ATCC. All the isolates tested were inhibitory against the pathogens employed (Table 5). Among all, LHI4 and LHI7, LMI5 and LKI2 showed highest zone of inhibition against all the tested pathogens. Highest potency against tested pathogens was exhibited by LHI4 and LHI7. This indicates that the Lactobacillus strains exhibited inhibitory activity against enteric pathogens and may have similar effect over other pathogens. Klebsiella sp. and S. typhimurium were inhibited by all the test isolates at high or moderate level. However, E. coli showed resistance against three isolates viz. LKI1, LKI2, LKI3.

Table 4: Adhesion to Caco-2 cells (%).

<table>
<thead>
<tr>
<th>Lactobacillus isolates</th>
<th>Adhesion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHI7</td>
<td>68.7 ± 0.53</td>
</tr>
<tr>
<td>LHI6</td>
<td>65.4 ± 0.15</td>
</tr>
<tr>
<td>LMI5</td>
<td>30.2 ± 0.30</td>
</tr>
<tr>
<td>LKI1</td>
<td>12 ± 1.18</td>
</tr>
</tbody>
</table>

Values are presented in triplicate, ± SEM.

Figure 6: Caco-2 cell adhesion of shortlisted LAB isolates. Error bars represents ± SEM.
Zone of inhibition (mm) > 10 mm = ++++, > 5 mm = ++, < 5 mm = +

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Klebsiella sp.</th>
<th>Salmonella typhimurium</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHI1, LHI2, LHI3, LMI5, LKI3</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>LHI4, LHI7, LKI1, LKI2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>LHI5</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>LHI6, LMI2, LMI4</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 5: Antimicrobial activity.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>AK</th>
<th>AMP</th>
<th>CIP</th>
<th>CET</th>
<th>C</th>
<th>CD</th>
<th>E</th>
<th>NV</th>
<th>K</th>
<th>OXT</th>
<th>CXM</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHI1, LMI4</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
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<tr>
<td>LHI2</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
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<tr>
<td>LHI3</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>LHI4</td>
<td>LHI6, LHI7, LKI3</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>LMI1</td>
<td>LMI2</td>
<td>LMI3</td>
<td>LMI5</td>
<td>LMI6</td>
<td>LKI1</td>
<td>LKI2</td>
<td>LMI4</td>
<td>LMI1</td>
<td>42.07 ± 0.83</td>
<td>LMI2</td>
<td>59.27 ± 0.65</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>46.49 ± 0.95</td>
<td>LMI3</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>32.84 ± 0.82</td>
<td>LMI4</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60.49 ± 0.76</td>
<td>LMI5</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>36.04 ± 0.34</td>
<td>LMI6</td>
</tr>
<tr>
<td>39.61 ± 0.61</td>
<td>42.07 ± 0.83</td>
<td>59.27 ± 0.65</td>
<td>60.49 ± 0.76</td>
<td>72.83 ± 0.82</td>
<td>59.27 ± 0.65</td>
<td>76.48 ± 0.71</td>
<td>72.83 ± 0.82</td>
<td>43.42 ± 0.42</td>
<td>54.37 ± 0.68</td>
<td>47.23 ± 0.59</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented in triplicate, ± SEM

Table 6: Antibiotic susceptibility.

Based on these preliminary findings, majority of test strains possess antimicrobial activity and may be utilized for applications in food and dairy products. Our results can be justified with earlier [73-76] and recent studies [77-79] reporting antimicrobial activity of probiotic Lactobacillus sp. Although different isolates has not been identified at species and strain level, but our results can be substantiated with reports describing antimicrobial activity as common parameter for most LAB that may be attributed to production of organic acids, antimicrobial peptides and bacteriocins [79,80]. Studies are required to elucidate the actual antimicrobial component of LAB metabolism. The difference in activity may be attributed to the varied metabolic profile of different Lactobacillus isolates [81] and also to the distinct response of test pathogen against different antimicrobial components.

**Antibiotic susceptibility**

All the isolates were tested for their susceptibility to 12 commonly administered antimicrobial agents, against whom variable responses were observed. It is evident that all the isolates were uniformly sensitive to AK, AM, AMP, C, CD, E, OXT and CXM. However, against other antimicrobials; sensitive, intermediate and resistant profiles were observed (Table 6). In contrast to results showing resistance of few strains (LHI3, LMI3) towards novobiocin and kanamycin. Anandharaj and Sivasankari [2], reported that all the isolates were sensitive to both the antibiotics. Zhou et al. [82] also reported high level of kanamycin resistance in Lactobacillus isolates. These report supports that antimicrobial susceptibility or resistance is highly variable at strain level, and it is important to screen isolates for their susceptibility profile. Among all isolates, LHI14, LHI16, LHI17 and LKI2 were sensitive to all the 12 antimicrobials. Only LHI5 showed multiple drug resistance against AK, CIP and KN. Our results are supported with several such reports mentioning variable antibiotic susceptibility profiles of different isolates from different host [83] and geographical regions [2,61,83,84]. It is a matter of debate, whether probiotics, having GRAS status should be resistant or sensitive against commonly used antimicrobials. As hypothesized by Cebeci and Gurakan [86] and Kim and Austin [87], resistance to specific antimicrobials, enhances application of probiotics, as these can be administered along with antibiotic therapy, secondly, these help to recover the gastrointestinal microflora quickly. On the contrary, these safe strains showing antimicrobial resistance possess risk of transferring resistance to pathogens, making them resistant to antibiotic therapy. In this regard, further safety parameters are to be studied, before making these eligible for human consumption or product development. Strains exhibiting natural resistance to antibiotics are not considered as a risk to animal or human health [61]. However, the probiotics must be safe for human consumption and should not possess transmissible antibiotic resistance determinants.

**Antioxidative potential**

Many lactobacilli possess antioxidant activities that are able to decrease the risk of accumulation of reactive oxygen species during cellular metabolism in body. LAB isolates were studied for their antioxidative potential following ABTS assay. Against ABTS radical cation, the inhibition ranged from 32.84-85.79% (Table 7). Highest inhibitions of 85.79 and 78.82% were recorded with LH16 and LMI3, respectively, and the least 32.84% with LIH3 (Figure 7). These differences could be due to different proteolytic activity of individual cultures, which results in release of antioxidative peptides [88,89]. Several studies have reported antioxidative potential of live, heat killed and cell free extracts of LABs [90]. This property may be attributed to enzymatic processes such as coupled NADH oxidase/peroxidase and catalase [91,92]. Keeping in view, high antioxidative potential, these isolates can be considered as health promoting probiotics.

**Bile salt hydrolase assay**

BSH activity is important for bacteria to grow in and colonize in the intestine [93] by deconjugating bile salts, which are readily excreted in GIT. The relevant physiological concentrations of human bile range from 0.3 to 0.5% [58]. All the isolates precipitated TC (sodium
taurocholate) and TDC (sodium taurodeoxycholate) to a great extent (Table 8) but none of the isolates either not precipitated TGC or at a slighter extent. It can be inferred from results that LHI3, LHI6, LHI7, LMI3, LMI5 and LKI1 not only tolerated the toxicity of these salts but also carryout BSH mediated deconjugation of TC and TDC, which helps their colonization in intestine. Growth of our isolates in presence of high bile salts in medium suggested that these produced BSH activity specific to TC, TGC (sodium tauroglycocholate) and TDC hydrolysis. The inhibition of common intestinal bacteria has been related to the presence of free (deconjugated) bile acids rather than conjugated ones [94]. McAuliffe et al. [95] had identified genes responsible for BSH activity in L. acidophilus NCFM, which further support BSH activity in our cultures. Tanaka et al. [96] accounted that certain strains of a species do not possess deconjugation activity, while others show that bacteria without this enzyme can either survive in this environment or survive the passage through it.

Conclusion

Our preliminary findings highlights that Lactobacillus isolates, viz. LHI7, LHI6, LMI5 and LKI2 fulfils the primary requirements of a probiotic and may be a reliable candidate for further validation in vitro and animal models. However, studies are required to characterize them at molecular level, their safety assessment before exploring for any functional product development.

Acknowledgement

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References

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Figure 7: Anti-oxidative activity of LAB isolates assessed through ABTS assay.

Error bars represents ± SEM.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>DC</th>
<th>TC</th>
<th>TGC</th>
<th>Isolate</th>
<th>DC</th>
<th>TC</th>
<th>TGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHI1, LHI2, LMI1</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>LHI7, LMI3</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>LHI3, LKI1</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>LMI4</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>LHI4, LMI2</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>LMI5</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>LHI5, LMI6</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>LKI2</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>LHI6</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>LKI3</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*** Intense ppt; ** Moderate ppt; +Slight ppt; - No ppt

Table 8: Bile salt hydrolase activity.


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