ABSTRACT
Fifty two lactic acid bacteria (LAB) were isolated from milk of Desi and cross-breed cows of India and its functional characteristics were investigated on the basis of morphological and biochemical properties. Among them four bacterial isolates were screened on the basis of in vitro probiotic attributes. Isolate CP-12d and CP-8d which were isolated from Desi Indian cows showed high tolerance to low pH, bile, NaCl and certain antibiotics. These isolates exhibited the highest adhesion to hydrocarbons xylene, n-octane and n-heptane. Isolate CP-8d exhibited the highest auto-aggregation rate (62%). It was the most resistant isolate against different antibiotics. All the four isolates inhibited the enteric pathogens viz., Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae and Salmonella typhi but the isolate CP-12d and CP-8d exhibited high antagonistic activity against all pathogens. These two bacterial isolates exhibited good probiotic properties and also grew well in presence of inulin as compared to honey.

KEY WORDS: Lactic acid bacteria, Probiotics, Antibacterial activity, Prebiotics

INTRODUCTION
Probiotics are the live microbial food supplements which beneficially affect the host by improving its intestinal microbial balance. There are many benefits of probiotics including improved nutrition, growth and prevention of various gastrointestinal (GI) disorders. Traditionally, physicians used various antibiotics for treating gastrointestinal disorders. However, the incidence of antibiotic-associated diarrhea (AAD) and resistance of the pathogens to antibiotics require alternative strategies for treatment. AAD results from disruption of the normal microflora of the gut by antibiotics (Wistrom et al., 2001).

Lactic acid bacteria (LAB) are widely used in food and pharmaceutical industry, especially for the fermentation of milks and as medicine to improve the gastrointestinal health (Battcock and Azam-Ali, 1998). Some LAB strains can be used as probiotics for human and animals (Chou and Weimer, 1999). In general, LAB used as probiotic should be resistance to host gastrointestinal conditions, adhesion to host intestinal epithelium, and the prevention of growth or invasion of pathogenic bacteria, such as Salmonella spp. and Escherichia coli in the animal intestine (Chou and Weimer, 1999; Jin et al., 1996). Furthermore, certain LAB strains have been reported earlier for other health benefits, such as stimulation of the immune system of the human hosts (Schiffrin et al., 1997). Bacterial strains to be considered as probiotic should contain some essential properties, such as origin of strain, safety, acid, bile resistance, survivability during processing, and storage with beneficial effects (Saarela et al., 2000; Holzapfel and Schillinger, 2002). The growing competence in
characterizing and harnessing the potential of these minute, short-lived, health promoting microorganisms has added new dimensions to the understanding of their usefulness to humans.

Probiotics produce a variety of compounds responsible for their antimicrobial activity i.e., exopolysaccharides, organic acids and bacteriocins, etc. (Ouwehand et al., 1999). An effective probiotic should be viable and able to survive during the passage in GI tract (Casey et al., 2004; Singh et al., 2011). Probiotic bacteria should adhere and colonize on gut epithelial cells (Walker and Duffy, 1998). Bacterial Adhesion to Hydrocarbons (BATH) supports the adherence capability of bacteria to gut surfaces to enhance their interaction with the host (Kumar et al., 2012).

Prebiotics are the ‘non-digestible food’ ingredients that beneficially affect host by selectively stimulating the activity of probiotic bacteria and normal microflora residing in colon (Gibson et al., 2004). Milk of indigenous cows (also called Desi cows) has more nutritional value as compared to cross-breed cows (De et al., 2015). Therefore, the aim of this study was to investigate the comparative probiotic potential of LAB isolated from Desi and cross-breed cows and effect of prebiotics on its growth.

RESULTS AND DISCUSSION

In this study, a total of fifty two isolates were isolated from milk samples collected from Desi cows and cross-breed cows. Among them 22 were isolated from milk of Desi cows, and 30 were isolated from that of cross-breed cows. The isolates were identified as LAB that appeared white, creamish-yellow, Gram-positive and non-endospore forming. Probiotic bacteria have also been isolated earlier by Garabal et al. (2008) but we report the comparative probiotic potential of Desi and cross-breed cows milk first time. Milk is considered the most accessible and the best supplement for children and adults also. The nutritive value of milk also depends on the microbial composition and the benefits imparted by these microbes (Cromie et al., 1991; Brouillaud et al., 1997).

On the basis of the primary screening, two representative isolates from each referred source (CP-4c and CP-26c from cross-breed, while CP-12d and CP-8d from Desi/breed) were selected for further study. Primary screening for in vitro probiotic attributes revealed that the survival rate of bacterial isolates from Desi cow milk was high at low pH and in different concentration of bile as compared to that from cross-breed cow. Bacterial isolates from Desi breed were also able to tolerate the high salt concentration as compared to cross-breed cows.

![Figure 1: Auto-aggregation rate of bacterial isolates of Desi and cross-breed cow’s milk.](image)

The main in vitro selection criteria for any probiotic bacteria are acid and bile-resistance which indicate their ability to survive in GI tract (Pennacchia et al., 2004; Garabal et al., 2008). Before the entry in GI tract, these probiotic bacteria transit through the stomach where pH varied from 1.5 to 2.0. All the selected isolates were able to resist the low pH. The survival rate of isolate CP-12d and CP-8d was higher than that of CP-4c and CP-26c in acidic condition (Table 1).

![Figure 2: Antibacterial activity of selected of Desi and cross-breed cow’s milk bacterial isolates.](image)

The average bile concentration in GI tract remains around 0.3% (Gupta and Tiwari, 2014). Therefore, these probiotic bacteria must tolerate such bile concentration to survive in intestine and maintain its microflora. Isolate CP-12d and CP-
Table 1: Growth rate of lactic acid bacterial isolates at low pH

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Acid tolerance (viable counts, log cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 2.0</td>
</tr>
<tr>
<td></td>
<td>0h</td>
</tr>
<tr>
<td>CP-4^c</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>CP-26^c</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>CP-12^d</td>
<td>8.7 ± 0.4</td>
</tr>
<tr>
<td>CP-8^d</td>
<td>8.3 ± 0.3</td>
</tr>
</tbody>
</table>

Values are mean of triplicate ± standard error

Table 2: Effect of bile on lactic acid bacterial isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Bile tolerance (viable counts, log cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.50 %</td>
</tr>
<tr>
<td></td>
<td>0h</td>
</tr>
<tr>
<td>CP-4^c</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>CP-26^c</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>CP-12^d</td>
<td>9.6 ± 0.3</td>
</tr>
<tr>
<td>CP-8^d</td>
<td>10.3 ± 0.1</td>
</tr>
</tbody>
</table>

Values are mean of triplicate ± standard error

Table 3: Hydrophobicity (%) of lactic acid bacterial isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Hydrophobicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xylene</td>
</tr>
<tr>
<td>CP-4^c</td>
<td>13.5 ± 0.3</td>
</tr>
<tr>
<td>CP-26^c</td>
<td>26.2 ± 0.5</td>
</tr>
<tr>
<td>CP-12^d</td>
<td>38.3 ± 0.2</td>
</tr>
<tr>
<td>CP-8^d</td>
<td>54.6 ± 0.4</td>
</tr>
</tbody>
</table>

Values are mean of triplicate ± standard error
Table 4: Antibiogram of lactic acid bacterial isolates

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Conc. (mg)</th>
<th>CP-4&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CP-26&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CP-12&lt;sup&gt;d&lt;/sup&gt;</th>
<th>CP-8&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracyclin</td>
<td>25</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Chlormaphenicol</td>
<td>25</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>5</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Penicillin</td>
<td>1</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>5</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Meticillin</td>
<td>10</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>10</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

R- Resistance; S-Sensitive

8<sup>d</sup> grew well in presence of bile, though CP-8<sup>d</sup> displayed the highest (9.9 and 8.5 log cfu/ml) survival rate at 0.5% and 1.0% bile till 8h, respectively (Table 2). But the survival rate of bacterial isolates from cross-breed cows was not satisfactory.

BATH test explains the adhesion properties of bacterial isolates and hydrophilic and hydrophobic nature of the cell surface of bacteria (Lee et al., 2008). High adherence to xylene represents the hydrophobic nature of the bacterial cell surface. All the isolates followed the different range of adhesion with different hydrocarbons (Table 3). Isolate CP-12<sup>d</sup> and CP-8<sup>d</sup> showed highest percentage of hydrophobicity with each hydrocarbon used in this study. CP-12<sup>d</sup> and CP-8<sup>d</sup> have showed maximum (56% and 54%) percentage of hydrophobicity with n-hexane and xylene, respectively. Aggregation capability of bacterial isolates directly relates to their colonization potential in GI tract (Casena et al., 2001). Auto-aggregation rate of the isolates ranged between 35 and 65% (Fig. 1). Isolate CP-8<sup>d</sup> aggregated rapidly as compared to the other isolates followed by CP-12<sup>d</sup>. Thus LABs isolated from Desi cows showed higher adhesion and auto-aggregation rate as compared to LABs from cross-breed isolates. Isolates CP-4<sup>c</sup> and CP-26<sup>c</sup> were found sensitive to most of the antibiotics such as streptomycin, chlormaphenicol, novobiocin, erythromycin, penicillin and meticillin. CP-12<sup>d</sup> was sensitive to only novobiocin and fusidic acid, while CP-8<sup>d</sup> was resistant to all the antibiotics used in this study (Table 4). Therefore, LABs isolated from Desi cows were more resistant to antibiotics used during this study as compared to other isolates. Antibiotic susceptibility proves the safety of bacterial strain as probiotic (Herreros et al., 2005); hence CP-12<sup>d</sup> and CP-8<sup>d</sup> are safer to consume as probiotic than CP-4<sup>c</sup> and CP-26<sup>c</sup>.

Figure 3: Effect of inulin on the growth of bacterial isolates of Desi and cross-breed cow’s milk.

All the LABs exhibited antibacterial activity against the selected enteric pathogens. Similar research work has also been performed by Kos et al. (2008) to examine the inhibitory effect of some probiotic strains against food-borne pathogens. In this study, CP-8<sup>d</sup> has shown strong
antagonistic activity against each pathogen causing 10.5, 10.5, 8.5 and 9 mm zone of inhibition against *E. coli*, *K. pneumonia*, *S. aureus* and *S. typhi*, respectively (Fig 2). Antibacterial activity of LABs also increases their potential as food preservative for food industries (Gong *et al*., 2010). Both the isolates from *Desi* cow breed inhibited each pathogen efficiently which make them more suitable to treat various gastrointestinal disorders as compared to CP-4c and CP-26c.

**Figure 4:** Effect of honey on the growth of bacterial isolates of *Desi* and cross-breed cow’s milk

Inulin and honey modulates the growth of LABs by improving the quality and sensory characteristics of dairy products.

They also enhance the physical properties such as firmness and viscosity of the probiotic product (Kristo *et al*., 2003; Donkor *et al*., 2007; Oliveira *et al*., 2009). During this study, all the isolates grew well in presence of inulin as compared to honey (Fig 3 and 4). Isolate CP-12d and CP-8d showed the high growth rate in presence of different concentration of inulin as compared to other LABs isolates. Thus these isolates can be effectively used with inulin as synbiotic.

It may be concluded that the LABs isolated from *Desi* cows has high probiotic potential as compared to the cross-breed cows and they also shown satisfactory growth in presence of prebiotic.

**MATERIALS AND METHODS**

**Isolation and characterization of LAB**

Milk samples of lactating cows of both *Desi* (Tharparker, Badri, Kankrej) and cross-breed (Jersey, Holstein fries) were collected aseptically from different area of Haridwar district (Uttarakhand). Fifty two lactic bacteria were isolated from 14 milk samples by using serial dilution method. Selected dilutions of the milk samples were spread on the MRS medium and purified by following the method of De Man *et al*. (1960). The isolates were priorly identified on phenotypic traits according to the Bergey’s Manual of Determinative Bacteriology (Holt *et al*., 1994). Finally, isolates were identified, following different morphological and biochemical tests.

**Acid, Bile and NaCl tolerance**

Probiotic characteristics, such as tolerance to low pH, bile and NaCl were evaluated by following the method of Romos *et al*. (2013). The overnight grown cultures were separately harvested and suspended in 3 ml MRS broth having pH 2.0, 3.0 and 6.5, and MRS broth supplemented with 0.5 and 1.0% oxgall (Himedia) and NaCl. All the tubes were incubated at 37°C for 24 h and absorbance was measured at 600 nm using a spectrophotometer (Shimadzu, Japan).

**Antibiotic resistance**

Determination of the resistance of bacterial isolates against different antibiotics was carried out following the method described of Zonenschain *et al*. (2009). Freshly grown cultures of all isolates were separately spread on the MRS agar plates and antibiotic discs (Himedia) were placed on agar surface and incubated at 37°C for 48 h.

**Auto-aggregation assay**

All the isolates were analyzed for auto-aggregation ability qualitatively as well as quantitatively following the method of Rhaman *et al*. (2008). The overnight grown cultures were harvested by centrifugation, re-suspended in PBS and adjusted to an absorbance between 0.5 and 1.0 at 600 nm and incubated at 37°C. One ml of upper phase was removed carefully after 2 h and the absorbance was measured at 600 nm. Auto-aggregation rate was measured by using the following formula:

Auto-aggregation (%) = OD<sub>initial</sub> - OD<sub>final</sub> / OD<sub>initial</sub> × 100

**Bacterial adhesion to hydrocarbons (BATH) test**

Adhesion potency of bacterial isolates to different hydrocarbons i.e. xylene, n-hexane and n-octane was determined by the modified method of Reniero *et al*. (1992). Bacterial isolates were harvested in log phase by centrifugation at 8000 rpm at 4°C for 3-5 min. Cell pellets of isolates were washed 2-3 times in phosphate urea magnesium (PUM) buffer (pH 6.5) and absorbance of bacterial
suspension was adjusted near to 1.0 at 600 nm. Aliquots of 3 ml bacterial suspension were separately transferred into different tubes containing each hydrocarbon (1ml). The tubes containing the mixture of bacterial suspension and hydrocarbons were incubated at 37°C for 10-15 min, and further vortex for 1min. Tubes were kept undisturbed for 1 h to allow the phase separation. Therefore, aqueous phase was separated and transferred carefully into another tube, and absorbance was measured at 600 nm by using spectrophotometer (Shimadzu, Japan). Hydrophobicity was calculated by the following formula:

\[
\text{Hydrophobicity} \% = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{X 100}}
\]

**Antagonistic activity of isolates**

Antimicrobial activity of the LAB isolates against enteric pathogens viz., Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 27853, Klebsiella pneumoniae MTCC 432 and Salmonella typhi MTCC 733 (procured from IMTECH, Chandigarh, India) was determined by agar well diffusion method using Mueller Hinton agar (MHA) plates following the method of Ridwan et al. (2008). Wells of MHA plates containing pathogens were filled with suspension of LAB. Plates were incubated at 37°C for 24-48 h and the zone of inhibition was measured.

**Compatibility of isolates with prebiotics**

Compatibility of bacterial isolates with prebiotic was determined following the method of Dhewa et al. (2009) with little modification. In MRS medium, glucose was replaced with different concentration of inulin and honey. Thereafter, active bacterial culture was inoculated in the modified MRS medium and viable cells were enumerated at different time intervals as earlier. The medium containing glucose as sole energy source acted as control.

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