Screening of Raw Buffalo’s Milk from Karnataka for Potential Probiotic Strains

Nannu Shafakatullah* and M. Chandra
Dept. of Bio-Sciences, Mangalore University, Mangalagangothri-574199, Mangalore, Karnataka, INDIA

Available online at: www.isca.in, www.isca.me
Received 21st June 2014, revised 20th July 2014, accepted 15th August 2014

Abstract
Buffalo's milk was cultured with appropriate dilution on MRS media for the isolation of potential probiotics and pure cultures were obtained by sub-culturing. Purification of cultures were confirmed by Gram’s staining and catalase test and identified based on morphological, cultural, physiological and different biochemical characteristics as presented in Bergey’s Manual of Systematic Bacteriology. During lactic acid bacterial (LAB) transit through the gastrointestinal tract, ingested microorganisms were exposed to successive stress factors, including low pH in the human stomach and bile salt. These isolates were examined for survival in bile salt, acidic pH, different NaCl concentrations, anti pathogenic activity, as well as survival at different storage temperatures. These stress factors can be used as criteria for the evaluation of probiotic strains. Isolated strains of Lactobacilli spp. and Bifidobacterium spp. showed satisfactory probiotic potentials.

Keywords: Bifidobacterium spp., buffalo’s milk, lactic acid bacteria, Lactobacilli spp., probiotics.

Introduction
The history of consuming fermented milk and foods containing live microbes for maintaining good health and restoring healthy intestinal balance is thousands of years old. Historically it is known that the association of lactic acid bacteria with milk and milk products is linked with the good health of human beings. Hundred years ago, Elie Metchnikoff (a Russian scientist, Nobel laureate, and professor at the Pasteur Institute in Paris) stated that lactic acid bacteria offer health benefits which are capable of promoting long life. In the recent years Probiotic based products have gained a lot of attention due to their health promoting prospects.

Intestinal pathogens are effectively controlled by organic acids and proteins like substance produced by the probiotics, competition for nutrients and attachment sites on intestinal mucosa, altered enzyme activity, increased antibody levels and increased macrophage activity. Certain probiotic bacteria produce inhibitory compounds called Bacteriocin, which is antagonistic to various degrees against intestinal pathogens and also has antitumour and anticholesterol activity. The transition of LAB in the GI tract is helpful in delivering enzymes and other substances into the intestine which play an important role in monitoring intestinal microbiota. The lactic acid bacteria also got anti oxidative activity.

All Lactobacillus strains which are being used as probiotic agents do not possesses all of the necessary properties that will make it a potential probiotic. It is necessary to choose a strain that possesses essential characteristics that help them to survive and establish under various intestinal environmental conditions. Lack of pathogenicity, tolerance to GI environment, ability to colonize at the gastrointestinal mucosa and competition with pathogens are some of the important criteria that have been used for the selection of probiotics.

Material and Methods
Isolation of Bacteria: Three fresh samples of buffalo milk were collected from local vendors in sterile containers and used within 24 hrs. 1ml of each sample was serially diluted to 10^-5, 10^-6 using sterile saline (0.85% NaCl), and 0.1ml was spread on to sterile de-Mann, Rogosa and Sharpe (MRS) agar plates. The plates were incubated at 37°C for 48 hours an-aerobically. Morphologically distinct and well isolated colonies were picked and transferred to new MRS agar plates by streaking. Finally, pure colonies were obtained. Only catalase -ve and gram +ve colonies were selected and inoculated on fresh media for further identification. The isolates were maintained in MRS broth, stock cultures were stored on agar slants in refrigerator.

Morphology: The colony characteristics (size, shape, margin and colour) on solid medium, growth pattern in broth and agar slants was recorded. Motility, Indole test, Spore formation, type and arrangement of cells, Starch Hydrolysis, Arginine hydrolysis has been studied.

Growth curve: It was important to ascertain the growth time of the isolates in order to determine the multiplication time of the organisms. After 1% of the activated LAB had been inoculated into the MRS broth, the growth rates were assessed at 0, 8, 16, 24, 32, 40, 48, 56, 60 and 80h by taking the OD at 660nm.

Bile Tolerance: Bile plays an important role in the survival of bacteria in the small intestine. Food remains in the small
Antimicrobial Activity Test: Agar well diffusion method was used to determine the inhibitory capacity of the isolated LAB against pathogenic strains such as Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus and Bacillus subtilis. The isolates and pathogenic strains were incubated in MRS agar medium at 37°C for 24 to 48 h.

Results and Discussion

Physiological and Biochemical Characterization: After Gram staining and catalase test only Gram positive and catalase negative isolates were selected for further identification. The isolates BM1, BM2 and BM3 were Gram positive and catalase negative. Inoculated tubes containing Durham’s tubes were observed for 5 days for gas production from glucose. Isolate BM1 showed gas production while BM2 and BM3 showed no gas production. Ability to grow at different temperatures is also used for the identification of the isolates. After 5 days observation it has been found that all of the isolates grown well at 15-45 °C. The isolates BM2 and BM3 have shown growth at 50 °C, however they cannot grow at 10°C. Growth at different NaCl concentrations was observed. All of the isolates have shown good growth at 2-6% NaCl concentration. Isolate BM1 and BM2 shown growth at 8.0% NaCl. Arginine hydrolysis by the isolates is another criterion used to identify them. The bright orange colour indicated the positive arginine hydrolysis where as the yellow colour indicated negative test. All isolate shown -ve for Arginine hydrolysis. Hydrolysis of starch was negative in all isolates. All of the isolates were non motile, non spore forming. The carbohydrate fermentation test is the most useful test for the identification of different strains. Seventeen (other than glucose) different carbohydrates were used for identification. They gave different fermentation patterns which are shown in table 3. Cultural, morphological, physiological and biochemical characteristics showed that the following genera and species of LAB and probiotic bacteria were present in the Buffalo’s milk examined. They are BM1- Lactobacillus acidophilus, BM2- Lactobacillus rhamnosus and BM3- Bifidobacterium longum.

Growth curve: During incubation of isolates the viable cell count was monitored. The results revealed that Lactobacillus acidophilus and Lactobacillus rhamnosus achieved stationary phase at 48 hours (figure 3), whereas Bifidobacterium longum was in log phase until 80 hours of incubation.

Resistance to Low pH: The isolates BM1 and BM2 have shown survival at pH 2 till 3 hours. Isolates BM3 has shown survival at 1.5 hours at pH 2, but all organisms died at pH 2 at 3rd hour.

Bile Tolerance: All the strains were screened for their ability to survive at different bile salt concentration. Strains were inoculated in 0.3%, 0.5% and 1.0% bile salt and allowed to grow till 3 hours. From the results it has been found that, all the isolates were resistant to 0.3% and 0.5% bile salt. Isolate BM1
was more tolerant than BM2 and BM3. Whereas all the isolates shown sudden death at 1.0% of bile.

**Anti Bacterial Activities:** Antimicrobial activity helps to select the potential probiotics strains. Antimicrobial activity usually targets the intestinal pathogens\(^5\). The isolates were examined for their antibacterial activity. The isolated strains were grown with indicator microorganisms, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus* and *Bacillus subtilis*. The antibacterial effect on the indicator microorganisms was determined by diameter of inhibition zones. *Lactobacilli acidophilus* have a high ability to inhibit pathogenic growth and multiplication through competition with other pathogenic microorganisms for nutritional requirements\(^6-18\). But *Bacillus subtilis* has shown resistance to *L. acidophilus*. *Klebsiella pneumonia* has shown resistance to *L. Rhamnosus*. All test pathogens shown 100% sensitivity to *B. Longum*, whereas *B. Subtilis* shown lesser sensitivity.

### Table-1

**Morphological, cultural and physiological characteristics of the isolates**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Isolate No.</th>
<th>Catalase test</th>
<th>Size(mm)</th>
<th>Shape</th>
<th>Margin</th>
<th>Gram’s staining</th>
<th>Motility</th>
<th>Spore formation</th>
<th>Arginine utilization</th>
<th>Starch Hydrolysis</th>
<th>Growth in broth</th>
<th>Growth on slants</th>
<th>Nacl-2%</th>
<th>NaCl-4%</th>
<th>NaCl-6%</th>
<th>NaCl-8%</th>
<th>NaCl-10%</th>
<th>Indole test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BM1</td>
<td>-</td>
<td>&lt;1</td>
<td>Circular</td>
<td>Entire</td>
<td>+ Rods pairs/ chains</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>Beaded</td>
<td>Beaded</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>BM2</td>
<td>-</td>
<td>1</td>
<td>Circular</td>
<td>Entire</td>
<td>+ Rods</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>Smooth</td>
<td>Smooth</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BM3</td>
<td>-</td>
<td>&lt;1</td>
<td>Circular</td>
<td>Entire</td>
<td>+ Small rods</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>Beaded</td>
<td>Beaded</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

### Table-2

**Physiological characteristics of the isolates**

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Isolate No.</th>
<th>Growth at different temperature (°C)</th>
<th>Growth at different pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15 30 37 45 50 55 60 2 3 4 5 6 7 8 9</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>BM1</td>
<td>++ +++ +++ +++ +++ + - - + + +++ +++ +++ ++ ++</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>BM2</td>
<td>++ +++ +++ +++ +++ + - - + + +++ +++ +++ + +</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BM3</td>
<td>++ +++ +++ +++ +++ - - - + + +++ +++ +++ + +</td>
<td></td>
</tr>
</tbody>
</table>

(++) Luxurious growth, (++) Moderate growth, (+) less growth, (-) No growth

### Table-3

**Biochemical characteristics of the isolates by utilization of carbon sources**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Isolate No.</th>
<th>Fructose</th>
<th>Galactose</th>
<th>Cellulose</th>
<th>Esculin</th>
<th>Imulin</th>
<th>Rhamnose</th>
<th>Melibiose</th>
<th>Mannitol</th>
<th>Maltose</th>
<th>Mannose</th>
<th>Ribose</th>
<th>Trehalose</th>
<th>Arabinose</th>
<th>Lactose</th>
<th>Sucrose</th>
<th>Xylose</th>
<th>Salicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BM1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>BM2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>BM3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Positive reaction (+), negative reaction (-)
Effect of different concentration of bile salt on the growth of the isolates

Table 4
Antibacterial susceptibility of the isolates

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>L. acidophilus</th>
<th>L. Rhamnosus</th>
<th>Bifidobacterium longum</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>100% S</td>
<td>100% S</td>
<td>100% S</td>
</tr>
<tr>
<td>E. coli</td>
<td>100% S</td>
<td>100% S</td>
<td>100% S</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>100% S</td>
<td>R</td>
<td>100% S</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>100% S</td>
<td>100% S</td>
<td>100% S</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>R</td>
<td>100% S</td>
<td>75% S</td>
</tr>
</tbody>
</table>

Conclusion
The present research revealed the presence of Lactobacillus acidophilus, Lactobacillus rhamnosus and Bifidobacterium longum in the buffalo’s milk. All these isolates are potential probiotics strains. Their acid, bile, and alkaline stability will allow them to survive in the stomach and proliferate in the intestine. This will help strains to reach the small intestine and colon and contributing to the balance of intestinal microflora. All the strains also possessed high antibacterial activity, thus might potentially help to alleviate diarrhoea and other intestinal infections. Additional experiments concerning the adhesive capability and the role of these strains in the prevention and cure of colon cancer is in progress.
Figure-2
Effect of acidic pH on the growth of the isolates

Figure-3
Growth Pattern of the isolates

A. Lactobacillus acidophilus, B. L. Rhamnosus, C. Bifidobacterium longum
Acknowledgement

The authors are thankful to Indian Council of Medical Research (ICMR) for financial assistance. No. 3/1/2/40/Nut./2013 dated 20.11.2013

References

2. Reuter G., Probiotics—Possibilities and limitations of their application in food, animal feed, and in pharmaceutical preparations for men and animals. *Berl Munch Tierarztl Wochenschr*, 114(11-12), 410-9 (2001)