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Isolation and characterization of probiotic microorganism from milk and milk product

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Abstract
This study was envisaged to analyz the probiotic properties of isolated lactic acid bacteria from curd and milk sample. Cow's milk and curd sample was cultured on MRS Agar media with appropriate dilution for the isolation of potential probiotics strains and pure cultures were obtained by continuous sub-culturing. Identication of lactic acid bacteria was done by Gram's staining and catalase test and further confirmation was based on morphological, cultural, physiological and different biochemical tests. A total four isolates viz. *Lactobacillus fermentum,* L. *casei,* L. *acidophilus and Bifidobacterium longum* were identified after different biochemical analysis which also exhibited reliable probiotic properties. These isolates were examined for probiotic properties including tolerance to different concentrations of bile salt, pH and NaCl. Selected isolates from curd and cow's milk sample fulfilled the most common criteria of probiotic bacteria, so numerous probiotic products can be developed from cow's milk which will enhance further research on therapeutic use of probiotics.

Keywords: milk, probiotics, *Lactobacillus acidophilus* and *Bifidobacterium* sp

Introduction
There are several species of microorganisms neighboring us in which some are beneficial and some are harmful to our health. The word 'probiotic' comes from Greek word to describe substance secreted by one microorganism that stimulates the growth of another, first used by Lilley and Stillwell [1]. The history of probiotics began with the history of man by consuming fermented foods that is well known in Greeks and Romans [2, 3]. The small and large intestines naturally encompass bacteria, often referred to as our 'normal flora'. Our normal flora contains more than 400 species of bacteria that endow with many beneficial functions [4]. So Probiotics can be simply defined as naturally occurring microorganisms consumed as a food component or dietary supplement that ensures good health. A large amount of probiotic bacteria is present in milk or milk products which are mainly lactic acid bacteria (LAB). Lactic acid bacteria (LAB) including *Lactobacillus* spp. are Generally Recognized as Safe (GRAS) bacteria that have been used in the processing of fermented food for centuries [5]. LAB can ferment different carbohydrate and generate lactic acid that helps in decreasing pH and in addition lactic acid production has beneficial effect on formation of texture, aroma and flavor in different milk products [6]. Most probiotic bacteria belong to the group of lactic acid bacteria (LAB) and among them Lactobacilli and Bifidobacteria apparently play a significant role in sustaining the intestinal bionetwork and in stimulating the immune system of the host [7]. Development of various probiotic products, such as fermented milk drinks, yoghurt, cheese, ice-cream etc. with defined probiotic culture would be able to confer health benefits. The main objectives of the study was isolation and characterization of lactic acid bacteria from milk and curd sample through different biochemical tests and through analysis of probiotic properties of those isolates.

Materials and Methods
Sample collection
Two milk samples and two curd sample were brought from different places in Coimbatore. After collection, the samples were stored aseptically at 4°C in refrigerator to defend from deterioration and contamination. Sample was collected in autoclaved plastic vials. At each time of collection, precaution was taken to prevent or avoid cross contamination.

Isolation lactic acid bacteria (LAB)

One gram of sample was dissolved in 9 ml of 0.15% buffered peptone water solution and diluted up to ten logarithmic (10^{-10}) fold. The diluted sample was then inoculated into the Lactobacillus MRS Agar plate by ensuring the criteria of pH 6.5, incubation temperature of 37 °C and incubation time of 48 hr. After incubation single colony was obtained by streaking. Well isolated bacterial strains were picked up and stored in MRS broth for further studies.

Bacterial characterization

Morphological characterization

Bacterial colonies were further purified by sub culturing continually on MRS agar media, and the colony morphologies (color, shape and size) were examined in nude eye, however microscopic observation was needed to separate one colony to another. According to the protocol of Erkus, gram staining was done with some modifications [8]. At first single colony was taken aseptically then smeared on to a clean dry slide and heat-fixed. Heat fixed smear was flooded with crystal violet solution for 30 sec and rinsed with water for 5 sec. Then iodine solution was used to cover over the slide for 1minute and rinsed with tap water for 5 sec. The slide was then decolorized with 95% ethanol for 15 to 30 second and rinsed for 5 second. Finally safranin was used as counter stains for 60-80 seconds and rinsed with water, and were examined under light microscope.

Catalase Test

Slide method was used to perform catalase test. In this method a clean glass slide was divided into two sections with lubricant pencil, one was labeled as test and the other as control. A small drop of normal saline on each area was placed with a sterilized and cooled inoculating loop and a small amount of the culture from the petri plate was picked up. One or two colonies were emulsified on each drop to make a level suspension. One drop of hydrogen peroxide was spread over the test smear and the other drop on control part. The fluid over the smears was observed for the appearance of gas bubbles.

Probiotic properties analysis

Determination of Sugar Fermentation

Sugar fermentation tests were done according to modifed protocol of Erkus [8]. Five different sugars; Glucose, Fructose, Sucrose, Maltose and Lactose were used for sugar fermentation assay. At first every sugar was dissolved in deionized water at a final concentration of 5% (w/v), then sterilization of sugar solutions were done by microfiltration with 0.22 µm pore diameter. MRS broth (pH 6.5) was taken into screw cap test tube and phenol red (0.01 g/L) was added into the tube as a pH indicator. Inverted Durham's tubes were placed and the medium was autoclaved at 121 °C for 15 min. 1 ml of different sugar solutions were inoculated into different tubes and 200 µl overnight liquid cultures were inoculated into the broth medium. Incubation was performed anaerobically at 37 °C for 24 h. Sugar fermentation was observed, as the acid production changes the color of medium from its original to yellow and formation of gas in test tubes.

Assay for NaCl Tolerance

For the determination of NaCl tolerance of isolated LAB, test tubes containing MRS broth were adjusted with different concentrations (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% fresh overnight culture and incubated at 37 °C for 24 h. After 24 h of incubation their growth were determined by observing their turbidity [9].

Bile salt tolerance test

The isolated lactic acid bacteria from selected samples were assayed using the protocol by Zinedine and Faid with some modification [10]. MRS broths with different concentrations (0.05%, 0.15% and 0.3%) of bile oxgall (Sigma Laboratories, UK) were used to find out the tolerance and growth rate of isolated culture.

Survivability in acid condition

Final pH of the medium was adjusted to different pH levels viz., 2.0, 3.0, 4.0, and 6.8 and autoclaved at 121 °C. Then 1% overnight culture of isolated LABs were inoculated into the MRSO (MRS-Oxgall) broth medium and incubated at 37°C for twenty four hours under anaerobic condition. The survival rates of the isolates were measured by taking absorbance at 620 nanometer of the MRSO with bacterial culture by spectrophotometer.

Results

Growth morphology

A total four probiotic strains were isolated named as CLB1, CLB2 CLB3 and CLB4 that formed round, creamy white colonies on MRS agar plate. Morphological, cultural, physiological and biochemical characteristics of the isolates were examined for their identification and further experiments. Among four isolates all were gram positive but CLB1, CLB2 revealed rod shaped while CLB3 and CLB4 were cocci shaped.

Table 1: Colony characteristic of isolated cultures.

S.no	Isolates	Gram reaction	Shape	Size	Colony character
1.	CLB1	+	Long rods	Small	Circular, dry appearance, entire margin, white colonies.
2.	CLB2	+	Small rods	Small	Circular, cream colonies, erupted margin cream colonies.
3.	CLB3	+	Long rod in chain	Medium	Raised, slimy, white colonies.
4.	CLB4	+	Small rod in chain	Small	Circular, submerged colonies.

Biochemical characterization

Four isolates were tested for catalase test in which all of them reveled negative result. Sugar fermentation test was performed to consider their capability to ferment different sugars. In this experiment five sugars maltose, sucrose, fructose, lactose and glucose were used. The results of sugar fermentation test are described in Table 2 that confirmed all the isolates were able to ferment given sugars.

Table 2: Catalase and Sugar Fermentation Test of Isolates.

Isolates	catalase	glucose	fructose	sucrose	maltose	lactose
CLB1	-	++	++	+	+/-	++
CLB2	-	++	++	+	+/-	++
CLB3	-	++	+/-	+	+/-	++
CLB4	-	++	+/-	+	+/-	++

Probiotic properties analysis

Nacl and pH Test

In this study all the isolated probiotic cultures were able to grow at 1-6% NaCl concentration, whereas fair growth was observed at 7% concentration but completely failed at 8-10% NaCl concentration (Table 3). The isolated probiotic were screened for their ability to endure different pH environment and it is elucidated in figure 1.

Table 3: Survivability of isolates in different Nacl concentration.

NaCl concentration	CLB1	CLB2	CLB3	CLB4
1%	+	+	+	+
2%	+	+	+	+
3%	+	+	+	+
4%	+	+	+	+
5%	+	+	+	+
6%	+	+	+	+
7%	+\-	+	+/-	+
8%	_	_	_	_
9%	_	_	_	_
10%	_	_	_	_

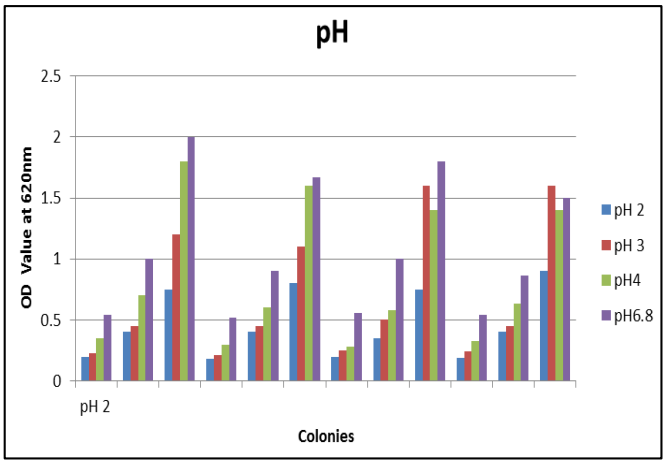

Fig 1: Survivability test in different pH.

Bile salt tolerance test

The isolates were screened for their capacity to tolerate bile salt, although the bile concentration of the human gastro intestinal tract varies. Experimented isolates was able to grow at 0.05%, 0.15% and 0.50% of bile salt concentrations. The optical density was measured by spectrophotometer after 2 hr, 4 hr and 24 hr intermission. Their ability to tolerate different concentrations of bile salt in different time interval illustrated in Figure 2.

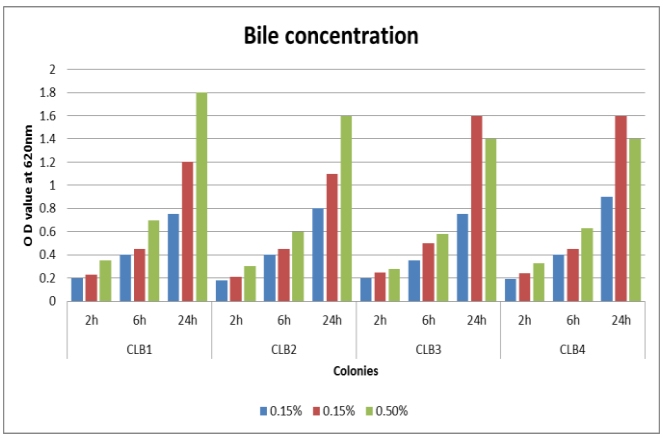

Fig 2: Survivability test in different bile salt concentration.

Discussion

The study was designed for characterization and determination of probiotic properties of some lactic acid bacteria (LAB) isolated from milk and curd samples. MRS agar media was used for cultivation of lactic acid bacteria (LAB). After growing the isolates on MRS agar media they were subcultured and colonies were selected for physical and biochemical characterization. After which four isolates were selected for determination of probiotic properties. Selected isolates were gram positive which was confirmed by gram staining and catalase negative.

L. fermentum and *L. casei* were rod shaped, gram positive, facultative anaerobic, non-endospore forming, non-motile and catalase negative bacteria that occurred in chains, pairs or singly [6, 12]. In this study a similar result was observed for CLB1 and CLB2 isolates after morphological and biochemical study. *Bifidobacterium longum* and *L. acidophilus* isolated from milk was found to be coccus shaped, gram positive, facultative anaerobic, non-endospore forming, non-motile, catalase negative that occurs in chains, pairs or singly [13, 14]. In this study similar result was observed for CLB3 and CLB4 isolate after morphological and biochemical study.

The isolated probiotic bacteria were capable to grow optimally at 37 °C. Pundir *et al*. isolated lactic acid bacteria from fermented foods which were able to grow at 25, 37 and 40 °C [15]. All probiotic isolates were capable to grow at 1-6% NaCl concentration but failed at 7-10% NaCl concentration. Lactobacillus strains from river buffalo milk cheese [16], which as well found to tolerate 1-7% NaCl. The bile concentration of the human gastrointestinal territory varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the residing time is suggested to be 4 h [17]. According to our findings, every probiotic isolates were able to grow up in 0.05 to 0.3% bile salt concentrations. Biochemical description of probiotic strains revealed that resistant and tolerance to bile salts was not dependent on species; however, they are different among the strains of the same species as reported by additional researchers [18-20]. Resistance to bile salt toxicity is one of the criteria used to select probiotic strains that would be potentially capable of performing effectively in human gastrointestinal tract [21]. From all the conducted experiments it was observed that, isolated lactic acid bacteria could be used as an excellent probiotics and finally for probiotic product development.

Conclusion

Recently it has been discovered that probiotics have detect on anticancer agent. Therefore, some future studies should be performed to use the seisolates reliably including molecular techniques like 16S rRNA sequencing or Maldi-TOff for accurate identification of lactic acid bacterial species and multiplex RAPD-PCR technique could be used to reveal the complete metabolic potential of each of the probiotic strain which opens future research works to study for better efficacy and advancement of food biotechnological research in the food and dairy industries.

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