Identification and Characterization of Probiotics from New Sources

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Abstract: *Background: Probiotics are live microbial feed supplements, which positively affect the host animal by improving its intestinal microbial balance. Studies have shown probiotic activities of Lactobacillus isolated from dairy foods, which include the ability to inhibit the growth of other bacteria and the reduction of cholesterol. However, there is limited documented work on the probiotic activity. Natural foods are always accepted in an Indian context than any pharmaceutical preparation hence this study contributes to the repertoire of natural food for health, and can be formulated for consumption of humans. Hence, probiotic organisms are produced on a large scale and the cells are dried and employed as capsules that can reestablish the lost normal enteric flora, improving the microbial balance. Aim: A simple and reliable method for the identification of the beneficial strains has become important because of its increasing applications. This work was aimed at isolating and characterizing the lactobacillus strain that can be directly taken in diet. Results: In this study we could isolate and purify the culture of lactobacillus strains from cow's milk and camel milk- a new source in search for probiotics which may reduce the cholesterol levels. Then we characterized it morphologically and biochemically at the species level. Attributes of the Probiotics were also studied. Antibiotic sensitivity test was done for different isolated cultures. Crude samples were subjected to ion exchange chromatography by using a CMC exchanger. Estimation of the Protein was determined by Lowry's method. Conclusion: It is concluded that the new source of probiotics could be camel milk as it has all the attributes of probiotics, as well as cholesterol lowering capacity.*

Keywords: *Lactobacillus acidophilus* ATCC 43121, Probiotics, Cholesterol, Antibiotic Resistance, Camel milk

1. Introduction

Probiotics are commonly defined as living microorganisms which, when administered in adequate amounts, confer health benefit to the host. Probiotics are demonstrated that probiotics can prevent pathogen colonization of the gut and reduce the incidence or relieve the symptoms of various diseases caused by dysregulated immune responses. Probiotics seem to function by influencing both intestinal epithelial and immune cells of the gut, but the details of these effects are still being unravelled. Therefore, probiotics, through their effects on the host immune system, might ameliorate diseases triggered by disordered immune responses. Caveats remain and, because the beneficial effects of probiotics can vary between strains, the selection of the most suitable ones will be crucial for their use in the prevention or treatment of specific diseases [1]

In order to exert their functional properties, probiotics need to be delivered to the desired sites in an active and viable form. The viability and activity of probiotics in the products have been frequently cited as a prerequisite for achieving numerous beneficial health benefits [2].

Lactic acid bacteria make up an extremely important group of probiotic bacteria and are already used in many probiotic dairy products [3]. This group of bacteria is non-pathogenic, acid resistant; bile tolerant and produce antimicrobial substances, including, organic acids and hydrogen peroxide and bacteriocins (biologically active proteins) [4]

Today, there is a growing need for new strains of lactic acid bacteria that carry the probiotic traits mentioned above and with favorable health effects on human and animals. This can be obtained from other natural ecological niches which remain unexploited. Camel's milk is much more nutritious than that from a cow. It is lower in fat and lactose, and higher in potassium, iron and Vitamin C. It is normally drunk fresh, and the warm frothy liquid, heavy and sweet, is usually an acquired taste for the Western palate.

Camel milk is used therapeutically against dropsy, jaundice, problems of the spleen, tuberculosis, asthma, anaemia, and piles .Patients with chronic hepatitis had improved liver function after being treated with camel milk. The camel milk works also as a laxative on people unaccustomed to drinking this milk. The numerous nutrients in camel milk help promote our body natural defenses [5].Camel milk has anti-tumor properties. The presence of high-level of insulin-like protein in camel milk (about 52 micro unit/ml) helps reduce the effect of diabetes. Camel milk has antiviral properties that help reduce liver inflammation. The milk also has many nutrients that are required for healthy liver function. It is rich in immunoglobulin that will strengthen the body's autoimmune system. This will help treat and heal autism.

In the present study, Camel milk was brought from a place in Hyderabad, where camels are being maintained for commercial activities such as camel rides and/or selling milk for medicinal purposes. *Lactobacillus* strains were isolated from camel milk and were investigated for various biochemical tests, acidic pH values, and Antibiotic resistance as well as for bacteriocin production. Our goal is the selection of potential probiotic strains from camel milk. [6]

2. Materials and Methods

2.1 Bacterial strains and media

Camel milk was collected from Moosarambagh a place in Hyderabad whereas the Cow milk was collected from a farm

in Hyderabad. The samples were taken for isolating the bacterial strains by Serial dilution method. Serial dilution is an easier method to where we can spread bacteria over a wide area on the agar petriplates containing the media and count the number of colonies that grow. The purpose of serial dilution is to determine the number of bacteria per unit volume in the original culture. When the bacteria are spread out enough, each bacterial cell should produce a single colony. MRS (DeMan, Rogosa and Sharpe Agar) media was used for the isolation and enumeration of *lactobacillus* species. This medium was designed to favour the luxuriant growth of *Lactobacilli* for lab study.

Serial dilution was done by dissolving 1 ml sample (camel milk/cow milk) into 5 ml of distilled water. It was mixed properly. From the stock solution prepared, 1 ml was transferred in 4 ml of distilled water containing different concentration of test tubes (10^{-1}, 10^{-2}, 10^{-3}… 10^{-10}).Each dilution was plated onto MRS agar plate aseptically.All plates were then incubated at 37˚C for 3 days.

2.1.1 Motility test
Hanging drop method was done and the slide was observed under light microscope under 40X for motility.

2.2 Identification of the Bacterial Strains

The tentative LAB isolates grown onto MRS agar were randomly selected and inoculated onto MRS slant agar for biochemical tests. The tentative LAB isolates were examined for their morphology cells and colonies, Gram test, catalase, CO2 production from glucose, ability to grow at temperature of 15°C and 45°C, sugar fermentations and carbohydrate fermentation profiles. The growth of bacterial strains at 35°c, 40°c and 45°c was visually confirmed by the changes in turbidity of MRS broth after 24, 48 and 72h of incubation. For evaluation of citrate utilization, MR-VP agars were used.MRS broth containing inverted Durham's tubes was used for evaluation of gas production was done in MRS agar. Based on Bergey's Systemic Determinative Classification of Bacteria the above tests were performed. For checking the attributes of probiotics the following tests like bile salt tolerance test, and acid tolerance test were performed.

2.3 Attributes of Probiotics

2.3.1 Growth at Different P^H.
A single isolated colony was subcultured in MRS agar adjusted to different P^H by adding NaOH (1.0M) and HCl(1.0M) and incubated at 37 degrees for 24hrs to observe the ability of the growth of *L.acidophilus* under P^H Values.

2.3.2 Acid Tolerance
2ml of supernatant of the two strains from each source were centrifuged for 10min at 10,000rpm and the suspension containing the cellular sediment was re-suspended again in 2ml of MRS broth adjusted with Hydrochloric acid to P^H values of 2.0, 3.0 and 4.0 respectively. The cultures were incubated at 37 degrees for 24hrs.1ml was taken at 0hrs and 3hrs and viable colonies in each sample was determined at 540nm. Simultaneously a control at optimal growth p^H was used.

2.3.3 Bile Salt Tolerance test
In order to determine the bile resistance, 2ml of supernatant of the two strains from each source were centrifuged for 10min at 10,000rpm and the suspension containing the cellular sediment was re-suspended again in 2ml of MRS broth with 0.2% and 0.4% of Bile salts. Broth was poured into three tubes. The control were incubated in MRS broth without bile salts and other tubes contains 0.2% and 0.4% bile salts respectively kept for incubation and OD was taken at 540nm.

2.3.4 Cholesterol Reduction Assay
Cholesterol measurement was done by the method described by Searcy and Bergusst (1960) .The Lactobacillus species were grown in a media containing MRS broth supplemented with 0.3% bile salts. 10mg of cholesterol was dissolved in 500 µL of ethanol was added to 100ml of MRS broth with bile salt. The cultures were grown for 24 hrs at 37 degrees. Cells were harvested by Centrifugation at 8,000rpm for 10min at 4°c.The spent broth was collected and used for assaying of cholesterol. The broth which is uninoculated is used as control. To the 1ml of spent broth 3ml of 95% Ethyl alcohol was added followed by 2ml of Potassium hydroxide was added and the contents were mixed after addition of each component. Then the tubes were heated for 10min at 60 degrees. The tubes were heated for ten minutes at 60degree in water bath, after cooling 5ml of Hexane was dispensed to all tubes and vortexed for 5 min. Then 3ml of water was added and mixed thoroughly. Tubes were allowed to stand for 20min at 30 degrees to permit the phase separation.2.5ml of hexane layer was transferred to a fresh tube and it was allowed to dry completely.1.5ml Ferric chloride reagent was added and was allowed to stand for 10min. one ml of concentrated sulphuric acid was added from the sides of the test tubes. The mixture was vortexed and allowed to stand for 45min at 30 degrees. The optical density was measured at 540nm in UV spectrophotometer (Lambda Scientific, Australia) .The concentration of cholesterol was calculated by using the Cholesterol Assimilation formula.

$$\text{Assimilation (\%)} = \frac{\text{Conc. of cholesterol in control} - \text{Conc. of cholesterol in sample}}{\text{Conc. of cholesterol in control}}$$

2.4 Screening of Lactic acid bacteria for Antimicrobial Activity

Disk Diffusion method proposed by Bauer Et al was followed for Antibiotic susceptibility test. Each strain was inoculated into MRS broth, which was Plates were made with Nutrient agar media and allowed to solidify. By spread plate technique the cultures were inoculated in the plates using sterile swab. The antibiotic disks of Chloramphenicol, Penicillin, Vancomycin, Neomycin, Azithromycin, (Himedia) were placed in the plates. Agar plates with Antibiotic disks were then incubated for 24h.The diameter of the inhibition zone was measured by ruler. The results were expressed as Sensitive(S) and Resistance(R).

3. Results and Discussion

In the present study the isolated strains of probiotics were identified based on Bergey's Manual of Determinative Bacteriology. The isolated bacteria were observed by light microscope. The bacteria were gram positive (Fig.1) rod shaped. The gram staining results indicated that the isolated bacteria could be identified as *Lactobacilli*. [7] Hanging drop method showed that the bacteria were non-motile and the non-motile behavior is a characteristic of *Lactobacillus acidophilus*. [8] In performing the Catalase test no bubble was observed. (Fig.2) It is well known that *lactobacillus acidophilus* is Catalase negative. [9, 10] The carbohydrate fermentation test was to investigate the ability of bacteria to ferment different types of carbohydrates. Table-1 shows that the isolated bacteria could ferment Lactose, Maltose, Glucose and sucrose, thus the results obtained coincided with *L.acidophilus* strain characteristic. (Fig.3) [11, 12]

Table 1: Biochemical (Fermentation) results

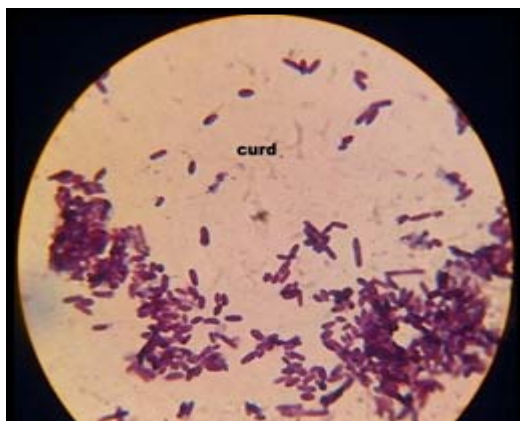| Test	Results
Lactose	Fermentation (acid production)
Maltose	Fermentation (acid production)
Glucose	Fermentation (acid production)
Sucrose	Fermentation (acid production)

Figure 1: Shows Gram positive bacteria (Camel Milk**)**

Figure 2: Shows Catalase negative result (No bubbles)

Figure 3: Shows Carbohydrate Fermentation results

Table 2: Effects of PH & Effect of different Concentration of bile salts on the growth of L.acidophilus

P^H	Result
3	-
4	++
5	+++
6	+++
7	++
8	-
9	-
Bile Salt Concentration (%)	Bile Tolerance (O.D at 540nm)
0	100
0.1	90.1
0.3	75.5
0.5	69.5
1	54

(-) No growth, (+) slightly growth, (++) good growth, (+++) very good growth.

3.1 Bile salt Tolerance

The bacteria which are to be used as probioitcs should be able to resist inhibitory factors in the GI tract such as bile salts. [13], for this reason the effect of different Concentration of bile salts were used to check the growth of L.acidophilus , results are represented in (Table:2).It showed that the viable count of the bacteria decreased with an increase of bile salt concentration when compared to the control. The ability of L.acidophilus to resist bile salts was studied by other researchers. [14, 15]. Tolerance to bile salts is a prerequisite for colonisation and metabolic activity of bacteria in the small intestine of the host (Havenaar et al., 1992).This will help LAB to reach the small intestine and colon and contribute in balancing the intestinal microflora (Tambekar and Bhutada, 2010).

3.2 Acid Tolerance

Before reaching the intestinal tract, probiotic bacteria must first survive the acidity of the stomach. The PH used were ranging from 3.0 to 9.0, taking into account that stomach acidity varies from person to person, either naturally or due to several factors (Dunne et al., 2001; Goldin et al., 1992). The results showed that resistance to low pH is strain dependent. The strains tested were resistant to pH 4.0, 5.0, 6.0 & 7.0 (Table: 2)

Table 3: Cholesterol Assimilation by the isolates

Cholesterol Reduction (%)	Isolate
Greater than 70%	++

Some of the strains were capable of reducing the cholesterol levels naturally and shows anti cholesterol activity.Table:3 shows the results of Cholesterol reduction by isolates from camel milk in the presence of bile salts.

3.3 Screening of Lactic acid bacteria for Antimicrobial Activity

Table 5: Results showing the Resistance or Senitivity of the strain towards the Antibiotics

Antibiotics	*Result*
Vancomycin	R
Penicillin	R
Chloramphenicol	S
Neomycin	R
Azithral	S

4. Discussion

One of the main criteria to be fulfilled by a Probiotic organism is it should be non-pathogenic. (Dubois et al., 1956; Ljungh and Wadstrom, 2006). Elevated level of certain blood lipids are a greater risk of cardiovascular disease. A few research reports describe the use of *L.acidophilus* to decrease the serum cholesterol levels in human and Animals. (Lee et al., 1992). As per studies by Hyeong –Jun et al.(2004) *Streptococcus* HJS-1, *Lactobacillus* HLJ-37 and *Bifidobacterium* HJB-4 had the best Hypercholesterolemic activity. (57%. 64.4% & 58.8% respectively) in MRS broth with soluble cholesterol containing 0.3% Oxgall.

There are reports that lactic acid bacteria can reduce the serum cholesterol level up to 50% in presence of bile salts in 48hrs (Guslandi et al.,2003). In the present study the strain *L.acidophilus* has the ability to reduce the cholesterol level up to 70% and this is an important finding. It has been reported that the ability of an organism to reduce the cholesterol level was due to the assimilation of cholesterol within bacterial cell and increased excretion of bile salts is due to the deconjugation by bile salt hydrolase .(Salminen et., al 2003).

5. Conclusion

In conclusion, the LAB strains used in this study showed a high resistance to low pH values and to bile salts. These features may enable them to survive in the stomach and intestine, or even to compete with other bacterial groups in this environment and to colonize the GIT of the host. The results of our present study, together with our previous studies concerning the antibacterial activity against pathogenic bacteria, showed that *L.acidophilus* might be a rich source of LAB strains with a high potential for their use as probiotic strains.

6. Future Scope of Study

Camel milk is used therapeutically against dropsy, jaundice, problems of the spleen, tuberculosis, asthma, anaemia, and piles. Patients with chronic hepatitis had improved liver function after being treated with camel milk. The camel milk works also as a laxative on people unaccustomed to drinking this milk. The numerous nutrients in camel milk help promote our body natural defenses .Camel milk has anti-tumor properties. The presence of high-level of insulin-like protein in camel milk (about 52 micro unit/ml) helps reduce the effect of diabetes. Camel milk has antiviral properties that help reduce liver inflammation. The milk also has many nutrients that are required for healthy liver function. It is rich in immunoglobulin that will strengthen the body's autoimmune system. This will help treat and heal autism. Research on camel milk should be encouraged more for therapeutically which will be helpful to the society.

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