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

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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 being[1-2]. 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[3] 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[4-6] and also has antitumour and anticholestrol 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[7]. The lactic acid bacteria also got anti oxidative activity[8].

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 [9-10].

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 37^0C 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

intestine for around 4 hours[11] till it gets absorbed. All the strains were screened for their survival at different bile concentrations. Cultures were inoculated into 10 ml MRS broth in test tubes and incubated at 37^0C overnight in anaerobic condition. 100µl of active culture was inoculated into fresh MRS broth tubes with pH 6.5 containing 0.3%, 0.5% and 1.0% bile (CDH India). The bacterial survival was measured by MRS agar colony count with taking 100µl culture for 0, 30, 60, 90 and 180 min and aliquots spread onto MRS agar plates to calculate the CFU/ml. The experiment was determined in triplicate to calculate intra-assay variation. CFU/ml was recorded.

NaCl Tolerance: The tolerance of culture isolates against different NaCl concentrations was recorded by inoculating the pure culture isolates in MRS broth having 2.0%, 4.0%, 6.0 %, 8% and 10% (w/v) sodium chloride. After 48 h of incubation at 37°C, broths were examined for growth and results were recorded.

Growth at different Temperature: The growth behavior of culture isolates were observed at different temperatures to differentiate between mesophilic and thermophilic organisms. Inoculation of pure culture isolates were made in MRS broth and incubation was done at 15^0C, 30^0C, 37^0C, 45°C, 50^0C, 55^0C and 60^0C for 48-72 hrs. During these incubation time cells growth was observed and results were recorded.

Growth at different pH: The growth behavior of culture isolates were observed at different pH to find the acid and alkali tolerance capacity of organisms. Isolates were inoculated in MRS broth with pH 2, 3, 4, 5, 6, 7, 8 and 9 and incubated at 37^0C for 48-72 hrs. During these time cells growth was observed and results were recorded.

Resistance to acidic pH: Resistant to acidic pH is considered as another major selection criterion for potential probiotic strains[12]. Microbes should pass through the acidic environment of stomach[13] to reach small intestine. Stomach, pH is as low as 1.0 but in vitro assays pH 3.0 has been preferred. The isolates were inoculated into MRS broth maintained at pH 2 and pH 3. The rate of survival of the isolates has been examined by plating 0.1ml of cultures at 0, 1.5 and 3hours.

Sugar fermentation tests: The isolates were examined for their ability to ferment different sugars like fructose, galactose, cellobiose, esculin, inulin, rhamnose, melibiose, mannitol, maltose, mannose, ribose, trehalose, arabinose, lactose, sucrose, xylose, salicin as described by Harrigan (1998). Enteric fermentation (SRL) broth was prepared with 1% of each sugar along with Bromecresol purple indicator (0.01%) solution. Durham's tubes were inverted in to test tubes containing 5 ml of MRS broth and then sterilized. Tubes were inoculated with active culture and incubated at 37°C for 48-72hrs. Results were observed by change in the media color and gas formation.

Antimicrobial Activity Test: Agar well diffusion method[14] 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[15]. 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[16-18]. 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

Sl. No.	Isolate No.	Catalase test	Size(mm)	Shape	Margin	Gram's staining	Shape	Motility	Gas from glucose	Spore formation	Arginine utilization	Starch Hydrolysis	Growth in broth	Growth on slants	Nacl-2%	NaCl-4%	NaCl-6%	NaCl-8%	Nacl-10%	Indole test
1	BM1	-	<1	Circular	Entire	+	Rods pairs/ chains	-ve	+ve	-ve	-ve	-ve	Turbidity	Beaded	+++	+++	++	+	-	-
2	BM2	-	1	Circular	Entire	+	Rods	-ve	-ve	-ve	-ve	-ve	Turbidity	Smooth	+++	+++	++	+	-	-
3	BM3	-	<1	Circular	Entire	+	Small rods	-ve	-ve	-ve	-ve	-ve	Turbidity	Beaded	+++	++	+	-	-	-

Table-2
Physiological characteristics of the isolates

Sl.No.	Isolate No.	Growth at different temperature (^{0}C)							Growth at different pH							
		15	30	37	45	50	55	60	2	3	4	5	6	7	8	9
1	BM1	++	+++	+++	+++	+	-	-	+	+	+	+++	+++	+++	++	++
2	BM2	++	+++	+++	+++	+	-	-	-	+	+	+++	+++	+++	+	+
3	BM3	++	+++	+++	+++	-	-	-	-	-	+	++	+++	+++	+	+

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

Table-3
Biochemical characteristics of the isolates by utilization of carbon sources

Sl. No	Isolate No.	Fructose	Galactose	Cellobiose	Esculin	Inulin	Rhamnose	Melibiopse	Mannitol	Maltose	Mannose	Ribose	Trehalose	Arabinose	Lactose	Sucrose	Xylose	Salicin
1	BM1	+	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+
2	BM2	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+
3	BM3	+	+	+	+	+	-	+	-	+	-	+	-	+	+	+	+	-

Positive reaction (+), negative reaction (-)

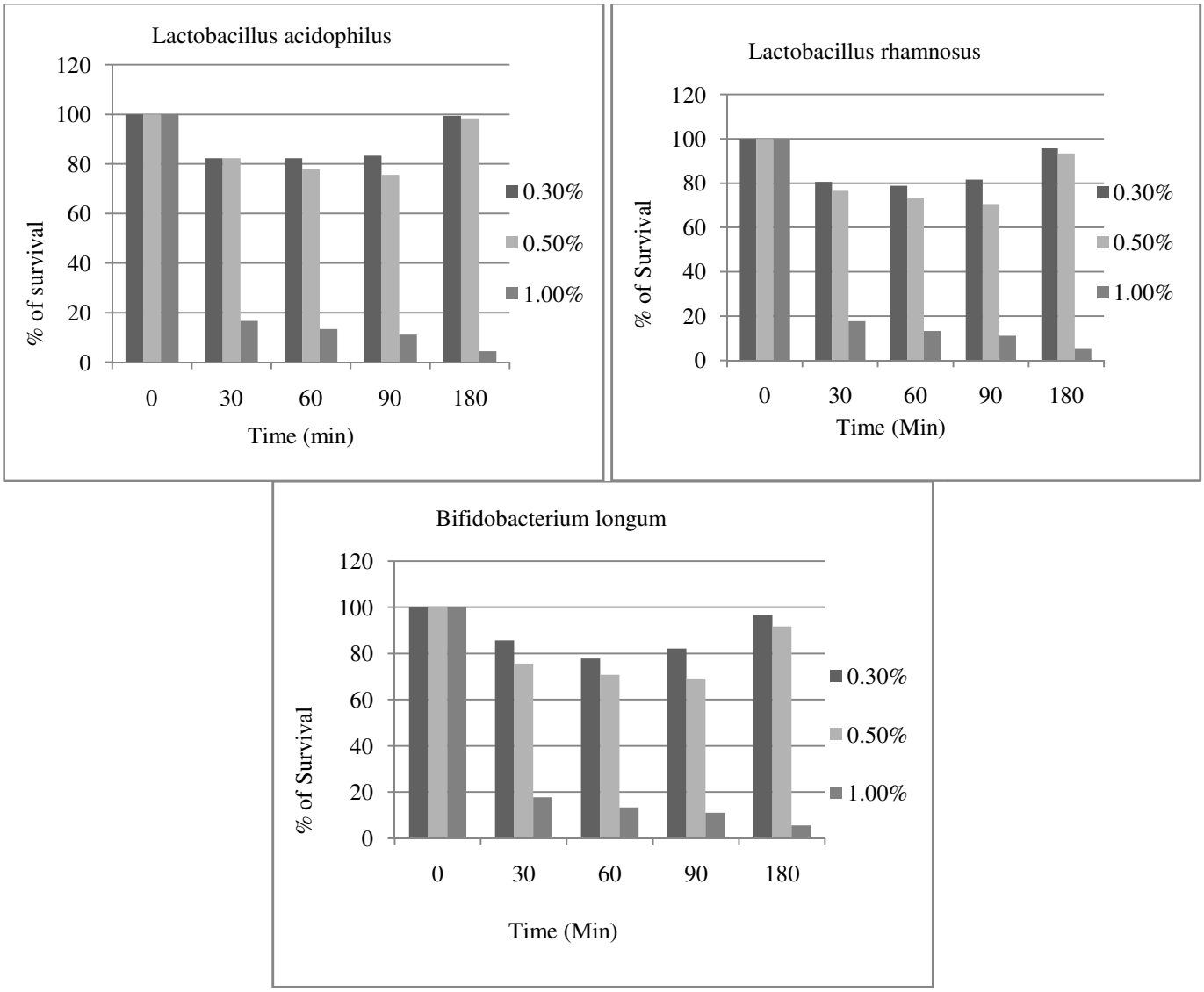


Figure-1
Effect of different concentration of bile salt on the growth of the isolates

Table-4
Antibacterial susceptibility of the Isolates

Pathogens	*L. acidophilus*	*L. Rhamnosus*	*Bifidobacterium longum*
P. aeruginosa	100% S	100% S	100% S
E. coli	100% S	100% S	100% S
Klebsiella pneumonia	100% S	R	100% S
Staphylococcus aureus	100% S	100% S	100% S
B. subtilis	R	100% S	75% S

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.

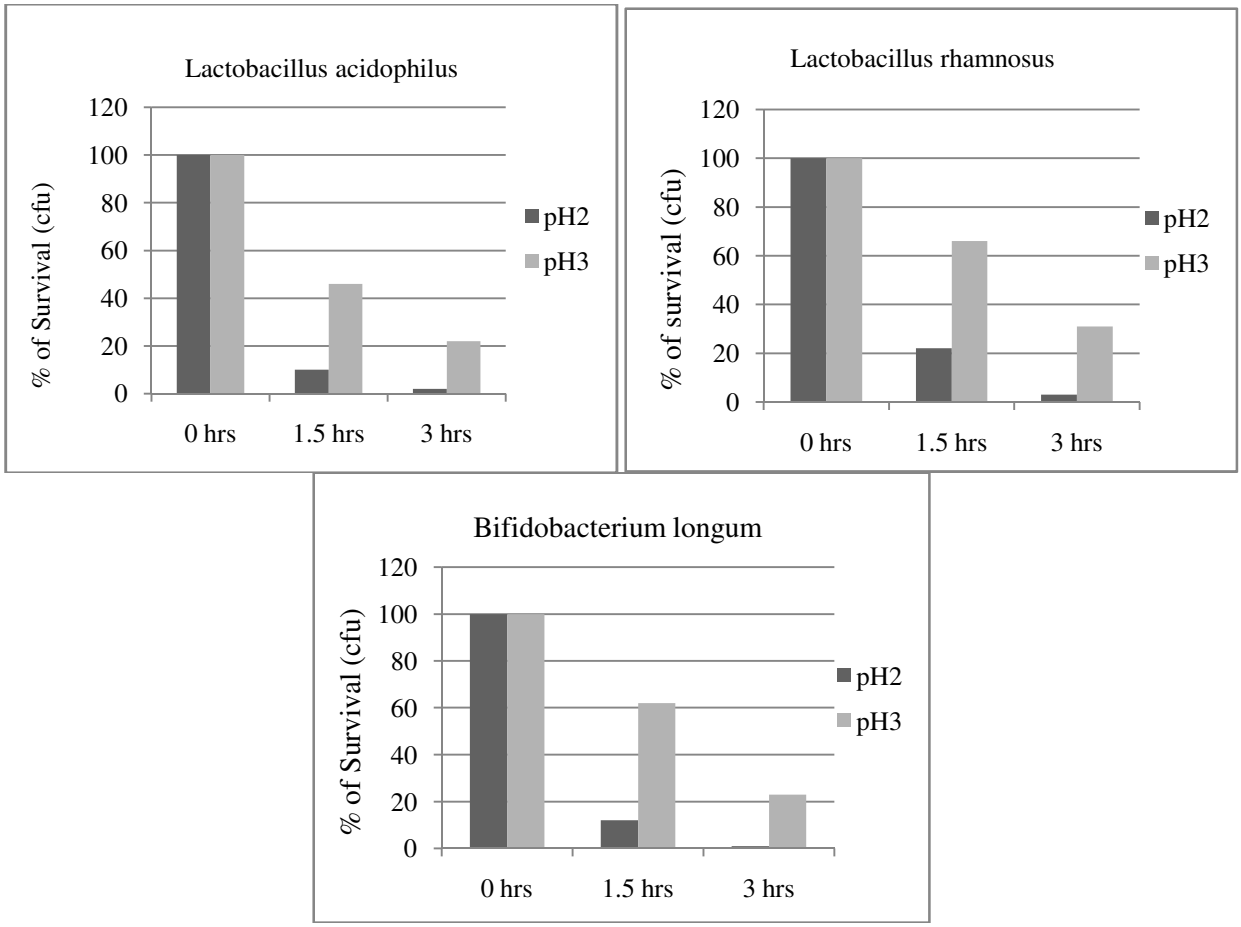
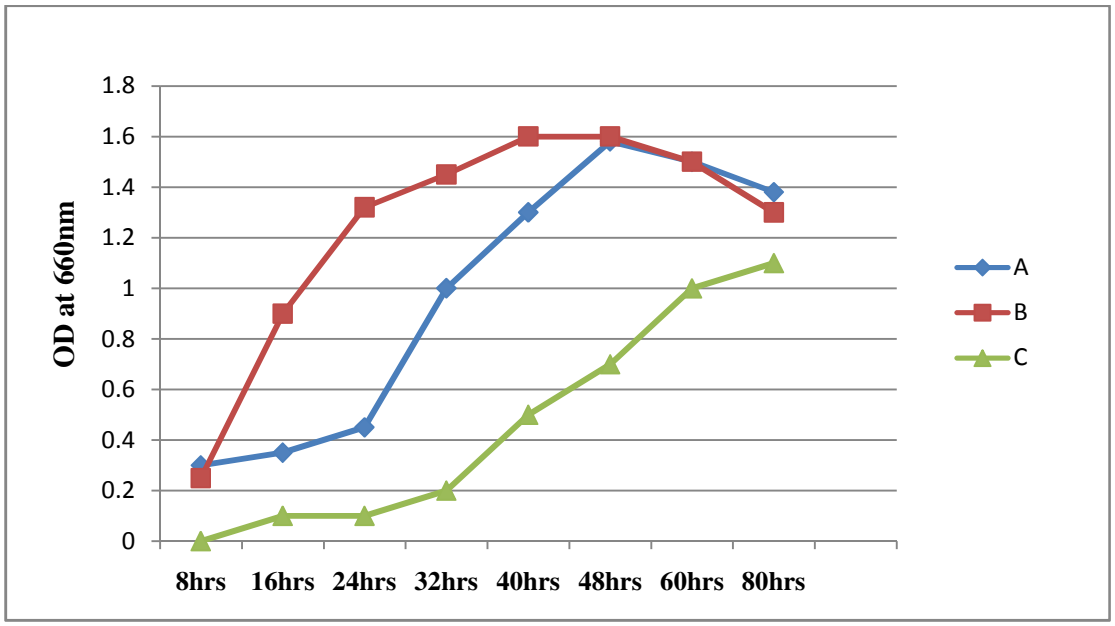


Figure-2
Effect of acidic pH on the growth of the isolates



A. ***Lactobacillus acidophilus*****. B.** ***L. Rhamnosus*****. C.** ***Bifidobacterium longum***
Figure-3
Growth Pattern of the isolates

Acknowledgement

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