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AN APPROACH TO DIFFERENTIATE, ENUMERATE, ISOLATE, CHARACTERISE LACTOBACILLUS SP. FROM NATURAL SOURCES VS ARTIFICIAL SOURCE

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ABSTRACT
Rapid scientific and technological advances have allowed for a more detailed understanding of the Lactobacillus sp., its relevance as beneficial probiotic organism and the entire body-wide micro biome, to human health and well-being. Unknowingly, recent time's public have started consuming fermented dairy products containing live bacteria to cure intestinal discomforts. Many studies have provided suggestive evidence that probiotics (Lactobacillus sp.) can influence, prevent against IBS, Crohn's disease and ulcerative colitis, the three of which are powerfully harmful, as well as in eradicating basic digestive problems like bloating, diarrhoea and constipation. So a need to develop, characterise good bacteria from various natural and artificial sources including breast milk, cow's milk, pasteurised milk, yoghurt, curd etc., are becoming popular. Based on this urge, an approach has been made in this study, to differentiate and characterise lactobacillus sp. from different sources.

KEYWORDS: Lactobacillus sp., Probiotics, breast milk, cow's milk, pasteurised milk, yoghurt, curd.

INTRODUCTION
There is an increasing scientific and commercial interest in the use of beneficial microorganisms, or "probiotics," for the prevention and treatment of diseases. The word "probiotic" is derived from the Greek, meaning "for life." Probiotics are live bacteria that can resist the rigors of the human digestive system, compete with pathogens, and that help to improve the gut flora balance. (Macfarlane and Cummings, 1999; Hozapfel et al., 1998) In other words, probiotics are homo/ heterogenous culture of live microbes that help a host to nourish nutritionally by improving the percentage of indigenous beneficial microbes in host gut through competitive exclusion and antagonism (Fuller, 1989) by improving feed ingestion and digestion (Nahanshon et al., 1993) and by varying bacterial metabolism (Jin et al., 1997). "Let food be thy medicine and medicine be thy food" as Hippocrates said, is the principle of Probiotics.

In ancient times, the benefits and health potential of foods containing live bacteria were recognized and fermented foods were quite common. During the beginning of the 20th century, Metchinkoff (1907) proposed a scientific rationale for the beneficial effects of bacteria in yogurt and attributed the long life of Bulgarian peasants to their intake of yogurt containing *Lactobacillus* sp. Despite improvements in public health and economic wealth, the incidence of intestinal infections remained high in the developed world and continues to be an important clinical problem with relevant morbidity (Hutt et al., 2006). Thus, recognition of the importance of the intestinal microbiota to health has led to increasing interest in manipulating the composition and activity of the microbiota to improve both adult and child health.

Many medical microbiologist and scientists proposed that many diseases could be prevented if an optimal gut microflora was maintained. Especially, selected beneficial strains of lactic acid bacteria are generally recognised as safe (GRAS) organism, (Hasan and Frank, 2001) that could survive passage through the digestive system and used them to develop fermented milk products (Cripps and Gleason, 1999) and can be used for various medical and veterinary applications (Gardiner et al., 2002). Based on the analysis of previous studies, concerning isolation of probiotic bacteria, indicating that they can be found in dairy and fermented products, an approach is made to enumerate and characterise Lactobacillus sp.

MATERIALS AND METHODS
Collection of samples
The samples of human breast milk were collected from two healthy mother volunteers in a private hospital, and cow's milk directly from cow by following proper collection methods. These samples were considered as natural sources. The pasteurized milk, yoghurt and curd were brought from commercial shops with recent manufacturing date. These samples were considered as

artificial sources. All the samples were collected in sterile carriers and stored on ice until delivery to the laboratory.

Enumeration and isolation of bacteria

The samples of *natural and artificial sources* were analyzed for the enumeration of bacterial count using serial dilution and by spread plate technique of10^{-4}, 10^{-5} and 10^{-6} dilutions on to MRS agar medium. All the plates were incubated at 37°C for 24 hours and the colony forming units were counted based on the standard formula.

Morphological identification

The morphological characteristics were identified by Grams staining, motility and endospore testing. It is characterised by means of several biochemical test such as indole, MR, VP production, citrate utlilization, urease, catalase, oxidase and carbohydrate fermentation test.

Determination for Nacl tolerance

For the determination of NaCl tolerance of isolated lactobacillus 10 test tube containing MRS broth were adjusted with different concentration (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% (v/v) fresh overnight culture of *Lactobacillus* spp. and incubated at 37°C for 24 h. After 24 h of incubation their growth were determined by observing turbidity. Maximum growth were indicated as double positive sign (+ +), normal growth as single positive sign (+) and no growth were indicated as negative sign (-).

Determination for Bile salt tolerance

For the determination of Bile salt tolerance of isolated lactobacillus, MRS broth was prepared with different concentrations of bile salt such as 0.05, 0.1 and 0.3%. After sterilization, each test tube was inoculated with 1% (v/v) fresh overnight culture of *Lactobacillus* spp. and incubated at 37°C for 24 h. After 24 hours of incubation their growth were determined by observing turbidity using spectrophotometer.

Isolation of opportunistic pathogen E.coli from urinary infection patients

The urine samples were collected as a clean midstream urine specimen in a sterile wide mouthed leak proof container. The collected samples (n=5) were brought to the laboratory in ice box. The urine samples were mixed thoroughly, centrifuged to remove any suspended particles. A calibrated sterile Nichrome wire loop for the semi-quantitative method was used for the plating. It has a 4.0mm diameter to deliver 0.01ml. A loopful of the well mixed urine sample was inoculated on Eosin methylene blue agar plates specially designed for E.coli isolation. The plates were then incubated at 37°C aerobically for 24 hours. They were examined for bacterial growth and then pure culture isolate is made by again quadrant streaking onto EMB plates.

Antagonistic effect of Lactobacillus sp. Vs opporunisitic E.Coli

A suspension was prepared in concentration of 0.5 McFarland by adding of 2-3 colonies from +grown bacteria to 10 mL sterile distillated water. For this the petriplates of diameter of 90 mm were poured 20 ml of Muller Hinton media, swabbed with the suspensions of turbity M.F.S. # 0.5 for E. coli incubated at 37ºC for 15 minutes. Sterile blotting paper disc of 6mm diameter were soaked with the 20 µl of probiotic suspension of turbidity equal to M.F.S. 1.0 (3×10^8 cfu/ml) and the serial suspension of 1/10 ($3x10^7$cfu/ml) and 1/100 ($3x10^6$ cfu/ml) so the disc now contained 6×10^6 cfu/disc (for M.F.S. 1.0), 6×10^5 cfu/disc (M.F.S. 1/10) and $6x10^4$ cfu/disc (M.F.S. 1/100).

Determination of death kinetics

Lactobacilli strains were cultured in MRS broth Medium, centrifuged in 12000 rpm for 7 min to determine pathogenic bacteria death kinetic. Then a suspension were prepared in 0.5 McFarland concentrations from pathogenic bacteria that had grown in Caso agar. One ml of this suspension was added to Caso broth Medium in three separated parts. Lactobacilli species in concentration of 2, 5 and 10% were added to these suspensions at the same time. Then OD (optical density) of pathogenic bacteria was measured each hour. There was one control with pathogen and without *Lactobacilli*.

RESULTS

15 species of various microorganisms were isolated altogether from both natural and artificial samples. In which five samples showed Gram positive with grape like structure in Gram staining and catalase positive indicating Staphylococcus sp. There were 2 more species showing Gram positive and Indole positive, urease positive, citrate negative, Triple sugar ion test as acid butt and alkaline slant indicating it as Enterococcus. These biochemical test and sugar fermentation test indicated and isolated pure cultures of Lactobacillus sp and differentiated from other organisms (Table no. 1A and 1B). The colony morphologies of Lactobacillus sp showed very small circle shaped and non-transparent colonies (Fig no. 1A) and Appearance of Gram staining(Fig no. 1B).

The micro organisms were grown in MRS broth with different concentration of Nacl from (1-10%) indicated that 2-3% of Nacl concentration is optimal for maximum growth of the organisms (Table no.2). In other experiment, 0.05%, 0.1% and 0.3% of bile salt concentrations were added, incubated for 24 hours and the optimal growth were checked periodically for 3,6, 12 and 24 hours. For maximum growth within less time than 0.3% of bile salt concentration can be added, but for an optimal and constant growth 0.1% of bile salt concentration is suggested (Table no. 3).

For the isolation of opportunistic pathogen, samples were collected from urinary infection patients, indicating

symptoms of burning sensation while urinating, frequent urination, fever and body pain. Streaking of samples on EMB agar plate indicated green metallic sheen with blackish red colonies confirming E.coli. (Fig no. 2).

Readymade antibiotic disc streptomycin was taken as a positive control and sterile distilled water as negative control. These discs were placed on Muller Hinton media and kept at 4ºC for 1 hour for proper diffusion. Now the plates were kept at 37ºC for 24 hours and zones were measured. All the tests were done twice and best was used for readings. Antagonistic activity of E.coli showed significant zone of inhibition of 18mm at M.F.S of 1.0 (3 x 10^{8} cfu/ml). No zone was seen in the serial suspension of M.F.S. 1/100, except in the sample 6 and 7, 8. The following serial suspension showed serially decrease in the inhibition zone (Table no.4).

In this study, the assay of death kinetic of E.coli pathogens indicated that 5% concentration of Lactobacillus decreased OD of both E.coli within 2 and 3 h after contact time. At zero time, OD of E.coli was 0.055, 0.050 and 0.005 but it showed reduction to 0.049 0.03, 0.015 after 3 h (Fig no. 3). In 10% concentration of Lactobacillus, OD of E. coli decreased from 0.005 to 0 during 2 h after contact time.

DISCUSSION

All the strains were screened initially for antimicrobial activity using antibiotic sensitive pattern against the test opportunistic pathogenic organisms, E. coli. Preliminary screening of bacterial isolates showed that Lactobacillus sp (sample no. 6) and Lactobacillus sp. sample no.5 had antibacterial activity against E. coli in comparison to Enterococcus sp. at sample no.1,3,4. Overall, Lactobacillus sp., had antimicrobial effect, while Enterococcus seemed to be passive.

Lactobacilli and E. coli coexist in the human gut, but sometimes, E. coli being an opportunistic enteric pathogen can result in enteric diseases like Traveller's Diarrhoea, urinary tract infection. Moreover, E. coli and Pseudomonas aeroginosa are the most common contaminants of water and indicators of faecal contamination in drinking water. In these reports, effect of antimicrobial therapy using Lactobacilli isolated from natural and artificial sources against Gram negative organisms in natural sources were proved (Reza and Khudaverdi, 2015). Therefore, further mass screening, evaluation, characterisation of lactobacilli strains will be of both industrial and medical significance.

Recent studies indicate that the antibiotic taken for urinary tract infection causes observable effect like stomach discomfort, gastritis, shiverings, fatique etc., on humans and rarely these antibiotics indiscriminately destroys both beneficial and pathogenic bacteria in the body thereby leading to imbalance of the microflora with very negative effect (Prema, 2013). Thus, consuming of Lactobacillus sp. in the form of curd, milk etc., can regain the microflora and their antibacterial property will inhibit the attachment and proliferation of potentially putrefactive pathogenic organisms.

Table no. 1 A Biochemical tests to identify microbes

Isolates	Indole	Methyl red	VP test	Citrate	Catalase	Oxidase	Urease	TSI	Identification
1	+	+	+	-	-	-	+	A/K	*Enterococcus sp.*
2	-	+	-	+	-	-	-	A/A+G	*Lactobacillus sp*
3	+	+	+	-	-	-	+	A/K	*Enterococcus sp.*
4	+	+	+	-	-	-	+	A/K	*Enterococcus sp.*
5	-	+	-	+	-	-	-	A/A+G	*Lactobacillus sp*
6	-	+	-	+	-	-	-	A/A+G	*Lactobacillus sp*
7	-	+	-	+	-	-	-	A/A+G	*Lactobacillus sp*
8	-	+	-	+	-	-	-	A/A+G	*Lactobacillus sp*
9	Grape like structure in Grams staining and Catalase positive								*Staphylococcus sp.*

Table no. 1 B Sugar fermentation test for microbes

Isolates	Mannitol	Ribose	Sorbitol
1	+	+	-
2	-	+	-
3	+	+	-
4	+	+	-
5	+	+	-
6	+	+	-
7	+	+	+/-
8	+	+	+/-

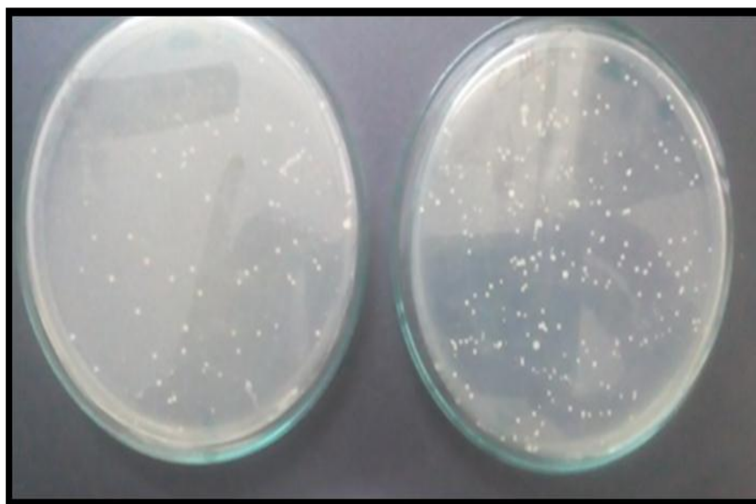
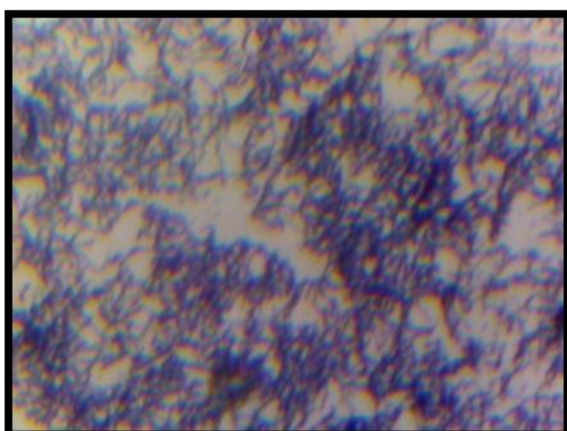
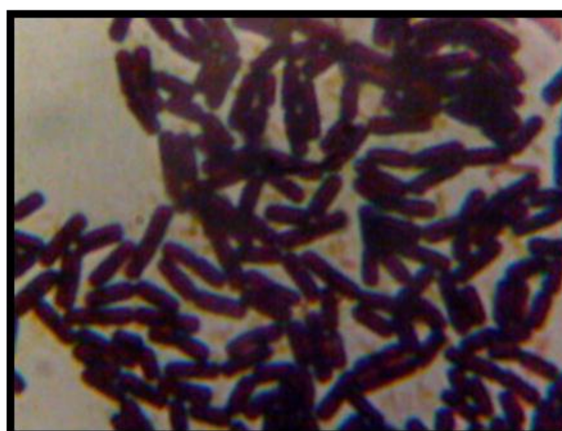


Figure no. 1 A Isolation of Lactobacillus



10 X Objective



100 X Objective

Figure no. 1 B Gram s Staining

Table no. 2 Tolerance Testing - Nacl

Nacl (%)	1,3,4	2	5	6	7,8
1	+++	+++	+++	+++	+++
2	+++	+++	+++	+++	+++
3	+++	+++	+++	++	+++
4	++	++	++	++	++
5	++	++	++	++	++
6	++	+	+	+	+
7	+	+	+	+	+
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	-	-

- +++ Maximal growth, ++ - good growth, + minimal growth, - no growth 1,3,4 – Enterococcus sp, rest all are Lactobacillus sp.

Table no. 3 Bile Salt Tolerance Testing Concentration of Bile Salt (0.05%)

Isolates	Incubation Time (hr)	O.D
1, 3, 4	3	1.002
	6	1.724
	12	2.001
	24	2.225
2	3	1.110
	6	1.954
	12	2.020
	24	2.325
5	3	1.005
	6	1.625
	12	2.001
	24	2.175
6	3	1.105
	6	1.743
	12	2.030
	24	2.225
7,8	3	1.004
	6	1.876
	12	2.045
	24	2.321

Concentration of Bile Salt (0.1%)

Isolates	Incubation Time (hr)	O.D
1, 3, 4	3	0.962
	6	1.525
	12	1.950
	24	2.126
2	3	0.875
	6	1.427
	12	1.876
	24	2.025
5	3	0.866
	6	1.432
	12	1.881
	24	2.120
6	3	0.925
	6	1.543
	12	2.113
	24	2.345
7,8	3	0.975
	6	1.632
	12	1.975
	24	2.224

Concentration of Bile Salt (0.3%)

Isolates	Incubation Time (hr)	O.D
1, 3, 4	3	1.016
	6	1.654
	12	2.010
	24	2.175
2	3	1.120
	6	1.920
	12	2.010
	24	2.500
5	3	1.015
	6	1.715
	12	2.010
	24	2.225
6	3	1.115
	6	1.542
	12	2.220
	24	2.310
7,8	3	1.114
	6	1.920
	12	2.110
	24	2.416

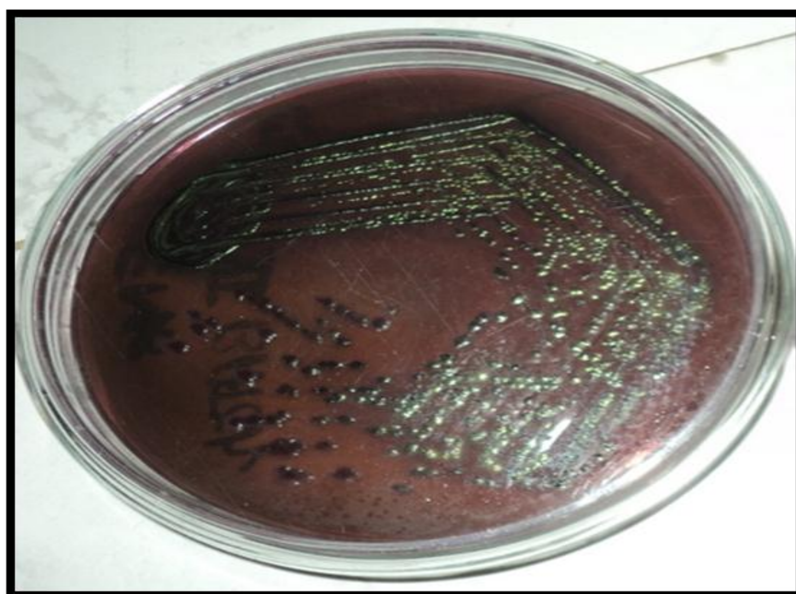


Fig no. 2 E.coli on EMB agar

Table no. 4 Antagonistic activity of Lactobacillus sp. against opportunistic pathogen E.coli

S.No.	Sample nos.	1,3, 4	2	5	6	7,8
	Antibiotic/probiotic disc	Diameter of zones of inhibition (mm)				
1	1.0 (3×10^8)	11	15	16	18	15
2	1/10 (3×10^7)	8	10	8	11	9
3	1/100 (3×10^6)	0	6	0	7	4
4	Distilled water	0	0	0	0	0
5	Streptomycin	20	23	23	24	23

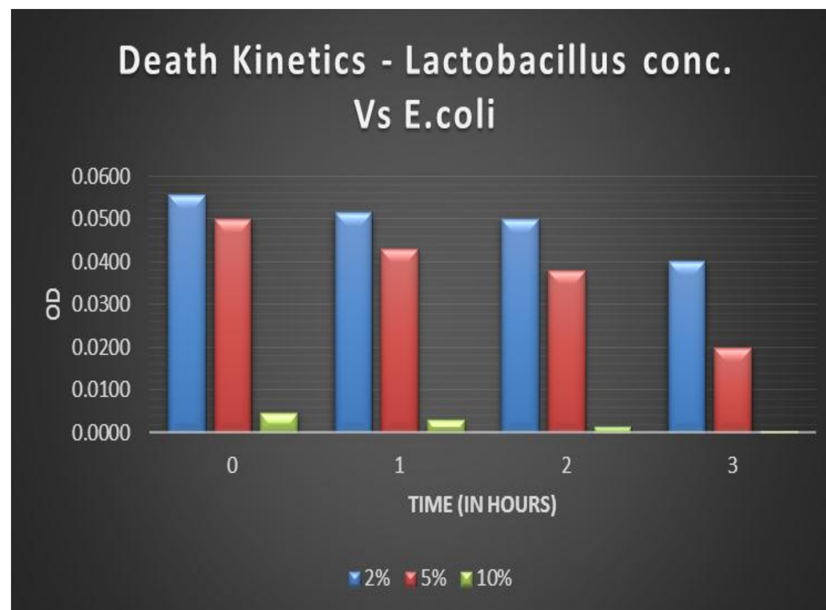


Fig no. 3 Death kinetics of opportunistic pathogen

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