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Evidence for probiotic properties of *Lactobacillus fermentum* and *Lactobacillus reuteri* isolated from human breast milk

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The present study was conducted to determine the probiotic properties of *Lactobacillus fermentum* and *Lactobacillus reuteri* isolated from human breast milk. The samples were inoculated with MRS medium and incubated for 48 hrs at 37^{0}C under anaerobic incubation. The identification of the culture was based on characteristic of Lactobacilli as presented in the Bersey's Mannual of Determinative Bacteriology, carrying out morphology, gram stain, catalase, oxidase and other biochemical tests, growth at 15^0, 37^{0}C and 45^{0}C and fermentation of different carbon sources. Selection of the strain included various criteria such as agreement with biosafety aspects, antibiotic susceptibility test, tolerance to low pH, bile and NaCl concentration, temperature, Hemolytic activity and antimicrobial activity. This result suggests that these two strains are favorable for use as probiotics. It should be suitable strains for probiotic use of human being and animals. *L. fermentum* had high probiotic activity when compared to *L.reuteri*.

Key words : MRS medium, Probiotic, Antimicrobial therapy, *Lactobacillus*

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INTRODUCTION

Growing human population urges the immense need to exploit the existing live stocks resources to meet an animal protein requirement (Saavedra, 2001). In contrast to "antibiotic" the term "probiotic" was coined to describe a substance produced by one microorganism that stimulates the growth of another microorganism. The term "probiotic" was derived from the Greek word meaning "for life". (Reid *et al.,* 2003). An expert panel commissioned by FAO (Food and Agriculture Organization) and WHO (World Health Organization) defined probiotic as "live microorganism", which when administrated in adequate amounts confers a health benefit on the host. (FAO and WHO 2001).Various bacterial genera most commonly used in probiotic preparations are *Lactobacillus, Bifidobacterium, Enterococcus, Bacillus* and *Streptococcus*. Some fungal strains belonging to saccharomyces have also been used. (Jin *et al.,* 2000; Gibson and Roberfroid, 1995).

The probiotic meaning for life is derived from the greek language. It was first used by Lilly and Stillwell in 1965 to describe "Substances secreted by one microorganism which stimulates the growth of another" and thus was constructed with the term antibiotic. Probiotic for human use will require substantiation of efficacy with human trails. Appropriate target specific *in vitro* tests that correlate with *in vivo* test results are recommended. Currently used *in vitro* tests for study of probiotic strains are resistance to gastric acidity, bile salt resistance, adherence to mucous and/ or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria, ability to reduce pathogens adhesion to surface, bile salt hydrolase's activity, resistance to spermicides (applicable to probiotics for vaginal use. (Dash, 2009).

Probiotics can be bacteria, moulds, yeast but most probiotics are bacteria. Among bacteria, lactic acid bacteria are more popular. *L.acidophillus, L.casei, L.lactis, L.helviticus, L.salivarius, L.plantarum, L.bulgaricus, L.rhamnose, L.johnsonii, L.reuteri, L.fermentum, L.delbruckii, Streptococcus thermophillus, Enterococcus faecaum, E.faecalis, Bifidobacterium bifidum, B.breve, B.longum* and *Saccharomyces boulardii* are commonly used bacterial probiotics. A number of clinical studies have been

performed on the ability of probiotic strains to prevent or treat gastro intestional infections. The most common strains belong to the two genera *Lactobacillus* and *Bifidobacterium*, but other micro organisms including *Enterococcus*, *Streptococcus*, *E.coli* and *Saccharomyces species* have also been used. The result from well performed, double-blind, placebo-controlled studies suggested that *Lactobacillus* strains are most promising strain in the prevention of diarrhea. Only for non-breast fed childrens in one of these age groups was the probiotic strain of any advantages (Szajewsk *et al.,* 2001 and Oberhelman *et al.,* 1999).

Because of inhibitory effect selected lactobacilli may be used as probiotic and biological preservative, so the aim of this study was to present some data on isolation, antimicrobial activity, proteolytic enzyme activity, tolerance to acidic pH, tolerance to bile, stability of heat, lactic acid production, hemolytic activity, resistance to antibiotics, aggregation assays and bacterial viability during storage. The ability of the probiotic *Lactobacillus* to help and prevent pathogenic bacteria from proliferating and healthy bacteria from becoming toxic is well documented (Reid, 2001 and Famularo *et al.,* 2001).

RESEARCH METHODOLOGY

Isolation and identification:

The *Lactobacillus* strains were isolated from human breast milk on solid Man-Rogosa-Sharpe media (MRS)(g/l, peptone 10.0, meat extract 8.0, yeast extract 4.0, D(+)glucose 20.0, dipottassium hydrogen phosphate 2.0, tween 80 - 1.0, di-ammonium hydrogen citrate 2.0, sodium acetate 5.0, magnesium sulphate 0.2, manganese sulphate 0.04, supplemented with 14.0 g agar, respectively). Strains purity has been verified by three successive subculture from the single colony. The culture has been grown at 37^0C in microaerophillic condition with out shaking. The *Lactobacillus* strains were identified according to the method described by Michael (1981) and growth on MRS agar (pH 7.2), cell morphology, Gram stain, catalase activity, oxidase activity, indole production, nitrate reduction, gelatin liquefaction, etc., for long term preservation an MRS broth supplemented with 10% glycerol.

Carbohydrate fermentations:

Further identification of the species of this lactobacillus was performed according to the sugar fermentation patterns described as Bergey's manual of systemic bacteriology. Isolates were characterized according to their fermentation profiles of ability to ferment 14 different carbohydrates. Each sugar solution was prepared at a final concentration of 10% (w/v) with phenolphthalein red indicator, and the solutions were sterilized. After preparation steps the procedure was applied. All the reactions were performed twice. Also positive and negative controls were used to indicate any contamination. After overnight incubation at 37^0C, the turbidity and the colour change from red to yellow was recorded as positive fermentation, results were compared with the positive and negative controls. (Roos *et al.,* 2005).

Gas production from glucose:

The production of gas during glucose fermentation was observed by placing an inverted Durham's tube in MRS broth and inoculated with 1% overnight fresh cultures. Then the test tubes were incubated at 37 °C for 24 hrs. Gas occurrence in Durham tubes was observed during 24 hrs which is the evidence for CO_2 production from glucose (Davis, 1995).

Growth at different temperatures and NaCl concentrations:

Temperature test media, MRS containing bromecresol purple indicator, was prepared and transferred into tubes as 5 ml. Then fifty µl of overnight cultures inoculated to tubes and incubated for 5 days at 15 °C, 37^0C and 45 °C. During these incubation time cells growth at any temperatures was observed by the change of the colour from purple to yellow. Isolates were tested for their tolerance against different NaCl concentrations. For this purpose 1%, 2% and 3% NaCl concentrations were selected. Test mediums containing bromecresol purple indicator were prepared according to the appropriate concentrations and transferred into tubes in 5 ml. These tubes were inoculated with 1% overnight cultures and then incubated at 37^0C for 5 days. The change of the colour from purple to yellow was proofed the cell growth.

Antimicrobial activity:

Microbial antibiotic resistance was determined on MRS agar medium by use of 13 different antibiotic discs (Hi-media, India). The results (average of 3 readings) was expressed as Sensitive (S), Intermediate (I) and Resistance (R), thanks to the standard disc diffusion method (National Committee for Clinical Laboratory Standards, 1999).

Antimicrobial activity of bacteriocin:

Antimicrobial action of all lactobacilli species against indicator bacteria was determined by agar well diffusion method as described by Flemming *et al.* (1985). A total of 11 clinical isolates were obtained from department of microbiology, SRM Medical College Hospital and Research Centre. Out of these twelve, four were Gram positive (*Staphylococcus aureus, MRSA (Methicillin Resistant Staphylococcus Aureus). Streptococcus pneumoniae and Enterococcus spp.*) and seven were Gram negative (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus spp., Salmonella typhi, Shigella dysentriae, and Vibrio cholera*) were used as a indicator bacteria. Supernatant of lactobacilli sp were monitored for antibacterial activity against indicator bacteria inoculated on Muller Hinton Agar (MHA). A volume of 50 µl of cell free supernatant was filled in 5 mm diameter sealed wells cut in the MHA agar. The diameter of the zone of inhibition was measured with caliper after 24 hr of incubation. (Todorov and Dicks, 2004).

Haemolytic activity:

Blood haemolysis was evaluated on nutrient agar plate supplemented with 5% sheep blood which was incubated at 37^{0}C for 24 hrs. (Lombardi *et al.,* 2004).

Resistance to low pH:

Resistance to pH 3 is often used *in vitro* assays to determine the resistance to stomach pH. Because the foods are staying during 3 h, this time limit was taken into account. For this purpose, active cultures (incubated at 37^{0}C for 16-18 h) were used. Cultures were inoculated in 10 ml of 0.05 M sodium phosphate buffer adjusted to pH 2.0 to 7.0 with 1N HCl and samples were incubated at 37^{0}C for 3 h. Cells were serially diluted to 10 fold dilution by phosphate buffer pH 7.0. Viable microorganisms were enumerated by pour plate techniques. Appropriate dilutions were done and plates were incubated at 37^{0}C under anaerobic conditions for 48 h. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial cell concentration. Each experiment was performed in triplicate.

Bile salt resistance:

The ability of isolated species to grow in the presence of 0.3%, 0.6%, 0.9% and 1.2% bile salts was determined in MRS broth, as described by Dunne *et al.* (2001). The growth was examined after 24 hours under anaerobic conditions of incubation at 37^{0}C by plate count method. Viable cells in the presence of bile salt were compared to without bile salt. The experiment was performed in triplicate.

RESULTS AND ANALYSIS

Human breast milk samples were obtained from lactating women age ranging from 20-36 years. The samples were collected 6 to 32 days after delivery. Isolated lactobacillus strains proved to be microaerophilic, Gram positive, catalase negative, oxidase negative, non-spore forming and non-capsulated rod. As it is microaerophilic, when cultivated in liquid (MRS broth) media, this strain forms turbidity and sediments. Microscopically cells consist of short to long rods that appear as single cells, in pairs and in short chains. Surface colonies on MRS agar plate are 0.5 to 2 mm in dm, circular, lenticular, creamy-white. The most useful test for the

Table 1: Antibiotic resistance profiles of the tested probiotic strains (Antibiotic Conc. range, µg/ML)

Antibiotics (µg)	L.f 1	L.f 2	L.f 3	L.f 4	L.r 1	L.r 2	L.r3	L.r 4
Ampicillin (10)	S	S	S	S	S	S	S	S
Amikacin (32)	S	S	S	S	S	S	S	S
Cefoxitin (30)	S	S	R	S	S	S	S	S
Clindamycin (2)	S	S	S	S	S	S	S	S
Ciprofloxacin (30)	S	S	S	S	S	S	S	S
Cloramohenicol (30)	S	S	S	S	S	S	S	S
Gentamycin (5)	S	S	S	S	S	S	S	S
Kanamycin (30)	R	R	R	R	R	S	R	R
Oxacillin (1)	S	S	S	R	R	S	S	S
Rifampicin (5)	S	S	S	S	S	S	S	S
Tetracycline (30)	R	R	R	S	R	S	S	S
Vancomycin (30)	R	R	R	R	R	R	R	R

L.f- *Lactobacillus fermentum*, L.r- *Lactobacillus reuteri*, S- sensitive, R- resistant

determination of strain differences is carbohydrate fermentation. According to the biochemical test results both species produced gas from glucose. When these biochemical test results were compared with the literature information, it seems that *Lactobacillus reuteri* and *Lactobacillus fermentum.*

Table 1 shows the result obtained for antibiotic susceptibily of the two species. Both the species were resistance to Vancomycin and Sensitive to Ampicillin, Chloramphenicol, Clindamycin, rifampicin, Amikacin, Gentamycin and ciprofloxacin. Most species showed resistance to 3 of the 13 antibiotics tested *i.e.* to Vancomycin, tetracycline and kanamycin. Four spp (three *L.fermentum* and one *L.reuteri*) have showed a multiple resistance to 3 different antibiotics, resistant to cefoxitin, oxacillin and kanamycin. In addition present results showed that 4 strains were tetracycline resistant which is in accordance with other reported studies. (Halami *et al.,* 2000 and Coppola *et al.,* 2005). It is well known that Vancomycin is an antibiotic belongs to glycopeptide antibiotics inhibits the peptidoglycan synthesis which is an important structural components of the bacterial cell wall. Therefore, Gram positive bacteria including lactic acid bacteria are especially vulnerable to Vancomycin treatment (Reynolds, 1989). In our case, all the strains tested were resistance to Vancomycin.

Present study reveled that all lactobacilli inhibited the growth of *S.aureus*, MRSA (Methycillin resistant *Staphylococcus aureus*), *Enterococcus* spp, *E.coli*, *Klebsiella pneumonia*. *Pseudomonas aeruginosa*, *Proteus spp*, *Salmonella typhi*, *Shigella* spp, *Vibrio chlorae*. The strongest antimicrobial effect was shown by *L.fermentum* against indicator bacteria when compared to *L.reuteri*. (Fig. 1). The antimicrobial action is due to the potential of LAB to produce lactic acid and bacteriocins. It is also reported that these bacteria produce peptides having inhibitory properties (Strus *et al.,* 2001).

Another criterion for the selection of probiotic was the ability of growth at different temperatures. From the results of 5 days observation, all of the isolates can grow at 15, 37 and 45^{0}C. Growth at different NaCl concentrations was observed. All of the isolates have the ability to grow at 2% NaCl concentration. It tolerated only up to %2 NaCl concentrations and grew at 37^{0}C and 45^{0}C but slightly grew at 15^{0}C. *Lactobacillus* isolated from human breast milk and used in this work, no haemolysis activity was observed (Table 2). However, absence of hemolytic activity should be a selection criteria for (bacteriocin producing) starter strain for probiotic use and the *Lactobacillus* indicates that these bacteria are non-virulent. (De Vuyst *et al.,* 2003).

The effect of pH ranging from 2.0 to 7.0 on the *L.fermentum* and *L.reuteri* was studied. It was found that two spp could survive approximately in pH 2.0. When the pH was raised to 3.0 to 7.0 the survival rate exhibited higher. Resistant to low pH is one of the major selection criteria for probiotic strains (Quwehand *et al.*, 1999, Çakýr, 2003). Since, to reach the small intestine they have to pass through from the stressful conditions of stomach (Çakir, 2003). For selection the strains resistant to low pH, MRS broth pH-adjusted to 2,3,4, and 5 was used. The time that takes during the digestion in the stomach is 3 hours. So the two isolates were detected whether they were resistant to pH 3 during 3 hours. According to this experiment both the isolates were resistant to low pH. Experiments were run twice. Results, cfu (colony forming units) values were shown as graphics (Fig. 2).

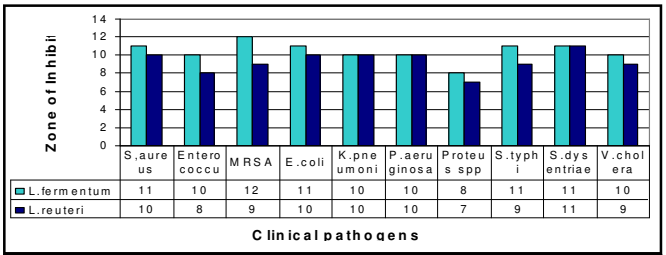

Fig. 1: Antimicrobial activity of culture supernatant against pathogens

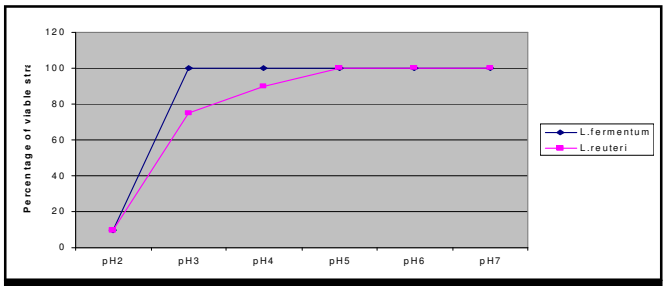

Fig. 2: Resistance to low pH

Table 2: Resistance to temperature, bile and hemolysin production

	Resistance to Temp.			Resistance of NaCl IN %			Type of Hemolysis
	15^{0}C	37^{0}C	45^{0}C	1%	2%	3%	
L.fermentum	SG	G	G	G	G	NG	NH
L.reuteri	SG	G	G	G	G	NG	NH

SG- slight growth, G-growth, NG- no growth, NH- non hemolytic

The effect of bile on the viability of the *L.fermentum* and *L.reuteri* was studied Fig 3. These strains were able to survive for 5 h in MRS media supplemented with 0.3% bile. Optical density data showed that slow growth of *L.reuteri* when compared to *L.fermentum* occurred over a 24 h period in the presence of 0.3% bile-containing MRS (Fig. 3). Optical density results suggest that slow growth of *L.fermentum* occurred in the presence of 0.3% bile containing MRS media, as compared to the control (MRS broth without added bile). Bile tolerance has been described as an important factor for the survival and growth of LAB in the intestinal tract**.** (Gilliland *et al.*, 1990). While both strains involved in this study could tolerant 0.3% oxgall bile, there were some minor differences. In general, the required concentration of bile salts considered necessary to screen for resistant strains for human use is 0.3%.

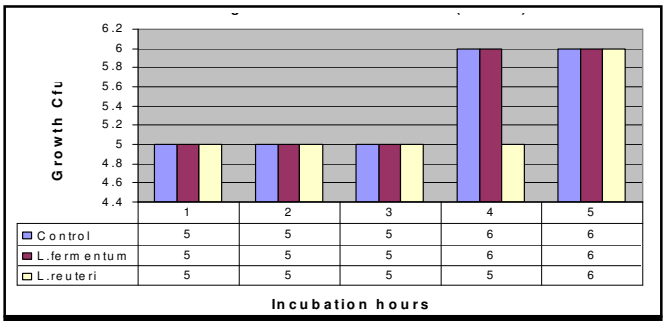

Fig. 3: Bile tolerence (Cfu/ml)

Conclusion:

This review will report on recent efforts involving the use of such strains with a particular focus on emerging probiotic therapies for human and livestock's. The area of study involves the use of both *in vitro* and *in vivo* studies aimed at determining the impact of probiotic properties on the ability to provide a positive health benefit to the host. This review has highlighted the most promising of these studies, including those involving human and animal applications. It could be concluded that *Lactobacillus* is one of the normal bacterial flora of the breast milk of human beings. *L.fermentum* and *L.reuteri* were the most predominant isolates and showed high probiotic effect against the most predominant pathogens isolated from clinical specimen. The ability to survive acidic conditions, bile tolerance and the production of gas may have potential applications. This will help these two strains to reach the small intestine and colon and contributing the balance of the intestinal mocrobiota. In addition most strains were susceptible to antibiotics tested. This belonged to the major classes of antibiotics used in human clinical therapy. The absence of antibiotic resistance can be considered a positive trait for bacteria used in probiotic food productions. This result suggests that these two strains are favorable for use as probiotics. It should be suitable strains for probiotic use of human being and animals.

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