Synopsis of the thesis on

Study of *Lactobacillus* isolates from human sources with regard to their beneficial physiological attributes

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Introduction:

Probiotics are microorganisms that are known to provide health benefits when consumed (Rijkers et al. 2011). The term probiotic is currently used to signify ingested microorganisms associated with beneficial effects to humans and other animals. A significant expansion of the potential market for probiotics has led to increase the requirement for scientific substantiation of putative beneficial effects conferred by the microorganisms. According to FAO of UN and WHO, probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (WHO 2001). The genus *Lactobacillus* comprises a large heterogeneous group of low-G+C content Gram-positive, non-sporulating, and facultative anaerobes. There are several reports available which provide evidence for the health promoting properties of lactobacilli. These organisms are known to favourably alter the intestinal microflora balance, promote intestinal integrity and mobility, inhibit the growth of pathogens and increase resistance to infection (Veldman 1992). They should be able to survive in the gastrointestinal (GI) tract and have to be safe for the consumer (Tuomola et al. 2001; De Vries et al. 2006). Survivability and colonization in the digestive tract are desirable properties for any probiotic, which depends on several factors including the ability of the bacteria to tolerate acidic pH, bile toxicity of the digestive tract and on the adhesion of bacteria to intestinal cells and mucus. Several probiotic effects are mediated through immune regulation, particularly through establishing and maintaining a balance between pro- and anti- inflammatory cytokines (Winkler et al. 2007). Hence the study of immunomodulatory properties of the probiotics is also high on priority. Lactobacilli have been shown to activate monocytes, macrophages and PMNs, which are important in antigen processing, presentation and activation of antigen-specific immune response, i.e. cell-mediated immunity. Probiotics can modulate macrophage function with outcome dependent on the macrophage subset present (Benoit et al. 2008).

Adhesion is considered as a potential probiotic feature along with other desirable attributes for screening of novel probiotic lactobacilli (Kleeman and Klaenhammer 1982; Conway et al. 1987). Several *in vitro* models have been established to study bacterial adhesion and

competitive inhibition ability to screen probiotic strains (Laparra and Sanz 2009). The human intestinal epithelial cell lines- Caco-2 and HT-29 have been extensively used to study adhesion ability of lactobacilli. This is primarily because the Caco-2 cells express morphological and functional differentiation of mature enterocytes including polarization and functional brush border *in vitro* (Sambuy et al. 2005), whereas HT 29 cell-line is derived from human intestinal mucus secreting goblet cells as reported by Lesuffleur et al. (1991).

Lactic acid bacteria are normal components of the intestinal microflora in both humans and animals and have been associated with various health-promoting properties. For this reason, there has been much interest in developing food products containing these bacteria as dietary adjuncts (Salminen et al. 1998). Lactobacilli are frequently used in products for human consumption and can be found as probiotics in infant foods, cultured milks, and various pharmaceutical preparations. The health claims of ingesting live cells of lactobacilli could be due to several possible mechanisms, which may include restoration of normal intestinal flora and removal of pathogens, accumulation of their metabolites including organic acids in the intestine and enhancement in the normal functioning of digestive tract (Corral et al. 2006). One of the beneficial effects that have been suggested to result from human consumption of LAB is amelioration of symptoms of lactose intolerance. One of the glycosidases is β-galactosidase an enzyme that is widely used in dairy industry and it is produced by most lactobacilli (Karasova et al. 2002). Low activity of β-galactosidase causes digestive insufficiency, called lactose intolerance in most cases (Karasova et al. 2002). The addition of lactobacilli producing high β-galactosidase enzyme as probiotic can be used for improvement of lactose digestion in dairy products. Another beneficial effect is, their α-galactosidases, in order to ascertain the potential use of probiotics as suppliers of α-galactosidase in processed pulse and soya-based foods. α-galactosidases are the exoglycosidases that helps to cleave the α-1, 6 linked galactose residues from α-D-galactosides such as melibiose, raffinose and stachyose, and branched polysaccharides such as galactomannans and galactoglucomannans (Naumoff 2005). Since humans lack α-galactosidases in the intestinal mucosa, there is a chance for the formation

of flatus due to the fermentation of such oligosaccharides by intestinal microflora (Gote et al. 2004). Therefore it is necessary to remove the oligosaccharides from food with the help of α-galactosidases. Some studies have shown that adding probiotics to pulse products can indeed reduce gastrointestinal discomfort due to gas (LeBlanc et al. 2008). Other beneficial effect that has been suggested to result from human consumption of LAB is a reduction in serum cholesterol levels, as suggested by the results of several human and animal studies (Pereira and Gibson 2002; Pereira et al. 2003). The *in vitro* experiments have demonstrated that many LAB have an ability to reduce cholesterol level in the growth medium containing bile salts. These cholesterol-lowering effects can be partially ascribed to Bile salt hydrolase (BSH) activity (Klaver and Van Der Meer 1993; Hosono 1999). Bile salt hydrolase enzyme of lactobacilli plays a significant role in cholesterol removal by deconjugating the bile salts (Smet et al. 1994). Deconjugated bile salts are less soluble and less efficiently reabsorbed from the intestinal lumen than their conjugated counterparts, which results in excretion of larger amounts of free bile acids in feces, also, free bile salts are less efficient in the solubilization and absorption of lipids in the gut (Reynier et al. 1981). Therefore, deconjugation of bile acids by LAB bacteria could lead towards a reduction in serum cholesterol either by increasing the demand of cholesterol for de novo synthesis of bile acids to replace that lost in faeces or by reducing cholesterol solubility and, thereby, absorption of cholesterol throughout the intestinal lumen. *Lactobacillus* strains also have the ability to remove cholesterol by co-precipitation of cholesterol with free bile salts. The cholesterol-reducing ability also may be due to the assimilation of cholesterol by cells or attachment of cholesterol to the surface of *Lactobacillus* cells. Moreover, Gilliland et al. (1985) have observed a significant relationship between cholesterol assimilation by lactobacilli and their degree of bile deconjugation.

Under the present study, the probiotic properties of *Lactobacillus* isolates have been studied for their immunomodulatory role and possible application in human health and disease and have been compared with standard stain *Lactobacillus rhamnosus* GG (LGG) to help select an isolate with superior health promoting features. In order to achieve this, the objectives of this study were the following:

Objectives:

1. Isolation, identification and characterization of *Lactobacillus* strains from human sources.
2. Study production of β-galactosidase and α-galactosidase and cholesterol removal by *Lactobacillus* strains.
3. Study immunomodulating potential of *Lactobacillus* strains on PBMCs, PMNs and macrophages.

Objective 1

Isolation, identification and characterization of *Lactobacillus* strains from human gut origin.

Isolation of *Lactobacillu*s has been carried out from human gut origin. Lactobacilli were isolated on Rogosa SL agar (selective media for lactobacilli) and propagated on MRS agar. The primary screening was carried out on the basis of Gram staining and biochemical character like presence of catalase. The isolates which showed Gram positive nature and catalase negative phenotype were further analysed with molecular technique to identify them at species level. The 16-23S rRNA gene intergenic region of the isolates was amplified using 16-1A and 23-1B specific primers and the amplification profile was analysed on agarose gel electrophoresis as reported by Tannock et al. (1999). The two bands obtained corresponding to large and small 16-23S rRNA gene intergenic regions were compared with that of standard *Lactobacillus* strains – *Lactobacillus rhamnosus* GG (LGG) and ATCC 8014. At least one isolate from each sample which showed similar profile compared to that of standard where further processed for sequencing. The sequence obtained was submitted to NCBI database and isolates were identified on the basis of maximum sequence homology in BLAST analysis. Standard strains *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus plantarum* ATCC 8014 were obtained as kind gift from Dr. Shira Doron, MD, Department of Medicine, Tufts–New England Medical Center, USA and Food and Drugs Laboratory (FDL), Vadodara, India respectively. For all the analysis related to probiotic properties, LGG was considered as the established probiotic strain and results were compared with that of LGG.

Table 1: 16-23S sequence analysis and GenBank submission of selected isolates.

No.	Isolate	Source (Human isolate of gut origin)	Accession number	16S–23S sequence based species identification	Similarity of 16S–23S sequence to that of reference strain in GenBank (%)
1	GPI-1(S)	Human gut isolate	JX118837	*L. salivarius*	86
2	GPI-4	Human gut isolate	JX118830	*L. salivarius*	91
3	GRI-2	Human gut isolate	JX118835	*L. plantarum*	99
4	GPI-1(B)	Human gut isolate	JX118836	*L. fermentum*	98
5	GPI-6	Human gut isolate	JX118833	*L. fermentum*	99
6	GPI-7	Human gut isolate	JX118831	*L. fermentum*	94

Table 2: Some other lab isolates used for the present study.

No.	Isolate	Accession number	Source
1	*L. fermentum* GKI-1	JX118832	Human gut isolate
2	*L. fermentum* GPI-3	JX118834	Human gut isolate
3	*L. plantarum* CS24.2	FJ870560	Child gut isolate
4	*L. fermentum* ASt1	FJ899642	Human gut isolate
5	*L. delbrueckii* M	FJ899641	Curd of buffalo milk
6	*L. casei* CS5.2	FJ899643	Child gut isolate
7	*L. plantarum* CS23	FJ899639	Child gut isolate
8	*L. rhamnosus* CS25	FJ899640	Child gut isolate
9	*L. rhamnosus* SCA	JX118842	Child gut isolate
10	*L. rhamnosus* SCB	JX118841	Child gut isolate
11	*L. fermentum* FA-5	KT337435	Fermented soybean seeds (Agya)
12	*L. fermentum* FA-1	KT337434	Fermented bamboo shoot (Iku)
13	*L. helveticus* FA-7	KT337436	Fermented rice (Nyogrin)
14	*L. fermentum* IIS11.2	KT337437	Child gut isolate

The isolates were analysed for their probiotic properties which includes tolerance to acid and bile, antimicrobial activity, antibiotic susceptibility assay and as well as their cell surface properties like cell adhesion assays. The different *Lactobacillus* strains used in this study were able to tolerate bile up to 3% (w/v) in MRS medium, most of the isolates had equal or better ability to grow in presence of bile. As compared to the standard strain LGG, many isolates showed better tolerance to 1% bile concentration in MRS broth. Isolate GPI-6 showed best survival rate (91% at 0.3% bile salt, 89% at 1% bile salt) in contrast to

standard strain LGG (81% at 0.3% and 1% bile salt). These isolates exhibit the property of bile tolerance which is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of host. In order to survive in the GI tract, the organisms need to survive at low pH and as evident from the data they have required ability to survive acidic conditions, which is a critical factor in selecting a potential probiotic. Survival rate of lactobacilli in acidic buffer (pH 2.5) was examined by the difference in viable cell counts following 0 h and 2 h incubation. All the isolates showed better tolerance to acidic pH of 2.5 as compared to standard strain LGG (73%), isolates GRI-2 and GPI-4 showed no reduction in viability while isolate GPI-1(B) and GPI-1(S) showed 84% and 79% survival rate respectively in acidic pH. In the next set of experiment, antimicrobial activity of the isolated lactobacilli was examined against test pathogens which includes *Shigella dysenteriae*, *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC25668), *Salmonella typhi* (MTCC 733), *Proteus vulgaris* and *Escherichia coli* O26:H11 (*E. coli*) by agar spot test. Isolates GPI-7 and GPI-1(B) had the highest zone of inhibition against both Gram-positive and Gram-negative bacteria, followed by GPI-1(S), GPI-4 and GRI-2. Later on the tests for antibiotic susceptibility were performed by disc diffusion method and all the isolated strains of *Lactobacillus* were found to be resistant to Kanamycin and Ofloxacin antibiotic. However, *L. salivarius* isolates GPI-1(S) and GPI-4, *L. fermentum* isolates GPI-1(B) and GPI-6 were found to be resistant (no zone of inhibition) to all the tested antibiotics.

Adhesion ability of *Lactobacillus* strains to intestinal epithelial cell lines and adhesion inhibition of enteropathogen.

The adhesion ability of *Lactobacillus* strains was assayed by their binding to intestinal epithelial cell lines Caco-2 and HT-29. Isolates GPI-4 and GPI-7 showed significantly higher adhesion to HT-29 than that of the LGG. When the same study was performed with Caco-2 cells, none of the isolates showed better binding to the Caco-2 than LGG. All the isolates except GPI-6 and GRI-2, were found to have similar adhesion to Caco-2 cells as seen with that of LGG. Further the strains were studied for their ability to inhibit adhesion of *E. coli* to the two intestinal cell lines (Caco-2 and HT-29). To mimic the *in vivo*

condition, the assay was done three ways, where 1) *Lactobacillus* were allowed to adhered first followed by *E. coli* adhesion (Adhesion inhibition assay), 2) *E. coli* was first incubated to the cell-line followed by *Lactobacillus* adhesion (Displacement inhibition assay) or 3) both *Lactobacillus* and *E. coli* were simultaneously allowed to adhere (competitive inhibition assay). When compared to LGG, some of the isolates (GPI-7, GPI-6 and GPI-1(B)) showed significantly higher reduction in *E. coli* adhesion to HT-29 cells in all the three assays. When the same study was done using Caco-2 cell-line, compared to the standard strain LGG, significantly higher reduction in *E. coli* adhesion to Caco-2 cells was observed with GPI-1(S), GPI-6 and GRI-2 in all three assays.

Objective 2

Study production of β-galactosidase and α-galactosidase and cholesterol removal by *Lactobacillus* strains.

β-galactosidase production by different *Lactobacillus* strains.

Lactobacillus isolates were allowed to grow on MRS-X-gal agar plate. Most of the cultures except M, ASt-1, CS25, SCA and SCB were able to produce blue coloured colonies, indicating their ability to produce β-galactosidase enzyme. Further β-galactosidase activity in whole cells was determined. Overnight grown cultures of the *Lactobacillus* strains were harvested by centrifugation, washed twice in PBS (pH 7.0) and inoculated (1% v/v) in MRS-lac broth. Cultures were incubated at 37°C for 24 h. Cells were then harvested and washed with PBS and A_{560} nm was adjusted to approximately 1.0 with the same buffer. Cell suspension was permeabilized with toluene/acetone (1:9 v/v) solution, vortexed for 7 min and immediately assayed for β-galactosidase activity. β-galactosidase activity was calculated in miller units as follows. Most of the cultures showed higher values than both standard strains LGG and ATCC 8014. Excellent levels were found for GPI-1(S), GPI-6 and GPI-3 which were about 2 folds compared to LGG and ATCC 8014.

α-galactosidase production by different *Lactobacillus* strains.

All of the organisms were assessed for the α-galactosidase activity. Cells of the *Lactobacillus* culture were inoculated into sterile MRS broth and incubated at 37°C for 24 h. After 24 h incubation, the cells were harvested by centrifugation. The supernatant was discarded and the pellet was washed with cold 50 mM sodium phosphate buffer (pH 5.5). Finally, cells were resuspended in the same buffer and sonicated. The cell debris was removed by centrifugation. The resultant supernatant was used as a crude enzyme extract. Crude enzyme extracts from the organisms were assayed for α-galactosidase activity. The α-galactosidase activity was determined by the rate of hydrolysis of PNPG. Most of the cultures showed better α-galactosidase activity as compared to both standard strains LGG (0.074 U mg protein^{-1}) and ATCC 8014 (0.157 U mg protein^{-1}). *L. salivarius* GPI-1(S) (12.939 U mg protein^{-1}) showed highest level of α-galactosidase activity followed by *L. fermentum* FA-5 (9.627 U mg protein^{-1}) and *L. helveticus* FA-7 (8.150 U mg protein^{-1}).

Cholesterol removal by different *Lactobacillus* strains.

The cells were grown overnight in MRS broth followed by centrifugation and washed with PBS. 1×10^8 cells were suspended in 1 ml of 0.3% oxbile MRS broth containing cholesterol. Cells were allowed to grow for 24 h at 37°C. Cells were pelleted down by centrifugation. Following which the supernatant was used for cholesterol estimation by colorimetric analysis. This assay was done with help of cholesterol estimation kit (Reckon Diagnostics, Baroda, India). The cholesterol removal percentage was calculated in supernatant, most of the cultures showed good cholesterol removal in supernatant than both standard strains LGG and ATCC 8014, excepting CS25, IIS11.2 and GKI-1. However SCB (78.76%) showed best cholesterol lowering ratio amongst all, while strains CS24.2 (50.21%), CS23 (45.42%), GPI-1(S) (45.35%) and M (45.43%) were better than LGG (21.13%) and ATCC 8014 (30.90%).

Objective 3

Study immunomodulating potential of *Lactobacillus* strains on PBMCs, PMNs and macrophages.

Isolation of mononuclear cells was done from blood of healthy individual by using HISTOPAQUE®-1077. A buffy coat layer (PBMCs) obtained below the plasma and above the HISTOPAQUE®-1077 solution was aspirated out and washed with Dulbecco's PBS (DPBS). The cells were resuspended in RPMI-1640 media (with 10% FBS v/v) and the cell-density was adjusted to 10^6 cells ml^{-1} in RPMI-1640 media. Further, the cells were transferred to a 24 well-plate. For macrophages, plate was kept at 37°C, 5% CO_2 for 2 h to select for adherent macrophages. Following this the PBMCs and macrophages were incubated with different lactobacilli cells for 12-16 h at 37°C in 5% CO_2. After incubation the media was removed and the cells were washed twice with DPBS. PBMCs and differentiated monocytes i.e, macrophages were extracted and were further used for RNA isolation and subsequently cDNA was prepared. Stability of cDNA prepared was confirmed by β-actin gene amplification. Gene expression of M1 and M2 markers were studied using semi quantitative RT-PCR. GAPDH is used as an internal control. Strains which were able to stimulate M1 macrophages will lead to generation of pro-inflammatory cytokines, whereas those strains which were able to stimulate M2 macrophages will lead to generation of anti-inflammatory cytokines. Generation of anti-inflammatory cytokines is considered to be beneficial at the state of an infection. PBMCs and macrophages both showed high level of M2 marker expression and low level of M1 marker expression after treated with *L. salivarius* strains GPI-1(S) and GPI-4. Same procedure will be used to studying immunomodulatory role of these isolated lactobacilli co-incubated with PMNs.

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