Synopsis of the thesis on

Studies on native *Lactobacillus* isolates for their potential use against gut dysfunction

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INTRODUCTION:

Probiotics are living microorganisms which confer health benefits on the host when administered in sufficient amounts. For probiotics to be successful, they must possess certain properties such as resistant to bile salts and acidic environment of the GIT, colonizing ability to the intestine, antibacterial property, safe to the host upon consumption etc. Strains of *Lactobacillus* and *Bifidobacterium* are the most commonly applied probiotics in food products. *Lactobacillus* is a gram positive, non-pathogenic, non-spore forming and rod shaped non-motile bacteria. They are widely used to process foods and beverages, including beer, fermented olives and dairy products (Lourens- Hattingh *et al.,* 2001). In the human body, lactobacilli may colonize three anatomic regions: the oral cavity, the intestines and the vaginal tract. It has been widely explored for their health promoting application under the concept of "Probiotics". The health promoting effect of lactobacilli includes stabilization of indigenous microflora, protection against intestinal infection (Roberfroid MB., 2002), non-specific enhancement of the immune system, protection against epithelial barrier dysfunction induced by pathogens, alleviation of lactose tolerance (DeVerse *et al.,* 1992), reduction of serum cholesterol (Noh *et al.,* 1997) etc. *Lactobacilli* are increasingly being viewed as an alternative therapeutic agents or food supplements for providing health benefits to individuals.

Bacteria used as probiotic adjuncts are commonly delivered in a food system and, therefore, begin their journey to the lower intestinal tract via the mouth. As such, probiotic bacteria should have the ability to resist the digestion process in the stomach and the intestinal tract. Cellular stress begins in the stomach, which has a pH value as low as 1.5 to 2. After the bacteria pass through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. The concentration of bile in the human gastrointestinal system is variable and is difficult to predict at any given moment (Lankaputhra and Shah, 1995). After travelling through this harsh environment, the organism colonizes the epithelium of the lower intestinal tract (Conway et al., 1987). The survival of probiotic organisms in the gut depends on the colonization factors that they possess organelles which enable them to resist the antibacterial mechanisms that operate in the gut. In addition to the antibacterial mechanisms, they need to avoid the effects of peristalsis, which tend to flush out bacteria with food. This can be achieved either by immobilizing themselves or by growing at a much faster rate than the rate of removal by peristalsis. Thus, strains selected for use as probiotic bacteria should be able to

tolerate acid and bile acids, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits.

The epithelial cells are tightly bound together by intercellular junctional complexes that regulate the paracellular permeability and are crucial for the integrity of the epithelial barrier. The Tight junction complex comprises of integral membrane proteins including occludin, claudins, and junction adhesion molecule that interact with the zonulins zonula occludens (ZO)-1, ZO-2, and ZO-3 that are in turn bound to the perijunctional ring of cytoskeletal actin (Paris L. *et al.,* 2008; Schneeberger E.E.*et al.,* 2004). Increased intestinal permeability or "leaky gut" is recognized as having a role in the pathophysiology of a variety of gastrointestinal disorders and is observed in inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), celiac disease, and early stages of colon cancer (Clayburgh D.R. *et al.,* 2004). Altered permeability also causes cytokine induced changes in the tight junction and a vicious cycle of mucosal barrier dysfunction and inflammation, increase in the load of bacterial and dietary antigens in the lamina propria (Breuwer M. *et al*., 2006; Mankertz J. *et al.,* 2007). Lactobacilli have been demonstrated to improve barrier integrity in mouse model of colitis (Johan D. *et al.,* 2012) as well as human patients with Crohn's disease (Gionchetti *et al.*,2003; Rembacken *et al.,* 1999) or chemically induced barrier dysfunction, while reversing the effect of pathogens on intestinal barrier function. Therefore, the modulation of epithelial permeability is a highly relevant target for novel therapeutic or prophylactic treatments against a range of diseases. Several lactobacilli have been used as a therapeutic agent against gut dysfunction. These mitigating effects of lactobacilli are mediated through increased expression of plaque proteins such as ZO or claudin or redistribution of TJ associated proteins (Zyrek A.A. *et al*.,2007; Ewaschuk J.B. *et al*., 2010) indicating that different probiotics may use different pathways to modulate barrier function.

The present study thus aims to isolate the native *Lactobacillus* from various sources and characterize them for their probiotic properties. The isolates having probiotic properties are further studied for their potential use against gut dysfunction.

OBJECTIVES:

1. Isolation, identification and biochemical characterization of *Lactobacillus* isolates from various sources.
2. Adhesion ability of *Lactobacillus* isolates to intestinal epithelial cell lines.

3. Neutralization effect of *Lactobacillus* isolates on *E.coli* induced epithelial barrier dysfunction.
4. *In-vivo* studies on ability of *Lactobacillus* isolates to improve the effect of colitis in mice induced by Dextran Sodium Sulphate (DSS).

RESULTS:

Isolation, identification and biochemical characterization of *Lactobacillus* isolates from various sources.

Lactobacilli were isolated from healthy human stool samples and fermented food samples using Rogosa SL agar medium followed by their growth on MRS agar. They were further analyzed for their gram positive nature and absence of catalase enzyme. The molecular identification was carried out by 16s-23s rRNA intergenic spacer region amplification using primers specific for 16-1A and 23-1B region. The amplified product was analysed as reported by Tannock et al. (1999). Two bands corresponding to the large and small 16S–23S rRNA gene intergenic region were obtained of which the smaller band was eluted and re-amplified using the same set of primers and the resultant amplicon was sequenced. The sequences were compared with the 16S–23S rRNA gene intergenic small spacer region sequences of GenBank and same sequences of the isolates were submitted to GenBank and their accession numbers are listed in Table. Standard strain Lactobacillus rhamnosus GG (LGG) was obtained as a kind gift from Dr. Shira Doron (MD, Department of Medicine, Tufts Medical Centre, Boston, MA, USA).

Table 1. Identification of Lactobacillus isolates based on similarity to 16S–23S rRNA small intergenic spacer region sequences with database in GenBank.

No.	Isolate	Source	Accession number	16S–23S sequence based species identification
1	FA-1	Fermented bamboo shoot (Iku)	KT337434	*Lactobacillus fermentum*
2	FA-5	Fermented soybean seeds (Agya)	KT337435	*Lactobacillus fermentum*
3	FA-7	Fermented rice (Nyogrin)	KT337436	*Lactobacillus helveticus*
4	GPI-3	Faeces of adult human	JX118834	*Lactobacillus fermentum*
5	IIS11.2	Faeces of human infant	KT337437	*Lactobacillus fermentum*

6	GKI-1	Faeces of adult human	JX118832	*Lactobacillus fermentum*

Table 2: Some other lab Isolates used in the present study

No.	Isolate	Source	Accession number	16S–23S sequence based species identification
1	GPI-1(S)	Faeces of adult human	JX118837	*Lactobacillus salivarius*
2	GPI-4	Faeces of adult human	JX118830	*Lactobacillus salivarius*
3	GRI-2	Faeces of adult human	JX118835	*Lactobacillus plantarum*
4	GPI-1(B)	Faeces of adult human	JX118836	*Lactobacillus fermentum*
5	GPI-6	Faeces of adult human	JX118833	*Lactobacillus fermentum*
6	GPI-7	Faeces of adult human	JX118831	*Lactobacillus fermentum*

The lactobacilli strains were analyzed for their probiotic properties such as acid and bile tolerant, antibiotic susceptibility and antimicrobial activity against pathogens. To study the survival of these isolates under bile or acidic condition, the isolates were incubated in the MRS broth containing 0.3% and 1% bile salt or MRS broth adjusted to 2.5pH for 2 h and their survival was studied by measuring CFUs at 0 h and 2 h incubation. The isolates FA-1, FA-5 and GKI-1 (85%, 83% and 90% at 0.3% bile; and 82%, 83% and 83% at 1 % bile concentration, respectively) were having better survival than the standard strain LGG (81%) in both 0.3% and 1% bile salt containing MRS broth. Except FA-1 and GKI-1, all the other strains had better survival (upto 81%) at pH 2.5 than the standard strain LGG (73%). The survival of the bacteria in the acidic and bile containing environment of the GIT is important for their own endurance in the gut as well as to aid beneficial effect(s) to the host. The antimicrobial activity of the isolates against *E. coli* serotype O26:H11 (EPEC), *Salmonella entericas*erovar Typhi (MTCC 733), *Shigella dysentery*, *Staphylococcus aureus*, *Proteus vulgaris* and *Pseudomonas aeruginosa* were analysed by agar spot assay. Amongst all the isolates, GKI-1 exerted maximum antimicrobial activity against all the pathogens as measured by zone of inhibition. Furthermore, the antibiotic sensitivity assay was carried out by disc diffusion method. The isolates FA-1, FA-5 and GPI-3 were found to be resistant to all the antibiotics tested.

Adhesion ability of *Lactobacillus* isolates to intestinal epithelial cell lines.

To study the adhesion ability of the isolates to the intestinal epithelial cell-lines, the lactobacilli isolates were incubated with Caco-2 and HT-29, individually, and the cells adhered to these cell-lines were measured by CFU on MRS agar plate. The adhesion ability of these isolates were compared with that of the standard strain LGG and it was observed that isolate FA-1 is having better adhesion ability to HT-29 cells compared to LGG, whereas, the adhesion ability of GKI-1 to HT-29 cells was similar to that of the LGG. However, when the same study was carried out using Caco-2 cell-line, none of the isolates had better adhesion than LGG. The adhesion ability of all the isolates was similar to that of the standard strain LGG except isolates FA-1 and GKI-1. Furthermore, the isolates were studied for their antagonistic effect on adhesion of pathogenic *E.coli* to the Caco-2 and HT-29 cell-lines. To mimic the in-vivo condition, the assay was performed in three different ways: Adhesion inhibition (AI) assay in which monolayer was first incubated with lactobacilli followed by *E.coli* incubation, Displacement inhibition (DI) assay wherein *E.coli* is allowed to adhere first to the monolayer, followed by lactobacilli adhesion, and competitive inhibition (CI) assay in which lactobacilli and *E.coli* were allowed to adhere simultaneously to the monolayer. When monolayer was incubated with IIs11.2 and GKI-1, lower *E.coli* adhesion was observed in all the three assay conditions compared to incubation with LGG. However, when Caco-2 cell-line was used to study displacement inhibition assay, *E.coli* reduction was higher with all the isolates (except FA-1 and FA-5) compared to LGG. None of the isolates were having the better antagonistic effect on *E.coli* adhesion than LGG in all three assays.

Neutralization effect of *Lactobacillus* isolates on *E.coli* induced epithelial barrier dysfunction.

The neutralization effect of the lactobacilli isolates on epithelial barrier dysfunction was studied by measurement of TEER and permeability for macromolecules across the monolayer at different time interval. Decrease in the TEER observed after *E.coli* infection was reversed by lactobacilli treatment. Using Caco-2 monolayer, reversal of TEER reduction was observed after 2 h incubation with *L. fermentum* FA-1 and LGG, whereas with the other strains, it was observed after 4 h of incubation which was continued at 16 and 18 h of treatment with lactobacilli. Whereas, using HT-29 cell-line, reversal of TEER reduction was observed by some of the lactobacilli strains (LGG, *L. fermentum* strains GPI-3 and IIs11.2, *L. plantarum* GRI-2 and *L. salivarius* GPI-1(s)) following 4 h of their exposure to *E. coli* infected monolayer. Similarly, the increased permeability of the monolayer induced by *E.coli* was also reversed after lactobacilli treatment. Maximum reduction (3.8 fold and 3.5 fold) in the beads

translocation was observed when monolayer was treated with LGG and *L. fermentum* GPI-3 respectively, for 4 h. Later on the study was conducted to analyze the effects of these isolates on tight junction (TJ) protein expression (claudin-1, claudin-4, JAM-1, ZO-1 and occludin) in *E.coli* infected monolayer by quantitative real-time PCR and immunoflourescence assay. Isolate FA-7 was able to increase the mRNA expression of ZO-1 and claudin-1 to 2.5 and 3 fold, whereas the other isolates had no effect on the mRNA expression of tight junction proteins. Immunoflourescence assay revealed that the distribution of TJ proteins in the Caco-2 monolayer was altered after *E.coli* infection. FA-1 treated Caco-2 monolayer exhibited the well-defined staining pattern at the cell periphery for all the TJ proteins. This redistribution of TJ proteins was also observed in *L. plantarum* GRI-2 treated Caco-2 cells except for JAM-1 protein although JAM-1 protein was less disturbed than *E.coli* infected monolayer.

Work to be done:

1. Histopathological analysis of colon samples from lactobacilli treated DSS-induced colitis mice.
2. Quantitative real time PCR analysis of TJ proteins from colon samples to study the effect of lactobacilli on DSS induced colitis in mice.

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