CHAPTER 2

Isolation, Identification and Screening of Probiotic Lactobacilli of Human Origin

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Introduction

"Probiotic are live microorganisms which when taken in adequate amount confer the health benefit on the host" (FAO/WHO). In fact, probiotics have been used for as long as people have eaten fermented foods. However, it was Metchnikoff at the turn of the 20th century who first suggested that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract. He hypothesized that lactobacilli were important for human health, longevity, and promoted yogurt and other fermented foods as healthy (Metchnikoff, 1907). Lactic acid bacteria (LAB) are Gram-positive, non-spore forming cocci, coccobacilli or rods and most genera have a DNA base composition of less than 50% G+C, lack catalase, grow under microaerophilic or anaerobic conditions, and typically ferment glucose mainly to lactic acid (homo-fermentative), but can also lave lactic acid, CO_2, and ethanol/acetic acid as end product (hetero-fermentative). The genus *Lactobacillus* belongs to the large group of lactic acid bacteria. The genus *Lactobacillus* belongs phylogenetically to the phylum *Firmicutes* (Garrity *et al*., 2004). The family *Lactobacillaceae* comprises the main family in the order *Lactobacillales* which itself belongs to the class Bacilli. Lactobacilli can be found in a variety of ecological niches, such as plants (fruits, vegetables, cereal grains) or plant-derived materials, silage, fermented foods (yogurt, cheese, olives, pickles, salami, etc.), as well as in the oral cavities, gastrointestinal tract, and vaginas of human and animals. The prescribed sources are provides the sites for isolation of novel probiotics. The bacteria that occupy a niche in the GIT are residents or autochthonous (i.e., found where they are formed). Other bacteria are just a "get a lift" through the gut and are allochthonous (i.e., formed in another place). Autochthonous strains have a long-term association with a particular host, and they form stable populations of a characteristic size in a particular region of the gut. It is often difficult to determine whether or not a particular microorganism is truly autochthonous to a particular host (Tannock, 2004). *Lactobacillus* strains of human origin are most suitable candidates for potential probiotic candidate, because they are well adapted to the condition prevailing in the gastrointestinal tract and

therefore be more competitive than probiotics from other sources. The health promoting effects of lactobacilli have been widely explored and include maintaining the microbial homeostatis, protection against intestinal infection, alleviation of lactose intolerance, increased nutritional value of foods, reduction of serum cholesterol level and immunomodulation. Several lactobacilli which act as probiotic bacteria are currently being explored as novel bio therapeutic agents (Mercenier *et al*., 2003). *In vitro* screening for probiotic properties is important because *in vivo* efficacy is expensive and time-consuming. The present study aimed to isolate and characterize for probiotic properties of *Lactobacillus* strains of human origin.

Materials and Methods

Isolation and identification of lactobacilli

Lactobacillus strains were isolated from the infant feces and vaginal mucosa of healthy female. Approximately 1 g fecal sample and vaginal swabs were enriched in the de Man Rogosa Sharpe (MRS; Himedia, Mumbai, India) medium for 48 h at 37°C (De Man *et al*., 1960). 0.1 ml of the enriched culture was serially diluted, plated on MRS agar, and incubated at 37°C until sufficient growth was observed. Individual spindle shaped chalky white colonies were randomly picked, inoculated in MRS medium, and subjected to microscopic observation. Catalase negative, Gram-positive rods were characterized for sugar fermentation, gas production, and their ability to grow at 15°C and 45°C.

Maintenance of cultures

Lactobacillus strains were maintained in 10% skim milk at 4°C. Stock culture was maintained in 20% glycerol at -20°C.

Phenotypic characterization of lactobacilli

Growth at 15°C and 45°C and gas production from glucose

0.1 ml of activated cultures were inoculated in 5 ml MRS medium and incubated at 15°C and 45°C. MRS medium containing glucose was placed with inverted Durham's vial and inoculated with activated cultures. The tubes were observed for CO_2 production after incubation at 37°C for 24 h.

Sugar fermentation profile

Sugar fermentation was studied by inoculating 1% activated culture in sterile basal MRS medium (without glucose and beef extract) containing 0.1% andrade's indicator (Himedia, India) and 1% sugar. The tubes were incubated at 37°C overnight. Positive reaction was recorded by the development of pink color indicates the acid production. Appropriate inoculated and un-inoculated controls were also included.

Screening of probiotic lactobacilli

Lactobacillus strains were inoculated in MRS medium, MRS modified with bile salt (2-4%), NaCl (8-10%), pH (2-3) adjusted with 0.1 N HCl and skim milk with phenol (0.4-0.6% phenol) and incubated at 37°C for overnight. For skim milk-phenol medium, curdling of the milk was considered as positive growth. Viability of the isolates was evaluated by inoculating 0.1 ml from the above modified MRS media into MRS medium and incubated overnight at 37°C and observed for the growth.

Antimicrobial activity of cell free culture filtrates of lactobacilli

Preparation of CFC

100 ml MRS medium containing in Erlenmeyer flask was inoculated with activated cultures of *Lactobacillus* strains and incubated at 37°C for 24 h. The entire content of the flask was centrifuged (5000 rpm, 20 min, 4°C), and supernatant was passed through 0.45 µm Millipore filter and used as Cell Free Culture Filtrate.

Antimicrobial activity by well-diffusion agar assay

Antimicrobial activity of CFC filtrate was determined by well-diffusion agar assay (British Standards, 1974) against the organisms mentioned (Table 4 and b). 100 µl of 18 h old cultures of test organisms were inoculated in molten nutrient agar and poured in sterile Petri plates. Wells (7 mm) were made using cup borer and 100 µl of CFC filtrate, was loaded in the wells and pre-incubated at 4°C for 2 h and incubated overnight at 37°C. Appropriate controls were also included.

Antibiotic susceptibility test

Antibiotic susceptibility of lactobacilli was performed according to Charteris *et al.* (1998b) 0.1 ml of 18 h old culture (10^8 cfu/ml) of lactobacilli grown in MRS medium was used to inoculate 25 ml molten MRS agar (1.5% w/v) and poured in sterile petri plates. Antibiotics octadisc (Himedia, India) were placed on MRS agar plates and incubated at 37°C for 24 h and inhibition zones were measured.

Results

Isolation and identification of lactobacilli

Lactobacillus strains isolated from vaginal mucosa and infant feces by pour plate method. A typical colony characteristic of isolated *Lactobacillus* strain is shown in Table 1. The 43 isolates that were Gram-positive rods, arranged singly or in pairs, non-motile and catalase negative were considered LAB.

Phenotypic characteristics of lactobacilli

All the isolates were able to grow at 15°C and slight growth at 10°C but not at 45°C. According to the classification scheme they belong to the group *Streptobacterium* and facultative heterofermenter (Orla Jensen, 1943). All the strains fermented glucose, lactose, ribose and rhamnose. Isolates exhibited diversity in their ability to ferment fructose, cellobiose, maltose, mannitol, mannose and galactose (Table 2). According to sugar fermentation pattern the isolates were tentatively identified as *Lactobacillus rhamnosus* strains. The selected strains Fb and Vc were identified by 16S rDNA sequence analysis.

Table 1. Typical colony characteristics of the *Lactobacillus* strains growing on MRS medium

Parameters	**Results**
Size	Medium
Shape	Circular
Opacity	Opaque
Texture	Smooth
Margin	Entire
Elevation	Raised
Pigmentation	Chalky white

Table 2. Phenotypic characteristics of *Lactobacillus* strains

Isolates	glucose	lactose	fructose	mannitol	mannose	maltose	galactose	cellobiose	ribose	rhamnose	gas production	growth at 15°C	growth at 45°C
Vc1	+	+	+	+	+	+	+	+	+	+	+	+	-
Vc2	+	+	-	+	+	+	+	+	+	+	-	+	-
Vc3	+	+	-	+	+	+	+	+	+	+	+	+	-
Vc4	+	+	-	+	+	+	+	+	+	+	+	+	-
Vc5	+	+	+	+	+	+	+	-	+	+	-	+	-
Vc6	+	+	+	+	+	+	+	-	+	+	+	+	-
Vc7	+	+	+	-	-	+	+	-	+	+	+	+	-
Vc8	+	+	+	-	-	+	+	-	+	+	d	+	-
Vc9	+	+	+	-	-	+	+	+	+	+	d	+	-
Vc10	+	+	+	-	-	+	+	+	+	+	-	+	-
Vc11	+	+	+	-	-	+	+	+	+	+	-	+	-
Vc12	+	+	+	-	-	+	+	-	+	+	+	+	-
Vc13	+	+	+	-	-	+	+	-	+	+	d	+	-
Vc14	+	+	+	-	-	+	+	-	+	+	+	+	-
Vc15	+	+	+	+	+	+	+	-	+	+	-	+	-
Vc16	+	+	+	+	+	+	+	-	+	+	-	+	-
Vc17	+	+	+	+	+	+	+	-	+	+	+	+	-
Vc18	+	+	-	+	+	+	+	-	+	+	-	+	-
Vd1	+	+	-	+	+	+	+	-	+	+	-	+	-
Vd2	+	+	-	+	+	+	+	+	+	+	-	+	-
Vd3	+	+	-	+	+	+	+	+	+	+	d	+	-
Vd4	+	+	-	+	-	+	+	+	+	+	-	+	-
Vd5	+	+	-	+	-	+	+	+	+	+	-	+	-
Vd6	+	+	-	+	-	+	+	+	+	+	+	+	-

d-variable + or -

Table 2. Phenotypic characteristics of *Lactobacillus* strains (Continue…)

Isolates	glucose	lactose	fructose	mannitol	mannose	maltose	galactose	cellobiose	ribose	rhamnose	gas production	growth at 15°C	growth at 45°C
Vd7	+	+	-	-	-	+	+	+	+	+	-	+	-
Vd8	+	+	-	-	+	+	+	+	+	+	-	+	-
Vd9	+	+	-	-	+	+	+	+	+	+	-	+	-
Vd10	+	+	-	-	+	+	+	+	+	+	d	+	-
Vd11	+	+	+	-	+	+	+	+	+	+	d	+	-
Vd12	+	+	+	-	+	+	+	+	+	+	-	+	-
Vd13	+	+	+	+	+	+	+	+	+	+	-	+	-
Vd14	+	+	+	+	-	+	+	+	+	+	-	+	-
Vd15	+	+	+	+	-	+	+	+	+	+	-	+	-
Vd16	+	+	+	+	-	+	+	+	+	+	+	+	-
Vd17	+	+	+	+	-	+	+	+	+	+	d	+	-
Vd18	+	+	+	+	+	+	+	+	+	+	+	+	-
Vd19	+	+	+	-	+	+	+	+	+	+	-	+	-
Vb1	+	+	+	-	+	+	+	+	+	+	+	+	-
Vb2	+	+	+	-	+	+	+	+	+	+	+	+	-
Vb3	+	+	+	+	+	+	+	+	+	+	+	+	-
Vb5	+	+	+	+	+	+	+	+	+	+	+	+	-
Fb	+	+	+	+	+	+	+	+	+	+	d	+	-
Fc	+	+	+	+	+	+	+	+	+	+	-	+	-
Fe	+	+	+	+	+	+	+	+	+	+	-	+	-

d-variable + or -

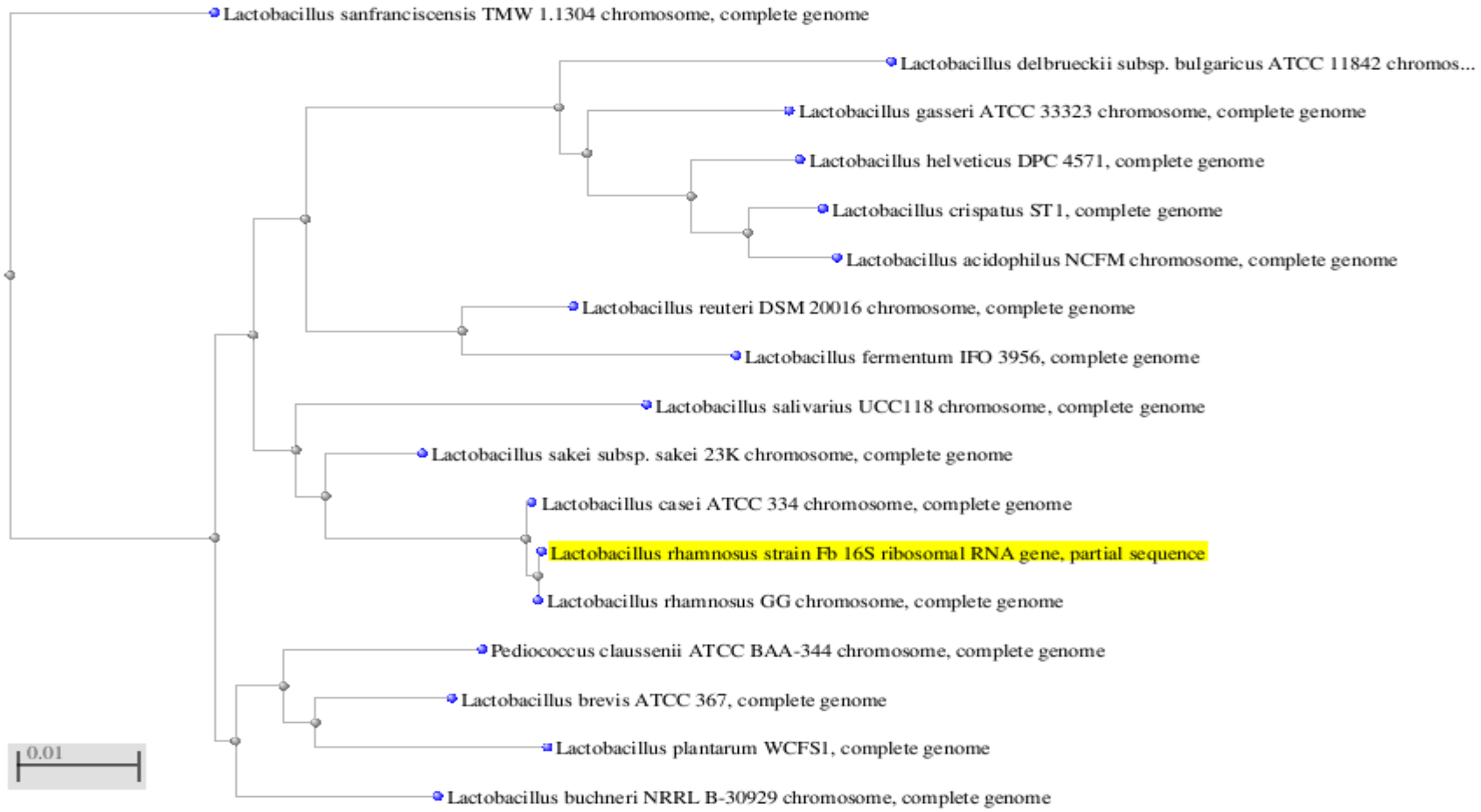

Fig. 1 Phylogenetic tree of *Lactobacillus rhamnosus* Fb (JX406746) showing relationship of the strain nearest in BLAST based on 16S rDNA gene sequence and constructed using neighbor-joining method.

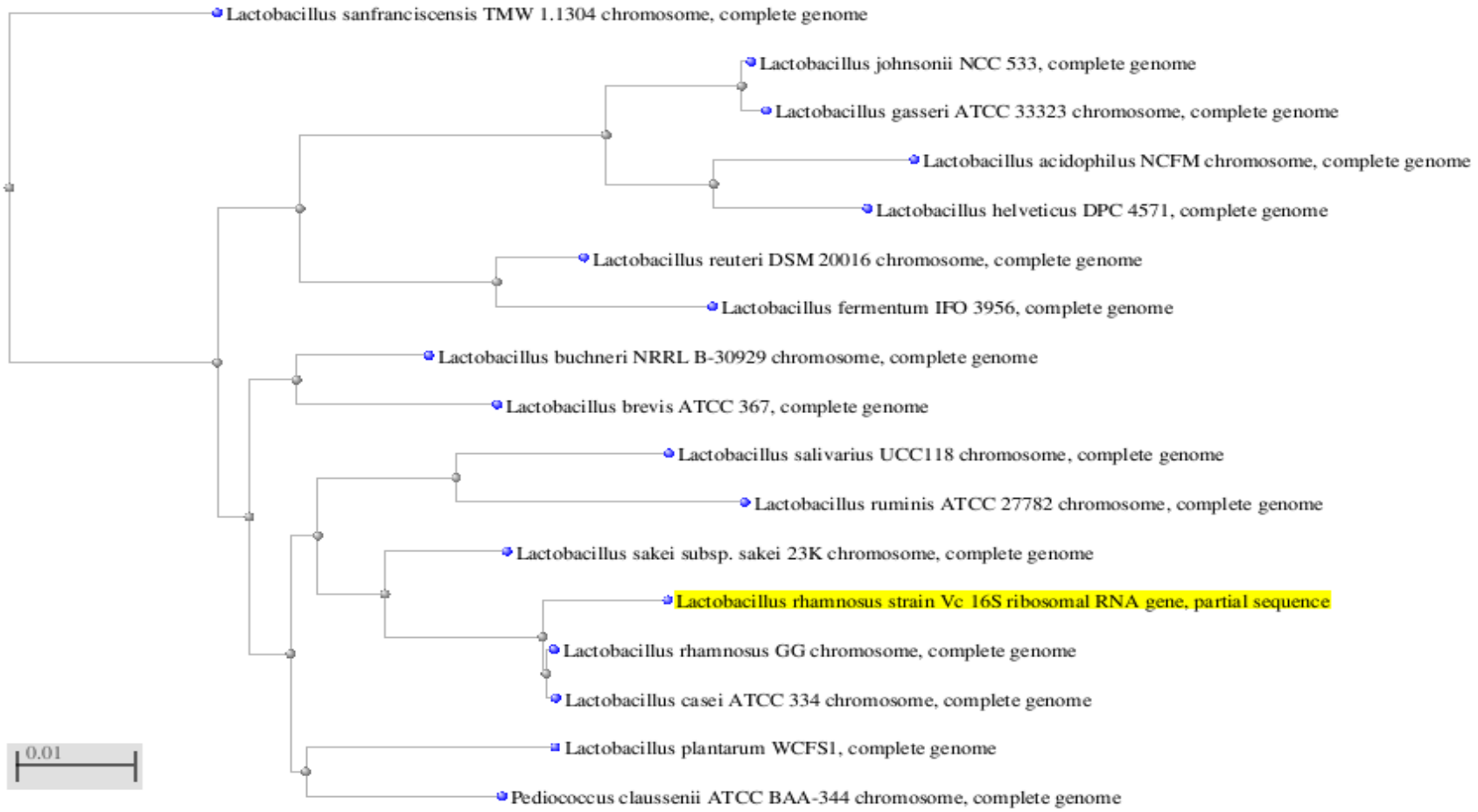

Fig. 1 Phylogenetic tree of *Lactobacillus rhamnosus* Vc (JX406745) showing relationship of the strain nearest in BLAST based on 16S rDNA gene sequence and constructed using neighbor-joining method.

Molecular identification of isolates

Stains Fb and Vc were identified as *Lactobacillus rhamnosus* strains by 16S rDNA sequence analysis and sequences submitted to NCBI (National Center for Biotechnology Information). The 16S rDNA sequence of the isolates showed over 100% query coverage and 99% similarity with the nucleotide sequence database of NCBI GenBank. Gen Bank accession numbers are Fb-JX406746, and Vc-JX406745. Phylogenetic tree of Fb and Vc constructed using BLAST (Fig. 1).

Screening of probiotic lactobacilli

Out of 43 isolates, 3 isolates obtained from vaginal samples Vc3, Vd2, Vd6 and Fb isolated from human infant fecal sample exhibited tolerance to all the prescribed conditions pH 2, bile salt (4%), and phenol (0.6%) (Table 3), whereas 14 isolates were able to tolerate pH 2, 8 isolates were able to tolerate 4% bile salt, 26 isolates were able to tolerate 0.6% phenol. All the isolates were able to grow in the presence of 6% NaCl.

Antimicrobial activity of cell free culture filtrates of lactobacilli

Cell Free Culture filtrate (CFC) of *Lactobacillus* strains grown in MRS medium exhibited broad antimicrobial spectrum against Gram positive and Gram negative organisms (Table 4 a and b) although extent of activity varied with the strains. The extent of inhibition was higher against *B. cereus*, *P. aeruginosa* followed by *Staph. aureus* and *Ent. aerogenes.*

Antibiotic susceptibility test

All the isolates were susceptible to ampicillin, cephalothin, chloramphenicol, clindamycin, cloxacillin, erythromycin, gentamicin, lincomycin, novobiocin, oxacillin, penicillin, and tetracycline (Table 5). However, the same strains were not susceptible to amoxicillin, cephalexin, ciprofloxacin, co-trimoxazole, fusidic acid and vancomycin.

Table 3. Screening of *Lactobacillus* strains for their probiotic properties *i.e*. ability to grow at low pH, and in the presence of bile salt and phenol

	pH		Bile salt (%)		Phenol (%)	
Isolates	2	3	2	4	0.4	0.6
Vc1	-	+	slight	-	+	+
Vc2	+	+	+	-	+	+
Vc3	+	+	+	+	+	+
Vc4	+	+	+	-	+	+
Vc5	-	+	+	-	+	+
Vc6	-	+	+	-	+	+
Vc7	+	+	+	-	+	+
Vc8	+	+	+	-	+	+
Vc9	-	+	+	-	+	+
Vc10	-	+	+	-	+	+
Vc11	-	+	+	-	+	+
Vc12	+	+	+	-	+	+
Vc13	-	+	+	-	+	+
Vc14	-	+	+	-	+	+
Vc15	-	+	+	-	+	+
Vc16	-	+	+	-	+	+
Vc17	-	+	+	-	+	+
Vc18	-	+	+	-	+	+
Vd1	+	+	+	-	+	+
Vd2	+	+	+	+	+	+
Vd3	+	+	+	-	+	+
Vd4	+	+	+	-	+	+
Vd5	+	+	+	-	+	-
Vd6	+	+	+	+	+	-
Vd7	-	+	+	-	+	-
Vd8	-	+	+	-	+	-
Vd9	-	+	+	-	+	+
Vd10	-	+	+	-	+	+
Vd11	-	+	+	-	+	-
Vd12	-	+	+	-	+	-
Vd13	-	+	+	-	+	-
Vd14	-	+	+	-	+	-
Vd15	-	+	+	-	+	-
Vd16	-	+	+	-	+	-
Vd17	-	+	+	-	+	-
Vd18	-	+	+	-	+	-
Vd19	-	+	+	-	+	-
Vb1	-	+	+	+	+	-
Vb2	-	+	+	+	+	-
Vb3	+	+	+	+	+	-
Vb5	-	+	+	+	+	-
Fb	+	+	+	+	+	+
Fc	-	+	+	-	+	+
Fe	-	+	+	-	+	+

+ growth observed, - no growth

Table 4a. Antimicrobial activity of CFC filtrates of 24 h *Lactobacillus* cultures grown in MRS medium at 37°C against selected food-borne Gram-positive pathogens

Lactobacillus strains	**Zone of inhibition*(mm)**			
	B. subtilis	*B. megaterium*	*B. cereus*	*Staph. aureus*
Vc1	19	18	23	16
Vc2	18	15	22	15
Vc3	20	18	20	16
Vc4	19	18	22	15
Vc5	15	15	19	14
Vc6	17	14	19	18
Vc7	13	14	16	14
Vc8	13	14	15	14
Vc9	15	13	16	15
Vc10	16	-	20	15
Vd1	-	-	19	10
Vd2	-	-	19	11
Vd3	-	-	19	10
Vd4	-	-	18	10
Vd5	-	-	20	-
Vd6	-	-	19	10
Vd8	-	-	20	10
Vd9	-	-	21	10
Vd10	-	-	22	10
Vb1	-	-	23	12
Vb2	-	-	24	11
Vb3	-	-	23	11
Vb5	-	-	11	10
Va3	-	-	23	-
Va4	-	15	23	15
Fb	11	14	20	19

*****including 7 mm bore diameter

Table 4b. Antimicrobial activity of CFC filtrates of 24 h *Lactobacillus* cultures grown in MRS medium at 37°C against selected Gram-negative GIT pathogens

Lactobacillus strains	**Zone of inhibition*(mm)**				
	E. coli	*Ent. aerogenes*	*Ps. aeruginosa*	*S. typhi*	*K. pneumoniae*
Vc1	14	15	23	16	-
Vc2	14	13	23	-	-
Vc3	14	14	23	-	10
Vc4	14	12	21	14	-
Vc5	14	12	21	-	-
Vc6	14	18	22	-	-
Vc7	14	14	20	-	10
Vc8	14	13	18	-	-
Vc9	14	15	21	-	-
Vc10	14	13	22	-	-
Vd1	-	17	22	11	-
Vd2	-	19	22	11	-
Vd3	-	13	23	-	-
Vd4	-	19	22	14	-
Vd5	-	15	21	14	-
Vd6	-	-	21	14	11
Vd8	-	-	23	15	-
Vd9	-	-	23	12	-
Vd10	-	-	21	11	-
Vb1	20	16	23	-	-
Vb2	20	-	23	-	-
Vb3	19	-	23	-	-
Vb5	15	-	18	-	-
Va3	-	-	23	-	-
Va4	-	-	22	-	-
Fb	10	13	21	13	12

*including 7 mm bore diameter

Table 5. Antibiotic susceptibility profile of the *Lactobacillus* strains

Antibiotics (µg)	Zone of inhibition (mm)						
	Vc1	Vc3	Vc6	Vd3	Va3	Va4	Fb
Amoxycillin (10)	-	-	-	10	-	-	10
Ampicillin (2)	-	-	-	10	-	10	-
Ampicillin (10)	10	10	10	11	11	11	-
Cephalexin (30)	-	-	10	11	-	-	-
Cephalothin (5)	11	12	11	13	10	12	10
Chloramphenicol (30)	-	16	13	17	17	16	16
Ciprofloxacin (5)	-	11	11	12	11	-	-
Clindamycin (2)	10	20	20	19	19	20	20
Cloxacillin (5)	10	14	-	16	16	15	12
Co-trimoxazole (25)	-	-	-	10	-	-	9
Erythromycin (5)	14	13	10	16	10	14	9
Erythromycin (15)	-	17	16	19	16	16	16
Fusidic acid (10)	-	-	-	-	-	-	-
Gentamicin (10)	-	11	10	12	11	11	11
Lincomycin (2)	18	14	14	21	13	21	16
Novobiocin (5)	19	19	14	23	20	19	12
Oxacillin (1)	-	10	10	10	10	10	11
Penicillin-G (1 unit)	17	16	18	19	18	17	17
Tetracycline (25)	20	20	17	22	16	22	16
Tetracycline (30)	11	17	19	24	13	15	19
Vancomycin (30)	-	-	-	-	-	-	-

(-) indicates no zone of inhibition, not susceptible

Discussion

A focus of the study was to screen potential probiotic *Lactobacillus* strains isolated from the infant feces and vaginal mucosa of healthy females. The basic consideration in selecting the isolates was their natural habitat, since they were selected from the normal flora of vaginal mucosa and infant feces as suggested by Gilliland (1990).

Forty-three isolates that were Gram-positive rods, non-motile, non-spore forming and catalase negative were considered Lactic acid bacteria and further identified by the sugar fermentation pattern. All the strains are facultative heterofermenter and belong to the group *Streptobacterium*, as they grow at 15°C but not at 45°C and on the basis of gas production in glucose fermentation test (Orla Jensen, 1943). All were identified as *Lactobacillus rhamnosus*, predominant species in infant feces as well as vaginal

mucosa. The potential probiotic organism is preferred to be of human origin so that it can lead us to a candidate probiotic eventually targeted for human consumption.

First, probiotic candidates should be of healthy human origin and non-pathogenic; lactobacilli have a long history of being safe for humans and have been conferred GRAS status. Second, the strain has to survive in harsh condition during gastrointestinal transit; gastric fluid is the crucial barrier to overcome prior to reaching the site of action (Dunne *et al*., 2001), which enable their viable passage through the gastrointestinal tract allow them to establish and multiply in the existing nutritional and ecological conditions (Huis in't *et al*., 1996)

Isolates Fb and Vc were selected for detailed studies on the basis of their tolerance to pH 2, bile salt 4%, phenol 0.6% and NaCl 6% and broad spectrum antimicrobial activity against food spoilage organisms and gastrointestinal pathogens. The additional positive virtues of the isolated strains were their tolerance to acid, bile salt and phenol which enable their viable passage through gastrointestinal tract and allow them to establish and multiply there, in the existing nutritional and ecological conditions. *Lactobacillus* isolates show broad antimicrobial spectrum covering gram-positive and gram-negative bacteria such as *E. coli, Ent. aerogenes, S. typhi, K. pnemoniae, Ps. aeruginosa, Staph. aureus* and *Bacillus* spp. which are major food spoilage organisms and human pathogens. Antimicrobial activity is higher against *B. cereus, Staph. aureus* and *Ps. aeruginosa,* the activity is attributed to extracellular diffusible metabolites of *Lactobacillus* strains. Vc3, Vc1 and Fb shown activity against all the test organisms, are more potent. Current evidence indicates that probiotic activities are strain-specific, therefore a beneficial effect attributes to one strain cannot be assumed to be provided by another strain even when it belongs to the same species. Lactobacilli exert antimicrobial action through the production of organic acids (lactic and acetic acid), H_2O_2, and or other antibacterial molecules such as bacteriocins, and low molecular peptides.

Antimicrobial activity of the *Lactobacillus* strains is useful in treating fermented food to prevent the growth of food spoilage organisms, albeit the acid formation during fermentation may inhibit the growth of food spoilage organisms. The isolated *Lactobacillus* strains possess potential in development of functional foods, starter culture in food fermentation and food ensiling.

Antibiotic susceptibility profile shows that non-susceptibility and sensitivity of *Lactobacillus* strains to a range of antibiotics is important. Antibiotic susceptibility profile of the isolated strains is similar in terms of their non-susceptibility to vancomycin, and fusidic acid. The non-susceptibility of *Lactobacillus* strains to certain cell wall synthesis inhibitors has been reported previously (Temmerman *et al*., 2003; Danielsen *et al*., 2003; Zhou *et al*., 2005). Considering these results, the non-susceptibility of *Lactobacillus* strains is innate and not a case of acquired resistance. Strains that show resistance to a specific antibiotic can be given at the time of antibiotic treatment. In the treatment of urogenital tract infection and traveller's diarrhea, better management is obtained when concurrent therapy was made with probiotic lactobacilli and antibiotic to which they are intrinsically resistant. By doing so intestinal microflora can recover more quickly (Cebeci and Gurakan, 2003) . An important drawback of antibiotic resistance is possible transfer of antibiotic resistance genes; this may result in highly antibiotic resistant pathogenic bacteria. The *L. rhamnosus* strains used in this study are susceptible to a broader range of antibiotics making them safer for use as probiotics.

Concisely, the study was to screen new probiotic candidates to be of human origin. The *in vitro* characterization of this study showed some *Lactobacillus rhamnosus* Fb, Vc3, Vd4 strains to meet several functional features to be considered as suitable probiotics. These strains have been selected as they belong different habitats, after preliminary screening, further *in vitro* studies have been carried out with reference to standard strains *L. rhamnosus* GG, *L. rhamnosus* 231 and *L. casei* Actimel for probiotic potential of the isolated strains.