Chapter-3

Materials & Methods
3. MATERIALS AND METHODS

The materials used and various methods applied in this study to isolate potential probiotic lactobacilli from indigenous and exogenous sources for development of a synbiotic formula are presented below.

3.1. Collection of Samples and Isolation of Lactobacilli

Food samples (curd) for isolation of lactobacilli were collected from various Dairies. Fresh fecal specimens were obtained from 10 healthy elderly Indian people. Each specimen was taken from a single subject in a sterile container and put in a polythene bag. A specimen of curd and human fecal of approximately 1g was immediately placed in aseptic conditions, suspended in 9mL of normal saline (0.85%) and homogenized manually. One mL of each homogenized sample was enriched in 9mL of sterile Lactobacillus selection MRS broth (Hi Media, Mumbai) for 24h at 37°C. The enriched curd and fecal samples were streaked on the Petri plates containing Lactobacillus selection MRS agar (Hi Media, Mumbai) with the help of calibrated inoculating loop (Hi Media, Mumbai) and incubated aerobically at 37°C for 48h.

3.1.1 Identification and characterization of lactobacilli

The identification and further characterization of isolates was done with the help of following tests:

- Microscopic examination
- Catalase test
- Physiological characterization
- Biochemical characterization
3.1.1.1 Microscopic examination

The purity and tentative morphological identification of the isolates as lactobacilli was confirmed microscopically by Gram staining.

3.1.1.2 Catalase test

The catalase test was performed as per slide method. Using an inoculating needle, culture from a typical colony was placed onto a clean glass slide. A drop of 3% hydrogen peroxide solution was added to this culture and closely observed for the evolution of bubbles. The production of bubbles indicates positive catalase reaction.

3.1.1.3 Physiological characterization

a) Growth at 15 and 45°C

The isolates were tested for their ability to grow in MRS broth at 15 °C for 7d and 45°C by incubating for 24-48h. For this 10mL of MRS broth tubes were inoculated at 1% of lactobacilli cultures. Development of turbidity was recorded as the ability to grow at 15 °C and 45°C.

3.1.1.4. Biochemical characterization of lactobacilli

a) Gas from glucose

Sterile test tubes of 10mL glucose broth containing Durham’s tube (inverted and dipped), were inoculated with lactobacilli cultures at 1% and incubated at 37°C for 24h. Gas production in the form of hollow space in the total inoculated Durham’s tube was recorded.

b) Arginine hydrolysis

Autoclaved arginine hydrolysis broth tubes were inoculated with the isolated cultures (1%) and incubated at 37°C for 48h. After
incubation, 3-5 drops of the Nessler's reagent were added to each test tube and observed for the change in colour.

c) Aesculin hydrlosis

Bile aesculin agar plates were streaked with the isolated cultures and incubated at 37°C for 24h. After incubation, plates were examined for the presence of a dark brown to black halo around the bacterial growth.

d) Nitrate reduction test

The isolates were incubated at 37°C for 24h. After incubation, 0.5mL each of sulphanilic acid (0.8%, in 5N Acetic acid) and α-naphthylamine (0.5%, in 5N Acetic acid) were added into the tubes. The appearance of red or pink colour indicated the positive test for nitrate reduction.

e) Carbohydrate fermentation pattern

Different sugars were used to determine the fermentation profile of Lactobacillus isolates. For this, Lactobacillus cultures were subjected to sugar fermentation reactions using CHL medium for knowing their sugar fermentation pattern. Small test tubes (12 x 75mm) were used for the production of acid from different sugars. CHL medium was used as basal medium. Four mL of the medium was taken in each tube and sterilized by autoclaving. One sugar disc (Hi-media, Mumbai) was aseptically added to each tube. Each tube was inoculated with 0.1mL of inoculum, incubated at 37°C for 24-48 hand the results of colour change were recorded as positive or negative. A control using 0.1mL sterile water as inoculum was used to compare the color change. Different sugars used to determine the fermentation profile of Lactobacillus isolates.
3.2 Standard Bacterial Cultures

Following organisms were used as indicator strains (Pathogens) for antimicrobial activity, procured from National Collection of Dairy Cultures (NCDC), DM Division, NDRI Karnal and Microbial Type Culture Collection (MTCC) Chandigarh. E. coli MTCC443, B. cereus NCDC240, Enterococcus faecalis MTCC439, S. typhimurium NCDC113 and S. aureus MTCC87. The standard cultures of Mycobacterium smegmatis MTCC6 and L. acidophilus NCDC15 were used as positive and negative cultures of cell surface hydrophobicity experiment, respectively.

3.3 Maintenance and Propagation of Cultures

The lactobacilli cultures were maintained in chalk litmus milk at refrigeration temperature after their growth at 37°C for overnight. The cultures were sub-cultured at regular intervals in chalk litmus milk and stored under refrigeration conditions before use the cultures were activated in MRS broth. The indicator were maintained and stored at refrigeration temperature in nutrient agar slant. Before use cultures were activated in nutrient broth and checked for purity by microscopic examinations. The culture of M. smegmatis MTCC6 was maintained at refrigeration temperature in Lowenstein-Jensen Medium.

3.4 Purity and Confirmation of Cultures

The cultures of Lactobacillus species and indicator strains were regularly tested for their purity by Microscopic examination and Catalase test.

3.5 In Vitro Evaluation of Probiotic Attributes

The isolates of lactobacilli were further subjected for evaluating the functional probiotic attributes.
3.5.1. Tolerance to simulated pH of human stomach

The acid tolerance of *Lactobacillus* species was studied in different pH solutions. For this, the solution of hydrochloric acid (Rankam, Chemicals) in autoclaved distilled water was adjusted to pH levels of 1.5, 2.0, 2.5 and 3.0. Sterile distilled water (pH 6.4) served as the control (Mishra and Prasad, 2005). The prepared solutions were transferred to test tubes in 10mL volumes, sterilized at 121°C for 15min and stored at room temperature until used.

Ten mL of each pH solution was taken in autoclaved test tubes. Each of the five pH solutions (pH 1.5, 2.0, 2.5, 3.0 and 6.4) were inoculated with active culture of lactobacilli containing approx. $10^{10}$ cfu/mL. Contents of the tubes were mixed thoroughly and one mL of culture from each tube was taken immediately (0h) and after 1, 2 and 3h of aerobic incubation at 37°C. Serial dilutions were prepared in 0.85% normal sterile saline. Appropriate dilutions were pour plated in sterile Lactobacillus selection MRS agar (Hi Media Laboratories Ltd., Mumbai). The plates were incubated in inverted position aerobically at 37°C and colonies developed were counted after 48-72h (Clark *et al.*, 1993).

3.5.2 Tolerance to simulated bile concentrations of the human small intestine

The ability of lactobacilli strains to survive in the presence bile salts is an absolute need for selecting them as a probiotic bacterium. Solutions of different ox bile (Hi Media Laboratories Ltd., Mumbai) concentrations (0, 1.0, 1.5, 2.0 and 3.0%) were prepared in autoclaved distilled water and sterile distilled water without ox bile (pH 6.4) was used as control (Mishra and Prasad, 2005). Ten mL of each solution was transferred aseptically in sterile test tubes, were inoculated with active lactobacilli cultures containing approx. $10^{10}$ cfu/mL. One mL of
culture from each tube was taken immediately (0h) and after 3 and 12h of aerobic incubation at 37°C. Serial dilutions were prepared in 0.85% normal sterile saline. Appropriate dilutions were pour plated in sterile Lactobacillus selection MRS agar (Hi Media Laboratories Ltd., Mumbai). The plates were incubated in inverted position aerobically at 37°C and colonies developed were counted after incubation period (Gilliland and Walker, 1990).

3.5.3. Cell surface hydrophobicity

The ability of organism to adhere selected hydrocarbons was determined by the method of hydrophobicity assay (Rosenberg et. al., 1982) with some modifications.

The test bacterium was grown in LA PTg broth and harvested after 24h by centrifugation at 12000 resolutions per minutes (RPM) for 5min at 5°C (Model No. C 24 Remi, Mumbai) washed twice in 50 mM KH₂PO₄ (pH 6.5) buffer and finally suspended in same buffer. The optical density (OD) of cell suspension was adjusted to approximately 1.0 at 600 nm of with buffer. In a sterile centrifuge tube, 3mL of bacterial suspension and 1mL of test hydrocarbons (n-hexadecane, xylene and toluene) was added. The mixtures were vortexes for 90sec. The tubes were allowed to stand for separation of two phases and OD₆₀₀ nm of aqueous phase was measured. Hydrophobicity was calculated from percentage decrease in optical density of original bacterial suspension due to partitioning was calculated using equation:

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\text{Percent Hydrophobicity (H %)} = \frac{(\text{O.D}_{600 \text{ before mixing}} - \text{O.D}_{600 \text{ after mixing}})}{(\text{O.D}_{600 \text{ before mixing}})} \times 100
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3.5.4. Antimicrobial activity against indicator organisms

The antimicrobial activity of Lactobacillus species was measured by agar well diffusion assay given by Anand et al. (1984) with some
modification. Nutrient agar containing 0.1% Tween-80 was seeded with 100μL of 24h old culture of indicator organism's, stirred gently and poured into the glass plates. These were then allowed to solidify. The plates were marked into five different zones to represent four lactobacilli cultures and one for the control in central region. Five wells (6mm dia) were bored at equal distance on the solidified agar medium in each of those plates. A sterile cylindrical hollow stainless steel gel-cutter (6mm dia) was used for this purpose. Then holes were filled (except central hole) with 50μL of the 24h old culture of lactobacilli grown in MRS broth. The central hole was used as a control, filled with sterile water. The plates were kept at room temperature until the liquid was absorbed, then incubated at 37°C for 48h and the diameter (mm) of inhibition zone measured.

3.6 The Growth of Lactobacilli Cultures in Presence of Prebiotics

3.6.1 Agar plate assay

A first screening for the growth of the Lactobacillus species was studied in different energy sources, including prebiotics, performed by an agar plate assay (Kaplan and Hulkins, 2000) with some modifications. Briefly, modified MRS agar medium containing the appropriate energy source (1% w/v) and Bromocresol Purple BCP, (30 mg/L) as a color indicator was used. The Lactobacillus strains were propagated twice in modified MRS broth and cultures obtained after 24h of growth at 37°C were centrifuged (at 5,500 RPM for 10min). The pellet was washed once with phosphate buffered saline and resuspended in it, followed by spotting of 10μL of this suspension on solidified modified MRS agar plates. The plates were incubated at 37°C for 48h. After incubation plates were checked for color change around the developing colonies. Development of a yellow color around bacterial spotting was considered positive for provided energy source.
3.6.2 Effect of inulin and gum acacia on the growth of lactobacilli cultures

Carbohydrate free modified MRS broth containing BCP (30 mg/L) was used as the basal medium to study the utilization pattern of different prebiotics (inulin and gum acacia at 0.5, 1.0, 3.0, and 5.0%) by *Lactobacillus* species. Ten mL of each medium was transferred aseptically in sterile test tubes and tubes were inoculated with active lactobacilli cultures containing approx. $10^{10}$ cfu/mL. In this experiment medium containing glucose as sole energy source has been referred to as control. The inoculated tubes were incubated aerobically at 37°C for 24h. At interval of 6h, 18h, 24h pH and viable counts of *Lactobacillus* species were determined. For viable counts, One mL of culture from each tube was taken immediately and serial dilutions were prepared in 0.85% sterile saline. Appropriate dilutions were pour plated in sterile Lactobacillus selection MRS agar (Hi Media Laboratories Ltd., Mumbai). The plates were incubated in inverted position aerobically at 37°C and colonies developed were counted after incubation period. The specific growth rate ($\mu$) and mean doubling time (Td) for lactobacilli grown in inulin and gum acacia were calculated (Kaplan and Hutkins, 2003).

3.7 Antibacterial Activity of the Selected Lactobacilli Culture against Enteric Organisms in the Presence of Prebiotics

The antimicrobial activity of the selected *Lactobacillus* cultures grown in modified MRS containing inulin and gum acacia was determined and compared with that grown in modified MRS containing glucose by agar well method (Anand *et al.*, 1984). Nutrient agar containing 0.1% Tween-80 was seeded with 100µL of 24h old culture of indicator organism, stirred gently and poured into the plates. These were then allowed to solidify. The plates were marked...
into five different zones to represent four lactobacilli cultures and one for the control in central region. Five wells (6mm dia) were bored at equal distance on the solidified agar medium in each of those plates. A sterile cylindrical hollow stainless steel gel-cutter (6mm dia) was used for this purpose. Then holes were filled (except central hole) with 50\(\mu\)L of the 24h old culture of lactobacilli grown in modified MRS containing either glucose or inulin and gum acacia as the sole carbon source was added into them. The central hole was used as a control, filled with sterile water. The plates were kept at room temperature until the liquid was absorbed, then incubated at 37°C for 48h and the diameter (mm) of inhibition zone measured.

3.8 Development of a Lyophilized Synbiotic Preparation Containing Lactobacilli and Prebiotics

The freeze-dried synbiotic formulation was developed on the basis of the method followed by Crittenden et al., (2006); Collins and Hall (1984) with some modifications. For this, the selected lactobacilli cultures were inoculated into modified MRS broth @ 2% and incubated at 37°C for 48h under aerobic conditions. The cells were harvested by centrifugation at 12000 RPM for 10min at 4°C, washed once in sterilized distilled water and resuspended at the rate of \(10^{10}\) cfu/mL in a sterilized mix containing 5% Skim milk powder, 15 and 20% inulin or gum acacia, 8% sucrose and 1.5% gelatin (gelatin was separately sterilized and mixed with the solution containing other components under aseptic conditions). The mix thus obtained was poured into sterile Petri plates and freeze-dried at room temperature; the condenser was cooled to ~55°C. A lyophilized synbiotic preparation was developed as per the flow diagram (Fig.3.1), to develop a suitable carrier for the probiotic cultures.
Fig. 3.1: Flow chart for development of freeze-dried synbiotic formulation

3.9 Storage Study of Synbiotic Product

In order to carry out storage studies the synbiotic product was aseptically transferred into sterile moisture proof 30mL glass roll tubes and stored at room and refrigeration temperatures. The viability of probiotic organisms was determined on 0, 7, 14, 21, 30, 60 and 90 days (d) of storage. Freeze-dried samples taken at random. For viable counts, 1g of synbiotic product was immediately placed in aseptic conditions, suspended in 9mL of normal saline (0.85%) and manually homogenized. One mL of culture from each tube was taken immediately and serial dilutions were prepared in 0.85% sterile saline. Appropriate dilutions were pour plated in sterile Lactobacillus selection MRS agar (Hi Media Laboratories Ltd., Mumbai). The plates were incubated in inverted position aerobically at 37°C and colonies
developed were counted after incubation period. The degree of survival was expressed in cfu/mL.

### 3.10. Statistical Analysis

The data obtained in the present study for the evaluation of cell surface hydrophobicity and antimicrobial activity of recovered isolates were statistically analyzed by calculating the Standard Deviation as per Snedecor and Cochran (1980) and presented as ± of the average values of the five replicates.