Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Chemicals
The media and chemicals used are listed in Annexure-I.

3.1.2 Reference strains
Two probiotics strains *Lactobacillus casei* Shirota strain SAA 182 and *Lactobacillus rhamnosus* GG SAA 183 from the Dept. of Food and Environmental sciences, Viikki Biocentre, University of Helsinki, Finland.

3.2 Isolation of lactic acid bacteria

3.2.1 Sample collection
The samples of fermented food/beverage products (pickle, dahi, khamir, *chhang*, *churrpe*, fermented broth of apple and grapes etc.) were collected from different areas of Himachal Pradesh in sterilized vials. Five ml of each sample was transported to laboratory within 6 h. The samples were processed for the isolation of lactic acid bacteria (LAB).

3.2.2 Isolation of Lactic acid bacteria
One g or ml of samples was serially diluted from $10^{-1}$ to $10^{-8}$ with 9 ml of physiological saline. 0.1 ml of diluted sample was spread on MRS (de Mann Rogosa Sharpe agar) (modified Hi-media), acidified with glacial acetic acid to pH 5.7 for isolation of LAB, and incubated at 30 °C for 24 h.

3.2.3 Identification of LAB
Identification of predominant bacterial colonies was done on the basis of morphological and biochemical characteristics according to Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994).

Colonies with typical characters (such as pin pointed, whitish transparent etc.) were randomly selected from plates and tested for Gram-character, cell morphology, catalase activity and sugar fermentation.

3.2.4 Maintainance of pure culture
The selected isolates were successively streaked on MRS agar plates for purity. Pure culture plates were closed with paraffin film and stored in refrigerator. Some isolates
were propagated in Elliker’s broth (for 24 h) and then pour plates were prepared and stored at 4 °C.

3.2.5 Preparation of stock culture

The MRS broth vials with pure cultures were incubated for 24 h at 30 °C and stored with 15% glycerol (v/v) at 4 °C.

3.3 Morphological examination

3.3.1 Colony morphology

Isolates were further purified by streaking repeatedly on MRS agar plates, and the colony morphologies (color, shape and size) were examined.

3.3.2 Gram staining

The above selected LAB isolates were examined morphologically under the microscope. Gram staining was performed. LAB are known to be Gram positive and the blue-purple color indicates the Gram positive nature of the bacteria.

Smear of LAB cultures were made on the glass slide by heat fixing. Crystal violet was flooded on the slide for one minute and washed with distilled water. After that it was exposed to iodine solution for one minute. Washed with tap water and then with 95% alcohol for 30 seconds. Smear was counterstain with safranin for 30 seconds. Washed, drained and examined under light microscope. Gram-positive bacteria stained deep violet to blue and gram-negative bacteria stained pink to red.

3.3.3 Catalase test

Catalase is an enzyme produced by many organisms and therefore the lack of catalase is a significant diagnostic characteristic. The enzyme breaks down hydrogen peroxide into water and oxygen which evolved as gas bubbles.

LAB are known as catalase negative. Hence, in order to confirm catalase status of the isolates, catalase test was performed. For this purpose, overnight cultures of isolates grown on MRS agar plates at 30 °C were used. Catalase activity was investigated by dropping 3% hydrogen peroxide solution (one drop) onto randomly chosen colony. The formation of gas bubbles therefore indicates the presence of catalase enzyme:

\[ 2\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2 \]
3.3.4 Endospore stain

To check the presence of bacterial endospores, the isolates were stained according to the standard endospore staining procedure. Vegetative cells stain red, while endospores appear green. Since *Lactobacillus* is a non-sporo forming bacteria, it would produce red/brown stained cells.

Smears of 2-3 days old LAB culture were prepared. Smears were covered with a piece of absorbent paper which was cut to fit in the slide. Paper was saturated with malachite green holding the Bunsen burner in the hand, heated the slide until steam can be seen rising from the surface. Slides were kept for steaming for about three minutes. Paper was removed with tweezers and rinsed the slide thoroughly with tap water. Slide was drained and counterstained for 45 seconds with 0.5% safranin. Washed, blotted, and examined under microscope. The vegetative cells appear red and the spores appear green under light microscope.

3.4 Biochemical and technological identification

3.4.1 Gas production from glucose

In order to further determine homo or hetero-fermentative isolates, CO₂ production from glucose test was performed. For this, phenol red glucose (dextrose) broth and inverted Durham tubes were used. Fifty μl of overnight cultures were transferred into the 8 ml of phenol red glucose (dextrose) broth medium. After incubation for 5 days at 30 °C, gas accumulation in Durham tubes was taken as the evidence for CO₂ production from glucose.

3.4.2 Growth at different temperature

Fifty μl of overnight LAB cultures were transferred into the tubes which contain 5 ml medium containing Elliker broth and bromocresol purple. After inoculation, tubes were incubated for 7 days at 10 °C, 37 °C and 45 °C. Cells growth at any of these temperatures was measured by the change in the color of the cultures, from purple to yellow.

3.4.3 Growth at different NaCl concentration

Fifty μl of overnight cultures were transferred into the tubes which contained 5 ml NaCl test medium in Elliker broth containing 0.04 g/L of bromocresol purple. Isolates
were tested for growth at 2, 4 and 6.5 % NaCl concentrations. These were incubated for 7
days at 30 °C and the change of the color from purple to yellow was taken as evidence for
cell growth.

3.4.4 Arginine hydrolysis

For arginine hydrolysis test, arginine MRS broth containing various ingredients as
given in Annexure-I was used. Fifty μl overnight cultures were inoculated into 5 ml
arginine test medium, and were then incubated for 5 days at 30 °C. After the incubation,
ammonia production was detected by using Nessler reagent. For this purpose, 100 μl of
culture broth was pipetted into tubes and 100 μl of Nessler’s reagent were added.
Immediate orange colour formation was taken as the indication for ammonia production.
No color change indicated that the isolate is negative for arginine hydrolysis.

3.4.5 Citrate utilization

In order to perform this test, 8 ml of Reddy broth (Annexure-I) and inverted
Durham tubes were used. Fifty μl of overnight cultures were inoculated into the Reddy
broth and were incubated for 5 days at 30 °C. The breakdown of the citrate results in
production of carbon dioxide. Gas accumulation in the inverted Durham tubes indicated
citrate utilization.

3.4.6 Carbohydrate fermentations

LAB isolates were inoculated into carbohydrate fermentation medium containing
different sugars (sucrose, raffinose, trihalose, xylose, maltose, fructose, galactose, ribose,
dextrose, mannitol and lactose). Incubation was done at 30 °C and examined after 24 h.
Phenol red was used as an indicator. A change in color in the tube from red to yellow
indicates that the organism has fermented that particular carbohydrate, producing acid as
end products.

3.4.7 Growth at different pH values

Sterile tubes containing 8 ml of MRS broth, adjusted to different pH (2.5, 3.5, 8.5
and 9.5) were inoculated with approximately 3 log CFU/ml of LAB and incubated at 30
°C for 2 days. Tubes of MRS broth (pH 6.0), inoculated with LAB were used as positive
controls. Growth was observed after 2 days of incubation.
3.4.8 Resistance to acetic and lactic acid

Assays were performed in MRS broth added with 0.6% of lactic acid and 0.15% of acetic acid inoculated with LAB (3 log CFU/ml) and incubated at 30 °C for 2 days. MRS broth without acetic and lactic acid, inoculated with LAB was used as control. Cell growth was observed after 2 days of incubation.

3.5 Selection and characterization of a potential probiotic LAB isolates

3.5.1 Tolerance to acidic pH

The method used was according to Maragkoudakis et al. (2006). Bacterial cells from overnight (18 h) cultures were harvested (10,000 x g, 5 min, 4 °C), washed twice with PBS buffer (pH 7.0), and adjusted to pH 2, 3 and 7 (control). Resistance was assessed in triplicates in terms of viable colony counts and enumerated on MRS agar after incubation at 30 °C for 3 h, reflecting the time spent by food in the stomach.

3.5.2 Resistance to bile salt

The method used was according to Maragkoudakis et al. (2006). Tolerance to bile salt was tested at 30 °C by inoculation of fresh cultures in MRS broth, enriched with 1, 2 and 3% (w/v) Oxgall (Ox-Bile, HiMedia). Resistance was assessed in terms of viable colony counts, enumerated after incubation for 24 h. Bacterial growth was expressed in CFU/ml and survival percentage (%) of strains in bile was then calculated by comparing with control.

3.5.3 In-vitro cholesterol assimilation

Cholesterol assimilation of bacterial strains was done according to Liong and Shah (2005). Freshly prepared MRS broth was supplemented with 0.30% oxgall, cholic acid and taurocholic acid as a bile salt. Cholesterol (polyoxyethanyl cholesteryl sebacate) was filter-sterilized and added to the broth at a final concentration of 70 to 100 μg/ml, inoculated with each isolates (at 1%), and incubated anaerobically at 30 °C for 20 h. After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the broth was determined using a modified colorimetric method as described by Rudel and Morris (1973). One milliliter of the aliquot was added with 1 ml of KOH (33 % w/v) and 2 ml of absolute ethanol, vortexed for 1 min, and heated at 37 °C for 15 min. After cooling, 2 ml of distilled water and 3 ml of hexane were added and vortexed for 1 min. One ml of the hexane layer was transferred into a glass tube and evaporated under
nitrogen. The residue was immediately dissolved in 2 ml of o-phthalaldehyde reagent. After complete mixing, 0.5 ml of concentrated sulphuric acid was added and the mixture was vortexed for 1 min. Absorbance was read at 550 nm after 10 min. All experiments were replicated twice.

3.5.3.1 Cholesterol removal by dead and resting cells

Freshly prepared MRS broth containing 0.3 % oxgall was inoculated with each LAB isolate and incubated at 30 °C for 20 h. Cells were harvested after the incubation period by centrifuging at 10,000 x g at 4 °C for 10 min. The cell pellet was washed twice with sterile distilled water. For preparation of heat-killed cells, the cell pellet was suspended in 10 ml of sterile distilled water and autoclaved for 15 min at 121 °C. The heat-killed cells were suspended in MRS broth containing 0.3 % oxgall and water-soluble cholesterol. For preparation of resting cells, the cell pellet was suspended in 10 ml of sterile 0.05 M phosphate buffer (pH 6.8) containing oxgall and water-soluble cholesterol. All strains were incubated at 30 °C for 20 h. The spent broth was assayed for cholesterol content as mentioned above in 3.5.3. Cholesterol assimilation by growing, resting, and dead cells was expressed in dry weight to obtain uniformity in all treatments. The following equation was used:

\[
\text{Cholesterol assimilation} = \frac{(C_1 - C_2)}{(W_2 - W_1)},
\]

where \( C_1 \) and \( C_2 \) were the amount of cholesterol present in the fermentation broths at time = 0 and 20 h, respectively, and \( W_1 \) and \( W_2 \) were the dry weight of the individual culture at time = 0 and 20 h, respectively, for all treatments studied. The experiments were repeated twice.

3.5.4 Lysozyme resistance

Survival of isolates in the presence of different concentration of lysozyme (50, 100 and 150 \( \mu \)g/ml) was determined. The overnight culture (200 \( \mu \)l) grown in MRS broth was inoculated into 5 ml of fresh MRS broth supplemented with different lysozyme concentration and incubated at 30 °C for 24 h. The culture was centrifuged at 1,500 x g for 15 min at 4 °C. The pellet was washed twice in PBS, serially diluted in saline and plated onto MRS plates. After incubation at 30 °C overnight, the colonies (CFU/ml) were counted.
3.5.5 Antibiotic susceptibility

Bacterial antibiotic resistance was determined on MRS agar by the use of different antibiotic discs (Turchi et al., 2013). Disc diffusion method was used to screen for the antibiotic susceptibility of isolates with 14 discs containing Penicillin G (P) 10 Units, Clindamycin (CD) 2μg, Co-trimoxazole (COT) 25μg, Erythromycin (E) 15μg, Vancomycin (VA) 30μg, Ampicillin (A)10μg, Carbenicillin (CB)100μg, Cefotaxime (CTX) 30μg, Chloramphenicol (C) 30μg, Co-trimazine (CM) 25μg, Gentamicin (GEN) 10μg, Norfloxacain (NX) 10μg and Oxacilin (OX) 5μg. Diameters of inhibition-zones were measured after incubation at 30 °C for 24 h. The results were expressed as sensitive (S) or resistance (R) on the basis of diameter of inhibition zone.

3.5.6 Antimicrobial activity against pathogens

Antimicrobial effects of LAB isolates on *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginos*, *Bacillus subtilis*, *Shigella dysenteriae*, *Listeria monocytogenes* and *Yersinia enterocolitica* as test organisms were determined by the bit disc and agar well diffusion method.

The test organisms were obtained from the IGMC (Indira Gandhi Medical College) Shimla and IMTECH (Institute of Microbial Technology) Chandigarh. Approximately $10^5$-$10^7$ CFU/ml of the bacteria to be tested for sensitivity against indicator bacteria were spread on nutrient agar petri plates. For the detection of antibacterial activity of the LAB, ten ml of broth was inoculated with each isolates and incubated at 30 °C for 48 h. After incubation, a cell-free broth was obtained by centrifuging (6000 x g for 15 min) the culture, followed by filtration of the supernatant through a 0.22 μm pore size (Schleicher & Schuell, Germany) cellulose acetate filter. A portion of supernatant was neutralized with 1 N NaOH to pH 6.5, and the inhibitory effect of the hydrogen peroxide was eliminated by the addition of catalase (5 mg/ml). Unneutralized (general inhibitory effect) and neutralized (bacteriocin and bacteriocin-like metabolites) supernatants of the LAB were checked for antibacterial activity against indicator bacteria in inoculated nutrient agar. Then 100 μl of cell free broth was filled in 6-mm diameter sealed wells cut in the nutrient agar. The petri plates were stored for 2 h in refrigerator. The inoculated plates were incubated for 24 h at 30 °C, and the diameter of the inhibition zone was measured in millimetres (mm).

3.5.7 Autoaggregation assay

Autoaggregation abilities were determined as described by Reniero et al. (1992) as
the autoaggregation percentage. Lactic acid bacteria were harvested after 18 h of growth, washed twice with PBS and resuspended in the same buffer. Absorbance of cells at 600 nm was adjusted to 0.5 in order to standardize the number of bacteria (10^7-10^8 CFU/ml). Then the bacterial suspensions were incubated at 30 °C and were monitored during different times (0, 5 and 24 h). Autoaggregation percentage was determined using the below equation:

\[ 1 - \frac{A_{\text{upper suspension}}}{A_{\text{total bacterial suspension}}} \times 100. \]

### 3.5.8 Coaggregation assay

The coaggregation test was performed as a method described by Handley et al. (1987). Briefly, bacterial suspensions were prepared as described for autoaggregation. Equal volumes of cells (500 μl) of the different LAB isolates and *L. monocytogenes* strains were mixed and incubated at 30 °C without agitation. The absorbance (A_600 nm) of the mixtures described above was monitored during 6 and 24 h of incubation. Absorbance was determined for the mixture and for the bacterial suspensions alone. The coaggregation (%) was calculated according to the equation described by Malik et al. (2003) as:

\[ \frac{(A_{\text{pat}} + A_{\text{probio}}) - (A_{\text{mix}})}{(A_{\text{pat}} + A_{\text{probio}})} \times 100, \]

where \( A_{\text{pat}} + A_{\text{probio}} \) represent \( A_{600} \) of the mixed bacterial suspensions at time 0 min and \( A_{\text{mix}} \) represents \( A_{600} \) of the mixed bacterial suspension at 6 and 24 h.

### 3.5.9 Cell surface hydrophobicity

The bacterial adhesion to hydrocarbons (BATH) test was performed according to Rosenberg et al. (1980). Lactic acid bacteria were harvested after 18 h of growth, washed twice with PBS and resuspended in the same buffer to an optical density of 0.8 at 600 nm. Then, equal proportions of viable bacterial suspension and solvents (n-hexadecane, xylene, octane and toluene) were mixed by vortexing for 5 min. The two-phase system appeared and the aqueous phase was removed after 1 h of incubation at room temperature and its absorbance was measured. Hydrophobicity was calculated as the percentage of decrease in the OD of the bacterial suspension due to partitioning of cells into the hydrocarbon layer. Results were reported as a percentage from 3 replicates according to the formula:

\[ \text{BATH} \% = \left( \frac{A_0 - A}{A_0} \right) \times 100, \]

where \( A_0 \) and \( A \) are absorbance before and after mixing with solvents at \( A_{600} \) nm.
3.5.10 Exopolysacharride production

Exopolysacharride production was evaluated as reported by Mora et al. (2002). Overnight cultures were streaked on the surface of plates containing ruthenium red milk (10 % w/v, skim milk powder, 1 % w/v, sucrose and 0.08 g/L ruthenium red, 1.5 % w/v agar). After incubation at 37 °C for 24 h, non-ropy isolates gives red colonies due to the staining of the bacterial cell wall, while ropy strains appeared as white colonies.

3.5.11 Haemolytic activity

For haemolytic activity tests, the isolates were subcultured in MRS and then streaked on Columbia agar plates, containing 5 % of sheep blood. The plates were incubated for 24 h at 30 °C. As suggested by Maragkoudakis (2009), the isolates that produced green-hued zones around the colonies or did not produce any effect on the blood plates were considered non hemolytic. The strains showing blood lysis zones around the colonies were classified as hemolytic (β-hemolysis).

3.5.12 Proteolytic activity

The isolates were tested for their proteolytic activity on skim milk agar according to Essid et al. (2009). An overnight culture of each LAB isolates (MRS broth, 24 h) was centrifuged at 13,000 rpm for 5 min and pellet was suspended in 20 mM phosphate buffer, pH 7.0. 50 microliter of each suspension was filled in the well on the surface of skim milk agar (10 % of skim milk, 0.5 % yeast extract, 1.5 % agar) incubated at 30 °C for 24 h. The proteolytic activity was determined by the measurement of the diameter of clear zones around the spots (mm).

3.5.13 Bile salt hydrolase activity

Overnight cultures were spotted on MRS agar plates containing 0.37 g/L CaCl₂ and 0.5% sodium salt of taurodeoxycholic acid (TDCA). Plates were incubated at 30 °C for 72 h. Presence of halos around colonies or a white opaque colony indicated positive bile salt hydrolase activity.

3.5.14 Phosphoketolase assay

The principle of this assay is based on the ability of studied isolates to transform fructose-6-phosphate into acetyl-phosphate and erythrose by enzyme phosphoketolase.

Isolates were grown in MRS broth for 48 h at 30 °C and centrifuged for 6000 rpm for 5 min and cells were washed twice with PBS buffer (pH 6.5) containing 0.25 % L-cysteine. Cells were then suspended in 2 ml of a lysozyme solution (10 mg/ml) and
incubated at 30 °C. After pre-treatment, 0.25 ml of a solution containing sodium fluoride (NaF, 3 mg/ml) and potassium or sodium iodoacetate (5 mg/ml) in water was added. To that, 0.25 ml of sodium fructose-6-phosphate (80 mg/ml in water) was added, the solution was vortexed and then incubated at 37 °C for 30 min. Following incubation, 1.5 ml of a solution of hydroxylamine-HCl (13 g/100 ml in water, pH 6.5) was added and allowed to incubate at room temperature for 10 min. 3 ml of a solution of trichloroacetic acid (TCA at 15 % w/v), 1.0 ml of 4N HCL and 1.0 ml of ferric chloride (FeCl₂, 6 H₂O, 5% w/v in 0.1 N HCl) were added and incubated at room temperature for 10 min. A reddish-violet colour develops immediately with the addition of ferric chloride if the culture contains phosphoketolase activity. The reaction mix without phosphoketolase activity develops a light yellow colour.

3.5.15 Resistance to H₂O₂

LAB isolates were cultivated in Elliker broth for 24 h at 30 °C. The tubes containing MRS broth having different concentrations of H₂O₂ (50, 100, 200 µg/ml) were seeded with LAB isolates. Tubes were incubated for 24 h at 37 °C. The culture was centrifuged at 1,500×g for 15 min at 4 °C. The pellet was washed twice with PBS, serially diluted in saline and plated onto MRS agar plates. After incubation at 30 °C overnight, the colonies (CFU/ml) were counted.

3.5.16 Coexistence test

The screening procedures comprised the evaluation of LAB isolates antimicrobial activity against each other. However, in the probiotic mixtures, the isolates with antagonism might cause the loss of viability of other isolate and lead to diminished efficacy. The finally selected isolates were examined by a cross streaked method. The isolates were streaked perpendicularly and across each other on MRS agar plate. The plates were incubated at 30 °C for 48 h to observe their antagonism against each other.

3.5.17 Statistical analysis

Statistical analysis was carried out with SPSS Inc. software (version 19.0). One-way analysis of variance (ANOVA) was used to study significant difference between means, with significance level at \( p < 0.01/0.05 \). 2-sided Tukey’s HSD test was used to perform multiple comparisons between means. All data presented are mean values of two determinations and three replicates, unless stated otherwise.
3.6 Principal Component Analysis (PCA) for the selection of promising probiotic isolates

Statistical differences among the isolates were pointed out through the Principal Component Analysis. The relationship among the isolates was determined by Hierarchical Cluster Analysis (HCA) and Principal Component Analysis (PCA) using XLSTAT\textsuperscript{TM} software. The cases introduced in the analysis were the 40 identified lactobacilli isolates along with the two standard probiotic strains while the discriminating variables were acid and bile tolerance, hydrophobicity, auto and co-aggregation, antimicrobial and antibiotic assay. Results of the qualitative and quantitative probiotic characterization were converted into three coded values (0, 1 and 2), as reported in Table 3.1, and used as input data for PCA.

Table 3.1: Codes for the PCA to select the most promising probiotic isolates.

<table>
<thead>
<tr>
<th>pH tolerance</th>
<th>Bile tolerance</th>
<th>Hydrophobicity</th>
<th>Auto/coaggregation</th>
<th>Cholesterol assimilation</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI&lt;50%</td>
<td>VI&lt;60%</td>
<td>HI&lt;20%</td>
<td>PA&lt;25%</td>
<td>CA&lt;20%</td>
<td>-</td>
</tr>
<tr>
<td>50%&lt;VI&lt;80%</td>
<td>60%&lt;VI&lt;80%</td>
<td>20%&lt;HI&lt;40%</td>
<td>25%&lt;PA&lt;50%</td>
<td>20%&lt;CA&lt;40%</td>
<td>+1</td>
</tr>
<tr>
<td>VI&gt;80%</td>
<td>VI&gt;80%</td>
<td>40%&lt;HI&lt;60%</td>
<td>50%&lt;PA&lt;80%</td>
<td>40%&lt;CA&lt;60%</td>
<td>+2</td>
</tr>
</tbody>
</table>

VI-Viability Index; HI- Hydrophobic Index, PA- Percent aggregation; CA-Cholesterol assimilation; *-proteolytic, exopolysacharride and haemolytic assay (- for negative; + for positive result).

PCA was done by using varimax rotation. For the selection of the number of principal components of factors, the Kaiser criterion mentioned by Jolliffe (1986) was followed, and only factors with eigenvalues higher than 1.00 were retained.

3.7 Genotypic identification

3.7.1 Genomic DNA isolation

3.7.1.1 Alkaline lysis of lactic acid bacterial cultures

To the cell suspension of lactic bacterial cultures lysozyme was added to a concentration of (1 mg/ml) and incubated for 1 h at 58 °C. Alkaline SDS (10 % w/v in 1 M NaOH) was added to lysozyme treated cells to 2 % SDS and 0.2 M NaOH. The contents were gently mixed over ice till the formation of highly viscous lysate.

3.7.1.2 Phenol: chloroform: isoamyl alcohol extraction

Equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added into the viscous lysate and mixed by inverting the tubes several times. The content of the tubes were centrifuged at 12,000 g for 20 min at 4 °C. The upper aqueous phase was gently
pipetted into a fresh centrifuge tube and it was once again extracted with phenol: chloroform: isoamyl alcohol (25:24:1).

3.7.1.3 Chloroform extraction

Equal volume of chilled chloroform was added to the aqueous phase obtained from the above step and mixed immediately by inverting the tube. The contents were centrifuged at 12,000 g for 20 min at 4 °C and the upper aqueous phase was collected.

3.7.1.4 Precipitation of nucleic acid using ethanol

To the aqueous phase, sodium acetate (3M, pH 4.8) corresponding to 1/10 volume of the aqueous phase was added, which was followed by addition of 2.5 volume of chilled ethanol (95 % v/v) for the precipitation of nucleic acid. The contents were mixed by inverting the tube and incubated at -20 °C for 30 min. The precipitated nucleic acids were centrifuged at 15,000 g and washed with 70 % ethanol at room temperature. The pellet was dried at room temperature and finally suspended in TE buffer (pH 8.0).

3.7.1.5 Purification of genomic DNA

The DNA suspension from the above step was treated with RNase A at a concentration of 10 µg/ml and 10 µg/ml Proteinase K and incubated at 37 °C for 1 h. The steps 3.7.1.2-3.7.1.4 was repeated and the DNA pellet obtained was washed with 70% ethanol. After drying the pellet the DNA was suspended in TE buffer (pH 8.0).

3.7.1.6 Quantification of DNA

DNA suspension was diluted appropriately and its absorbance was recorded at 260 nm and 280 nm (Sambrook et al., 1989). The A_{260} was used to determine the concentration of DNA in solution (A_{260}=50 µg/ml of ds DNA). The A_{260}/A_{280} ratio was used to determine the purity of the DNA sample.

3.7.2 Agarose gel electrophoresis (Sambrook et al., 1989)

Agarose gel electrophoresis was used to visualize the genomic DNA and PCR products.

Reagents

Ethidium bromide stock solution (5 mg/ml)

250 mg ethidium bromide

45 ml double distilled water
Final volume made to 50 ml by adding double distilled water and stored at 2-8°C in brown bottle.

**50X TAE buffer (1000 ml)**

- 242 g Tris base
- 57.1 Glacial acetic acid
- 100 ml 0.5 M EDTA (pH 8.0)

Final volume made to 1000 ml by adding double distilled water and then autoclaved.

**6X Gel loading dye**

- 30 % (v/v) glycerol
- 0.25 % (w/v) Xylene cyanol FF
- 0.25 % (w/v) Bromophenol blue

3.7.3 Preparation of agarose gel

One gram agarose was added to 100 ml of 1X TAE buffer and dissolved by heating in a microwave oven and cooled to 60 °C. Ethidium bromide to a concentration of 0.5 μg/ml was added mixed by gentle swirling and poured into a gel casting tray.

3.7.4 Sample preparation for agarose gel electrophoresis

The polymerase chain reaction (PCR) amplified samples were mixed with the gel loading dye in a ratio of 5:1 and slowly transferred in to the wells of agarose gel submerged in 1X TAE.

3.7.5 Electrophoresis

The samples loaded in the wells of agarose gel were electrophoresed at a constant voltage of 100 V till the required migration of tracking dye was achieved. The gel was visualized in a UV trans illuminator and image was captured using Alpha Digi Doc system.

3.7.6 PCR amplification

The gene of interest, the 16S ribosomal gene, was amplified using the standard PCR protocol and the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Thomas, 2004) for lactic acid bacteria.

PCR was carried out with 50 μl of the reaction mixture containing 1μl of DNA, 5.0 μl of PCR buffer (10X) (Promega, A3511, USA), 1.0 μl of 5 mM dNTP (Promega,
U1240, USA), 1.0 μl each of forward and reverse primers 5 mM, 1 μl of Dynazyme (Promega) and 33 μl of autoclaved deionized water (Millipore). The amplification was performed with a total of 34 cycles in a thermal cycler. The cycling program was started with an initial denaturation at 94 °C for 5 min followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C and elongation at 72 °C for 1 min/30 sec. The PCR was terminated with a final extension at 72 °C for 7 min and then amplified product was cooled at 40 °C. The products of PCR reactions were visualized on 1.0 % agarose gel containing ethidium bromide to confirm fragment and was send to Xcelris Labs Ltd, Ahmedabad for DNA sequencing.

3.7.7 Bioinformatics analysis of amplified sequence

16S DNA sequence obtained from Xcelris Labs Ltd. was analysed with the Bioedit software and the resulted contig sequence was compared with already sequenced organisms by BLAST (Basic Local Alignment Search Tool) program on the NCBI website [http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) (Altschul et al., 1990). Sequence was submitted in National Center for Biological Information (NCBI) using BankIt sequence submission tool (Nucleic Acids Research, 2013). Multiple sequence analysis was done with Clustal Omega (Sievers et al., 2011). Phylogenetic tree was constructed using software Mega blast 6 (Morgulis et al., 2008) and similarities among the sequences was compared by the Neighbor-joining method. Bootstrap analysis was used to evaluate the tree topology of the data by performing 1,000 resamplings.

3.8 Detection of enzymatic activities of finally selected LAB isolates

3.8.1 β-Galactosidase activity

Bacterial culture was exponentially grown in Elliker broth. Cells were permeabilized with 50 μl of SDS and 100 μl of chloroform. Cells (100 μl) were treated with o-nitrophenyl β-galactopyranoside (200 μl; 0.012 M) (ONPG, Hi-Media Pvt Ltd, India) at 37 °C for 10 min. After the incubation period ice cold sodium carbonate (500 μl; 0.6 M) was added to stop the reaction and absorbance was recorded in a spectrophotometer at 420 nm. Reaction mixture without cells was used as blank. One unit of the enzyme activity was defined as the amount of β-galactosidase that released 1 μmol of o-nitrophenol from the substrate ONPG per millilitre per minute under assay conditions.
3.8.2 β-Glucosidase activity

Bacterial culture was exponentially grown in Elliker broth. Cells were permeabilized with 50 μl of SDS and 100 μl of chloroform. Cells (100 μl) were treated with p-nitrophenyl β-galactopyranoside (200 μl; 0.012 M) (p-NPG, Hi-Media Pvt Ltd, India) at 37 °C for 10 min. After the incubation period ice cold sodium carbonate (500 μl; 0.6 M) was added to stop the reaction and absorbance was recorded in a spectrophotometer at 420 nm. Reaction mixture without cells was used as blank. One unit of the enzyme activity was defined as the amount of β-glucosidase that released 1 μmol of p-nitrophenol from the substrate p-NPG per millilitre per minute under assay conditions.

3.8.3 Amylase activity (Bernfield, 1955)

Reagents:

a) DNSA reagent
b) 40% Rochelle’s salt solution (sodium potassium tartarate)
c) 0.1 M Potassium phosphate buffer (pH 6.6)
d) Standard: Maltose 100-1000 μg/ml

Procedure:

Overnight grown LAB isolates were centrifuged at 10,000 ‘g’ for 10 min. Supernatant was used for amylase assay. To 1 ml of substrate (starch), 1 ml of enzyme was added and incubation was done for 30 min at 30 °C. The reaction was stopped with the addition of 2 ml of reagent ‘a’ and then kept in boiling water bath for 5 min and after that reagent ‘b’ was added. After cooling the absorbance of dark red colour was recorded at 560 nm. For the estimation of amylase activity, maltose standard was prepared in the range of 100-1000 μg/ml and absorbance was recorded at 560 nm. One unit of enzyme activity was defined as the amount of enzyme required to release one μmole of maltose/mg/min under the assay conditions.

3.8.4 Proteolytic activity (Manachini et al., 1988)

Reagents:

a) Casein substrate: 0.5 % (w/v) of casein dissolved in 50 mM Tris HCl buffer (pH 7.0)
b) Trichloroacetic acid (TCA) solution: 5 % (w/v) in distilled water.

Procedure

To 4 ml of substrate solution, 1 ml of enzyme was added and incubated for 20 min at 30 °C. The reaction was stopped with 5 ml of the stopping reagent and vortexed. The
reaction mixture was centrifuged at 10,000 x g for 5 min and the optical density (OD) was taken at 275 nm (UV-160 Shimadzu Spectrophotometer). The standard curve was prepared with tyrosine (10-100 μg/ml). One unit of the enzyme activity is defined as the amount of enzyme required to release one μg of tyrosine/ml under assay conditions.

3.8.5 Phytase activity (Bae et al., 1999)

The phytase activity of LAB strains was detected using a specific method described by Bae et al. (1999). The LAB isolates were grown in sodium phytate containing medium at 30 °C and examined after 48 h of incubation for clearing zones around the spots. To eliminate false positive results caused by microbial acid production, the petri plates were flooded with 2 % (w/v) aqueous cobalt chloride solution. After 5 min of incubation at room temperature, the cobalt chloride solution was removed and then freshly prepared solution containing equal volumes of a 6.25 % (w/v) aqueous ammonium molybdate solution and a 0.42 % (w/v) ammonium vanadate solution were added. Following 5 min of incubation, the ammonium molybdate/ammonium vanadate solution was removed and the plate was examined for zones of phytate hydrolysis.

3.8.6 Urease activity (Fawcett and Scott, 1960)

Reagents (1000 ml):
Solution A: Phenol (10 g) and Sodium nitroprusside (0.05 g)
Solution B: NaOH (5 g) and Sodium hypochlorite (40 %)

Procedure

For Urease assay, 400 μl of reaction supernatant and 1 ml of solution A were mixed and kept for 5 min at room temperature. It was followed by the addition of 1 ml solution B. The contents were mixed and kept in a boiling water bath for 5 min. The absorbance was recorded at 640 nm against the blank. One unit of urease activity is defined as that amount of enzyme, which released 1 μmol of ammonia per min under standard assay condition.

3.9 In vitro adhesion assay of selected isolates

3.9.1 Propagation and maintenance of cell line

The human adenocarcinoma cell line namely HT-29 (mucus secreting) for adhesion assay were procured from National Centre of Cell Science, Pune. Cell line was cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma,
supplemented with 10 per cent (v/v) heat-inactivated (30 min, 56 °C) foetal bovine serum (Sigma, USA), 25 mM HEPES (Sigma, USA), 100 U/ml penicillin (Sigma, USA), and 100 μg/ml streptomycin (Sigma, USA) in 25 cm² culture flask at 37 °C in an atmosphere of 5 per cent CO₂/95 per cent air. The culture was fed with fresh medium every alternate day. When reached about 80 per cent confluency, cells were harvested by incubating adhered cells with 3 ml of 0.25 per cent Trypsin-EDTA solution (Sigma, USA) at 37 °C.

3.9.2 Adhesion assay

Adhesion assay was carried out after 60-90 passages for HT-29 cell line. Adhesion of the *Lactobacillus* cultures was measured as per the method described by Jacobsen et al. (1999). The cell suspension with 1x10⁵ cells prepared in 4 ml complete DMEM medium was transferred to each well of six-well tissue culture plates. The medium was changed every alternate day. When cells reached 80 per cent confluency, the medium was replenished each day consecutively for 20 days for the cell lines. The spent medium was completely removed 24 h before adhesion assay and cells were fed with DMEM medium lacking antibiotics. The cells were washed twice with 3 ml phosphate-buffered saline (PBS, pH 7.4). An aliquot of 2 ml of DMEM (without serum and antibiotics) was added to each well and incubated at 37 °C for 30 min. Different LAB cultures (1x10⁉ CFU/ml) suspended in 1 ml DMEM medium (without serum and antibiotics) were added to different wells. The plates were incubated at 37 °C in 5 per cent CO₂-95 per cent air for 4 h. The monolayers were washed five times with sterile PBS (pH 7.4). The adhesion score was measured by enumerating adhered bacteria per 20 different microscopic fields. Per cent adhesion was determined by plating method.

3.9.3 Adhesion score

Methanol was added to each well of six-well plate at the rate of 3 ml followed by incubation for 10 min at room temperature. Methanol was completely removed and fixed cells were stained with Giemsa stain (0.72 % w/v) for 20 min at room temperature. The wells were washed with ethanol to remove excess stain. The plates were air dried and examined under oil immersion microscope. The number of bacteria was counted in 20 random microscopic fields and were grouped into non adhesive (<40 bacteria), adhesive (41-100 bacteria) and strongly adhesive (>100 bacteria). Adhesion of *Lactobacillus* was expressed as the number of bacteria adhering to 100 HT-29 cells.
3.9.4 Per cent adhesion

Cells from monolayers were detached by trypsinization. One ml 0.25 % trypsin-EDTA solution (Sigma, USA) was added to each well of six-well plate and plate was incubated for 15 min at room temperature. The detached cells were repeatedly but gently aspirated to make homogenous suspension. The cell suspension was then serially diluted with saline solution and plated on MRS agar. The plates were incubated for 24-48 h at 37 °C and colonies were counted (B₁ CFU/ml). Bacterial cells initially added to each well of six-well plates were also counted (B₀ CFU/ml). The adhesion percentage was then calculated as:

\[
\% \text{ adhesion} = \left( \frac{B_1}{B_0} \right) \times 100
\]

3.10 Screening of LAB isolates for inhibitory action against *Listeria monocytogenes*

*L. monocytogenes* strain 839 was procured from Institute of Microbial Technology (IMTECH), Chandigarh. Fresh 18-24 h culture of *L. monocytogenes* in Brain Heart Infusion (BHI) broth at 30 °C was used as the indicator organism. Well diffusion assay was employed to evaluate inhibition of *Listeria* by lactic acid bacterial isolates.

Culture broths were centrifuged at 10,000 g for 15 min, at 4 °C. The clear supernatants (CFS) were sterilized by membrane filtration (0.22 μm). The pH of the cell-free supernatants were adjusted to 6.5 with NaOH (1 N) and then treated with catalase (Sigma, Germany) and trypsin (Sigma), for 1 h at 37 °C. Cell-free supernatant, neutralized cell-free supernatant treated with catalase and neutralized cell-free supernatant treated with catalase and trypsin, were spotted against the *L. monocytogenes*. 50-100 μl of CFS was placed in 5 mm diameter sealed wells which has been cut into a BHI agar plate. The supernatants were allowed to diffuse into the agar for 4-6 h at 20 °C. BHI soft agar (8 ml) was seeded with 8 μl of a *Listeria* culture and poured over the surface of the agar well plate. The plates were incubated aerobically at 30 °C overnight and zones of inhibition in the *Listeria* cell lawns were examined.

3.11 Evaluation of efficacy of probiotic formulations (whey permeate) for the inhibition of *Listeria monocytogenes* in raw vegetables and meat by dipping method

Contamination of ready-to-eat (RTE) meat products with *Listeria monocytogenes* is a major concern to the meat processing industry and needs to be addressed in order to enhance the safety of these products.

The objective of this work was to examine the behaviour of *L. monocytogenes* in RTE meat products and sliced raw vegetables, surface treated (dipped) with the whey fermentate.
Chapter 3  
Materials and Methods

3.11.1 Screening of LAB strains with antilisterial activity at refrigeration temperature

Screening of selected isolates from 3.10 with potential antagonistic activity toward *L. monocytogenes* at refrigeration temperatures (4 °C) for 28 days in MRS media were accomplished with the agar spot test method described by Harris et al. (1989). Antilisterial activity on these tests were determined by measuring the clear or translucent zone around the colonies, considering a diameter of 0.5 mm or greater inhibitory toward the pathogen. Agar spot tests were done in triplicate.

3.11.2 Preparation of probiotic LAB formulation in whey permeate

Whey permeate (WP), the greenish translucent liquid which is the by-product of dairy industry was procured from the local dairy of Shimla. Fat and precipitated proteins were removed from whey permeate by centrifugation at 15,000 g for 15 min at 4 °C and pH was adjusted to 6.5 (by adding NaOH). Finally it was filter sterilized by using membrane filter (Millipore, USA) of 0.22 μm pore size for complete removal of intrinsic microflora. The fermentation medium prepared in this way was used for the growth of lactic acid bacteria.

The overnight isolates of LAB were seeded in WP at the 1% (v/v) rate supplemented with 2.5 g/L of yeast extract and incubate at 30 °C for 24 h and the probiotic formulation was prepared.

3.11.3 Quantification of antilisterial activity in selected LAB strains

Finally selected two LAB isolates was inoculated to sterile MRS broth to obtain approximately 1x10^7 CFU/ml final concentration respectively of each isolate. The each inoculated broth was split into two equal portions; one of each was inoculated with *L. monocytogenes* to obtain final concentration of approximately 1x10^5 CFU/ml, and the other was used as a control. Treatments were stored at 4 °C, and samples were collected on days 0, 7, 14, 21, and 28 to determine the numbers of LAB and *L. monocytogenes*. The total number of *L. monocytogenes* present was determined by the pour plate method with appropriate dilutions onto PALCAM agar plates. Samples were also plated onto MRS agar to determine if the selected LAB grew under refrigeration conditions. The pH of each treatment was determined at each sampling time. Experiments were done in triplicate.
3.11.4 Efficacy of selected LAB strains for inhibition *L. monocytogenes* on cooked meat

This study was conducted as a completely randomized design, with four treatments and five sampling intervals over time. The treatments were:

(i) products containing only the pathogen (control)
(ii) products containing both the pathogen and LAB
(iii) products containing only LAB
(iv) background control containing neither the pathogen nor LAB.

Experiments were done in triplicates.

Meat (mutton) was purchased from local meat market of Shimla. Meat was cut into small pieces of approximately 1 cm² and autoclaved for 10 minutes to eliminate the effect of background microflora. The meat pieces were placed on aluminium foil and inoculated by *Listeria monocytogenes* at $10^5$ CFU/ml on one side (0.1 ml from the appropriate dilution) under a biological safety cabinet. The inoculum was spread over the entire surface of each sample with a sterile bent glass rod. The inoculated slices were left to stand for 15 min at 4 °C for attachment and then the procedure was repeated for the other side. After that infected meat samples were dipped into the whey fermentate fermented with probiotic lactic acid isolates having antagonistic activity against *Listeria monocytogenes* taking meat dipped with distilled water as control. Dipping time was varied to 5 min and store at freezing temperature (4 °C) in the sterile plastic vials and total viable cell count was checked after each step. Microbiological analyses for total microbial counts (CFU/ml) of treated and untreated (uninoculated) were performed on days 0, 7, 14, 21 and 28 for samples stored at 4 °C after the inoculation of LAB and *L. monocytogenes* and pH was measured in each sample.

For microbiological analysis, each sample (1 g) was removed and homogenized aseptically and placed in sterile vials containing 9 ml of sterile physiological saline solution (0.85 % NaCl). Ten-fold serial dilutions were made with sterile physiological saline and surface plated on TSAYE for the enumeration of total microbial populations; PALCAM agar for the enumeration of *L. monocytogenes* and MRS for the viable counts of lactic acid bacteria. After incubation (30 °C, 72 h for TSAYE and 30 °C, 48 h for PALCAM and MRS) the colonies with or without antimicrobials in the formulation, inoculated with the pathogen (except uninoculated), dipped or not dipped in antimicrobial formulations were counted manually.
3.11.5 Efficacy of selected LAB strains for inhibition *L. monocytogenes* on RTE (ready to eat) salads

Carrots and cabbages were purchased from the local Subzi Mandi of Shimla. The four outer leaves of cabbage were discarded, and inner leaves were cut in pieces of approximately 1 cm$^2$ and carrots were peeled and cut into pieces of approximately 1 cm$^2$ and both vegetable were mixed to prepare RTE salads. RTE were disinfected by immersion in a 0.1% hypochlorite water solution for 10 min, followed by 2 consecutive 10 min washes of distilled sterile water (Frances et al., 2006) and were exposed to room temperature to remove excess water. Dipping with LAB isolates and the inoculation with *L. monocytogenes* were done as the same method described previous in section 3.11.4.

Samples for bacterial enumeration of LAB and pathogen test bacteria were taken at 0 and 7 day after inoculation of the pathogen, using the same methods and media described above. All experiments were run in triplicate and controls consisting in water-inoculated products were also included to check for cross-contamination of samples.

3.12 Preparation of probiotic product

3.12.1 Preparation of probiotic fermented soymilk

Preparation and fermentation of soymilk were performed according to the procedure described by Wang et al. (2002). Soybeans (*Glycine max*) variety Harit soya P-4-2 was purchased from Y.S Parmar University, Solan. Whole soybeans were washed and soaked overnight in distilled water. After decanting the soaking water, the soybeans were blended with 10 times their weight of distilled water for 3 min. The resultant slurry was filtered through a double-layered cheese cloth to obtain soymilk. Soymilk was dispensed into containers and was sterilized by heating for 15 min at 121 °C.

When fermentation was to be carried out, 100 ml of autoclaved soymilk was placed in a 150-ml screw-cap Erlenmeyer flask and were inoculated with 0.1 ml cultures of LAB. The initial population of lactic acid bacteria was 3-4 log CFU/ml in soymilk. Soymilk containing LAB was incubated at 30 °C till the maximum population of lactic acid bacteria could be obtained. The fermented soymilk drinks were held at 5 °C for 10 days. During the storage period, the pH and viable counts of lactic acid bacteria of the fermented soymilk drink were determined

3.12.2 Effect of prebiotics on viability of LAB

Autoclaved soymilk was supplemented with 10 g/L of individual prebiotics
including mannitol, pectin, FOS, inulin and maltodextrin. Each bacterial culture (50 ml/L) was washed twice with phosphate-buffered saline (pH 7.4) prior to adjustment of the cell density (optical density of 1.0 at 600 nm) in the same buffer. After centrifugation at 12 000×g for 10 min at 4 °C, the cell pellet was inoculated into 10 ml of soymilk and fermented at 30 °C for 24 h. The control sample was inoculated soymilk without prebiotic supplementation.

3.12.3 Microbiological and chemical analysis of product

3.12.3.1 Viability of LAB

The growth of lactobacilli cultures was determined by the pour plate method using MRS agar (Hi Media) and incubated at 30 °C for 48 h. One millilitre of appropriate serial dilutions of each sample was pour-plated onto the appropriate MRS medium. After 48 h of incubation at 37 °C, the colonies that appeared on the plates were counted and the CFU/ml was calculated.

3.12.3.2 pH measurements

Changes in pH were monitored during fermentation of soymilk at 0, 12, 24, 36, and 48 h using a pH meter.

3.12.3.3 Titratable acidity

Titratable acidity was determined using the method of AOAC (1984) by titration with 0.01N NaOH solution and expressed as percent lactic acid.

\[
\text{Titrable acidity (\% Lactic Acid)} = \frac{\text{Volume of NaOH} \times N \times \text{Mol wt of acid}}{\text{Volume of sample}} \times 100
\]

Mol. wt of acid: lactic acid = 0.0090

3.12.3.4 Determination of moisture (or total solids) content

Principle:

When a thin layer of food products is subjected to convicting air at 100 °C temperature, the free moisture present in products gets evaporated. The weight lost by the fermented soymilk due to the evaporation of water is determined and expressed as % moisture.

Procedure:

Cleaned, dried and cooled (at 100 ± 1 °C) moisture dishes were weighed accurately. 4-5 ml of fermented soymilk was weighed into moisture dishes and these were
transferred into the oven maintained at 100±1 °C and dry it for 2-3 h. Dishes with dried samples were cooled and weighed and expressed as % moisture in the sample.

Observations:
1) Weight of empty moisture dish = W₁ g
2) Weight of dish + sample = W₂ g
3) Weight of dish + dried sample = W₃ g

Calculations: % Moisture = \( \frac{W₂ - W₃ \times 100}{W₂ - W₁} \)

3.12.3.5 Determination of ash content

Principle
A known weight of fermented soymilk weighed into a silica dish is incinerated over a burner and then in a furnace maintained at 550 °C till light grey residue is obtained. This inorganic residue is weighed and expressed as percentage total ash.

Procedure
Weight of empty silica dishes (crucibles) was noted. 5 ml of well mixed fermented soymilk was added into crucibles and weighed. Samples were first incinerated on slow flame of burner and then ignited in a muffle furnace (550±20 °C) until light grey ash is obtained (about 4 to 6 h). Dishes were cooled and weighed and the results were expressed as percentage total ash.

Calculations: % ash = \( \frac{(W_{ash}/W_{wet\ sample}) \times 100}{W} \)

W- weight in grams (g)

3.12.3.6 Total carbohydrate (Dubois et al., 1956)

Reagents:
a) Phenol 5 %
b) H₂SO₄ 96 %
c) Glucose standard 10-100 μg/ml

Procedure:
100 μl of soymilk was blended with 5 ml of distilled water. To 1 ml of properly diluted sample, 1 ml of reagent ‘a’ and 5 ml of reagent ‘b’ was added. After 10 min, the content of tube was vortexed and placed in water bath at 30 °C for 20 min. Absorbance was read at 490 nm.
3.12.3.7 Minerals content

To analyse the minerals content in a food sample, wet ashing method was used (see Content 3.12.3.5). Organic matrix in food sample is breakdown and the minerals are left in aqueous solution.

Weigh dried food sample in a flask containing strong acids and oxidizing agents (e.g., nitric, perchloric and/or sulfuric acids) and then heated for 10-20 minutes at 150 °C till organic matter is completely digested and leaving the mineral oxides in solution. The resulting solution is then analysed by atomic absorption spectroscopy (AAS) using the suitable lamp for corresponding elements i.e. Zn, Ca, Fe and Mg.

3.12.4 Determination of enzymatic activity of fermented soymilk

3.12.4.1 Proteolytic activity

Proteolysis during fermentation of soymilk was determined by measuring free NH$_3$ using the o-phthaldialdehyde (OPA) method (Donkor et al., 2005). Briefly, 3 ml aliquots of fermented soymilk were mixed with 0.75 % (w/v) trichloroacetic acid (TCA) and filtered. The filtrate was consequently mixed with 3 ml of OPA reagent and left at room temperature (20 °C) for 2 min. The absorbance of the solution was measured spectrophotometerically at 340 nm. The relative proteolytic activity of these organisms was expressed as the absorbance of free amino groups, measured using the untreated soymilk as a blank. Triplicate aliquots from each TCA filtrate were analysed.

3.12.4.2 β-Galactosidase activity

β-Galactosidase activity was determined according to the method of Bhowmik and Marth (1989). After 24 h of soymilk fermentation, cells were harvested by centrifugation at 4000 X $g$ for 10 min at 4 °C. The cell pellet was washed twice with 20 ml of cold 50 mM sodium citrate buffer (pH 5.5) and centrifuged at 4000 g for 10 min. Finally, the cell pellet was resuspended in 10 ml of the same buffer, placed in an ice bath and sonicated twice for 15 min. Cell debris was removed by centrifugation at 10,000 g for 30 min at 4 °C. The supernatant was used as the crude enzyme extract. Cell free supernatant (1 ml) was treated with o-nitrophenyl β-galactopyranoside (1 ml; 0.012 M) (ONPG, Hi- Media Pvt Ltd, India) at 37 °C for 30 min. After the incubation period ice cold sodium carbonate (2 ml; 0.6 M) was added to stop the reaction and absorbance was recorded in a spectrophotometer at 420 nm. Reaction mixture without cells was used as
blank. Specific activity was then expressed as the amount of o-nitrophenol released per mg of protein.

3.12.4.3 β-Glucosidase activity

Fifty milliliters of aliquot was withdrawn aseptically from each sample at 0, 12, 24, 36 and 48 h of incubation and β-glucosidase activity was determined using a modified method of Tsangalis et al. (2002) by measuring the rate of hydrolysis of 1 mM of p-nitrophenyl-β-D glucopyranoside (p-NPG). Cells were prepared by the method done in Section 3.12.4.2. One thousand microliters of 5 mM (p-NPG) prepared in 100 mM sodium phosphate buffer (pH 7) was added to 10 ml cells of each aliquot and incubated at 37 °C for 30 min (Scalabrini et al., 1998). Five hundred microliters of 1M cold sodium carbonate was added to stop the reaction. The aliquots were centrifuged at (14000xg) for 30 min. The amount of p-nitrophenol released was measured at 420 nm. One unit of the enzyme activity was defined as the amount of β-glucosidase that released 1 micromole of p-nitrophenol from substrate p-NPG per ml per min under assay conditions. The specific activity was expressed as unit of enzyme per microgram of the protein.

3.12.5 Determination of antioxidative activity

3.12.5.1 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was measured by the method of Pick and Keisari (1980) with minor modification. Briefly, 1 ml of sample or distilled water (control) was first mixed with 1 ml of 5 mM H₂O₂ solution and incubated at room temperature for 20 min. It was then supplemented with 2 ml of horseradish peroxidase-phenol red solution (100 mM phosphate buffer containing HRPase 300 mg/ml and phenol red 4.5 mM). After another 10 min of incubation, the sample absorbance at 610 nm was monitored by an automated microplate reader. The scavenging effect was then calculated according to the following equation:

\[
\text{Scavenging effect} \% = \left[ 1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \right] \times 100
\]

3.12.5.2 DPPH free radical scavenging activity

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was assessed according to Moon and Terao (1998). To 1.0 ml DPPH (500 μM in ethanol), 200 μl aliquot was added, and the reaction mixture was made to 2.0 ml with Tris-HCl buffer
(100 mM, pH 7.4). The mixture was shaken vigorously and incubated at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm. Reaction mixture without DPPH was used as control.

Scavenging effect % = \[1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\] \times 100

3.12.5.3 Determination of total flavonoids

The total flavonoids of the soybean extracts were assayed according to the method described by Yang et al. (2009). An aliquot of 0.25 ml of soybean extract was mixed with 1.25 ml of distilled water and 75μl of 5 % sodium nitrite. After 6 min, 150 μl of 10 % aluminum chloride were added and kept for 5 min at room temperature prior to mixing of 0.5 ml of 1 M sodium hydroxide and 775 μl of distilled water. The absorbance of the solution was determined at 510 nm. Quercetin standard in range of (5-50 μg/ml) was made. The result of total flavonoid contents in the soybean extracts were expressed as milligram quercetin equivalent /ml of extract.

3.12.5.4 Determination of polyphenols

Polyphenols were determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). The sample (0.1 ml) was mixed with 0.9 ml of distilled water and was extracted for 2 h at room temperature on a mechanical shaker. To this, 1 ml of Folin-Ciocalteu reagent (1:2 dilution) and 2 ml of 10 % Na₂CO₃ was added. The mixture was centrifuged at 20,000×g for 20 min, and the supernatant was decanted and filtered through Whatman No. 1 filter paper. The absorbance of the clear supernatant solution was measured at 765 nm. Gallic acid was used as a standard. Each sample was analysed twice with duplicates. Results were expressed as milligram tannic acid equivalent per 100 g dry weight.

3.12.6 Viability of LAB during storage at 4 °C

The final product was stored for 14 days in a refrigerator at 4 °C. During storage of the fermented products the changes in pH-value and viable cells count of *L. casei* PLa5 were periodically observed.