CHAPTER 3

Isolation, Identification and Probiotic Characterization of Folate Producing Lactic Acid Bacteria

3.1. Introduction

Folate plays a key role in one carbon metabolism. The bioavailability of folate has great impact on the efficiency of DNA replication, repair and methylation (Jacob, 2000; Pompei et al., 2007). Humans, being auxotrophic to folate, require a continuous supply of this vitamin through diet. The impending risks associated with high intake of folic acid, an artificial analogue of natural folate used for food fortification kindled an interest in probiotic bacteria. Studies show that the elevated levels of folate in certain fermented foods are due to the folate production by the fermenting microorganisms mainly lactic acid bacteria (LAB). The centuries old tradition of safe use of LAB in various fermented foods and the associated health benefits make them important candidates as probiotics. Hence, folate producing LAB with probiotic characteristics could be used for *in situ* folate fortification of fermented foods (LeBlanc et al., 2007; Pompei et al., 2007).

Probiotics are the "good bacteria" which improve the host health when administered in ample amounts and hence the consumption of probiotic food is very popular worldwide. LAB are the most important group of microorganisms commercially used for the manufacture of probiotic foods. Although a number of probiotic strains have been isolated and characterized, search for more effective strains still continue. Criteria for selection of probiotic strains include resistance to the enzymes in the oral cavity, survival through the gastric acid environment along with the exposure of bile and pancreatic juice in the upper small intestine to reach at the site of action in a viable physiological state and susceptibility to antibiotics. Colonization of the intestine by binding to the intestinal epithelial cells and production of antimicrobial compounds are other important properties to be considered during probiotic strain selection because it provides competitiveness against enteric pathogens and prevent or reduce their existence (Divya et al., 2012; Kilic et al., 2013; Lim & Im, 2009; Williams, 2010).

Present study focused on the isolation of folate producing LAB and *in vitro* probiotic characterization of substantial folate producers.

3.2. Materials and Methods

3.2.1. Microorganisms and Culture Conditions

Lb. casei NCIM 2364 was used for folate detection by microbiological assay. The culture growth and maintenance were mentioned in section 2.2.1.f. The LAB isolates were routinely sub cultured in MRS medium.

3.2.2. Isolation and Identification of LAB

LAB were isolated from various sources including butter, curd, cow's milk, leaves of green and red amaranthus, chekkurmanis leaves, cabbage, fish gut and pickle. For LAB isolation 1g each of the sample was weighed and kept in citrate buffer (0.2M, pH 4) for 7 days. Samples were then serially diluted and plated onto MRS agar supplemented with 1% (w/v) $CaCO_3$ and incubated at 37 ºC for 48 h. Colonies with clear zone around them were selected. Pure colonies were streaked onto MRS agar and glycerol stocks (40% v/v) were prepared for long term storage at -80 ºC. Preliminary characterization was done by gram staining and catalase test. The isolates were also checked for lactic acid production as per the protocol described in section 2.2.2.b.

3.2.3. Screening for Folate Producers

Initially, LAB isolates were qualitatively screened for folate production by checking their ability to grow in folate free FAA medium. For quantitative determination of folate production, a single colony of each isolate was inoculated into 2 mL FAA medium supplemented with 2 µL nutrient solution and incubated at 37 ºC for 18 h. This was used as the pre-inoculum for folate production. The nutrient solution was composed of (mg ⁄ mL) (L-Glutamate-30.0, L-Alanine-20.0, L-Glycine-20.0, L-Histidine- 20.0, L-Serine-10.0, L-Threonine-10.0, L-Cysteine-10.0, L-Arginine-20.0, L-Asparagine- 10.0, L-Isoleucine- 20.0, L-Methionine-10.0, L- Valine- 10.0, Ascorbic acid – 50.0, Nicotinamide-20.0, Calcium pantothenate -20.0, Pyridoxal HCl -20.0, Riboflavin-20.0, Biotin -10.0, PABA-1.0). The folate production was determined after 7 h of fermentation using 2% (v/v) of the pre-inoculum by microbiological assay as per the method explained in section 2.2.2.a.

The isolates selected on the basis of folate production in FAA medium were further evaluated for their efficacy for fermentative production of folate in skim milk. 4% (w/v) skim milk was fermented using 1% (v/v) inoculum for 7 h at 37 ºC. For the isolates

coded as G4 and P8, 0.5% (w/v) glucose was added to the skim milk for appropriate growth. The extracellular folate was extracted and quantified by microbiological assay (2.2.2.a).

3.2.4. Biochemical and Molecular Identification of the Selected Folate Producers

The selected isolates were differentiated on the basis of fermentation profile to homo- and heterofermentative using Lactic Bacteria Differential Broth (2.2.1.c). Carbohydrate utilization tests were carried out using HiCarbo Kit as described in section 2.2.1.e.

Genomic DNA was isolated from the selected LAB isolates as per the protocol described in section 2.2.3.a. The 16S rRNA gene sequence was amplified using the universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T-3') (Soto et al., 2009). PCR reactions were carried out to a final volume of 50 µL containing a reaction mix of 5 µL 10X PCR buffer for *Taq* DNA polymerase (Sigma), 1.5 mM $MgCl_2$ (Fermentas), 300 µM dNTP mix (Fermentas), 0.4 µM of each primer, 2 U of *Taq* DNA Polymerase (Sigma) and 1 µL of template DNA. The conditions for PCR were 30 s of denaturation (94 °C), 30 s of annealing (55 °C), and 1 minute of extension (72 °C), for 35 cycles. The reaction was started by a denaturation step (4 min at 94 °C) and ended by a 7 min extension step at 72 °C. The PCR-amplified samples were subjected to electrophoresis. Amplicons of about 1500 bp were gel eluted and sequenced. The sequences obtained were compared with those deposited in the GenBank (National Centre for Bio-technology Information) database using BLAST algorithm.

3.2.5. Probiotic Characterization of the Selected Isolates

The LAB isolates selected on the basis of folate production were evaluated for their probiotic properties.

3.2.5.1. Tolerance to Inhibitory Conditions

The LAB isolates were checked for their ability to survive in growth inhibitory conditions like presence of phenol, bile salts, NaCl and low pH. Tolerance studies were done by growing the selected isolates in MRS broth containing varying amounts of phenol (0.3, 0.4, 0.5 and 0.6% (w/v)), bile salts (0.3, 0.5 and 0.8% (w/v)) and NaCl (4, 6, 8 and

12% (w/v)) for 24 h at 37 ºC. The cultures were diluted and A_{620} nm was measured after 24 h incubation at 37 ºC.

For determining the tolerance to low pH, overnight culture was pelleted (3000 x g for 5 min at 4 °C) and then washed twice in 50 mM potassium phosphate buffer (pH 6.5). It is then resuspended in the same buffer. 1 mL of the washed cell suspension was harvested by centrifugation (3000 x g for 5 min at 4 °C) and resuspended in 10 mL simulated gastric solution (pepsin – 0.3% w/v, NaCl- 0.5% w/v) of pH 2.0 and 3.0 respectively. Total viable counts were taken before and after an incubation period of 3 h at 37 °C. Results were expressed in CFU/mL (Charteris et al., 1998).

3.2.5.2. Antibiotic Susceptibility

Antibiotic susceptibility was determined by disc diffusion method (Temmerman et al., 2003). Overnight culture (50 µL) of each of the LAB isolate (OD_{620} adjusted to 0.6) was plated evenly over the entire surface using sterile cotton wool swab. Antibiotic discs (Himedia, Mumbai) were applied to the surface of MRS agar plates and incubated at 37 ºC overnight, and diameters of the zone of inhibition around the discs were measured.

3.2.5.3. Antimicrobial Activity

Sterile MRS broth (10 mL; pH 6.0) was inoculated with 1% v/v (10^9 CFU / mL) viable culture of each LAB isolate and incubated at 37 ºC for 24 h. The culture was centrifuged (8160 × g, 15min) and the crude clear supernatant was dried under vacuum at 45 ºC using a rotary evaporator, resuspended in one-tenth of the original volume and filtered through sterile 0.45-mm membrane filters. To detect antimicrobial activity of the preparations, *E. coli* MTCC 739 and *Staphylococcus aureus* MTCC 96 (test organisms) were grown in nutrient broth at 37 ºC for 24 h. Active culture of the test organisms was swabbed onto nutrient agar plates. A 0.5 mm wide ditch was cut in the agar across the centre of the dish. Ten times concentrated test material (100 µL) was added on to the ditch and incubated at 4 ºC for 60 min to allow the test material to diffuse in the agar and then incubated at 37 ºC for 18 h. After incubation, the presence of clear zone indicated the antagonistic effect of the isolates.

3.2.5.4. Antioxidant Activity

The antioxidant activity was determined by inhibition of ascorbate autoxidation (Mishra & Kovachich, 1984). The culture was grown at 37 °C in freshly prepared

modified MRS (mMRS) medium in which magnesium sulphate, manganese sulphate, and dipotassium phosphate were eliminated from the formula (Lin & Yen, 1999). The isolates were propagated at least three times in mMRS prior to use in antioxidant activity assays.

Preparation of intracellular cell-free extract (ICFE)

ICFE was prepared as per the protocol described by Ahire et al. (2013). Bacterial cells were harvested by centrifugation at 3000 × g for 10 min after 18 h of incubation in mMRS broth at 37 °C. Cell pellets were then quickly washed twice with deionised water and resuspended in same and sonicated at 130 W with 30% amplitude for 5 min in 50 s on/10 s off cycle while keeping it in ice bath. Cell debris was removed by centrifugation at 6000 × g for 10 min and the supernatant was used for the assay.

Inhibition of ascorbate autoxidation

0.1 mL of ICFE or phosphate buffer which served as control was mixed with an ascorbate solution (0.1 mL, 5 mM) and phosphate buffer (9.8 mL, 0 .2 M, pH 7.0). Incubated at 37 °C for 10 min and absorbance at 265 nm was measured. The ascorbate autoxidation inhibition was then calculated as:

% Inhibition effect = $[1 - A / A'] \times 100$, where A is the test and A ′ is the control

3.2.5.5. Surface Binding Properties

a. Cell Surface Hydrophobicity

The cell surface hydrophobicity of the selected LAB isolates was measured by the method described by Thapa et al. (1998). Overnight culture (10 mL) was pelleted and the cell pellet was washed and resuspended in 10 mL Ringer solution (w/v) (6% NaCl, 0.0075% KCl, 0.01% $CaCl_2$ and 0.01% $NaHCO_3$). The absorbance at 600 nm was measured. Cell suspension was thoroughly mixed separately with equal volume of n-hexadecane, xylene, toluene, chloroform and ethyl acetate by vortexing for 2 min. The two phases were allowed to separate for 30 min, and absorbance at 600 nm of the lower phase was recorded. The percentage hydrophobicity of strain adhering to the solvent was calculated as:

$$\text{Hydrophobicity (\%)} = \frac{OD_{600}\text{ (initial)} - OD_{600}\text{ (final)}}{OD_{600}\text{ (initial)}}$$

b. Autoaggregation and Coaggregation Assay

Autoaggregation assay was performed according to Del Re et al. (2000) with certain modifications. Overnight culture was pelleted and washed twice in PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) and resuspended in the same to get an OD of 0.5 at 600 nm. The cell suspension (4 mL) was taken and vortexed for 10 s and incubated at room temperature for 5 h. The upper suspension (0.1 mL) was pipetted and mixed with 900 µL of PBS and the absorbance at 600 nm was measured. Percentage of autoaggregation was calculated as:

Autoaggregation (%) = $1 - (A_t/A_0) \times 100$

Where At is Absorbance at time t = 1, 2, 3, 4, and 5 h and A_0 = Absorbance at 0 h

Coaggregation ability of the selected LAB isolates with pathogens (*E. coli* MTCC 739 and *Raoultella planticola* (isolate from NIIST) was also studied. Preparation of cell suspension is same as described for autoaggregation. Equal volumes (2 mL) of each cell suspension were mixed together in pairs and vortexed for 10 s. Incubated at room temperature for 5 h and absorbance at 600 nm was measured. Percentage of coaggregation is calculated according to Handley et al. (1987) **as**:

$$\text{Coaggregation (\%)} = \frac{(Ax + Ay)/2 - A(x + y)}{Ax + Ay/2} \times 100$$

Where x and y represent each of the two strains in the control tubes and (x + y) in the mixture.

c. Mucin Binding Assay

Isolates were grown at 37 ºC for 24 h in MRS medium supplemented with 0.1% (w/v) mucin to induce binding. Microtitre plate wells were coated with mucin (150 µL/well – 100 µg mucin in 50 mM Na_2CO_3 buffer; pH, 9.7) and incubated at 4 ºC with slow rotation. It was then blocked with PBS (with 1% (v/v) tween 20) for 1 h and washed with PBST (PBS with 0.05% (v/v) tween 20; pH, 7.3) (Roos & Jonsson, 2002). The bacterial strains were washed once in PBST and diluted to an absorbance of 0.5 ± 0.02 at 595nm in the same buffer. Bacterial suspensions (100 µL) was added to each well and incubated for 1 h at 30 ºC. The wells were washed with PBST and the mucin binding percentage was determined by measuring the absorbance at 450 nm.

d. Adhesion to Intestinal Cell Lines by SEM Analysis

Human intestinal cell lines, HT-29 and Caco-2 propagated *in vitro* were used for the study. Caco-2 cell line was maintained in Eagle's minimum essential medium (ATCC), supplemented with 20 % (v/v) foetal bovine serum, penicillin/streptomycin (100 U/mL). HT-29 cells were maintained in McCoy's 5a Medium (ATCC) supplemented with fetal bovine serum to a final concentration of 10%, penicillin/streptomycin (100 U/ mL). The cells were grown at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air until a confluent monolayer was obtained.

The adhesion studies were performed as per the method described by Kaushik et al. (2009). Monolayers of HT-29 and Caco-2 cells at a concentration of 2.5 x 10^6 cells/mL were seeded over pre-sterilized cover glass in six well tissue culture plate. The 24 h old bacterial cells were washed twice in phosphate buffered saline (PBS; pH 7.4) and resuspended in the respective growth medium without antibiotics to a final concentration of approximately 10^9 CFU/mL. This cell suspension (1 mL) was added to each well of the tissue culture plate. After 90 min of incubation with the isolates, the monolayers were washed two times with PBS to remove non adherent bacteria. The adhesion of the LAB strains to the intestinal epithelial cell lines were determined qualitatively by SEM analysis. The cells were fixed with 4% (v/v) glutaraldehyde and washed with increasing concentration of ethanol (20, 50, 70 and 90% (v/v)) and dried. The cover glass with bacterial cells adhered to epithelial cells were coated with platinum and viewed under scanning electron microscope (JSM 7400 SEM, JEOL, Japan). The isolates CM22 and CM28 were selected for the adhesion studies based on folate production as well as probiotic properties. Though the other two isolates P8 and G4 also exhibited good probiotic properties, extracellular folate production in skim milk medium was significantly lower when compared to CM22 and CM28 and hence were omitted from further studies.

All the experiments were carried out in triplicates and the results were expressed as mean ± standard deviation (SD).

3.3. Results and Discussion

3.3.1. Isolation of LAB

Bacterial colonies that showed clear zone in MRS-$CaCO_3$ (1% w/v) plates were selected randomly for further characterization. 58 cultures were isolated from the selected

sources and two strains previously isolated by our group from cow's milk were used for further studies. **Table 3.1** shows the isolates and their source.

Table 3.1. LAB isolates and their source of isolation

Source of isolation	**Isolates**
Pickle	P1, P2, P3, P4, P5, P6, P7, P8
Fish gut	F1, F2, F3, F4, F5, F6, F7, F8, F9
Chekkurmanis leaf	G1, G2, G3, G4, G5, G6, G7, G8, G9
Butter	B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11
Cabbage	Cb1, Cb2, Cb3, Cb4
Green amaranthus	Gr1, Gr2, Gr3, Gr4
Red amaranthus	R1, R2, R3, R4
Curd	C1, C2, C3, C4, C5, C6, C7, C8, C9
Cow's milk	CM22, CM28

LAB are widely found in nature including gastrointestinal tract of various animals, dairy products, fermented foods, seafood products, soil and on some plant surfaces (Makarova et al., 2006; Matamoros et al., 2009). All the isolates were gram positive, catalase negative rods or cocci. **Fig. 3.1** shows acid producing isolates from curd. The acid production is indicated by the clear zone around the colonies in MRS-$CaCO_3$ plate.

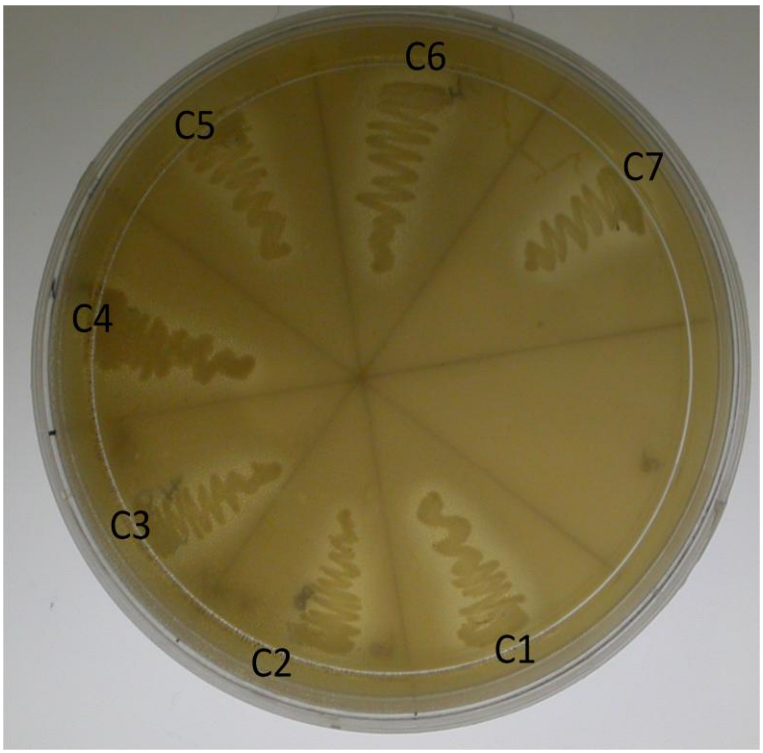

Fig. 3.1. Isolates from curd in MRS – $CaCO_3$ plate

The lactic acid production by the isolates selected based on its growth in folate deficient medium, was monitored and is represented in **Fig. 3.2.** The isolates from fish gut F4 (30.45 mg/mL) and F1 (28.8 mg/mL) were the highest producers among the others. The isolates C2, P4, F6, G4, C5 and CM28 also produced significant amount of lactic acid ranging from 19 - 25 mg/mL.

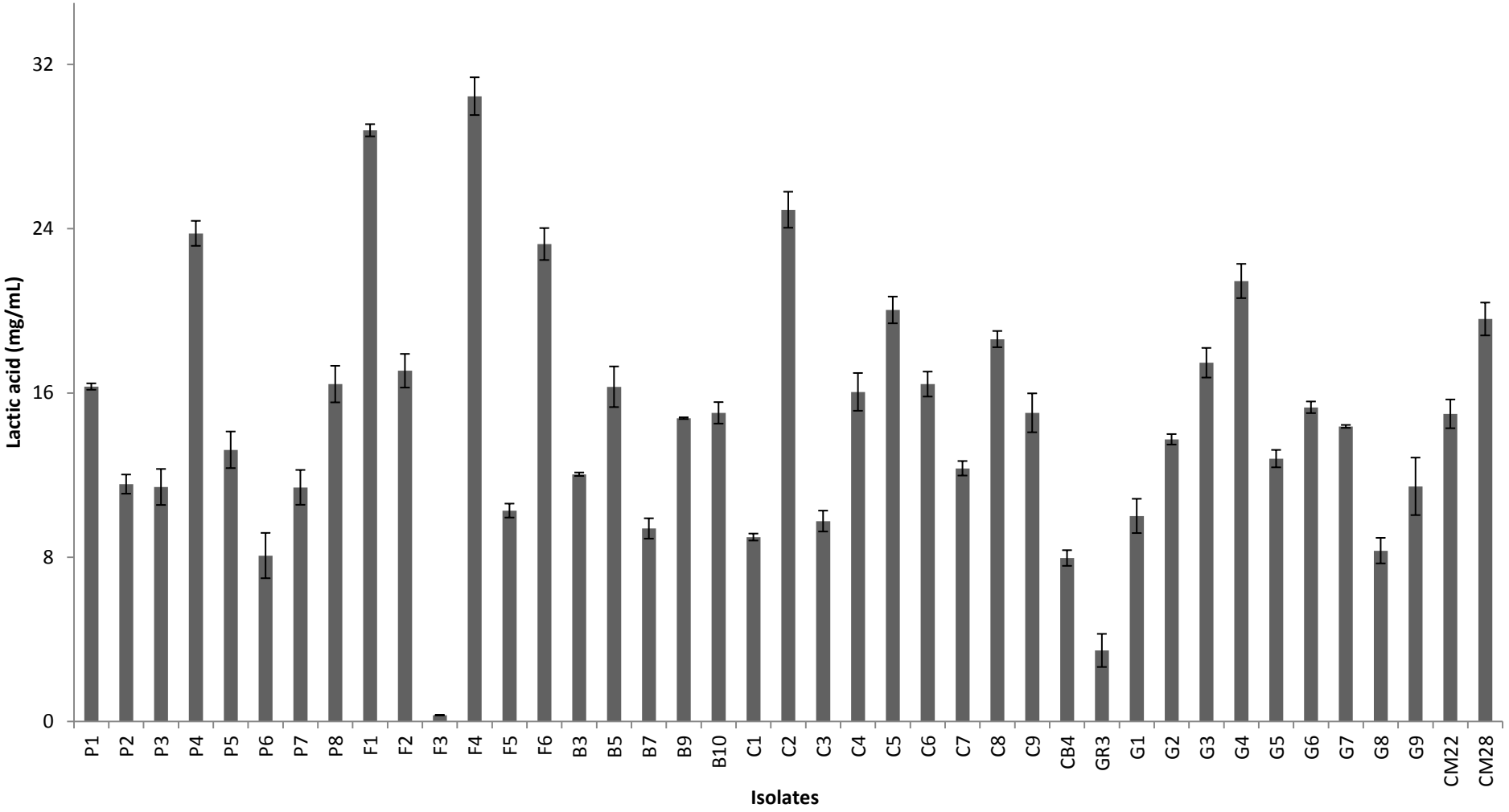

Fig. 3.2. Lactic acid production by the isolates

Lactic acid is the major metabolic end product of carbohydrate fermentation in LAB. Homofermentative LAB produce more than 85% lactic acid from glucose whereas heterofermeters produce ethanol, acetic acid, carbon dioxide in addition to lactic acid (Pot & Tsakalidou, 2009). Lactic acid and other organic acids produced by LAB reduce the pH which contributes towards its ability to prevent food spoilage and inhibition of pathogens (Olaoye et al., 2008). De Keersmaecke et al. (2006) reported that the strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella* was mediated by lactic acid.

3.3.2. Primary Screening for Folate Production and Folate Quantification

The isolates were checked for their ability to grow in folate deficient FAA medium. FAA medium contains all the other essential nutrients for the growth of bacteria and only those bacteria that could grow in FAA medium might produce folate. Among the 58 isolates, 39 could grow in FAA medium. These 39 isolates were selected for further studies on folate quantification and 21 isolates including CM22 and CM28 produced considerable amount of folate (above 3 µg/mL). P8 produced 12.58 ± 0.11µg/mL folate and G4 produced 11.2 ± 0.26 µg/mL while CM28 and CM22 produced 13.5 ± 0.04 µg/mL and 12.65 ± 0.31 µg/mL respectively (**Fig. 3.3**).

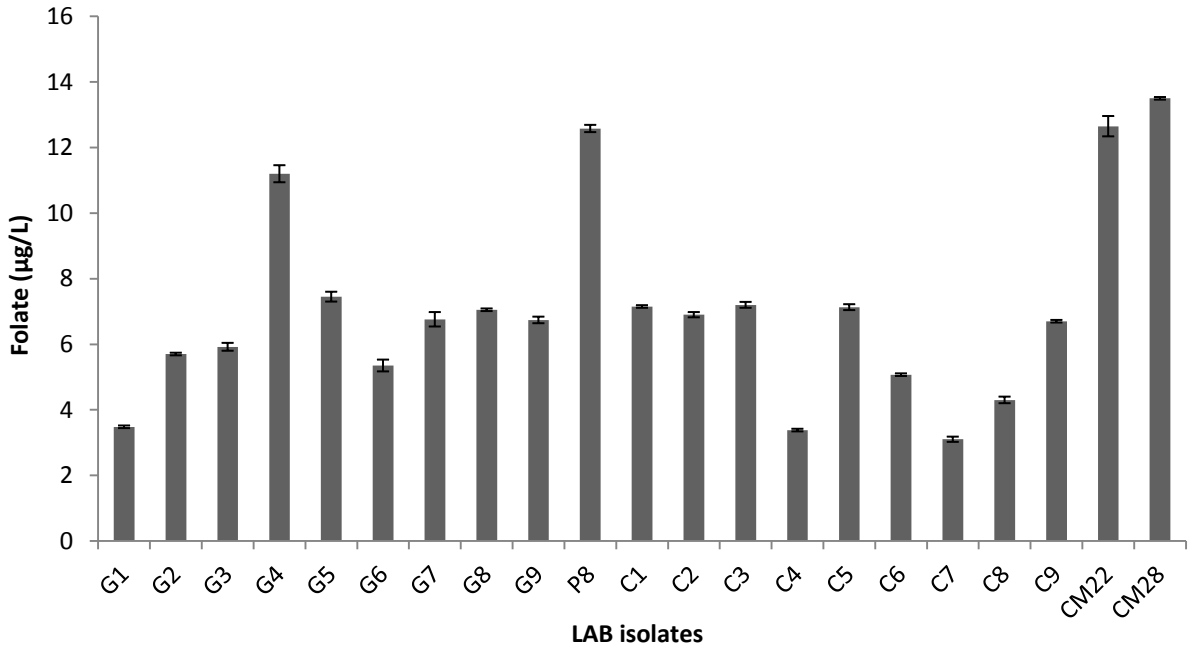

Fig. 3.3. Folate production by the isolates in FAA medium

The isolates CM22, CM28, P8 and G4 were further evaluated for folate production in skim milk medium and the extracellular folate production by CM28 followed by CM22 was higher compared to the other two isolates (**Fig. 3.4**). For G4 and P8, the skim milk medium was supplemented with 0.5% (w/v) glucose for attaining proper growth and fermentation. Folate content in milk is very low and hence could be used as a basal medium for folate fortification studies. Several LAB including *Streptococcus thermophilus*, *Lactococcus lactis*, and *Leuconostoc* spp. were reported to produce intracellular and/or extracellular folate while most *Lactobacillus* spp., with the exception of *Lb. plantarum*, were not able to produce folate (Sybesma et al., 2003b). Folate fortified functional foods can be developed by incorporating these folate producing bacteria.

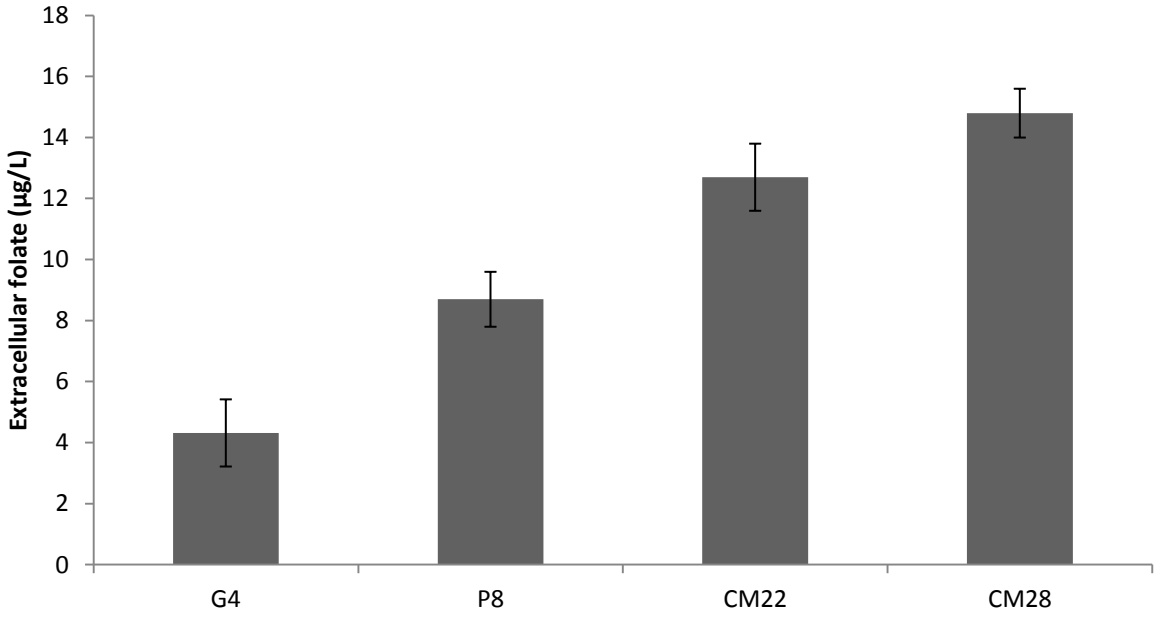

Fig. 3.4 Extracellular folate production by the selected isolates in skim milk

3.3.3. Identification of the Selected Isolates

Both phenotypic and genotypic identification are important in the selection of potential probiotic bacteria. The isolates were initially characterized on the basis of phenotypic characteristics and acid production from carbohydrates. Cell morphology and colony characteristics were observed in MRS agar plates. CM22, CM28 and P8 were cocci and G4 appeared as short rods when observed microscopically (Leica DM2000, Leica Microsystems, CMS GmbH) (**Fig. 3.5**). **Table 3.2** summarizes the morphological and biochemical characteristics of the isolates.

Table 3.2. Morphological and biochemical characteristics of the isolates

Isolates	**CM28**	**CM22**	**P8**	**G4**
Source of isolation	Milk	Milk	Pickle	Chekkurmanis leaves
Gram staining	Gram positive	Gram positive	Gram positive	Gram positive
Cell type	Cocci	Cocci	Cocci	Short rods
Cell arrangement	Single, pairs or short chains	Single, pairs or short chains	Single, pairs or short chains	Single, pairs or short chains
Catalase test	Negative	Negative	Negative	Negative
Colony morphology in MRS agar	Creamy white pin point colonies	Creamy white pin point colonies	Creamy white pin point colonies	Large, creamy white , slimy colonies
Fermentation pattern	Homofermentor	Homofermentor	Homofermentor	Heterofermentor
Citrate utilization	Negative	Negative	Negative	Negative
Acid Production from				
Lactose	+	**w**	-	-
Xylose	+	**w**	-	+
Maltose	**w**	**w**	+	+
Fructose	+	+	+	+
Dextrose	+	+	+	+
Galactose	+	**w**	+	**w**

Raffinose	w	w	+	w
Trehalose	w	w	+	+
Melbiose	+	w	+	w
Sucrose	+	+	+	+
L-Arabinose	+	+	w	+
Mannose	+	+	+	+
Inulin	+	+	+	w
Sodium gluconate	-	-	-	-
Glycerol	-	-	-	-
Salicin	w	-	+	w
Dulcitol	-	-	w	w
Inocitol	-	-	w	w
Sorbitol	w	-	w	w
Mannitol	w	-	w	w
Adonitol	-	-	w	w
Arabitol	-	-	w	w
Erythrytol	-	-	w	+
α-methyl D glucoside	-	-	+	+

+, positive; - , negative; w, weak reaction

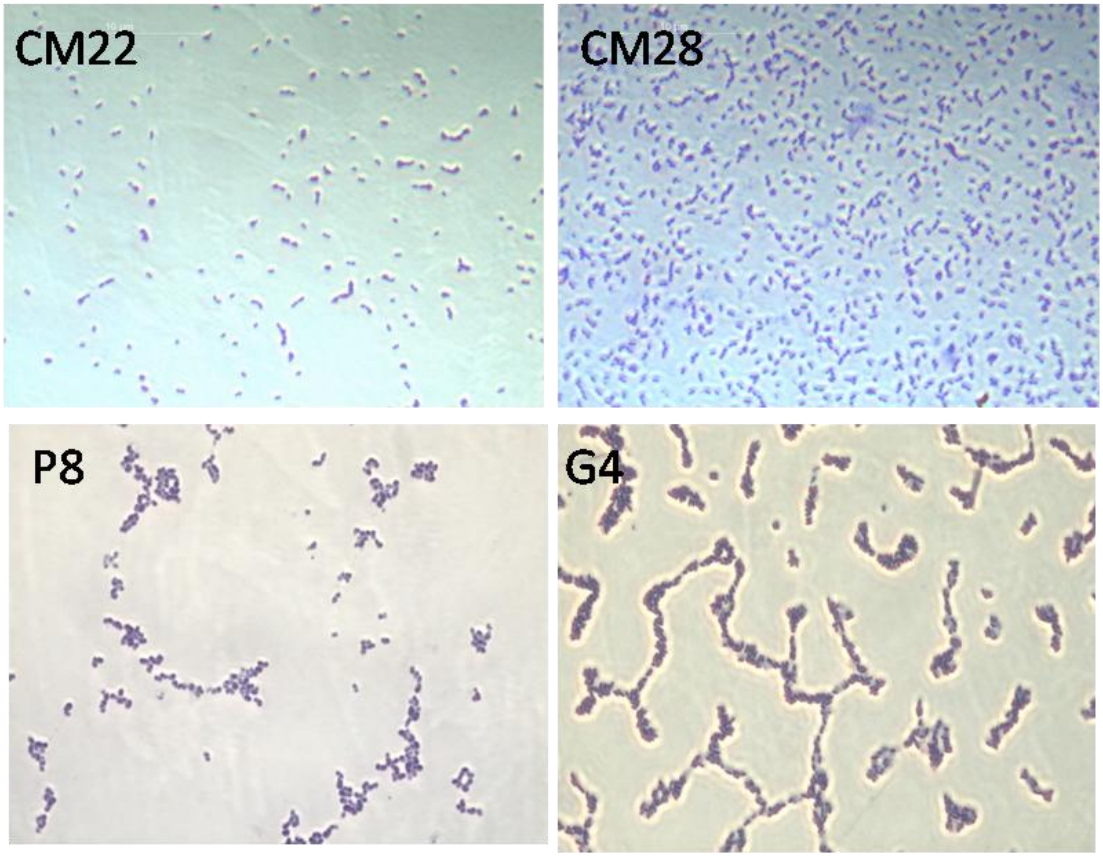

Fig. 3.5. Gram staining images of the isolates showing the cell morphology

After PCR amplification using universal primers targeting 16S rDNA and the following sequence analysis by BLAST the isolates CM22 and CM28 were identified as *Lactococcus lactis* strains, G4 was identified as *Weissella cibaria* and P8 as *Enterococcus* sp. All the isolates were identified by partial 16S rRNA gene analysis had an alignment identity of 99 – 100%. The 16S rRNA gene sequence and the NCBI GenBank accession number of the respective isolates are given in the following tables (**Table 3.3 to Table 3.6**).

Table 3.3. 16S rRNA gene sequence of *Lactococcus lactis* CM28 (1349 bp)

GenBank Accession No: KJ676682

CGTGGGGAATCTGCCTTTGAGCGGGGGAAACACTTGGAAACGAATGCTAATACCGCATAAAAACTTTAAACA
CAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCACAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAG
GTAAAGGCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACG
GCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGC
GTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCA
TCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCG
AGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGC
TCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGG
TGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCT
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGATGTAG
GGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC
CTTACCAGGTCTTGACATACTCGTGCTATTCCTAGAGATAGGAAGTTCCTTCGGGACACGGGATACAGGTGG
TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTA
GTTGCCATCATTAAGTTGGGCACTCTAACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACAGTG
ATGTTTAGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAA
TCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC
ACGGGAGTTGGGAGTACCCGAAGTAGGTTGCCTAACCGCAAGGAGGGCGCTTC

Table 3.4. 16S rRNA gene sequence of *Lactococcus lactis* CM22 (903 bp)

GenBank Accession No: KJ742708

GGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTG
TATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGT
AGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGATGTAGGGAGCTATAA
GTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT
CTTGACATACTCGTGCTATTCCTAGAGATAGGAAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCA
TTAAGTTGGGCACTCTAACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATG
CCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACAGTGATGTTTAGCT
AATCTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAA
TCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGGGAGTTG
GGAGTACCCGAAGTAGGTTGCCTAACCGCAAGGAGGGCG

Table 3.5. 16S rRNA gene sequence of *Weissella cibaria* G4 (1447 bp)
GenBank Accession No: KJ742706

GCAAGTCGAACGCTTTGTGGTTCAACTGATTTGAAGAGCTTGCTCAGATATGACGATGGACATTGCAAAGAG
TGGCGAACGGGTGAGTAACACGTGGGAAACCTACCTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAAT
ACCGTATAACAATAGCAACCGCATGGTTGCTACTTAAAAGATGGTTCTGCTATCACTAAGAGATGGTCCCGC
GGTGCATTAGTTAGTTGGTGAGGTAATGGCTCACCAAGACGATGATGCATAGCCGAGTTGAGAGACTGATCG
GCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGA
AAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTAAGAGAAGAAT
GACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGGAACGGCTAAATACGTGCCAGCAGC
CGCGGTAATACGTATGTTCCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTTATTTAAG
TCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAATTGCTTTGGAAACTGGATGACTTGAGTGCAGTAGAGGAA
AGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTTTCTG
GACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATGCGGATACCCTGGTAGTCCACACCG
TAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAGCTAACGCATTAAGCACTCCGCC
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CGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGATTGTAGGC
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GTCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAACCTTCGGGAGC
CAGCCGT

Table 3.6. 16S rRNA gene sequence of *Enterococcus* sp. P8 (1417 bp)
GenBank Accession no: KJ742707

CATGCAGTCGTACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACGGGTGAGT
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AACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGT
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TGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCC
CCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGT
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CGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTG
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GTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTT

3.3.4. Probiotic Characterization of the Selected Isolates

The isolates P8, G4, CM22 and CM28 were studied for their probiotic properties *in vitro.*

3.3.4.1. Tolerance to Inhibitory Conditions

Tolerance to adverse growth conditions like low pH, presence of bile, phenol and high sodium chloride concentration are very important characteristics to be fulfilled by probiotic strains. Probiotics, before reaching the intestine must first survive transit through the stomach where the pH can be as low as 1.5 to 2 (Dunne et al., 2001). All the isolates showed good survival at pH 3.0 while CM28 and CM22 exhibited better tolerance at pH 2.0 when compared to others after 3 h incubation at 37 ºC. The results are presented in **Table 3.7**.

Table 3.7. Tolerance of isolates to low pH

Isolates	0 h	3h; pH 3.0	3h; pH 2.0
CM22	3.8×10^9	2.1×10^7	1.0×10^4
CM28	4.6×10^9	4.0×10^7	1.6×10^4
P8	3.5×10^9	1.2×10^6	2.6×10^3
G4	7.8×10^8	2.0×10^6	3.0×10^2

Tolerance of the isolates to bile NaCl and phenol was also checked and the results are given in **Table 3.8.**

Table 3.8. Bile, NaCl and phenol tolerance of the isolates

Isolate	Bile (% w/v)			NaCl (% w/v) OD at 620 nm			Phenol (% w/v)			
	0.3	**0.5**	**0.8**	**4.0**	**6.0**	**8.0**	**0.3**	**0.4**	**0.5**	**0.6**
G4	2.013	0.655	0.503	2.678	1.9	0.341	0.101	0.095	0.081	0.071
P8	2.363	1.958	1.329	2.531	1.88	0.908	2.128	0.100	0.083	0.048
CM22	0.692	0.557	0.242	0.791	0.695	0.044	0.462	0.129	0.070	0.046
CM28	0.573	0.393	0.219	0.998	0.610	0.029	0.483	0.226	0.087	0.058

The resistance of probiotics to bile salts is a prerequisite for their colonization and metabolic activity in the small intestine of the host (Bhutada & Tambekar, 2010). The physiological concentration of human bile ranges from 0.1 to 0.3% (Dunne et al., 2001). Numerous studies showed extreme variability in bile tolerance that can be found within a species or genus and hence can be concluded that bile tolerance is a strain specific trait and tolerances of species cannot be generalized (Begley et al., 2005). So it is necessary for a probiotic strain to grow and survive in the presence of bile salts up to 0.3%. Thus, acid and bile tolerance help probiotic microorganism to survive and colonize the gastrointestinal tract by enterocyte adhesion (Zavaglia et al., 1998). It was observed that all the isolates could grow and survive in the presence of bile salts ranging from 0.3 to 0.8%. It has been reported that the viability of probiotics in the gut can be further enhanced by microencapsulation using alginate and prebiotics (Chavarri et al., 2010).

Phenolic compounds are important constituents of food products of plant origin. They contribute towards the sensory characteristics of foods like flavour, astringency, and colour and are also associated with certain health benefits. To get desirable properties on plant derived fermented food products, LAB has to be adapted to the presence of phenolic compounds (Rodríguez et al., 2009). Phenol can also be formed by deamination of some aromatic amino acids by some bacteria and could exert bacteriostatic effect (Suskovic & Matosic, 1997). Hence, resistance to phenol is an important probiotic property. LAB could go on through this bacteriostatic effect and inhabit the intestine. *Lactobacillus brevis,*

Lactobacillus plantarum, and *Pediococcus* could decarboxylate the phenolic carboxylic acids, ferulic and *p*-coumaric acids (Cavin et al., 1993). Phenol tolerance of some LAB strains up to 0.5% (w/v) has been reported (Aswathy et al., 2008). The survival rate of the LAB isolates was more in 0.4% (w/v) phenol which then gradually decreased with increased phenol concentration. It was observed that the isolates were able to tolerate a maximum of 0.6% (w/v) phenol.

LAB generally tolerate a high salt concentration that helps to begin metabolism (Aswathy et al., 2008) which produces acid and further inhibits the growth of non-desirable organisms in pickled food products. NaCl tolerance is an indicator of osmotolerance level of the strain. During industrial fermentation, as lactic acid is being produced by the cells, alkali would be pumped into the broth to prevent excessive reduction in pH. Thus, the free acid would be converted to its salt form which would in turn increase the osmotic pressure on the cells. Therefore, a LAB strain with high osmotolerance would be desirable as an industrial strain (Adnan & Tan, 2007). All the four LAB isolates could carry on well through a sodium chloride concentration of up to 6% (w/v). A further increase in NaCl concentration resulted in a marked reduction in the growth and survival of the isolates CM22 and CM28 while P8 and G4 showed better survival.

3.3.4.2. Antibiotic Susceptibility

Resistance against certain antimicrobial agents can be inherent in a bacterial species and is referred to as intrinsic resistance or "natural resistance" (Mathur & Singh, 2005). Susceptibility of the isolates against 20 antibiotics was checked (**Fig.3.6**). G4 was resistant to vancomycin (30 µg), P8 was resistant to amoxyclav (30 µg), and CM22 was resistant to co trimoxazole (25 µg) while CM28 was sensitive to all the tested antibiotics. The results are shown in **Table 3.9**.

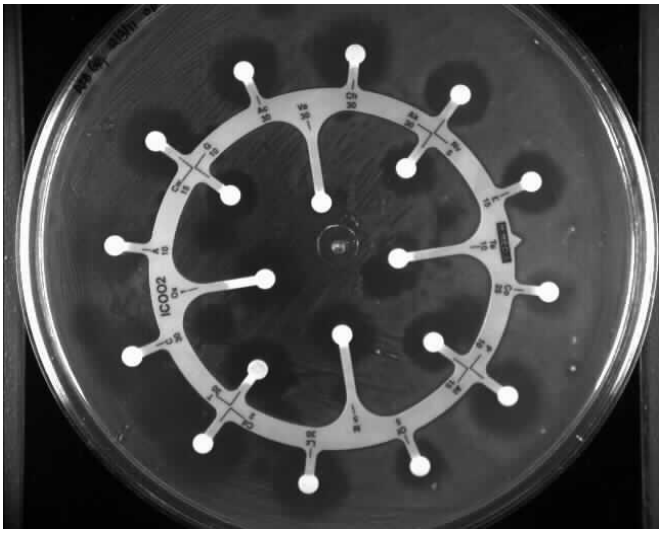


Fig. 3.6. Antibiotic susceptibility of G4

Table 3.9. Antibiotic susceptibility of the isolates

	Diameter of zone of inhibition (cm)			
Antibiotic/Isolates	P8	G4	CM22	CM28
Ox	2.7	1.9	1.9	2.0
A	3.0	2.2	2.5	3.0
Cw	1.5	2.3	2.5	3.0
G	2.1	1.7	2.6	2.7
Ac	**R**	2.3	2.1	2.1
Va	2.1	**R**	2.0	2.0
Ch	1.0	2.4	2.0	2.6
Ak	1.5	1.2	1.8	1.8
E	1.4	2.0	2.1	2.1
Te	2.1	1.7	2.0	1.7
Co	1.7	1.2	**R**	0.8
P	2.5	2.3	2.2	2.4
At	1.4	1.7	2.7	2.2
Of	2.0	1.4	2.1	1.7
M	1.7	2.0	1.7	1.9
Lz	3.1	2.3	2.6	2.1
Cd	3.0	2.3	3.1	3.1
T	3.4	1.8	3.1	2.6
C	2.8	1.3	2.9	1.8
Nv	2.5	2.0	2.1	2.0

Ch- Cephalothrin (30 µg), Cd – Clindamycin (2 µg), Co – Co-trimoxazole (25 µg), E – Erythromycin (15 µg), G – Gentamycin (10 µg), Of – Ofloxacin (5 µg), P – penicillin (10 units), Va – Vancomycin (30 µg), A – Ampicillin (10 µg), C – Chloramphenicol (30 µg), Ox – Oxacillin (1 µg), Lz – Linezolid (30 µg), At – Azithromycin (15 µg), Ak – Amikacin (30 µg), Cw – Clarithromycin (15 µg), Te – Teicoplanin (10 µg), M – Methicillin (5 µg), Ac – Amoxyclav (5 µg), Nv – Novobiocin (5 µg), T – Tetracycline (30 µg)

Antibiotic resistance is a health concerning issue since genetic elements carrying resistance genes are mobile and as such possibly transmitted to pathogens. LAB have a long history of safe use with very few indications of resistance gene transfer to other species (Canzek Majhenic & Bogovic Matijasic, 2001; Marteau, 2001). LAB are intrinsically resistant to several antibiotics. Vancomycin resistance in LAB is usually intrinsic, that is, chromosomally encoded and non transmissible (Zhou et al., 2000). LAB such as *Lactobacillus, Pediococcus, Leuconostoc and Weissella* species are known to be intrinsically resistant to vancomycin. (Abriouel et al., 2015; Kamboj et al., 2015). The popular probiotic *Lb. casei* Shirota is resistant to gentamicin, pefloxacin and trimethoprim sulfamethoxazole (Melgar-Lalanne et al., 2014). Co-trimoxazole resistance by *Lb. plantarum* has been reported in a previous study (Bhutada & Tambekar, 2010). It is desirable that probiotics are sensitive to commonly prescribed antibiotics at low concentrations. At the same time resistance of the probiotic strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections (El-Naggar, 2004). A combinational therapy of probiotics and antibiotics may provide higher antimicrobial activity and thus may reduce the antibiotic dose in addition to the replenishment of intestinal flora (Rybak & McGrath, 1996). It was reported that a cell free extract of *Lb. plantarum* and co-trimoxazole act synergistically against *Salmonella typhimurium* (Rishi et al., 2011).

3.3.4.3. Antimicrobial Activity

LAB produce variety of antimicrobial substances which made them a preferable choice for dairy starter cultures and also as food bio preservatives. Probiotic strains with antagonistic properties can also be made use to repress potential pathogens. The antagonistic activities of the isolates against *E. coli* and *S. aureus* were tested. The antagonistic activities of ten times concentrated supernatant of P8 and G4 against *S. aureus* is indicated by a zone of inhibition (**Fig. 3.7**). Concentrated culture supernatant of all isolates showed antagonistic activities against the test pathogens. The results are summarized in **Table 3.10**.

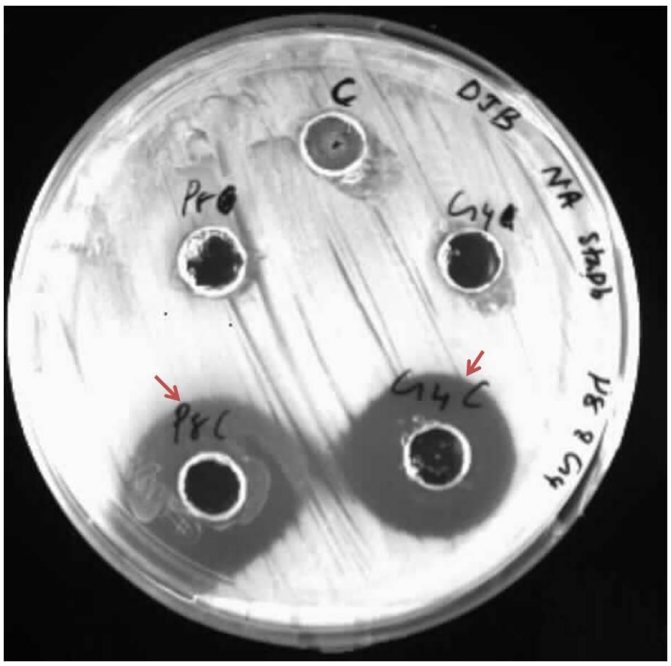


Fig. 3.7. Antagonistic activities of G4 and P8 against *S. aureus*

P8 C and G4 C – concentrated culture supernatants, P8 and G4 – culture supernatant, C – control (MRS broth)

Table 3.10. Antimicrobial activity of LAB isolates

Isolates	**Diameter of zone of inhibition (mm)**	
	E. coli	*S. aureus*
CM22	6.0 ± 0.20	2.0 ± 0.07
CM28	8.0 ± 0.07	2.0 ± 0.20
G4	11 ± 0.07	35 ± 0.10
P8	15 ± 0.10	33 ± 0.07

The antibacterial activity of LAB has been attributed to the production of H_2O_2, organic acids (lactic, acetic and formic acid), proteinaceous compounds and cyclic dipeptides (Cabo et al., 2002; Magnusson et al., 2003; Ström et al., 2002). Lactic acid also reportedly functions as a permeabilizer of the outer membrane of gram negative bacteria and may act as a potentiator of the effects of other antimicrobial substances (Alakomi et al., 2000; Cálix-Lara et al., 2014). The antagonistic effects of most LAB against *S. aureus*

were reportedly due to the low pH resulting from the production of organic acids (Hor & Liong, 2014; Radovanovic & Katic, 2009). The inhibition of growth of *S. aureus* was also found to be dependent on bacteriocin production by LAB. LAB bacteriocins are generally considered safe and can be used as natural food preservative and also as therapeutics (Perez et al., 2014).

3.3.4.4. Antioxidant Activity of the Isolates

Reactive oxygen species (ROS) that are continuously generated in the body can cause damage to DNA, proteins and membrane phospholipids. Probiotic strains that present antioxidant properties could provide protection against oxidative stress, decrease the accumulation of ROS and improve the total antioxidant status of the body. The inhibition of ascorbate autoxidation is commonly used to determine the total antioxidant activity of probiotics (Amaretti et al., 2013; Su et al., 2015). The ICFE of all the LAB isolates except G4 exhibited inhibition of ascorbate autoxidation (**Table 3.11**). The intracellular extract over death of microbial cells can provide protection against the free radical species (Kaushik et al., 2009). Inhibition of ascorbate autoxidation by *Oenococcus oeni* strains ranging from 13.75 to 23.51 % for the cell-free extracts was reported by Su et al. (2015). Ahire et al. (2013) reported 27.5 ± 3.7 % inhibition of ascorbate autoxidation by ICFE of *Lb. helveticus* CD6.

Table 3.11. Inhibition of ascorbate autoxidation by intracellular cell free extract of the LAB isolates

Isolates	Inhibition of Autoxidation (%)
CM22	21.0 ± 3.2
CM28	14.06 ± 1.8
P8	26.5 ± 2.6
G4	Not detected

3.3.4.5. Surface Binding Properties

a. Cell Surface Hydrophobicity

The microbial adhesion to hydrocarbons has been widely used to measure the cell surface hydrophobicity of bacteria (Orlowski & Bielecka, 2006; Vinderola & Reinheimer, 2003). Adherence to the host cell surface which determines the colonization capability of bacteria is a crucial step in the establishment of probiotics in the intestine. Through this adhesion property, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage on cell receptors (Otero et al., 2004). In the present study, the adhesion ability of the isolates to hydrocarbons as a measure of their hydrophobicity was evaluated. The hydrophobicity of the isolates was studied using chloroform, ethyl acetate, n-hexadecane, xylene and toluene and all the isolates exhibited different degree of hydrophobicity. The results are shown in **Table 3.12**.

Table 3.12. Hydrophobicity of the isolates

Isolates	**Hydrophobicity (%)**				
	Chloroform	Ethyl acetate	Xylene	Toluene	Hexadecane
CM22	81 ± 1.1	61 ± 0.8	36 ± 1.3	11± 0.6	45 ± 2.1
CM28	89 ± 1.2	27 ± 1.6	33 ± 2.1	10 ± 1.4	45 ± 1.4
G4	54 ± 1.8	16 ± 2.3	39 ± 1.04	48 ± 1.4	44 ± 1.5
P8	89 ± 2.0	25 ± 1.1	62 ± 1.5	65 ± 2.2	49 ± 1.6

The microbial adhesion to hexadecane reflects cell surface hydrophobicity or hydrophilicity because electrostatic interactions are absent. The adhesion of cells to xylene, a non polar solvent also demonstrated hydrophobic cell surface properties (Collado et al., 2007). All the isolates showed above 40% hydrophobicity in n-hexadecane. The cell surface hydrophobicity ranging from 2 to 95% has been reported for different probiotic bacteria (Rijnaarts et al., 1993; Schillinger et al., 2005). Studies on various strains of bacteria showed huge variation in the surface hydrophobic properties even among strains of a given species. Strains with hydrophobicity as low as 2% was

found to be able to adhere very well with the mucus producing HT-29 MTX cells. Hence it was suggested that hydrophobicity may not be the only criteria but an important component in a complex interplay between specific and non-specific factors which enable a microorganism to bind and persist in the host gut for an extended period (Kaushik et al., 2009; Schär-Zammaretti & Ubbink, 2003). The values obtained with the two other solvents, chloroform and ethyl acetate, were regarded as a measure of electron donor/basic and electron acceptor/acidic characteristics of bacterial surface, respectively (Martín et al., 2005). All the isolates had stronger affinity for chloroform, which is an acidic solvent and electron acceptor, than for ethyl acetate, which is a basic solvent and electron donor. The differences in cell surface hydrophobicity could be due to variation in the expression levels of cell surface proteins among strains of a species as well as due to environmental conditions which could affect the expression of surface protein (Kaushik et al., 2009).

b. Autoaggragation and Coaggregation of the Isolates

Bacterial aggregation between microorganisms of the same strain (autoaggregation) or between different species and strains (coaggregation) is of considerable importance in several ecological niches, especially in the human gut where probiotics are to be active (Jankovic et al., 2003). As shown in **Fig. 3.8**, all isolates except G4 exhibited more than 50% autoaggregation after 5 h incubation. Among the isolates CM28 and P8 showed maximum autoaggregation of 61.02 ± 1.3% and 60.9 ± 0.7% respectively after 5 h. The autoaggregation ability is one of the key factors that determine the ability of the probiotic strain to adhere to the oral cavity, gastrointestinal tract and urogenital tract and coaggregation ability helps to form a barrier that prevent colonization by pathogens. Lactobacilli with aggregation ability and hydrophobic cell surface could have more chance for adhesion to intestinal cells (Nikolic et al., 2010). In a previous study it was demonstrated that aggregation phenotype of *Lb. crispatus* M247 is important for the persistence of the strain in the colon since its aggregation-deficient isogenic mutant MU5 was not recovered from faeces and colonic mucosa (Voltan et al., 2007).

All the isolates also demonstrated coaggregation abilities against the tested pathogens where coaggregation with *R. planticola* was significantly higher than with *E. coli*. Coaggregation with *R. planticola* was highest for the isolate G4 (86%) and CM22 was better in terms of coaggregation with *E. coli* (22.91 ± 2.7%). **Fig 3.9** shows the coaggregation abilities of the isolates with pathogens after 5 h incubation at room

temperature. It has been suggested that LAB producing inhibitor molecules, which coaggregate with pathogens, may constitute an important host defence mechanism against urogenital infections and a similar protective mechanism could work in the gastrointestinal tract thus contributing towards the probiotic attributes of these bacteria (Kos et al., 2003).

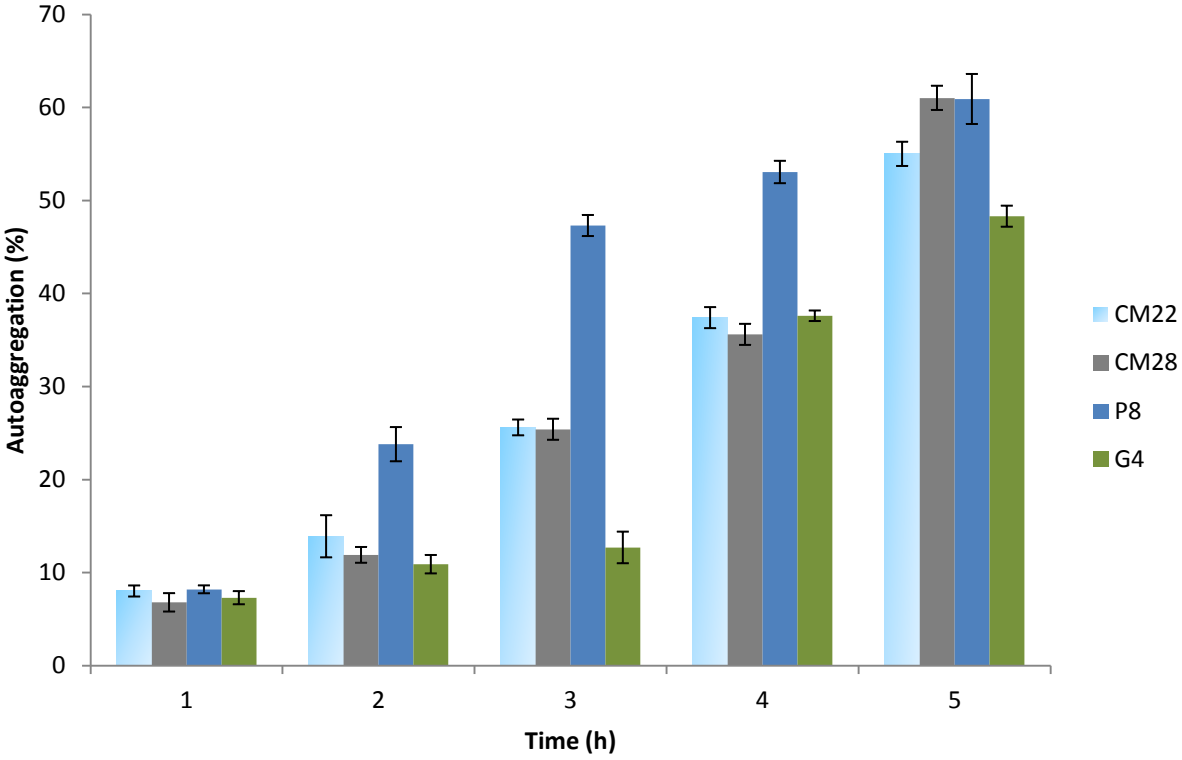


Fig. 3.8. Autoaggregation of the isolates

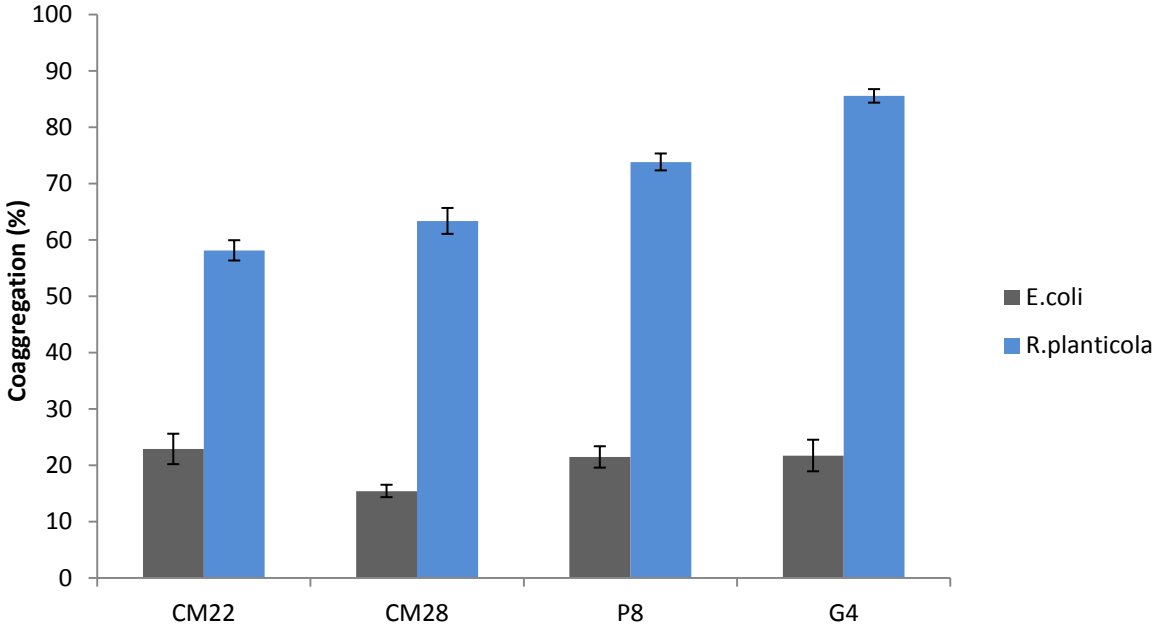


Fig. 3.9. Coaggregation of the isolates with pathogens (5 h)

c. Mucin Binding

All isolates demonstrated mucin binding ability (**Fig. 3.10**) and the isolate CM22 exhibited 60% mucin binding. Mucus layer in the intestinal epithelium acts as a protective barrier and harbours the normal mucosa associated microbial flora (Ljungh et al., 2002). Adherence to intestinal or gastric mucosa has a significant role in the competitive exclusion of pathogenic bacteria (Martín et al., 2005). This interaction with epithelial cells in the intestinal tract, may also promote retention and host-bacterial communication. Mostly surface proteins of LAB have been proposed to be involved in colonization of gastrointestinal epithelial cells and mucosa of mammals (Rojas et al., 2002).

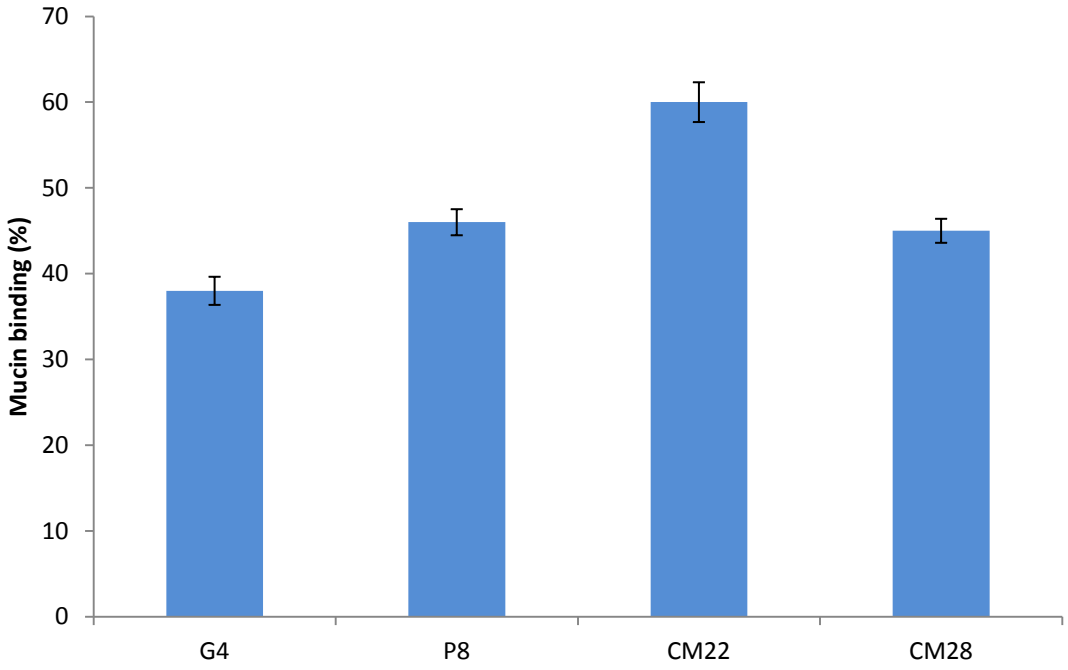
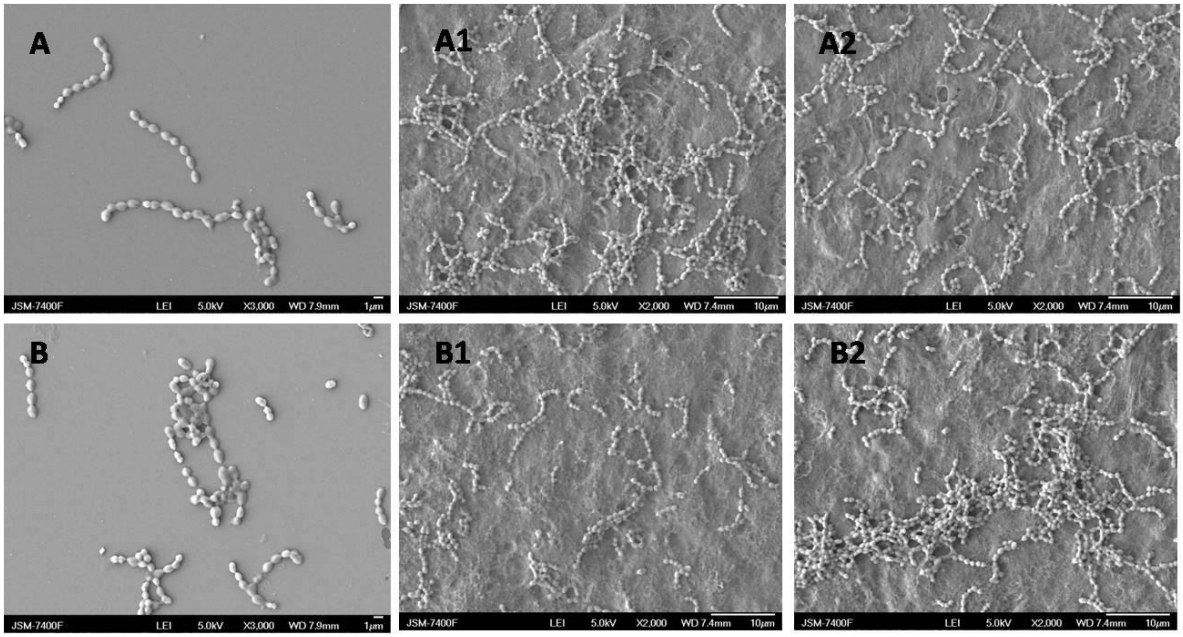


Fig. 3.10 Mucin adhesion of the isolates

d. Adhesion to Intestinal Epithelial Cells

Effective colonization of intestinal mucosa by the strains is considered as a potential probiotic marker along with other desirable attributes. This complex process of cell adhesion involves contact between the bacterial cell membrane and interacting surfaces. Due to the difficulties in studying bacterial adhesion *in vivo*, especially in humans *in vitro* models were developed for preliminary screening of potentially adherent strains (Duary et al., 2011). The human intestinal cell lines HT-29 and Caco-2 derived from colon adenocarcinomas expressing morphologic and physiologic characteristics of normal human enterocytes have been extensively used for *in vitro* studies (Blum et al.,

1999; Duary et al., 2011). The results of adhesion assay showed that *L. lactis* CM22 and *L. lactis* CM28 could adhere to the intestinal epithelial cells HT-29 and Caco-2. The LAB strains exhibited strong adherence to HT-29 while compared to Caco-2 cells. The micrographs illustrate the adherence of the LAB strains to intestinal epithelial cells (**Fig. 3.11** and **Fig. 3.12**).



3.11. Adhesion of the LAB isolates on HT-29 cells by SEM analysis

A- Free cells of *L. lactis* CM22, A1 and A2 – monolayer of HT-29 cells with *L. lactis* CM22;
B – Free cells of *L. lactis* CM28, B1 and B2 – monolayer of HT-29 cells with *L. lactis* CM28

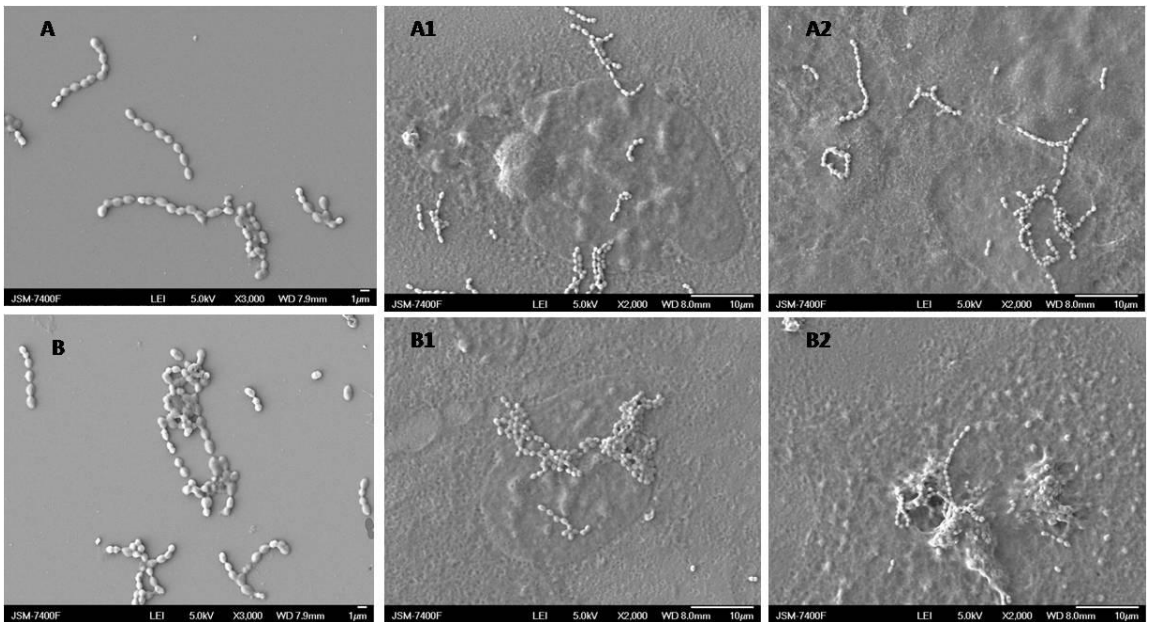


Fig. 3.12. Adhesion of isolates to Caco-2 cells

A- Free cells of *L. lactis* CM22, A1 and A2 – Adherence of *L. lactis* CM22 to Caco-2 cells

B – Free cells of *L. lactis* CM28, B1 and B2 – Adherence of *L. lactis* CM28 to Caco-2 cells

3.4. Conclusion

The chapter describes the isolation of folate producing LAB with probiotic characteristics. Among the LAB isolates, two *L. lactis* strains (CM22 and CM28), one *Weissella cibaria* (G4) and one *Enterococcus* sp. (P8) were significant folate producers. All the isolates exhibited essential probiotic characteristics as well. The probiotic features varied among the individual isolates and also found to be strain dependent. However, considering the extracellular folate production in skim milk medium (a medium of choice for optimization and scale up) the isolates CM22 and CM28 were selected for further detailed studies.