ISSN Online: 2349-7785

Probiotic Characterization and Quantification of Folates Produced by *Bacillus* Strains Isolated from Infant Faecal Matter

Smita Hasini Panda [1]*, Jyothsna Khanna Goli [2], Sushrirekha Das [1], Nakulananda Mohanty [1]

[1] Department of Microbiology, North Orissa University, Baripada, Odisha-757003, India
[2] Department of Microbiology, Osmania University, Hyderabad- 500007, Andhra Pradesh, India

Received 28 October 2013; accepted in revised form 12 December 2013

Abstract: The aim of the present study was to characterize the probiotic qualities and to study the efficacy of the selected isolates to produce folic acid by microbiological assay. Three isolates, IFM22B, IFM24B and IFM25B were selected for folate production in folic acid casei medium and the net folate yield was 59 ng/ml, 56 ng/ml and 49 ng/ml, respectively. The three isolates showed more than 98 % similarity to *Bacillus* spp. by 16S rRNA sequencing. Spores of these strains showed excellent tolerance in partially simulated gastrointestinal tract conditions and exhibited antimicrobial activity against organisms such as *Staphylococcus aureus*, *Klebsiella* and *Escherichia coli*. Importantly, these isolates were susceptible to the most of the antibiotics tested, in conflict that they would not donate resistance determinants if administered in the form of probiotic preparations.

Key words: Probiotic, lactic acid bacteria, *Bacillus*, folic acid, infant faecal matter, microbiota, gut.

Introduction

Folate is a B-group vitamin that cannot be synthesized by humans and must be obtained exogenously. Folate is involved in essential functions of cell metabolism, such as DNA replication, repair, and methylation; synthesis of nucleotides, vitamins, and some amino acids [34]. In nature, folate is present in different forms and matrixes that differ significantly in stability and bioavailability and tetrahydrofolate is its active form [32]. In bone marrow, folic acid is required for the normal production of the red blood cells and for RNA synthesis. Folic acid is in demand during lactation to improve the concentrations of choline, creatine, creatinine and carnitine, as well as milk proteins in milk [50,53]. Increase in the folic acid requirements were also seen in active proliferative cells such as those from the placenta, embryo and the fetus or the newborn [7,32]. Folic acid is necessary for normal cell division and multiplication.

Vitamins have been characterized to enhance resistance to infection by increasing migration and proliferation of phagocytic cell and vitamin B6 have been found to affect immune responses of fish [4,17]. Folate deficiency has been observed in a wide variety of disorders from Alzheimer's to coronary heart diseases, osteoporosis, increased risk of breast and colorectal cancers, poor cognitive performance, hearing loss [30.31]. Folate deficiency is associated with increased risk for malforma-tions in early embryonic brain and spinal cord development, neural tube defects (NTDs) [51]. Megaloblastic anemia is considered as the typical sign of FA deficiency. In most animals and in humans folic acid deficiency is

*Corresponding author (Smita Hasini Panda)
E-mail: < panda.smita@gmail.com >

© 2014, Har Krishan Bhalla & Sons

characterized by impaired hematopoiesis [33,54].

In many countries fortification programmes were recommended by Health organizations [52], USA (US Food and Drug Administration, 1996 and Chile [18]) have mandatory fortification of flour and uncooked cereal-grain products. However, many countries did not choose fortification because of the potential link between high doses of synthetic folic acid and the development and progression of certain cancer forms [3,23], as well as masking of vitamin B_{12} deficiency and thereby the risk of neuropathy [5]. Natural folates, in contrast to synthetic folic acid, do not mask vitamin B_{12} deficiency and are probably of lesser risk with respect to overdosing and cancer [29]. Therefore, biofortification with natural folates produced by selected microorganisms may be an alternative to fortification with synthetic folic acid.

Probiotics are defined as "live microorganisms when administered in adequate amounts confer beneficial health effects on the host [35]. Various groups of microbes have been chosen for probiotic action including many species of genera *Lactobacillus* and *Bifidobacterium* which were originally isolated from the human gastro-intestinal tract. These genera are the most copious and well established in probiotic containing food products but species of *Enterococcus*, *E. coli*, *Bacillus*, *Brevibacillus*, etc have also been recommended for probiotic effects [24,35]. Out of more than 100 *Bacillus* spp. known, only a few like *B. subtilis, B. licheniformis, B. clausii, B. coagulans, B. cereus, B. pumilus,* and *B. laterosporus* possess common probiotic characteristics, such as gut viability, resistance to bile and acid, and the ability to synthesize various compounds useful to humans, hence they are increasingly used in different food products as probiotics for human consumption, [26,44].

Probiotics *Bacillus* can serve as prophylactic agents in humans and as an alternative to antibiotics in farming and aquaculture to enhance growth and resistance to diseases [38,49]. They are also used as therapeutic agents for the treatment of urinary tract infections. Probiotic *Bacillus* became popular as they exhibit functionalities such as production of antibiotics, which provide protection against wide range of pathogenic bacteria [28], production of essential amino acids, vitamins and enzymes, decrease the levels of serum blood cholesterol, reduce blood clotting by fibrinolysis, antimutagenic effects, and stimulation of the immune system**.** Probiotic therapy gained importance because of its effective and non-invasive approach, and capacity to restores the natural flora [27].

Spore forming *Bacillus* gain more importance over non-spore-formers, As spore forming *Bacillus* can survive at high temperature and pressure during foods processing, survive better under gastrointestinal tract, possess a long shelf-life and remain viable both at room temperature and refrigerated conditions throughout their shelf-life and they can be used in low dose as probiotic supplements due to their better survivability [12,14].

Products containing *Bacillus* endospores are used commercially as probiotics containing a single dose of 10^9 spores/g or 10^9 spores/ml [31]. Further, the composition of currently used probiotics varies from those containing a mixture of many strains to those containing a single strain. The probiotic Biosporine®is made up of *B. subtilis* and *B. licheniformis* cultures and it is commercially available in Russia and Ukraine [43].

Materials and methods

Isolation for folate production

Samples were collected from infant faecal matter of different age groups i.e from 6 months to 2 year old healthy infants. They were diluted by serial dilution method and plated using MRS agar media (Mann Rogassa Sharpe) [42] by spread plate and pour plate method. Further, plates were incubated at 37°C for 48h. Based on the morphological appearance, the colonies were picked and characterized by different biochemical tests. The isolates were preserved as frozen glycerol stocks and maintained on MRS agar slants at 4°C. Working cultures were prepared by propagating them in MRS broth.

Screening of folate producers

Isolates were screened for folate production, by inoculating a singly colony from MRS agar plates into folic acid assay medium (2 ml) and

incubated for 18 h at 37°C. Isolates showing growth in the assay medium were selected as folate producers and quantitative determination of folate was carried out by microbiological assay.

Microbiological assay for quantification of folate

To quantify the folate, microbiological assay was performed using an folate auxotrophic mutant cryoprotected *L.casei* NCIM 2364 as standard. The organism was activated by growing in folic acid assay medium with supplementation of 30 ρg of calcium folinic acid and 15 mg of ascorbic acid and incubating for a period of 18 h at 37°C. Various concentrations of folate standard ranging from 50 pM to 300 pM were used for standardization. The sample reaction mixture contained working buffer (16 % sodium ascorbate in 50 mM phosphate buffer pH 6.1), varying concentrations of working standard solution (0.9 nM of calcium salt of folinic acid), double strength folic acid casei medium, sterile water for making up the volume and the culture inoculum. All the above reagents were added in a 96-well microtiter plate and incubated for 18 h at 37°C and the absorbance of the supernatant was read at 655nm using UV-VIS spectro-photometer. Further the isolates producing maximum extracellular folate were identified by 16SrRNA sequencing.

Qualitative analysis of folate by HPLC (High pressure liquid chromatography)

To determine different forms of folate produced by the isolates, the isolates were grown in folic acid assay medium for 7 h. Further the samples were collected, filtered through 0.2 micron filters and analysed by HPLC (Schimadju HPLC system). The C_{18} Nova-Pak (25cm x 4.6 mm, 4 µ spherical packing) was used as the analytical column and 0.2 micron filtered HPLC grade acetonitrile (2.5 %) in 20 mM sodium phosphate buffer pH 6.2 was used as mobile phase. Flow rate through the column was 1 ml/min. A UV-Visible detector was used at 280 nm. Different forms of folate such as tetrahydrofolate (THF), 5-methyl tetrahydrofolate (5-MeTHF) and 5-formyl tetrahydrofolate (5-FTHF) were procured from Sigma and used as standards.

Analysis of 16S ribosomal RNA sequence

Identification of isolates producing maximum extracellular folate was carried out by complete 16S rRNA gene sequence analysis and phylogenetic studies (Macrogen Inc., Korea). Universal primers 518F (52-CCAgCAg CCg Cgg TAATACg-32) and 800R (52-TACCAggg TAT-CTAATCC-32) were used for the amplification for 16SrRNA gene of the isolates. Evolutionary analyses were conducted in MEGA 5 software [47]. Evolutionary history was inferred using the Neighbor-Joining method [41] and the evolutionary distances were computed using the Tajima-Nei method [46].

Characterization of probiotic properties of isolates

Acid tolerance test

For this purpose, overnight incubated active cultures of isolates were used. Cells were harvested by centrifugation for 15 min at 8,000 rpm and 4°C. Pellets were washed once in phosphate-saline buffer (PBS at pH 7.2), resuspended in 1 ml PBS (pH 3) and the strains were further diluted 1:100 in PBS at pH 1, 2, 3 and 4 and incubated at 37°C. Surviving micro-organisms were enumerated at 0, 1, 2, and 3 h by plating on MRS agar and the growth count was expressed in colony forming units (CFU) per milliliter ($\log_{10}$ cfu/ml) The survival rate was calculated.

Bile salt tolerance

Bile salt tolerance was determined by inoculating 100 µl overnight grown culture of the isolates into 900 µl MRS broth supplemented with 0.3 %, 0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 %, 3 %, 3.5 % and 4 % bile salt (Ox gall, Hi-media) and was incubated at 37µC for 24 h. The viable bacteria were enumerated by plating 100 µl of culture onto the MRS agar plates incubated at 37°C for 24 h. Growth of bacteria was expressed in colony forming units per millilitre ($\log_{10}$ cfu /ml) and survival % of strain was then calculated.

Phenol tolerance

Phenol tolerance was determined by inoculating 100 µl overnight grown culture of the isolates

into 900 µl MRS broth supplemented with 0.1-0.5 % of phenol and was incubated at 37°C for 24 h. Tolerance of isolates was analyzed by measuring the absorbance at 600 nm and MRS broth without phenol was taken as reference and further the survival % of strain was calculated.

NaCl tolerance

NaCl tolerance was determined by inoculating 100 µl overnight grown culture of the isolates into 900 µl MRS broth supplemented with 2, 4, 6, 8, 10 and 12 % of NaCl and was incubated at 37°C for 24 h. Tolerance of isolates was analyzed by measuring the absorbance at 600 nm and MRS broth without phenol was taken as reference and further the survival % of strain was calculated.

Antibiotic susceptibility test

The susceptibility of isolates to antibiotics was determined by disk diffusion assay according to the Clinical and Laboratory Standards Institute (CLSI) guideline [11]. Types of antibiotic disktested were tetracycline, erythromycin, ampicillin, gentamycin, penicillin, chloramphenicol, cefuroxime, cefoperazone, levofloxacin, norfloxacin, Hi-Media, Mumbai) were placed on the surface of the MRS agar medium aseptically. Plates were incubated for 24 h at 37°C and observed for zones of inhibition.

Screening for antimicrobial activity

Antimicrobial activity of isolates was determined by Colony Overlay assay against the indicator strains. Five microliters of an overnight culture of each isolates were spotted on isolation media and incubated at 37°C for 24 h. Inoculum of the indicator strains, enteric pathogens like and *Staphylococcus aureus* NCIM 5021, *E.coli* NCIM 6145, *Klebsiella* and *Pseudomonas aerugenosa* was prepared by calibrating the overnight culture to a turbidity of 0.5. Then, 0.1 ml of the inoculum was mixed with 5 ml medium containing 0.7 % agar and the mixture was subsequently overlaid on the spots and incubated at 37°C for 24 h. Any isolate showing inhibition zone around the spot was considered to have antimicrobial activity.

Autoaggregation assay

Autoaggregation assay was performed according to Del Re *et.al*., [13] with certain modifications. Isolates were grown over night at 37°C in MRS broth. The cells were pelleted and washed twice with PBS (pH 7.3) and resuspended in PBS to get an OD of 0.5 at A_{600} and considered as $A_{0.}$ Four ml of culture was mixed by gentle vortexing for 10s and incubated at 37°C for 1h. After incubation absorbance of upper suspension was measured as At. Autoaggregation % was expressed as: A_0 - (A_t /A_0) x100, where A_t represents the absorbance at time t = 1 h and A_0 the absorbance at t = 0 h.

Bacterial adhesion to hydrocarbons

Bacterial adhesion ability of the cultures were assessed according to the procedure described by Rosenberg *et.al*., [40]. This test was conducted using hydrocarbon xylene, acetone and heptane. 3 ml of overnight grown culture was taken and centrifuged at 8,000 rpm for 15 min at 4°C. The collected pellet was washed with phosphate-buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4, pH 7.2) and resuspended in in 0.1 mol/l KNO_3 (pH 6.2) and absorbance was measured at $A_{600,\ as}$ A_0 by using spectrophotometer (UV-VIS 1601 Spectrochem, Mumbai). To this cell suspension, 1.0 ml of solvent (xylene, acetone and heptane) was added and after 10 min pre-incubation at room temperature, the two phase system was thoroughly mixed by vortexing for 3 min. Aqueous phase was removed after incubation for 20 min at room temperature and its absorbance was measured at A600, as A_1. Adhesion percentage was calculated using the formula, (A_0- A_1 /A_0) x 100.

Hemolysis of red blood cells

Each isolates was streaked on MRS agar supplemented with 5 % sheep blood and incubated at 37°C for 24h. The presence of clear zone around colonies indicated lysis ability of those colonies and was considered as the positive result.

Statistical analysis

To find out the linear association and to compare the factor level difference among the variables

such as tolerance to bile salt, phenol and sodium chloride by isolates correlation analysis was performed. All the analysis was carried out by using SPSS software for windows release 19.0 version (SPSS Inc., IBM, NewYork, USA).

Results

Isolation and identification of folate producing lactic acid bacteria

Selection of isolates was based on the macroscopic differences in the colony morphology and also on the collection of samples (Infant faecal matter) from 6 months to 2 year old healthy infants. The isolates were qualitatively analyzed for the ability to produce folate by observation of growth of the isolates in the modified folic acid assay media. Among the 60 isolates analyzed, 19 isolates (RM1, 2,3 5, 6,7,8,9,10,11, IFM7, DB8, JC14, PM15, 16, IFM18, IFM22B, IFM24B, IFM25B) were found to grow in folic acid assay media indicating their ability to grow without any supplementation of folate (Figure 1). Among the above isolates the significant folate producers are IFM22B, 24B and 25B (59 ng/ml, 56 ng/ml and 49 ng/ml, respectively), hence choosed for carrying out further probiotic studies.

Biochemical analysis

Morphologically isolate, IFM22B, 24B and 25B appeared yellowish roughed surfaced with irregular margins. Microscopically all the three isolates appeared as Gram +ve rods and cocci. Results of biochemical analysis were in close agreement with 16S rRNA analysis of the isolates. IFM 22B, 24B, 25B were catalse positive, Voges-progeskaur positive, spore forming and found to be indole negative, arginine dihydrolase negative and positive for citrate utilization and for sugars like; mannose, xylose, fructose, glucose and arabinose. The above results closely resemble the biochemical characters of *Bacillus* spp. [25] (Results not shown).

Strain identification

Genetic analysis performed using 16S r-RNA gene analysis resulted isolates with expected base pairs 996, 1179, 978 bp for IFM22B, 24B and 25B, respectively. After performing a BLAST search isolates IFM22B, 24B and 25B exhibited close association with known *Bacillus* sp. with GC content of 54 %, 55 % and 55 %, respectively. These results were further confirmed by constructing a phylogenetic tree (Figure 2). The optimal tree with the sum of branch length for isolate IFM22B, 24B and 25B is 0.20663937. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 31 nucleotide sequences, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position and there were a total of 44 nucleotide sequences and a total of 878 positions in the final dataset for IFM22B, 24B and 25B. The gene sequences of the isolates IFM22B have been submitted to NCBI gene bank data based

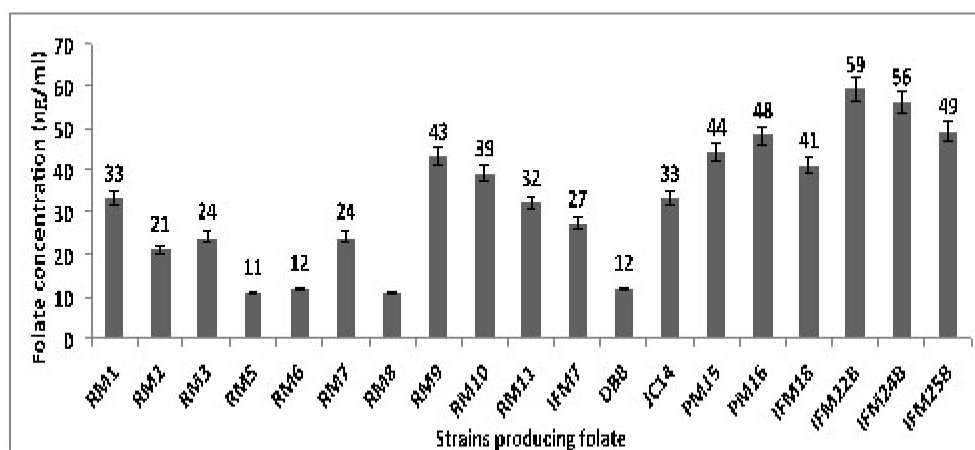

Figure 1. Folate production by different isolates

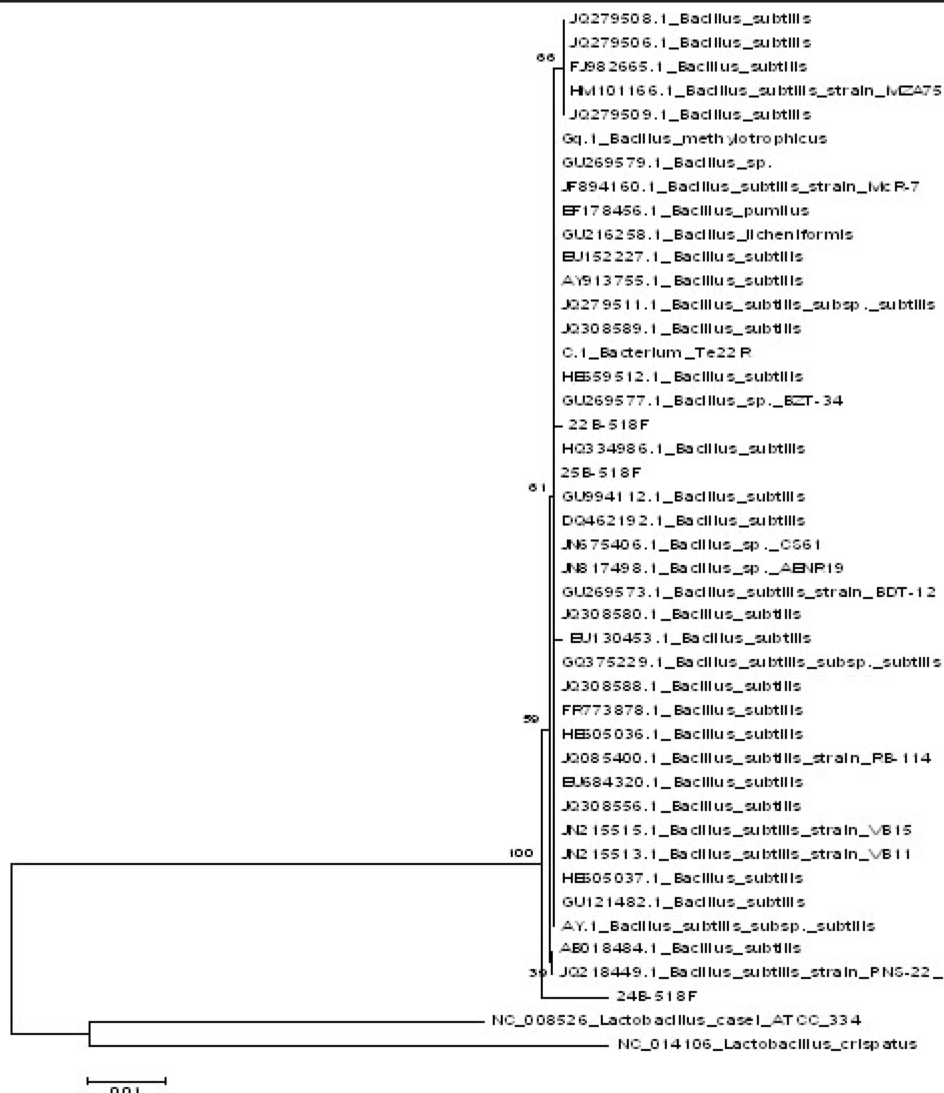

Figure 2. Phylogeny tree of IFM 22B, 24B and 25B

under accession number KC545699, respectively.

Qualitative determination of forms of folate

Microorganisms mainly synthesize different forms of folate such as THF, MTHF and N5FTHF. Extracellular folate produced by isolate IFM 22B, 24B and 25B (*Bacillus* spp) was detected by HPLC and compared with the standards tetrahydrofolate and 5-methyl tetrahydrofolate, which have shown the retention time of 2.798 and 4.112, respectively. The chromatogram suggested that IFM 22B, 24B and 25B synthesized THF and MTHF forms of folate (Figure 3 a, b). Our study was in close agreement with Gangadharan *et.al.* [19], who also reported major forms of folate production by lactic acid bacteria as THF and MTHF.

Probiotic properties

Acid tolerance

Among the isolates evaluated for acid tolerance more than 80 % viability was observed at pH 3 and nearly 70 % viability at pH 2 after 120 min of exposure (Figure 4).

Bile salt tolerance

Viability of the three isolates when tested for bile salts (0-4 %) it was found that a constant survival rate of 50-80 % was maintained in the bile salt range of 0.3 % - 2 % and the viability

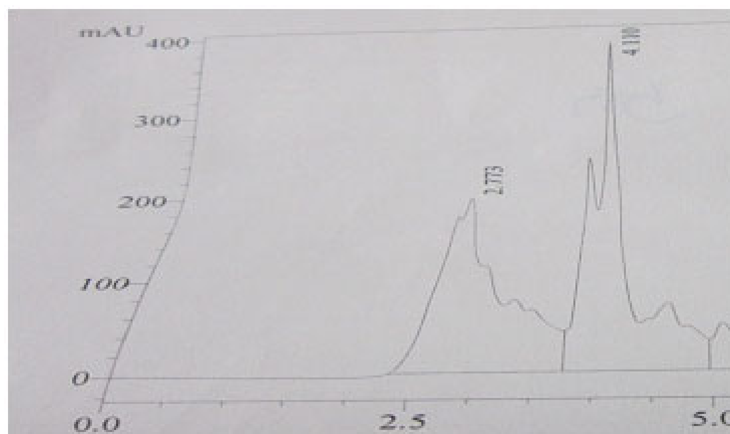

Figure 3a. Chromatogram of standard folate derivatives; peak 1= tetrahydrofolate, peak 2= 5-methyl tetrahydrofolate

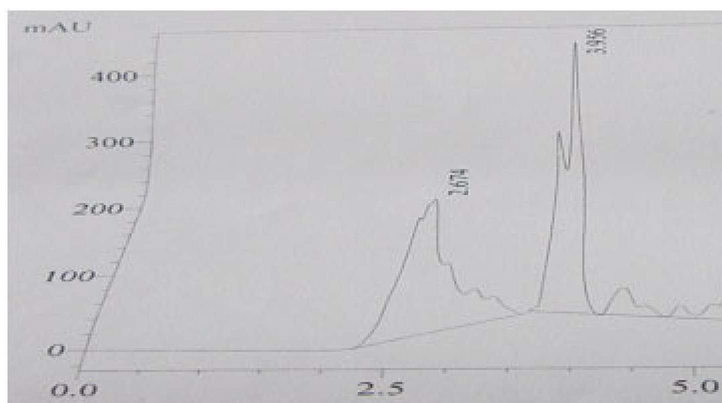

Figure 3b. Chromatogram of tetrahydrofolate and 5-methyl tetrahydrofolate produced by isolate IFM22B, IFM24B and IFM25B

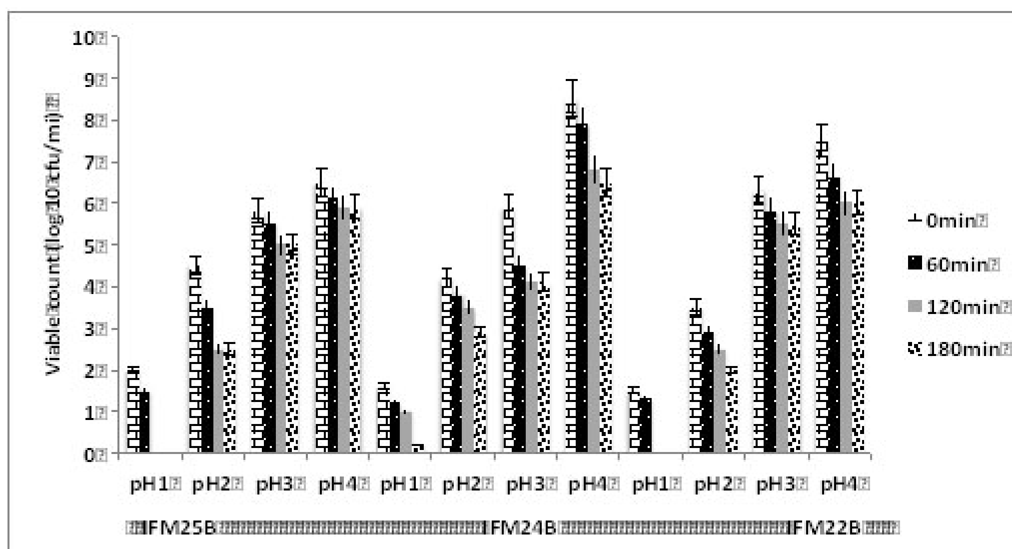

Figure 4. Acid tolerance and viable count of isolates at different pH and at different time of exposure

declined as the concentration of bile salt increased (Figure 5).

Phenol tolerance

Results of phenol resistance exhibited relatively high survival rate at 0.2 % while the three isolates showed 60 % survival at 0.4 % phenol and gradually decreased to less than 20 % at 0.5 % phenol (Figure 6).

NaCl tolerance

The results of salt tolerance studies in the Figure 7 showed 59-99 % viability at 2 % and 4 % salt concentration by IFM22B, 24B and 25B. The viability reduced to 50 % and 30 % at 6 % and 8 %, respectively and the growth was totally inhibited at 12 % NaCl.

Antibiotic resistance study

IFM22B, 24B and 25B were moderately susceptible to the entire antibiotic tested (Table 1).

Antimicrobial activity

Isolate IFM22B,24B and 25B showed moderate antimicrobial activity with a zone of 1.5 mm, 1 mm 0.5 mm against *S. aureus*, *Klebsiella* and *E. coli*, respectively.

Autoaggregation assay

Autoaggregation is an important property of probiotic bacteria because they reflects its adhesion ability to enterocytic cellular lines and also provides resistance to peristaltic elimination [32]. Autoaggregation was investigated on the basis of sedimentation characteristics of isolates. The

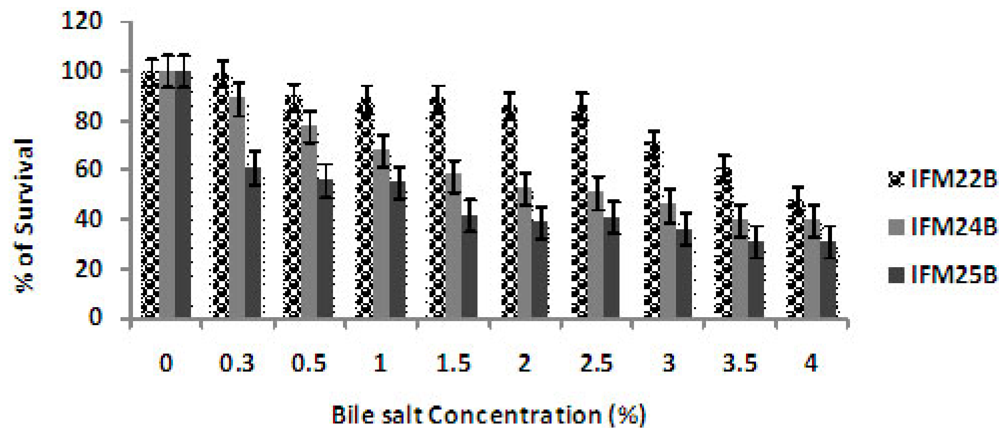

Figure 5. Bile salt tolerance of survival of isolates at bile salt range of 0-4%

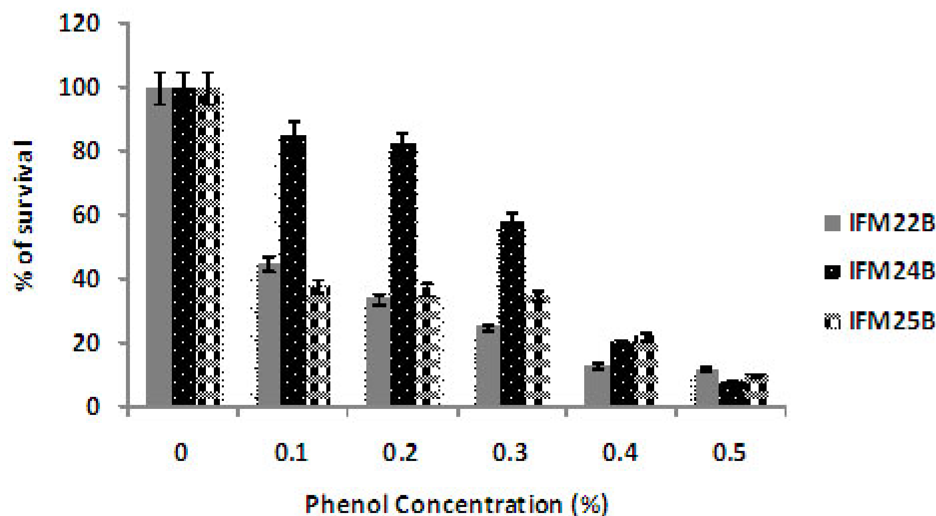

Figure 6. Phenol tolerance and % of survival of isolates at a range of 0- 0.5 %

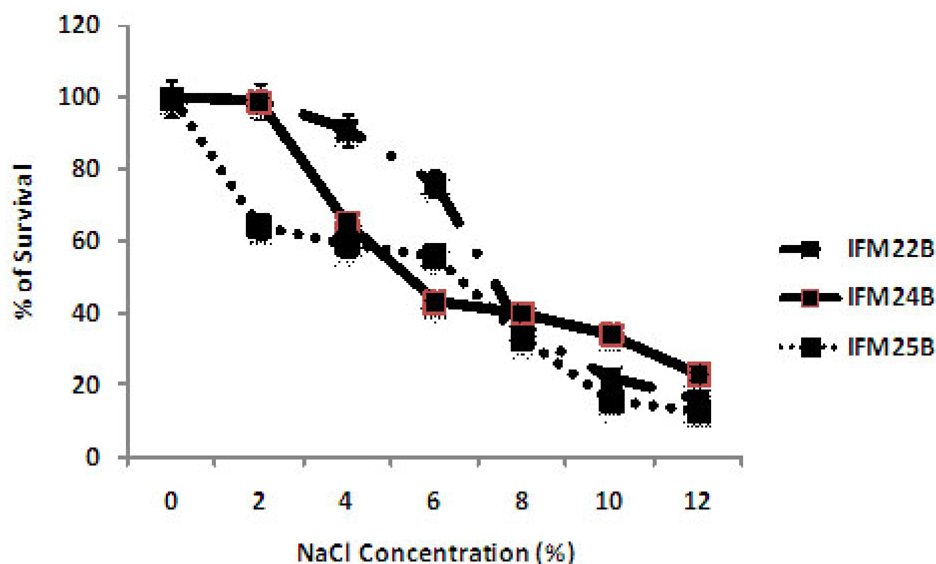

Figure 7. NaCl tolerance and % of survival of isolates at a range of 0- 12%

Table 1. Antibiotic sensitivity tests of different isolates

Isolates	Name of the Antibiotic (mcg)	Diameter of inhibition zone (mm)	Sensitivity
IFM22B	Norfloxacin (10)	31	+++
	Erythromycin (15)	19	++
	Tetracyclin (10)	21	++
	Gentamycin (10)	36	+++
	Chloramphenicol (30)	28	+++
	Cefuroxime (30)	19	++
	Ampicillin (10/10)	31	+++
	Levofloxacin (5)	22	++
IFM24B	Levofloxacin (5)	33	+++
	Cefuroxime (30)	23	++
	Norfloxacin (10)	14	++
	Erythromycin (15)	15	++
	Ampicillin (10/10)	20	++
	Gentamycin (10)	16	++
	Chloramphenicol (30)	29	+++
IFM25B	Norfloxacin (10)	24	++
	Ampicillin (10/10)	31	+++
	Tetracyclin (10)	32	+++
	Gentamycin (10)	17	++
	Chloramphenicol (30)	24	++
	Erythromycin (15)	17	++
	Levofloxacin (5)	30	+++
	Cefuroxime (30)	24	++

results are depicted in Table 2.

Hydrophobicity of strains

The hydrophobicity of the isolate IFM 22B and 24B was higher with acetone(> 40 %). However, for isolate IFM 25B hydrophobicity was higher with xylene(Table 3).

Haemolytic activity

The three isolates IFM22B, 24B and 25B were found to be non-haemolytic (γ-haemolysis) on 5 % sheep blood agar. The absence of haemolytic nature is considered to be a positive trait for bacteria to be used as a probiotic strain.

Statistical analysis

The ANOVA results revealed that folic acid production by IFM22B, 24B and IFM25B was highly significant at pH7 [$F (4, 14) = 433.400$ and 542.067, respectively; $P<0.001$], inoculum volume at 15 % [$F (4, 14) = 426.375$ and 593.250, respectively; $P<0.001$], agitation speed at 150 rpm [$F (3, 11) = 338.800$ and 220.00, respectively; $P<0.001$] and incubation period at 72 h [$F (4, 14) = 439.0$, 543.0, respectively; $P<0.001$]. The post hoc analyses revealed that all the factors such as pH at 7, incubation period at 72 h, inoculum volume at 15 % and agitation speed at 150 rpm had significant effect on folic acid production ($P< 0.05$; Tukey HSD).

Pearson's correlation coefficient (r2) analysis was used for the measurement of linear association between the survibility of all the three isolates and variables like bile salt, phenol and NaCl (Table 4 a, b and c). The analysis showed that all the above variables had significant negative correlation with the three isolates i.e when the concentration of bile salt, NaCl and phenol increased the survibility rate of the isolates decreased. For example- IFM24B - bile salt tolerance (-0.947, $P< 0.01$), IFM24B-phenol tolerance (-0.977, $P< 0.01$) and IFM24B- NaCl tolerance (-0.960, $P< 0.01$).

Discussion

Lactobacillus and *Bifidobacterium* are safer and well-recognized genera of probiotic, available in commercially probiotic products. Use of spore formers as probiotics has not been established well because of their non-indigenous

Table 2. Percent of Autoaggregation by different isolates

Isolates	Initial OD at 600 nm	Final OD at 600 nm	% of Autoaggregation
IFM22B	0.478	0.318	33
IFM24B	0.333	0.231	31
IFM25B	0.404	0.260	36

Table 3. Percent hydrophobicity of different isolates against different solvents

Isolates	Solvents	Initial OD at 600 nm	Final OD at 600 nm	% of Hydrophobicity
IFM22B	Heptane	0.239	0.179	25
	Xylene	0.329	0.229	4.1
	Acetone	0.239	0.129	46
IFM24B	Heptane	0.168	0.155	7.7
	Xylene	0.168	0.148	11.9
	Acetone	0.168	0.099	44
IFM25B	Heptane	0.193	0.163	15.5
	Xylene	0.193	0.183	51.8
	Acetone	0.193	0.112	41.9

Table 4 a. Correlation coefficient analysis between bile salt and survibility of three isolates

Bile salt concentration (%)	IFM24B	IFM25B	IFM22B
1.000	-0.947**	-0.829**	-0.933**
	1.000	0.930**	0.961**
		1.000	0.973**
			1.000

** Correlation is significant at the 0.01 level (2-tailed)

Table 4 b. Correlation coefficient analysis between phenol concentration and survibility of three isolates

Phenol concentration (%)	IFM24B	IFM25B	IFM22B
1.000	-0.977**	-0.856**	-0.888**
	1.000	0.819**	0.747**
		1.000	0.522**
			1.000

** Correlation is significant at the 0.01 level (2-tailed)

Table 4 c. Correlation coefficient analysis between NaCl concentration and survibility of three isolates

NaCl (%)	IFM24B	IFM25B	IFM22B
1.000	-0.960**	-0.967**	-0.959**
	1.000	0.893**	0.975**
		1.000	0.996**
			1.000

** Correlation is significant at the 0.01 level (2-tailed)

origin, and it is scarcely studied in the various vital aspects like folate synthesis. The inherent resistance of spores to environmental stress is an attractive attribute for commercial probiotic preparations. Moreover, there are some of the functional food like natto of Japan, which comprises the use of bacilli in it [35]. One aspect must be considered for *Bacillus* probiotic that non-intestinal sites like mouth, stomach, vaginal tract, etc., where probiotic attribute do not employ, are connected to native flora and adherence to intestinal epithelial cell. *Bacillus* probiotics must be relevant, since these shows higher protease activity and antimicrobial property against various Gram-positive and Gram-negative pathogens [35,38]. High acidity in the stomach and the high concentration of bile components in the proximal intestine are the first host factors, which affect the strain selection.

Major characteristic used for *in vitro* screening of probiotic bacteria is its resistance to acidity of the stomach, lysozyme, bile, pancreatic enzymes and antibiotics used in therapy [45] to exert their beneficial effects in the gut [8,36]. The probiotic strains are likely to be buffered by food or other carrier molecules when exposed to extremes of pH in the stomach. It has been reported that the survival rate in the stomach might increase in the presence of foodstuff, which may protect the LAB from the effect, by pepsin and acid [10,22]. Acidity

of human gastrointestinal tract varies from 1.5 to 4.5 but the *in-vitro* studies were mostly performed at pH 3 as the viability below pH 3 is very low [2].

Gangadharan *et.al.*, [19] reported 80 % viability of *Lactococcus* spp. at pH 3 and 60 % viability at pH 2. Probiotic strains need to survive bile salts in the duodenum to exert their beneficial effects in the gut. Hence bile salt tolerance is considered one of the most important properties of probiotic microorganism as it allows them to survive and colonize the gastrointestinal tract by enterocytes adhesion [1,35]. Bile salt tolerance studies were mostly carried out using Oxgall bile salt because of their similarity to human bile juice. Bhakta *et.al.*, [8] reported lactic acid bacteria (LAB) have the highest bile salt (4g/l) tolerance. The *Lactobacillus* strains can grow in MRS agar supplemented with 3 g/l bile salt [37]. The strains *Pediococcus acidilactici* (P2), *Lactobacillus curvatus* (RM 10) and *Lactobacillus sake* (L2), were resistant to 3 g/l bile salt at pH 6 [15].

Resistance to phenol is also an important factor for probiotic bacteria as some aromatic amino acids are derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols [40]. Phenolic compounds can exert bacteriostatic effects, thus testing for resistance to phenol generates information on potential for survival in the gastrointestinal conditions, thereby proving to be the best probiotic strain. Gangadharan *et.al.*, [19] has reported relatively high survibility at 0.2 % phenol that has been decreased to 50 % at 0.4 % phenol and to 10 % survival at 0.6 % phenol by *Lactococcus* spp. Tolerance to high salt concentrations is useful to help in the initiation of metabolism [6]. Gomes *et.al.*, [20] reported that *E. faecium* and related species were resistant to ampicillin, tetracycline, chloramphenicol, trimethoprim/ sulfamethozaxole, quinolones and streptogramins. Antimicrobial activity may be as a result of organic acids i.e. lactic acid, acetic acid and formic acid produced or because of diecetyl, hydrogen peroxide and CO_2 alone or in combination [19,21]. Gomes *et.al.*[20] reported antimicrobial activity of *Enterococcus* spp against Gram +ve bacteria such as *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus* and also against some Gram –ve bacteria spp.

Qing *et. al.*, [39] has reported wider range (5-30 µg/ml) of antibiotic resistance by *Enterobacter cloacae* against ampicillin, erythromycin, kanamycin and rifampicin. The antibiotic resistant properties also indicated that the isolated LAB strains would be able to survive in the environment and intestinal milieu by with standing the undesirable situation occurred because of occasional high antibiotic concentrations.

Autoaggregation is an important property of probiotic bacteria because they reflects its adhesion ability to enterocytic cellular lines and also provides resistance to peristaltic elimination [34]. Autoaggregation was investigated on the basis of sedimentation characteristics of isolates. Ahire *et.al.*, [2] reported 18.23 %. Colonization of the mucosal surfaces and adhesion of bacteria to gastrointestinal host epithelial cells and extracellular matrix proteins is dependent on the cell surface hydrophobicity [19]. The colonization of the tissues by probiotic microbes can prevent pathogen access by steric interactions or specific blockage on cell receptors [16]. High hydrophobicity indicates the presence of hydrophobic molecules on the bacterial surface like array proteins, wall intercalated proteins, cytoplasmic membrane protein and lipids. Kos *et. al*.[30] reported the hydrophobicity of *Lactobacillus* towards xylene and chloroform as 70.96 % and 36.06 %, respectively and *Enterococcus* towards chloroform as 61.21 %. The three strains have been proved to be potent probiotic strains due to their survival under highly acidic conditions, higher tolerance to bile salt, NaCl and phenol. In addition, its colonization efficiency was proved by exhibiting high autoaggregation and hydrophobicity. Further absence of haemolytic nature and antibiotic resistance with maximum siderophore production made them to be considered as potent probiotic strains, but their applications have to be determined by conducting proper animal and human trials.

Acknowledgements

We authors are very much thankful to SERB-FTYS Scheme, New Delhi for giving us the financial support to carry out this study. The authors are also thankful to Macrogen Inc., Korea for the confirmation of our isolates by 16S rRNA sequencing.

References

1. **Ahire, J.J., Patil, K.P., Chaudhari, B.L., Chincholkar, S.B. (2011a).** A potential probiotic culture ST2 produces siderophore 2, 3-dihydroxybenzoylserine under intestinal conditions. Food Chemistry. 127: 387-393.
2. **Ahire, J.J., Patil, K.P., Chaudhari, B.L., Chincholkar, S.B. (2011b).** *Bacillus* spp. of Human Origin: A potential siderophoregenic probiotic bacteria. Applied Biochemistry Biotechnology. 164: 386-400.
3. **Aimmo, M.R.D., Mattarelli, P., Biavati, B., Carlsson, N.G., Andlid, T. (2012).** The potential of bifidobacteria as a source of natural folate. Journal of Applied Microbiology. 112: 975-984 .
4. **Albrektsen, S., Sandnes, K., Glette, J., Waagbø, R. (1995).** Influence of dietary vitamin B6 on tissue vitamin B6 contents and immunity in Atlantic salmon, Salmo salar L. Aquaculture Research. 26: 331-339.
5. **Asrar, F.M., O'Connor, D.L. (2005).** Bacterially synthesized folate and supplemental folic acid are absorbed across the large intestine of piglets. Journalof Nutritionand Biochemistry. 16: 587-593.
6. **Aswathy, R.G., Ismail, B., John, R.P., Nampoothiri, K.M. (2008).** Evaluation of the probiotic characteristics of newly isolated lactic acid bacteria. Applied Biochemistry Biotechnology 151: 244-255.
7. **Barkow, B., Matte, J.J., Bo'Ehme, H., Flachowsky, G. (2001).** Influence of folic acid supplements on the carry-over of folates from the sow to the piglet. British Journal of Nutrition. 85: 179-184.
8. **Bhakta, J.N., Ohnishi, K., Munekage, Y., Iwasaki, K., Wei, M.Q. (2012).** Characterization of lactic acid bacteria-based probiotics as potential heavy metal sorbents. Journal of Applied Microbiology. 112: 1193-1206.
9. **Berrada, N., Lemeland, J.F., Laroche, G., Thouvenot, P., Piaia, M. (1991).** Bifidobacterium from fermented milks: survival during gastric transit. Journal of Dairy Science. 74: 409-413.
10. **Clinical and Laboratory Standards Institute, (2006).** Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard, Seventh Edition. CLSI document M7-A7 (ISBN 1-56238-587-9). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2006
11. **Cutting, S.M. (2011).** *Bacillus* probiotics. Food Microbiology 28: 214-220.
12. **Del Re, B.S., Miglioli, B., Palenzona, M.D. (2000).** Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Letter in Applied Microbiology. 31: 438-442.
13. **Durkee, D.L. (2010).** Coming out of the dairy case: New developments in shelf stable probiotic foods. http://www.foodmaster.com.
14. **Erkkila, S., Petaja, E. (2000).** Screening of commercial meat starter cultures at low pH in the presence of bile salts for potential probiotic use. Meat Science 55: 297-300.
15. **Espeche, M.C., Otero, M.C., Sesma, F., Nader-Macias, M.E.F. (2009).** Screening of surface properties and antagonistic substances production by lactic acid bacteria isolated from the mammary gland of healthy and mastitis cows. Vetenary Microbiology. 135 (3-4): 346-357.
16. **Feng, L., He, W., Jiang, J., Liu, Y., Zhou, X.Q., (2010).** Effects of dietary pyridoxine on disease by intestinal bacteria of carp. Mem. Fac. Fish. Kagoshima University. 20: 185-189.
17. **Freire, W.B., Hertrampf, E., Cortes, F. (2000).** Effect of folic acid fortification in Chile: preliminary results. European Journal of Pediatrics Surgeon. 10: 42-43.
18. **Gangadharan, D., Sivaramakrishnan, S., Pandey, A., Nampoothiri, K.M. (2010).** Folate-producing lactic acid bacteria from cow's milk with probiotic characteristics. International Journalof Dairy Technology. 63(3): 339-348.
19. **Gomes, B.C., deMelo, F.B.D.G., DeMartinis, E.C.P. (2010).** Dualistic aspects of *Enterococcus* spp. in foods. Current Research Technology Education topics Applied Microbiology

Biotechnology. 1119-1125.

20. **Gonzalez, L., Sandoval, H., Sacristan, N., Castro, J.M., Fresno, J.M., Tornadijo, M.E. (2007).** Identification of lactic acid bacteria isolated from Genestoso cheese throughout ripening and study of their antimicrobial activity. Food Control 18: 716-722.
21. **Gangadharan, D., Nampoothiri, K.M. (2011).** Folate production using *Lactococcus lactis* ssp *cremoris* with implications for fortification of skim milk and fruit juices. LWT - Food Science and Technology 44: 1859-1864
22. **Hirsch, S., Sanchez, H., Albala, C., de la Maza, M.P., Barrera, G.,Leiva, L., Bunout, D. (2009).** Colon cancer in Chile before and after the start of the flour fortification program.
23. **Hoa, N.T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.H., Ammendola, S., Ricca, E., Cutting, A.S. (2000).** Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Applied Environmental Microbiology. 66: 5241-5247.
24. **Holt, J.G., Krieg, R.N., Sneath, P.H.A., Staley, J.T., Williams, S.T. (1994).** Bergey's Manual of Determinative Bacteriology, 9th edn. Williams and Wilkins, Baltimore.
25. **Hong, H.A., Duc, L.H., Cutting, S.M. (2005).** The use of bacterial spore formers as probiotics. FEMS Microbiology Review 29: 813-835.
26. **Hong, H.A., Huang, J.M., Khaneja, R., Hiep, L.V., Urdaci, M.C., Cutting, S.M. (2008).** The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. Journal of Applied Microbiology. 105: 510-520.
27. **Inatsu, Y., Nakamura, N.,Yuriko, Y., Fushimi, T.,Watanasiritum, L.,Kawamoto, S. (2006).** Characterization of *Bacillus subtilis* strains in Thua nao, a traditional fermented soybean food in northern Thailand. Letter in Applied Microbiology. 43: 237-242.
28. **Kim, T.H., Yang, J., Darling, P.B., O'Connor, D.L. (2004).** A large pool of available folate exists in the large intestine of human infants and piglets. Journal of Nutrition. 134: 1389-1394.
29. **Kos, B., Suskovic, J., Vokavic, S., Simpraga, M., Frece, J., Matosic, S. (2003).** Adhesion and autoaggregation ability of probiotic strain *Lactobacillus acidophilus* M92. Journal of Applied Microbiology. 94: 981-987.
30. **LeBlanc, J.G., Savoy de Giori, G., Smid, E.J., Hugenholtz, J., Sesma, F. (2007).** Folate production by lactic acid bacteria and other foodgrade microorganisms. In: Méndez-Vilas A. (ed) Communicating current research and educational topics and trends in applied microbiology. Formatex Research Center, Badajoz, pp 329-339
31. **LeBlanc, J.G., Taranto, M.P., Molina, V., Sesma, F. (2010a).** B-group vitamins production by probiotic lactic acid bacteria. In: Mozzi F, Raya R, Vignolo G (eds) Biotechnology of lactic acid bacteria: novel applications. Wiley-Blackwell, Ames, pp. 211-232.
32. **Laiño, J.E., LeBlanc, J.G., Savoy de Giori, G. (2012).** Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts Canadian. J. Microbiol 58: 581-588
33. **Mazza, P. (1994).** The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. Boll. Chim. Farm. 133: 3-18.
34. **Moreno, F., Sarantinopoulos, M.R., Tsakalidou, P.E., De Vuyst, L. (2006).** The role of application of Enterococci in food and health. International Journal of Food Microbiology. 106: 1-24.
35. **NRC (National Research Council).(1993).** Nutrient Requirements of Fish. National Academy Press, Washington, DC, USA. 114 pp.
36. **Patel, A.K., Ahire, J.J., Pawar, S., Chaudhari, B.L., Chincholkar, S.B. (2009b).** Comparative accounts of probiotic characteristics of *Bacillus* spp. isolated from food wastes. Food Research International. 42: 505-510.
37. **Patel, A.K., Ahire, J.J., Pawar, S., Chaudhari, B.L., Shouche, Y.S., Chincholkar, S.B.**

(2010). Evaluation of the probiotic characteristics of siderophoregenic *Bacillus* spp. isolated from dairy waste. Applied Biochemistry Biotechnology. 160: 140-155.

38. **Patel, A.K., Deshattiwar, M.K., Chaudhari, B.L., Chincholkar, S.B. (2009a).** Production, purification and chemical characterization of the catecholate siderophore from potent probiotic strains of *Bacillus* spp. Bioresearch Technology. 100: 368-373.
39. **Pennacchia, C., Ercolini, D., Blaiotta, G., Pepe, O., Mauriello, F., Villani, F. (2004).** Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics. Meat Science. 67: 309-317.
40. **Pe´rez-Miranda, S., Cabirol, N., George-Te´llez, R., Zamudio, L.S., Ferna´ndez, F.J. (2007).** O-CAS, a fast and universal method for siderophore detection. J. Microbiol Methods, 70: 127-131.
41. **Pinchuk, I.V., Bressollier, P., Verneuil, B., Fenet, B., Sorokulova, I.B., Megraud, F., Urdaci, M.C. (2001).** *In vitro* anti- Helicobacter pylori activity of the probiotic strain *Bacillus subtilis* is due to secretion of antibiotics. Antimicrobial Agents Chemotherapay. 45: 3156-3161.
42. **Qing, H., Min-Na, D., Hong-Yan, Q., Xiang-Ming, X., Guo-Qiang, Z., Min, Y. (2007).** Detection, isolation, and identification of cadmium-resistant bacteria based on PCR-DGGE. Journal of Environmental Science. 19: 1114-1119.
43. **Rosenberg, M., Gutnick, D., Rosenberg, E. (1980).** Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiology Letter. 9: 29-33.
44. **Saitou, N., Nei, M. (1987).** The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology Evolution. 4: 406-425.
45. **Sharpe, M., Elizabeth-pyer, T.F. (1996).** Identification of lactic acid bacteria. In: Gibbs, B.M., Skinner, F.A. editors. Identification Methods for Microbiologists.London, New York: Academic Press; 65-79.
46. **Sorokulova, I.B. (1996).** Outlook for using bacteria of the genus *Bacillus* for the design of new biopreparations. Antibiot Khimioter. 41: 13-15.
47. **Sorokulova, I.B., Pinchuk, I.V., Denayrolles, M., Osipova, I.G., Huang, J.M., Cutting, S.M., Urdaci, M.C. (2008).** The safety of two *Bacillus* probiotic strains for human use. Digestion Disease Science. 53: 954-963.
48. **Stadler, M., Viernstein, H. (2003).** Optimization of a formulation containing viable lactic acid bacteria. International Journal of Pharmaceutics. 256: 117-122.
49. **Tajima, F., Nei, M. (1984).** Estimation of evolutionary distance between nucleotide sequences. Molecular Biology Evolution. 1: 269-285.
50. **Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007).** MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology Evolution. 24: 1596-1599.
51. **Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W. (2000).** Probiotic bacteria as biological control agents in aquaculture. Microiology Moleular Biology Review. 64: 655-671.
52. **Sheng-Ping, W., Yu-Long, Y.,Yin, Q., Feng-Na, L.L.L.L., Bi-E, T., Xiang-Shan, T., Rui-Lina, H. (2011).** Effects of folic acid on the performance of suckling piglets and sows during lactation. Journal of Science Food Agriculture. 91: 2371-2377.
53. **Wickramasinghe, S.N. (2006).** Diagnosis of megaloblastic anaemias. Blood Review. 20: 299-318.
54. **Wright, A.J.A., Finglas, P.M., Southon, S. (2001).** Proposed mandatory fortification of the UK diet with folic acid: have potential risks been underestimated. Trends Food Science Technology. 12: 313-321.
55. **Xue, G.P., Snoswell, A.M. (1985).** Regulation of methyl group metabolism in lactating ewes. Biochemistry International. 11: 381-385.
56. **Lin, Y.H., Lina, H.Y., Shiau, S.Y. (2011).** Dietary folic acid requirement of grouper, Epinephelus malabaricus, and its effects on non-specific immune responses. Aquaculture. 317: 133-137.