MATERIALS AND METHODS

CULTURE MEDIA USED

(1). Arginine Hydrolysis Medium (Thornley, 1960)

Peptone	10.0 g
Yeast extract	5.0 g
D (+) glucose	0.5 g
$K_2HPO_4.3H_2O$	2.0 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.05 g
Sodium acetate	5.0 g
Tri-sodium citrate	20.0 g
Tween 80	1.0 ml
Arginine	0.3 %
Phenol red	0.01 g
Distilled water	1000 ml
pH	5.0

(2). Ascospore Agar (M804, HiMedia, Mumbai)

(3). *Bacillus cereus* Agar Base (M833, HiMedia, Mumbai)

(4). Bacteriocin Screening Medium (Tichaczek *et al.*, 1992)

Peptone	10 g
Beef extract	5.0 g
Yeast extract	5.0 g
Glucose	2.0 g
K_2HPO_4	2.0 g
Tween 80	1.0 g
Diammonium citrate	2.0 g
Sodium acetate	5.0 g
$MgSO_4$	0.1 g
$MnSO_4$	0.05 g
Distilled water	1000 ml
pH	6.5
Agar	12 g

(5). Baird Parker Agar Base (M043, HiMedia, Mumbai)

(6). Biogenic Amine Sub-culturing Medium (Bover-Cid and Holzapfel, 1999)

MRS Broth (M369, HiMedia, Mumbai)	52.2 g
D-Tyrosine (RM 1520, HiMedia, Mumbai)	1.0 g
L-Histidine monohydrochloride (Merck)	1.0 g
L-Lysine monohydrochloride (Merck)	1.0 g
L-Ornithine monohydrochloride (Merck)	1.0 g
Pyridoxal-5-Phosphate (RM 1554, HiMedia)	0.001 g
Distilled water	1000 ml
pH	6.00

(7). Biogenic Amine Screening Medium (Joosten and Northold, 1989; modified by Bover-Cid and Holzapfel, 1999)

Tryptone	5.0 g
Yeast extract	5.0 g
Meat extract	5.0 g
Sodium chloride	2.5 g
Glucose	0.5 g
Tween 80	1.0 g
K_2HPO_4	2.0 g
Ammonium citrate	2.0 g
Calcium carbonate	0.1 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.05 g
$FeSO_4.7H_2O$	0.04 g
Thiamine	0.001 g
Pyridoxal-5-phosphate	0.005 g
Bromocresol purple	0.05 g
Agar	22.0 g
Amino acid	5.0 g
Distilled water	1000 ml

Amino acids used were D-Tyrosine (pH 5.3) (RM 1520, HiMedia, Mumbai); L-Histidine monohydrochloride (pH 5.0) (Merck, Germany); L-Lysine monohydrochloride (pH 5.15) (Merck, Germany); L-Ornithine monohydrochloride (pH 5.0) (Merck, Germany).

(8). Egg Yolk Emulsion (FD045, HiMedia, Mumbai)

(9). Egg Yolk Tellurite Emulsion (FD046, HiMedia, Mumbai)

(10). Fermentation Basal Medium for yeasts (Wickerham, 1951)

Yeast extract	4.5 g
Peptone	7.5 g
Distilled water	1000 ml

Bromothymol blue (Till sufficiently dense green colour appears)

(11). Lactate Configuration Medium (Tamang and Holzapfel, unpublished)

Peptone from casein	10.0 g
Yeast extract	4.0 g
Glucose	20.0 g
Di-potassium hydrogen phosphate	2.0 g
Tween 80	1.0 g
Di-ammonium hydrogen phosphate	2.0 g
Magnesium sulphate	0.2 g
Manganese sulphate	0.04 g
Distilled water	1000 ml

(12). *Listeria* Identification Agar Base (PALCAM) (M 1064, HiMedia, Mumbai)

(13). *Listeria* Selective Supplement (FD 061, HiMedia, Mumbai)

(14). Malt Extract Agar (M137, HiMedia, Mumbai)

(15). MRS Agar (M641, HiMedia, Mumbai)

(16). MRS Broth (M369, HiMedia, Mumbai)

(17). Nitrate Broth (Gordon *et al.*, 1973)

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	1.0 g
Distilled water	1000 ml
pH	7.0

(18). Nutrient Agar (MM012, HiMedia, Mumbai)

(19). Nutrient Broth (M002, HiMedia, Mumbai)

(20). Phytase Degrading Screening Medium (Holzapfel, 1997)

Ca-Phytate	5.0 g
Glucose	2.0 g
Na-Citrate	0.1 g
$MgSO_4$	0.075 g
$MnSO_4$	0.010 g
$FeSO_4$	0.005 g
L-Glutamate	0.150 g
DL-Alanine	0.20 g
L-Arginine-HCl	0.05 g
L-Asparagine	0.20 g
L-Cysteine-HCl	0.20 g
L-Phenylalanine	0.04 g
L-Histidine	0.05 g
L-Isoleucine	0.06 g
L-Leucine	0.06 g
L-Lysine-HCl	0.05 g
L-Methionine	0.05 g
L-Proline	0.04 g
L-Tyrosine	0.002 g
L-Threonine	0.025 g
Thiamine	0.005 g

L-Tryptophan	0.025 g
L-Valine	0.015 g
Adenine	0.025 g
Guanosine	0.025 g
Thymidine	0.025 g
Uracil	0.025 g
Vitamin B12	0.0001 g
Biotin	0.0001 g
Ca-Pantothenate	0.01 g
Folic acid	0.001 g
Niacin	0.01 g
Riboflavine	0.005 g
Agar Agar	18 g
Distilled water	1000 ml
pH	6.0

(21). Plate Count Agar (M091, HiMedia, Mumbai)

(22). Potato Dextrose Agar (M096, HiMedia, Mumbai)

(23). *Salmonella–Shigella* Agar (M108, HiMedia, Mumbai)

(24). Skim Milk Powder (RM1254, HiMedia, Mumbai)

(25). Sucrose Broth (Garvie, 1960)

Tryptone	10 g
Yeast extract	5.0 g
K_2HPO_4	5.0 g
Trisodium citrate	5.0 g
Sucrose	50 g
Distilled water	1000 ml

Ingredients were dissolved in distilled water by heating in a steamer and sterialised by autoclaving.

(26). Tryptone Soya Agar (M290, HiMedia, Mumbai)

(27). Violet Red Bile Glucose Agar w/o Lactose (M581, HiMedia, Mumbai)

(28). Yeast-Malt Extract (YM) Agar (M424, HiMedia, Mumbai)

(29). Yeast Malt Extract (YM) Broth (M425, HiMedia, Mumbai)

(30). Yeast Morphology Agar (M138, HiMedia, Mumbai)

(31). Yeast Nitrogen Base (M139, HiMedia, Mumbai)

REAGENTS

(1). Acidic Ninhydrin

1-Butanol/water saturated	465 ml
Acetic acid	35 ml
Ninhydrin	2.5 ml

(2). Burke's Iodine Solution (Bartholomew, 1962)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

(3). Gram's Crystal Violet (S012, HiMedia, Mumbai)

(4). Malachite Green (S020, HiMedia, Mumbai)

(5). Nessler's Reagent

Potassium iodide	50.0 g
Mercuric chloride (saturated)	35.0 ml
Distilled water (ammonia free)	25.0 ml
Potassium hydroxide (50 %)	400.0 ml

Potassium iodide was dissolved in 35 ml of distilled water followed by addition of saturated aqueous solution of mercuric chloride till the appearance of precipitate. Then, 400 ml of potassium hydroxide was added and made the final volume to 1000 ml by adding distilled water. The solution was left for a week, the supernatant was decanted and stored in capped amber bottle at 4° C.

(6). Nitrate Reduction Test Reagent

Solution A	
Sulphanilic acid	0.8 g
5 N acetic acid	100 ml
(Glacial acetic acid: water, 1:2.5)	

Solution B	
∝-Napthylamine	0.5 g
5 N acetic acid	100 ml

The solutions A and B were mixed in equal quantities just before use.

(7). Phenolphthalein (I009, HiMedia, Mumbai)

(8). Ringer solution (Merck, Germany)

(9). Safranin (S027, HiMedia, Mumbai)

Reference Strains

Reference Strains	Origin	Purpose in this experiment
Bacillus cereus CCM 2010	CCM	Indicator strain for antimicrobial activity
Enterobacter agglomerans BFE 154	BFE	Indicator strain for antimicrobial activity
Enterobacter cloacae BFE 282	BFE	Indicator strain for antimicrobial activity
Enterococcus faecium DSM 20477	DSM	Indicator strain for antimicrobial activity
Klebsiella pneumoniae subsp. *pneumoniae* BFE 147	BFE	Indicator strain for antimicrobial activity
Lactobacillus brevis DSM 20054	DSM	Molecular identification
Lactobacillus buchneri LMG 8692	LMG	Molecular identification
Lactobacillus collinoides DSM 20595	DSM	Molecular identification
Lactobacillus diolivorans DSM 14421	DSM	Molecular identification
Lactobacillus hilgardii DSM 20176	DSM	Molecular identification
Listeria innocua DSM 20649	DSM	Indicator strain for antimicrobial activity
Listeria monocytogenes DSM 20600	DSM	Indicator strain for antimicrobial activity
Lactobacillus paraplantarum LTH 5200	LTH	Molecular identification
Lactobacillus pentosus DSM 20314	DSM	Molecular identification
Lactobacillus plantarum DSM 20174	DSM	Standard strain for *meso*-diaminopimelic acid determination (DAP); molecular identification
Lactobacillus sakei DSM 20017	DSM	Ammonia from arginine
Pediococcus acidilactici DSM 20333	DSM	Molecular identification
Pseudomonas aeruginosa BFE 162	BFE	Indicator strain for antimicrobial activity
Pediococcus pentosaceus MTCC 3817	MTCC	Molecular identification
Staphylococcus aureus S1	FMR	Indicator strain for antimicrobial activity
Streptococcus mutans DSM 6178	DSM	Indicator strain for antimicrobial activity

Originally, these reference strains were obtained from DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), CCM (Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia), BFE (Instutute of Hygiene and Toxicology, Karlsruhe, Germany), MTCC (Microbial Type Culture Collection, Chandigarh, India), LTH (Universität Hohenheim, Germany) and FMR (Food Microbiology Laboratory, Sikkim Government College, Gangtok, India).

Listeria monocytogenes DSM 20600, *Listeria innocua* DSM 20649, *Staphylococcus aureus* S1, *Bacillus cereus* CCM 2010, *Enterobacter agglomerans* BFE 154, *Enterobacter cloacae* BFE 282, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147, *Pseudomonas aeruginosa* BFE 162 were propagated in standard nutrient agar. *Enterococcus faecium* DSM 20477, *Streptococcus mutans* DSM 6178, *Lactobacillus plantarum* DSM 20174, *Lactobacillus sakei* DSM 20017, *Lb. brevis* DSM 20054 were cultivated in MRS broth. The cultures were maintained as frozen stocks at –20° C in 15% glycerol.

METHODS

Traditional knowledge

Detailed information on various types of traditional fermented vegetable products including wild edible bamboo shoot products was documented from different villages located in Sikkim, Arunachal Pradesh and Manipur of North East India. Indigenous knowledge on methods of preparation including biopreservation methods of perishable and seasonal vegetables practised by the ethnic and tribal people of North East India, the nature and use of the traditional fermented products were documented based on the information sought from the local people of the respective places.

Collection of samples

Twenty five samples of gundruk and twenty one samples of sinki were collected from local markets of Sikkim. Twenty samples of goyang and twenty five samples of khalpi were directly obtained from the producers in different rural areas in Sikkim. A total of forty six samples of mesu were collected from different places of Sikkim during rainy seasons when bamboo shoots were plenty. Six samples of inziangsang were obtained from Bishnupur market of Manipur. Sixteen samples of ekung, thirteen samples of eup and fifteen samples of hirring were collected from different places of Arunachal Pradesh. Twelve samples of soibum, eleven samples of soidon and eight samples of soijim were obtained from Bishnupur and Imphal of Manipur. All samples were collected aseptically

in sterile bottles and poly-bags, which were kept in an ice-box container, and transported to the laboratory for analyses.

Microbiological analysis

Ten g of sample were homogenised with 90 ml of 0.85 % (w/v) sterile physiological saline in a stomacher lab-blender (400, Seward, UK) for 1 min. A serial dilution (10^{-1} to 10^{-8}) in the same diluent was made. Lactic acid bacteria (LAB) were isolated on MRS agar (M641, HiMedia, Mumbai) supplemented with 1 % $CaCO_3$, and incubated under anaerobic condition in an Anaerobic Gas-Pack system (LE002, HiMedia, Mumbai) and incubated at 30° C for 48-72 hour. Aerobic mesophilic counts were determined using plate count agar (M091A, HiMedia, Mumbai) incubated at 30° C for 48-72 hour. Moulds and yeasts were isolated on potato dextrose agar (M096, HiMedia, Mumbai) and yeast-malt extract (YM) agar (M424, HiMedia, Mumbai), supplemented with 10 IU/ml benzylpenicillin and 12 µg/ml streptomycin sulphate, respectively, and incubated aerobically at 28° C for 72 hour. Colonies were selected randomly or all sampled if the plate contained less than 10 colonies, according to Leisner *et al.* (1997). Purity of the isolates was checked by streaking again on fresh agar plates of the isolation media and sub-culturing on corresponding broths/agar, followed by microscopic examinations. Microbiological data obtained were transformed into logarithms of the numbers of colony forming unit (cfu) per g of sample. Identified strains of LAB were preserved in MRS broth using 15 % (v/v) glycerol at –20° C.

Characterisation of Bacterial Isolates

Cell morphology

Smear of a 24 hour-old bacterial culture was made in a grease free slide, air-dried (not heated-fixed), stained for 30 sec with safranin (S027, HiMedia, Mumbai), washed in water, air-dried (Harrigan, 1998) and observed under oil-immersion objective. Cell dimensions were measured with a standardized ocular micrometer.

Gram staining

All isolates were Gram-stained following the method of Bartholomew (1962). A suspension of a 24 hour-old bacterial culture on slant was prepared, and smear was made in a grease-free slide. Then heated-fixed, flooded by crystal violet stain for 1 min, and washed for 5 sec with water. The smear was flooded with Burke's iodine solution, allowed to react for 1 min, and washed again for 5 sec with water. Holding the slide against a white surface, 95% ethanol was poured drop-wise from the top edge of the slide until no more colour came out from the lower edge of the slide. After washing with water, the smear was stained with safranin for 1 min and washed again with water. The slide was air-dried and observed under oil-immersion objective.

Motility

A hanging drop of a 24 hour-old culture in MRS broth was prepared in a cavity slide following the method of Harrigan (1998). The prepared

culture was observed in a phase contrast microscope (Olympus CH3-BH-PC, Japan) for motility of the strains

Production of catalase

The production of gas bubbles by the isolates were observed by adding 0.5 ml of 10 % hydrogen peroxide solution (Merck) to the cultures indicating the presence of catalase (Schillinger and Lücke, 1987).

Ammonia from arginine

Tubes of 5 ml arginine hydrolysis medium (Thornley, 1960) were inoculated with 24 hour-old culture. The tubes were incubated at 30° C for 3 days and formation of ammonia from arginine was detected by spotting 100µl culture onto a white porcelain tile and adding equal volume of Nessler's reagent. Appearance of dark orange colour indicated presence of ammonia. *Lactobacillus sakei* DSM 20017 was used as ammonia positive reference strain (Schillinger and Lücke, 1987).

Gas ($C0_2$) production from glucose

Tubes of 10 ml MRS broth without citrate and containing inverted Durham tubes was inoculated with 24 hour-old cultures and incubated at 30° C (Schillinger and Lücke, 1987). Accumulation of gas in the inverts indicated positive result.

Growth at different pH

The pH of MRS broth was adjusted to 3.9 and 9.6 using 1 N HCl or 10% w/v NaOH. The medium was distributed into tubes containing 5 ml in each. They were autoclaved, cooled to room temperature and inoculated with 24 hour-old MRS broth culture. The tubes were incubated at 30° C for 24-72 hour and observed for growth (Dykes *et al.*, 1994).

Growth at different temperatures

MRS broth were inoculated with 24 hour-old cultures and incubated at 10° C and 15° C for 7 days, and 45° C for 3 days, respectively and observed for growth (Dykes *et al.*, 1994).

Growth in different NaCl concentrations

Salt tolerance was tested by inoculating a loop-full of culture in MRS broth supplemented with 6.5 %, 10.0 % and 18.0 % NaCl, respectively, and incubated for 3 days at 30° C in a slanting position to improve aeration (Schillinger and Lücke, 1987). Cultures were observed for growth after incubation.

Acid from carbohydrates

The method was based on Schillinger and Lücke (1987). Tubes of 5 ml MRS broth without beef extract, containing 0.5% w/v of different carbohydrates instead of glucose and 0.004% phenol red indicator were inoculated and incubated at 30° C for 2-5 days. Colour change from red to yellow indicated acid production.

Production of dextran from sucrose

Production of dextran in sucrose broth (Garvie, 1960) was tested exclusively for leuconostoc isolates following the method described by Kelly *et al.* (1995).

Lactic acid configuration

The configuration of lactic acid produced was determined enzymatically using D-lactate and L-lactate dehydrogenase kits (Boehringer-Mannheim GmbH, Cat. No. 1112821, Germany). The LAB strains were grown in lactate configuration medium at 30° C overnight. One ml culture was centrifuged in a microcentrifuge (Heraeus, Germany) at 8,000 *g* for 5 min. The 20 µl of the supernatant was mixed with 980 µl of redistilled water to obtain 1:50 sample dilution. The 1 ml of Solution (1), 0.2 ml of Solution (2), 0.02 ml of Suspension (3), 0.1 ml sample solution and 0.9 ml of redistilled water was pipetted into a cuvette, followed by gentle swirling to mix the contents of the cuvette after closing it with parafilm. Similarly, a blank was prepared by adding all the reagents except the sample solution being replaced with 1.0 ml of redistilled water. After 5 minutes the absorbance of the solutions (A_1) was measured in UV-VIS Spectrophotometer (Analytik Jena, Germany) at 340 nm. The absorbance differences (A_2-A_1) for both, blank and sample was determined and the difference of the absorbance difference of the blank from that of the sample ($\Delta A_{D\text{-lactic acid}}$) was calculated. The reaction was started by adding 0.02 ml of Solution (4) to the sample as well as to the

blank. The cuvettes were swirled gently to mix the contents by closing it with parafilm. After 30 minutes the absorbance (A_2) of the sample and the blank were measured immediately one after another at 340 nm. The 0.02 ml of Solution (5) was added to both the sample and the blank followed by mixing. These were allowed to stand for 30 minutes. The absorbance (A_3) was measured immediately one after another for the sample as well as for the blank at 340 nm. The absorbance differences (A_3-A_2) for both, blank and sample was determined and the difference of the absorbance difference of the blank from that of the sample ($\Delta A_{L\text{-lactic acid}}$) was calculated. The lactic acid isomer concentration was calculated as: $c = (V \times MW \times \Delta A)/(\varepsilon \times d \times v \times 1000)$ (g/l), where, V = final volume (ml), v = sample volume (ml), MW = molecular weight of lactic acid = 90.1 (g/mol), d = light path = 1 cm, ε = extinction coefficient of NADH at 340 nm = 6.3 (l/m mol × cm). The result was multiplied by the dilution factor.

meso-Diaminopimelic acid (DAP)

The presence of *meso*-diaminopimelic acid in the cell walls of LAB was determined using thin-chromatography on cellulose plate (Tamang *et al.*, 2000). Cells were harvested from 48 hour old MRS broth culture by centrifuging 5 ml culture at 8,000 *g* for 5 min, and washed with 3 ml of distilled water. The sediment was resuspended in 1 ml of 6 N HCl and transferred to screw-capped tubes. The cells were hydrolysed overnight at 100° C in a water-bath. The contents of the tubes were blow-dried while immersed in boiling water. The sediment was resuspended in 1 ml of

distilled water and blow dried again and oven dried for 1 hour. Finally, the sediment was suspended in 0.1 ml of distilled water and each sample (5 µl) was spotted on thin-layer chromatography plates on cellulose plates (Merck, Germany). Descending one-dimensional chromatography was done by keeping the plates in a TLC chamber in a solvent solution containing methanol: pyridine: 10 N HCl: water (32:4:1:7). The solvent solution was prepared 1 hour before use. After keeping for 4-5 hour the plates were dried with a hair drier and the chromatograms were developed by spraying acidic ninhydrin and when almost dried, placed for 5 min in 100° C oven. Spots representing *meso*-diaminopimelic acid appeared dark green to grey and turned yellow within 24 hour. *Lactobacillus plantarum* DSM 20174 was used as standard (*meso*-DAP positive).

API Tests

Carbohydrate fermentation patterns of LAB were determined using API 50 CHL and API 20 STREP test strips (bioMérieux, France) according to manufacturer's instructions as well as the method described by Tamang and Holzapfel (1999). Cultures were grown on MRS agar at 30° C for 48 hour. The growth was harvested in 2 ml sterile normal NaCl solution which was used to prepare suspensions, corresponding to 10^7 cells/ml. The incubation box was prepared by distributing about 10 ml of sterile water into the honeycombed base of the 50 CHL trays. The strips were unpacked, placed them in the trays and the tubes were filled with the

bacterial suspensions. The inoculated strips were kept slightly tilted and incubated at 30° C for 48 h. The results were read by referring to the manufacturer's interpretation table at 24 hour and 48 hour, respectively. All spontaneous reactions were recorded. The APILAB PLUS database identification software (bioMérieux, France) was used to interpret the results.

Biolog system

The Biolog microplate bacterial identification system (Biolog Inc., USA; Oxoid GmbH, Wesel, Germany) based on the utilisation of 95 single carbon sources was used for the identification of *Leuconostoc* species. The metabolism of the substrates in the wells of the microplates results in a reduction of tetrazolium dye producing a colour change, and a specific "metabolic fingerprint" was obtained for each strain and compared with the data of the Biolog MicroLog database software (Biolog Inc.). Before inoculation of the Biolog AN microplates, strains were grown anaerobically on BUA (Biolog Universal Anaerobe) agar (Oxoid GmbH, Germany) with 5 % sheep blood at 30° C for 48 hour. The bacterial cells were swabbed from the surface of the agar and suspended in AN Inoculating fluid (Biolog Inc.) at the recommended cell density. 100 µl of the bacterial suspension was pipetted in each well of the Biolog AN microplate. The microplates were incubated in an anerobic jar with a hydrogen-free anaerobic atmosphere (Oxoid AnaeroGen; Oxoid GmbH) at 30° C for 48 hour and then read with the Biolog Microstation Reader (Biolog Inc.).

Phenotypic identification

Bacterial species were identified following the taxonomic keys of Bergey's Manual (Sneath *et al.*, 1986), Simpson and Taguchi (1995), Wood and Holzapfel (1995), and by APILAB PLUS Database software of API tests (bioMérieux, France).

Genotypic Characterization of LAB Strains

DNA extraction

Total genomic DNA of some selected strains of LAB was extracted from 2-ml samples of overnight cultures grown in MRS broth at 30° C according to the methods of Pitcher *et al.* (1989). Reference strains were obtained from DSMZ, LTH and LMG, as mentioned earlier in this chapter.

RAPD-PCR analysis

The primer M13 (5'-GAG GGT GGC GGT TCT-3') (Huey and Hall, 1989) was used for randomly amplified polymorphic DNA (RAPD)-PCR. Conditions of PCR reactions and amplification were performed as described by Schillinger *et al.* (2003). The PCR amplification was conducted with a Primus 96 Plus thermal cycler (MWG Biotech, Ebersberg, Germany).

Repetitive element (rep)-PCR analysis

Repetitive element (rep)-PCR analysis of LAB strains was carried out using reaction and amplification conditions as previously described by Gevers *et al.* (2001). For rep-PCR, the primer $(GTG)_5$ (5'-GTG GTG GTG

GTG GTG-3') recommended for LAB identification by Gevers *et al.* (2001) was used.

Species-specific PCR

For identification of *Lactobacillus brevis*, a species-specific PCR was applied. The oligonucleotide primer 5'-CTTGCACTGATTTTAACA-3' and 5'-GGGCGGTGTGTACAAGGC-3' were used as forward and reverse primers, respectively (Guarneri *et al.*, 2001). The PCR conditions were as described by Guarneri *et al.* (2001). To verify the identity of the PCR product, amplified fragments were digested with P*st*I (New England Biolabs, Frankfurt, Germany) in a 15 µl reaction mixture containing 11.5 µl of the PCR product, 1.5 µl incubation buffer and 10 U P*st*I (1 h, 37° C).

Gel electrophoresis

Amplification products from RAPD-PCR and rep-PCR were subjected to electrophoresis in 1.8 % agarose gels in 1 x TBE buffer for 15-17 hour at 48 and 55 V, respectively. The products resulting from the *Lactobacillus brevis* specific PCR were separated on 1.2 % agarose for 2 hour at 100 V. A DNA molecular mass marker (100 and 500 bp molecular ladders from Biorad, München, Germany) was used as a standard. After electrophoresis, the gels were stained in ethidium bromide and after washing visualised with a UV transilluminator. Gels were photographed using the Fluorchem Imager 5500 system (Alpha Innotech, USA) and the digitised images were analysed and processed using the Bionumerics software (Applied Maths, Kortrijk, Belgium). Groupings of the fingerprints

were performed by means of the Pearson product-moment correlation coefficient and the unweighted pair-group method using arithmetric averages clustering algorithm (UPGMA).

Characterisation of yeast isolates

Cell morphology

Cell morphology and mode of vegetative reproduction of yeast was observed following the method of Yarrow (1998). Sterile yeast morphology agar (M138, HiMedia, Mumbai) slants were inoculated with an actively growing (24 hour-old) yeast culture and incubated at 28° C for 3 days. Dimensions of cells were measured with a standardized ocular micrometer.

Pseudo- and True-mycelium

For observation of pseudo-mycelium and true-mycelium of yeast isolates, the slide culture method described by Kreger-van Rij (1984) was followed. A petri-dish, containing U-shaped glass rod supporting two glass slides, was autoclaved at 121° C for 20 min. The glass slides were quickly removed from the glass rod with a flame sterialized pair of tweezers, and were dipped into the molten potato dextrose agar (M096, HiMedia, Mumbai) after which they were replaced on the glass rod support. The solidified agar on the slides was inoculated very lightly with yeast isolates in two lines along each slide. Four sterile coverslips were placed over part of the lines. Some sterile water was poured into the petri-dish to prevent the agar from drying out. The culture was then incubated at 28° C for 4

days. The slides were taken out of the petri-dish and the agar was wiped off from the back of the slide. The edges of the streak under and around the coverslips were examined microscopically for the formation of pseudo-mycelium or true-mycelium.

Characteristics of asci and ascospore

Sterile ascospore agar (M804, HiMedia, Mumbai) slants were streaked with actively grown yeast cultures, incubated at 28° C for 3 days and examined at weekly intervals up to 4 weeks for observation of asci and ascospores. A heat fixed smear was flooded with 5 % w/v aqueous malachite green (S020, HiMedia, Mumbai) for 30 to 60 sec, heated to steaming 3 to 4 times over the flame of a spirit lamp and counterstained with safranin (S027, HiMedia, Mumbai) for 30 sec and observed under the microscope (Yarrow, 1998).

Reduction of nitrate

Yeast cultures were grown in 5 ml nitrate broth incubated at 28° C. After 3, 7 and 14 days, 1 ml of the culture was mixed with 3 drops of the reagent for nitrate reduction test and observed for the development of a red or yellow colour, indicating the presence of nitrate. A small amount of zinc dust was added to the tube that was negative even after 14 days and observed for the development of red colour, indicating the presence of nitrate, i.e. absence of reduction (Yarrow, 1998).

Growth at 37° C

Slants of malt-extract agar (M137, HiMedia, Mumbai) were inoculated with cells of actively grown yeast isolates and incubated at 37° C for 4 days and observed for growth (Yarrow, 1998).

Sugar fermentation

Yeasts isolates were grown at 28° C on yeast-malt extract (YM) agar (M242, HiMedia, Mumbai) slants for 3 days. Tubes of 10 ml of fermentation basal medium (Wickerham, 1951) supplemented with 2 % w/v sterile sugars containing inverted Durham tubes, were inoculated with the above yeast culture and incubated at 28° C and were shaken regularly to observe gas accumulation in the inverts (Yarrow,1998).

Sugar assimilation

Yeast isolates were grown at 28° C on yeast-malt extract (YM) agar (M242, HiMedia, Mumbai) slants for 3 days. Tubes containing 5 ml mixture of yeast nitrogen base (M139, HiMedia, Mumbai) and carbon source were inoculated with cultures and incubated at 28° C for 3 to 7 days. Control test tube was made by adding 0.5 ml of yeast nitrogen base in 4.5 ml of sterilized distilled water (devoid of any carbon source). Assimilation of carbon sources was observed by comparing with the control (Yarrow, 1998).

Identification of Yeast

Yeast isolates were identified to the genus level according to the criteria laid down by Kreger-van Rij (1984), Kurtzman and Fell (1998) and Yarrow (1998).

Pathogenic contaminants

Enumeration of pathogenic contaminants from the samples were done in selective media such as *Bacillus cereus* agar base (M833, HiMedia, Mumbai) for *Bacillus cereus*, Baird Parker agar base (M043, HiMedia, Mumbai) for *Staphylococcus aureus* and Violet Red Bile Glucose agar w/o lactose (M581, HiMedia, Mumbai) for enterobacteriaceae (Han *et al.*, 2001). *Salmonella-Shigella* Agar (M108, HiMedia, Mumbai) was used for the detection of *Salmonella* and *Shigella* and *Listeria* identification agar base (M1064, HiMedia, Mumbai) with *Listeria* selective supplement (FD 061, HiMedia, Mumbai) for *Listeria* in the samples following the standard method of Metaxopolous *et al.* (2001). Ten g of sample were blended with 90 ml of peptone-physiological saline (0.1% neutral peptone, 0.85% NaCl) and homogenized in a stomacher lab-blender 400 (Seward, UK) for 1 min. Serial decimal dilution series was prepared in the same diluent in duplicates.

***Bacillus cereus*:** Selective enumeration was carried out on spread plates of *Bacillus cereus* agar base (M833, HiMedia, Mumbai) with appropriate additions of Polymyxin B Selective Supplement (FD003, HiMedia, Mumbai) and Egg yolk emulsion (FD045, HiMedia, Mumbai). The

inoculated plates were incubated at 30° C for 24-48 hour. Characteristic turquoise to peacock blue colonies surrounded by zone of precipitate of the same colour were regarded as presumptive *Bacillus cereus*.

***Staphylococcus aureus*:** Spread plates of Baird Parker agar base (M043, HiMedia, Mumbai) with appropriate additions of Egg yolk tellurite emulsion (FD046, HiMedia, Mumbai) was used for selective enumeration of *Staphylococcus aureus*. After serial dilution plates were overlaid with the medium and incubated at 30° C for 24-48 hours. The black colonies surrounded by clear zone extending 2-5 mm into the opaque medium appeared were regarded as presumptive *Staphylococcus aureus*.

Enterobacteriaceae: Sample dilutions in tryptone soya broth (M011, HiMedia, Mumbai) were allowed to resuscitate on thinly plated tryptone soya agar (M290, HiMedia, Mumbai) plates for 1-2 hour at 27° C, followed by a thick overlay of selective Violet Red Bile Glucose agar (without lactose) (M581, Himedia, Mumbai) medium and incubated at 30° C for 20 hour. Pink colonies appeared were regarded as presumptive enterobacteriaceae.

***Listeria* sp:** *Listeria* identification agar base (M1064, HiMedia, Mumbai) with *Listeria* selective supplement (FD061, HiMedia, Mumbai) media were used for detection of *Listeria* in the samples following the standard method of Metaxopolous *et al.* (2001). Inoculated plates were incubated

at 30° C for 48 hour and observed in dark background for smooth glistening colonies indicating presence of *Listeria*.

***Salmonella* and *Shigella*:** *Salmonella-Shigella* (SS) agar (M108, HiMedia, Mumbai) was used for the detection of *Salmonella* and *Shigella* in samples following the method of Metaxopolous *et al.* (2001). After serial dilution plates were inoculated, followed by an overlay of the SS agar and incubated at 37° C for 48 hour and observed in dark background for presumptive colonies. *Salmonella* colonies appear dark-centred while colourless colonies are regarded as presumptive *Shigella*.

Technological Properties of LAB Isolates

Acidification and coagulation

Effect of acidification and coagulation of the LAB isolates was assayed by inoculating 10 % skim milk with 24 hour old cultures (RM1254, HiMedia, Mumbai) (centrifuged at 8,000 *g* for 20 min and sterialized at 110^0 C for 10 min) at 1 % level, and incubated at 30^0 C. Observation was made for commencement of clotting, and pH was measured after 72 hour of incubation (Olasupo *et al.*, 2001).

Antimicrobial and bacteriocin activity

The LAB isolates were screened for antimicrobial activity by agar spot method of Schillinger and Lücke (1989). The indicator strains used for antagonisms included: *Listeria innocua* DSM 20649, *Listeria monocytogenes* DSM 20600, *Bacillus cereus* CCM 2010, *Staphylococcus*

aureus S1, *Enterococcus faecium* DSM 20477, *Streptococcus mutans* DSM 6178, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147, *Enterobacter cloacae* BFE 282, *Enterobacter agglomerans* BFE 154 and *Pseudomonas aeruginosa* BFE 162. Cell-free neutralized supernatants fluids of LAB isolates were screened for bacteriocin production by agar spot test method described by Uhlman *et al.* (1992), using the bacteriocin screening medium of Tichaczek *et al.* (1992).

Bacteriocin activity was quantified based on an agar diffusion assay as described by Cabo *et al.* (1999). The quotient between the inhibition zone area and the sensitivity of the indicator bacterium used to compare different bacteriocins. The twofold serial dilutions of the neutralized supernatants of bacteriocin are spotted (10 µl) onto the surface of lawns containing the required indicators bacteria. Bacteriocins titres were expressed as the reciprocal of the highest dilution exhibiting a zone of inhibition and were reported in activity units (AU) per ml.

Enzymatic profile by API-zym

The enzymatic profile of LAB were assayed following the method of Arora *et al.* (1990) using API-zym (bioMérieux, France) galleries by testing for the activity of the following 19 enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine-, valine- and cystine- arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-

glucosaminidase, α-mannosidase and α-fucosidase. Cultures were grown on MRS agar and cells were harvested in 2 ml sterile normal saline which was used to prepare suspension of 10^7 cells/ml. The strip was unpacked and 2 drops of cell suspensions was inoculated in each cupule of the strip containing ready-made enzyme substrates and incubated at 30° C for 6 hour. After incubation, 1 drop of ready-made zym-A and zym-B reagents was added and observed for colour development based on the manufacturer's colour chart.

Phytic acid degradation

Ability of LAB isolates to degrade phytic acid was determined on a synthetic phytic acid screening medium (Holzapfel, 1997), containing calcium phytate (Sigma, USA) as sole phosphate source. Control was prepared without calcium phytate. In preparing the medium, phytate and salts are added separately. After adding glucose, Na-citrate, magnesium sulfate, manganese sulfate and ferrous sulfate to the phytate solution, the pH was adjusted to 6.0 and the medium was autoclaved. Vitamins, amino acids and nucleotides were filter sterialised and added to the medium before plating. The pH of the medium was finally adjusted between 5.8-6.0. The plates were streaked with 24 hour-old broth culture and incubated aerobically at 30° C for 5 days. Clear zone around the colony of the test organism indicated a positive reaction.

Degradation of oligosaccharides

Screening of LAB for degradation of oligosaccharides such as stachyose and raffinose were performed in MRS broth without beef extract (pH 6.4) containing 2 % stachyose and 1 % raffinose (instead of glucose), respectively, and 0.004% chlorophenol red as indicator. Inoculation was followed by incubation at 30° C for 3 days (Holzapfel, 1997).

Biogenic amine

The ability of LAB isolates to produce biogenic amines was determined qualitatively on an improved screening medium as described by Bover-Cid and Holzapfel (1999) using a 'cocktail' of four precursor amino acids (histidine, lysine, ornithine and tyrosine). Cultures previously grown and subcultured twice in biogenic amine sub-culturing medium were spotted onto the plates containing screening medium. Change of the bromocresol purple indicator to purple was considered as index of significant amino acid decarboxylase activity, corresponding to >350 mg of a particular amino acid/litre (Olasupo *et al.*, 2001).

Hydrophobicity assay

Bacterial adhesion to hydrocarbons was determined and results were expressed according to Rosenberg (1984) and Perez *et al.* (1998), modified as follows. Fresh cultures were grown in MRS broth at 30° C for 24 hour and centrifuged at 8,000 *g* for 5 min. The pellet was washed three times with 9 ml of Ringer solution (Merck, Germany), and thoroughly

mixed in a vortex. The 1 ml of the suspension was taken and the absorbance at 580 nm was measured. Then, 1.5 ml of the suspension was mixed with equal volume of n-hexadecane (RM 2238, HiMedia, Mumbai) in duplicates and mixed thoroughly in a vortex. The phases were allowed to separate for 30 min at room temperature, after which aqueous phase was carefully removed and absorbance at 580 nm was measured. The percentage hydrophobicity was expressed as follows: hydrophobicity % = $[A_0\text{-}A/A] \times 100$, where A_0 and A are the absorbance values of the aqueous phase before and after contact with n-hexadecane. The percent hydrophobic index greater than 70 % was arbitrarily classified as hydrophobic (Martin *et al.*, 1989; Nostro *et al.*, 2004).

Fermentation Dynamics *in situ*

Two types of gundruk were prepared following the traditional method, 'rayo-saag' gundruk, prepared from leaves of 'rayo-saag' [*Brassica rapa* L. sub-sp. *campestris* (L.) Clapham variety *cumifolia* Roxb.], and 'mula-saag' gundruk, prepared from leaves of radish. Leaves were wilted in the sun for 1 day, then crushed and soaked in hot water. About 400 g of crushed leaves were put into sterile 250 ml-bottle, pressed with sterile pestle to remove excess water, and loosely capped. Then, bottles were tightly capped and fermented at room temperature (20-25° C) for 16 days. Samples were taken at every one day interval till 16th day for analyses.

Ripened cucumber was collected from Gangtok market (Sikkim) and khalpi was prepared following the traditional method. Cucumber was

washed and cut into pieces and sun dried for 1 day. About 400 g of pieced cucumbers were filled into sterile 250 ml bottles and fermented at room temperature (20-25°C) for 3 days. Samples were taken at every 6 hour interval till 72 hours for analyses.

The pH and titratable acidity of the fermenting substrates were also determined. Changes in microbial population of major microbial groups were analysed as described in microbial analysis section. Temperature of each sample was recorded before taking sample for analysis.

Preparation of Gundruk and Khalpi using Selected Strains of LAB

'Rayo-saag' leaves of *Brassica rapa* L. sub-sp. *campestris* (L.) Clapham variety *cumifolia* Roxb. were purchased from Gangtok market. Leaves were washed thoroughly in sterile distilled water and wilted in oven (~30° C) for 6 hour. Leaves were crushed and put into sterile warm water (about 90° C) for 5 min. Leaves were transferred into another sterile glass container. Excess water in the leaves was removed by squeezing and then, about 400 g of crushed leaves of 'rayo-saag' were distributed aseptically into each sterile 250 ml capped-bottles, totalling 6 bottles for samplings. Each bottle was inoculated by a mixture (1 ml each) of actively grown culture strains of *Lactobacillus plantarum* GLn: R1 and *Pediococcus pentosaceus* GLn:R2, previously isolated from market samples of gundruk. Bottles were tightly capped and incubated at 20°, 25° and 30° C, respectively for 6 days. Samplings were done on 3rd and 6th days for organoleptic test followed by determination of pH and acidity.

Ripened cucumber (*Cucumis sativus* L.) was collected from Sadam village in Sikkim. Cucumber was cleaned, washed and cut into pieces and wilted at ~30° C in an oven for 6 hour. About 400 g of wilted pieced cucumbers were transferred into each sterile 250 ml bottles, totaling 9 bottles for samplings. Each bottle was inoculated by a mixture (1 ml each) of pure culture strains of actively grown *Lactobacillus plantarum* KG:B1, *Lactobacillus brevis* KG:B2 and *Leuconostoc fallax* KB:C1, previously isolated from traditionally prepared khalpi samples. Bottles were tightly capped and incubated at 20°, 25° and 30° C, respectively for 72 hours. Samples were taken on 24, 48 and 72 hours, respectively for sensory evaluation and also for determination of pH and acidity of the fermenting cucumbers.

Sensory evaluation

Sensory evaluation of gundruk and khalpi, prepared by selected starter cultures was evaluated in terms of aroma, taste, texture, colour and general acceptability as described by Meilgaard *et al.* (1990). Gundruk was organoleptically evaluated by a panel of 7 judges with score rate of 1, bad and 5, good considering market gundruk as control with scoring rate of 3, moderate (Table B). Similarly, khalpi was also evaluated organoleptically by a panel of 7 judges with score rate of 1, bad and 5, good considering market khalpi as control with scoring rate of 3, moderate (Table B).

Table B. Format for sensory evaluation of gundruk/khalpi produced by selected strains of LAB

Please use market gundruk/khalpi as a control with scoring rate of 3 (moderate)

Sample code:..... Name:.....

Attribute	Score					Comment
Aroma	Weak				Strong	
	1	2	3	4	5	
Taste: Mild acidic	Weak				Strong	
	1	2	3	4	5	
Acidic (sour)	Weak				Strong	
	1	2	3	4	5	
Texture:	Hard				Soft	
	1	2	3	4	5	
Colour:	Bad				Good	
	1	2	3	4	5	
General Acceptability:	Bad				Good	
	1	2	3	4	5	

Proximate Composition

pH

Ten g of sample were mixed with 20 ml carbon dioxide-free distilled water in a blender for 1 min and the pH of the slurry was determined directly (AOAC, 1990) using a digital pH meter (Model 361, Systronics, India) calibrated with standard buffer solutions (Merck).

Titratable acidity

Titratable acidity of sample was calculated by titrating the filtrates of a well blended 10 g sample in 90 ml carbon-dioxide free distilled water with 0.1 N sodium hydroxide to end point of phenolphthalein (0.1 % w/v in 95 % ethanol) (AOAC, 1990).

Moisture

Moisture content of sample was calculated by drying 2.5–3.0 g of well-mixed sample at 135 ± 1° C for 2 hour to constant weight (AOAC, 1990).

Ash

A sample (~ 2 g) was accurately weighed into a previously dried and weighed porcelain crucible and placed in a muffle furnace, preheated to 550° C for 3 hour. The crucible was transferred directly to a desiccator, allowed to cool to room temperature and weighed immediately (AOAC, 1990). The process of heating for 30 min, cooling and weighing was

repeated until the difference between two successive weighing was ≤ 1 mg.

Fat

Fat content was determined by ether extraction using glass soxhlet (AOAC, 1990). Flat-bottomed flask was oven dried and kept in a desiccator for cooling. The weight (W_1) of the round-bottomed flask was taken. A cellulose thimble (dry and fat free) was taken and in which ~ 2 g of sample was placed and put in the soxhlet. Fat was extracted by using petroleum ether with boiling range 40-60° C, on a heating mantle at 60° C for 5 hour. The flat bottomed flask was dried for 1 h at 100° C to evaporate ether and moisture, cooled in desiccator and weighed (W_2). Fat was calculated: Fat (%) = $W_2 - W_1$/Sample weight × 100

Protein

Total nitrogen of sample was determined following the method described in AOAC (1990). Approximately 1 g of sample was taken in a digestion flask, 0.7 g catalyst ($CuSO_4$: K_2SO_4, 1:9) and 25 ml of concentrated H_2SO_4 were added to it. The flask was heated gently until frothing ceased, boiled briskly until the solution became clear and then continued the boiling for about 1 hour. The solution was transferred quantitatively to a round-bottomed flask, and mixed with approximately 100 ml of distilled water and 25 ml 4 % w/v aqueous Na_2S to precipitate mercury. A pinch of zinc granules to prevent bumping and a layer of 40 %

w/v NaOH were added carefully. The flask was immediately connected to a distillation apparatus and the tip of the condenser was immersed in standard 0.1 N H_2SO_4 containing about 5 drops of methyl red indicator (I007, HiMedia, Mumbai). The flask was rotated to mix the contents thoroughly and heated until all the ammonia had distilled. The receiver was removed and the tip of the condenser was washed with distilled water. The remaining acid in the receiver was titrated with standard 0.1 N NaOH solution. The blank determination on reagents was considered for correction. Nitrogen was calculated in percentage. Total nitrogen (%) = [(ml of standard acid × N of standard acid) – (ml of standard NaOH – C.F.) × N of standard NaOH] ×1.4007/weight of sample (g). Correction factor (C.F.) = (titre of standard NaOH against blank – ml of standard acid).

Protein content was determined by multiplying total nitrogen value with 6.25 (for plants) (AOAC, 1990). Protein (%) = Total Nitrogen (%) × 6.25

Carbohydrate

The carbohydrate content of the samples was calculated by difference: 100 – (% protein + % fat + % ash) (Standal, 1963).

Nutritive Value

Nutritive value of each sample was determined by multiplying the protein, fat and carbohydrate contents by the factors 4, 9 and 4,

respectively, and adding all the multiplication values to get kcal per 100 g (Indrayan *et al.*, 2005).

Minerals

The method of AOAC (1990) for determination of mineral was followed. The ash after heating the sample at 550° C for 3 hour was dissolved in 5 ml of 20 % HCl. The solution was evaporated to dryness on a hot plate at a temperature of 100-110° C and in an oven at 110° C for 1 hour. The minerals in the dried residue were dissolved in about 10 ml of 100 % HCl and the solution was heated on a hot plate at a temperature of 100-110° C for 3-4 times. The solution was made up to 100 ml with 1 % HCl. Calcium, sodium and potassium were estimated in flamephotometer (Model CL 361, Elico, India) at 623, 589 and 766 nm, respectively following the method of Ranganna (1986).