MATERIALS AND METHODS

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)

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

(4). Baird Parker Agar Base (M043, HiMedia)

(5). Egg Yolk Emulsion (FD045, HiMedia)

(6). Egg Yolk Tellurite Emulsion (FD046, HiMedia)

(7). 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)	

(8). Lactate Configuration Medium (Tamang *et al.*, 2005)

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

(9). *Listeria* Identification Agar Base (M 1064, HiMedia)

(10). *Listeria* Selective Supplement (FD 061, HiMedia)

(11). Malt Extract Agar (M137, HiMedia)

(12). MRS Agar (M641, HiMedia)

(13). MRS Broth (M369, HiMedia)

(14). 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

(15). Nutrient Agar (MM012, HiMedia)

(16). Nutrient Broth (M002, HiMedia)

(17). Plate Count Agar (M091, HiMedia)

(18). Potato Dextrose Agar (M096, HiMedia)

(19). *Salmonella-Shigella* Agar (M108, HiMedia)

(20). Skim Milk Powder (RM1254, HiMedia)

(21). Sucrose Agar (Garvie, 1984)

Tryptone	10 g
Yeast extract	5.0 g
K_2HPO_4	5.0 g
Trisodium citrate	5.0 g
Sucrose	50 g
Agar	15 g
Distilled water	1000 ml

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

(22). Tryptone Soya Agar (M290, HiMedia)

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

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

(25). Yeast Malt Extract (YM) Broth (M425, HiMedia)

(26). Yeast Morphology Agar (M138, HiMedia)

(27). Yeast Nitrogen Base (M139, HiMedia)

REAGENTS

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

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

(2). Gram's Crystal Violet (S012, HiMedia)

(3). Malachite Green (S020, HiMedia)

(4). 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.

(5). 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.

(6). Phenolphthalein (I009, HiMedia)

(7). Safranin (S027, HiMedia)

Reference Strains

Reference Strains	Origin	Purpose in this experiment
Bacillus cereus CCM 2010	CCM	Indicator strain for antimicrobial activity
Klebsiella pneumoniae subsp. *pneumoniae* BFE 147	BFE	Indicator strain for antimicrobial activity
Listeria monocytogenes DSM 20600	DSM	Indicator strain for antimicrobial activity
Staphylococcus aureus S1	FMR	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 (Institute of Hygiene and Toxicology, Karlsruhe, Germany), and FMR (Food Microbiology Laboratory, Sikkim Government College, Gangtok, India).

Listeria monocytogenes DSM 20600, *Staphylococcus aureus* S1, *Bacillus cereus* CCM 2010, and *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147 were propagated in standard nutrient agar. The cultures were maintained as frozen stocks at –20° C in 15 % glycerol.

METHODS

Survey

Survey was conducted in randomly selected 214 households in villages located in all four districts of Sikkim – North (54 households), West (48 households), South (42 households) and East (70 households), representing the major ethnic communities, namely the Nepalis, Bhutias and Lepchas. Information was collected on consumption of *Selroti* using questionnaire (Table B) during 2001-2005. Amount of *Selroti* consumed in every meal by each person was weighed directly by a portable weighing balance (Ishida, Germany) and daily per capita consumption was estimated as g/capita/day. Feeding frequency of *Selroti* by each family was also recorded in percentage. A data base was developed from the field data collection and analysed using statistical software (SPSS) at the Indian Statistical Institute, Kolkata. Indigenous knowledge on traditional method of *Selroti* batters practised by the ethnic people of the Sikkim Himalaya, their mode of consumption, socio-economy and ethnical importance of the product was also documented based on the information sought from the local people of the respective places.

Table B. Questionnaire on survey on *Selroti* consumption of Sikkim

- Name of the Informant:
- Ethnic Group:
- Name of (i) Village/Revenue Block:; (ii) Sub-division; (iii) District:
- Approximate number of population of the Village:
- Nearest Market:
- Kindly provide information on fermented product:

Fermented product	Local name	Raw material	Consume (daily/times per week/occasionally)	Whether prepare at home or market purchase/both
Selroti (deep-fried)				

- Traditional method of preparation:
- Mode of consumption:
- Socio-economy of the product:
- Ethnical importance:

Collection of samples

A total of 78 samples of home-made *Selroti* batters were collected directly from different villages located in the Darjeeling hills and Sikkim. Similarly, 36 market-samples of *Selroti* batters were collected from different restaurants, local food stalls and canteens located in Gangtok. Samples of *Selroti* batters prepared at our laboratory following the traditional method were also included. 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) plates supplemented with 1 % $CaCO_3$, and were incubated at 30° C under anaerobic condition kept in an Anaerobic Gas-Pack container (LE002, HiMedia) for 48-72 hour. Aerobic mesophilic counts (AMC) were determined in plate count agar (M091A, HiMedia) plates and incubated at 30° C for 48-72 hour. Moulds and yeasts were isolated on potato dextrose agar (M096, HiMedia) and yeast-malt extract (YM) agar (M424, HiMedia), 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

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

Gram staining

Bacterial 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 minute, and washed for 5 second

with water. The smear was flooded with Burke's iodine solution, allowed to react for 1 minute, and washed again for 5 second 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 minute 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).

Arginine hydrolysis

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 100 µl cultures onto a white porcelain tile and adding equal volume of Nessler's reagent. Appearance of dark orange colour indicated presence of ammonia (Schillinger and Lücke, 1987).

Gas 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 the 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

The 24 hour-old cultures were inoculated in MRS broth 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 % and 18 % 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

Dextran production of *Leuconostoc* isolates were tested by growth on the 5 % (w/v) sucrose agar (Garvie, 1984) and observed for mucoid appearance on the agar plates (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 (Tamang *et al.*, 2005) at 30° C overnight. One ml culture was centrifuged in a microcentrifuge (Heraeus, Germany) at 8,000 *g* for 5 minutes. 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.

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 hour. 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.

Identification of Bacteria

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).

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) 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 minutes. 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) 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) 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) for 30 to 60 second, heated to steaming 3 to 4 times over the flame of a spirit lamp and counterstained with safranin (S027, HiMedia) for 30 second 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) were inoculated with 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) 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) slants for 3 days. Tubes containing 5 ml mixture of yeast nitrogen base (M139, HiMedia) 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) for *Bacillus cereus*, Baird Parker agar base (M043, HiMedia) for *Staphylococcus aureus* and Violet Red Bile Glucose agar w/o lactose (M581, HiMedia) for enterobacteriaceae (Han *et al.*, 2001). *Salmonella-Shigella* Agar (M108, HiMedia) was used for the detection of *Salmonella* and *Shigella* and *Listeria* identification agar base (M1064, HiMedia) with *Listeria* selective supplement (FD 061, HiMedia) 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 minute. 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) with appropriate additions of Polymyxin B Selective Supplement (FD003, HiMedia) and Egg yolk emulsion (FD045, HiMedia). 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 was regarded as presumptive *Bacillus cereus*.

***Staphylococcus aureus*:** Spread plates of Baird Parker agar base (M043, HiMedia) with appropriate additions of Egg yolk tellurite emulsion (FD046, HiMedia) was used for selective enumeration of *Staphylococcus aureus*. After serial dilution plates were overlaid with the medium and incubated at 30° C for 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) were allowed to resuscitate on thinly plated tryptone soya agar (M290, HiMedia) plates for 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) with *Listeria* selective supplement (FD061, HiMedia) 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* agar (M108, HiMedia) 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 Isolates

Acidification and coagulation

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

Enzymatic profile by API-zym

The enzymatic profile of LAB and yeast isolates 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. LAB cultures were grown on MRS broth, and yeast cultures were grown on YM broth, respectively and cells were harvested in 2 ml sterile distilled water 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.

Antimicrobial and bacteriocin activity

Agar Spot Test: The LAB isolates were screened for antimicrobial activity against some of the pathogenic bacteria by agar spot method of Schillinger and Lücke (1989). Cultures were grown on the respective broth media for 24 hour. Sterilized Petri-plates were plated with MRS agar (containing 0.2 % glucose) and allowed to dry. These were spotted with a drop of the broth culture of the producer strain and incubated at 30° C for 24 hour. The indicator

strains *Bacillus cereus* CCM 2010, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147, *Listeria monocytogenes* DSM 20600 and *Staphylococcus aureus* S1 were propagated in standard nutrient agar (M002, HiMedia). The 0.1 ml of an overnight culture (~10^7 cells) of each indicator strain was inoculated into 7 ml of soft MRS agar (containing 0.7 % agar) and poured over the plate on which the producer was grown, respectively. These were incubated at 30° C for 24 hour. After incubation, the plates were checked for inhibition zones (clearing of the medium) around the producer colony.

Bacteriocin Activity: Bacteriocin activity of the LAB isolates was estimated using an agar spot assay as described by Uhlman *et al.* (1992) and Schillinger *et al.* (1993). The antimicrobial-positive strains were grown in MRS broth at 30° C for 24 hour and a cell-free extract was obtained by centrifuging the culture in a microcentrifuge (Heraeus, Germany). The supernatant was heated at 100° C for 5 minutes in block-thermostat (Staurt Scientific, UK). The cell-free supernatant was adjusted to pH 6.5 by addition of 1 N NaOH. Agar plates overlaid with 7 ml soft MRS agar (containing 0.7 % agar) were inoculated with 0.1 ml of an

overnight culture of the indicator strains (as mentioned above), respectively. After incubation at 25° C for 24 hour, 0.01 ml of the culture supernatant was spotted onto the agar surface. The plates were incubated at 30° C for 24 hour and subsequently examined for zones of inhibition.

Successional studies during *Selroti* batter fermentation

Preparation of Selroti batters at Laboratory

Selroti batter was prepared in the laboratory following the traditional method based on the combination of three popular methods being practiced at different places in Sikkim. Rice (*Oryza sativa* L.) local variety 'attey' was purchased from Gangtok market. One kg of rice was sorted, washed and soaked overnight at ambient temperature (20-22° C). Water was decanted from the soaked rice, spread over a tray for 1 hour, pounded and sieved to get rice flour. The rice flour was thoroughly mixed with 250 g of wheat flour (refined), 250 g sugar, 100 g butter and 25 g powdered spices including large cardamom (*Amomum subulatum* Roxb.), cloves (*Syzygium aromaticum* Merr.), coconut (*Cocos nucifera* L.), fennel (*Foeniculum vulgare* Mill), nutmeg (*Myristica fragrans* Houtt.), cinnamon (*Cinnamomum zeylanicum* Breyn.), and

small cardamom (*Elletaria cardamomum* Maton.). Milk was added to the mixture and kneaded into soft dough. More milk was added to make the batter with easy flow during preparation. Equal volumes of batter were distributed in 11 sterile 500 ml Duran bottles and were loosely capped, labeled and incubated at 28° C. Successional studies were carried at every 1 hour interval within a range of 0 to10 hours.

Microbial changes

Samplings were made at one hour interval till 10th hour for microbial changes during *in situ* fermentation. LAB, yeasts, *Bacillus cereus, Staphylococcus aureus* and enterobacteriaceae isolated from fermenting batters in every hour (0-10 hour) were analysed for their microbial load.

Physico-chemical changes

Samplings were made at every one hour interval till 10th hour for analysis of physico-chemical (pH, titratable acidity, temperature of fermenting batters, batter volume and batter weight) changes during *in situ* fermentation.

Sensory evaluation

Sensory properties of product was evaluated in terms of aroma, taste, texture, colour and general acceptability as method described by Meilgaard *et al.* (1990). *Selroti* batters were prepared at laboratory following the traditional method as described above. Fermenting batters were taken out in every hour and deep-fried in hot edible oil to make a *Selroti.* Deep-fried *Selroti* prepared from every hour fermenting batters were served to 10 judges for sensory evaluation with score rate of 1 as bad (hard texture) and 5 as excellent (soft texture); the market deep-fried *Selroti* was considered as control with scoring rate of 3, moderate (Table C).

Table C. Format for sensory evaluation of *Selroti*

Please use market Selroti as a control with scoring rate of 3 (moderate)

Sample code:

Name:

Attribute	Score					Comment
Aroma	Bad				Excellent	
	1	2	3	4	5	
Taste	Bad				Excellent	
	1	2	3	4	5	
Texture:	Hard				Soft	
	1	2	3	4	5	
Colour:	Bad				Excellent	
	1	2	3	4	5	
General Acceptability:	Bad				Excellent	
	1	2	3	4	5	

Date: Signature of the judge

Requirements for high grade *Selroti*

Aroma: Typical *Selroti* flavour
Taste: Sweet
Texture: Soft
Colour: Golden brown

Testing of strain(s) for *Selroti* Preparation

Starter cultures preparation

A loopful of LAB culture was inoculated in 5 ml MRS broth (M369, HiMedia) and incubated overnight at 30° C. The 1 ml of each culture was centrifuged (Biofuge pico, Heraeus, Germany) at 8,000 *g* for 5 minutes, the supernatant was discarded, 1 ml of sterile distilled water was added to the pellet, cells were resuspended and again centrifuged at 8,000 *g* for 5 minutes. Cells were again suspended in 1 ml sterile distilled water. This procedure achieved an inoculum size containing 10^8 cfu/ml, and was checked as viable count in MRS agar (M641, HiMedia) plates. A loopful of different yeast cultures were inoculated in 5 ml YM broth (M425, HiMedia) separately and incubated overnight at 28° C. These cultures were centrifuged and washed as described previously and an inoculum containing 10^7 cfu/ml, was made.

Sensory evaluation of Selroti prepared by starter culture

Pounded rice along with all ingredients are mixed thoroughly and soft dough was made as described earlier. Equal volumes (nearly 350 ml) of batter were distributed in 14 sterile 500 ml Duran bottles with screw-caps. *Selroti*

batter samples were inoculated with 1 ml of LAB and yeast inocula per 100 g of batter. Different starter cultures were used. In mixed fermentation, 1 ml of each inoculum was used for the same batter sample. Inoculated samples were mixed thoroughly by sterile spatula, loosely capped, labeled and incubated at 28° C. The pH and titratable acidity of the fermenting batters were determined at 0 hour, 4 hour and 6 hour. Fermenting batters were taken out at 4 hour and 6 hour and deep-fried in hot edible oil to make a *Selroti.* Deep-fried *Selroti* prepared from fermenting batters were served to 10 judges for sensory evaluation with score rate of 1 as bad (hard texture) and 5 as excellent (soft texture); the market deep-fried *Selroti* was considered as control with scoring rate of 3, moderate (Table C).

Consumers' Preference Trial

Freshly fried *Selroti* purchased from Gangtok market as well as fried *Selroti* prepared from batters made in the laboratory by using a mixture of selected isolates were served to 50 consumers who were familiar with *Selroti.* The 9-point scale used in consumers' preference trial ranged from score 1 (dislike extremely) to score 9 (like extremely) (IS, 1971).

Physical and Biochemical Analysis

pH

Ten g of sample were mixed with 20 ml carbon dioxide-free distilled water in a blender for 1 minute 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).

Determination batter volume

The volume in the batter during fermentation was determined following the method described by Thyagaraja *et al.* (1992). The batter (50 ml) was transferred to a sterile measuring cylinder of 100 ml capacity, covered with aluminium foil and incubated at 30° C. The increase in volume at different stages of fermentation was recorded and expressed as the percent volume increase over the initial volume.

Determination of batter weight

Batter weight (~25 g) was taken at every hour during fermentation using a digital balance (Lutron GM-500,

Taiwan) to observe an increase/decrease on weight of fermenting batter.

Batter temperature

The temperature (°C) change of the fermenting substrates during *Selroti* batter fermentation was recorded directly by a thermometer (Thapa, 2001).

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).

Reducing sugar

Reducing sugar content of the sample was determined by modified colorimetric method of Somogyi (1945) using glucose as standard solution. To 1 ml of sample extract in a 20 ml capped glass tube, 1 ml of Reagent D was added and heated in a vigorously boiling water-bath for 20 minutes. This was allowed to cool for 5 minutes in running tap water and 1

ml of Reagent C was added and the test tube shaken until no bubbles evolved. After standing for 20 minutes, this was diluted to 25 ml with distilled water and absorbance was measured at 520 nm in UV-VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). Reducing sugar was calculated in percentage.

Glucose (%) = $(As-Ab)/(Ag-Ab) \times [G] \times 10^{-3} \times V_1/1 \times 250/V_2 \times 100/10$

As = absorbance of sample

Ab = absorbance of blank

Ag = absorbance of glucose

[G] = concentration of glucose solution (μg/ml)

10^{-3} = mg to g

V_1 = total dilution volume for reaction (ml)

1= 1 ml for reaction

V_2 = pipetting volume of extract for dilution (ml)

250 = total volume of extract (ml)

100 = %

10 = sample size for preparation of extract

Total Sugar

Total sugar of the sample was estimated by determining reducing sugar in hydrolysed sample with HCl (AOAC, 1990). In a 300 ml conical flask fitted with condenser, 2 g of sample was blended in 20 ml of distilled water to which 160 ml of distilled water and 20 ml of HCl (25 %) were added. It was heated in vigorously boiling water bath for 3 hour, cooled in a running tap water, neutralized with 10 % NaOH using pH

meter (Type 361, Systronics, India) and diluted to 500 ml with distilled water. It was filtered and the filtrate was taken for determining reducing sugar as described above. Total sugar was calculated in percentage.

Total sugar (%) = $(As-Ab)/(Ag-Ab) \times [G] \times 10^{-3} \times V_1/1 \times 500/V_2 \times 100/S$

As= absorbance of sample

Ab = absorbance of blank

Ag = absorbance of glucose

[G]= concentration of glucose solution (µg/ml)

10^{-3} = mg to g

V_1 = total dilution volume for reaction (ml)

1= 1 ml for reaction

V_2 = pipetting volume of extract for dilution (ml)

500 = total volume of extract (ml)

100 = %

S = sample size for preparation of extract

Moisture

Moisture content of the batters 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 minutes, cooling and weighing was repeated until the difference between two successive weighing was ≤ 1 mg.

Fat

Fat content of the sample 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 hour 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 the 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). 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 (AOAC, 1990). Protein (%) = Total Nitrogen (%) × 6.25

Water-soluble nitrogen

For estimation of water-soluble nitrogen, homogenised samples was centrifuged at 7,000 *g* for 10 minutes and 10 ml of accurately weighed supernatant was digested and distilled as described for total nitrogen (Tamang and Nikkuni, 1996).

TCA-soluble nitrogen

For determination of TCA-soluble nitrogen, homogenised sample was added with equal volume of 8 % trichloroacetic acid (TCA) (w/v) and kept at room temperature for 60 minutes. Mixture was centrifuged at 7,000 *g* for 10 minutes and 10 ml of accurately weighed samples was digested and distilled as described above (Tamang and Nikkuni, 1996).

Carbohydrate

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

Food Value

Food value of each batter 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 flame-photometer (Model CL 361, Elico, India) at 623, 589 and 766 nm, respectively following the method of Ranganna (1986).

Statistical analysis

The data were analysed by determining standard deviation (SD), standard error of measurement (SEM) and analysis of variance (ANOVA) (Snedecor and Cochran, 1989).