3

Materials and Methods

3.1. Materials

3.1.1. Reference organisms

The reference bacteria, used as control, were *Bacillus cereus* ATCC9139 (courtesy: Dr M.J.R. Nout, Wageningen University, Wageningen, The Netherlands), and *Clostridium perfringens* MTCC450, *Staphylococcus aureus* MTCC96, *Escherichia coli* MTCC118, *Salmonella typhi* MTCC733 and *Shigella flexneri* MTCC1457 (purchased from Microbial Type Culture Collection and Gene bank (MTCC), Institute of Microbial Technology, Chandigarh, India).

3.1.2. Culture media

***Bacillus cereus* selective agar**

Bacillus cereus agar base (M833; HiMedia Laboratories Pvt Limited, Mumbai, India)

Polymyxin B selective supplement (HiMedia FD003)
Egg yolk emulsion (HiMedia FD045)

Baird-Parker medium
Baird-Parker agar base (HiMedia M043)
Potassium tellurite 3.5% (wv^{-1}) (HiMedia FD047)
Egg yolk emulsion (HiMedia FD045)

Bismuth sulphite agar
(HiMedia M027) - melted (not autoclaved)

Brain heart infusion broth
(HiMedia M210)

Brilliant green bile broth 2%
(HiMedia M121)

Coagulase mannitol broth base
(HiMedia M227)

Cooked meat medium
(HiMedia M149)

DNase test agar with toluidine blue
(HiMedia M1041) - autoclaved at 0.9 kg cm^{-2} for 15 min

Fluid selenite cystine medium
(HiMedia M025A) - heated in a boiling water-bath for 10 min (not autoclaved)

Fluid thioglycolate medium
(HiMedia M009)

Fortified nutrient agar (Kim and Goepfert 1971)

Nutrient agar	23.0 g
Agar	5.0 g
NaCl	8.0 g
Glucose	0.1 g
$CaCl_2.2H_2O$	0.08 g
$(NH_4)_2SO_4$	0.08 g
$MnCl_2.4H_2O$	0.008 g
Modified mineral solution	50 ml
Distilled water	950 ml
pH 7.0	

***Lactobacillus* MRS agar**
(HiMedia M641)

Lactose gelatin medium, modified
(HiMedia M987) - autoclaved at 1.1 kg cm^{-2} for 10 min

Lysine iron agar
(HiMedia M377)

MacConkey agar
(HiMedia M082)

Medium for acid and gas production from glucose (Claus and Berkeley 1986)

$(NH_4)_2HPO_4$	1.0 g
KCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
Yeast extract	0.2 g
Glucose (autoclaved separately)	5.0 g
Agar	15.0 g
Bromocresol purple	0.006 g
Distilled water	1000 ml

pH 7.0

Milk agar
(HiMedia M163)

Motility nitrate medium, buffered
(HiMedia M630I)

MRS-0.2 agar (MRS broth containing 0.2% (w v^{-1}) glucose)

Proteose peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
Dextrose	2.0 g
Polysorbate 80	1.0 g
Ammonium citrate	2.0 g
Sodium acetate	5.0 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.05 g
Dipotassium phosphate	2.0 g
Agar	15.0 g
Distilled water	1000 ml

PH 6.5

MR-VP (methyl red - Voges-Proskauer) medium
(HiMedia M070)

Mueller Hinton agar
(HiMedia M173)

Nitrate broth (Claus and Berkeley 1986)

Peptone	5.0 g
Beef extract	3.0 g
KNO_3	1.0 g
Distilled water	1000 ml

pH 7.0

Nutrient agar
(HiMedia M561)

Nutrient broth
(HiMedia M002)

Perfringens agar
Perfringens agar base (OPSP) (HiMedia M579)
Perfringens supplement I (sodium sulphadiazine) (HiMedia FD011)
Perfringens supplement II (oleandomycin phosphate and polymyxin B) (HiMedia FD012)

Plate count agar
(HiMedia M091)

Purple agar
Purple agar base (HiMedia M098)
Glucose (5-10 g l^{-1})

Raffinose gelatin medium

Tryptose	15.0 g
Yeast extract	10.0 g
Raffinose	10.0 g
Na_2PO_4	5.0 g
Phenol red	0.05 g
Gelatin	120.0 g

pH 7.5

Rappaport-Vassiliadis medium
(HiMedia M880) - autoclaved at 0.7 kg cm^{-2} for 15 min

Starch agar
(HiMedia M107)

Tributyrin agar base
(HiMedia M157)

Triple sugar iron agar
(HiMedia M021)

Tryptone soya agar
(HiMedia M290)

Tryptone soya broth
(HiMedia M011)

Tryptone water
(HiMedia M463I)

Violet red bile glucose agar without lactose
(HiMedia M581) - not autoclaved

Voges-Proskauer broth (Claus and Berkeley 1986)

Proteose peptone	7.0 g
Glucose	5.0 g
NaCl	5.0 g
Distilled water	1000 ml

pH 6.5

Yeast malt agar
(HiMedia M424)

All the media mentioned above were sterilized by autoclaving at 1.1 kg cm^{-2} pressure for 15 min, unless mentioned otherwise.

3.1.3. Reagents

Acrylamide solution

Acrylamide (SRL 014022)	300 g
N,N,-methylene bisacrylamide (SRL 134985)	8 g
Deionized water	1000 ml

Buffered peptone water
(HiMedia M614)

Coomassie brilliant blue solution

Coomassie brilliant blue R250 (SRL 024018)	0.4 g
Methanol (SRL 132977)	90 ml
Glacial acetic acid (Merck 60006325001046)	20 ml
Deionized water	90 ml

Destaining solution

Methanol	40 ml
Glacial acetic acid	10 ml
Deionized water	50 ml

Gram's crystal violet solution
(Merck 9218)

Gram's iodine solution
(Qualigens 38753)

Gram's safranine solution
(Merck 9217)

Kovac's reagent strip
(HiMedia DD019)

Modified mineral solution (Kim and Goepfert 1971)

$MnSO_4.H_2O$	1.0 g
$MgSO_4$	4.0 g
$CuSO_4.5H_2O$	1.0 g
$ZnSO_4.7H_2O$	0.1 g
$FeSO_4.7H_2O$	0.01 g
Distilled water	1000 ml

Nisaplin® Nisin preparation for foodstuffs
Activity 1,000,000 IU g^{-1} = 25 mg nisin g^{-1} Nisaplin (Delves-Broughton *et al.* 1992)
Aplin & Barrett Ltd, Beaminster, Dorset, England
(Courtesy: Vasta Marketing in Chennai - Indian agency of Aplin & Barrett)

Nitrate reagent (Norris *et al.* 1981)

Solution A	Sulphanilic acid	0.8 g
	5N Acetic acid	100 ml
	(Glacial acetic acid : water, 1 : 2.5)	
Solution B	α-Naphthylamine	0.5 g
	5 N Acetic acid	100 ml

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

Peptone physiological saline (Nout *et al.* 1998)

Neutral peptone	0.1 g
NaCl	0.85 g
Distilled water	100 ml
pH 7.2	

Phenolphthalein
1 g (SD's 30088) l^{-1} 95% (v v^{-1}) ethanol

Phosphate buffer (0.2 M, pH 7.0)

$NaH_2PO_4.2H_2O$ (HiMedia RM3694), 0.2 M	39.0 ml
Na_2HPO_4 (HiMedia RM463I), 0.2 M	61.0 ml

- diluted to a total of 200 ml

Resolving gel buffer

Tris (hydroxymethyl) aminomethane (Tris-HCl; SRL RM262)	3.0 M
pH 8.9	

Sample buffer 2X

Sodium lauryl/ dodecyl sulphate (SDS; SRL 1948101, 10% w v^{-1})	4 ml
Stacking gel buffer	2.5 ml
Glycerol (SRL 072438)	1.5 ml
Bromophenol blue (SRL 0240168)	10.0 mg
β-mercaptoethanol (SRL 1327198)	2.0 ml

Stacking gel buffer

Tris-HCl	0.5 M
pH 6.8	

Tank buffer

Tris-HCl	0.025 M
Glycine (Merck India 4201)	0.192 M
SDS	10% (w v^{-1})
pH 8.3	

All the chemicals used were of the highest purity grade available.

3.1.4. Antimicrobial susceptibility test discs

Ampicillin (10 μg $disc^{-1}$)
(HiMedia SD002)

Bacitracin (10 units $disc^{-1}$)
(HiMedia SD003)

Carbenicillin (100 μg $disc^{-1}$)
(HiMedia SD004)

Cephalothin (30 μg $disc^{-1}$)
(HiMedia SD050)

Chloramphenicol (30 μg $disc^{-1}$)
(HiMedia SD006)

Ciprofloxacin (10 μg $disc^{-1}$)
(HiMedia SD080)

Cloxacillin (10 μg $disc^{-1}$)
(HiMedia SD143)

Erythromycin (15 μg $disc^{-1}$)
(HiMedia SD013)

Kanamycin (30 μg $disc^{-1}$)
(HiMedia SD017)

Metronidazole (5 μg $disc^{-1}$)
(HiMedia SD020)

Nalidixic acid (30 μg $disc^{-1}$)
(HiMedia SD021)

Penicillin G (10 units $disc^{-1}$)
(HiMedia SD028)

Polymyxin B (300 units $disc^{-1}$)
(HiMedia SD029)

Rifampicin (15 μg $disc^{-1}$)
(HiMedia SD128)

Streptomycin (10 μg $disc^{-1}$)
(HiMedia SD031)

Tetracycline (30 μg $disc^{-1}$)
(HiMedia SD037)

Trimethoprim (10 μg $disc^{-1}$)
(HiMedia SD093)

Vancomycin (10 μg $disc^{-1}$)
(HiMedia SD163)

3.2. Experimental

3.2.1. Sampling of foods

Samples of legume-based traditional fermented foods were purchased from randomly selected retail outlets and restaurants scattered over different districts of the State. The unpackaged samples, each having an average weight of 150 g, were collected in sterile Nasco sampling bags (HiMedia PW389), and kept in an ice-box. Those as well as packed (in sealed pouches made with low-density polyethylene film) samples were transported to the laboratory immediately and analysed as early as possible.

3.2.2. Biochemical analysis

3.2.2.1. Moisture content

Approximately 10 g of sample was accurately weighed using a Sartorius CP224S (Sartorius AG, Gõttingen, Germany) balance into a cooled and weighed Petri dish, previously heated to 105 ± 1 °C. The sample was uncovered and allowed to dry for 48-72 h at 105 ± 1 °C in a hot air oven. The dish was covered while still in oven, transferred to a desiccator, and weighed soon after reaching room temperature. The process of drying, cooling and weighing was repeated until the two successive weighings reached a constant value. Moisture content was calculated by subtracting the final weight from the initial weight (AOAC 1990; Banerjee and Sarkar 2003).

3.2.2.2. pH

A 10 g-sample of food was blended with 20 ml carbon dioxide-free deionized water, and pH of the slurry was determined using a pH meter (type 335; Systronics, Naroda, India). pH of batter was determined taking 20 g of undiluted batter .

3.2.2.3. Titratable acidity

Titratable acidity of the batter samples was determined by titrating the filtrates of well-blend 10 g samples in 90 ml carbon dioxide-free distilled water with 0.1 N sodium hydroxide to end point of phenolphthalein (AOAC 1990).

3.2.3. Bacteriological analysis

3.2.3.1. Isolation of bacteria from foods

Representative 10 g portions of samples were aseptically weighed and homogenized with 90 ml sterile peptone-physiological saline using a Stomacher lab-blender 400 (Seward Medical, London, UK) at 'normal' speed (2 min for wadi, 1 min for others). Serial decimal dilutions were prepared with the same diluent, and duplicate counting plates were prepared using appropriate dilutions. For pour plating, 1 ml of the dilutions were mixed with molten (45 °C) media and poured into plates. For surface seeding, 0.1 ml of the dilutions were spread on the surface-dried plates. After incubation at appropriate temperatures, the colonies appearing on the selected plates (having 50-300 colonies per plate) were counted as colony forming units (cfu) per gram fresh weight sample. The representative colonies of each type were picked and diluted by streaking out on plates of appropriate media. After microscopic examination, the purified colonies were grown on slants or in broths of suitable media and stored at 4 °C (Banerjee and Sarkar 2003; FDA 1984; Speck 1984).

3.2.3.1.1. Total aerobic mesophilic bacteria

The standard plate count for total aerobic mesophilic bacteria was carried out in pour-plates of plate count agar, incubated at 35 °C for 18-24 h (Banerjee and Sarkar 2003).

3.2.3.1.2. Mesophilic bacterial spores

In order to estimate mesophilic bacterial spores, 100 ml sample l^{-1} suspension was heated at 80 °C for 30 min, suitably diluted, and spread on plate count agar plates followed by incubation at 30°C for 72 h (for aerobic sporeformers) and pour-plated with perfringens agar followed by incubation for 48 h at

37 °C in an anaerobic jar with AnaeroHiGas pack (HiMedia LE002A) (for anaerobic sporeformers) (Banerjee and Sarkar 2003; Kneifel and Berger 1994).

3.2.3.1.3. *Bacillus cereus*

Selective enumeration of *B. cereus* was made on spread-plates of *Bacillus cereus* selective agar which were incubated at 35 °C for 24-48 h (HiMedia 1998). A representative number of presumptive isolates were purified on *B. cereus* selective agar and finally on nutrient agar (Banerjee and Sarkar 2003).

3.2.3.1.4. *Clostridium perfringens*

Isolation of *C. perfringens* was carried out in pour-plates of perfringens agar, incubated anaerobically at 37 °C for 18-48 h (HiMedia 1998). The representative presumptive colonies were purified on perfringens agar (Banerjee and Sarkar 2003).

3.2.3.1.5. *Staphylococcus aureus*

Selective enumeration of *S. aureus* was carried out on spread-plates of Baird-Parker medium following incubation at 35 °C for 24-48 h (HiMedia 1998). Representative presumptive colonies were purified on the same medium and finally on nutrient agar (Banerjee and Sarkar 2003).

3.2.3.1.6. Enterobacteriaceae

Estimation of Enterobacteriaceae was carried out by mixing 1.0 ml of appropriate dilutions of food samples with tryptone soya agar and incubating the plates for 1-2 h at room temperature (26 ± 2°C) followed by a thick overlay of violet red bile glucose agar without lactose and incubated at 35 °C for 18-24 h (HiMedia 1998). The representative colonies were purified on tryptone soya agar and finally on nutrient agar (Banerjee and Sarkar 2003).

3.2.3.1.7. *Escherichia coli*

Selective isolation of *E. coli* was made on spread-plates of MacConkey agar incubated at 35 °C for 24 h. The representative presumptive colonies were purified on MacConkey agar and finally on nutrient agar.

3.2.3.1.8. *Salmonella/Shigella*

For qualitative detection of *Salmonella* and *Shigella*, 25 g-samples were added to 225 ml buffered peptone water (pre-enrichment medium) followed by incubation at 35-37 °C for 16-20 h. Ten and 0.1 ml of this pre-enrichment culture were added to fluid selenite cystine medium and Rappaport-Vassiliadis medium, respectively. The former was incubated at 35 °C for 18-24 h, whereas the latter at 42 °C for 18-48 h. Bismuth sulphite agar plates, streaked with the enriched broth cultures, were incubated at 35-37 °C for 24-48 h. The representative colonies were purified on bismuth sulphite agar and finally on nutrient agar (Adams and Moss 1995).

3.2.3.2. Maintenance of pure cultures

All the isolates, excepting *C. perfringens* (which were stored in cooked meat medium), were maintained on nutrient agar and tryptone soya agar slants at 4 °C (Banerjee and Sarkar 2003).

3.2.3.3. Confirmation of taxonomic status of the isolates

3.2.3.3.1. *Bacillus cereus*

The presumptive isolates of *Bacillus cereus* were confirmed on the basis of motility, endospore formation, glucose fermentation, acetylmethylcarbinol production and nitrate reduction following the methods of Claus and Berkeley (1986).

3.2.3.3.1.1. Motility

A 24 h-old culture in nutrient broth was used to prepare a hanging drop in a cavity slide. The drop was observed using a phase-contrast microscope (model BH2-PC-PA-1; Olympus, Tokyo, Japan).

3.2.3.3.1.2. Endospore

Six day-old cultures on nutrient agar at 30 °C were examined on a slide freshly coated with a thin layer (approximately 0.5 mm) of 20 g agar l^{-1} water for endospore production using a phase-contrast microscope.

3.2.3.3.1.3. Glucose fermentation

Tubes of 10 ml medium for acid and gas production from glucose were stabbed with the 24 h-old isolates and incubated at 30 °C for 7 d. Any change in colour of the media from purple to yellow indicated acid production and cracking of the media was the indication of gas production.

3.2.3.3.1.4. Nitrate reduction

Cultures (24 h-old) were grown in 10 ml nitrate broth containing inverted Durham tubes at 30 °C. After 3, 7 and 14 d, 1 ml of the cultures were mixed with 3 drops of nitrate reagent and observed for development of red or yellow colour indicating the presence of nitrite. A small amount of zinc dust was added to the tube that was negative even after 14 d and observed for any development of red colour indicating the presence of nitrate i.e. absence of reduction.

3.2.3.3.1.5. Voges-Proskauer reaction (acetylmethylcarbinol production)

Tubes of 10 ml Voges-Proskauer broth were inoculated with the 24 h-old cultures. After 3, 5, and 7 d incubation at 30 °C, 3 ml of 400 g sodium hydroxide l^{-1} and 0.5-1 mg creatine were added, shaken thoroughly for 30-60 min at room temperature for the production of any red colour indicating a positive Voges-Proskauer reaction.

3.2.3.3.2. *Clostridium perfringens*

Presumptive *C. perfringens* isolates were confirmed by testing motility, reduction of nitrate, liquefaction of gelatin and fermentation of lactose and raffinose (Adams and Moss 1995).

3.2.3.3.2.1. Motility and nitrate reduction

Motility nitrate medium, buffered (10 ml) was stabbed with a 48 h-old pure culture from fluid thioglycolate medium and incubated at 35 °C for 24 to 48 h. A centrifugal growth beyond the stab line was the indication of motility. Production of any red colour by adding nitrate reagent was the indication of nitrate reduction (HiMedia 1998).

3.2.3.3.2.2. Gelatin liquefaction and lactose fermentation

Pure culture (48 h-old) from fluid thioglycolate medium was grown in 10 ml of lactose gelatin medium, modified. After incubation at 35 °C for 24 to 72 h, a change in colour from red to yellow and cracking of the medium indicated acid and gas production, respectively, from lactose. Liquefaction of gelatin was checked after keeping the tubes at 5 °C for 1 h (HiMedia 1998).

3.2.3.3.2.3. Raffinose fermentation

Raffinose gelatin medium (10 ml) was stabbed with a 48 h-old culture from fluid thioglycolate medium. The inoculated tube was incubated at 35 °C for 24-48 h. A change in colour from red to yellow and cracking of the medium were the indication of positive reaction.

3.2.3.3.3. *Staphylococcus aureus*

The presumptive isolates were confirmed by the production of coagulase, thermostable DNase, fermentation of mannitol and production of acetylmethylcarbinol (Adams and Moss 1995; Schleifer 1986).

3.2.3.3.3.1. Coagulase and mannitol fermentation

Coagulase mannitol broth base (5 ml), added with pretested EDTA-treated rabbit plasma (150 ml plasma l^{-1} broth base), was inoculated with 2 drops of 24 h-old culture in nutrient broth and incubated at 35 °C. Coagulase activity (opaque due to coagulated plasma) was examined after 2-5 h incubation. Mannitol fermentation (a change in colour from red-orange to yellow) was tested after 18-48 h at 35 °C (HiMedia 1998).

3.2.3.3.3.2. Thermostable DNase

Thermostable deoxyribonuclease activity was tested with 10 min-boiled culture supernatant by spotting on DNase test agar with toluidine blue. After incubation at 35 °C for 18-24 h, formation of a bright pink zone around the colony was considered positive reaction (HiMedia 1998).

3.2.3.3.3.3. Voges-Proskauer reaction

Same as in section 3.2.3.3.1.5.

3.2.3.3.4. Enterobacteriaceae

Presumptive Enterobacteriaceae isolates were confirmed on the basis of glucose fermentation and oxidase production (Nout *et al.* 1998).

3.2.3.3.4.1. Glucose fermentation

Tubes of 10 ml-purple agar containing 5-10 g sterile glucose l^{-1} were stabbed with 24 h-old cultures and incubated at 35 °C for 18-48 h. A change in colour form purple to yellow indicated acid production, and cracking of the medium indicated gas production (HiMedia 1998).

3.2.3.3.4.2. Oxidase

Oxidase reaction was carried out by touching and spreading a well-isolated colony on oxidase disc (HiMedia DD018) .The reaction (formation of any deep purple blue colouration) was observed within 2 min at 25-30 °C (HiMedia 1998).

3.2.3.3.4.3. Lactose fermentation in presence of bile

Coliform was confirmed by inoculating Enterobacteriaceae isolates into 10 ml brilliant green bile broth 2% in tubes containing inverted Durham tubes, incubating those at 37 °C for 24-48 h and examining for growth and gas production (Nout *et al.* 1998).

To confirm the presence of faecal coliform, the BGBB tubes (containing inverted Durham tubes) were inoculated with confirmed coliforms and incubated at 44 ± 0.5 °C for 24 h (Nout *et al.* 1998) and examined for growth and gas production.

3.2.3.3.4.4. Indole production

Confirmed faecal coliforms were tested for the production of indole by inserting Kovac's reagent strip between plug and inner wall of the tube, above the inoculated tryptone water and incubating at 35°C for 18-24 h. When negative, incubation was prolonged for 3-7 d. A pink colour at the lower portion of the strip was the indication of positive reaction (HiMedia 1998).

3.2.3.3.5. *Escherichia coli*

The presumptive isolates were confirmed by the ability to ferment lactose in presence of bile and produce indole.

3.2.3.3.5.1. Lactose fermentation in presence of bile

As in section 3.2.3.3.4.3. (at 44 ± 0.5°C)

3.2.3.3.5.2. Indole production

As in section 3.2.3.3.4.4.

3.2.3.3.6. *Salmonella* and *Shigella*

Presumptive isolates were confirmed by testing sugar fermentation, hydrogen sulphide production, lysine decarboxylation/ deamination, motility, nitrate reduction, indole production (Brenner 1984; Adams and Moss 1995).

3.2.3.3.6.1. Sugar fermentation and hydrogen sulphide production

Triple sugar iron agar slants, having 2-3 cm butt, were inoculated with 24 h-old cultures and incubated at 35 °C for 18-24 h to determine fermentation of glucose, sucrose and lactose and production of hydrogen sulphide (HiMedia 1998).

3.2.3.3.6.2. Decarboxylation/ deamination of lysine and hydrogen sulphide production

Lysine iron agar slants, having 2-3 cm butt, were inoculated with 24 h-old cultures and incubated at 35 °C for 18-24 h to determine their ability to decarboxylate and deaminate lysine and to form hydrogen sulphide (HiMedia 1998).

3.2.3.3.6.3. Motility and nitrate reduction

Isolates screened as *Salmonella* from triple sugar iron agar and lysine iron agar were further confirmed by examining motility and nitrate reduction using motility nitrate medium (same as in 3.2.3.3.2.1).

3.2.3.3.6.4. Indole production

Same as in 3.2.3.3.4.4.

3.2.4. Susceptibility to antimicrobials

Antimicrobial susceptibility was determined by the disc agar diffusion method (HiMedia 1998). About three colonies of 24 h-old culture were transferred to about 5 ml tryptone soya broth and incubated at 37 °C for 6-8 h until the broth became moderately turbid. A sterile cotton swab (HiMedia) was dipped into the inoculum and applied evenly onto Mueller-Hinton agar plate (4 mm thick). After drying for 15 min, various antimicrobial susceptibility test discs were applied aseptically. The discs were deposited with their centres at least 30 mm apart. The plates were incubated at 37 °C for 14-19 h. The zones showing complete inhibition were measured.

3.2.5. Thermal inactivation of sporeformers

3.2.5.1. *Bacillus cereus* (Kim and Goepfert 1971)

3.2.5.1.1. Preparation of spore suspension

Dried plates of fortified nutrient agar were surface-seeded with a suspension of *B. cereus* grown overnight in tryptone soya broth at 30 °C on a shaker (200 rpm). The inoculated plates were kept upright at 30 °C for 24 h, and inverted for an additional 24 h at 30 °C. The plates were then held at 4°C for 24 h. The growth on each plate was suspended in 10 ml cold sterile distilled water by scraping the surface with a bent glass rod. The suspensions were centrifuged (model R-24; Remi Instruments, Mumbai, India)

eight times at 9500 g for 8 min each. Between each centrifugation, the pellets were resuspended in 60 ml cold sterile distilled water. Final pellets were suspended with an appropriate amount of water. The working spore suspensions were subjected to heat-shock at 80 °C for 30 min, and then cooled to 50 °C (Johnson *et al.* 1982).

3.2.5.1.2. Determination of *D*-values

Tubes of 9 ml brain heart infusion broth with 10 g glucose l^{-1} were placed in a water-bath. When the temperature of the monitored uninoculated broth reached 100 °C, 1 ml of the *B. cereus* spore suspension, held at 50 °C (in order to minimize cooling effect), was added to each tube of the test broth and allowed to wait for 35 s in order to return to 100 °C. The tubes in batches were removed from the bath after the specified time interval. Following cooling at room temperature, the tubes were diluted serially at successive decimal levels using ice-cold sterile distilled water. Appropriate dilutions (0.1 ml) were used for plate count on nutrient agar following incubation at 35 °C for 24 h. The *D*-value was computed from the negative reciprocal of the slope of individual trials using the linear portion of the curve of log cfu against time, plotted on a semi-log scale (Johnson *et al.* 1982).

3.2.6. Production of extracellular enzymes by *B. cereus*

Each of the *B. cereus* isolates was grown in 20 ml brain heart infusion broth at 37 °C for 20 h, and centrifuged at 9500 g for 30 min. The supernatant was filtered (0.2-μm cellulose acetate; Sartorius) and stored in a pre-sterilized screw-capped glass tube at 4 °C. A 50-μl aliquot of it was used for determining the activities of different extracellular enzymes using well-assay plate method in suitable media. Production of protease, lipase and amylase was determined using milk agar, tributyrin agar base added with 1.0% v v^{-1} tributyrin (Fluka 91,012), and starch agar, respectively. The incubated starch agar plates were flooded with Gram's iodine solution. The results were expressed as clear zone diameter (including well diameter of 5 mm).

3.2.7. Influence of pH on growth

The pH of sterile nutrient broth was adjusted to different levels using 2N HCl or 2N NaOH and a pH meter (model 335; Systronics, Ahmedabad, India). The broths (10 ml in each) were poured into sterile tubes (for *S. aureus*, *E. coli* and *Salmonella*) and Erlenmeyer flasks (for *B. cereus*). A 24 h-old culture (0.1 ml) was added to each of the tubes and flasks. The flasks (kept on a shaker; 200 rpm) and the tubes were incubated at 35 °C for 24 h. The growths were measured at 580 nm using a spectrophotometer (Systronics type 103).

3.2.8. Influence of food preservatives on growth

3.2.8.1. Determination of minimum inhibitory concentrations (Banerjee and Sarkar 2004a)

3.2.8.1.1. Sodium chloride

Plates of nutrient agar supplemented with different concentrations of sodium chloride were spotted (5 spots per plate) using a 2 mm-diameter loop with 18 h-old cultures. The inoculated plates were incubated at 35 °C and observed for any growth after 18 h.

3.2.8.1.2. Benzoic acid

Molten (45 °C) nutrient agar was mixed with filter-sterilized (0.2-µm cellulose acetate; Sartorius) benzoic acid (1.00134.0005; E. Merck (India) Ltd, Mumbai, India) (stock solution, 3 mg ml^{-1}) to prepare plates having different concentrations of benzoic acid. Cultures (18 h-old) of *B. cereus, S. aureus, E. coli* and *Salmonella* were spotted on the plates (5 spots per plate). The inoculated plates were incubated at 35 °C for 18 h and checked for growth.

3.2.8.1.3. Sorbic acid

Filter-sterilized (0.2-µm cellulose acetate; Sartorius) sorbic acid (1,947,109; Sisco research Laboratories, Mumbai, India) (stock solution, 2 mg ml^{-1}) in different volumes was mixed with molten (45 °C) nutrient agar to prepare plates having different concentrations of sorbic acid. Fresh cultures (18-20 h-old) of *B. cereus, S. aureus, E. coli* and *Salmonella* were spotted on the plates (5 spots per plate). The inoculated plates were incubated at 35 °C for 18 h and observed for growth.

3.2.8.1.4. Nisin

A sterile stock solution containing 1 mg nisin ml^{-1} was prepared by dissolving 0.4 g Nisaplin in 10 ml of 0.02 N HCl (pH 1.85), and the pH was adjusted to 3.0 followed by autoclaving at 0.7 kg cm^{-2} for 20 min and filtration through a Whatman No. 1 paper (Bell and De Lacy 1987; Davies *et al.* 1998). Molten (45 °C) nutrient agar was mixed with appropriate volume of nisin stock solution to get desired concentrations and poured into plates. Fresh cultures (18 h-old) were spotted on the plates (5 spots per plate). The inoculated plates were incubated at 35 °C for 18 h and checked for growth.

3.2.8.2. Combined effect of pH, sodium chloride, benzoic acid and nisin on growth of *B. cereus*

Nutrient broth supplemented with different concentrations of sodium chloride was sterilized by autoclaving. Filter-sterilized (0.2-µm cellulose acetate; Sartorius) benzoic acid (stock solution, 3 mg ml^{-1}) and autoclaved (0.7 kg cm^{-2}, 20 min) and aseptically filtered (Whatman No. 1 paper) nisin (stock solution, 1 mg ml^{-1}) were added separately to sterile nutrient broth to get desired concentrations of them. Each broth was inoculated with 0.1 ml of 18 h-old culture of *B. cereus* 37-B1 in nutrient broth and incubated on a shaker (200 rpm) at 30 °C for 24 h. The growth was measured turbidimetrically at 580 nm.

Next, four variables (pH, sodium chloride, benzoic acid and nisin) with three levels of each (selected from the linear slopes of growth against individual hurdle), 19 different combination sets were prepared as per Hoke's response surface design (Thompson 1982). Sodium chloride was added to nutrient broth to get appropriate concentrations. After autoclaving, sterile benzoic acid and nisin solutions were added to the sterile sodium chloride-supplemented nutrient broth, and the final pH was adjusted with sterile 2 N HCl/ NaOH. Inoculation and incubation of different sets and monitoring of growth were the same as described earlier.

3.2.8.3. Combined effect of pH, sodium chloride and benzoic acid on growth of *Salmonella*

Nutrient broth supplemented with different concentrations of sodium chloride was sterilized by autoclaving. Filter-sterilized (0.2-µm cellulose acetate; Sartorius) benzoic acid (stock solution, 3 mg

ml^{-1}) was added separately to sterile nutrient broth to get a desired concentration of them. Each broth was inoculated with 0.1 ml of 18 h-old culture of *Salmonella* 1-S4 in nutrient broth and incubated at 35 °C for 24 h. The growth was measured turbidimetrically at 580 nm.

To study combined effects of three variables (pH, sodium chloride and benzoic acid) with three concentration levels of sodium chloride and benzoic acid (selected from the linear slopes of growth against each hurdle) at two different pHs, 18 different combination sets were prepared as per Hoke's response surface design (Thompson 1982). Sodium chloride was added to nutrient broth to get appropriate concentrations. After autoclaving, sterile benzoic acid solution was added to the sterile sodium chloride-supplemented nutrient broth, and the final pH was adjusted with sterile 2 N HCl/ NaOH. Inoculation and incubation of different sets and monitoring of growth were the same as described earlier.

3.2.9. Whole-cell protein fingerprinting

SDS-PAGE was carried out following the method described by Laemmli (1970) in a vertical gel electrophoresis system (Cat No. 05-03, Bangalore Genei, Bangalore, India). A 10% (w v^{-1}) solution of running gel was prepared by taking appropriate volume of acrylamide solution, resolving gel buffer, 10% (w v^{-1}) SDS solution and deionized water. Excess N,N,N',N'-tetramethyl ethylenediamine (TEMED; SRL 202788) and a pinch of ammonium persulphate (APS; SRL 0148134) were added to set the gel suitably. When the resolving gel set in, 4% (w v^{-1}) stacking gel, prepared by taking appropriate volume of acrylamide solution, stacking gel buffer, 10% SDS, deionized water, TEMED and APS, was poured over the resolving gel.

Samples were prepared by taking a loopful of culture from a 24 h-old (37 °C) nutrient agar plate and washing it three times in phosphate buffer by centrifugation at 3000 g for 15 min each. The pellet was suspended in stacking gel buffer and boiled in a water-bath for 10 min after adding equal volume of 2 x sample buffer. A discontinuous buffer system was used. Samples were stacked at constant current of 15 mA and resolved at 25 mA until the tracking dye reached the bottom of the gel. After electrophoresis, the gel was fixed in 10% (v v^{-1}) glacial acetic acid for 30 min, stained with coomassie brilliant blue solution for 12 h and washed in a destaining solution until the protein bands became clearly visible in a colourless gel matrix. The gels were photographed and then processed using the NTSYS pc. 2.0 software for generation of the cluster analysis in a dendrogram based on Dice's similarity coefficient (S_D) and the unweighted pair group method using arithmetic averages (UPGMA).

3.2.10. Antagonistic activity in vitro

The antibacterial activity was studied following the 'agar spot' test (Schillinger and Lücke 1989) and 'spot-on-the-lawn' method (Lewus and Montville 1991). Plates of MRS-0.2 agar were dried overnight, spotted with producing cultures (5 spots per plate) and incubated in an anaerobic jar with AnaeroGas pack for 18-24 h at 30°C. Brain Heart Infusion (BHI) broth, supplemented with 10 g agar l^{-1}, termed BHI soft agar, was tempered to 45 °C and seeded with 18 h old 10^5-10^6 cells ml^{-1} of indicator bacteria. The spotted plates were overlaid with 8 ml of the seeded BHI soft agar, incubated for 18-24 h at 30 °C and observed for the formation of any inhibition zone.

3.2.11. Microbial challenge testing during fermentation

3.2.11.1 Preparation of food, and intentional inoculation with indicator pathogenic bacteria

3.2.11.1.1. Dhokla

Bengalgram dal and white polished rice, procured from a local market, were washed and soaked in excess tap water for 10 h. Whereas the dal was ground to a smooth paste, rice was coarsely ground using a waring blender (Bajaj, India). The two slurries (4 : 1) were mixed thoroughly along with commercial common salt (8 g kg^{-1}). The thick batter, in 100 ml aliquots, was dispensed into pre-sterilized 250 ml beakers, the mouth of which was then wrapped with aluminum foil. The batches of batter were incubated at 30 °C for 15 h. The fermented batter was then dispensed in greased cups and steamed for 15 min to prepare dhokla.

B. cereus 34-B1 and *S. aureus* 34-S1 used in the challenge study were the isolates from a market sample of dhokla, while *E. coli* 61-E2 was an isolate from idli. The cultures were maintained at 4 °C on nutrient agar slants with subculturing after every six months. Before use, the organisms were activated by streaking them on nutrient agar and allowing them to incubate at 37 °C. Cultures (24 h-old) were suspended in sterile distilled water. A suspension of 10^7-10^8 total cells ml^{-1} was prepared by using a Neubauer's counting chamber and a phase-contrast microscope. A measured volume of this suspension was mixed with the freshly prepared batter to obtain an inoculation level of approximately 10^5-10^6 total cells ml^{-1}. Sampling was done at 0 h and every 3 h-interval during fermentation.

3.2.11.1.2. Idli

White polished rice and blackgram dal, procured from a local market, were washed and soaked in excess of non-sterile tap water for 10 h. After decanting excess water, the rice and dal were ground separately to coarse slurry and smooth mucilaginous paste, respectively. These two components (2:1) of idli were then mixed along with salt (8 g kg^{-1}). The thick batter, in 40 ml aliquots, was dispensed into pre-sterilized 100 ml beakers having the mouth wrapped with aluminum foil. The batches of batter were incubated at 32 °C for 18 h. The fermented batter was then dispensed in cups (7 cm in diameter having holding capacity of 40 ml) of pre-sterilized idli pan, and steamed for 15 min to prepare idli cakes.

Beside *S. aureus* 34-S1, in this study, *B. cereus* 94-B1 and *E. coli* 61-E2 which were isolated from market samples of idli, were used as indicator strains. The cultures were maintained at 4 °C on nutrient agar slants with subculturing in the same medium after every six months followed by incubation at 37 °C. Before use, the organisms were activated by streaking them on nutrient agar and allowing them to incubate at 37 °C. Cultures (24 h-old) were suspended in sterile distilled water. A suspension of 10^7-10^8 total cells ml^{-1} was prepared. A measured volume of this suspension was mixed with the freshly prepared batter to obtain an inoculation level of approximately 10^6 total cells ml^{-1}. Sampling was done at 0 h and every 6 h-interval during fermentation.

3.2.11.1.3. Wadi

Blackgram dal was soaked in excess of tap water for 10 h and ground to a smooth mucilaginous dough using a wet grinder. The dough was incubated in a closed container at 32 °C for 10 h. After hand-beating continuously for 30 min, the dough was hand-moulded to small cones which were deposited on a greased bamboo mat and sun-dried (29-33 °C) for 8 h daily on three successive days with an interval of 16 h shade-drying at room temperature (28-30 °C).

Beside *S. aureus* 34-S1, in this study, *B. cereus* 2-B1 and *E. coli* 7-E2 which were isolated from market sample of wadi, were used. The cultures were maintained at 4 °C on nutrient agar slants with

subculturing in the same medium after every six months followed by incubation at 37 °C. Before use, the organisms were activated by streaking them on nutrient agar and allowing them to incubate at 37 °C. Cultures (24 h-old) were suspended in sterile distilled water. A suspension of 10^7-10^8 total cells ml^{-1} was prepared. A measured volume of this suspension was mixed with the freshly prepared dough to obtain an inoculation level of approximately 10^5 total cells ml^{-1}. Sampling was done at 0 and 10 h of fermentation, and at every 12 h-interval during drying.

3.2.11.2. Lactic acid bacteria

3.2.11.2.1. Isolation

Presumptive lactic acid bacteria were isolated by pour-plating suitably diluted sample using *Lactobacillus* MRS agar which was then incubated anaerobically at 35 °C for 48 h.

3.2.11.2.2. Characterization

The presumptive isolates of lactic acid bacteria were confirmed by determining their Gram positive and catalase negative reactions and nosporeformation and nonmotility (Tamang and Sarkar 1996).

3.2.11.2.2.1. Gram reaction

A suspension of 24 h-old bacterial culture on slant was prepared in distilled water. A drop of that suspension was taken on a grease-free slide, smeared and heat-fixed. The smear was then flooded by Gram's crystal violet stain for 1 min, and washed for 5 s with water. The smear was flooded with Gram's iodine solution, allowed to react for 1 min, and washed again for 5 s with water. Holding the slide against a white surface, 95% ethanol was poured dropwise 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 safranine for 1 min and washed again with water. The slide was air-dried and observed under oil-immersion objective (Bartholomew 1962).

3.2.11.2.2.2. Production of catalase

A 24-h old slant culture was flooded with 0.5 ml of 10% (v v^{-1}) hydrogen peroxide solution and observed for the production of gas bubbles, indicating the presence of catalase (Norris *et al.* 1981).

3.2.11.2.2.3. Endospore formation

Same as in 3.2.3.3.1.2.

3.2.11.2.2.4. Motility

Same as in 3.2.3.3.1.1.

3.2.11.3. Isolation of yeasts

Enumeration of yeasts was carried out on spread-plates of yeast malt agar supplemented with 10 IU

benzylpenicillin and 12 μg streptomycin sulphate ml^{-1}, incubated at 28 °C for 2-5 d. The representative colonies were checked for purity before counting.

3.2.12. Statistical analysis

Data were analysed by determining standard error of the measurements (SEM), two-way analysis of variance (ANOVA) and simple correlation, after converting the microbial counts to a logarithmic scale (Snedecor and Cochran 1989).