3

Materials and methods

3.1. Materials

3.1.1. Culture media

Anaerobic agar

(M228; HiMedia Laboratories Pvt Limited, Mumbai, India)

***Bacillus cereus* selective agar**

Bacillus cereus agar base (HiMedia M833)

Polymyxin B selective supplement (HiMedia FD003)

Egg yolk emulsion (HiMedia FD045)

Baird-Parker medium

Baird-Parker agar base (HiMedia M043)

Potassium tellurite 3.5% (w/v) (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 M277)

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

(HiMadia M009)

Fortified nutrient agar (Kim and Goepfert 1971a)

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

Lactose gelatin medium, modified

(HiMedia M987) - autoclaved at 1.1 kg cm^{-2} for 10 min

Lysine iron agar

(HiMedia M377)

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	0.4 g
Distilled water	1000 ml

pH 7.0

Modified Duncan Strong medium

(HiMedia M1237)

Motility nitrate medium, buffered

(HiMedia M630I)

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, M088)

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

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

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

3.1.2. Reagents

AnaeroHiGas Pack™

(HiMedia LE002A)

BCET-RPLA (*Bacillus cereus* enterotoxin - reversed passive latex agglutination) toxin detection kit

(Oxoid TD950; Unipath Limited, Basingstoke, Hampshire, UK)

Benzoic acid solution (3 mg ml^{-1})

Buffered peptone water

(HiMedia M614)

Kovac's reagent strip

(HiMedia DD019)

Methyl red reagent

Methyl red	0.1 g
Ethanol	300 ml

Modified mineral solution (Kim and Goepfert 1971a)

$MnSO_4.H_2O$	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
5N Acetic acid	100 ml

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

Oxidase discs

(HiMedia DD018)

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

Neutral peptone	0.1 g
NaCl	0.85 g
Distilled water	100 ml

pH 7.2

PET-RPLA (*Clostridium perfringens* enterotoxin - reversed passive latex agglutination) toxin detection kit

(Oxoid TD930)

Sodium chloride solution (8.5 g l^{-1})

Sorbic acid solution (2 mg ml^{-1})

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

3.1.3. Antimicrobial susceptibility test discs

Ampicillin (10 μg $disc^{-1}$)

(HiMedia SD002)

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

(HiMedia SD003)

Carbenicillin (100μg $disc^{-1}$)

(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)

Gentamicin (10 μg disc^{-1})
(HiMedia SD016)

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

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

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

Norfloxacin (10 μg disc^{-1})
(HiMedia SD057)

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. Reference strains

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 (obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India).

3.2.2. Sampling of spices

Spices were collected from retail markets scattered all over India. Approximately 150 g of unpacked samples of spices were collected in sterile sampling bags (Fig. 3A) or screw-capped glass bottles. Those as well as packed (in sealed pouches made with low-density polyethylene film) (Fig. 3B) samples were transported to the laboratory and analyzed as soon as possible.

Fig. 3. Sampling in sterile sampling bags (A) and as branded packs (B)

3.2.3. Moisture content

Approximately 10 g of sample was accurately weighed 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 were 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; Nout *et al.* 1998).

3.2.4. Bacteriological analysis

3.2.4.1. Isolation of bacteria from spices

Representative 10 g portions of spices 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 (1 min for powdered samples, 2 min for whole ones). 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 (FDA, 1984; Speck, 1984).

3.2.4.1.1. Total aerobic mesophilic bacteria

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

3.2.4.1.2. Mesophilic bacterial spores

In order to estimate mesophilic bacterial spores, 100 ml sample l^{-1} suspension were treated for 30 min at 80°C and there upon spread on PCA plates followed by incubation at 30°C for 72 h (for aerobic sporeformers) and pour-plated with perfringens agar (PA) followed by incubation for 48 h at 37°C in an anaerobic jar with AnaeroHiGas Pack (for anaerobic sporeformers) (Kneifel and Berger 1994).

3.2.4.1.3. *Bacillus cereus*

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

3.2.4.1.4. *Clostridium perfringens*

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

3.2.4.1.5. *Staphylococcus aureus*

Selective enumeration of *Staph. aureus* was carried out on spread-plates of Baird-Parker (BP) medium following incubation at 35°C for 24-48 h (HiMedia 1998). Representative presumptive colonies were purified on BP agar and finally on NA (Nout *et al.* 1998).

3.2.4.1.6. Enterobacteriaceae

Estimation of Enterobacteriaceae was carried out by mixing 1.0 ml of appropriate dilutions of spices with tryptone soya agar (TSA) and incubating the plates for 1-2 h at room temperature

(27°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 TSA and finally on NA (Nout *et al.* 1998).

3.2.4.1.7. *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 0.01 ml of 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 (BSA) plates, streaked with the enriched broth cultures, were incubated at 35-37°C for 24-48 h. The representative colonies were purified on BSA and finally on NA (Adams and Moss 1995).

3.2.4.2. Maintenance of pure cultures

All the isolates, excepting *Cl. perfringens* (which were stored in cooked meat medium), were maintained on NA and TSA slants at 4°C.

3.2.4.3. Confirmation of taxonomic status of the isolates

3.2.4.3.1. *Bacillus cereus*

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

3.2.4.3.1.1. Motility

A 24 h-old culture in nutrient broth (NB) 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).

3.2.4.3.1.2. Endospore

Six day-old cultures on NA 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.

3.2.4.3.1.3. 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.

3.2.4.3.1.4. 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 days, 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.

3.2.4.3.1.5. Voges-Proskauer reaction (acetylmethylcarbinol production)

Tubes of 10 ml Voges-Proskauer (VP) 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 and intermittently for 30-60 min at room temperature for the production of any red colour indicating a positive VP reaction.

3.2.4.3.2. *Clostridium perfringens*

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

3.2.4.3.2.1. Motility and nitrate reduction

Motility nitrate medium, buffered (10 ml) was stabbed with a 48 h-old pure culture from fluid thioglycolate medium (FTM) 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)

3.2.4.3.2.2. Gelatin liquefaction and lactose fermentation

Pure culture (48 h-old) from FTM 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) .

3.2.4.3.2.3. Raffinose fermentation

Raffinose gelatin medium (10 ml) was stabbed with a 48 h-old culture from FTM. 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.

3.2.4.3.3. *Staphylococcus aureus*

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

3.2.4.3.3.1. 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 NB 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).

3.2.4.3.3.2. 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).

3.2.4.3.3.3. Voges-Proskauer reaction

Same as in section 3.4.4.3.1.5

3.2.4.3.4. Enterobacteriaceae

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

3.2.4.3.4.1. 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 from purple to yellow indicated acid production, and cracking of the medium indicated gas production (HiMedia 1998).

3.2.4.3.4.2. Oxidase

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

3.2.4.3.4.3. Lactose fermentation in presence of bile

Coliform was confirmed by inoculating confirmed Enterobacteriaceae isolates into 10 ml brilliant green bile broth 2% (BGBB) 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.

3.2.4.3.4.4. 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).

3.2.4.3.5. *Salmonella* and *Shigella*

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

3.2.4.3.5.1. Sugar fermentation and hydrogen sulphide production

Triple sugar iron (TSI) 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).

3.2.4.3.5.2. Decarboxylation/deamination of lysine and hydrogen sulphide production

Lysine iron (LI) 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) .

3.2.4.3.5.3. Motility and nitrate reduction

Isolates screened as *Salmonella* from TSI agar and LI agar were further confirmed by examining motility and nitrate reduction using motility nitrate medium (same as in 3.2.4.3.2.1.). Isolates identified as *Shigella* from TSI agar and LI agar were further confirmed by observing motility (same as 3.2.4.3.I.1.).

3.2.4.3.5.4. Indole production

Same as in 3.2.4.3.4.5.

3.2.4.3.5.5. Methyl red and Voges-Proskauer reaction

Shigella were further confirmed by inoculating in MR-VP medium, incubating at 30°C for 48 h for VP reaction (same as in 3.2.4.3.1.5.) and 5 d for MR test. Development of red colour by adding a few drops of methyl red reagent was the indication of positive reaction (HiMedia 1998).

3.2.5. 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 (TSB) 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 (TSA for *Cl. perfringens*) 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 (in an AnaeroHiGas Pack system for *Cl. perfringens*). The zones showing complete inhibition were measured.

3.2.6. Thermal inactivation of sporeformers

3.2.6.1. *Bacillus cereus* (Kim and Goepfert 1971a)

3.2.6.1.1. Preparation of spore suspension

Fortified nutrient agar (FNA) plates were surface-seeded with an overnight suspension of *B. cereus* grown in TSB at 30°C on a shaker (200 rpm). The inoculated FNA 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. 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. Between each centrifugation, the supernatant fluids were discarded, and pellets were resuspended in 60 ml cold sterile distilled water. Final pellets were suspended with an appropriate amount of sterile distilled water (Johnson *et al.* 1982).

3.2.6.1.2. Determination of D and *z*-values

Tubes with 9 ml brain heart infusion broth with 10 g glucose l^{-1} (BHIG) were placed in a water-bath and heated to 91, 94, 97 and 100°C. The temperature of water-bath and an uninoculated tube were monitored. When the temperature of the monitored tube reached the required level, 1 ml of the spore suspension was added to the first tube. Every 5 min intervals, 1 ml of spore suspension was added to the following tubes in the water-bath. The zero-time control was the last one to which spore suspension was added. All the tubes were removed from the water-bath immediately after the addition of spore suspension to the zero-time control. After 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 NA which were then incubated at 35°C for 24 h.. The D-value was determined from the negative reciprocal of the slope of individual trials using the linear portions of the curve of log cfu against time, plotted on a semi-log scale (Johnson *et al.* 1982). The *z*-value was determined by plotting log D-values against respective temperatures.

3.2.6.2. *Clostridium perfringens*

3.2.6.2.1. Selection of medium for maximum sporulation

Plates of anaerobic agar, PA, cooked meat medium supplemented with 20 g l^{-1} agar and FTM with 20 g l^{-1} agar were surface-seeded with *Cl. perfringens* 47-C2 and incubated at 37°C for 5 d in an anaerobic jar (AnaeroHiGas Pack system). The jar was then kept at 4°C for another period of 5 d. Spore and vegetative cell count was done using a Neubauer counting chamber under phase-contrast objective. Medium showing the highest sporulation percentage was selected for the preparation of spore suspension.

3.2.6.2.2. Preparation of spore suspension

An anaerobic agar plate was surface-seeded with 2 d-old culture in FTM, incubated anaerobically first at 37°C for 5 d, and then at 4°C for another 5 d. The working spore suspension was prepared following the protocol as described in 3.2.6.1.1., and subjecting it to a heat shock at 80°C for 30 min.

3.2.6.2.3. Determination of D-value

Tubes with 9 ml FTM were placed in a water-bath and heated to 100°C. After heat treatment as described in 3.2.6.1.2., appropriate dilutions (0.1 ml) were used for plate count (spread plates of PA overlaid with the same). The plates were then incubated anaerobically at 35°C for 48 h.

3.2.7. Enterotoxins

Qualitative as well as semi-quantitative tests for enterotoxins were performed using BCET-RPLA and PET-RPLA kits for the detection of *B. cereus* enterotoxin (diarrhoeal type) and *Cl. perfringens* enterotoxin (type A), respectively.

3.2.7.1. Extraction

3.2.7.1.1. *Bacillus cereus* enterotoxin

The isolates (*B. cereus*) were inoculated into 10 ml BHIG and incubated at 33°C for 18 h on a shaker (200 rpm). For the isolation of enterotoxin from spice samples, a 10 g-sample was blended with 10 ml of 8.5 g sodium chloride l^{-1} (extra dilution done when needed) using Stomacher. The broth culture or blended sample (1.5 ml) was taken in an Eppendorf tube and centrifuged (Remi CM12) (900 g; 20 min for culture, 30 min for sample) at 4°C. For sample, the supernatant was filtered through a 0.2μm-cellulose acetate membrane (Sartorius AG, Göttingen, Germany). The supernatants/filtrates were retained for the assay of enterotoxin.

3.2.7.1.2. *Clostridium perfringens* enterotoxin

The *Cl. perfringens* isolates were grown at 37°C for 20 h in cooked meat medium. The cells were incubated by heating at 75°C for 20 min. Modified Duncan Strong medium (16 ml) was inoculated with a 0.8 ml-culture in cooked meat medium (taken from the base of the tube). The tubes were incubated at 37°C for 24 h. The culture was centrifuged at 900 g for 20 min at 4°C and the supernatant was used for the assay. Extraction of enterotoxin from food was done following the method as described in 3.2.7.1.1.

3.2.7.2. Assay

A microtitre plate (V-well) was arranged so that each row consisted of 8 wells. For each sample, two rows were used. A 25 μl-diluent (phosphate buffer saline containing bovine serum albumin) was dispensed in each well of the two rows, except the first well of each row. The test sample was added to the first and second wells of both the rows. Starting at the second well of each row, 25 μl of the mix were picked up and performed doubling dilutions along each of the rows until the seventh wells (the last well in each row contained diluent only). To each well in the first row 25 μl of sensitized latex (latex sensitized with specific *B. cereus/Cl. perfringens* anti-enterotoxin (rabbit IgG)), and to each well in the second row 25 μl of latex control (latex suspension sensitized with non-immune rabbit globulins) were added. The plate was covered with a lid, rotated for 5 min using a cyclomixer (Remi CM101), kept in a moisture box, and left it undisturbed on a vibration-free surface at room temperature for 24 h. Each well in each row was examined against a black background for agglutination pattern.

3.2.8. Microbial challenge testing in black pepper powder

Sample (100 g) of black pepper powder, where both *B. cereus* count and BCET titre were found below their respective limits of detection in 100% of the samples, was mixed well in a sterile screw-capped glass bottle with spores of *B. cereus* 120-B1 which was grown at 37°C for 72 h on sterile cellophane (British cellophane grade 325P, Avonmouth, UK) overlaid on NA plate. The bottle was left at room temperature (31-33°C). *Bacillus cereus* count and enterotoxin titre were monitored after 0 h, 7 d and 14 d of storage using BCSA and BCET-RPLA kit, respectively.

3.2.9. Safety of foods on storage

3.2.9.1. Seasoning/intentional inoculation of *aloo dam* (a potato-based food) with a spice and *Bacillus cereus* separately

3.2.9.1.1. Preparation of *aloo dam*

Ingredients:

Potato (2-4 cm dia)	1 kg
Cumin	¼ tsp.
Tejpat	2pcs
Cumin powder	2 tbsp.
Turmeric powder	½ tsp.
Grated fresh ginger	2 tsp.
Oil	4 tbsp.

Water	4 cups (600 ml)
Salt to taste	

Recipe:

Whole deskinned potatoes were fried in hot oil until golden brown and kept aside. Tejpat and whole cumin were sautéd followed by addition of turmeric powder, cumin powder and grated ginger. The mixture was fried till spices separated from oil. Previously-fried potatoes along with an appropriate amount of water were added to the mixture. Salt was sprinkled to taste and mixed well. The pan was covered and allowed to cook on low heat for 15 min until the potatoes were tendered and a typical aroma developed.

3. 29.1.2. Challenge study on *aloo dam*

Fifty grams of freshly prepared *aloo dam* were taken in each of three sterile glass bottles. *Aloo dam* of one bottle was seasoned with ground small cardamom (where *B. cereus* was detected in 100% of the samples analyzed), while another bottle was intentionally inoculated with *B. cereus* 120-B1 (an enterotoxigenic strain). All the three bottles were plugged with sterile cotton wool and kept at 30°C. Sampling from each bottle was done at 0 h and after 21 h for *B. cereus* count and BCET production using BCSA and BCET-RPLA kit, respectively.

3.2.9.2. Intentional inoculation of meat (goat) curry with *Clostridium perfringens*

3.2.9.2.I. Preparation of meat curry

Ingredients:

Minced meat	1 kg
Small cardamom	½ tsp.
Clove	½ tsp.
Cinnamon	½ tbsp.
Tejpat	4 pcs
Turmeric powder	2 ½ tbsp.
Red chilli powder	1 tsp.
Grated fresh ginger	3 tsp.
Grated garlic	3 tsp.
Grated onion	8 tbsp.
Oil	5 tbsp.
Water	5 cups (750 ml)
Salt to taste	

Recipe:

Minced goat meat, marinaded with grated onion, garlic and ginger, was refrigerated at 4-6°C for 1 h. Tejpat, clove, cinnamon and small cardamom were sautéd followed by addition of turmeric powder and red chilli powder. Marinaded minced meat was added in it and fried till the mass separated from oil. The fried meat along with water and salt was cooked for 15 min in a pressure cooker (1.1 kg cm^{-2}).

3.2.9.2.2. Chailenge study on gravy

Fifty grams of freshly prepared meat gravy were taken in a sterile screw-capped glass bottle and mixed well with spores of *Cl. perfringens* 16-C2 (an enterotoxigenic strain). An uninoculated bottle was taken as a control. Both the bottles were capped tightly and incubated at 37°C for 19 h. Cell counts and enterotoxin titres were measured at 0 h and after 19 h of storage using PA in AnaeroHiGas Pack system and PET-RPLA kit, respectively. They were again measured after boiling the cultured gravy for 15 min in a water bath.

3.2.10. Effect of garlic on growth and BCET production

3.2.10.1. Inhibitory activity of garlic slices

An 18 h-old broth culture of a test organism was spread on a surface-dried NA plate using a sterile cotton swab (HiMedia). After 15 min, a 1 mm-thick slice from a deskinned garlic clove, made using a kitchen slicer (Fig. 4) was placed at the middle of the plate. A gentle pressure was applied on the slice using a sterile forceps. The plates were incubated at 37°C for 18 h followed by measurement of inhibition zone.

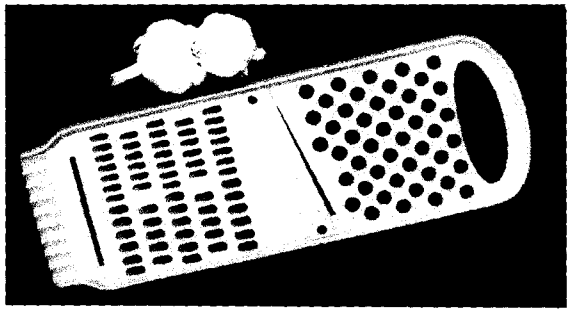

Fig. 4. Garlic and slicer

3.2.10.2. Preparation of garlic extract

Deskinned garlic cloves (50 g) were chopped and homogenized with 50 ml distilled water using Stomacher for 10 min at 'high' speed. The homogenate was centrifuged at 6500 g for 10 min. The supernatant was first filtered through a Whatman No. 1 paper and then through a 0.45-μm cellulose acetate membrane (Septrane, Cat. No. 71-45.02; Sewa Medicals Ltd, Mumbai, India). The sterile extract was used for inhibition studies within 1 h of its preparation (Al-Delaimy and Ali 1970).

3.2.10.3. Assay of antibacterial activity

The agar dilution method, used for the assay was based on Saleem and Al-Delaimy (1982). Molten (45°C) NA/PA was mixed with filter-sterilized garlic extract to prepare 15 ml-plates having different concentrations of garlic extract. Fresh (18 h-old) cultures were spotted on the plates (5 spots per plate). The inoculated plates were incubated at 37°C for 18-24 h (anaerobically for *Cl. perfringens*), and then observed for any growth.

For liquid assay, 10 ml-batches of BHIG containing garlic extract at different concentrations in 250 ml Erlenmeyer flasks were inoculated with 0.1 ml of an 18 h-old broth culture of *B. cereus* 120-B1. Following incubation at 33°C for 24 h on a shaker (approx. 200 rpm) the cultures were diluted appropriately and spread on NA plates. The plates were then incubated at 37°C for 24 h, and the colonies those appeared on the selected plates were counted as colony forming units (cfu) (Saleem and Al-Delaimy 1982).

3.2.10.4. Estimation of BCET

The procedure followed was as described in 3.2.7.1. and 3.2.7.2.

3.2.11. Effect of pH on growth

The pH of sterile NB/FTM was adjusted to different levels using 2N HCl or 2N NaOH and a pH meter (model 335; Systronics, Ahmedabad, India). The broths were poured (10 ml in each) into sterile tubes (for *Staph. aureus, E. coli, Salmonella* and *Shigella)* 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.

For *Cl. perfringens,* FTM was inoculated with a 48 h-old culture and incubated at 35°C for 48 h. The growths were measured turbidimetrically at 580 nm using a spectrocolorimeter (Systronics type 103).

3.2.12. Growth as influenced by food preservatives

3.2.12.1. Determination of minimum inhibitory concentrations

3.2.12.1.1. Sodium chloride

Plates of NA supplemented with different concentrations of sodium chloride were spotted (5 spots per plate) with 18 h-old cultures. The inoculated plates were incubated at 35°C and observed for any growth after 18 h . For *Cl. perfringens*, inoculated PA plates containing different concentrations of sodium chloride were incubated anaerobically at 35°C for 24 h.

3.2.12.1.2. Benzoic acid

Molten (45°C) NA (PA for *Cl. perfringens*) was mixed with filter-sterilized (0.2-μm cellulose acetate; Sartorius) benzoic acid solution (3 mg ml^{-1}) to prepare plates having different concentrations of benzoic acid. Cultures (18 h-old) of *B. cereus, Staph. aureus, E. coli, Salmonella* and *Shigella* were spotted on the plates (5 spots per plate). The inoculated plates were incubated at 35°C for 18 h and checked for growth. For *Cl. perfringens*, inoculated plates were incubated for 24 h at 35°C in an anaerobic atmosphere.

3.2.12.1.3. Sorbic acid

Filter-sterilized (0.2-μm cellulose acetate; Sartorius) sorbic acid solution (2 mg ml^{-1}) in different volumes was mixed with molten (45°C) NA (PA for *Cl. perfringens*) to prepare plates having different concentrations of sorbic acid. Fresh cultures (18-20 h) of *B. cereus, Staph. aureus, E. coli, Salmonella* and *Shigella* were spotted on the plates (5 spots per plate). The inoculated plates were incubated at 35°C for 18 h and observed for growth. For *Cl. perfringens*, inoculated plates were incubated anaerobically at 35°C for 24 h.

3.2.12.1.4. Nisin

A sterile stock solution containing 4 x 10^4 IU Nisaplin 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) NA (PA for *Cl. perfringens*) was mixed with appropriate volume of nisin stock solution to get a desired concentration 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. For *Cl. perfringens*, the inoculated plates were incubated for 24 h in an AnaeroHigas Pack system.

3.2.12. 2. Combined effect of pH, sodium chloride, benzoic acid and nisin on growth of *Bacillus 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 of 3 mg ml^{-1}) and autoclaved (0.7 kg cm^{-2}, 20 min) and aseptically filtered (Whatman No. 1 paper) Nisaplin (stock solution of 40,000 IU ml^{-1}) were added separately to sterile NB to get desired concentrations of them. Each broth was inoculated with 0.1 ml of 18 h-old culture of *B. cereus* 120-B1 in NB, and incubated on a shaker (200 rpm) at 30°C for 24 h.

The growth was measured turbidimetrically at 580 nm.

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

3.2.13. Statistical analysis

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