Occurrence and behaviour of foodborne bacterial pathogens in some legume-based traditional fermented foods marketed in West Bengal, India

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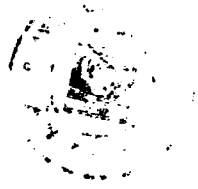

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Certified that the work presented in the thesis entitled, *'Occurrence and behaviour of foodborne bacterial pathogens in some legume-based traditional fermented foods marketed in West Bengal, India'* has been carried out by Mr Arindam Roy, MSc under my supervision at Microbiology Laboratory of the Department of Botany in the University of North Bengal. The results incorporated in the thesis have not been submitted for any other degree elsewhere.

Further certified that Mr Roy has followed the rules and regulations laid down by the University of North Bengal in carrying out this work.

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Siliguri
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Arindam Roy

Arindam Roy

Selling in a sweetmeat parlour at Siliguri

1

Introduction

The selection of foods best suited for promoting good health has been found by continuous trial and error by ancestral human beings (Swaminathan 1992). In the long way of journey of human civilization, cereals and legumes have become the two most important raw materials for preparation of plant food.

Edible leguminous seeds (pulses) are used in Asia to prepare human food much more than in any other continent (Padmaja and George 1999). These are an important source of proteins, B-vitamins and minerals in the dietaries of millions of people in the Asian countries. Legume proteins are, in general, a good source of lysine and threonine in which cereal proteins are deficient. They are, however, poor sources of sulphur-containing amino acids and tryptophan of which cereals are good sources. Hence, legume proteins effectively supplement cereal proteins. In the developing countries, like India, where problem of poverty, malnutrition and protein deficiency prevails, legumes offer a low-cost alternative source of protein. However, this property is underestimated due to some negative factors,

including unavailable carbohydrates, phytates, trypsin inhibitors, haemagglutinins, goitrogenic factors, cyanogenic glucosides and saponins present in legumes. Beneficial micro-organisms may play an important role in detoxifying these chemicals as well as fortifying foods with vitamins and minerals through fermentation (Khader 2001). However, pathogenic micro-organisms present in the foods may cause diseases due to infection, intoxication and toxicoinfection, causing severe gastrointestinal problem. Foodborne illnesses are a health problem in developing countries, particularly among infants and children. It is estimated that about 70% of the episodes of diarrhoeal diseases are foodborne, resulting from food contaminated by dirty utensils, poor processing, handling and storage conditions (Motarjemi and Nout 1996). Therefore, it is essential to check and improve the microbiological quality of foods through regular inspection.

A food is considered fermented when one or more of its constituents have been acted upon by micro-organisms to produce a considerably altered final product acceptable for human use (van Veen 1957). The use of micro-organisms to ferment foods goes back to prehistoric time. It is well approved that the science of fermentation is not an invention, rather a discovery. The credit goes solely to those ancient wise men who observed that when a few grains of barley are left in the rain, opportunistic micro-organisms ferment the starch-derived sugars into alcohols. Their patience of observation and capacity to harness and encourage these fermentations are worth-mentioning. According to some anthropologists, the knowledge of preservation of the otherwise perishable fruits and grains by fermentation changed the nomadic wanderers into settled farmers.

Descriptions of fermented foods go back as far in time as inscriptions are available. The art of food fermentation, used as a means of improving the keeping quality of food, probably originated around 7000 to 8000 BC in the tropical areas of Mesopotamia and the Indus Valley (Adams and Moss 1995). The Egyptians, Sumarians, Babylonians and Assyrians knew about the use of barley to produce alcoholic beverages; a cuneiform inscription on a Babylonian brick from 2800 BC gave a recipe for the production of barley wine (Borgstrom 1968). Records of soya sauce and also miso production in China go back to around 1000 BC with the transfer of knowledge of these production processes to Japan occurring around 600 AD (Yokotsuka 1985). Early Europeans were known to be making flat sourdough bread from rye in 800 BC. Around 100 BC, there were 250 bread bakeries operating in ancient Rome (Pederson 1979). Accounts of using dahi (curd) throughout India, dadhanvat (milk product) and kali (fermented rice) in southern part of India and panir (milk product) in Punjab are as old as 2000 BC (Om Prakash 1961). Records of dosa and idli go back to 1100 AD (Gode 1955). The history of using bhat bajraka (bajra flour product) in southern India, rabdi (maize flour product) in Rajasthan and rasgulla (milk product) in Bengal is found in 1500 AD (Pathak 1970). As soon as man started collecting milk from animals, sour milk became an item in his diet. Accounts of production of fermented dairy products can be found in early Sanskrit and Christian works, while recipes of sour fermented milks were given in Roman times around 200 AD (Oberman 1985). The origins of most fermentation technologies have been lost in the mists of history. Many fermented foods are now receiving world attention for their health-promoting or disease-preventing effects. Fermented foods are palatable and wholesome, prepared from raw or heated raw materials by microbial activity (Holzapfel 1997). The micro-organisms produce the proper enzymes, which bring about specific transformations of the substrate: improved flavour and appearance, destruction of undesirable components, improved keeping quality, enhanced nutritional value, improved digestibility, changing physical state of the product from raw material, imparting colour to the products, and providing dietary variety to otherwise monotonous meal. Moreover some fermented foods have medicinal importance, and the products require less cooking than the original substrate (Hesseltine 1965, 1979, 1983; Hesseltine and Wang 1980; Ramakrishnan 1979a; Steinkraus 1996) .

Traditional or indigenous fermented foods are those popular products that since early history have formed an integral part of the diet and that can be prepared in the household or in cottage industry using relatively simple techniques and equipment (Aidoo *et al.* 2006; Hesseltine and Wang 1980). Several plant foods are preferred in a fermented state, e.g. tempe to unfermented soya beans; and gari to unprocessed cassava tubers (Holzapfel 1997). Traditional fermented foods are essential for the well being of many people of the world, especially people of the Near East, southeast Asia, India, Far East, and Africa - south of the Sahara desert (Hesseltine and Wang 1980).

India is a treasure of different fermented foods. The plant-based fermented foods of India are mostly acid products prepared by bacterial and yeast fermentation of cereals such as rice supplemented with a protein source, like locally-grown legumes. Indeed, Indians are credited for developing the methods of souring and leavening cereal-legume batters (Padmaja and George 1999). Micro-organisms used in the production of legume-based traditional fermented foods of India are typically those present in or on the ingredients and are selected by adjusting the fermentation conditions. Fermentation processes of these foods have been developed mainly to add nutrition and flavour to a bland cereal-legume batter making organoleptically acceptable and easily digestible in addition to pertaining shelf-life enhancement. Today, consumer awareness for minimally processed, nutritionally rich and chemical preservative-free foods has paved the path for popularization of these fermented foods.

Fermented foods have generally been considered as less likely to be vehicles for foodborne infection or intoxication than fresh foods due to the competitive activity and metabolites of the functional microflora (Nout 1994). But due to unhygienic handling, external contamination, contaminated water and inferior quality of raw material, many of these foods may get contaminated by bacteria, such as *Bacillus cereus, Staphylococcus aureus, Clostridium perfringens, Escherichia coli, Salmonella, Shigella* and many other pathogens. In most of the fermented foods, especially in lactic acid bacterial fermented ones, the inhibition of growth of bacterial pathogens is common and can often ensure safety where levels of contamination are low (Adams and Nicolaides 1997). But with infectious pathogens, particularly those with a small low infectious dose, some degree of inactivation may be necessary to provide an acceptable level of safety (Beumer 2001).

Human illness from the consumption of foods contaminated with factors other than poisons or chemical toxic agents was recognized long before the understanding of the role of pathogens in foodborne diseases (Ray 2001). For healthy and well-nourished people, most food poisoning is an unpleasant episode from which recovery is normally complete after a few days (Adams and Moss 1995). But for the old and immunocompromised persons, foodborne illnesses can be fatal when caused by pathogens with low infectious doses. Moreover foodborne illnesses cause suffering, discomfort, and debilitation among the survivors. The economic losses from various factors could be high. The factors include medical treatment, lawsuits, lost wages and productivity, loss of business, recall and destruction of products, and investigation of the outbreaks (Garvani 1987). Statistics covering foodborne illnesses are notoriously unreliable. Many countries have no system for collecting and reporting data on gastrointestinal infections and even where these exist the reported data is acknowledged to represent only a fraction of the true number of cases. It is widely accepted that the real figure for foodborne illness may be 25-100 times higher than the recorded data due to factors such as under-reporting not seeking medical treatment. The World Health Organization (WHO) has estimated that only 10% of incidents occurring in most European countries are reported. Reported cases are most likely those who have sought medical attention and probably represent those who are most seriously ill or those who are most at risk such as the very young, the elderly and those with pre-existing illness or compromised immune systems. Unreported cases are usually those with mild illness or those with milder illness who treat themselves or who consult a doctor but for some reasons do not get reported (Adams and Moss 1995).

The increased incidence of foodborne diseases due to microbiological hazards is the result of a multiplicity of factors, all associated with our fast changing world. Demographic profiles are being altered, with increasing proportions of people who are most susceptible to micro-organisms in food. Changes in farm practices, more extensive food distribution systems and the increasing preference for meat and poultry in developing countries all have the potential to increase the incidence of foodborne illnesses. Extensive food distribution system raises potential for rapid, widespread distribution of contaminated food products. Changes in food products result in new types of food that may harbour less common pathogens. Changes in eating patterns, such as a preference for fresh and minimally processed food, the increasingly longer interval between processing and consumption of foods and the increasing prevalence of eating food prepared outside the home all contribute to the increased incidences of foodborne illness ascribed to micro-organisms. The emergence of new pathogens and pathogens not previously associated with food is a major public health concern. The results of these risk assessments will provide the scientific basis for undertaking measures to reduce illness from microbiological hazards in foods. Effective management of microbiological hazards is enhanced through the use of tools such as microbiological risk assessment (MRA) and hazard analysis and critical control point (HACCP) systems. Sound microbiological risk assessment provides an understanding of the nature of the hazard, and is a tool to set priorities for interventions. HACCP is a tool for process control through the identification of critical control points (CCPs). The ultimate goal is improvement of public health, and both MRA and HACCP are meant to that end.

Microbiological safety of fermented foods is an important issue in developing countries, including India. Processing technologies that ensure food safety are required at both the rural and urban levels, particularly in view of the frequently poor sanitary conditions and high ambient temperatures. As the population is increasing tremendously, lots of mobile shops, restaurants, hotels and cafeterias are mushrooming to cater the hungry mouth as well as to earn money to sustain in the ocean of unemployment. Most of the people working in these catering establishments have no basic knowledge of self-hygiene. Irregular disposal of sewage, scarcity of potable water, increasing pollution, warm and humid climatic condition also may contribute to the development of foodborne diseases. The production of safe foods is the responsibility of the producers. But the authorities need to regularly verify and validate those through inspection and product testing both at the site of production and at the point of sale. Originally the legume-based fermented foods in India were prepared traditionally in household level from a long time. Works on microbiology and biochemistry have established these foods as wholesome, nutritionally rich, easily digestible and organoleptically well-accepted. These attributes have made them popular as breakfast, tiffin and light midday meals resulting in their commercialization. However, incomplete fermentation, contaminated water, low-grade raw materials and unhygienic environmental condition may allow profuse growth of pathogenic contaminants in commercial products. Many traditional legume-based fermented foods including idli, dosa, dhokla and wadi contain considerable level of lactic acid lowering the pH to 4-5 that could prevent the survival or growth of pathogens in the cereal-legume batter or dough. However, bacteria like *Bacillus* and clostridia produce endospores, which can sustain unfavourable environmental conditions. Moreover, post-preparative contamination after cooking of batter or dough to make final product may occur from unhygienic storage condition and food handlers. The fact that several bacteria have a remarkable ability to survive different environmental stress conditions makes it very difficult for the food industry to exclude them from their products (Andersson *et al.* 1995).

In the State of West Bengal in India, fermented foods like amriti, papad and wadi are popular from time immemorial. In the passage of time, southern and western Indian fermented foods, like idli, dosa and dhokla have also become the choice for restaurant hunters as nutritious and delicious light

midday meal. The traditional method of preparation of amriti and appearance of the product are similar to those of jalebi, excepting that in pretzel-looked jalebi, blackgram dal (dehulled split beans) is replaced by refined wheat flour (*maida*) and the fermentation time is longer (Campbell-Platt 1987; Steinkraus 1996). The preparation of dhokla, dosa and idli are similar, excepting that in dosa and idli Bengalgram is substituted with blackgram, and while dosa is a highly seasoned griddled pancake, idli is a steamed pancake resembling dhokla. While all the aforementioned ones are ready-to-eat (RTE) foods, marketed papad and wadi are cooked before consumption. Though these foods are consumed throughout the country, no published data regarding the microbiological quality of these fermented foods except a few for idli and wadi have been found. In this context, necessity for checking the microbiological quality of the legume-based traditional fermented foods sold in markets and studies to better defined conditions affecting the behaviour of the isolates are of public health importance.

Hence, the protocol adopted to attain these objectives was as follows:

(i) observing hygienic status of the retailed outlets of these foods in West Bengal, and sampling a variety of foods through a cross-section of the retailers;

(ii) isolating major foodborne bacterial pathogens from the samples using their respective selective media;

(iii) characterizing the isolates in order to determine their taxonomic status;

(iv) enumerating the identified isolates to reveal the microbiological quality of the samples;

(v) generating antibiograms of the representative isolates against antimicrobials to find out their resistance patterns;

(vi) studying thermal inactivation of the sporeformers in order to minimize potential health hazards;

(vii) evaluating the production of extracellular enzymes to predict shelf-life evaluation;

(viii) examining the influence of different hurdles such as pH, sodium chloride, benzoic acid, sorbic acid and nisin on the growth of the isolates with a view to control a possible menace that may be caused by them;

(ix) protein-typing of the isolates to find out the diversity among them;

(x) studying antagonistic activity of lactic acid bacteria from legume-based traditional fermented foods against some isolated pathogens; and

(xi) determining survivability and growth following spiking of foods with isolated pathogens.

2

Review of literature

All over the world, people wage a continual battle against food contamination and the resulting foodborne diseases. The history of attempts in alleviating the devastating consequences of food contamination is lost in the mist of past. Besides cooking, smoking and simple sun drying, fermentation is one of the oldest technologies used for food preservation. Over the thousands of years, it has evolved and has been refined and diversified. Today, a large variety of foods, produced on an enormous scale, are derived from this technology, which is used in households, small-scale food industries and large-scale entreprises (Table 1). Since the days of Louis Pasteur, who pointed to the importance of hygiene in relation to fermentation, it is established that this technology is easily influenced by various factors during processing, and if not followed correctly, the safety and/or quality of the final product may be jeopardized, leading to even outbreaks of foodborne illnesses.

In the tropical developing regions, like India, fermentation is one of the main options for processing foods. It serves as an affordable and manageable technique for food preservation. Fermentation can also increase the safety of foods by removing their natural toxic components, or by preventing the growth of disease-causing microbes (Nout 2001).

Table 1. Group of fermented foods

Group	Examples
Cereal	Ang-kak, banku, breads, gari, kenkey, lao-chao, mawè, ogi, beer
Legume	Dawadawa, inyu, kinema, meitauza, natto, papad, sufu, tempe, wadi
Cereal-legume mixture	Dhokla, idli, miso, soy sauces, taoco
Dairy	Cheeses, dahi, kefir, koumiss, lassi, yogurt
Fish	Bagoong, izushi, katsuobushi, som-fak
Meat	Country-cured ham, nem, nham, salami, pepperoni
Fruits and vegetables	Gundruk, kanji, kimchi, pickles, mesu, sauerkraut, sinki, wines
Miscellaneous	Basi, bongkrek, kishk, kombucha, miang, tarhana, ugba, vinegar

The antimicrobial factors present in fermented foods may affect both the growth and the survival of bacterial pathogens that are present in raw materials. In most fermented foods, the inhibition of growth is more common and can often ensure safety where levels of contamination are low. But with infectious pathogens, particularly those with a small minimum infectious dose, some degree of inactivation may be necessary to provide an acceptable level of safety. So, although fermented foods are generally considered as safe, process failures and contaminated raw materials have resulted in their being involved in foodborne illness. Several outbreaks of foodborne illnesses have raised questions regarding the safety of fermented products.

2.1. Legume-based fermented foods

The Indian population depends heavily on several fermented cereal products; many products are also made from combinations of cereal grains and legume seeds. This gives a higher protein content, and provides a better balanced ratio of amino acids, overcoming the danger of lysine deficiency from cereals alone or sulphur-containing amino acid deficiency from legumes alone. These cereal-legume products include idli, dosa, dhokla, adai, vada, papadam and wadi (Padmaja and George 1999). The popularity of fermented foods is due to the desirable changes in texture and taste brought about during fermentation, not to mention the improvement in digestibility (Ramakrishnan 1979b). Although extensive study on the microbiology and biochemistry on fermentation of some of these foods have been done, no due attention has been paid to the microbiological quality in respect to the foodborne bacterial pathogens that might be present in those foods. Relevant information on microbiology and biochemistry of these foods found in the available literatures is summarized below.

2.1.1. Dhokla

Dhokla, one of the popular indigenous fermented foods of India, had its origin in Gujarat. It is liked as snacks all over the country because of its unique mild sour taste and a spongy texture. Traditionally, it is prepared by fermenting a mixture of Bengalgram (*Cicer arietinum*) dal and rice (*Oryza sativa*) (Nout *et al.* 2007).

The microbiota associated with the fermentation of dhokla batter consists of lactic acid bacteria and yeasts. The different types of lactic acid bacteria identified were *Lactobacillus fermentum, Lactococcus lactis, Lactobacillus delbrueckii* and *Leuconostoc mesenteroides*. However, *L. lactis and L. delbrueckii* become extinct after 12 and 15 h, respectively, of fermentation. The yeast identified was *Pichia silvicola*. The pH of dhokla batter falls from 5.3 at the start to 4.0 after 18 h of fermentation. Total titratable acidity increases indicating production of acids by the proliferating micro-organisms. Diacetyl and

acetoin content increase, but during prolonged fermentation diacetyl may be converted to 2-3-butanediol. The flavour of the product could be attributed to acetoin and volatile fatty acids, namely acetic acid, propionic acid, iso-butyric acid and iso-valeric acid. The lactic acid bacteria contribute lactic acid and acetoin. The yeast produces folic acid and raises the volume of the batter, imparting sponginess to the product (Joshi *et al.* 1989; Kanekar and Joshi 1993).

2.1.2. Dosa

Dosa is a fried, thin, fairly crisp, pancake-like food prepared from a mixture of milled rice and blackgram (*Phaseolous mungo*) dal having its origin in South India. Ordinarily the micro-organisms developed during the initial soak is sufficient to bring about the fermentation (Soni *et al.* 1985, 1986). Dosa batter is a slightly modified version of idli batter, where the proportion of rice is increased and both the ingredients are finely ground and the batter is made to a thinner consistency than the idli batter. Sometimes the batter is added with backslop (fermented batter of the previous batch which serves as a starter). Dosa is prepared by spreading the fermented batter on a hot greasy griddle (Aidoo *et al.* 2006; Padmaja and George 1999). The microbiota of the fermenting batter consists of either bacteria alone or, less frequentiy, bacteria in combination with yeasts. The loads of bacteria and yeasts after 24 h fermentation were 10^9 and 10^6, respectively, g^{-1} dry matter (Soni *et al.* 1985). *L. mesenteroides* is the most commonly encountered bacterium followed by *Enterococcus faecalis* and *L. fermentum, Bacillus amyloliquefaciens, L. delbrueckii, Bacillus subtilis, Pediococcus cerevisiae, Bacillus polymyxa and Enterobacter* sp. Of the yeasts, *Saccharomyces cerevisiae* is most predominant followed by *Debaryomyces hansenii, Pichia anomala, Trichosporon cutaneum, Oosporidium margaritiferum, Guehomyces pullulans, Kluyveromyces marxianus, Candida kefyr and C. krusei* (Padmaja and George 1999). However, yeasts are absent when the ingredients are ground without presoaking (Soni *et al.* 1986). The pH of dosa batter declines from 5.5 to 4.1 after 24 h of fermentation during which there is an increase in batter volume and contents of soluble solids, reducing sugars, non-protein nitrogen, soluble nitrogen, free amino acids, thiamine, riboflavin and cyanocobalamine (Padmaja and George 1999). Soluble protein and reducing sugar contents, though decline initially, increase significantly after 24 h of fermentation (Soni *et al.* 1985).

2.1.3. Idli

Though originated in the southern part of India, idli has gained rapid popularity all over the country (Ramakrishnan 1979a). It is a moist steamed tiffin food, prepared by fermenting a mixture of wet milled rice (raw or parboiled) and blackgram dal. During preparation, the ingredients are washed and soaked separately in water. The soaked rice is then coarsely ground, whereas the dal is ground finely to a smooth mucilaginous paste. Then two slurries (2:1) are mixed with salt, put in a closed container and left overnight to allow a definite leavening (a two- to three-fold increase in the original volume) and to develop a pleasant acid flavour. The fermented batter is poured in cups of an idli pan and steamed until the idli cakes are soft and spongy with a honeycomb structure inside (Aidoo *et al.* 2006; Nout *et al.* 2007; Steinkraus *et al.* 1967). The honeycomb structure develops due to escape of gases trapped by the abundant gluten present in blackgram. Variation is there in the ratio of the two ingredients depending on availability of raw materials and consumer preference. Proportions of rice to blackgram varies from 4:1 to 1:4 in various studies (Desikachar *et al.* 1960; Lewis and Johar 1953; Mukherjee *et al.* 1965). Higher amounts of rice imparts a starchy flavour to idli. Soaking period varies from 3-10 h. Lewis reported that the best quality idli was prepared when ingredients were soaked and ground with water at 80 °C (Lewis and Johar 1953). But it takes a longer fermentation period due apparenfly

to destruction of some of the micro-organisms initially present (Steinkraus *et al.* 1967). Fermentation period varies from 14 to 24 h, overnight being the best frequent time span for idli. Substitutes of blackgram include dehulled dark soya beans (*Glycine max*) and common beans (*Phaseolus vulgaris*). It produces satisfactorily idli with higher protein content and idli-like product that might be useful in international food studies (Steinkraus *et al.* 1967). A strain of *L. mesenteroides* was isolated from soya bean idli which secrets a α-N-acetyl-glucosaminidase and α-D-mannosidase which hydrolyze the haemagglutinin in soya bean (Ramakrishnan 1979b).

Idli is a natural fermented food, no inoculum is added. The nutritional value of idli depends on the micro-organisms inherent in the food material used, raw materials and conditions of fermentation (Ramakrishnan 1979b). The composition of microbiota involved in idli batter fermentation is a controversial point. The micro-organisms present on the blackgram were sufficient to produce the required leavening and acid within a reasonable time interval. Whereas no fermentation failures were encountered with added inocula of yeast and/or sour buttermilk, occasional failures were encountered without the inocula. Mukherjee *et al.* (1965) reported sequential changes of bacterial flora. The predominant micro-organisms responsible for souring as well as gas production was *L. mesenteroides* followed by *E. faecalis* and *P. cerevisiae* in succession. Steinkraus *et al.* (1967) reported an initial bacterial load of the batter of 10^3-10^5 cells ml^{-1}, which after 20-22 h of fermentation increased up to 10^8-10^9 ml^{-1}. Predominant micro-organisms in idli batter are *L. mesenteroides* and *E. faecalis*. *Pediococcus* is also infrequently encountered. Other bacteria involved include *L. fermentum* and *L. delbrueckii*. Besides lactic acid bacteria, yeasts, such as *Candida saitoana, G. pullulans and T. holmii* were isolated from ripe batter and apparently they play some role in idli fermentation (Batra and Millner 1974; Lewis and Johar 1953; Venkatasubbaiah *et al.* 1984). Venkatasubbaiah *et al.* (1985) have reported four genera of yeasts including *Hansenula, Candida, Torulopsis* and *Trichosporon* from batter samples procured from hotels. Soni and Sandhu (1991) are of opinion that *S. cerevisiae* enrichment in combination with natural bacterial flora of the ingredients is the best microbial factor for standardizing idli fermentation in terms of improved organoleptic characteristics, leavening and nutritional constituents During fermentation, predominant among the yeasts appearing first are *S. cerevisiae, D. hansenii, P. anomala* and *G. pullulans*; and *T. cutaneum* develops subsequently (Aidoo *et al.* 2006). Fermentation of the batter during preparation of idli is responsible for increase in total acids and soluble solids but decrease of soluble nitrogen and total nitrogen (Steinkraus *et al.* 1967). The initial pH of 6.0 drops to 4.3-5.3. Though pH of 4.3-4.7 seemed desirable, idli prepared from batter with pH 4.1-5.3 had a satisfactory flavour when steamed. Venkatasubbaiah *et al.* (1984) found that yeast-fermented batter produced carbon dioxide, whereas batter fermented with *L. mesenteroides* produced hydrogen only.

2.1.4. Papad

Papad, popularly called papadam in southern parts of India, is a condiment prepared from blackgram dal. This thin, usually circular, wafer-like product is used to prepare curry or eaten by itself as a crackly snack or appetizer with meals after roasting or deep-frying in oil. Papad-making under controlled conditions has already developed into a cottage or small-scale industry (Aidoo *et al.* 2006). A variety of papads are produced from a great diversity of ingredients. They are made either using only cereal flour or a combination of it and pulse flour. Blackgram flour, lentil (*Lens culinaris*), redgram or greengram (*Vigna radiata*) flour is hand-kneaded with a small quantity of peanut oil, common salt (~ 8 g kg^{-1}), 'papad khar' (saltworts produced by burning a variety of plant species, or from very alkaline deposits in the soil) and water, and then pounded to a stiff paste. The dough (sometimes with a backslop and spices added) is left to ferment for 1-6 h. The fermented dough is shaped into small

balls which are rolled into thin, circular flat sheets (10-24 cm diameter, 0.2-1.2 mm thick) and generally dried in the shade to 12-17% (w w^{-1}) moisture content. (Aidoo *et al.* 2006; Saxena *et al.* 1989; Shurpalekar and Venkatesh 1975). The diameter and thickness of standard papad should be 5-23 cm and 0.1-1.2 mm, respectively (ISI 1972).

Candida krusei and *S. cerevisiae* are involved in the preparation of papad (Shurpalekar 1986). Moisture content of raw commercial papads ranges from 9.0 to 17.7%, total ash from 7.2 to 11.8%, acid insoluble ash from 0.18 to 0.52%, ether extractives from 2.2 to 5.8%, alkalinity from 3.4 to 6.0%, pH from 7.4 to 9.1, protein from 9.8 to 19.2%, free fatty acid content (as oleic acid) from 0.51 to 0.58% with diametric expansion on deep fat frying of papads from 4.8 to 17.8% (Kulkarni *et al.* 1996).

2.1.5. Wadi

Wadis, traditionally consumed in Punjab and Bengal of India, are now popular in many places of India, Pakistan and Bangladesh. Wadis are dried, hollow, brittle cones or balls (3-8 cm in diameter, 15-40 g in weight). It is used as a spicy condiment or adjunct for cooking vegetables, grain legumes or rice (Aidoo *et al.* 2006). To prepare wadi, generally blackgram dal is soaked, drained, ground into a smooth soft dough, left to ferment for 1-3 days and moulded into cones or balls which are deposited on bamboo or palm mats smeared with oil and sun-dried for 4-8 days. The surface of the cones or balls becomes covered with a mucilaginous coating which helps to retain the gas formed during their fermentation. The wadis look hollow, with many air pockets and yeast spherules in the interior and a characteristic surface crust. Sometimes spices like asafoetida, caraway, cardamom, cloves, fenugreek, ginger and red pepper are added to the fermenting dough (Aidoo *et al.* 2006; Sandhu and Soni 1989). Several bacteria and yeasts, constituting the natural flora of blackgram, spices and surroundings, are associated with wadi fermentation. The bacteria appearing initially in traditional wadi fermentation are *L. mesenteroides, L. delbrueckii, L. fermentum* and *B. subtilis* along with *Flavobacter* spp. *E. faecalis* develops subsequently. However, only *L. mesenteroides* and *L. fermentum* prevail at the end of fermentation. Among yeasts, the predominant ones are *T. cutaneum, S. cerevisiae, C. krusei, Pichia membranefaciens* and *P. anomola,* but eventually only *S. cerevisiae* and *T. cutaneum* persist. The development and prevalence of microbiota are affected by the seasons, summer being more favourable for bacteria and winter for yeasts. The lactic acid bacteria are mainly responsible for the acidification of dough, favourable conditions for the yeasts to grow and become active for leavening (Aidoo *et al.* 2006). During fermentation, the pH declines from 5.6 to 3.2 with the accompanying increase in total acid levels, dough volume and contents of soluble solids, non-protein nitrogen, soluble nitrogen, free amino acids, proteolytic activity and B-vitamins (Batra and Millner 1974; Sandhu and Soni 1989; Sandhu *et al.* 1986).

2.2. Foodborne bacterial pathogens

2.2.1. *Bacillus cereus*

B. cereus was originally isolated and described by Frankland and Frankland (1887). Anecdotal evidence of *B. cereus* food poisoning had existed in Europe since the turn of the last century. One of the earliest recorded episodes of *B. cereus* food poisoning was that of 1906 when Lubenau (1906) described an outbreak in a sanatorium in which 300 of 400 inmates and staff became ill with profuse diarrhoea, stomach cramps, and vomiting shortly after eating meatballs in the dinner. A large number of aerobic sporeforming *Bacillus* originally reported by him as *Bacillus peptonificans,* although most probably

should be identified as a strain of *B. cereus*, was isolated from the remnants of the dish. Later Seitz (1913), Brekenfeld (1926, 1929) and Trub and Wundram (1942) reported that *Bacillus*-contaminated foodstuffs stored for long periods at improper temperatures were able to cause illness when eaten. During the period 1936-1943, 117 of 367 cases investigated by the Stockholm Board of Health were suspected of being caused by aerobic sporeformers (Plazikowski 1947). Confusion was there in the early nomenclatures during reporting of *Bacillus*-related food poisoning. This reflected the disorder in *Bacillus* taxonomy as well as contributed to the slow recognition of pathogenicity among members of the genus *Bacillus* other than *B. anthracis*. Full credit goes to Smith *et al.* (1952) and Gordon *et al.* (1973) in bringing order to *Bacillus* taxonomy, and in providing a foundation of tests and interpretations on which rests our present understanding of this group of micro-organisms. However it was not until Hauge's remarkable experiments in the 1950s (Hauge 1950, 1955) that *B. cereus* was established as a cause of food poisoning. He presented the first classic description of *B. cereus* gastroenteritis in his discussion of four Norwegian outbreaks involving 600 persons resulting from vanilla sauce prepared and stored at room temperature for one day before being served. Later Hauge consumed vanilla sauce containing 10^7-10^8 cells ml^{-1}, and within 16 h he was suffering from profuse diarrhoea accompanied by cramping abdominal pain.

Cells of *B. cereus* are large (cell width, > 0.9 μm), gram-positive rods, and motile by means of peritrichous flagella. Single central to terminal, ellipsoid or cylindrical endospore without distention of the sporangium is present. *B. cereus* is able to metabolize glucose, fructose and trehalose, but not pentoses and many of the sugar alcohols. A small percentage of strains are urease-positive; the majority actively hydrolyzes starch, casein, and gelatin (Gordon *et al.* 1973; Granum 2007).

Growth and multiplication of vegetative *B. cereus* cells typically occur within 10-50°C, with the optimum between 28° and 30°C. However, psychrophilic variants identified in raw milk samples can grow at temperatures as low as 5 °C. An initial-stage spore germination has been demonstrated within wider limits, i.e., -1 °C, 30 °C and 59 °C being the minimum, optimum and maximum temperatures, respectively (Knaysi 1964). The range of pH permitting growth of *B. cereus* in laboratory media has been reported to be 4.9 to 9.3 when adjusted with mineral acids and alkalis (Fluer and Ezepchuk 1970; Kim and Goepfert 1971). Minimum water activity (a_w) for growth of *B. cereus* was reported to be 0.95, however in Cantonese style fried rice, the a_w ranged from 0.912 to 0.961 (Kramer and Gilbert 1989).

One of the most important aspects of study is the heat resistance of *B. cereus* spores because spore is a factor of primary concern to the food and pharmaceutical industries (Kramer and Gilbert 1989). Since spore inactivation is the principal concern in producing appertized foods, much higher temperatures are used in appertization processes and in the measurement of spore *D*-value (decimal reduction time). The *D*-value is defined as the time at a given temperature for the surviving population to be reduced by 1 log cycle, and *z*-value is defined as the temperature change which results in a 10-fold (1 log) change in *D* (Adams and Moss 1995).

D-values at 85 °C, 90 °C, 95 °C and 100 °C in phosphate buffer (pH 7.0) was reported to be 220 min, 71 min, 13 min and 8 min, respectively (Mol 1957). In contrast, $D_{121°C}$-values in soya bean oil and olive oil were 30 and 17.5 min, respectively, demonstrating that lipid material have a protective effect on the thermal resistance of *B. cereus* spores (Molin and Snygg 1967). In low acid foods (> pH 4.5), the $D_{100°C}$-value was found to be 5 min (Ingram 1969). A $D_{100°C}$-value of 2.7-3.1 min for *B. cereus* in skim milk was reported by Mikolajcik (1970). *z*-values ranging from 6.7 to 8.3°C were obtained in aqueous spore suspension by Gilbert *et al.* (1974). Strains (serotype H.1) producing emetic syndrome food poisoning are more heat resistant ($D_{95°C}$: 22.4-36.2 min) compared to routine isolates of *B. cereus* (random serotype) from samples of raw rice ($D_{95°C}$: 1.5-6.0 min) (Parry and Gilbert 1980). $D_{100°C}$ and $D_{92°C}$-values for six isolates in rice broth were 4.2-6.5 min and 16-36 min, respectively (Chung and Sun 1986). Rajkowski

and Mikolajcik (1987) reported $D_{100°C}$-values of *B. cereus* ranging from 0.6 to 27.0 min in demineralized water. The heat resistance at 90 °C for spores of the 32 *B. cereus* strains isolated from fresh vegetables and refrigerated minimally processed foods ranged from 1.4 to 21.2 min. $D_{90°C}$, $D_{95°C}$ and $D_{100°C}$ were 3.2-23.3, 0.7-5.2 and 0.4-1.1 min, respectively, in strains isolated from Spanish raw rice (Sarrias *et al.* 2002). Strains unable to hydrolyze starch were the most heat-resistant, with $D_{90°C}$-values higher than 10.8 min (Valero *et al.* 2002). Several studies have provided evidence of non-linear spore survivor curves associated with certain strains. Aging of spores affects the *D*-value (Collado *et al.* 2003). Banerjee and Sarkar (2004a) reported the $D_{100°C}$-values of *B. cereus* isolates from spices ranging from 3.5 to 5.9 min in glucose-supplemented brain-heart infusion broth.

Other factors that have been shown to exert a total inhibitory effect on the growth of *B. cereus* include 2 g sorbic acid kg^{-1} rice filling of Karelian pastry (Raevuori 1976), 500 µg benzoic acid ml^{-1} (pH 6.3) (Lueck 1980) and 6-10 mg garlic aqueous extract g^{-1} (Banerjee and Sarkar 2003). Application of nisin at the levels of 5 mg l^{-1} has been shown to act as an effective preservative giving significant increase in shelf-life and providing protection against the growth of psychrotrophic *B. cereus* (Delves-Broughton *et al.* 1992). Addition of nisin to a batter of crumpets at levels of 3.75-6.26 µg g^{-1} effectively prevented the growth to levels capable of causing food poisoning (Jenson *et al.* 1994).

The antibiotic susceptibility was tested for 66 isolates of *B. cereus* from rice in Taiwan against 12 different antibiotics. The isolates were 100% susceptible to chloramphenicol (30 µg $disc^{-1}$), erythromycin (15 µg $disc^{-1}$) and streptomycin (10 µg $disc^{-1}$), and 92.4% were sensitive to gentamicin (10 µg $disc^{-1}$). However, they were 100% resistant to penicillin G (10 units $disc^{-1}$) and polymyxin B (300 units $disc^{-1}$), 99% resistant to ampicillin (10 µg $disc^{-1}$) and carbenicillin (100 µg $disc^{-1}$) and 88% resistant to cephalothin (30 µg $disc^{-1}$) (Chung and Sun 1986). Shah *et al.* (1996) isolated *B. cereus* from about 300 samples of a variety of foods in which *B. cereus* was found in 20% spices. The antibiogram pattern of *B. cereus* was obtained with 50 isolates against nine antibiotics. All the isolates were resistant to ampicillin (10 µg $disc^{-1}$). A high resistance was found against trimethoprim (5 µg $disc^{-1}$) (92%), colistin (10 µg $disc^{-1}$) (86%) and rifampicin (5 µg $disc^{-1}$) (92%). All the isolate were sensitive to chloramphenicol (30 µg $disc^{-1}$) and ciprofloxacin (5 µg $disc^{-1}$), and 88% sensitivity was seen against streptomycin (10 µg $disc^{-1}$) and vancomycin (30 µg $disc^{-1}$).

B. cereus produces several phospholipases, eg. phospholipase C and egg yolk turbidity factor, with preferences for different phospholipids, including phosphatidylcholine (~ 23 kD), phosphatidylinositol (~ 29-35 kD), and a sphingomyelinase (~ 29 kD) (Drobniewski 1993).

B. cereus causes two different types of food poisoning: (i) the diarrhoeal type, first recognized after a hospital outbreak associated with vanilla sauce in Oslo, Norway, in 1948 (Hauge 1955), and (ii) the emetic type, described about 20 years later after several outbreaks associated with fried rice in London (Mortimer and McCann 1974). The emetic toxin, causing emesis (vomiting), is produced (preformed) by growing cells in the food (Kramer and Gilbert 1989), while the diarrhoeal type of food poisoning is caused by a complex enterotoxin produced during vegetative growth of *B. cereus* in the small intestine (Granum 1994). For both types of foodborne illness the food involved has usually been heat-treated, and surviving spores are the source of the food poisoning . The emetic toxin (1.2 kD) has been named cerulide, and consists of a ring structure of three repeats of four amino acids or oxy acids. Cerulide is resistant to heat (90 min at 121 °C), pH and proteolysis, but is not antigenic. Diarrhoeal disease is caused by at least three different enterotoxins, viz. haemolysin (Hbl), non-haemolytic enterotoxin (Nhe) and CytK. Two of the enterotoxins are multicomponent, one haemolytic and the other non-haemolytic, while the third (CytK) is a single protein (Granum 2007). The so-called 'diarrhoeal syndrome' is usually associated with proteinaceous foods, vegetables, sauces and puddings. The syndrome is characterized by an incubation period within the range of 8-16 h (av. 10-12 h) before the onset of abdominal pain, profuse watery diarrhoea, rectal tenesmus and occasional nausea that seldom

results in vomiting. Symptoms generally resolve within 12-24 h. In contrast, the 'emetic syndrome' form of illness is almost exclusively associated with farinaceous foods, particularly cooked rice, and is characterized by rapid onset (1-5 h) of nausea, vomiting and malise, in some cases followed by diarrhoea, of 6-24 h duration (Kramer and Gilbert 1989).

B. cereus is not a competitive micro-organism, but grows well after cooking and cooling (< 48 °C). The heat treatment causes spore germination, and in the absence of competing flora, *B. cereus* grows well, with a generation time as low as 12 min under optimal conditions (Borge *et al.* 2001). The infective dose of *B. cereus* sufficient to induce illness is variable. It was shown that count of *B. cereus* in foods ranging from 4.5-9.0 log cfu g^{-1} resulted in enteritis. Further, occurrence of *B. cereus* (> 10^4 cfu g^{-1}) in food has been considered as one of the epidemiological criteria for implicating *B. cereus* in food poisoning outbreaks (Concon 1988). Johnson (1984) reported a population of > 10^5 *B. cereus* cells g^{-1} is required for a food poisoning outbreak to occur.

Measures recommended for prevention of *B. cereus* gastroenteritis includes keeping foods at a temperature where the spores do not germinate and cells do not grow, proper sanitary measures during handling to prevent post-preparation contamination, and uniform reheating of a suspected food to above 75 °C before serving (Ray 2001).

2.2.2. *Clostridium perfringens*

The first description of *C. perfringens*, formerly known as *C. welchii*, was given by Welch and Nuttal (1892). However, it was not until 1940 that Knox and MacDonald in England confirmed *C. perfringens* as a cause of food poisoning. In addition to enteritis, *C. perfringens* is responsible for necrotizing tissue infections. Historically, the organism is best known for its association with gas gangrene (Adams and Moss 1995).

C. perfringens is a typical gram-positive, rod shaped (1 μm x 3-9 μm), anaerobic, sporeforming (oval in shape, subterminal in position) bacterium that is encapsulated and non-motile. Though catalase-negative, it survives and occasionally grows in the presence of oxygen (Labbe 1989). Growth of *C. perfringens* occurs at a temperature of 2-50 °C, although it is very slow below 20 °C. The most important characteristic of *C. perfringens* relative to food safety is the organism's ability to grow optimally at elevated temperatures ranging between 43 and 45 °C. However, Willardsen *et al.* (1978) found that one strain (NCTC 8238) has a shorter generation time of 7.1 min at 41 °C than at 45 °C. Minimum pH for growth is 5.0, whereas 6.0-7.5 is found to be optimum. Minimum a_w for growth is 0.95-0.97. Tompkin *et al.* (1974) found an inhibitory effect of sorbic acid on *C. perfringens*. Control of *C. perfringens* was achieved in Italian sausage by incorporating 5 μg nisin g^{-1} (Caserio *et al.* 1979). Growth of most strains of *C. perfringens* is prevented by sodium chloride at a level of 70-80 g kg^{-1}, although some inhibition occurs at a level of 50-60 g kg^{-1} (Roberts and Derrick 1978).

Food poisoning due to *C. perfringens* is usually self-limiting. Non-febrile illness characterized by nausea, abdominal pain, diarrhoea and, less commonly, vomiting usually occurs 8-24 h after the ingestion of food. The minimum required ingested dose of *C. perfringens* has been variously estimated at 10^6-10^8 cfu g^{-1}. However, the median count of the pathogen in foods implicated in outbreaks in UK was 7×10^5 g^{-1}. In otherwise healthy individuals, medical treatment is not usually required and recovery is complete within 1-2 d, although occasional fatalities occur in the very old or debilitated persons (Adams and Moss 1995; CDC 1985; Shandera *et al.* 1983).

C. perfringens is classified into five types, designated A through E, based on the production and expression of four (α, β, ϵ and ι) 'typing' toxins (McClane and Rood 2001). Type A strains are involved in foodborne intoxication. The enterotoxin associated with the foodborne disease is a heat-

labile protein. This intracellular protein is produced by the cells during sporulation in the intestine and released. There are some reports that, in addition to the intestine, sporulation and enterotoxin production to certain levels can also occur in some foods (Garvani 1987; Labbe 1988). The enterotoxin has been shown to be the major virulence factor in the common form of food poisoning. Stark and Duncan (1971) first showed that all clinically significant properties were linked to the enterotoxin. Human volunteer studies strengthened the theory (Skelkvåle and Uemura 1977), and gene deletion studies gave the definitive proof that the effects seen are solely due to the production of enterotoxin (Brynestad and Granum 2002).

C. perfringens is a common cause of food-poisoning outbreaks in USA (CDC 1985). There in the 1960s and 1970s, *C. perfringens* was involved in over 7% of the total foodborne outbreaks and over 10% of the total number of cases. In the year 1980s, the incidence dropped to about 3% of total outbreaks, affecting about 5% of the total cases. Most of the outbreaks generally occurred from cafeterias, restaurants, schools and banquets (Bean and Griffin 1990). Between 1980 and 1990, outbreaks in England and Wales have numbered between 46 and 69 each year with 896 to 1624 cases; corresponding figures for Scotland over the same period were 5 to 11 outbreaks and 75 to 364 figures (Adams and Moss 1995).

Growth of *C. perfringens* in food is influenced by a variety of environmental factors, including temperature, E_h, pH and a_w. The heat-resistance property of spores in part contributes to the ability of *C. perfringens* to cause food poisoning by enabling this bacterium to survive in undercooked foods. Spores of food poisoning isolates are typically much more heat-resistant than spores of *C. perfringens* isolates obtained from other sources; the extreme heat resistance of food poisoning isolate spores may contribute to foodborne virulence. It is also noteworthy that incomplete cooking of foods may not only fail to kill *C. perfringens* spores in foods but also actually favours development of *C. perfringens* type A food poisoning by inducing spore germination (McClane 2007). Roberts (1968) reported that spores of heat-resistant strains had $D_{90°C}$-values of 15-145 min and *z*-values of 9-16 °C, whereas spores of heat-sensitive strains had $D_{90°C}$-values of only 3-5 min and *z*-values of 6-8 °C. Furthermore, spores of heat-resistant strains required heat activation at 78-80 °C, whilst up to 50% of heat-sensitive strains grew without heat activation (Crowther and Baird-Parker 1984). *D*-values of spores of *C. perfringens* show a wide inter-strain variation with recorded values from 0.31 to > 38 min. The *D*-values of spores of NCTC 8798 at 95 °C, 105 °C and 120 °C were found to be 52.7 min, 2.5 min and < 0.01 min, respectively, for in water as the heating medium. At 90 °C and 100 °C, *D*-values for strain NCTC 10240 were found to be 15.5 min and 0.2 min, respectively, but for ATCC 3624, the values were 27.5 min and 0.22 min, respectively (Labbe 1989). Resistance of spores to heat is less in phosphate buffer than in water but greater in cooked meat than in water (Sutton 1966). Besides genetic difference among strains, lots of other factors including sporulating medium and heating medium, and contamination with vegetative cells and sporangia contribute to the variation in *D*-values (Labbe 1989). The antibiotic susceptibility of *C. perfringens* strains isolated from meat are similar to clinical isolates suggesting that meat is not a common source of resistant or multiple-resistant strains. However, porcine isolates from strains isolated from swine grown on farms where antibiotics are commonly used are often resistant to multiple antibiotics (Rood *et al.* 1978), one of which is transferable. Penicillin G has been the drug of first choice for treatment of gas gangrene by *C. perfringens*. Generally antibiotics are not used in case of human food poisoning. Recent reports of antibiotic-associated diarrhoea caused by *C. perfringens* are there.

The lowest a_w supporting growth of *C. perfringens* is 0.93 when other growth conditions are near-optimal. Although *C. perfringens* is an anaerobe, it does not require an extremely reduced environment to grow. Provided the environmental E_h is suitably low for initiating growth, *C. perfringens* can produce reducing molecules such as ferredoxin to modify the E_h of its environment and create

favourable growth conditions. In fact, the E_h of many common foods such as raw meats and gravies is often adequate to support the growth of *C. perfringens* which is pH sensitive, with optimal growth being at 6-7. It grows slowly, if at all, at pH values of < 5 and > 8.3 (Labbe 1989).

C. perfringens is widely distributed throughout the natural environment, including soil, foods, dust and the intestinal tract of humans and domestic animals (Labbe 1989). However, it is now understood that < 5% of global *C. perfringens* isolates carry the enterotoxin gene (*cpe*) necessary for causing *C. perfringens* type A food poisoning (McClane 2007).

In virtually all outbreaks the principal cause is failure to refrigerate properly previously cooked foods, specially when prepared in large portions. Rapid and uniform cooling of foods is therefore imperative. Gravies, broths and large pieces of meat should be cooled to < 10 °C within 2-3 h. Cooked, chilled foods should be related to minimal internal temperature of 75 °C immediately before serving to destroy vegetative cells. Cooked meat should be kept above 60 °C or below 10 °C. As most people harbour *C. perfringens* in their intestinal tract, preventing carriers from handling food is rather impossible. Similarly, the organism is present in a wide variety of foods. So, education of the food handlers remains a critical aspect of *C. perfringens*-related food poisoning control (Labbe 1989).

2.2.3. *Staphylococcus aureus*

The staphylococci were first described by Ogston (1881) as a pyogenic infection in humans. Staphylococcal food poisoning is among the most prevalent causes of gastroenteritis worldwide. It results from ingestion of one or more preformed staphylococcal enterotoxins in *Staphylococcus*-contaminated food. The etymological agents are members of the genus *Staphylococcus*, predominantly *S. aureus*. This form of food poisoning is called intoxication, and does not involve infection by, and growth of, the bacteria in the host. Staphylococcal toxin, the first true enterotoxin to be described, is not entirely destroyed by heating even for 30 min at 100 °C (Seo and Bohach 2007).

S. aureus cells are small (0.5-1.5 μm in diameter), gram-positive spheres. Cell division in more than one plane results in irregular clumps resembling bunches of grapes. They are catalase-positive, oxidase-negative, facultative anaerobic, and can ferment glucose. Most strains ferment mannitol and produce coagulase, thermonuclease, and hemolysin. The cells are killed at 66 °C in 12 min, and at 72 °C in 15 s. The suitable temperature for growth ranges from 7 to 48 °C, with fairly rapid growth in between 20 and 37 °C. Growth occurs optimally at pH 6.0-7.0, with minimum and maximum limits of 4.0 and 9.8-10.0, respectively. It grows rapidly in media containing sodium chloride at a level of 50-70 g kg^{-1}, and some strains are capable of growth in up to 200 g 100 kg^{-1} (Adams and Moss 1995). Lahellec *et al.* (1981) reported that the growth of *S. aureus* in brain heart infusion (pH 5.0) was inhibited by 10 g sorbate l^{-1}, but at pH 7.0 the organism grew in the presence of 50 g sorbate l^{-1}. Tompkin *et al.* (1974) reported inhibition of *S. aureus* by sorbic acid in cooked uncured sausage. The bacterium is sensitive to nisin (Thomas *et al.* 2000). It grows down to a_w 0.83 where it has a generation time of 300 min (Adams and Moss 1995).

S. aureus is wide spread, but occurs most frequently on the skin of higher primates. In humans, it is particularly associated with the nasal tract where it is found in 20-50% of healthy individuals. It can be isolated from faeces and sporadically from a wide range of other environmental sites such as soil, marine and fresh water, plant surfaces, dust and air (Adams and Moss 1995).

In USA, between 1983 and 1987, staphylococci accounted for 7.8% (47) of the 600 bacterial food poisoning outbreaks that were recorded. Equivalent figure for England and Wales over the same period was 1.9% (54) out of a total of 2815 outbreaks. Outbreaks of staphylococcal food poisoning inthe UK peaked during the 1950s at 150 outbreaks per year but have since declined to an annual levelof 10-20 outbreaks (Adams and Moss 1995).

Food poisoning by *S. aureus* is characterized by a short incubation period, typically 2-4 h. Nausea, vomiting, stomach cramps, retching and prostration are the predominant symptoms, although diarrhoea is also often reported and recovery is normally complete within 1-2 d. In severe cases, a marked dehydration may require treatment by intravenous infusion (Adams and Moss 1995).

The short incubation period is characteristic of toxins preformed in foods. *S. aureus* produces seven protein exotoxins designated A, B, C_1, C_2, C_3, D and E. The molecular mass of them are 27.1, 28.366, 27.496, 27.531, 27.438, 26.360 and 26.425 kD, respectively. The toxins are single-chain polypeptides each containing a single disulfide loop near the molecule's centre. As a result of their compact structure they are resistant to gut proteases and heat stable, being inactivated only by prolonged boiling. Toxin types A and D, either singly or in combination, are most frequently implicated in outbreaks of food poisoning. In the UK, type A is responsible for 52% of outbreaks, type D for 6%, types A and D combined for 19%, and types C and D combined for 9%. Susceptibility varies between individuals but it has been estimated that in outbreaks <1 µg of pure toxin has been required to elicit symptoms (Adams and Moss 1995).

Except for clinical isolates such as some community-acquired methicillin-resistant *S. aureus* (MRSA) strains exposed to antimicrobial therapy, most staphylococci are sensitive to β–lactams, tetracyclines, macrolides, lincosamides, novobiocin and chloramphenicol but are resistant to polymyxin and polyene (Stefani and Varaldo 2003).

Humans are the main reservoir for staphylococci involved in human disease. In humans, the anterior nares are the predominant site of colonization, although *S. aureus* can be present on other sites such as the skin or perineum. Dissemination of *S. aureus* among humans and from humans to food can occur through direct contact, indirectly through skin fragments, or through respiratory tract droplet nuclei (Seo and Bohach 2007).

Now-a-days, most sources of staphylococcal food poisonings are traced to humans who contaminate food during preparation. In addition to contamination by food preparers who are carriers, *S. aureus* may also be introduced into food by contaminated equipment used in food processing such as meat grinders, knives, storage utensils, cutting blocks and saw blades. A survey over 700 foodborne disease outbreaks revealed the following conditions most often associated with food poisoning: (i) inadequate refrigeration; (ii) preparation of foods far in advance; (iii) poor personal hygiene, e.g. not washing hands and instruments properly; (iv) inadequate cooking or heating of food; and (v) prolonged use of warming plates when serving foods (Bryan 1976).

To reduce the incidence of staphylococcal food poisoning, the aim will be to reduce initial load of *S. aureus* in a food by proper selection of raw materials and ingredients, sanitation of the food environments, and proper personal hygiene among the food handlers. Where possible, the products should be heat-treated to ensure killing of the live cells. Post-preparative contamination and temperature abuse should be avoided (Ray 2001).

2.2.4. Enterobacteriaceae

The term Enterobacteriaceae is derived from the Latin word enterobacterium, meaning an intestinal bacterium. The type genus for the family is *Escherichia* (Brenner 1984). The family Enterobacteriaceae includes a large biochemically and genetically related group of bacteria of heterogeneous ecology, pathogenic potential and host range. The organisms are facultative anaerobic, gram-negative straight rods of 0.3-1.5 µm in diameter that ferment glucose giving rise to acid and often gas. They are oxidase-negative and all, except *Erwinia chrysanthemi*, contain a common antigen. All the members of Enterobacteriaceae are catalase-positive, with a few exceptions, notably *Shigella dysenteriae*. They are

non-spore forming, non-acid fast and non-halophilic but tolerate the presence of bile salts (Brenner 1984).

Enterobacteriaceae are distributed worldwide. They are found in soil, water, fruits, vegetables, grains, flowering plants and trees, and in human and animal intestine (Brenner 1984). They are associated with many types of human infections, including more than 70% of urinary tract infections and nearly 50% of cases of septicemia. Until the 1940s, *Salmonella* and *Shigella* were considered to be the only gastrointestinal food or waterborne pathogens. In the late 1940s, enteropathogenic serotypes of *E. coli* were recognized as gastrointestinal pathogens, causing diarrhoea and vomiting in infants, and subsequently in adult volunteers.

Traditionally the group (designated as coliforms) has been chosen as an indicator of faecal pollution. These organisms are capable of fermenting lactose in the presence of bile at 37 °C. This group includes not only most strains of *Escherichia coli* but also organisms such as *Citrobacter* and *Enterobacter* which are not predominantly of faecal origin. The faecal coliforms, a more restricted group of organisms, are those coliforms which can grow and produce gas at 44-45 °C in suitable selective media (ICMSF 1978). One criticism of using coliforms and faecal coliforms is that their absence could give a false reassurance of safety when lactose-negative organisms predominate. The lactose-negative organisms include not only *Salmonella* and *Shigella*, but also enteropathogenic strains of *E. coli* itself such as O124. For this reason, tests for the whole of Enterobacteriaceae are increasingly being used. The Enterobacteriaceae includes even more genera of non-faecal origin than the coliforms, such as species of *Erwinia* and *Serratia* which are predominantly plant-associated. So, Enterobacteriaceae counts are used more generally as an indicator of hygienic quality rather than of faecal contamination and, therefore, say more about general microbiological quality than possible health risks posed by the product (Adams and Moss 1995).

Some important foodborne pathogens of this group, namely *E. coli, Salmonella* and *Shigella* are reviewed here.

2.2.5. *Escherichia coli*

Although *E. coli* was first isolated from children's faeces and described by Theodor Escherich in 1885, the first recognition of it as a cause of gastroenteritis in infants came in the early 1940s. Its common occurrence in faeces, ready culturability, generally nonpathogenic character, and survival characteristics in water led to the adoption of *E. coli* as an indicator of faecal contamination and the possible presence of enteric pathogens such as *Salmonella typhi* in water. This usage has been transferred to foods where greater circumspection is required in interpreting the significance of possible results (Adams and Moss 1995).

Until 1982, strains producing diarrhoea were classified into three types, viz. enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and enterotoxigenic *E. coli* (ETEC). However since 1982, *E coli* serotype 0157:H7, an enterohaemorrhagic type (EHEC) has been recognized as a cause of a number of outbreaks. The fifth type, enteroadherent *E. coli* has been implicated largely on epidemiological grounds. Its role in diarrhoea is under investigation (Adams and Moss 1995).

Cells of *E. coli* are gram-negative, catalase-positive, oxidase-negative, fermentative, short, non-spiral curved rods. *E. coli* is a typical mesophile growing from 7 to 50 °C with an optimum around 37 °C, although there have been reports of some ETEC strains growing at temperatures as low as 4°C. It shows no marked heat resistance, with *D*-value at 60°C of the order of 0.1 min, and can survivere frigerated or frozen storage for extended periods. A near-neutral pH is optimal for growth. Growth at pH 4.4 is possible under otherwise optimal conditions (Adams and Moss 1995).

The minimum a_w for growth is 0.95. Effect of 7.5 g sorbic acid l^{-1} (pH 5) on *E. coli* was reported by Doell (1962). Minimum inhibitory concentrations of 50-100 μg sorbic ml^{-1} (pH 5.2-5.6) and 50-1200 μg benzoic acid ml^{-1} (pH 5.2-5.6) were cited by Lueck (1980).

Symptoms of *E. coli* gastroenteritis include mild to severe diarrhoea. In severe cases, dehydration, prostration and shock may accompany the diarrhoea. Not all individuals show symptoms, but those who develop the symptoms shed the organisms in faeces after recovery (Doyle and Padhye 1989). EPEC strains typically cause infant diarrhoea in many tropical and developing countries, causing high mortality. ETEC strains cause travellers diarrhoea. Ingestion of 10^6-10^9 viable cells ml^{-1} by adults is necessary for the symptoms to occur within 24-72 h (Ray 2001).

Among 20 human and cattle isolates of *E. coli*, 80% were resistant to at least one antibiotic. Seventy percent were resistant to streptomycin, 65% to sulfonamide, 50% to tetracycline, 25% to ampicillin, 20% to sulfonamide/trimethoprim, 10% to chloramphenicol, 8% to cephalothin and 5% to gentamicin (Farina *et al.* 1996). Antibiotic resistance of 80% level for tetracycline, chloramphenicol, streptomycin and sulfonamide in some *E. coli* strains from mastitis infections in cows was reported by Teuber (1999).

The most important factor in the prevention of gastroenteritis in humans by pathogenic *E. coli* is to develop effective sanitation in water supplies, and treatment and disposal of sewage. Good personal hygiene and reheating of food before consumption should be practiced (Ray 2001).

2.2.6. *Salmonella*

Most salmonellae are regarded as human pathogens, though they differ in characteristics and severity of the illness they cause. Typhoid fever is the most severe, and consequently was the earliest salmonella infection to be reliably described. The typhoid bacillus was first observed by Eberth (1880). The paratyphoid bacilli, responsible for the clinically similar condition, paratyphoid fever, were first isolated by Achard and Bensaud (1896), and confirmed as culturally and serologically distinct from the typhoid bacilli by Schottmüller (1901). The genus *Salmonella* was created by Lignières in 1900 and named in honour of D.E. Salmon, the American veterinary pathologist who first described *Salmonella cholerae-suis* (now known as *Salmonella enterica* serovar Choleraesuis) isolated from swine suffering from hog cholera (Salmon and Smith 1885). The taxonomic nomenclature of the genus is rather different from that of other genera. On the basis of DNA-DNA hybridization, the genus *Salmonella* is now recognized to contain a single species, *S. enterica*, which comprises seven subspecies. Several classification schemes are currently used to clarify the biochemical and serological diversity within the *Salmonella* group (Chikami *et al.* 1985). In 1941 the scheme contained 100 serotypes, and the number has since risen to the current level of around 2000. The most recent proposal to introduce some taxonomic rectitude is to use the non-italicized serovar name after the species name so that *S. typhimurium* becomes *S. enterica* subsp. *enterica* serovar Typhimurium (Adams and Moss 1995).

Salmonellae are gram-negative, nonsporulating, facultative anaerobic rods (typically 0.5 μm x 1-3 μm) which are catalase-positive, oxidase-negative and generally motile with peritrichous flagella. They form gas while growing in media containing glucose (D'Aoust 1989). Generally, they ferment dulcitol but not lactose, utilize citrate as carbon source, produce hydrogen sulphide, decarboxylate lysine and ornithine, do not produce indole, and are negative for urease.

Salmonella spp. consist of resilient micro-organisms that readily adapt to extreme environmental conditions (D'Aoust and Maurer 2007). Growth occurs at temperature ranging from 5 to 47 °C with an optimum of 35-37 °C. Salmonellae are heat-sensitive and are readily destroyed by pasteurization temperatures (Adams and Moss 1995). Optimal pH for growth lies between 6.5 and 7.5 with possibilities

for growth at pH values ranging from 4.5 to 9.5 and slow death at more extreme conditions (Bryan *et al.* 1979). The growth of *Salmonella* is generally inhibited in the presence of 30-40 g sodium chloride l^{-1}. A higher temperature facilitated initiation of growth in the medium of greater salinity; high concentrations of sodium chloride protracted the lag period and decreased the rate of growth. Foods having a_w values of < 0.93 do not support the growth of salmonellae (D'Aoust 1989). Similar results were reported in a study of 23 strains of *Salmonella* grown at 10-30 °C in the presence of 20-80 g sodium chloride l^{-1} (Alford and Palumbo 1969). Inactivation of *Salmonella* by 7.5 g sorbic acid l^{-1} (pH 5.0) was reported by Doell (1962). Park and Marth (1972) found that 3 g sorbic acid l^{-1} (pH 5.0) inactivated *S. typhimurium* within 12 h in nutrient broth at 37 °C.

Salmonella has been recognized for its ability to harbour resistance to multiple antimicrobials (Besser *et al.* 1997; Breuil *et al.* 2000; CDC 1997; Cohen and Tauxe 1986). Bajaj *et al.* (2003) reported a very high level of resistance among 66 poultry egg isolates, particularly against penicillin (96.9%), vancomycin (83.3%), erythromycin (81.8%) and rifampicin (80.3%), a moderate resistance against trimethoprim (42.4%), chloramphenicol (28.7%) and streptomycin (24.2%), and a low resistance against gentamycin (3.0%), kanamycin (3.0%), furazolidone (16.6%) and tetracycline (9.0%). In another study on 91 *Salmonella enteritidis* isolates from broiler carcasses, food, human and poultry-related samples originated from South of Brazil, a high resistance was found against sulphonamides (75.8%) and nitrofurantoin (52.8%). Lower levels of resistance were found for tetracycline (15.4%), streptomycin (7.7%), nalidixic acid (7.7%), gentamicin (5.5%), norfloxacin (3.3%), trimethoprim (3.3%), cafalotin (2.2%), ampicillin (1.1%) and chloramphenicol (1.1%). In a study by the US Food and Drug Administration (FDA) on 502 *Salmonella* isolates from food samples, 247 (49.2%) were resistant to one or more antimicrobials of which 170 (68.8%) were resistant to one antimicrobial agent, 33 (13.4%) to two, 25 (10.1%) to three, 7 (2.8%) to four, 8 (3.2%) to five, and 2 (0.8%) each to six and seven antimicrobials (Kiessling *et al.* 2002). Study in two turkey processing plants in USA revealed that most *Salmonella* isolates were resistant to tetracycline, gentamicin, sulphamethoxazole, streptomycin, ampicillin, chloramphenicol and kanamycin (Olah *et al.* 2004). Similar effects have been reported by other researchers (Manie *et al.* 1998; Threlfall *et al.* 1997). Mayrhofer *et al.* (2004) reported highest resistance rate (42%) of *Salmonella* for nalidixic acid, followed by tetracycline (33%), streptomycin (27%), ampicillin, chloramphenicol (17%) and ciprofloxacin (9.6%). Of 52 *Salmonella* isolates from raw chilled retail poultry meats, 25 (48.0%) were resistant to one antibiotic, 5 (9.6%) were resistant to two, 4 (7.7%) were resistant to three, 6 (11.5%) were resistant to four, and 5 (9.6%) were resistant to five antibiotics. Two (3.8%) of the isolates were resistant to up to nine of the antibiotics tested. Fifty-one (98%) of the isolates were resistant to novobiocin, 18 (34.6%) to streptomycin, and 14 each (26.9%) to tetracycline and oxytetracycline (Jain and Chen 2006). In another study in USA on 208 *Salmonella* isolates recovered from imported foods, 23 (11%) isolates were resistant to at least one antimicrobial, and 7 (3.4%) to three or more antimicrobials. Resistance was most often observed to tetracycline (9%), followed by sulphamethoxazole (5%), streptomycin (4%), nalidixic acid (3%) and trimethoprim/sulphamethoxazole (2%). All the *Salmonella* isolates from betel leaves were sensitive to cotrimoxazole, and 97% of the isolates were sensitive to chloramphenicol, imipenem, ciprofloxacin, ceftriaxone and neomycin. Multidrug resistance (against 5-18 antibiotics) was common, particularly for nalidixic acid (65.8%), cephalothin (68.4%), cefoperazone (57.9%), sulphamethizole (52.6%), furazolidone (65.8%), kanamycin (68.4%), doxycycline (50.0%) and cefotaxime (44.7%) (Singh *et al.* 2006). For foodborne salmonellosis, an individual generally has to consume about 10^5-10^6 cells. However, there are some virulent strains where ingestion of fewer cells can cause the disease. Following ingestion of the pathogen, symptoms appear within 8-42 h. The general symptoms are abdominal cramps, diarrhoea, nausea, vomiting, chills, fever and prostration. It can be fatal to the sick, infants and the elderly (D'Aoust 1989).

Salmonellae are now established as one of the most important causes of foodborne illness worldwide. In Europe, in 1889, the annual incidence of salmonellosis was around 50 per 100,000 inhabitants. In USA, the incidence of salmonellosis continues to increase at a high rate: between 1969 and 1976 the average number of foodborne outbreaks was about 37 per year, while between 1983 and 1987 the number was over 68. Foods of animal origin including beef, chicken, turkey, pork, eggs, milk and products made from them have been associated with a large number of *Salmonella* outbreaks. Salmonellae have also been isolated from many foods of plant origin (due to use of sewage as fertilizer or washing products with polluted water), sea food, fin fish and crustaceans (Bean and Griffin 1990; Hatha and Lakshmanaperumalsamy 1997). *Salmonella* has frequently been implicated in foodborne outbreaks of illness with poultry meat being highlighted as a significant source of this pathogen (Bryan and Doyle 1995; Cloak *et al.* 1999).

Newborns, infants, the elderly and immunocompromized individuals are more susceptible to *Salmonella* infections than are healthy adults (D'Aoust 1989). Recent evidence suggests that 1-10 cells can constitute a human infectious dose (D'Aoust *et al.* 1985; Kapperud *et al.* 1990). Acute symptoms are nausea, vomiting, diarrhoea, fever and headache. Since birds, insects, handlers of infected food can all contaminate foods directly or indirectly, potential food vehicles for *Salmonella* are numerous.

Meat, milk, poultry and eggs are primary vehicles; they may be undercooked allowing the *Salmonella* to survive or these may cross-contaminate other foods which are consumed without further cooking.

Proper cooking of foods (minimum to pasteurization temperature and time such as 71.7 °C for 15 s or equivalent) and prompt cooling are important to control *Salmonella* infection. Prevention of cross-contamination of foods through cutting boards, equipment, utensils and hands should be practiced. Proper sanitation in the food environment and good personal hygiene can reduce the incidence. Refrigerated foods should be properly reheated before consumption (D'Aoust 1989). New knowledge that a single *Salmonella* cell can be infectious emphasizes the need for a greater stringency in food quality assurance programmes (D'Aoust *et al.* 1985; Adams and Moss 1995).

2.2.7. *Shigella*

The genus *Shigella* was discovered as the cause of bacillary dysentery by Shiga (1898). The genus contains four species which are serologically grouped based on their O-antigens, including *S. dysenteriae* (group A), *S. flexneri* (group B), *S. boydii* (group C) and *S. sonnei* (group D). Only humans and some primates are their hosts. The organisms are either transmitted directly through faecal-oral routes or indirectly through faecal-contaminated food and water. *S. dysenteriae* has been responsible for epidemics of severe bacillary dysentery in tropical countries, but is now rarely encountered in Europe and USA where *S. sonnei* is more common. *S. sonnei* causes the mildest illness, while that caused by *S. boydii* and *S. flexneri* is of intermediate severity (Adams and Moss 1995).

Shigellae are members of the family Enterobacteriaceae. They are non-motile, non-sporeforming, Gram-negative rods which are catalase-positive (with the exception of Shiga's bacillus, *S. dysenteriae* serotype 1), oxidase-negative and facultative anaerobes. They produce acid but no gas from glucose. The strains grow between 7 and 46 °C, with an optimum at 37 °C (Adams and Moss 1995). The cells survive for days under different physical and chemical stresses. These include refrigeration, freezing, 50 g sodium chloride l^{-1} and pH 4.5. They are killed by pasteurization. *Shigella* may survive in water up to 120 days. However, many food ingredients such as mayonnaise, vinegar, salt and sodium benzoate may be inhibitory. The strains are able to multiply in many types of food when stored at the growth temperature range (Smith 1987).

Isolation of *Shigella* from foods is not as facile as that from other sources. The physiological state of shigellae present in the food is a contributing factor in the successful recovery of this pathogen. *Shigella* may be present in low numbers or in a poor physiological state in suspected food samples. Under these conditions, special enrichment procedures are required for successful detection of *Shigella* (Andrews 1989).

Foodborne shigellosis is a neglected area of study on a global scale (Lampel and Maurelli 2007). The symptoms are the consequence of both the invasiveness of epithelial mucosa and the enterotoxin, and include abdominal pain, diarrhoea often mixed with blood, mucus and pus, fever, chills and headache. Generally, children are more susceptible to the disease than adults (Smith 1987). The infective dose is very low, about 10-1000 cells per person. Following ingestion of a contaminated food, the symptoms occur within 12 h to 7 days. In cases of mild infection, the symptoms last for about 5 or 6 days, but in severe cases the symptoms can linger for 2 to 3 weeks (Ray 2001).

Antibiotic therapy reportedly reduces the length of illness and of the carrier state for shigellae. However, wide use of antibiotics has resulted in selection of resistant strains and their resistance determinants. The best preventive measures for foodborne acquisition would be good personal hygiene and health education for food handlers. Proper treatment (chlorination) of water and sanitary disposal of sewage would prevent some foodborne as well as waterborne outbreaks of shigellosis (Doyle *et al.* 1985).

2.2.8. Occurrence of pathogenic bacteria in food

In spite of its undoubted value in nutrition, food has a long association with the transmission of disease. Regulations governing food hygiene can be found in numerous early sources such as the Old Testament, and the writings of Confucius, Hinduism and Islam. Such early writers had at best only a vague conception of the true causes of foodborne illness, and many of their prescriptions probably had only a slight effect on its incidence (Adams and Moss 1995). Even today, despite our increased knowledge, 'foodborne disease is perhaps the most widespread health problem in the contemporary world and an important cause of reduced economic productivity'. Foodborne disease has been defined by the WHO as 'a disease of infectious or toxic nature caused by, or thought to be caused by, the consumption of food and water'.

The cause of foodborne gastrointestinal disorders can be broadly divided into three groups: (a) from the consumption of food and water containing viable pathogenic micro-organisms or their preformed toxins; (b) from the ingestion of pathogenic algae, parasites and their preformed toxins through food; and (c) for reasons other than viable pathogens or their toxins including natural toxic chemicals in food, biological amines, allergic components, nutritional disorder and overeating (Adams and Moss 1995).

Steadily changing urban life style triggers the continuous rise in foodborne bacterial diseases. There is an increase in the number of woman working outside to make their living. Hence, eating outside has become more of a compulsion for the members of the metropolis family. The convenience of getting RTE food at work place or near the home makes many to eat outside (Kakar and Udipi 2002). The various steps involved in the preparation and distribution of these foods provide ample scope for contamination with pathogenic and spoilage micro-organisms. As a majority of these foods are cereal- or pulse-based and involve restricted heat treatments, the commonly encountered contaminants are mainly *Bacillus* species. In a study on eight market samples of idli, none was contaminated with *B. cereus* though other species of *Bacillus* were present (Varadaraj *et al.* 1992). In 25 samples of traditionally cooked pulses obtained from local markets and different household communities, *B. cereus*

was present in 32% of samples (Shah *et al.* 1996). In a study comprising 10 samples of soya bean wadi and 20 samples of pulses from different retail shops and food establishments in and around Bareilly in the State of Uttar Pradesh in India, *B. cereus* was confirmed in 20% percent of each type of samples (Meena *et al.* 2000). Earlier investigations of neutral-to-alkaline legume-fermented foods, e.g. tempe, revealed the presence of considerable levels of potentially objectionable micro-organisms such as *B. cereus*, *S. aureus* and members of Enterobacteriaceae (Samson *et al.* 1987). In dawadawa, the presence of *B. cereus* was observed by Antai and Ibrahim (1986). Out of the 15 market samples of kinema tested, 5 samples containing > 10^4 cfu of *B. cereus* g^{-1} and 2 samples containing > 10^5 cfu of *E. coli* g^{-1} were found (Nout *et al.* 1998). Han *et al.* (2001) reported the presence of *B. cereus* and low numbers of *C. perfringens* in their study on 23 samples of sufu.

Though literature on the occurrence and subsequent outbreak of foodborne bacterial pathogens in the traditional legume-based fermented foods are scanty, ample information is there on other foods which substantiate that in recent years, foodborne diseases of microbial origin have become the number-one food safety concern among consumers and regulatory agencies. This trend is also true for other developed and developing countries (Garvani 1987).

In a food survey conducted in the Netherlands, te Giffel *et al.* (1996) reported *B. cereus* to be present in 48% over 200 food samples tested, with contamination level ranging from 10^2 to 10^6 cfu g^{-1} (or ml^{-1}). *B. cereus* was the cause of 33% of the total cases of food poisoning (excluding virus) in Norway during 1988-93, 47% in Iceland during 1985-92, 22% in Finland in 1992, 8.5% in The Netherlands in 1991 and 5% in Denmark during 1990-92 (Granum and Lund 1997; Schmidt 1995). Much lower numbers have been reported before from other countries, such as England and Wales (0.7%), Japan (0.8%), USA (1.3%) and Canada (2.2%) (Kramer and Gilbert 1989). In the USA from 1983 to 1987, an average of 479 foodborne disease outbreaks were reported each year, involving 18,336 individuals. However, on average, only 38% of the outbreaks involving about 10,908 individuals were confirmed. Out of these outbreaks, bacterial pathogens caused about 66% of the outbreaks affecting 92% of the cases and 96% of the fatalities (Bean *et al.* 1990). Several factors may be involved in the high incidence caused by pathogenic bacteria: many pathogenic bacteria are found in the raw materials of animal and plant origin, many are present in the food environments, many grow very effectively in different foods, and many are not killed by the conditions used for processing different foods (Ray 2001).

Although many pathogenic bacterial species and viruses have been implicated in foodborne disease outbreaks, there are some that have occurred at higher frequencies than others. Among the two most common pathogens associated with foodborne intoxication from 1983 to 1987, the number of outbreaks as well as deaths was higher for *C. botulinum*, but the total number of cases was much higher for *S. aureus*. Among the enteric pathogens causing infection, the largest number of outbreaks, cases and fatalities resulted from the foodborne infections caused by *Salmonella* spp. Toxicoinfection outbreaks and number of cases were higher for *C. perfringens* than for *B. cereus*. Of all the three types of foodborne diseases, *Salmonella* was associated with the highest number of outbreaks, affecting the largest number of individuals and causing the most deaths (Bean *et al.* 1990).

The following information gives a quick glimpse of the microbiological quality of different foods. Anand and Singh (1987) analysed 102 samples of infant foods for the presence of enterotoxigenic staphylococci. A wide variation was observed in their incidence with an average log count of 3.5 g^{-1}. Staphylococci were present in 57.8% of the samples. Among the staphylococci isolates, 30% produced enterotoxin. Chyan *et al.* (1989) found coliforms and *E. coli* in 31% and 9%, respectively, of the 606 rice products. In the study by Fang *et al.* (1997), the isolation rates of *B. cereus* in regular instant cereals and cereal-mix were 26% and 38%, respectively. Coliforms were isolated from 2.7% of regular instant cereal products as well as from 7.4% of cereal-mix products. Kakar and Udipi (1998), in their studies

on microbiological quality of fried RTE foods sold in Mumbai city, found that 11% samples of samosa and batatawada, and 45% samples of patra had staphylococcal counts of more than 3 logs. Coagulase-positive *S. aureus* was detected in three samples of samosa and two of patra. *Salmonella, Shigella* and *Yersinia enterocolitica* were not detected in any of the samples.

Fang *et al.* (1999) analysed the microbiological quality of 320 samples of vegetarian food purchased from local markets in China. The incidence of *E. coli* and coliforms in these vegetarian food products were 28% and 33%, respectively, while 18% and 3% of the samples were found to contain *S. aureus* and *B. cereus*, respectively. The samples made from soya bean showed the highest detection rate (74.5%).

Mukhopadhyay *et al.* (2002) evaluated the microbiological quality of street-vended sliced papaya (*Carica papaya*) in Kolkata. In their study, the total aerobic plate count ranged from 3.3 to 6.52 log cfu g^{-1} with an average of 5.96 log cfu g^{-1}. Coliforms were detected in 70% of the samples with an average load being 13.5 g^{-1}. The presence of *E. coli* was confirmed in 48% of the samples positive for coliforms. *Salmonella* and *Vibrio cholerae* were detected in one sample each, and low levels of coagulase-positive *S. aureus* were detected in 17% of the samples.

Altug and Bayrak (2003) studied the microbiological quality of caviar from Russia and Iran. The relevant figures that were detected among 68 samples are as follows: standard plate count varied from 10^3-10^6 cfu g^{-1}, coliforms varied from $<10^1$-10^4 cfu g^{-1}, yeasts varied from 10^1 to 6×10^5 cfu g^{-1}, *E. coli* varied from $<10^1$-10^2 cfu g^{-1}. Only in one sample *S. aureus* was detected as 5×10^2 cfu g^{-1}.

Hanashiro *et al.* (2005) studied the occurrence of some foodborne bacterial pathogens in 40 popular street foods available in a restricted area in São Paulo of Brazil and concluded that 35% of the samples were unsuitable for consumption according to the microbiological criteria. Mankee *et al.* (2005) studied the microbiological quality of 196 samples of 'bara', 'channa', condiments/spices and RTE 'doubles' sold in Trinidad. *E. coli* was detected in 0%, 7.1%, 49% and 34.2%, respectively, of the samples. Staphylococci were isolated from 53.1%, 36.2%, 65.8% and 62.8%, respectively, of the samples, and *B. cereus* was recovered from 11.2%, 43.4%, 51% and 44.9%, respectively, of the samples.

2.2.9. Behaviour of foodborne bacterial pathogens during fermentation

Bacteria become acid-stressed at low pH levels, but can survive this stress when they have previously been exposed to a less severe acid shock by the induction of an acid tolerance response. Acidification is a common method of food preservation, such as pickling and fermentation (Browne and Dowds 2002). *B. cereus* grew to more than 10^7 cfu ml^{-1} in the mageu base, which was not fermented by lactic acid bacteria, and it was implied that, on its own, *B. cereus* could grow in maize porridge to potentially toxic levels but was reduced to smaller numbers within 24 h. The inhibition of *B. cereus* was achieved from 10^6 cfu ml^{-1} to 10^2 cfu ml^{-1} in the mageu base inoculated with both the starter culture and *B. cereus* after 24 h-fermentation (Byaruhanga *et al.* 1999; Svanberg *et al.* 1992).

Güven and Benlikaya (2005) found that in the boza base inoculated with both the starter culture of lactic acid bacteria and *B. cereus* and in control boza base to which no starter culture was added, the *B.cereus* count dropped to 1 log cfu ml^{-1} after 72 h-fermentation. In this study, they observed a pH of < 4.9 after 12 h of fermentation in all batches of fermentation of boza in which *B. cereus* number gave a sharp decrease, and this finding is very well correlated with the records of Goepfert and Kim (1975) and Hancioglu *et al.* (1999). Nout *et al.* (1987) have shown that *B. cereus* grew rapidly to 8-9 cfu g^{-1} tempe made from non-acidified soya beans. Hancioglu *et al.* (1999) found that *E. coli* 0157:H7 was not inhibited and survived during 32 h-fermentation at pH 3.7. However, *S. typhimurium* and *S. aureus* were inhibited after 12 h of fermentation at pH < 4.5.

The fate of *B. cereus* introduced in the lactic acid fermentation at different stages was investigated by Wong and Chen (1988). The growth of lactic acid bacteria was not affected by *B. cereus*, however *B. cereus* increased rapidly to about 10^8 cfu ml^{-1} from initial level of 10^4 cfu ml^{-1} when cells were added at the beginning of growth of lactic acid bacteria. However, it was inactivated slowly when added after 24 h and rapidly when added after 72 h of lactic acid bacterial growth.

During tempe manufacture, *S. aureus* was able to survive in newly started soya bean soaks (final pH 4.7), whereas it died during soaks with accelerated souring (final pH 4.0). Enterotoxin levels were highest after 48 h-fermentation (Nout *et al.* 1988). Metaxopoulos *et al.* (1981) reported that in Italian type dry sausage produced using 10^5 cfu g^{-1} *Lactobacillus* sp., *S. aureus* increased by 1.5-log and fermentation with 10^4-10^5 lactic acid bacteria g^{-1}, it is increased by 2.5-log under the same fermentation conditions. Turantas (1991) found out that in sucuks with starter culture, the count of *S. aureus* dropped below the detectable limit starting from the sixth day.

The inhibitory effect on *S. typhimurium* and *E. coli* of prolonged incubation (0-48 h) by finger millet (*Eleusine coracana*) flour fermented for varying time periods was tested by Antony *et al.* (1998). Glass *et al.* (1992) studied the fate of *E. coli* 0157:H7 in fermented dry sausage. They inoculated a commercial sausage batter with 4.8×10^4 *E. coli* 0157:H7 g^{-1} and fermented to pH 4.8 and dried until the moisture-protein ratio was < 1.9 : 1. The sausage chubs were then vacuum packaged and stored at 4°C for 2 months. The organisms survived but did not grow during fermentation, drying or subsequent storage at 4 °C and decreased by about 2-log cfu g^{-1} by the end of storage. Survival of *E. coli* 0157:H7 during the fermentation of Datta and Awaze, traditional lactic acid fermented Ethiopian condiments, was tested by Tsegaye *et al.* (2004). When fermenting Datta or Awaze were initially inoculated at low inoculum level (10^3 cfu g^{-1}), the test strains were not recovered after 24 h of fermentation. At higher initial inoculum level (10^6 cfu g^{-1}), however, the counts of the test strains in Datta at day 7 were less by about 1.5-log units than the initial inoculum level. In fermenting Awaze, all the test strains were completely eliminated after 7 days. The pH of the fermenting green and red Datta was reduced from 5.2 to 4.4 and that of Awaze dropped from 4.9 to 3.8 during this time. Tsegaye and Ashenafi (2005) studied the fate of *E. coli* 0157:H7 during the processing and storage of Ergo and Ayib, traditional Ethiopian dairy products. They found that when milk was inoculated with both lactic acid bacteria and *E. coli* 0157:H7 and fermented for 72 h, the pathogen increased up to 24 h till the pH was 3.5, however, decreased at 72 h when pH became 3.9. In absence of lactic acid bacteria, the pathogen increased steadily.

Tetteh *et al.* (2004) studied the survival and growth of acid-adapted and unadapted *Shigella flexneri* in a traditional fermented Ghanaian weaning food as affected by fortification with cowpea. They found that *S. flexneri* remained viable, but did not grow in porridge made from fermented corn dough of pH 4.07 held at 10 or 30 °C for 24 h.

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).

4

Results

4.1. Sampling

A total of 105 samples of six kinds of most popular and commonly used legume-based traditional fermented foods were purchased from 83 retail outlets scattered over selected 16 (out of 19) districts of West Bengal, a State in the eastern part of India (Fig. 1). The quality of the outlets, including sweet-meat parlours, restaurants, stationary and grocery shops, and roadside cafés, represented a cross-section of the standards available in the State. Amriti is a deep fat-fried pretzel-looked RTE product (Fig. 2a) which is stored even up to 5 days at ambient temperature and picked up using bare hands at the time of selling. Dosa is a fried pancake (Fig. 2c), prepared when ordered by the customers. Dhokla (Fig. 2b) and idli (Fig. 2d) are steamed cakes and kept even up to 2 days at ambient temperature. Grated coconut, coriander leaves, chilly, spices and leaves of *Muraya koennigii* are used for seasoning dhokla. Papad is a flat, thin product (Fig. 2e) which is sold mostly in packaged form. Wadi is a hollow, brittle, cone-shaped product (Fig. 2f) which is sold either locally packaged or open.

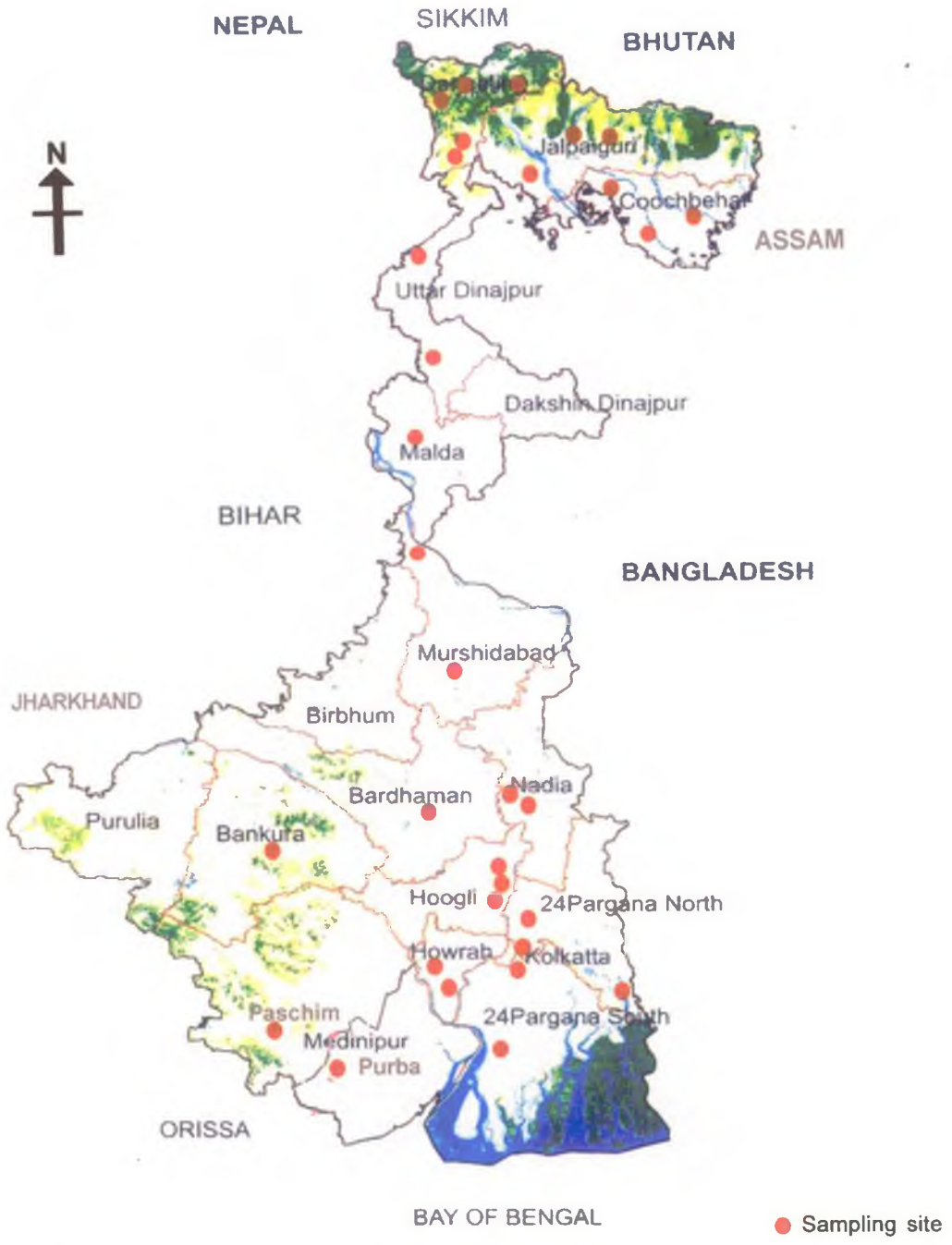


Fig. 1. Sampling sites of legume-based fermented foods in the State of West Bengal

The generalized traditional methods of production of these six foods are shown in Fig. 3. The collected information revealed that, now-a-days, in order to shorten the production time, in most cases dhokla is prepared from unfermented batter made up of Bengalgram flour (*besan*), lime juice and salt. Although dosa is prepared just prior to consumption, amriti, dhokla and idli are stored at ambient temperature even on open rack. Both raw and parboiled rice are added in different ratios in preparing dosa and idli batter. Most of the papad samples were branded, many of which were manufactured in other States of India. Packaged papads of different brands, namely Baba, Baba Lokenath, Bahurani, Duta, Ganesh, Jay, Kisan, Lazeez, Lijjat, Madhuri 777, Mahesh, MTR, Munmun, Nandan, Nimashi, Rajdhani, Ruchi, Sadhika, Shakti, Shrimati, Sona, Sonam, Super Shiv Ganga and Supreme, retailed in different markets of the State were analysed.

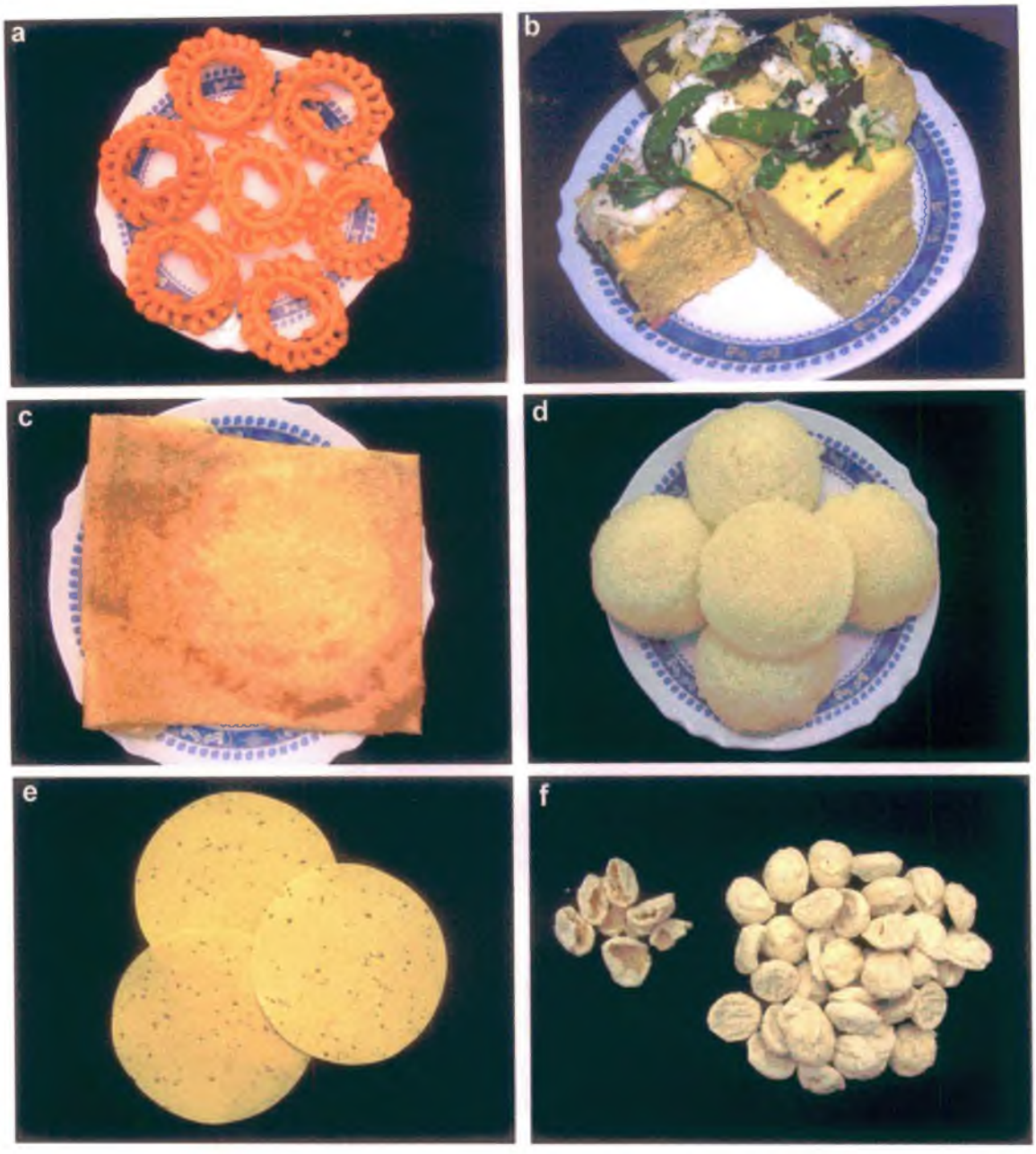
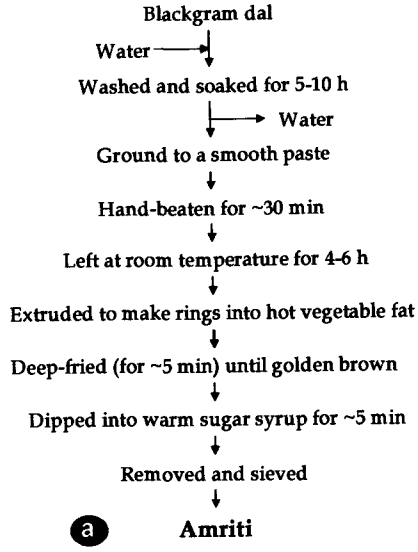
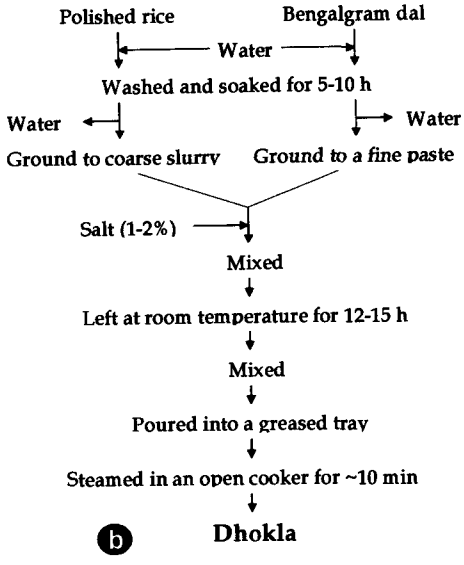
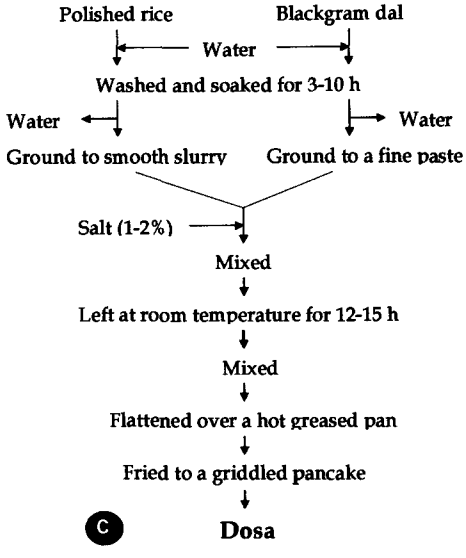


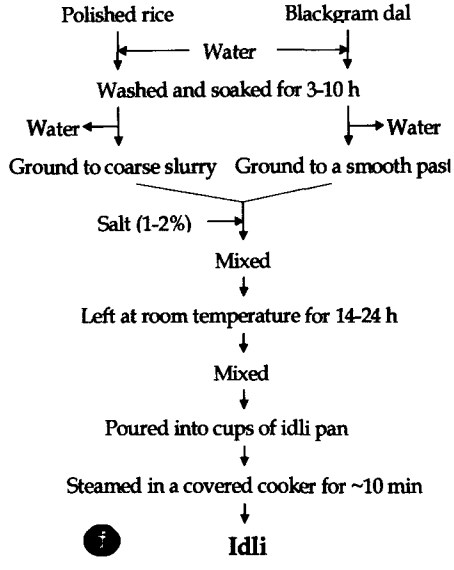
Fig. 2. Market samples of amriti (a), dhokla (b), dosa (c), idli (d), papad (e) and wadi (f)

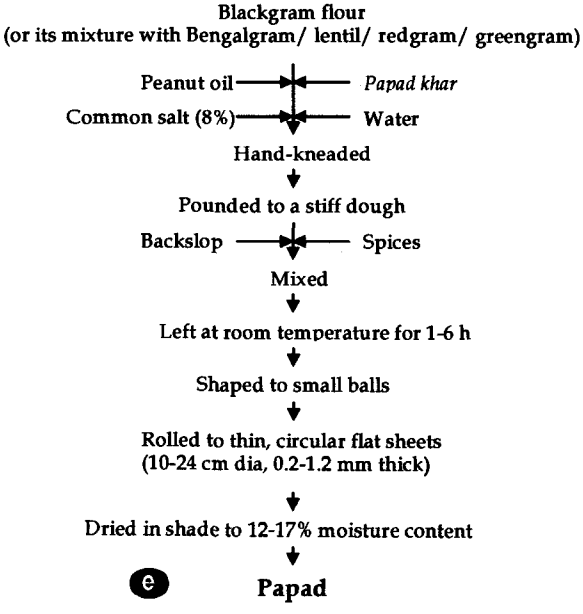
The general hygienic status of the retailers was not satisfactory. Amriti pieces, in all cases, were picked up with bare hands at the time of selling. Though spatula or forceps were used in some cases to pick up idli and dhokla pieces, in most cases bare hands were used. In some shops wadis were kept open in buckets, plastic jars or polyethylene bags. Those were picked up using bare hands to weigh for selling. In villages, wadis were commonly sold in periodic markets (locally called *haats*) where these were even kept displayed along with vegetables on polyethylene sheets. They used bare hands to handle both food and money simultaneously. Sixty-six samples were unpacked; and among the 39 packaged samples, 12 samples were locally packaged and the rest were branded packs (Table 2). Altogether 8 amriti, 5 dhokla, 16 dosa, 13 idli, 29 papad and 34 wadi samples were analysed for the presence of total aerobic mesophilic bacteria , mesophilic bacterial spores (aerobic as well as anaerobic), *B. cereus*, *C. perfringens*, *S. aureus* and Enterobacteriaceae (coliform, faecal coliform, *E. coli*, *Salmonella* and *Shigella*).











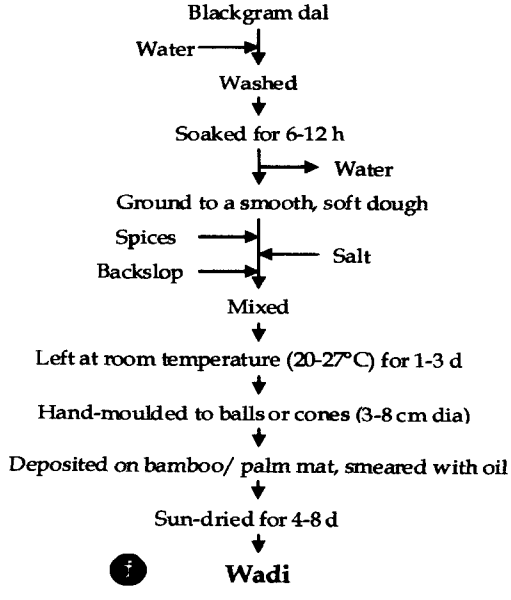


Fig. 3. Flow sheets for the preparation of amriti (a), dhokla (b), dosa (c), idli (d), papad (e) and wadi (f)

Table 2. Sampling of legume-based fermented foods from retailed outlets in West Bengal

Sple No.	Kind of sample	Date of purchase	Purchased from (locality, district)	Open/Pkd (L/B)*
S1	Wadi	20.10.03	Shivmandir, Darjiling	Open
S2	Wadi	28.10.03	Haldibari, Coochbehar	Pkd (L)
S3	Wadi	10.11.03	Jalpaiguri, Jalpaiguri	Pkd (L)
S4	Wadi	17.11.03	Malda, Malda	Open
S5	Wadi	22.12.03	Sealdah, Kolkata	Open
S6	Wadi	29.12.03	Tomluk, Purba Medinipur	Pkd (L)
S7	Wadi	29.12.03	Tomluk, Purba Medinipur	Pkd (L)
S8	Wadi	25.01.04	Kalimpong, Darjiling	Open
S9	Wadi	09.02.04	Siliguri, Darjiling	Pkd (L)
S10	Wadi	09.02.04	Bagdogra, Darjiling	Open
S11	Wadi	16.02.04	Mohitnagar, Jalpaiguri	Pkd (L)
S12	Wadi	16.02.04	Matigara, Darjiling	Open
S13	Idli	23.02.04	Bagdogra, Darjiling	Open
S14	Dosa	23.02.04	Bagdogra, Darjiling	Open
S15	Idli	01.03.04	Siliguri, Darjiling	Open
S16	Dosa	01.03.04	Siliguri, Darjiling	Open
S17	Wadi	08.03.04	Raiganj, Uttar Dinajpur	Open
S18	Papad	08.03.04	Shivmandir, Darjiling	Pkd (B)
S19	Idli	14.04.04	New Jalpaiguri, Jalpaiguri	Open
S20	Dosa	14.04.04	New Jalpaiguri, Jalpaiguri	Open
S21	Idli	22.04.04	Coochbehar, Coochbehar	Open
S22	Dosa	22.04.04	Coochbehar, Coochbehar	Open
S23	Papad	22.04.04	Coochbehar, Coochbehar	Pkd (B)
S24	Amriti	22.04.04	Coochbehar, Coochbehar	Open
S25	Amriti	22.04.04	Coochbehar, Coochbehar	Open
S26	Dosa	28.04.04	Raiganj, Uttar Dinajpur	Open
S27	Dhokla	28.04.04	Raiganj, Uttar Dinajpur	Open
S28	Amriti	28.04.04	Raiganj, Uttar Dinajpur	Open
S29	Dosa	05.05.04	Siliguri, Darjiling	Open
S30	Dosa	05.05.04	Siliguri, Darjiling	Open
S31	Dosa	05.05.04	Siliguri, Darjiling	Open
S32	Idli	05.05.04	Siliguri, Darjiling	Open
S33	Dhokla	12.05.04	Siliguri, Darjiling	Open
S34	Dhokla	12.05.04	Siliguri, Darjiling	Open
S35	Dhokla	12.05.04	Siliguri, Darjiling	Open
S36	Dosa	19.05.04	Jalpaiguri, Jalpaiguri	Open
S37	Dhokla	19.05.04	Siliguri, Darjiling	Open
S43	Wadi	31.05.04	Coochbehar, Coochbehar	Open
S44	Wadi	01.06.04	Maynaguri, Jalpaiguri	Pkd (L)
S45	Wadi	04.06.04	Garifa, Bardhaman	Pkd (L)
S46	Wadi	26.06.04	Aranghata, Nadia	Open
S47	Wadi	26.06.04	Krishnanagar, Nadia	Open
S48	Wadi	28.06.04	Shyamnagar, 24 Parganas (North)	Open
S49	Wadi	01.07.04	Bishnupur, Bankura	Open
S50	Wadi	04.07.04	Uttarpara, Hoogli	Open
S51	Wadi	04.07.04	Uttarpara, Hoogli	Open
S52	Papad	11.07.04	Islampur, Uttar Dinajpur	Pkd (B)
S53	Papad	11.07.04	Raiganj, Uttar Dinajpur	Pkd (B)
S54	Papad	16.07.04	Alipurduar, Jalpaiguri	Pkd (B)
S55	Dosa	27.07.04	Siliguri, Darjiling	Open
S56	Papad	27.07.04	Haldibari, Coochbehar	Pkd (B)
S57	Papad	27.07.04	Haldibari, Coochbehar	Pkd (B)
S58	Papad	27.07.04	Siliguri, Darjiling	Pkd (B)
S59	Idli	09.08.04	Sealdah, Kolkata	Open
S60	Dosa	09.08.04	Sealdah, Kolkata	Open

Sple No.	Kind of sample	Date of purchase	Purchased from (locality, district)	Open/Pkd (L/B)*
S61	Idli	09.08.04	Sealdah, Kolkata	Open
S62	Dosa	09.08.04	Sealdah, Kolkata	Open
S63	Idli	09.08.04	Sealdah, Kolkata	Open
S64	Dosa	09.08.04	Sealdah, Kolkata	Open
S65	Wadi	09.08.04	Howrah, Howrah	Open
S66	Wadi	09.08.04	Howrah, Howrah	Open
S67	Wadi	10.08.04	Jadavpur, Kolkata	Pkd (L)
S68	Wadi	10.08.04	Tolleyganj, Kolkata	Pkd (L)
S69	Papad	10.08.04	Vadreswar, Hoogli	Pkd (B)
S70	Papad	10.08.04	Srerampore, Hoogli	Pkd (B)
S71	Wadi	10.08.04	Nungi, 24 Parganas (South)	Open
S72	Wadi	10.08.04	Titagarah, 24 Parganas (North)	Open
S73	Papad	10.08.04	Nungi, 24 Parganas (South)	Pkd (B)
S74	Papad	10.08.04	Nungi, 24 Parganas (South)	Open
S75	Papad	11.08.04	Howrah, Howrah	Pkd (B)
S76	Papad	11.08.04	Howrah, Howrah	Pkd (B)
S77	Papad	11.08.04	Naihati, 24 Parganas (North)	Pkd (B)
S78	Papad	11.08.04	Titagarah, 24 Parganas (North)	Pkd (B)
S79	Papad	11.08.04	C.R.Avenue, Kolkata	Pkd (B)
S80	Papad	11.08.04	Krishnanagar, Nadia	Pkd (L)
S81	Papad	11.08.04	Srerampore, Hoogli	Pkd (B)
S82	Papad	11.08.04	Santoshpur, Kolkata	Pkd (B)
S83	Papad	11.08.04	Titagarah, 24 Parganas (North)	Pkd (B)
S84	Papad	11.08.04	Howrah, Howrah	Pkd (B)
S85	Papad	11.08.04	Naihati, 24 Parganas (North)	Pkd (B)
S90	Wadi	18.12.04	Kharagpur, Paschim Medinipur	Open
S91	Wadi	18.12.04	Kharagpur, Paschim Medinipur	Open
S92	Papad	18.12.04	Kharagpur, Paschim Medinipur	Pkd (B)
S93	Papad	18.12.04	Kharagpur, Paschim Medinipur	Pkd (B)
S94	Idli	27.12.04	Siliguri, Darjiling	Open
S95	Dosa	27.12.04	Siliguri, Darjiling	Open
S96	Idli	27.12.04	Siliguri, Darjiling	Open
S97	Dosa	27.12.04	Siliguri, Darjiling	Open
S98	Dosa	18.01.05	Malda, Malda	Open
S99	Amriti	18.01.05	Malda, Malda	Open
S100	Idli	18.01.05	Malda, Malda	Open
S101	Idli	18.01.05	Malda, Malda	Open
S102	Idli	18.01.05	Malda, Malda	Open
S103	Amriti	24.01.05	Siliguri, Darjiling	Open
S104	Amriti	24.01.05	Siliguri, Darjiling	Open
S105	Amriti	24.01.05	Siliguri, Darjiling	Open
S106	Amriti	24.01.05	Siliguri, Darjiling	Open
S107	Wadi	31.01.05	Malda, Malda	Open
S108	Wadi	31.01.05	Malda, Malda	Open
S119	Papad	31.01.05	Malda, Malda	Pkd (B)
S110	Papad	31.01.05	Malda, Malda	Pkd (B)
S111	Wadi	15.02.05	Farakka, Murshidabad	Pkd (L)
S112	Wadi	15.02.05	Farakka, Murshidabad	open
S113	Papad	15.02.05	Baharampur, Murshidabad	Pkd (B)
S114	Papad	15.02.05	Baharampur, Murshidabad	Pkd (B)

*Pkd, packaged; L, locally packaged; B, branded.

4.2. Isolation and confirmation of bacterial pathogens

Bacteria were isolated from the foods using selective media, and the presumptive isolates were confirmed morphologically and biochemically. Characteristic turquoise to peacock blue colonies

surrounded by a zone of precipitate of the same colour on *B. cereus* selective agar were regarded as presumptive *B. cereus* (Fig. 4). Confirmation of *B. cereus* was done according to Claus and Berkeley (1986). All the 81 presumptive isolates were endospore-forming (Fig. 5), and fermented glucose, however differed in their ability to reduce nitrate to nitrite, motility and production of acetylmethylcarbinol. A total of 48 strains were confirmed as positive following all these reactions (Table 3).

Fig. 4. *B. cereus* on *B. cereus* selective agar

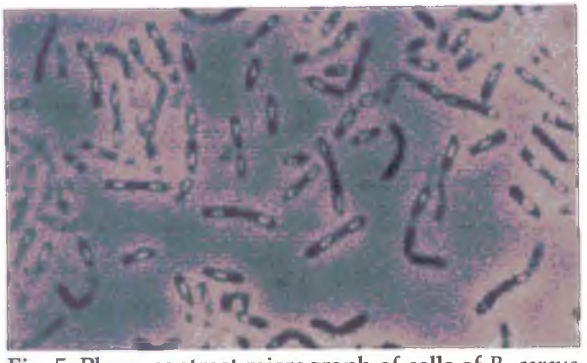


Fig. 5. Phase-contrast micrograph of cells of *B. cereus* 37-B1 (x 1250)

Table 3. Confirmation of the presumptive *B. cereus* strains grown on *B. cereus* selective agar plates*

Isolate code	Nitrate reduction	Motility	VP reaction	% +ve
1-B1, B2, B3	+, +, +	+, +, +	-, -, -	0
2-B1, B2, B3, B4, B5	+, +, +, +, +	+, +, +, +, +	+, -, +, -, -	40
3-B1, B2, B3, B4, B5	+, +, +, +, +	+, +, +, +, +	-, -, -, -, -	0
5-B1, B2, B3, B4, B5	+, +, +, +, +	+, +, +, +, +	-, -, -, -, -	0
6-B1, B2	+, +	+, +	-, +	50
16-B1	+	+	+	100
18-B1, B2, B3, B4, B5	-, +, +, -, +	+, +, +, +, +	+, +, +, +, +	60
33-B1, B2, B3	+, +, +	-, -, -	+, +, +	0
34-B1, B2, B3	+, +, +	+, +, -	+, -, +	33
35-B1, B2, B3	+, +, +	+, +, +	+, -, -	33
37-B1, B2, B3	+, +, +	+, +, +	+, -, -	33
46-B1, B2	+, +	-, +	+, +	50
49-B1, B2	+, +	+, +	+, +	100
52-B1, B2	+, +	+, +	+, +	100
54-B1	+	+	-	0
55-B1	+	+	+	100
57-B1, B2, B3, B4, B5	+, +, +, +, +	+, +, +, +, +	-, +, +, +, +	80
66-B1, B2, B3, B4, B5	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
70-B1, B2	+, +	+, +	+, +	100
80-B1, B2	+, +	+, +	-, -	0
93-B1, B2, B3	+, +, +	+, +, +	+, +, +	100
94-B1, B2, B3	+, +, +	+, +, +	+, +, +	100
98-B1, B2, B3	+, +, +	+, +, +	+, +, +	100
104-B1, B2, B3	+, +, +	+, +, +	+, +, +	100
105-B1, B2, B3	+, +, +	+, +, +	+, +, +	100
111-B1, B2, B3	+, +, +	+, +, +	+, +, +	100
113-B1, B2, B3	+, +, +	+, +, +	+, +, +	100

*All the isolates were endospore-forming, and fermented glucose.
+, positive reaction; -, negative reaction.

Perfringens agar (PA; OPSP) plates, incubated in an anaerobic jar containing AnaeroHiGas pack (Fig. 6), showed characteristic black colonies of presumptive *C. perfringens* (Fig. 7). Isolates, stored in cooked meat medium (Fig. 8), were examined microscopically and confirmed by the absence

Fig. 6. Anaerobic cultures for *C. perfringens*

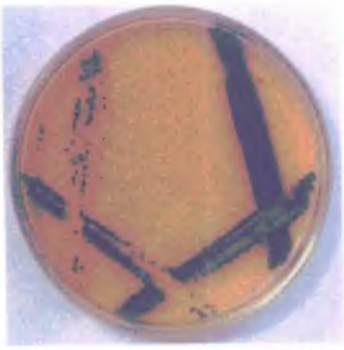


Fig. 7. *C. perfringens* in perfringens agar (OPSP)

Fig. 8. Storage of *C. perfringens* (Cp) in cooked meat medium. C, control

of motility, reduction of nitrate, fermentation of raffinose and lactose, and liquefaction of gelatin (Adams and Moss 1995). None of the 59 presumptive isolates was confirmed as *C. perfringens* (Table 4).

Table 4. Confirmation of the presumptive *C. perfringens* strains grown on perfringens agar (OPSP) plates*

Isolate code	Motility	Nitrate reduction	Raffinose fermentation	Gelatin liquefaction	% +ve
17-C1, C2, C3, C4, C5	+, +, +, +, +	+, +, -, -, -	+, +, +, +, +	+, -, -, -, -	0
18-C1	+	-	-	-	0
33-C1, C2, C3	+, +, +	-, -, -	-, -, -	-, -, -	0
35-C1, C2, C3	+, +, +	-, -, -	+, +, +	-, -, -	0
37-C1, C2, C3	+, +, +	-, -, -	+, +, +	-, +, +	0
46-C1, C2, C3	+, +, +	-, +, -	+, +, +	-, -, -	0
48-C1, C2	-, -	-, -	+, -	-, +	0
49-C1, C2	-, +	-, -	+, -	-, +	0
50-C1, C2	+, -	+, -	+, +	-, -	0
51-C1	+	-	+	-	0
52-C1, C2, C3	+, +, +,	+, +, +	+, +, +	+, +, -	0
54-C1	-	+	-	+	0
57-C1, C2, C3	+, +, -	-, +, -	+, +, -	+, -, +	0
65-C1, C2, C3	+, +, +,	+, +, +	+, +, -	+, +, -	0
66-C1, C2, C3	+, -, -	-, -, -	-, -, -	+, -, -	0
67-C1	+	+	-	-	0
69-C1	+	+	-	-	0
90-C1, C2, C3	+, +, +,	+, +, -	-, -, -	-, +, -	0
91-C1, C2, C3	+, -, -	+, +, +	+, +, +	+, -, -	0
92-C1, C2, C3	+, +, +,	-, -, +	+, +, +	-, +, +	0
93-C1, C2, C3	+, +, +,	+, +, -	-, +, +	+, -, +	0
103-C1, C2, C3	+, +, +,	+, -, +	+, +, -	+, +, +	0
108-C1	+	+	-	-	0
109-C1	+	+	-	-	0
110-C1, C2	+, -	-, -	-, -	+, -	0

*All the presumptive isolates fermented lactose.
+, positive reaction; -, negative reaction.

Characteristic grey-black shiny colonies surrounded by a clear zone on Baird-Parker agar were regarded as presumptive *S. aureus* (Fig. 9) which were confirmed by studying morphology (Fig. 10), and production of coagulase and thermostable nuclease (Fig. 11) (Adams and Moss 1995), fermentation of mannitol (HiMedia 1998) and production of acetylmethylcarbinol (Schleifer 1986). Out of three presumptive isolates, two were confirmed as *S. aureus* (Table 5).

Fig. 9. *S. aureus* on Baird-Parker agar

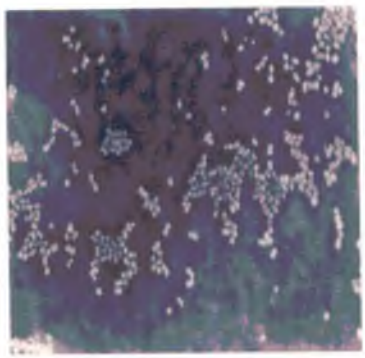


Fig. 10. Phase-contrast micrograph of cells of *S. aureus* 34-S1 (x 1200)

Fig. 11. *S. aureus* on DNase test agar

Table 5. Confirmation of the presumptive *S. aureus* strains grown on Baird-Parker agar plates

Isolate code	Coagulase	Mannitol fermentation	Thermostable DNase	VP reaction	% +ve
34-S1, S2, S3	+, +, -	+, +, -	+, +, -	+, +, +	67

+, positive reaction; -, negative reaction.

Pink colonies on tryptone soya agar-violet red bile glucose agar without lactose plates were considered as presumptive Enterobacteriaceae (Fig. 12). Those presumptive isolates were confirmed by testing for ability to ferment glucose and absence of oxidase (Nout *et al.* 1998). Out of 214 isolates, 154 were found confirmed members of Enterobacteriaceae. The isolates were then tested for their identity as coliform and faecal coliform by checking the production of gas at 37/44 °C in bile broth (Fig. 13). *Escherichia coli* (Fig. 14) was confirmed by the production of indole from tryptophan. A total of 72 strains of coliform and 19 strains of faecal coliform were found, of which only two were confirmed *E. coli* (Table 6).

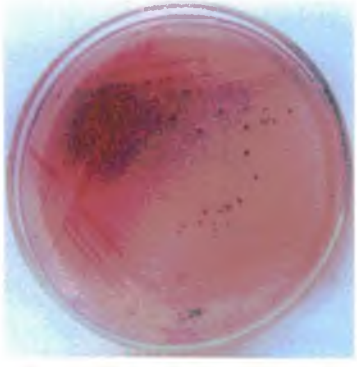


Fig. 12. Enterobacteriaceae on TSA-VRBGA

Fig. 13. Coliform/faecal coliform (2) in BGBB against control (1)

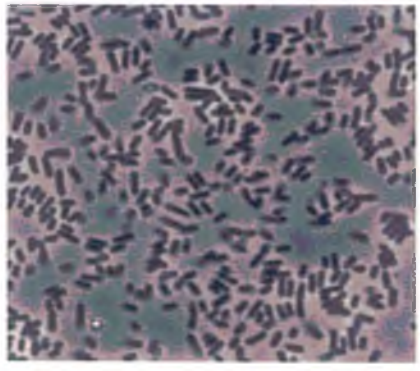


Fig. 14. Phase-contrast micrograph of cells of *E. coli* 7-E2 (x 1120)

Characteristic black colonies with metallic sheen (Fig. 15) and brown colonies (Fig. 16) on bismuth sulphite agar plates were regarded as presumptive *Salmonella* and *Shigella*, respectively. A total of 190 strains of *Salmonella/ Shigella* from bismuth sulphite agar plates were differentiated using their response to triple sugar iron agar (Fig. 17) and lysine iron agar (Fig. 18) (Adams and Moss 1995). The 33 strains of *Salmonella* were further confirmed by studying morphology (Fig. 19), the presence of motility, ability to reduce nitrate to nitrite, and inability to produce indole from tryptophan (Table 7). None of the isolates was confirmed as *Shigella* (Table 7).

Table 6. Confirmation of the presumptive Enterobacteriaceae strains isolated from TSA-VRBGA plates

Isolate code	Enterobacteriaceae			Coliform		Faecal coliform	
	Glucose fermentation	Oxidase	% +ve	Gas at 37°C	% +ve	Gas at 44°C	% +ve
2-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	-, +, -, -, -	20	x, -, x, x, x	0
3-E1, E2, E3	+, +, +	-, -, -	100	-, -, +	33	x , x, +	33
5-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, +, -, +, +	80	-, -, x, -, +	20
6-E1, E2, E3	+, +, +	-, -, -	100	-, -, -	0		
7-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, +, +, -, -	60	+, +, +, x, x	(60)
8-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	-, -, -, +, -	20	x , x, x, -, x	0
9-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, -, -, -, -	20	-, x, x, x, x	0
10-E1, E2, E3, E4, E5	+, +, +, +, +	-, +, -, -, -	80	+, x, +, -, -	40	+, x, +, x, x	40
13-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, +, -	80	-, +, +, x, +	60	x , -, -, x, -	0
14-E1, E2, E3, E4, E5	+, +, +, +, +	-, +, -, -, -	80	+, x, -, +, +	60	-, x, x, -, -	0
15-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	-, -, -, -, +	20	x , x, x, x, -	0
18-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, +, -, -, -	40	-, -, x, x, x	0
19-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, +, +, +, +	100	-, -, -, -, -	0
24-E1, E2	+, +	-, -	100	-, -	0		
25-E1, E2, E3, E4, E5	+, +, +, +, +	-, +, +, -, +	40	-, x, x, -, x	0		
32-E1, E2, E3	+, +, +	-, -, -	100	-, -, +	33	x, x, -	0
37-E1, E2, E3	+, +, +	-, -, -	100	+, +, +	100	+, +, -	67
43-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, +, +, +, +	100	-, -, -, +, -	20
44-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	-, -, -, +, +	40	x, x, x, -, -	0
45-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, +	80	-, -, -, -, x	0		
46-E1, E2, E3, E4, E5	+, +, +, +, +	+, +, +, -, +	20	x, x, x, -, x	0		
47-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, +, +, +	40	+, +, x, x, x	40	+, -, x, x, x	20
48-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, -, +, +, +	80	-, x, -, -, -	0
49-E1, E2, E3, E4, E5	+, +, +, +, +	+, -, +, -, -	60	x, +, x, +, +	60	x, -, x, -, -	0
51-E1, E2, E3, E4	+, +, +, +	+, +, +, -	25	x, x, x, +	25	x, x, x, +	25
52-E1, E2	+, +	-, +	50	-, x	0		
54-E1, E2	+, +	-, -	100	-, -	0		
61-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, +, -, +, +	80	+, +, x, +, +	(80)
65-E1, E2	+, +	-, -	100	-, -	0		
66-E1, E2	+, +	-, -	100	-, -	0		
67-E1, E2, E3, E4, E5	+, +, +, +, +	-, +, -, -, -	80	-, x, -, -, -	0		
68-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, +, +, -, +	80	-, +, -, x, -	20
72-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, +, -, -	80	-, -, x, -, -	0		
73-E1, E2, E3, E4, E5	+, +, +, +, +	-, -, -, -, -	100	+, +, +, -, -	60	-, -, -, x, x	0
75-E1, E2, E3	+, +, +	-, -, -	100	-, +, +	67	x, -, -	0
76-E1, E2, E3	+, +, +	-, -, -	100	+, +, +	100	-, +, -	33
78-E1, E2, E3	+, +, +	-, -, -	100	-, -, -	0		
83-E1, E2, E3	+, +, +	-, -, -	100	+, +, +	100	+, -, -	33
84-E1, E2, E3	+, +, +	-, -, -	100	-, -, -	0		
90-E1, E2, E3, E4	+, +, +, +	+, +, -, +	25	x, x, -, x	0		
91-E1, E2, E3, E4	+, +, +, +	+, +, -, +	25	x, x, -, x	0		
94-E1, E2, E3	+, +, +	-, -, +	67	+, +, x	67	-, -, x	0
96-E1, E2, E3	+, +, +	+, +, -	33	x, x, +	33	x, x, -	0
97-E1, E2, E3	+, +, +	-, +, -	67	+, x, -	33	-, x, x	0
98-E1, E2, E3	+, +, +	+, +, +	0				
104-E1, E2, E3, E4	+, +, +, +	+, -, +, -	50	x, -, x, +	25	x, x, x, -	0
105-E1, E2, E3, E4	+, +, +, +	+, +, +, -	25	x, x, x, -	0		
107-E1, E2, E3, E4, E5	+, +, +, +, +	+, -, -, +, +	40	x, -, -, x, x	0		
108-E1, E2, E3, E4, E5	+, +, +, +, +	-, +, -, -, +	60	-, x, -, -, x	0		
109-E1, E2, E3, E4, E5	+, +, +, +, +	+, +, +, +, +	0				
111-E1, E2, E3, E4, E5	+, +, +, +, +	+, +, +, +, +	0				
112-E1, E2, E3, E4, E5	+, +, +, +, +	+, +, +, +, +	0				

+, positive reaction; -, negative reaction; x, not determined; Circle indicates the presence of *E. coli*.

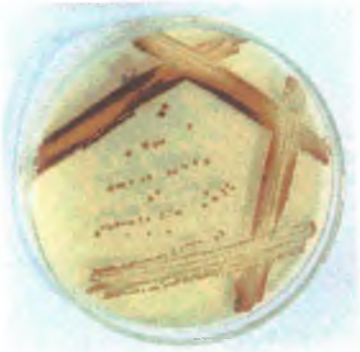


Fig. 15. *Salmonella* on bismuth sulphite agar

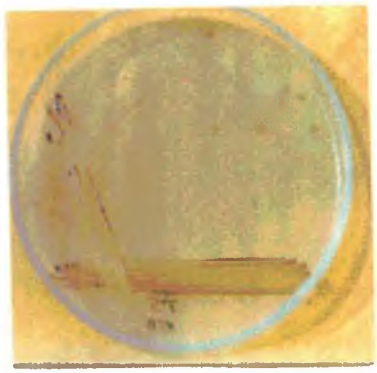


Fig. 16. *Shigella* on bismuth sulphite agar

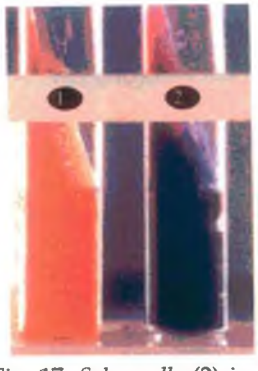


Fig. 17. *Salmonella* (2) in TSI agar against control (1)

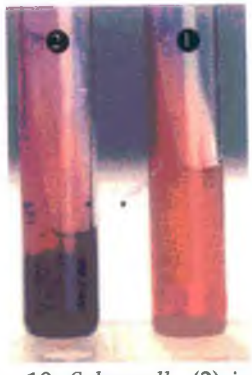


Fig. 18. *Salmonella* (2) in lysine iron agar against control (1)

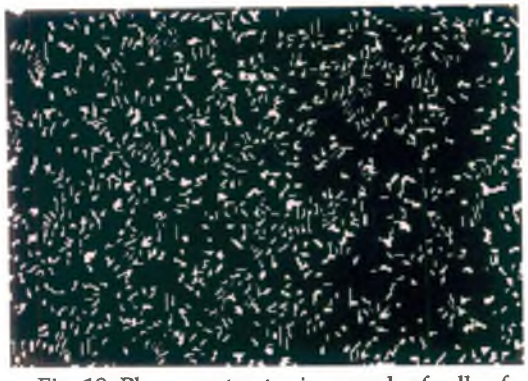


Fig. 19. Phase-contrast micrograph of cells of *Salmonella* 1-S4 (x 1200)

4.3. Biochemical profile and bacteriological quality of fermented foods

The results of moisture, pH and microbial analyses of 105 samples of the six kinds of foods are summarized in Table 8. While dhokla and idli were the high moisture-content (62 g (100 g)$^{-1}$) foods, others contained less moisture (14-27 g (100 g)$^{-1}$). Papad was alkaline (pH 8.7), whereas all the other foods were acidic (pH 4.4-5.8).

Thirty-eight percent (40 of 105) of the samples contained total aerobic mesophilic bacterial cells at a level of > 10^6 cfu g^{-1} (Table 8). Majority of the samples of each of the six foods, except dosa, had a high count (> 10^4 cfu g^{-1}) of these bacteria. While most of the samples of amriti, dosa, idli and papad contained total aerobic mesophilic bacteria in the range of 10^2-10^6 cfu g^{-1}, in most of the samples of dhokla and wadi their count was at a higher level (> 10^6 cfu g^{-1}).

Aerobic mesophilic bacterial spores were found in 88% (92 of 105) of the samples. All the samples of amriti, dhokla and papad contained these spores. A high count (> 10^5 cfu g^{-1}) of them was found in papad and wadi (10 of 63 samples). As dosa samples were freshly prepared ones, their load in the product was never more than 10^5 cfu g^{-1}. On the other hand, the load of their anaerobic counterpart was less; they occurred in 39% of the tested samples (41 of 105). Amriti, dosa and idli were free of them.

All the six types of foods contained *B. cereus*; this organism occurred in 20% of the samples (21 of 105). The potentially hazardous level (> 10^4 cfu g^{-1}) was observed in dhokla, papad and wadi. *C. perfringens* could not be detected from any of the 105 samples, and only one sample was found contaminated (at the load of 4 x 10^4 cfu g^{-1} dhokla) with *S. aureus*.

Table 7. Confirmation of the presumptive *Salmonella/ Shigella* strains isolated from bismuth sulphite agar plates

Isolate code	In triple sugar iron agar[a]				In lysine iron agar[b]			Motile	Indole production	Nitrate reduction	Confirmed identity
	Slant	Butt	Gas	H_2S	Slant	Butt	H_2S				
1-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	R, R, R	K, K, K	-, -, -	x[c], x, x	x, x, x	x, x, x	
1-S4, S5, S6	K, K, K	A, A, A	+, +, +	+, + +	K, K, K	A, A, A	+, +, +	+, +, +	-, -, -	+, +, +	*Salmonella*
1-S7, S8, S9	A, K, A	A, A, A	+, +, +	+, +, +	R, R, R	A, A, A	-, -, -	x, +, x	x, x, x	x, -, x	
1-S10, S11, S12	A, A, A	A, A, A	+, +, +	-, -, -	R, R, R	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
2-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	R, R, R	A, A, A	-, -, -	x, x, x	x, x, x	x, x, x	
2-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	R, R, R	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
3-S1, S2, S3	K, A, K	A, A, A	+, +, +	-, -, -	K, K, K	A, K, A	-, -, -	+, x, +	x, x, x	-, x, -	
3-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	R, R, R	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
4-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	R, R, R	A, A, A	-, -, -	x, x, x	x, x, x	x, x, x	
4-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	R, R, R	A, A, A	-, -, -	x, x, x	x, x, x	x, x, x	
4-S7, S8, S9	A, A, A	A, A, A	+, +, +	-, -, -	R, R, R	A, R, A	-, -, -	x, x, x	x, x, x	x, x, x	
5-S1, S2, S3	K, A, A	A, A, A	+, +, +	-, -, -	R, R, R	A, A, A	-, -, -	-, x, x	x, x, x	-, x, x	
5-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, +, +	R, R, R	A, A, A	+, +, +	x, x, x	x, x, x	x, x, x	
5-S7, S8, S9	A, A, A	A, A, A	+, +, +	-, -, -	R, R, R	A, A, A	-, -, -	x, x, x	x, x, x	x, x, x	
5-S10, S11, S12	A, A, K	A, A, A	+, +, +	-, -, -	R, R, R	R, A, A	-, -, -	x, x, +	x, x, +	x, x, +	
6-S1, S2, S3	K, K, K	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	+, +, +	x, +, +	-, +, +	
6-S4, S5, S6	A, K, A	A, K, A	+, -, +	-, -, -	K, K, K	A, K, A	-, -, -	x, x, x	x, x, x	x, x, x	
7-S1, S2, S3	K, K, K	A, A, A	+, +, +	+, +, +	K, K, K	A, A, A	+, +, +	+, +, +	+, -, -	+, +, +	*Salmonella*
7-S4, S5, S6	A, K, A	A, A, A	+, +, +	-, -, -	K, K, K	K, A, A	-, -, -	x, +, x	x, -, x	x, +, x	*Salmonella*
8-S1, S2, S3	K, K, A	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	-, -, x	x, x, x	-, -, x	
8-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, A, A	-, -, -	x, x, x	x, x, x	x, x, x	
9-S1, S2, S3	K, K, A	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	-, -, x	x, x, x	-, -, x	
9-S4, S5, S6	A, A, K	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	x, x, +	x, x, x	x, x, -	
10-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	x, x, x	x, x, x	x, x, x	
10-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
11-S1, S2, S3	A, K, A	A, A, A	+, +, +	-, -, -	K, K, K	K, A, A	-, -, -	x, +, x	x, -, x	x, +, x	*Salmonella*
11-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	A, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
12-S1, S2, S3	A, A, A	A, A, A	+, +, +	+, +, +	K, K, K	A, A, A	+, +, +	x, x, x	x, x, x	x, x, x	
12-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
13-S1, S2, S3	K, K, K	A, A, A	+, +, +	-, -, -	K, K, K	K, K, A	-, -, -	+, +, +	x, x, -	-, -, +	*Salmonella*
13-S4, S5, S6	R, R, R	K, A, K	-, -, -	- + -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
14-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	A, R, A	-, -, -	x, x, x	x, x, x	x, x, x	
14-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
15-S1, S2, S3	A, A, A	A, A, A	-, -, -	+, +, +	R, R, R	A, A, A	-, -, -	x, x, x	x, x, x	x, x, x	
15-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	x, x, x	x, x, x	x, x, x	

Isolate code	In triple sugar iron agar[a]				In lysine iron agar[b]			Motile	Indole production	Nitrate reduction	Confirmed identity
	Slant	Butt	Gas	H_2S	Slant	Butt	H_2S				
17-S1, S2, S3	K, K, K	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	+, +, +	-, -, -	+, +, +	*Salmonella*
17-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
21-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	+, +, +	+, +, +	-, -, -	+, +, +	*Salmonella*
21-S4, S5, S6	K, K, K	K, K, K	-, -, -	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
22-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
22-S4, S5, S6	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
23-S1, S2, S3	K, K, K	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	+, +, +	-, -, x	+, +, -	*Salmonella*
24-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
25-S1, S2, S3	A, A, A	A, A, A	-, +, +	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
26-S1, S2, S3	A, K, K	A, K, K	+, -, -	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
26-S4, S5, S6	K, K, K	K, K, K	-, -, -	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
27-S1, S2, S3	K, K, K	A, A, K	-, -, -	-, -, -	K, K, K	A, A, K	-, -, -	+, +, x	x, x, x	-, -, x	
29-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, A	-, -, -	x, x, x	x, x, x	x, x, x	
30-S1, S2, S3	R, R, R	K, K, K	-, -, -	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
32-S1, S2, S3	R, R, R	K, K, K	-, -, -	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
33-S1, S2, S3	K, K, K	A, A, A	+, +, +	+, +, +	K, K, K	A, A, A	+, +, +	+, +, +	x, x, x	-, -, -	
34-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	- + -	x, x, x	x, x, x	x, x, x	
36-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	x, x, x	x, x, x	x, x, x	
37-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	x, x, x	x, x, x	x, x, x	
43-S1, S2, S3	K, K, K	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	+, +, +	-, -, -	+, +, +	*Salmonella*
43-S4, S5, S6	K, K, K	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	+, +, +	-, -, -	+, +, +	*Salmonella*
43-S7, S8, S9	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	x, x, x	x, x, x	x, x, x	
43-S10	A	A	+	-	K	K	-	x	x	x	
62-S1, S2, S3	A, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, +	x, x, +	x, x, +	x, x, +	
68-S1, S2, S3	K, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	+, x, x	-, x, x	+, x, x	*Salmonella*
68-S4, S5, S6	K, A, A	A, A, A	+, +, +	-, -, -	K, K, K	K, K, K	-, -, -	+, x, x	-, x, x	+, x, x	*Salmonella*
83-S1, S2, S3	K, K, K	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	+, +, +	-, -, -	+, +, +	*Salmonella*
84-S1, S2, S3	K, K, K	A, A, A	+, +, +	-, -, -	R, R, R	R, R, A	-, -, -	+, +, +	-, -, -	+, +, +	*Salmonella*
85-S1, S2, S3	K, K, K	A, A, A	+, +, +	-, -, -	K, K, K	A, A, A	-, -, -	+, +, +	-, -, -	+, +, +	*Salmonella*

[a]A, acidic, yellow colour; K, alkaline, no change in colour; +, blackening (H_2S) positive reaction; -, no reaction.

[b]R, deep red, lysine deamination;, K, alkaline, no colour change; A, acidic, yellow colour; +, blackening of medium; -, no blackening of medium.

[c]x, not determined.

Enterobacteriaceae occurred in all the six types of foods studied (Table 8); these were detected in 46% (48 of 105) of the samples. Of the Enterobacteriaceae isolates, 92% were coliforms and 57% were faecal coliforms (Table 9). One sample each of idli (3.8×10^3 cfu g^{-1}) and wadi (3.2×10^4 cfu g^{-1}) were found contaminated with *E. coli*.

Salmonella was present in 11.4% (12 of 105) of the total samples analysed. It was not detected in amriti, dhokla and dosa. However, its prevalence in the other three foods is noteworthy; 15% (2 of 13), 14% (4 of 29) and 18% (6 of 34) of the samples of idli, papad and wadi, respectively, were found contaminated with this pathogen.

To demonstrate the variability of viable counts observed with single samples of different kinds of foods, the data ranges of positive samples are presented in Fig 20. The number of total aerobic mesophilic bacteria, mesophilic bacterial spores, *B. cereus*, *C. perfringens*, *S. aureus* and Enterobacteriaceae varied considerably. As to the total aerobic mesophilic bacteria, some samples of dhokla, papad and wadi contained a high load (> log 7.0 cfu g^{-1}) of them. The maximum load (log 11.4 cfu g^{-1}) with a wide range (log 4-11 cfu g^{-1}) of total aerobic mesophilic bacterial count occurred in wadi. As per aerobic mesophilic bacterial spores, some samples of papad and wadi exceeded log 5.0 cfu g^{-1}. A large variation

Table 8. Moisture content, pH and load of different components of microbiota (expressed as percentages of samples analysed) of legume-based traditional fermented foods

Parameter	Food					
	Amriti (n = 8)	Dhokla (n = 5)	Dosa (n = 16)	Idli (n = 13)	Papad (n = 29)	Wadi (n = 34)
Moisture[a], g (100 g) $^{-1}$	19.5bc±1.12	62.1a±0.86	27.3b±1.73	61.8a±1.58	18.0bc±0.41	14.4c±0.33
pH[a]	5.8b±0.07	4.9c±0.11	4.4d±0.08	4.6cd±0.07	8.7a±0.07	5.7b±0.04
Bacterial load (cfu g^{-1})[b]						
TAMB						
< DL[c]						
10^2-10^4	12.5		56.3	23.1	17.2	2.9
> 10^4-10^6	62.5		43.8	76.9	75.9	5.9
> 10^6-10^9	25	60			6.9	55.9
> 10^9 -10^{12}		40				35.3
aMBS						
< DL[d]			50	15.4		8.8
10^2-10^5	100	100	50	84.6	79.3	76.5
> 10^5-10^7					20.7	14.7
anMBS						
< DL[c]	100	40	100	100	44.8	35.3
10-10^4		60			55.2	47.1
> 10^4-10^5						17.6
B. cereus						
< DL[d]	75	40	81.3	92.3	79.3	82.4
10^2-10^3		20	12.5	7.7	10.3	8.8
> 10^3-10^4	25		6.3		6.9	2.9
> 10^4-10^6		40			3.4	5.9
Enterobacteriaceae						
< DL[c]	50	80	87.5	46.2	69	26.5
10-10^3	25			7.7	17.2	17.6
> 10^3-10^5	12.5		12.5	46.2	13.8	23.5
> 10^5-10^8	12.5	20				32.4

[a]Values are mean with standard error of measurements. Means with the same following letters, within rows, are not significantly different (P <0.05).

[b]TAMB, total aerobic mesophilic bacteria; aMBS, aerobic mesophilic bacterial spores, anMBS, anaerobic mesophilic bacterial spores.

[c]DL (detection limit), 10 cfu g^{-1}.

[d]DL, 100 cfu g^{-1}.

Table 9. Percentage of the different components of Enterobacteriaceae

Enterobacteriaceae component	Food						
	Amriti	Dhokla	Dosa	Idli	Papad	Wadi	Total
Coliform	49.1	100	58.7	82.6	49.2	59.8	91.9
Faecal coliform	0	66.7	0	15.5	12.5	18.6	56.9
E. coli	0	0	0	3.9	0	0.6	0.1

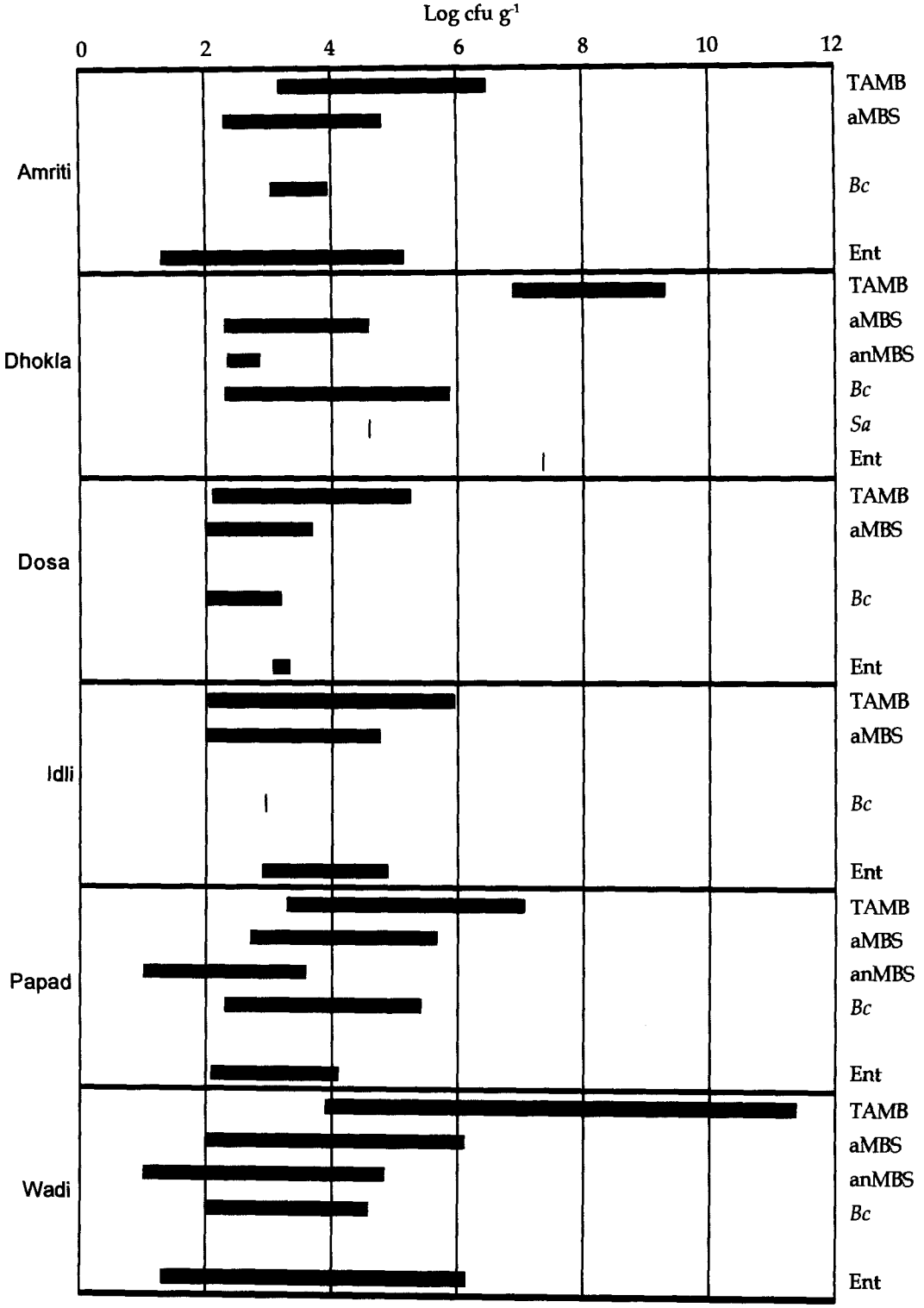


Fig. 20. Ranges of pathogenic bacterial load among the positive samples of food.
TAMB, total aerobic mesophilic bacteria; aMBS, aerobic mesophilic bacterial spores; anMBS, anaerobic mesophilic bacterial spores, *Bc*, *B. cereus*; *Sa*, *S. aureus*; Ent, Enterobacteriaceae

Table 10. Antibiogram of *the* isolates from legume-based fermented foods[a]

Mechanism of action	Antibiotics (disc^{-1})	Percent score								
		B. cereus (n = 48)			Enterobacteriaceae (n = 24)			*Salmonella* (n = 33)		
		S	I	R	S	I	R	S	I	R
Inhibition of cell wall synthesis	Ampicillin (10 µg)			100	50	33	17	9		91
	Bacitracin (10 U)		29	71	33	21	46	21	9	70
	Carbenicillin (100 µg)			100	8	21	71	42	16	42
	Cephalothin (30 µg)	2		98	58	13	29	21	3	76
	Cloxacillin (10 µg)			100	58		42	18		82
	Penicillin G (10 U)			100		8	92	15		85
	Vancomycin (10 µg)			100	4	29	67	9	6	85
Inhibition of protein synthesis	Chloramphenicol (30 µg)	96	4		75	4	21	100		
	Erythromycin (15 µg)		60	40	21	12	67			100
	Kanamycin (30 µg)	58	40	2	75	8	17	15	39	46
	Streptomycin (10 µg)	67	29	4	46	12	42	12	18	70
	Tetracycline (30 µg)	83	17		88	8	4	49	30	21
Damage to cell membrane	Polymyxin B (300 U)			100	54	42	4	45	55	
Inhibition of nucleic acid synthesis	Ciprofloxacin (10 µg)	98		2	79	4	17	79	15	6
	Nalidixic acid (30 µg)	38	58	4	54	21	25	73	18	9
	Rifampicin (15 µg)	6	25	69	12	17	71	3		97
	Metronidazole (5 µg)			100			100			100
Inhibition of folic acid synthesis	Trimethoprim (10 µg)			100	8	4	88	27		73

[a]S, sensitive; I, intermediate; R, resistant (the inhibition zone size, diameter in mm, interpretation was according to Banerjee and Sarkar 2004a).

(2-6 log cfu g^{-1}) in the counts of aerobic mesophilic bacterial spores was found in wadi. Large variation in the count of anaerobic mesophilic bacterial spores was found in papad and wadi only. Of the tested pathogenic bacteria, *B. cereus* was the most predominant one with a wide range of distribution in dhokla, papad and wadi. *S. aureus* was present in dhokla only and that at a low level. Enterobacteriaceae count was highest in dhokla. It varied widely in amriti and wadi. The results show that amriti and dosa were of better quality foods, compared to others.

4.4. Susceptibility to antimicrobials

Susceptibility to 18 antimicrobials, including β-lactams (5), benzene derivative (1), aminoglycosides (2), macrolides (2), peptides (2), glycopeptide (1), quinolones (2), nitro-imidazole (1), tetracycline and trimethoprim, are shown in Table 10. Each of the isolates of *B. cereus* showed multiple resistance. Out of the 48 isolates, 10% were resistant against 9 antibiotics, 21% against 10 antibiotics, 38% against 11 antibiotics, 29% against 12 antibiotics, and 2% against 13 antibiotics (Fig. 21). Out of the 24 Enterobacteriaceae strains, 4% each were resistant against 4, 11, 12, 13, 14 and 15 antibiotics, 25% against 5 antibiotics, 13% against 6, 7 and 9 antibiotics, and 12% against 10 antibiotics. Each of the tested 33 strains of *Salmonella* was multiple-antibiotic resistant. Six percent each were resistant against both 5 and 7 antibiotics, 3% each against both 6 and 8 antibiotics, 12% each against 9, 10 and 11 antibiotics, 28% against 12 antibiotics, and 9% each against both 13 and 14 antibiotics.

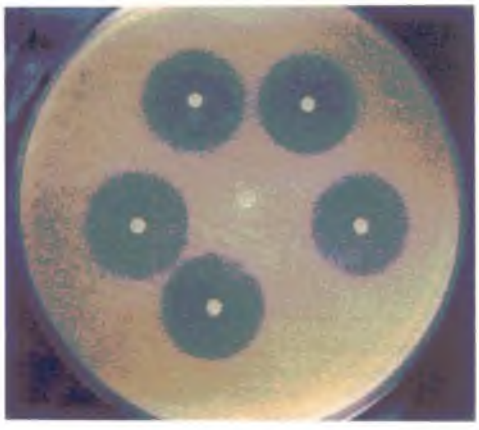


Fig. 21. Sensitivity of bacterial isolate to antimicrobial compounds

4.5. Thermal inactivation of sporeformers

The *D*-values were calculated from the regression analysis best-fit plot of the linear portion of the survivor curve (Fig. 22). In glucose-supplemented brain-heart infusion broth, the correlation coefficient (R^2) values of decimal reduction time curves for spore suspensions of 12 different isolates of *B. cereus* were at least 0.91. The mean $D_{100°C}$-values of 12 strains of *B. cereus* spores was 6.2 min (Table 11)

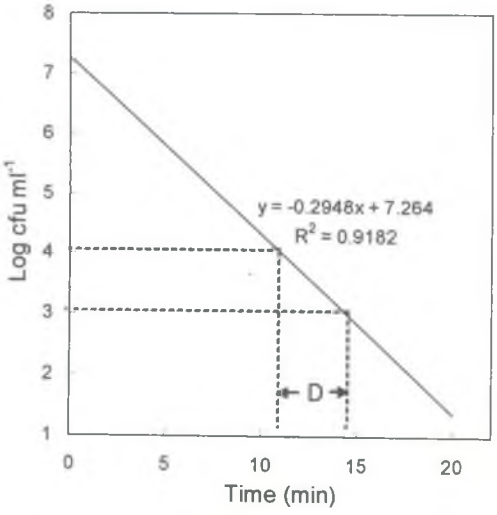


Fig. 22. Survivor curve of *B. cereus* at 100 °C

Table 11. Thermal inactivation of spores of *B. cereus* isolates from different food sources

Source	Isolate no.	$D_{100°C}$ (min)[a]
Amriti	104-B1	7.0 ± 0
	105-B1	5.2 ± 0.2
Dhokla	35-B1	5.3 ± 0.1
	37-B1	7.4 ± 0.1
Dosa	55-B1	5.6 ± 0.1
	98-B1	8.0 ± 0
Idli	94-B1	3.0 ± 0
	94-B2	4.8 ± 0.1
Papad	93-B1	9.2 ± 0.2
	113-B1	6.2 ± 0.2
Wadi	66-B1	6.0 ± 0.1
	111-B1	6.8 ± 0.2

[a]Values are mean ± SE of triplicate determinations.

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

The results on the production of three extracellular enzymes viz. protease, lipase and amylase are presented in Table 12. Proteolytic and amylolytic activities were found in 33% and 46%, respectively, of the isolates. However, lipolytic activity was found in only 27% of the isolates. Eleven (23%) isolates produced all the three enzymes, while 24 (50%) isolates did not produce any of these enzymes.

Table 12. Production of extracellular enzymes by *B. cereus* isolates (n = 48) from different food sources

Source	Isolate no[a]	Zone diameter (mm)[b]		
		Protease	Lipase	Amylase
Amriti	104-B3	27		30
	105-B2	26		11
	105-B3	32		33
Dhokla	34-B1	42	25	31
	37-B1			24
Dosa	55-B1		32	
Idli	94-B1			22
	94-B2			14
	94-B3	41	19	39
Papad	18-B2		22	47
	52-B2	41	36	55
	57-B2	41	26	39
	57-B3	42	30	41
	57-B5			23
	70-B1	42	28	41
	93-B2			12
	93-B3	37		44
	113-B2			12
	113-B3	40	22	35
Wadi	2-B1	40	26	35
	6-B2	45	26	38
	49-B1	32		
	66-B3	42	30	49
	111-B1	37	13	18

[a]Others had no activity.
[b]Includes diameter of the well (5 mm).

4.7. Influence of pH on growth

The effect of pH on the growth of *B. cereus* isolates, one each from the 6 different kinds of foods, is shown in Table 13. In nutrient broth, the minimum and maximum pHs permitting growth of *B. cereus* were 5.3 and 11.6, respectively. The optimum pH was 9.0-9.9.

S. aureus grew at pH range of 4.8-9.5, with an optimum being 6.1 in nutrient broth after 24 h at 35°C. The minimum and maximum pHs permitting growth of *E. coli* was 4.0 and 9.5, respectively, with an optimum of 6.1 in nutrient broth after 24 h at 35°C.

The minimum pH permitting growth of *Salmonella* was 4.3, while the maximum limit was 9.9 (Table 14), with an optimum of 7.3 in nutrient broth after 24 h at 35°C.

Table 13. Range of pH and MIC[a] of food preservatives against the growth of *B. cereus* isolates from different food sources

Source	Target strain	pH range for growth[b]	Preservative in nutrient agar[c]			
			NaCl (mg ml^{-1})	Benzoic acid (μg ml^{-1})	Sorbic acid (μg ml^{-1})	Nisin (μg ml^{-1})
Amriti	104-B1	5.3-11.6	65	400 (5.0)	500 (5.0)	175
	105-B1/B2	nd	80	650 (4.3)	500	nd
	104-B3, 105-B3	nd	85	550 (4.5)	500	nd
	104-B2	nd	85	650	500	nd
Dhokla	37-B1	5.4-11.1	50	400	500	>300
	34-B1, 35-B1	nd	85	550	500	>300
Dosa	98-B1	5.3-11.6	65	450 (4.8)	500	>300
	98-B2/B3	nd	80	650	500	nd
	16-B1, 55-B1	nd	85	600 (4.4)	500	nd
Idli	94-B1	5.3-11.6	65	450	600 (4.8)	>300
	94-B2	nd	85	450	600	nd
	94-B3	nd	85	600	500	nd
Papad	113-B1	5.3-11.6	70	400	500	>300
	57-B5	nd	80	650	500	nd
	113-B3	nd	85	550	500	nd
	18-B2/ B3/ B5, 52-B2, 57-B2/B3/B4, 70-B1/B2, 93-B1/B2/B3, 113-B2	nd	85	600	500	nd
	52-B1	nd	85	650	500	>300
Wadi	111-B1	5.3-11.6	70	450	500	175
	111-B2	nd	70	450	500	nd
	111-B3	nd	80	550	500	nd
	6-B2, 49-B2	nd	85	550	500	nd
	49-B1	nd	85	550	500	>300
	2-B3, 66-B2/B3/B4/B5	nd	85	600	500	nd
	46-B2	nd	85	650	500	>300
	66-B1	nd	85	650	500	nd
	2-B1	nd	85	700 (4.2)	500	nd

[a]MIC (minimum inhibitory concentration) signified minimum concentration of the preservative at which growth was completely inhibited.
[b]nd, not determined.
[c]Values within parentheses indicate pHs of media after the additions.

4.8. Influence of food preservatives on growth

The minimum inhibitory concentrations (MICs) of different food preservatives on the growth of 48 strains of *B. cereus* isolated from the six different kinds of foods are shown in Table 13. The growth was completely inhibited at 65-85 mg sodium chloride ml^{-1}, depending on the strains. The MICs of benzoic acid and sorbic acid against the growth were 400-700 μg ml^{-1} (pH 5.0-4.2) and 500-600 μg ml^{-1} (pH 5.0-4.8), respectively. The MICs of nisin against the growth of selected 10 isolates were determined; most (80%) of the strains were resistant to 300 μg ml^{-1} nutrient agar (pH 5.0).

The MICs of sodium chloride against *S. aureus* and *E. coli* were 110 and 90 mg ml^{-1} nutrient agar, respectively. The MIC of benzoic acid for *S. aureus* and *E. coli* was 650 μg ml^{-1} (pH 4.3). Both the strains of *S. aureus* were inhibited at 800 μg of sorbic acid ml^{-1} (pH 4.6), whereas for *E. coli*, it was 600 μg ml^{-1} (pH 4.8). One of the strains of *S. aureus* was resistant to 300 μg nisin ml^{-1} nutrient agar (pH 5.0).

The MICs of sodium chloride, benzoic acid and sorbic acid against the growth of *Salmonella* isolates were 70-95 mg ml^{-1}, 450-650 μg ml^{-1} (pH 4.8-4.3) and 500-700 μg ml^{-1} nutrient agar (pH 5.0-4.7), respectively (Table 14).

Table 14. Range of pH and MIC[a] of food preservatives against the growth of *Salmonella* isolates from different food sources

Source	Target strain	pH[b] range for growth	Preservative in nutrient agar[c]		
			NaCl (mg ml^{-1})	Benzoic acid (µg ml^{-1})	Sorbic acid (µg ml^{-1})
Idli	13-S3	4.3-9.0	80	600 (4.4)	600 (4.8)
	21-S1	nd	70	500 (4.7)	500 (5.0)
	21-S2	nd	90	600	500
	21-S3	nd	90	550 (4.5)	500
Papad	23-S1	4.8-9.7	90	550	600
	23-S2, 83-S1/S2/S3	nd	90	600	500
	84-S1/S2	nd	90	650 (4.3)	600
	84-S3, 85-S1/S2/S3	nd	90	600	600
Wadi	1-S4	4.3-9.9	70	650	500
	1-S5, 17-S1	nd	75	650	500
	1-S6	nd	75	600	500
	7-S2	nd	70	550	600
	7-S3	nd	75	550	600
	7-S5	nd	70	500	700 (4.7)
	11-S2	nd	70	450 (4.8)	500
	17-S2	nd	80	600	500
	17-S3	nd	90	600	700
	43-S1/S2/S4/S5	nd	95	600	500
	43-S3, 68-S4	nd	90	600	500
	43-S6	4.3-9.0	95	600	500
	68-S1	nd	95	600	600

[a]MIC (minimum inhibitory concentration) signified minimum concentration of the preservative at which growth was completely inhibited.
[b]nd, not determined.
[c]Values within parentheses indicate pHs of media after the additions.

The effects of sodium chloride, benzoic acid and nisin on the growth of *B. cereus* 37-B1 are presented in Fig. 23. In each of these cases, the growth declined with the increase in concentration of the preservatives. These three effects along with the effect of pH were subjected to Hoke's experimental design. The selected three points were on the trend lines of growth. While the lowest limits were zero in all the cases (excepting pH), the highest limits were judiciously chosen considering the sub-inhibitory concentration levels of the preservatives along with the recommended concentrations of them (Table 15). Growth of the strain against 19 different combinations of the four types of hurdles is shown in Table 16. There was no growth in 8 sets; all of which, excepting the set A, at least one of the three preservatives was at its maximum concentration tested.

Table 15. Levels of hurdles against *B. cereus* 37-B1 used for the Hoke's experimental design

Hurdle	Hurdle levels[a] tested		
	-1	0	+1
pH	5.6	6.4	7.2
Sodium chloride (mg ml^{-1})	0	20	40
Benzoic acid (µg ml^{-1})	0	300	600
Nisin (µg ml^{-1})	0	25	50

[a]+1, highest limit; 0, mid point; and -1, lowest limit of variable taken for study.

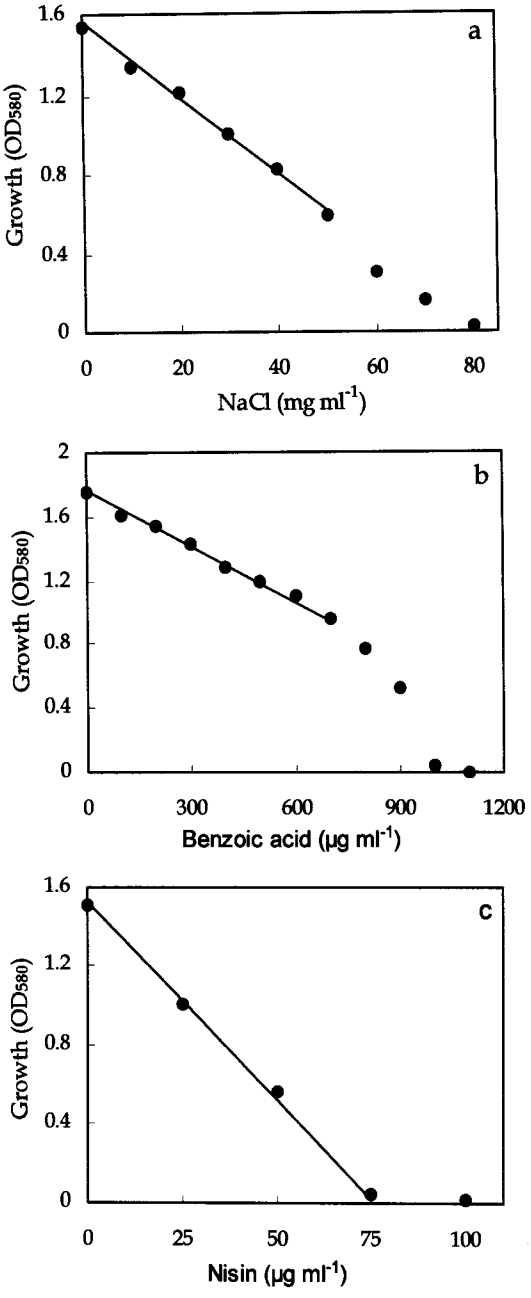


Fig. 23. The effect of sodium chloride (a), benzoic acid (b) and nisin (c) concentration on the growth of *B. cereus* 37-B1

Similarly, the effect of sodium chloride and benzoic acid on the growth of *Salmonella* 1-S4 is presented in Fig. 24. Growth of the strain against 18 different combinations of the three types of hurdles is shown in Fig. 25. There was no growth in one set only, at pH 5.4.

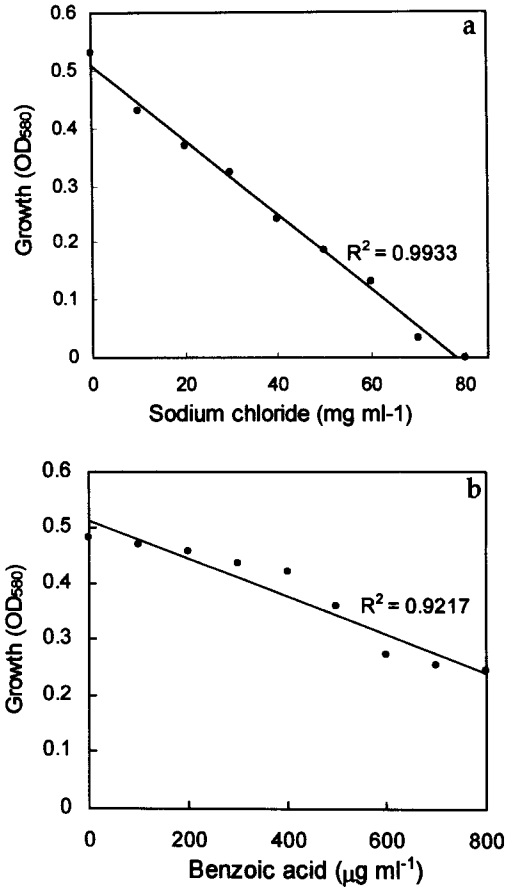


Fig. 24. The effect of sodium chloride (a) and benzoic acid (b) concentration on the growth of *Salmonella* 1-S4

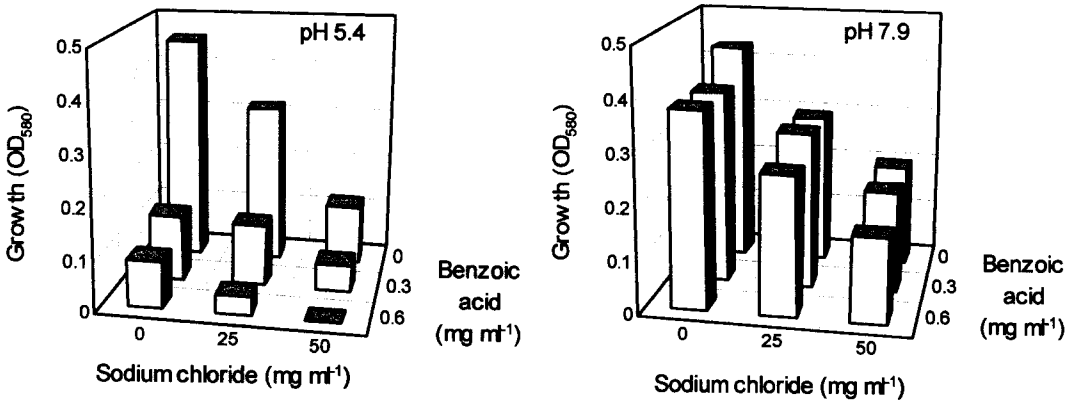


Fig. 25. Combined effect of sodium chloride and benzoic acid at two different levels of pH on the growth of *Salmonella* 1-S4 in nutrient broth at 35°C for 24 h

Table 16. Growth of *B. cereus* 37-B1 in nutrient broth as influenced by a combination of four independent variables (hurdles) following Hoke's response surface design

Hurdle combination no.	Independent variables				Growth (OD_{580}) Mean±SE[a]
	pH	Sodium chloride (mg ml^{-1})	Benzoic acid (µg ml^{-1})	Nisin (µg ml^{-1})	
A	5.6	20	300	25	0
B	6.4	0	300	25	0.32b ± 0.03
C	6.4	20	0	25	0.18d ± 0.02
D	6.4	20	300	0	0.43a ± 0.02
E	5.6	0	0	0	0.46a ± 0.04
F	5.6	40	600	50	0
G	7.2	0	600	50	0.18d± 0.01
H	7.2	40	0	50	0
I	7.2	40	600	0	0.25c ± 0.02
J	7.2	40	0	0	0.26c ± 0.01
K	7.2	0	600	0	0.30b± 0.02
L	7.2	0	0	50	0.24c ± 0.03
M	5.6	40	600	0	0.21d± 0.01
N	5.6	40	0	50	0
O	5.6	0	600	50	0.26c ± 0.02
P	6.4	40	600	50	0
Q	7.2	40	600	50	0
R	7.2	40	300	50	0
S	7.2	40	600	25	0

[a]Values with standard error (SE) were obtained from three replicates. Means within a column sharing a common letter are not significantly different ($P < 0.05$).

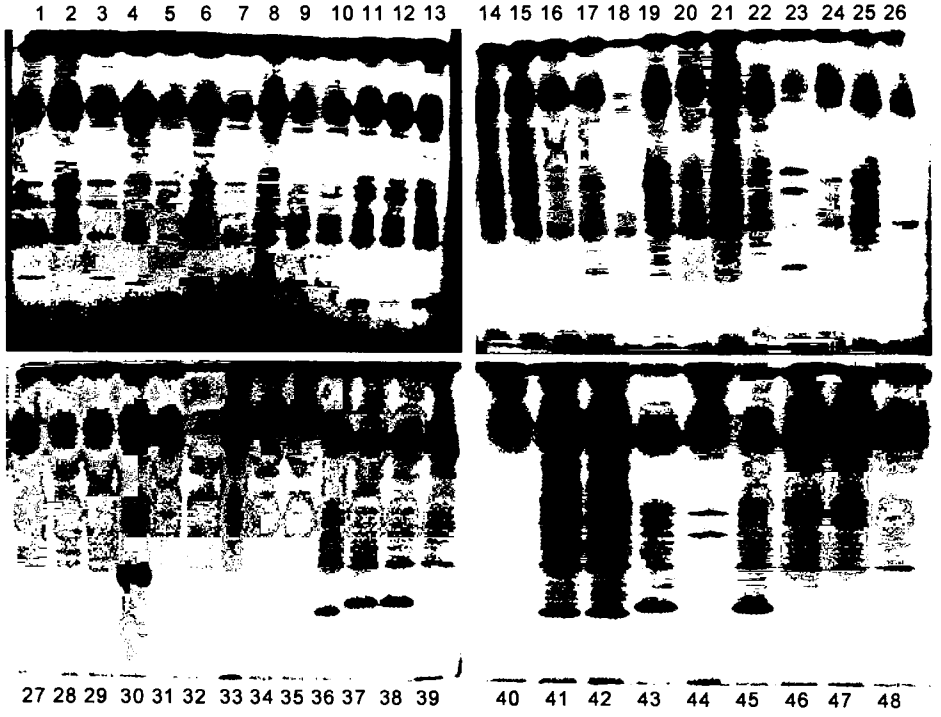


Fig. 26. SDS-PAGE profiles of whole-cell *B. cereus* strains. Lanes: 1, 111-B3 (A11); 2, 111-B2 (A11); 3, 111-B1 (A11); 4, 66-B4 (A9); 5, 66-B3 (C2); 6, 66-B2 (A9); 7, 66-B1 (C1); 8, 49-B2 (A5); 9, 49-B1 (A4); 10, 46-B2 (A4); 11, 6-B2 (A2); 12, 2-B3 (A3); 13, 2-B1 (A1); 14, 18-B2 (B1); 15, 18-B3 (B2); 16, 18-B5 (B3); 17, 52-B1 (B5); 18, 52-B2 (B14); 19, 57-B2 (B6); 20, 57-B3 (B6); 21, 57-B4 (B6); 22, 57-B5 (B15); 23, 70-B1 (B17); 24, 70-B2 (B7); 25, 93-B1 (B16); 26, 93-B2 (B16); 27, 93-B3 (B10); 28, 113-B1 (A12); 29, 113-B2 (B11); 30, 113-B3 (B4); 31, 34-B1 (B11); 32, 35-B1 (C3); 33, 37-B1 (B12); 34, 16-B1 (D); 35, 55-B1 (D); 36, 98-B1 (A7); 37, 98-B2 (A10); 38, 98-B3 (A10); 39, 66-B5 (A8); 40, 104-B1 (B8); 41, 104-B2 (B8); 42, 104-B3 (B8); 43, 105-B1 (A6); 44, 105-B2 (A13); 45, 105-B3 (A5); 46, 94-B1 (B9); 47, 94-B2 (B9); 48, 94-B3 (B13). Cluster/subcluster numbers are shown within parentheses (cf. Fig. 27).

4.9. Whole-cell protein fingerprinting

The whole-cell protein fingerprinting (WCPF) of the 48 isolates of *B. cereus* (Fig. 26) and 33 isolates of *Salmonella* (Fig. 28) yielded distinctly different band patterns. Majority of the strains isolated from the same kind of food were distinguished by their WCPF patterns. Fig. 27 and 29 show a simplified version of the dendrograms obtained from the strains of *B. cereus* and *Salmonella*, respectively. Basically, the WCPF profiles of *B. cereus* could be grouped into four major clusters emerging at a similarity level of 60%. These clusters, designated A through D, represented 40%, 50%, 6% and 2%, respectively, of the total strains. On the other hand, the WCPF profiles of *Salmonella* could be grouped into six major clusters emerging at a similarity level of 80%. These clusters, designated A through F, represented 12%, 15%, 55%, 12%, 3% and 3%, respectively, of the total strains.

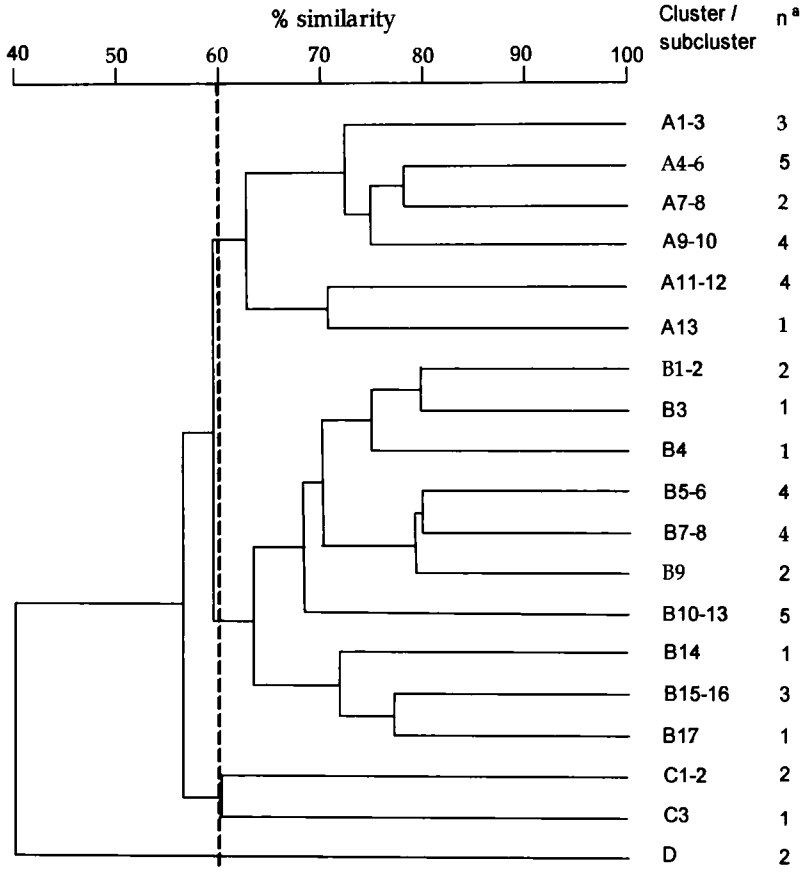


Fig. 27. Simplified dendrogram based on the UPGMA clustering of similarity coefficients (S_D) of whole-cell protein profiles of the 48 strains of *B. cereus* (as shown in Fig. 26). The fingerprint patterns were grouped into four major clusters, designated A through D, on the basis of 60% similarity (arbitrarily chosen) among the strains used.
[a] n, number of strains in cluster/ subcluster.

4.10. Antagonistic activity of lactic acid bacteria against food pathogens

A total of 84 strains of lactic acid bacteria isolated from the batter of dhokla, dosa and idli, and of dried wadi were tested for their antibacterial activity. No antagonistic activity against the *B. cereus*

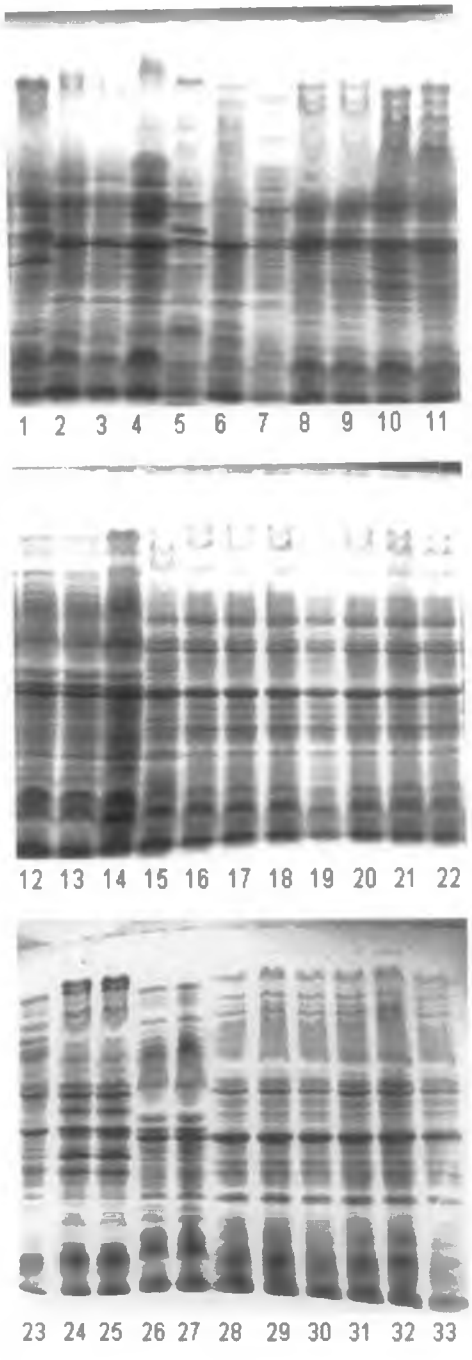


Fig. 28. SDS-PAGE profiles of whole-cell *Salmonella* strains. Lanes 1, 21-S1 (B4); 2, 17-S3 (B3); 3, 17-S2 (B2); 4, 17-S1 (B2); 5, 13-S3 (B1); 6, 11-S2 (F); 7, 7-S5 (E); 8, 7-S3 (C2); 9, 7-S2 (C6); 10, 1-S6 (C1); 11, 1-S5 (A1); 12, 83-S1 (D1); 13, 68-S4 (D1); 14, 68-S1 (C3); 15, 43-S6 (C3); 16, 43-S5 (C3); 17, 43-S4 (C3); 18, 43-S3 (C3); 19, 43-S2 (C3); 20, 43-S1 (C3); 21, 23-S2 (C3); 22, 23-S1 (C3); 23, 1-S4 (A3); 24, 21-S2 (A2); 25, 21-S3 (A2); 26, 83-S2 (D2); 27, 83-S3 (D2); 28, 84-S1 (C4); 29, 84-S2 (C4); 30, 84-S3 (C4); 31, 85-S1 (C4); 32, 85-S2 (C4); 33, 85-S3 (C5). Cluster/ subcluster numbers are shown within parentheses (cf. Fig. 29).

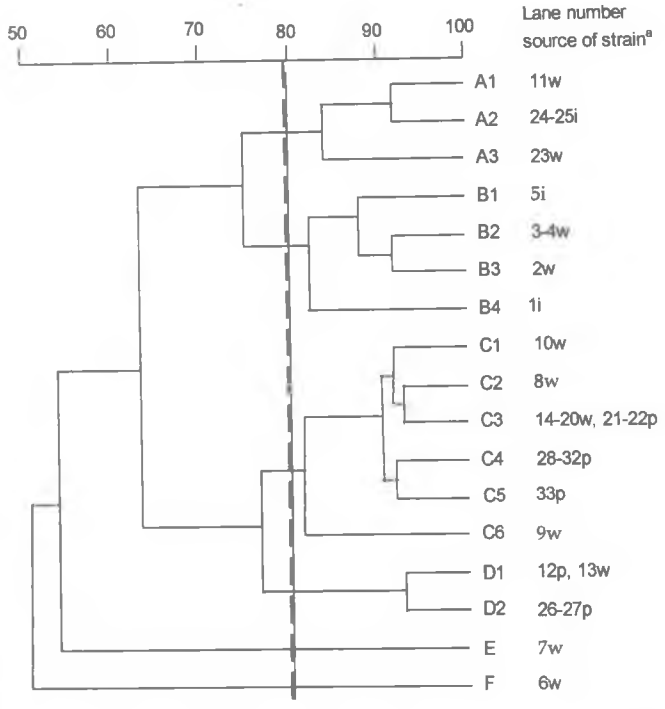


Fig. 29. Simplified dendrogram based on the UPGMA clustering of similarity coefficients (S_D) of whole-cell protein fingerprint profiles of the 33 strains of *Salmonella* (as shown in Fig. 28). The fingerprint patterns were grouped into six major clusters, designated A through F, on the basis of 80% similarity (arbitrarily chosen) among the strains used.
[a]Source: i, idli; p, papad; w, wadi.

isolated from wadi (strain S2-B1) or idli (strain S94-B1) could be found. The agar-spot assay revealed that none of the tested 15 strains of lactic acid bacteria isolated from laboratory-made fermenting batter of idli inhibited the growth of *B. cereus* 94-B1, *E. coli* 61-E2 and *S. aureus* 34-S1.

4.11. Microbial challenge testing

The quantitative changes in microbiota along with pH and volume of dhokla batter during natural fermentation are presented in Fig. 30. The count of total aerobic mesophilic bacteria occurring in the batter increased ($P < 0.05$) from initial 6.5 log cfu g^{-1} batter to 10.5 log cfu g^{-1} after 15 h of fermentation. The lactic acid bacterial count increased ($P < 0.05$) from initial 5.2 log cfu g^{-1} to 8.5 log cfu g^{-1} at the end. From an initial level of 3.6 log cfu g^{-1}, the yeast population reached 7.1 log cfu g^{-1} after 15 h of fermentation. When freshly prepared batter was intentionally inoculated

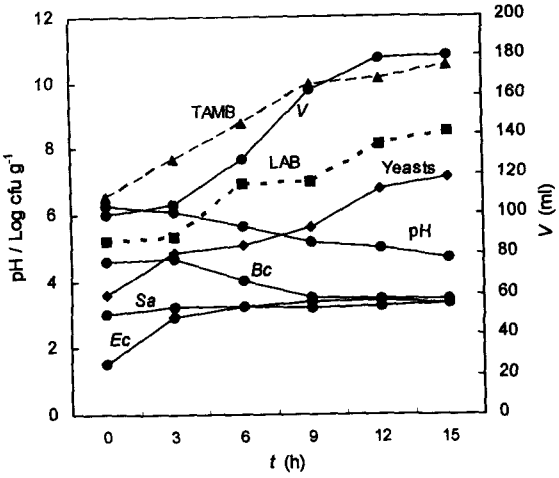


Fig. 30. Changes in pH, volume (*V*) and microbial cell count of dhokla batter during natural fermentation. Values are the means of nine batches of fermentations.
Abbreviations: TAMB, total aerobic mesophilic bacteria; LAB, lactic acid bacteria; *Bc, B. cereus; Sa, S. aureus; Ec, E. coli.*

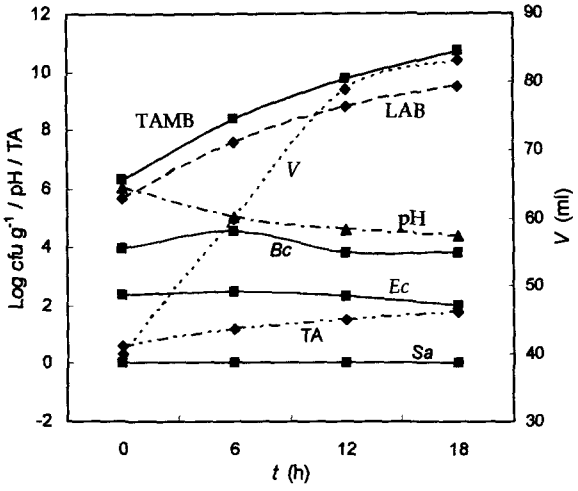


Fig. 32. Changes in pH, volume (*V*), titratable acidity (TA) and bacterial cell count of idli batter during natural fermentation. Values are the means of nine batches of fermentation.
Abbreviations: TAMB, total aerobic mesophilic bacteria; LAB, lactic acid bacteria; *Bc, B. cereus; Sa, S. aureus; Ec, E. coli.*

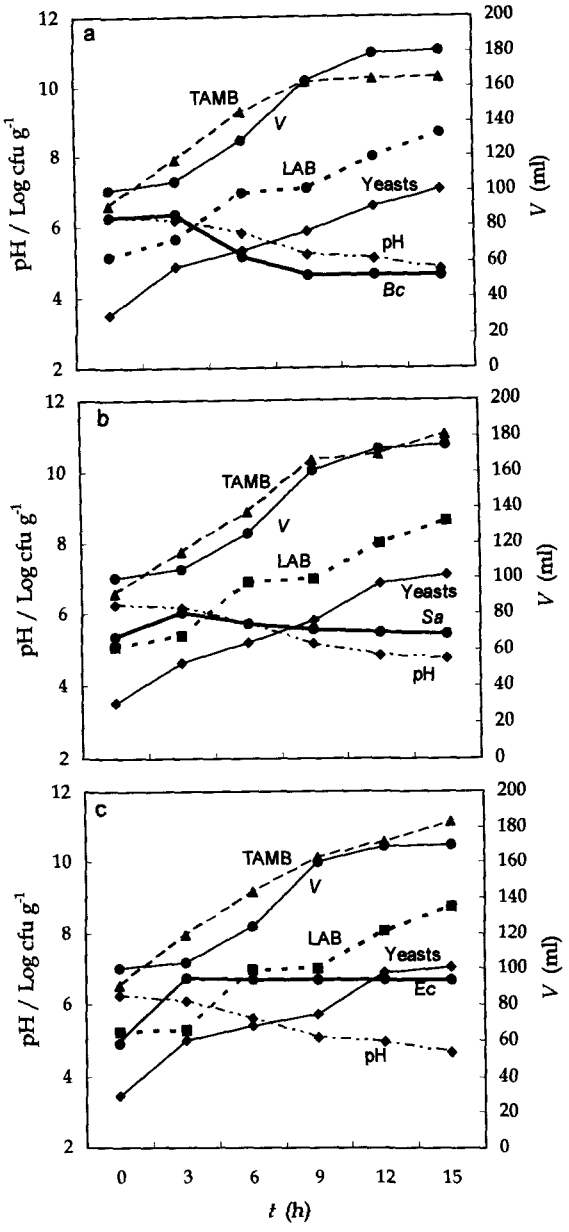


Fig. 31. Changes in pH, volume (*V*) and microbial cell count during fermentation of dhokla batter which was intentionally inoculated at the start with *Bc (a), Sa (b)* or *Ec (c)*. Values are the means of nine batches of fermentations.
Abbreviations: TAMB, total aerobic mesophilic bacteria; LAB, lactic acid bacteria; *Bc, B. cereus; Sa, S. aureus; Ec, E. coli.*

with *B. cereus* at a level of 6.2 log cfu g^{-1}, the pathogen remained unaffected ($P < 0.05$) during the first 3 h of fermentation, however the count decreased ($P < 0.05$) after 6 h and then again after 9 h of fermentation (Fig. 31a). *S. aureus*, although increased 1 log-cycle after 3 h, returned to the original level after 6 h and then remained unchanged ($P < 0.05$) till the end of fermentation (Fig. 31b). *E. coli*, on the other hand, exhibited a better ($P < 0.05$) growth during the fermentation (Fig. 31c). After steaming the uninoculated but fully fermented batter for 15 min in a pan, the load of *B. cereus, S. aureus* and *E. coli* in dhokla cakes reached below their detection limit (Table 17). However, in intentionally inoculated batter, *B. cereus*

Table 17. Viable count of indicator organisms in fermented batters and cakes of dhokla and idli, prepared following steaming for 15 min

Organism	Intentional inocualtion	log cfu g^{-1a} fresh wt			
		Dhokla		Idli	
		Before steaming	After steaming	Before steaming	After steaming
B. cereus	-	3.4	<dl	3.8	<dl
	+	4.6	2.6	4.1	2.0
S. aureus	-	3.3	<dl	<dl	<dl
	+	4.5	<dl	4.8	<dl
E. coli	-	3.3	<dl	2.0	<dl
	+	4.7	<dl	4.4	<dl

[a]dl, detection limit (2.0 log cfu g^{-1}).

reduced (P <0.05) but survived steaming at a level of 2.6 log cfu g^{-1} dhokla cakes. *S. aureus* and *E. coli* could not be detected in the cakes prepared from intentionally inoculated batter.

The quantitative changes in microbiota along with pH, titratable acidity and volume of idli batter during natural fermentation are presented in Fig. 32. When freshly prepared batter was intentionally inoculated with *B. cereus* at a level of 5.6 log cfu g^{-1}, the pathogen not only survived but also grew positively (P <0.05) during the first 6 h of fermentation, however the cell count reduced (P <0.05) by more than 1 log cycle after 12 h and remained unchanged (P <0.05) thereafter (Fig. 33a). A similar trend was noticed in cases of *S. aureus* (Fig. 33b) and *E. coli* (Fig. 33c). After steaming the naturally fermented batter for 15 min in an idli pan, the load of *B. cereus* and *E. coli* in idli cakes reached below their detection limit (Table 17). However, in intentionally inoculated batter at a much higher load, *B. cereus* reduced quantitatively but survived steaming at a level of 2.0 log cfu g^{-1} idli cakes. *S. aureus* and *E. coli* could not be detected in idli cakes prepared from intentionally inoculated fermented batter.

The quantitative changes in microbiota along with pH and moisture content of wadi dough during natural fermentation are presented in Fig. 34. The count (3 log cfu g^{-1}) of *B. cereus* decreased (P <0.05) during the first 10 h of fermentation, and again further during the first 12 h of drying; after 24 h of drying the count went below the limit of detection. *S. aureus* and *E. coli* could not be detected at any stage of fermentation and drying. Intentional inoculation of wadi dough at the onset of fermentation with *B. cereus*,

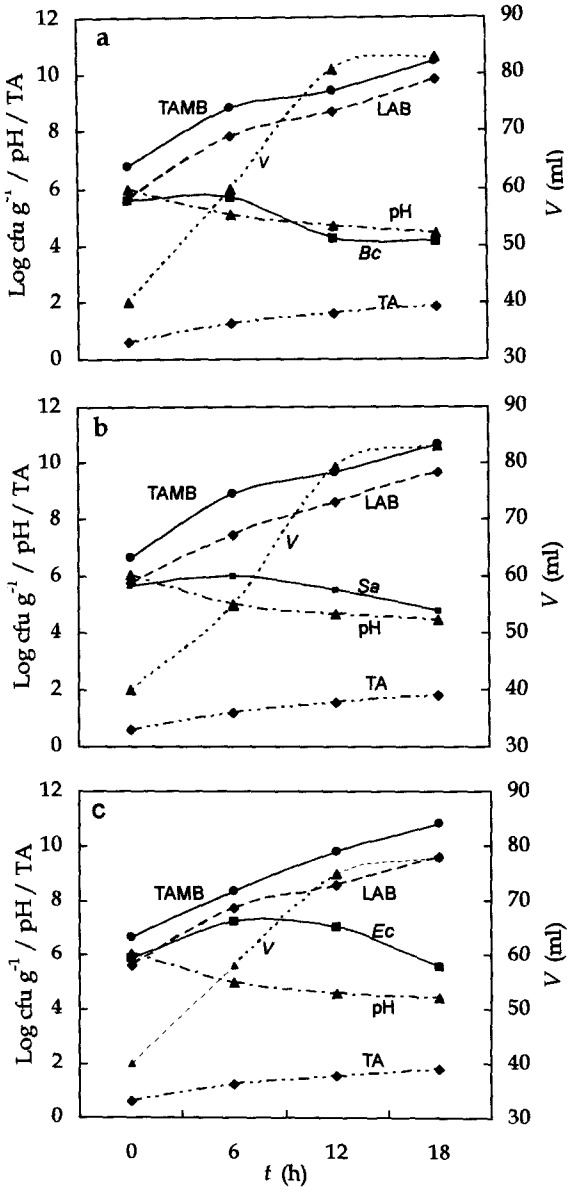


Fig. 33. Changes in pH, volume (*V*), titratable acidity and bacterial cell count during fermentation of idli batter which was intentionally inoculated at the start with *Bc* (a), *Sa* (b) or *Ec* (c). Values are the means of nine batches of fermentation.
Abbreviations: TAMB, total aerobic mesophilic bacteria; LAB, lactic acid bacteria; *Bc*, *B. cereus*; *Sa*, *S. aureus*; *Ec*, *E. coli*; TA, titratable acid (as % lactic acid).

S. aureus or *E. coli* cells had no apparent influence on the growth of inherent lactic acid bacteria, yeasts and total aerobic mesophilic bacteria, and changes in pH and dough volume, which are the cause and consequence of this autofermentation (Fig. 35). None of the pathogenic bacteria, either inherent or introduced to the dough at the start of fermentation, survived after 36 h of drying (Figs. 34 and 35). When freshly prepared dough was intentionally inoculated at a level of 5.2 log cfu g^{-1} (Fig. 35a), *B. cereus* could survive only for a while; the count reduced by 1 log cycle after 10 h of fermentation, and after 24 h of drying it could not be detected. After inoculation of dough with *S. aureus* at a level of 5.4 log cfu g^{-1} (Fig. 35b), the count remained unchanged ($P < 0.05$) during the first 10 h of fermentation, but decreased ($P < 0.05$) at every 12 h interval of drying, and went below the detection limit after 36 h. In contrast to the earlier two, the count of *E. coli* increased ($P < 0.05$) from 5.1 log cfu g^{-1} dough to 5.5 log cfu g^{-1} during the first 10 h of fermentation (Fig. 35c). The count then remained unchanged ($P < 0.05$) till 24 h of drying, however, *E. coli* cells could not be detected after 36 h.

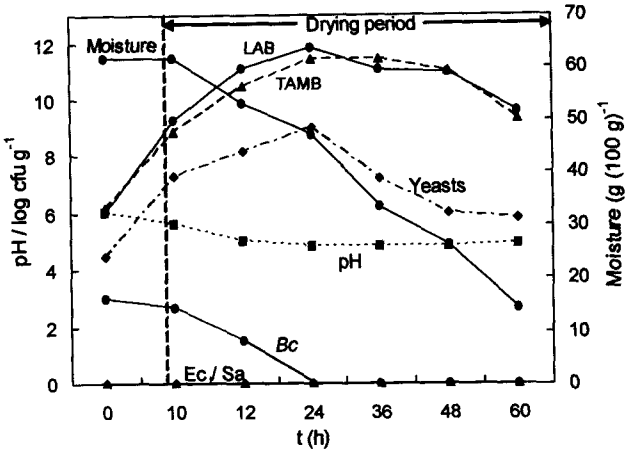


Fig. 34. Changes in pH, moisture and cell count of wadi dough during natural fermentation and drying. Values are the means of nine batches of fermentations.
Abbreviations: TAMB, total aerobic mesophilic bacteria; LAB, lactic acid bacteria; *Bc*, *B. cereus*; *Sa*, *S. aureus*; *Ec*, *E. coli*.

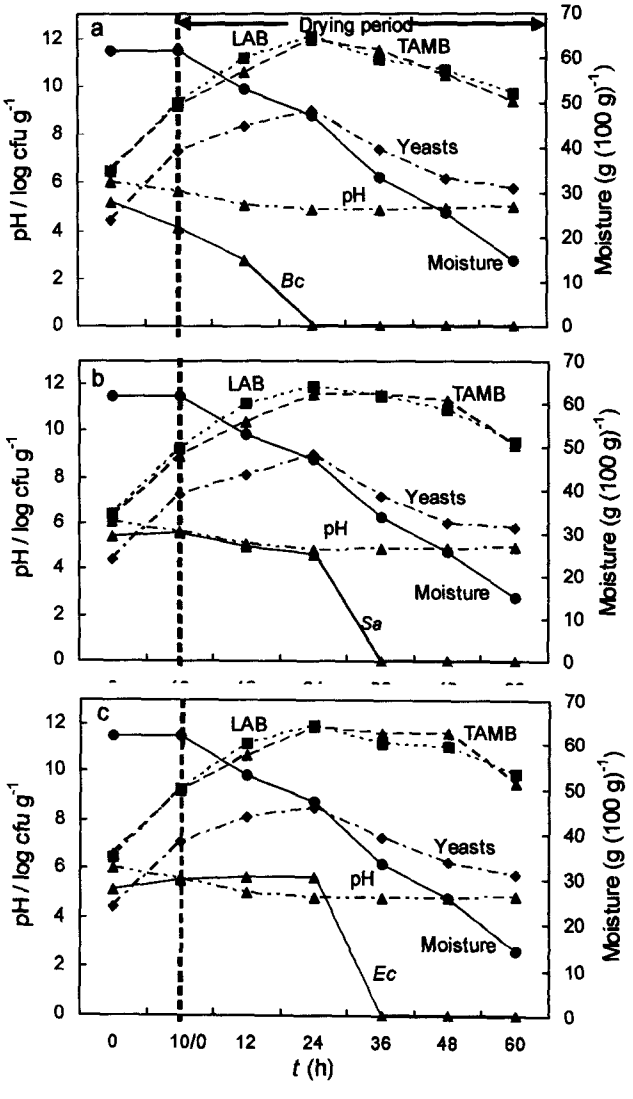


Fig. 35. Changes in pH, moisture and cell count during fermentation and drying of wadi dough which was intentionally inoculated at the start with *Bc* (a), *Sa* (b) or *Ec* (c). Values are the means of nine batches of fermentations.
Abbreviations: TAMB, total aerobic mesophilic bacteria; LAB, lactic acid bacteria; *Bc*, *B. cereus*; *Sa*, *S. aureus*; *Ec*, *E. coli*.

5

Discussion

5.1. Occurrence of foodborne bacterial pathogens in foods

Besides nutritional upliftment, fermentation of legume alone, or in combination with cereal, exhibits the capability of transforming a regular diet into delicacy. Though generally considered safe from the microbiological point of view due to activity of fermenting micro-organisms, these foods can become a vehicle of dreaded foodborne bacterial pathogens. Degree and frequency of contamination of the final product are influenced by hygienic condition of production area, personal hygiene as well as contaminated substrates. It is well approved that raw rice and pulses provide a hospitable environment for the growth of *B. cereus* and others (Chung and Sun 1986; Goepfert *et al.* 1972; Johnson 1984; Meena *et al.* 2000; Sarrias *et al.* 2002; Shah *et al.* 1996).

The moisture profile of legume-based traditional fermented foods (Table 8) varied greatly due to differences in the processes of their preparation. Because fermented batter was subjected to steaming, dhokla and idli contained a high moisture (62 g $(100\ g)^{-1}$). Amriti and dosa were intermediate- moisture foods (IMFs; 20-27 g $(100\ g)^{-1}$), because they were prepared by frying the fermented batter in oil. As prolonged air- and sun-drying were the final steps in the preparation of papad and wadi, they contained least moisture (14-18 g $(100\ g)^{-1}$).

The total aerobic mesophilic bacterial count in the foods varied greatly (Table 8 and Fig. 20). Its high count (> 10^4 cfu g^{-1}) in majority of the samples of all the tested foods, except dosa, indicates a lapse in good hygienic practices followed during preparation. While most of the samples of amriti, dosa, idli and papad contained these bacteria in the range of 10^2-10^6 cfu g^{-1}, in all the samples of dhokla and most (91%) of the samples of wadi the count was at a higher ($P < 0.05$) level (>10^6 cfu g^{-1}). Since marketed amriti, dosa and idli are either fried or steamed at the final stage of their preparation, death of most of the functional micro-organisms and associated microbiota (excepting those occurring in 'cool pockets') and a consequent low count of total aerobic mesophilic bacteria were expected in those samples. Although dhokla is a steamed product, their high count (> 10^6 cfu g^{-1}) in 100% of the samples might be mostly due to post-preparation contamination introduced from seasoning ingredients, including spices, chilly, grated coconut and curry (*Murraya koenigii*) leaves accompanied with a high moisture content of the product. Plate count agar is a non-selective complex medium commonly used for enumerating total microbial content in foods. So the viable count in the samples of papad and wadi, which do not pass through any heat treatment process, was likely of fermenting micro-organisms along with associated contaminating microbiota. Detection of the presence of a high count of total aerobic mesophilic bacterial cells in majority of the samples indicates that either highly contaminated substrates were used or poor processing practices, e.g. inappropriate handling or unhygienic condition were involved, as was observed during a study on sufu (Han *et al.* 2001). Considering that no sign of spoilage was recorded at the time of samplings, it might be assumed that most mesophilic bacterial spores either did not germinate or were not metabolically active in these products.

The external surface of the cereal grains is heavily contaminated with saprophytes acquired during development of the plants along with contaminants from soil, air, animals and also humans (Sarrias *et al.* 2002). *Bacillus* spp. are important as food-spoilage organisms, and can be isolated from a variety of animal and plant products (Johnson 1984). All the samples of amriti, dhokla and papad contained aerobic mesophilic bacterial spores (Table 8 and Fig. 20). A high count (> 10^5 cfu g^{-1}) of them was found in papad and wadi (10 out of 63 samples). As dosa samples were freshly prepared ones, their load in the product was never more than 10^5 cfu g^{-1}. On the other hand, the load of their anaerobic counterpart was less (Table 8 and Fig. 20); they occurred in 39% of the tested samples (41 of 105). However, amriti, dosa and idli samples were free of them.

All the six kinds of foods contained *B. cereus* in an overall load of 20% (21 of 105) of the total samples analysed (Table 8 and Fig. 20). Most of the samples (60%) of dhokla were contaminated with this pathogen, probably due to post-preparative contamination and contaminated seasoning ingredients. Only 8% (1 of 13) of the samples of idli contained this pathogen. The total number of *B. cereus* organism required to be ingested to produce illness is likely in the range of 10^5-10^6 viable cells or spores. For the lower dose it is likely that only spores, which all survive the stomach acid barrier, can cause the disease. Hence, food containing > 10^3 *B. cereus* cells g^{-1} spores cannot be considered completely safe for consumption (Granum 2007). The potentially hazardous level (>10^4 cfu g^{-1}) was observed in dhokla (2 of 5 samples), papad (1 of 29 samples) and wadi (2 of 34 samples). *B. cereus* is a common soil saprophyte and is easily spread to many types of foods, especially of plant origin (Granum 2007). The presence of considerably high levels of *B. cereus* was recorded in several legume-fermented foods, such as Indonesian tempe (Samson *et al.* 1987), African dawadawa (Antai and Ibrahim 1986) and Indian kinema (Nout *et al.* 1998). The presence of this bacterium at high levels suggests a potential risk of these foods to the health of consumers, because of the subsequent production of toxin associated with food poisoning (Banerjee and Sarkar 2004b). However, it was found in a legume food (kinema) that in the presence of functional bacterium (*Bacillus subtilis*), the growth of *B. cereus* was adversely affected and the production of enterotoxin was ceased (Nout *et al.* 1998). *C. perfringens* could not be

detected in any of the 105 samples, and only one sample was found contaminated (at the load of 4×10^4 cfu g^{-1} dhokla) with *S. aureus*. The latter organism was not detected in kinema also (Nout *et al.* 1998). Possibly, the lack of initial contamination, or the impact of competition and/or antagonistic reactions would have prevented its proliferation. So, considering the presence of foodborne bacterial pathogens, marketed samples of dhokla pose significant health risk to the consumers.

Enterobacteriaceae counts are considered more generally as an indicator of hygienic quality rather than of faecal contamination, and therefore, say more about general microbiological quality as than possible health risks posed by the product (Adams and Moss 1995). Enterobacteriaceae occurred in all the six kinds of foods studied (Table 8 and Fig. 20). Majority of the samples of idli (54%) and wadi (75%) were found contaminated with Enterobacteriaceae. Coliform and faecal coliform were detected from 29% (30 of 105) and 11% (12 of 105), respectively, of the samples (Table 9). The occurrence of these micro-organisms in a food is considered a reflection of the process practised during its preparation and/or subsequent handling under inefficient hygienic condition (ICMSF 1978). The presence of a high count of faecal coliform in dhokla, papad, wadi and even freshly prepared idli indicates a high risk that other pathogenic micro-organisms have also contaminated the food. One sample each of idli (3.8×10^3 cfu g^{-1}) and wadi (3.2×10^4 cfu g^{-1}) were found contaminated with *E. coli*. The prevalence of Enterobacteriaceae in these foods could be considered as undesirable and emphasizes an improvement of general hygienic condition of processing environment as well as personal hygiene.

Although the presence of *Salmonella* in foods of animal origin has been well documented, very limited studies are available on vegetarian foods, particularly the processed ones (Yadav *et al.* 2000). More than 95% of the cases of infections caused by *Salmonella* are foodborne, and these infections account for about 30% of deaths resulting from foodborne illnesses (Hohmann 2001). *Salmonella* was present in 11.4% of the total (12 of 105) samples analysed. It was not detected in amriti, dhokla and dosa. However, its prevalence in the other three foods is noteworthy; 15% (2 of 13), 14% (4 of 29) and 18% (6 of 34) of the samples of idli, papad and wadi, respectively, were found contaminated with this pathogen. Since marketed idli is RTE, the presence of *Salmonella* in idli is alarming. However, it is likely to be killed during heat-processing for consumption of papad and wadi. Interestingly, *Shigella* could not be detected in any of the samples.

The microbial composition of these products indicates that their manufacturing processes did not support survival and/ or growth of *S. aureus* and *Shigella*. Detection of the presence of a high count of total aerobic mesophilic bacteria, *B. cereus* and Enterobacteriaceae in all these foods suggests that a better control is needed and that some changes in the manufacturing practices, storage, distribution and service should be made to enhance their microbial safety. However, due to absence of any standard guidelines of these marketed foods, a comparative statement could not be generated. In general, out of these six kinds of marketed foods, amriti and dosa were found to be of relatively better microbiological quality.

Among the critical control points (CCPs) are raw materials, water, beating or mixing batter or dough with bare hands, utensils, drying environment (in case of papad and wadi), post-preparative storage conditions, and dish cloth. Many times, uncleaned raw materials (rice, blackgram and Bengalgram) are used. In most cases, due to lack of running water availability producers store water under vulnerable conditions subject to contamination. Personal cleanliness is another aspect which can reduce foodborne hazards; use of gloves at the time of beating or mixing batter or dough reduces the chance of cross-contamination. Utensils represent an important source of food contamination, since the same utensils are used in different batches of preparation without any in-between cleaning. Most of the fermented foods under study are taken RTE for sale and are, therefore, most susceptible to microbial growth in view of the longer length of time between preparation and consumption under

improper temperature conditions (30-38 °C). Open-air drying of papad and wadi leads exposure of these foods to aggravating environmental conditions, such as the presence of insects, rodents, other animals and dust. The dish cloth used in several tasks represents another hazard to the safety of foods. Hence, training of people, and producers and sellers in particular, for a cultural change would be one of the most effective interventions to reach a safer food.

5.2. Behaviour of pathogenic bacterial isolates from foods

5.2.1. Susceptibility to antimicrobials

Study was undertaken to evaluate the extent of prevalence of antibiotic resistance patterns in the food isolates (Table 10). Antibiotic sensitivity study shows that all the *B. cereus* isolates were multi-drug resistant; each of these was resistant to at least nine different antibiotics. Most of the antibiotics against which the isolates showed resistance belonged to different groups, including β-lactam (ampicillin, carbenicillin, cephalothin, cloxacillin and penicillin G), glycopeptide (vancomycin), peptide (bacitracin and polymyxin B) and trimethoprim. Most of these antibiotics inhibit synthesis of prokaryotic cell wall. As expected, metronidazole, an antiprotozoal drug, had no action on any of the isolates. All these isolates, enriched on *B. cereus* selective medium (which contained 100 U polymyxin B ml^{-1}), were resistant against even a higher concentration (300 U $disc^{-1}$) of polymyxin B. However, an earlier study (Banerjee and Sarkar 2004a) reported susceptibility of only 8% of the 84 *B. cereus* isolates from spices to this higher concentration of polymyxin B. The presence of such a high number of multiple-antibiotic resistant strains of *B. cereus* in foods is a matter of concern. Although use of antibiotics is not the rule of treating gastroenteritis, it is a common therapeutic measure taken (e.g., vancomycin) to combat acute necrotizing gastritis caused by *B. cereus*, particularly in immunocompromised patients (Le Scanff *et al.* 2006).

The most potent groups of antibiotics against which 75% or more of the Enterobacteriaceae strains were sensitive were benzene derivative (chloramphenicol), quinolone (ciprofloxacin), aminoglycosides (kanamycin) and tetracycline. These antibiotics act by inhibiting protein and nucleic acid syntheses. All the 24 strains of Enterobacteriaceae were multiple-antibiotic resistant. As expected, 100% of the strains were resistant to nitro-imidazole (metronidazole). Maximum number (> 50%) of the strains were resistant to a combination of β-lactams (carbenicillin, penicillin G), nitro-imidazole (metronidazole), macrolides (erythromycin, rifampicin), glycopeptide (vancomycin) and trimethoprim.

All the 33 strains of *Salmonella* were sensitive to benzene derivative (chloramphenicol). The next potent group of antibiotics against which 73-79% of the isolates were sensitive was quinolones (ciprofloxacin and nalidixic acid). However, 100% of the strains were multiple-antibiotic resistant, against at least two antibiotics (erythromycin and metronidazole); 9.1% of the isolates were resistant against as many as 14 antibiotics. A high level of resistance (> 80%) was found against nitro-imidazole (metronidazole), macrolides (erythromycin and rifampicin), glycopeptide (vancomycin) and most of the β-lactams (ampicillin, cloxacillin and penicillin G). This reflects the probability of abusive uses of antibiotics in bacterial infections leading to selection and stability of antibiotic resistant genes followed by their subsequent transfer. This trend is alarming because the isolates may transfer the resistant genes to other members of Enterobacteriaceae. Multiple-antibiotic resistance in *Salmonella* is now the norm in strains originating in the Indian Subcontinent and South-east Asia (Threlfall 2002). The World Health Organization noted an alarming increase in the incidence of antibiotic-resistant *Salmonella* strains isolated from animals and humans (Brisabois *et al.* 1997). Our results, indicating that all the *Salmonella* strains were resistant to erythromycin and metronidazole, support the observation

of Tripathi (1999) and Banerjee and Sarkar (2004a). Genes for resistance and molecular transfer mechanisms have been shown to be the same in bacteria from food and from pathogenic (animal and human) samples. This situation led to scientific and political efforts to handle the problems of antibiotic resistance in food (Teuber *et al.* 1999). Further research is needed to elucidate genetic and biochemical bases of antimicrobial resistance in these bacterial pathogens and the potential role of antimicrobial resistance in the outbreaks of foodborne illnesses.

5.2.2. Thermal inactivation of sporeformers

Foods are often subjected to thermal process in a number of different ways such as cooking, baking, boiling, frying, pasteurization and appertization. As a side effect, these processes often destruct, at least partially, the micro-organisms present in the substrate or the additives. The source of contamination is from spores, naturally present in food, that are able to survive normal cooking procedures. Detection of *D*-values for isolated spores helps to understand the hazardous potential of this organism which can survive the cooking processes.

In glucose-supplemented brain heart infusion broth, the correlation coefficient (R^2) values of decimal reduction time curves were in all cases at least 0.91. The $D_{100°C}$-values of the spore suspensions of 12 different *B. cereus* isolates (two from each of the six kinds of foods) were 3.0-9.2 min (Table 11), which suggests that time-temperature exposure at an appropriate level during cooking may destroy heat-sensitive spores, but not the heat-resistant ones. However, the response of *B. cereus* spores to heating is strain-dependent and is influenced by medium composition (Chung and Sun 1986). $D_{100°C}$-values of 2.7-3.1 min in skimmed milk (Mikolajcik 1970), 0.6-27.0 min in demineralized water (Rajkowski and Mikolajcik 1987) and 3.5-5.9 min in glucose-supplemented brain heart infusion broth (Banerjee and Sarkar 2004a) were reported for *B. cereus* spores. The spores of *B. cereus*, which may survive heat treatment (e.g., cooking, steaming and frying) during final step of preparation of these foods, germinate when kept at room temperature before consumption.

The data represented here can be used as an aid to predict the time required at 100 °C to achieve a certain number of log-cycle reductions of this potentially dreadful sporeformer.

5.2.3. Production of extracellular enzymes

Microbial enzymes are the major causes of quality deterioration and food spoilage (Braun *et al.* 1999). Activity of the enzymes, like protease, lipase and amylase indicates spoilage potentiality of the producing organisms. While 50% of the 48 isolates were capable of producing at least one of these three enzymes, 23% could produce all these enzymes in vitro (Table 12). From the results obtained, it can be concluded that many of the strains of *B. cereus* present in fermented foods have the potentiality of causing food spoilage also.

5.2.4. Influence of hurdles on growth

All the food isolates except, *B. cereus*, grew optimally at near-neutral pH. While the *Salmonella* isolates grew optimally at pH 7.3, the optimum pH for the growth of *S. aureus* and *E. coli* isolates was 6.1. This implies that if these pathogens contaminate the raw material well before fermentation has acidified the substrate, their number may reach a hazardous level.

Now-a-days, the use of natural antimicrobial compounds to preserve foods are widely used because of the consumers' demand for additive-free, fresher and more natural tasting food products,

while maintaining microbiological safety (Gould 1996). For the last several years, the use of chemical compounds in food products has come under increased criticism (Sofos and Busta 1981). So, this study was carried out to determine the effect of different levels of sodium chloride, weak acid preservatives and nisin on the growth of different food isolates.

The addition of salt to foods have been known for centuries. As common salt acts chiefly by reducing the water activity (a_w) of foods, its spectrum of action is governed by the demands imposed on a_w by the various micro-organisms (Lueck 1980). *S. aureus* tolerated highest salt concentration, up to 110 mg ml^{-1} which supports a halotolerant character of the bacterium. Others tolerated up to 95 mg sodium chloride ml^{-1}, and that is why salty foods contain a low number of pathogenic bacteria. However, since the a_w-value of saturated common salt solution is only about 0.75 and a number of micro-organism varieties continue to grow below this limit, it is impossible to protect a foodstuff reliably from all microbial attack by using common salt alone, quiet apart from the virtually unacceptable restrictions imposed on taste (Lueck 1980).

For thousands of years, the use of decreased pH has enhanced microbiological stability. In most cases weak organic acids, like benzoic acid and sodium benzoate, have been permitted for food preservation for many years. Apart from a few exceptions, the maximum permissible quantities are between 1500-2500 μg ml^{-1} (Lueck 1980). Benzoic acid is used as an acid or sodium salt at a concentration of 500-2000 μg ml^{-1} in many low pH products (Ray 2001). All the isolates tested were inhibited by benzoic acid concentration within permissible range (Tables 13 and 14).

Sorbic acid and sorbates are permitted in all countries of the world for the preservation of many foods, like margarine, cheese, dried and bakery foods using 500-2000 μg g^{-1} (Ray 2001). The maximum permissible quantity, other than in exceptional situations, is between 1000 and 2000 μg ml^{-1}. In the US, sorbic acid is a GRAS (generally recognized as safe) substance and its use is permitted in any food product to which preservatives may be added (Liewen and Marth 1985; Lueck 1980). In this study all the tested strains of pathogenic bacteria were inhibited at 800 μg sorbic acid ml^{-1}, the level which is lower than the one (900 μg ml^{-1}) reported by Del Torre *et al.* (2001). As the undissociated form of benzoic and sorbic acids is primarily responsible for antimicrobial activity, and it is highly pH dependent (Jay 1996), they would be much effective in controlling pathogenic bacteria in lactic fermented foods.

The toxicological data reveal that nisin can be considered as safe for human health (Frazer *et al.* 1962). Nisin exhibits a wide range of inhibitory effects against Gram positive sporeformers and pathogens, while it shows little or no inhibitory effects against Gram negative bacteria (Hurst 1981). However, in the present study it had a low level of inhibitory activity (≥ 175 μg ml^{-1}) against the strains tested. This result was consistent with the finding of Banerjee and Sarkar (2004a) who have reported MICs of ≥125 μg ml^{-1} for the *B. cereus* strains tested. Nisin is often used in acidic food, but it is effective in products across a wide range of pH value (3.5-8.0). Nisin seems to be a very effective preservative in liquid egg, which generally has a pH of 7.3-7.8 (Thomas *et al.* 2000). The use of nisin as the sole preservative for a food product would probably be unwise, as multiple exposures of a pathogen to nisin would greatly increase the probability of generating stable resistant mutants. However, coupling nisin with several other common food preservation strategies greatly reduces the frequency at which resistance arises (De Martinis *et al.* 1997). In fact, in most applications, nisin serves as one part of a multiple-barrier inhibitory system.

For centuries, foods have been preserved by heating chilling, drying, salting, conserving, acidification, oxygen-removal, fermenting and adding various preservatives. Often these methods were applied in combinations. More recently, the underlying principles of these traditional methods have been defined and effective limits of factors for microbial growth, survival and death were established (Leistner and Gould 2002). The microbial stability and safety of most foods are based on a

combination of several preservative factors (hurdles), which micro-organisms present in the food are unable to overcome. Using an intelligent combination of hurdles it is possible to improve not only the microbial stability and safety but also the sensory and nutritive quality as well as economic aspects of a food (Leistner 1985, 1987, 1992, 1994).

Food preservation implies exposing micro-organisms to a hostile environment in order to inhibit their growth, shorten their survival or cause their death. The effect of combination of pH, sodium chloride, benzoic acid and nisin on the growth of *B. cereus* 37-B1 (Table 16) and the effect of combination of pH, sodium chloride and benzoic acid on the growth of *Salmonella* 1-S4 (Fig. 25) were investigated in order to understand the scientific basis for an efficient application of hurdle technology in preservation of food which can be contaminated by these pathogens.

Prior to undertaking the in vitro multiple-hurdle preservation strategy, the effects of individual hurdles, namely pH, sodium chloride, benzoic acid and nisin on the growth of *B. cereus* 37-B1 were studied separately (Fig. 23). Out of 19 different combinations, there was no growth in eight sets (Table 16). The judicious combination found for the cessation of growth of *B. cereus* 37-B1 was 20 mg sodium chloride ml^{-1} in association with 300 µg benzoic acid and 25 µg nisin ml^{-1} at pH 5.6 (set A), in which all the three preservatives were in moderate concentrations. The same effect was achieved by the omission of benzoic acid, however only at higher concentrations of the other two preservatives (sets H and N). Harris *et al.* (1991) found that the effectiveness of nisin was slightly enhanced by supplementing 25 mg sodium chloride ml^{-1} in a nisin solution.

The effects of individual hurdles, namely pH, sodium chloride and benzoic acid on the growth of one isolate were also studied separately (Fig. 24). The correlation coefficient (R^2) values of the survival curves were in all cases > 0.92. Out of 18 different combinations, there was no growth in only one set (Fig. 25). Hence, the logical combination found for the cessation of growth of *Salmonella* 1-S4 was 50 mg ml^{-1} sodium chloride and 600 µg ml^{-1} benzoic acid at pH 5.4. The results of this study will be the basis for an efficient application of hurdle technology in preserving legume-based fermented foods.

5.2.5. Diversity in terms of whole-cell protein fingerprinting

On the basis of whole-cell protein fingerprinting (WCPF; Fig. 26) analysis, the 48 isolates of *B. cereus* were found to belong to 34 subclusters under four major clusters emerging at a similarity level of 60% (Fig. 27). All the subclusters, excepting two (A5 and B11) were source (food)-wise homogeneous. The isolates from wadi were restricted to clusters A (12 isolates out of 14 grouped under 'A') and C (2 isolates out of 14 grouped under 'C') only, and those from papad were confined to clusters A (1 isolate out of 17 grouped under 'A') and B (16 isolates out of 17 grouped under 'B'). Cluster D was homogeneous, containing isolates from dosa only. All the isolates from idli were confined to cluster B.

On the basis of WCPF (Fig. 28) analysis, the 33 isolates of *Salmonella* were found to belong to 17 subclusters under six major clusters emerging at a similarity level of 80% (Fig. 29) all of which, excepting two (C3 and D1), were source (food)-wise homogeneous. This study revealed a diversity of the organisms at a subspecies level (in case of *B. cereus*) and a relative preference of the WCPF subclusters to particular kinds of legume-based fermented foods.

5.2.6. Microbial challenge testing

The fermentation of dhokla batter is essentially an autofermentative process where lactic acid bacteria predominate and have a major functional role. During the fermentation, an increase ($P < 0.05$) in the

number of lactic acid bacterial and yeast cells resulted in the lowering of pH and a definite leavening (Fig. 30). While the pH of the batter decreased ($P < 0.05$) at every 3 h-interval from the initial 6.3 to finally 4.7, the batter volume increased ($P < 0.05$) at every 3 h-interval, resulting in a total of 80% ($v\ v^{-1}$) rise. Spiking of dhokla batter at the onset of fermentation with *B. cereus*, *S. aureus* or *E. coli* cells had no adverse effect on the growth of inherent lactic acid bacteria or yeasts, and changes in pH and batter volume (Fig. 31), which are the cause and consequence of this autofermentation. *S. aureus* and *E. coli*, either inherent or introduced to the batter at the start of fermentation, showed an increase ($P < 0.05$) in the count above their initial level after some time of fermentation. However, the *B. cereus* count decreased ($P < 0.05$) after 6 h of fermentation in both the control and the spiked batter. This behaviour of *B. cereus* is in conformity with that observed in mageu, a sour maize beverage and fish sausage, where initial growth was observed, but when the pH decreased due to the growth of lactic acid bacteria, subsequent inhibition occurred that was correlated with the rate of decrease in pH (Aryanta *et al.* 1991; Byaruhanga *et al.* 1999). Increase ($P < 0.05$) in the number of *S. aureus* and *E. coli* cells during fermentation was probably due of the fact that the lactic microbiota could not create adverse situation for the survival and growth of these pathogens. According to Beumer (2001), the minimum pHs for the growth of *B. cereus*, *S. aureus* and *E. coli* are 5.0, 4.0 and 4.4, respectively. Survival of a low level of *B. cereus* cells (2.6 log cfu g^{-1}) after steaming spiked dhokla batter for 15 min (Table 17) might be because of the heat resistance property of their endospores and possible occurrence of some 'cool pockets' in the steaming batter as their escape route. This is in agreement with the view of Davies and Wilkinson (1973) that although cooking inactivates most contaminating micro-organisms, heat-resistant bacterial endospores may survive or even be stimulated to germinate. Since > 4.0 log cfu g^{-1} of *B. cereus* is required for gastroenteritis, freshly prepared dhokla is considered safe for consumption. However, superior quality raw materials, good hygienic conditions, and proper steaming should be maintained to ensure the best quality product.

Like earlier, the fermentation of idli batter is also an autofermentative process where lactic acid bacteria predominate and have a major functional role. *L. mesenteroides* is the most commonly encountered bacterium, followed by *L. fermentum*, *E. faecalis* and *P. dextranicus* (Mukherjee *et al.* 1965). During fermentation, along with *L. mesenteroides*, yeasts such as *S. cerevisiae*, *D. hansenii*, *P. anomala* and *T. pullulans* are predominant, while *T. cutaneum* develops subsequently (Soni and Sandhu 1991). The major functions of this fermentation include the leavening of batter and improvement of flavour and nutritional value. The role of lactic acid bacteria is to reduce the pH of the batter from an initial 6.0 to an optimum level (4.1-4.5) for yeast activity. Yeasts help in the degradation of starch, a process that cannot be carried out by *L. mesenteroides*, into maltose and glucose (Nout *et al.* 2007).

During the 18 h-fermentation, an increase ($P < 0.05$) in the number of lactic acid bacterial cells at every 6 h-interval led to the lowering of pH, increase ($P < 0.05$) in titratable acidity, and a definite leavening (Fig. 32). Spiking of idli batter at the onset of fermentation with *B. cereus*, *S. aureus* or *E. coli* cells had no apparent influence on the growth of inherent lactic acid bacteria and changes in the aforesaid response parameters (Fig. 33), which are the cause and consequence of this autofermentation. None of the pathogenic indicator micro-organisms, either inherent or introduced to the batter at the start of fermentation, increased ($P < 0.05$) above their initial level after 18 h-fermentation, however all of them survived. Increase ($P < 0.05$) in their cell number during the first 6 h of fermentation was probably because of the fact that the initial pH of the batter was around 6 and the initial load of competing lactic and associated microbiota could not create an adverse situation for the growth of these pathogens. A decrease ($P < 0.05$) in the number of indicator organisms was observed after the initial 12 h of fermentation when the pH was < 4.7. The minimum pHs required for the growth of *B. cereus*, *S. aureus* and *E. coli* isolated from legume-based Indian fermented foods were found to be 5.3, 4.8

and 4.8, respectively, in our study. Since in the agar-spot assay the tested lactic acid bacterial isolates did not exhibit any antagonistic activity against these indicator organisms, the decrease in their cell numbers could not be attributed to the decrease in pH, increase in titratable acidity or the production of starter-derived inhibitors such as bacteriocins, hydrogen peroxide, ethanol, diacetyl and carbon dioxide. Rather, the said inhibition in the fermenting batter could possibly be attributed to the nutrient depletion and crowding of the competing total aerobic mesophilic bacteria, either solely or in combination with lactic acid bacteria. Jama and Varadaraj (1999) noticed a progressive increase in the growth of *B. cereus*, *S. aureus* and *E. coli* during natural fermentation in idli batter. They concluded that the reduced pH level during idli batter fermentation could not retard the growth of indicator bacterial species.

Though the cell number of the said pathogens decreased ($P < 0.05$), complete inhibition of their growth could not be achieved in the batter even at the end of fermentation. When idli cakes were prepared after steaming the fermented batter for 15 min according to tradition, the cakes were found free from both *S. aureus* and *E. coli* (Table 17). However, a low level of *B. cereus* (2.0 log cfu g^{-1}) survived the steaming process because of the heat resistance property of their endospores and possible occurrence of some 'cool pockets' in the steaming batter as their escape route. Since a load of > 4.0 log cfu of *B. cereus* g^{-1} is required for developing gastroenteritis, freshly prepared idli is considered safe for consumption. However, superior quality raw materials, good hygienic conditions, and proper steaming should be maintained to ensure best quality product.

Fermentation of wadi dough is also an autofermentative process where lactic acid bacteria and yeasts predominate and have a major functional role (Sandhu and Soni 1989). Under the present experimental conditions of preparation of wadi, which mimics most of the traditional processes, the dough was allowed to ferment first for 10 h at 32 °C in a closed container, and then in concomitant with drying of wadi, at ambient temperature in an alternate period of 8 h under sun and 16 h in shade. During the first 10 h of fermentation the moisture content remained unchanged ($P < 0.05$), but the volume of the dough increased by 1.3 times (Fig. 34). During the subsequent period of drying for 60 h, the moisture content decreased ($P < 0.05$) at every 12 h interval. The pronounced effect of fermentation is the change in pH; it declined ($P < 0.05$) during the first 10 h from 6.0 to 5.6, and then at every 12 h-interval till 24 h of drying to reach 4.9. During this period, the change in pH was negatively correlated ($r = 0.92$, $P < 0.05$) with the changes in the counts of lactic acid bacteria, yeasts and total aerobic mesophilic bacteria. After 24 h of drying although each of the counts decreased ($P < 0.05$) at every 12 h interval, the pH remained unchanged ($P < 0.05$). The results indicate that wadi dough fermentation is possibly achieved due to the activities of both lactic acid bacteria and yeasts whose cell count increased by approximately 6 and 4 log cycles, respectively, till 24 h of drying. Both lactic acid bacteria and yeasts are found to occur in raw blackgram (Soni and Sandhu 1991).

Considering the correlation between the rate of decrease in viable cell numbers of the three pathogens and that of pH and moisture content, the decrease in the cell count could mainly be attributed to the increase in the intensity of these two hurdles. According to Beumer (2001), the minimum pHs for the growth of *B. cereus*, *S. aureus* and *E. coli* are 5.0, 4.0 and 4.4, respectively. In this study, the maximum pHs of dough which did not support survivability of these three respective indicator organisms were 4.9, 4.9 and 4.8 (Fig. 35). The results indicate that a_w in the dough, in combination with pH, might have caused the death of the pathogens. None of these pathogens could grow in the fermenting dough having moisture content of 14-47 g (100 g)$^{-1}$. This range of moisture, which is within the one for intermediate moisture foods (IMFs), prohibits the growth of Gram negative as well as a large number of Gram positive bacteria, and yeasts (Adams and Moss 1995). It is also possible that the inhibition of pathogenic bacteria is due to the contribution of certain other less potent hurdles, like nutrient depletion

and crowding, and the presence of starter-derived inhibitors such as bacteriocins, hydrogen peroxide, ethanol, diacetyl and carbon dioxide (Adams and Nicolaides 1997). *S. aureus* is generally regarded as a poor competitor and its growth in fermented foods is generally associated with a failure of normal flora (Beumer 2001; Nychas and Arkoudelos 1990). The specific reason for holding the pathogens in check during this fermentation is an area requiring further study.

The most important factor which stands in the way of wider acceptance of many of these foods is the non-availability or inadequacy of standards of checking and assuring their quality. This also prevents modernization or modification of the methods of their preparation or production, as there is no way to establish the equivalence of the product made by the modified method with that of the original product. Quality assurance of a fermented food and of a preparation thereof is not just an analytical operation, it does not end with the finding of foodborne pathogens therein, rather it embodies total information and controls including documentation which are necessary to guarantee consistency of nutritional value and safety. Quality assurance includes quality standardization, quality production, quality testing and quality monitoring of a product.

Our findings of diverse multidrug-resistant strains of foodborne bacterial pathogens from legume-based fermented foods call for an attention of food scientists and technologists as well as food handlers to apply good hygienic practices (GHPs), good manufacturing practices (GMPs), and hazard analysis and critical control points (HACCP) during the production, storage and selling of these foods. The kinds of food studied here have the potentiality of boosting the country's economy. However, Indian food industry is still awaiting its metamorphosis from traditional to the most advanced technology facets. The endeavours envisaged in this dissertation are footsteps towards itemizing them as a safe nutritious delicacy.

6

Summary

Legume-based traditional fermented foods that impart variety, nutrition and delicacy to the diet, may become an important carrier of various micro-organisms implicating possible health hazards for consumers. This project was undertaken with the objective of evaluating microbiological quality of legume-based traditional fermented foods marketed in West Bengal, India and understanding some behavioural patterns of the pathogenic isolates from those. A total of 105 samples of six different kinds of foods collected from 83 retail outlets scattered over 16 districts of the State of West Bengal was investigated to determine their microbiological quality status. Majority of the samples of each of the six kinds foods, except dosa, had a high count (> 10^4 cfu g^{-1}) of these bacteria, indicating a lapse in good hygiene practices followed in preparing these foods. Every kind of foods was contaminated with *Bacillus cereus*, Enterobacteriaceae and coliforms. *Staphylococcus aureus* was present in only one sample of dhokla (4×10^4 cfu g^{-1}). Faecal coliforms were present in dhokla, idli, papad and wadi. One sample each of idli (3.8×10^3 cfu g^{-1}) and wadi (3.2×10^4 cfu g^{-1}) were found contaminated with *Escherichia coli*. *Salmonella* was present in 11.4% (12 of 105) of the total samples analysed. It was present in idli, papad

and wadi. *Clostridium perfringens* and *Shigella* could not be detected in any of the samples. *B. cereus*, Enterobacteriaceae, coliforms and faecal coliforms were present in 20, 46, 28, and 11%, respectively, of the samples analysed. among the critical control points (CCPs) are raw materials, water, beating or mixing batter or dough, utensils, drying environment (in case of papad and dough), post-preparative storage conditions, and dish cloth used at the time of serving.

Strains of foodborne bacterial pathogens that are resistant to a variety of antimicrobial agents have become a major health concern. The extent of prevalence of antimicrobial resistance among the food isolates were determined. The tested strains of *B. cereus*, Enterobacteriaceae and *Salmonella* were found resistant to at least nine, four and five antimicrobials, respectively. Most of the antibiotics against which these isolates showed resistance inhibit synthesis of prokaryotic cell wall, however were sensitive to those inhibiting protein and nucleic acid syntheses. This situation invites a challenge to tackle the problem of antibiotic resistance in foods

The *D*-values for spores help to understand the hazardous potential of these organisms which can survive the cooking processes. In glucose-supplemented brain heart infusion broth, the mean $D_{100°C}$-values for 12 different isolates *B. cereus* spores ranged from 3.0-9.2 min, suggesting that time-temperature exposure at an appropriate level during cooking may destroy heat-sensitive spores, but not the heat-resistant ones. The data can be used as an aid to predict the time required at 100 °C to achieve a certain number of log-cycle reductions of this pathogen.

Activity of the enzymes, like protease, lipase and amylase indicates spoilage potentiality of the producing organisms. So, it was found that 50% of the tested strains of *B. cereus* present in fermented foods had the potentiality of causing food spoilage too.

All the representative isolates, except *B. cereus*, grew optimally at a pH range of 6.11-7.26. The The minimum inhibitory concentrations (MICs) of sodium chloride were 65-110 mg ml^{-1} and of benzoic acid for *B. cereus*, *S. aureus*, *E. coli* and *Salmonella* were 400-650 µg ml^{-1}. Similarly, the MICs of sorbic acid for all the tested isolates were 500-800 µg ml^{-1}. Most (80%) of the tested 10 strains were resistant to 300 µg nisin ml^{-1} nutrient agar. The effects of combination of pH, sodium chloride, benzoic acid and nisin on the growth of *B. cereus* 37-B1, and of pH, sodium chloride and benzoic acid on *Salmonella* 1-S4 were investigated in order to understand the scientific basis for an efficient application of hurdle technology in the preservation of food which can be contaminated by these micro-organisms. The best combination found for the cessation of growth of *B. cereus* was 20 mg sodium chloride ml^{-1}, 300 µg benzoic acid and 25 µg nisin ml^{-1} at pH 5.6. The logical combination for the cessation of the growth of *Salmonella* was 50 mg ml^{-1} sodium chloride and 600 µg ml^{-1} benzoic acid at pH 5.4. The results will be the basis for an efficient application of hurdle technology in preserving the legume-based fermented foods.

Whole-cell protein fingerprinting (WCPF) analysis of 48 strains of *B. cereus* and 33 strains of *Salmonella* revealed a diversity of these organisms and a relative preference of the WCPF subclusters to particular types of legume-based fermented foods.

Challenge study by intentional inoculation of a batter or dough with pathogenic bacteria revealed that fermentation alone could not completely inhibit the growth of the inoculated strains of *B. cereus*, *S. aureus* and *E. coli* from the fermenting batter of dhokla and idli, and dough of wadi. Steaming of the fully fermented batter for 15 min according to the traditional culinary process of making dhokla and idli, completely inhibited *S. aureus* and *E. coli*. However, a low level of *B. cereus* survived the steaming process, indicating that although cooking inactivates most contaminating micro-organisms, heat-resistant bacterial endospores may survive. None of the inoculated pathogenic bacteria could survive in wadi after 36 h of sun- and air-drying of fermented dough. Good hygienic and manufacturing practices are advised to ensure best quality products.

7

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List of Publications

Roy, A., Moktan, B. and Sarkar, P.K. (2004) Microbiological quality of dosa marketed in West Bengal, India. Proceedings of the National Seminar on '*Emerging Trends in Applied Botany, Seed Science and Technology*, University of Mysore, Mysore, Nov. 4-6, 2004

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Microbiological quality of legume-based traditional fermented foods marketed in West Bengal, India

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Abstract

A total of 105 samples of six different types of legume-based popular fermented foods, namely amriti, dhokla, dosa, idli, papad and wadi, purchased from retail outlets in West Bengal, was analysed to determine their microbiological safety status. While dhokla and idli were of high-moisture foods (62 g (100 g)$^{-1}$), others had a lower moisture level (14–27 g (100 g)$^{-1}$). Papad was alkaline (pH 8.7), whereas all the other foods were acidic (pH 4.4–5.8). Every sample was found contaminated with total aerobic mesophilic bacteria (detection limit, 10 cfu g^{-1}); 38% (40/105) of the samples contained more than 10^6 cfu g^{-1}. Aerobic mesophilic bacterial spores were found in 88% (92/105) of the samples (detection limit, 100 cfu g^{-1}), whereas their anaerobic counterparts were present in 39% (41/105) of the samples (detection limit, 10 cfu g^{-1}). Although all the samples, excepting one, were free from *Staphylococcus aureus* (detection limit, 100 cfu g^{-1}), 20% (21/105) of the samples were found contaminated with *Bacillus cereus* (detection limit, 100 cfu g^{-1}). Enterobacteriaceae were found in 46% (48/105) of the samples (detection limit, 10 cfu g^{-1}). Of the Enterobacteriaceae isolates, 92% were coliforms and 57% were faecal coliforms. *Escherichia coli* (detection limit, 10 cfu g^{-1}) was found in only one sample each of wadi and idli, at a load of 10^3–10^4 g^{-1}. *Salmonella* (detection limit, 1 cell (25 g)$^{-1}$) occurred in 12 samples of wadi, idli and papad, however was absent in the other three products. *Clostridium perfringens* (detection limit, 10 cfu g^{-1}) and *Shigella* (detection limit, 1 cell (25 g)$^{-1}$) could not be detected. The results obtained in the present study indicated that these foods were manufactured using poor-quality starting materials, processed under unhygienic conditions, or/and temperature-abused during transportation and storage. Based on these results, a guideline is recommended for obtaining safe products.

Keywords: Legume-fermented food; Foodborne pathogens; Microbiological safety

1. Introduction

Fermentation, an old and economical method of producing and preserving food, is widely practised in Asia and Africa (Campbell-Platt, 1987; Steinkraus, 1996). Fermented foods have generally been considered as less likely to be vehicles for foodborne infection or intoxication than fresh foods due to the competitive activity and metabolites of the functional microflora (Nout, 1994). But due to unhygienic handling, external contamination, contaminated water and inferior quality of raw material, many of these foods may get contaminated by bacteria such as *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella*, *Shigella* and many other pathogens. In most of the fermented foods, especially in lactic acid bacterially fermented ones, the inhibition of growth of bacterial pathogens is common and can often ensure safety where levels of contamination are low (Adams & Nicolaides, 1997). But with infectious pathogens, particularly those with a small low infectious dose, some degree of inactivation may be necessary to provide an acceptable level of safety (Beumer, 2001).

In India, legumes alone or in combination with cereals, like rice constitute the basic ingredient of many fermented

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Table 1
Legume-based traditional fermented foods marketed in West Bengal

Food	Substrate	Functional microflora[a]	Product marketed as	Nature of the product	Mode of consumption	Reference
Amriti	Legume	No data available	Ready-to-eat	Syrup-filled, ring-shaped confectionery	Snack	Nil
Dhokla	Legume	LAB, yeast	Ready-to-eat	Steamed, spongy cake	Snack	Desai and Salunkhe (1986); Joshi et al. (1989); Aidoo et al. (2006); Nout et al. (in press)
Dosa	Legume-rice mixture	LAB, yeast	Ready-to-eat	Thin, highly seasoned, griddled pancake	Snack	Soni and Sandhu (1999); Aidoo et al. (2006); Nout et al. (in press)
Idli	Legume-rice mixture	LAB, yeast	Ready-to-eat	Steamed, spongy cake	Snack	Soni and Sandhu (1999); Aidoo et al. (2006); Nout et al. (in press)
Papad	Legume	Yeasts	Raw	Thin, circular wafer	Deep-fried in oil or roasted, or made to condiment	Shurpalekar (1986); Aidoo et al. (2006); Nout et al. (in press)
Wadi	Legume	LAB, yeasts	Raw	Hollow, brittle, balls or cones	Briefly fried in oil, and made to condiment	Sandhu and Soni (1989); Aidoo et al. (2006); Nout et al. (in press)

[a] LAB, lactic acid bacteria.

foods. In the State of West Bengal in India, fermented foods like amriti, papad and wadi are popular from time immemorial. In the passage of time, southern and western Indian fermented foods, like idli, dosa and dhokla have also become the choice for restaurant hunters as nutritious and delicious light midday meal (Table 1). The traditional method of preparation of amriti (Fig. 1) and appearance of the product are similar to those of jalebi, excepting that in pretzel-looked jalebi, blackgram dal (dehusked split beans) is replaced by refined wheat flour (*maida*) and the fermentation time is longer (14–16 h) (Campbell-Platt, 1987; Steinkraus, 1996). The preparation of dhokla (Fig. 2), dosa and idli are similar, excepting that in dosa and idli Bengalgram is substituted with blackgram, and while dosa is a highly seasoned griddled pancake, idli is a steamed pancake resembling dhokla. While all the aforementioned ones are RTE (ready-to-eat) foods, marketed papad (Fig. 3) and wadi (Fig. 4) are cooked before consumption.

Microbiological safety of fermented foods is an important issue in developing countries, including India. Processing technologies that ensure food safety are required at

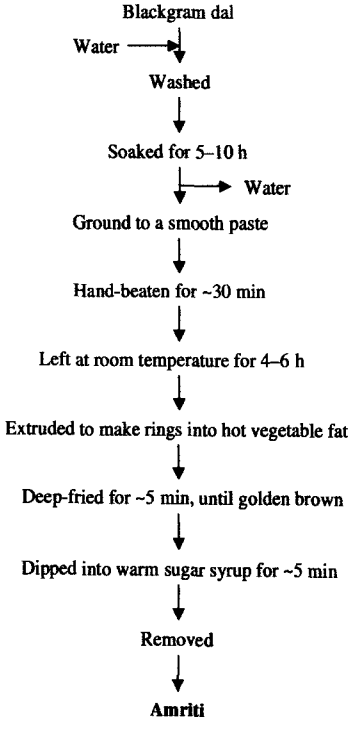

Fig. 1. Flow sheet for amriti production.

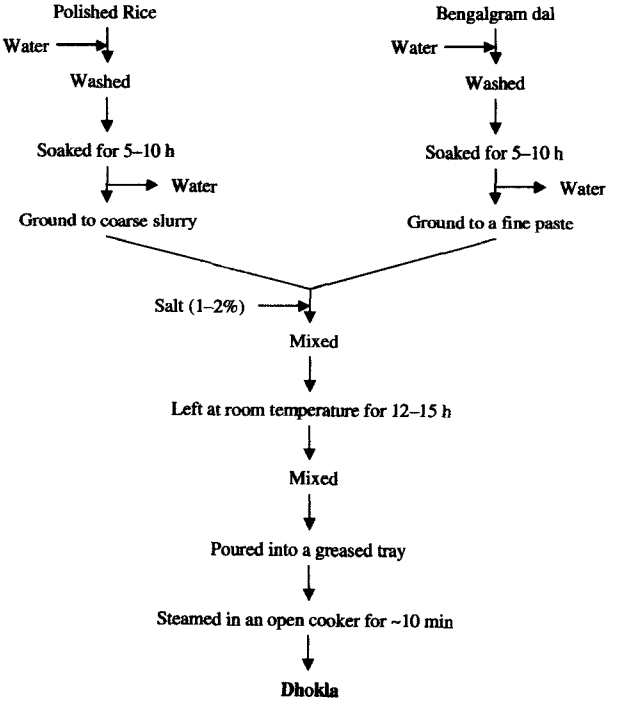

Fig. 2. Flow sheet for dhokla production.

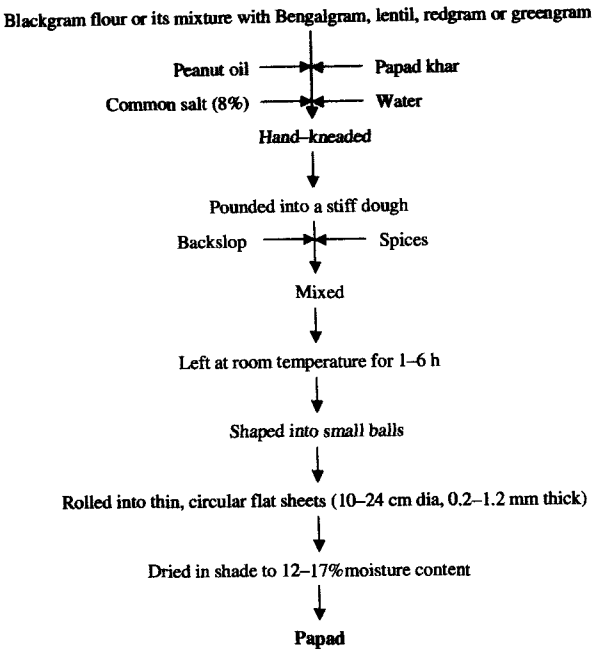

Fig. 3. Flow sheet for papad production.

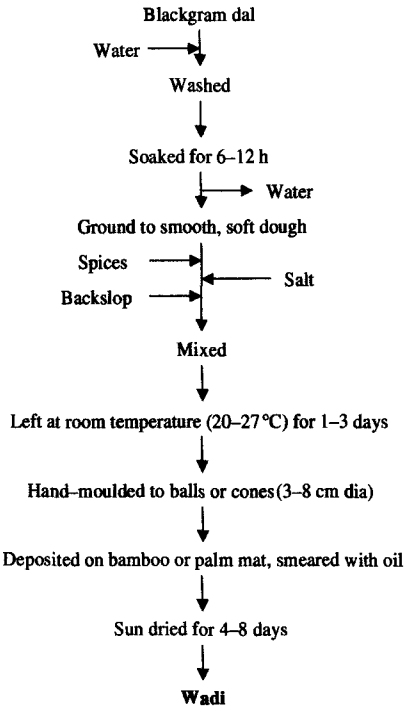

Fig. 4. Flow sheet for wadi production.

both the rural and urban levels, particularly in view of the frequently poor sanitary conditions and high ambient temperatures. The production of safe foods is the responsibility of the producers. But the authorities need to regularly verify and validate those through inspection and product testing both at the site of production and at the point of sale. These foods have not so far been studied with regard to their microbial safety. The present study was hence undertaken to evaluate the microbiological quality of legume-based fermented foods retailed in West Bengal, and to suggest a safety guideline for these products on the basis of the explored status.

2. Materials and methods

2.1. Materials

The culture media, chemicals and sampling bags used were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India, unless mentioned otherwise.

2.2. Reference organisms

B. cereus ATCC9139 was obtained from Dr. M.J.R. Nout, Wageningen University, The Netherlands, and *S. aureus* MTCC96, *C. perfringens* MTCC450, *E. coli* MTCC118, *Salmonella typhi* MTCC733 and *Shigella flexneri* MTCC1457 were purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

2.3. Sampling

A total of 105 samples belonging to six different kinds of legume-based traditional fermented products were purchased from randomly selected retail outlets and restaurants scattered over different places of the State (Table 2). The unpackaged samples, each having an average weight of 150 g, were collected in sterile Nasco sampling bags (PW389), and kept in an ice-box. The samples of amriti, dhokla, dosa and idli were found to be sold unpackaged. Dosa was found to be sold ready prepared and, hence, sampled immediately after its preparation. Samples of wadi and papad, sold as their sealed packets made with low-density polyethylene film, were also collected. The samples were transported to the laboratory immediately and analysed as early as possible.

2.4. Moisture content and pH

The moisture content was determined by drying approximately 10 g sample of foods at 105 ± 1 °C in a hot air oven to constant weight using a Sartorius CP224S (Sartorius AG, Göttingen) balance (Banerjee & Sarkar, 2003).

A 10 g sample 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).

2.5. Microbiological analysis

The methods used were based on those followed by FDA (1984), Speck (1984), Nout, Bakshi, and Sarkar (1998) and Banerjee and Sarkar (2003). Representative samples (10 g) were homogenized with 90 ml sterile peptone-physiological saline (0.1% w v^{-1} neutral peptone, 0.85% w v^{-1}

Table 2
Sampling of legume-based fermented foods from retail shops in different districts of West Bengal

Food	District[a] of collection	No. of samples analysed		
		Total	Unpackaged	Packaged[b]
Amriti	C, D, M_1, N_3	8	8	L-0, B-0
Dhokla	D, N_3	5	5	L-0, B-0
Dosa	C, D, J, K, M_1, N_3	16	16	L-0, B-0
Idli	C, D, J, K, M_1	13	13	L-0, B-0
Papad	C, D, H_1, H_2, J, K, M_1, M_2, N_1, N_2, N_3, S, W	29	1	L-1, B-27
Wadi	B_1, B_2, C, D, E, H_1, H_2, J, K, M_1, M_2, N_1, N_2, N_3, S, W	34	23	L-11, B-0

[a] B_1, Bankura; B_2, Bardhaman; C, Cooch Behar; D, Darjiling; H_1, Hooghly; H_2, Howrah; J, Jalpaiguri; K, Kolkata; M_1, Malda; M_2, Murshidabad; N_1, North 24 Parganas; N_2, Nadia; N_3, North Dinajpur; S, South 24 Parganas; W, West Midnapore.
[b] L, locally packaged; B, branded.

sodium chloride, pH 7.2) using a Stomacher lab-blender 400 (Seward Medical, London) at 'normal' speed (2 min for wadi, 1 min for others). Duplicate counting plates were prepared using appropriate dilutions. For pour-plating, 1 ml of the dilution was mixed with molten (45 °C) medium. For spread-plating, 0.1 ml of the dilution was spread on the surface of a dried plate. After incubation, the colonies appearing on the selected plates were counted and calculated as colony forming units (cfu) per gram fresh weight sample. The representative colonies of each type were picked up and diluted by streaking out. After microscopic examination, the purified colonies were grown on slants or in broths of suitable media and stored at 4 °C.

Total aerobic mesophilic bacteria were enumerated by pour-plating using plate count agar (M091) and incubating at 35 °C for 18–24 h. For the estimation of mesophilic bacterial spores, 10% (w v^{-1}) sample suspension was heated at 80 °C for 30 min, suitably diluted, and spread on PCA plates followed by incubation at 30 °C for 72 h (for aerobic sporeformers) and pour-plated with perfringens agar (M579, FD011 and FD012) followed by incubation at 37 °C for 48 h in an anaerobic jar with AnaeroHiGas pack (LE002A) (for anaerobic sporeformers) (Banerjee & Sarkar, 2003).

Enumeration of *B. cereus* was made on spread-plates of *B. cereus* selective agar (M833, FD003 and FD045), incubated at 35 °C for 24–48 h. A representative number of presumptive isolates was confirmed on the basis of motility, endospore formation, glucose fermentation, acetylmethylcarbinol production and nitrate reduction.

Isolation of *S. aureus* was carried out on spread-plates of Baird-Parker agar (M043, FD047 and FD045), incubated at 35 °C for 24–48 h. Representative isolates were confirmed by the production of coagulase and acid from mannitol using coagulase mannitol broth base (M277) with appropriate addition of sterile pre-tested coagulase plasma, thermostable DNase using DNase test agar with toluidine blue (MI041) and production of acetylmethylcarbinol.

Selective enumeration of *C. perfringens* was done in pour-plates of perfringens agar, incubated at 37 °C in an anaerobic jar for 18–48 h. The representative isolates, maintained in cooked meat medium (M149), were confirmed by testing motility and nitrate reduction using motility nitrate medium (M630I), raffinose fermentation using raffinose gelatin medium (M987, substituting lactose with raffinose), and lactose fermentation and gelatin liquefaction using modified lactose gelatin medium (M987).

Enterobacteriaceae members were isolated by mixing appropriate dilutions of samples with tryptone soya agar (M290) and incubating the plates at room temperature (27 °C) for 1–2 h followed by a thick overlay of violet red bile glucose agar without lactose (M581) and incubated at 35 °C for 18–24 h. The presumptive isolates were confirmed on the basis of cytochrome oxidase using oxidase disc (DD018) and glucose fermentation in stab cultures of purple agar base (M098) supplemented with 1% w w^{-1} D (+) glucose (Merck 17809).

For the detection of coliforms, brilliant green bile broth, 2% (M121) with inverted Durham tubes was inoculated with confirmed Enterobacteriaceae isolates, incubated at 37 °C for 24–48 h, and examined for gas formation. For tests of faecal coliforms, inoculated broth tubes were incubated at 44 °C for 24 h. The presence of *E. coli* was confirmed on the basis of indole production by using tryptone water (M463I) and Kovac's reagent strip (DD019).

For qualitative detection of *Salmonella* and *Shigella*, 25-g samples were added to 225 ml buffered peptone water (M614), followed by incubation at 35 °C for 20 h. Ten and 0.1 ml of this pre-enrichment culture were added to fluid selenite cystine medium (M025A) and Rappaport–Vassiliadis medium (M880), respectively. The former was incubated at 35 °C for 24 h, whereas the latter at 42 °C for 18–48 h. Bismuth sulphite agar (M027) plates, streaked with the enriched broth cultures, were incubated at 35 °C for 24–48 h. The presumptive isolates were confirmed on the basis of acid and gas production by using triple sugar iron agar (M021), lysine iron agar (M377), motility by using motility nitrate medium (M630I), production of acid from glucose by using MRVP medium (M070), and production of indole by using tryptone water and Kovac's reagent strip.

2.6. Statistical analysis

Statistical treatment of the data was performed using SPSS 12.0 for Windows for standard error of measurements (SEM) and analysis of variance (ANOVA).

3. Results and discussion

The results of moisture, pH and microbial analyses of 105 samples of six different kinds of legume-based traditional fermented foods marketed in West Bengal are summarized in Table 3. While dhokla and idli were the high-moisture (62 g $(100\,g)^{-1}$) foods, others contained less moisture (14–27 g $(100\,g)^{-1}$). Papad was alkaline, whereas all the other foods were acidic.

Thirty-eight percent (40/105) of the samples contained total aerobic mesophilic bacterial cells at a level of $>10^6$ cfu g^{-1}. Majority of the samples of each of the six foods, except dosa, had a high count ($>10^4$ cfu g^{-1}) of these bacteria, indicating a lapse in good hygiene practices followed in preparing these foods. While most of the samples of amriti, dosa, idli and papad contained total aerobic mesophilic bacteria in the range of 10^2–10^6 cfu g^{-1}, in most of the samples of dhokla and wadi their count was at a higher level ($>10^6$ cfu g^{-1}). Since marketed amriti, dosa and idli are either fried or steamed at the final stage of their preparation, death of most of the fermenting organisms and associated microflora (excepting those occurring in 'cool pockets') and a consequent low count of total aerobic mesophilic bacteria were expected in those samples. Although marketed dhokla is a steamed product, their high count ($>10^6$ cfu g^{-1}) in 100% of the samples might be mostly due to post-preparation contamination introduced from seasoning ingredients, including spices, chilly, grated coconut and curry (*Murraya koenigii*) leaves accompanied with a high moisture content of the product. Plate count agar is a non-selective complex medium commonly used for enumerating the total microbial content in foods. So, the viable count in the samples of papad and wadi, which did not pass through any heat treatment process, was likely of fermenting microorganisms along with associated contaminating microflora.

The external surface of the cereal grains is heavily contaminated with saprophytes acquired during development of the plants along with contaminants from soil, air, animals and also humans (Sarrias, Valero, & Salmerón, 2002). *Bacillus* spp. are important as food-spoilage organisms, and can be isolated from a variety of animal and plant products (Johnson, 1984). Aerobic mesophilic bacterial spores were found in 88% (92/105) of the samples. All the samples of amriti, dhokla and papad contained these spores. A high count (10^5 cfu g^{-1}) of them was found in papad and wadi (10/63 samples). As dosa samples were freshly prepared ones, their load in the product was never more than 10^5 cfu g^{-1}. On the other hand, the load of their anaerobic counterpart was less; they occurred in 39% of the tested samples (41/105). Amriti, dosa and idli were free of them.

Table 3
Moisture content, pH and levels of microflora (expressed as percentages of samples analysed) of legume-based traditional fermented foods marketed in West Bengal

Parameter	Amriti ($n=8$)	Dhokla ($n=5$)	Dosa ($n=16$)	Idli ($n=13$)	Papad ($n=29$)	Wadi ($n=34$)
Moisture, g $(100\,g)^{-1}$[a]	19.5bc ± 1.12	62.1a ± 0.86	27.3b ± 1.73	61.8a ± 1.58	18.0bc ± 0.41	14.4c ± 0.33
pH[a]	5.8b ± 0.07	4.9c ± 0.11	4.4d ± 0.08	4.6cd ± 0.07	8.7a ± 0.07	5.7b ± 0.04
Bacterial load (cfu g^{-1} fresh weight)						
Total aerobic mesophilic bacteria						
<Detection limit (10)						
10^2–10^4	12.5		56.3	23.1	17.2	2.9
$>10^4$–10^6	62.5		43.7	76.9	75.9	5.9
$>10^6$–10^9	25	60			6.9	55.9
$>10^9$–10^{12}		40				35.3
Aerobic mesophilic bacterial spores						
<Detection limit (100)			50	15.4		8.8
10^2–10^5	100	100	50	84.6	79.3	76.5
$>10^5$–10^7					20.7	14.7
Anaerobic mesophilic bacterial spores						
<Detection limit (10)	100	40	100	100	44.8	35.3
10–10^4		60			55.2	47.1
$>10^4$–10^5						17.6
Bacillus cereus						
<Detection limit (100)	75	40	81.3	92.3	79.4	82.4
10^2–10^3		20	12.4	7.7	10.3	8.8
$>10^3$–10^4	25		6.3		6.9	2.9
$>10^4$–10^6		40			3.4	5.9
Enterobacteriaceae						
<Detection limit (10)	50	80	87.5	46.2	69	26.5
10–10^3	25			7.6	17.2	17.6
$>10^3$–10^5	12.5		12.5	46.2	13.8	23.5
$>10^5$–10^8	12.5	20				32.4

[a] Values are mean with standard error of measurements. Means with the same following letters, within rows, are not significantly different ($P<0.05$).

Table 4
Percentage of Enterobacteriaceae isolates containing coliform, faecal coliform and *E. coli*.

Enterobacteriaceae component	Food						
	Amriti	Dhokla	Dosa	Idli	Papad	Wadi	Total
Coliform	49.1	100	58.7	82.6	49.2	59.8	91.9
Faecal coliform	0	66.7	0	15.5	12.5	18.6	56.9
E. coli	0	0	0	3.9	0	0.6	0.1

All the six types of foods contained *B. cereus*; this organism occurred in 20% of the samples (21/105). The potentially hazardous level ($>10^4$ cfu g^{-1}) was observed in dhokla, papad and wadi. The presence of considerable levels of *B. cereus* was recorded in several legume-fermented foods, such as Indonesian tempe (Samson, Van Kooji, & De Boer, 1987), African dawadawa (Antai & Ibrahim, 1986) and Indian kinema (Nout et al., 1998). The presence of this organism at high levels suggests a potential risk of these foods to the consumer, because of the subsequent production of toxin associated with food poisoning (Banerjee & Sarkar, 2004). However, it was found in a legume food (kinema) that in presence of fermenting microorganisms (*B. subtilis*), the growth of *B. cereus* was adversely affected and the production of enterotoxin was ceased (Nout et al., 1998). *C. perfringens* could not be detected from any of the 105 samples, and only one sample was found contaminated (at the load of 4×10^4 cfu g^{-1}dhokla) with *S. aureus*. The latter organism was also not detected in kinema (Nout et al., 1998). Possibly, the lack of initial contamination, or the impact of competition and/or antagonistic reactions would have prevented its proliferation. It may also happen that legumes do not offer a suitable environment for the proliferation of *S. aureus*.

Enterobacteriaceae counts are considered more generally as an indicator of hygienic quality rather than of faecal contamination, and therefore say more about general microbiological quality than possible health risks posed by the product (Adams & Moss, 1995). Enterobacteriaceae occurred in all the six types of foods studied (Table 4); these were detected in 46% (48/105) of the samples. Of the Enterobacteriaceae isolates, 92% were coliforms and 57% were faecal coliforms. The occurrence of these microorganisms in a food is considered as a reflection of the process practised during its preparation and/or subsequent handling under inefficient hygienic condition (ICMSF, 1978). The presence of a high count of faecal coliforms in dhokla, papad, wadi and even freshly prepared idli indicates a high risk that other pathogenic organisms have also contaminated the food. One sample each of idli (3.8×10^3 cfu g^{-1}) and wadi (3.2×10^4 cfu g^{-1}) were found contaminated with *E. coli*.

Although the presence of *Salmonella* in foods of animal origin has been well documented, very limited studies are available on vegetarian foods, particularly the processed ones (Yadav, Zende, & Sharma, 2000). More than 95% of cases of infections caused by *Salmonella* are foodborne and these infections account for about 30% of deaths resulting from foodborne illnesses (Hohmann, 2001). *Salmonella* was present in 11.4% of the total samples analysed (12/105). It was not detected in amriti, dhokla and dosa. However, its prevalence in the other three foods is noteworthy; 15% (2/13), 14% (4/29) and 18% (6/34) of the samples of idli, papad and wadi, respectively, were found contaminated with this pathogen. Since marketed idli is RTE, presence of *Salmonella* in idli is alarming. However, it will be killed during heat-processing for consumption of papad and wadi. *Shigella* could not be detected in any of the samples.

The microbial composition of these products indicates that their manufacturing processes did not support survival and growth of *S. aureus*. On the other hand, a high level of total aerobic mesophilic bacterial cells in majority of the samples indicates either that highly contaminated substrates were used or that poor processing practices e.g., inappropriate handling or unhygienic condition were involved, as was observed during the study with sufu (Han, Beumer, Rombouts, & Nout, 2001). Considering that no sign of spoilage was recorded, it might be assumed that most mesophilic bacterial spores either did not germinate or were not metabolically active in these products.

Detection of the presence of a high count of total aerobic mesophilic bacteria, *B. cereus*, and Enterobacteriaceae in all these foods suggests that a better control is needed and that some changes in the manufacturing practices, storage, distribution and service should be made to enhance their microbial safety.

Among the critical control points (CCPs) are raw materials, water, beating or mixing batter or dough with bare hands, utensils, drying environment (in case of papad and wadi), post-preparative storage conditions, and dish cloth. Many times, uncleaned raw materials (rice, blackgram and Bengalgram) are used. In most cases, due to lack of running water availability producers store water under vulnerable conditions subject to contamination. Personal cleanliness is another aspect which can reduce foodborne hazards; use of gloves at the time of beating or mixing batter or dough reduces the chance of cross-contamination. Utensils represent an important source of food contamination, since the same utensils are used in different batches of preparation without any in-between cleaning. Most of the fermented foods under study are taken ready to eat for sale and are, therefore the most susceptible to microbial growth in view of the longer length of time between preparation and consumption under improper temperature conditions (30–38 °C). Open-air drying of papad and wadi leads exposure of these foods to aggravating environmental conditions, such as the presence of insects, rodents, other animals and dust. The

dish cloth used in several tasks represents another hazard to the safety of foods. Hence, training of people, and producers and sellers in particular, for a cultural change would be one of the most effective interventions to reach a safer food.

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Characteristics of *Bacillus cereus* isolates from legume-based Indian fermented foods

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Abstract

An antibiogram of 48 strains of *Bacillus cereus* isolated from 6 different kinds of legume-based Indian fermented foods (amriti, dhokla, dosa, idli, papad and wadi) was generated against 18 different antibiotics that are commonly used against foodborne diseases, mainly gastroenteritis. Each of the isolates was found to be resistant against at least nine different antibiotics. Production of extracellular enzymes, namely protease, lipase and amylase by 33%, 27% and 46%, respectively, of the isolates indicates their potentiality for food spoilage. In brain–heart infusion broth supplemented with glucose, the $D_{100\,°C}$-values of the tested 12 strains ranged from 3.0 to 9.2 min. In nutrient broth, the minimum and maximum pHs permitting growth of *B. cereus* were 5.3 and 11.6, respectively. The minimum inhibitory concentrations of sodium chloride, benzoic acid and sorbic acid for the growth of the isolates were 65–85 mg ml^{-1}, 400–700 μg ml^{-1} (pH 5.0–4.2) and 500–600 μg ml^{-1} (pH 5.0–4.8), respectively. Of the tested 10 strains, eight were resistant to 300 μg nisin ml^{-1} (pH 5.0). While studying the combined effect of selected hurdles on the growth of an isolate, the judicious combination considered was 20 mg sodium chloride, 300 μg benzoic acid and 25 μg nisin ml^{-1} at pH 5.6. The whole cell protein fingerprinting (WCPF) analysis using SDS–PAGE revealed a high level of diversity among the isolates. At ⩾60% similarity level, the WCPF profiles could be grouped into four major clusters which were divided into 34 subclusters. Most of the subclusters were source-wise homogeneous.

Keywords: *Bacillus cereus*; Antibiotic susceptibility; Extracellular enzyme; *D*-value; Natural preservative; Combined effect; Whole-cell protein fingerprinting; Diversity

1. Introduction

Bacillus cereus is widely distributed in the natural environment and is easily spread to many types of food, especially those of plant origin. It causes food spoilage and two distinct types of food poisoning: the diarrhoeal type and the emetic type. Whereas the former type is caused by complex enterotoxins produced during vegetative growth in the small intestine, the latter type is produced by growing cells in the food (Granum & Lund, 1997).

In spite of competition and antagonistic activity incurred by the dominant fermenting microflora, *B. cereus* has been reported in some legume-based traditional fermented foods, viz. African dawadawa, Indonesian tempeh, and Indian idli and kinema (Antai & Ibrahim, 1986; Nout, Bakshi, & Sarkar, 1998; Samson, van Kooij, & de Boer, 1987; Varadaraj, Keshava, Devi, Dwarakanath, & Manjrekar, 1992). The present authors isolated *B. cereus* from 20% of the samples of legume-based Indian fermented foods (Roy, Moktan, & Sarkar, 2006). This finding triggered them to study the behavioural patterns of *B. cereus* so that measures can be undertaken to control this dreadful pathogen.

Surveillance of antimicrobial resistance is essential for providing information on the magnitude and trends in resistance and for monitoring the effects of interventions, especially because the prevalence of resistance varies widely between and within countries, and over time (WHO, 2001).

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Strains of foodborne bacterial pathogens that are resistant to a variety of antibiotics have become a major health concern (Kiessling et al., 2002). Sixty years of increasing application of antibiotics have created an ecological imbalance – the enrichment of multiple antibiotic-resistant pathogenic bacteria. Finding out an antibiotic resistance profile of the isolated strains against commonly used antibiotics for treating gastroenteritis was one of the objectives of the present work.

Increasing interest by consumers and producers in food safety and quality gives shelf-life evaluation a new significance. Proteolytic, lipolytic and amylolytic activities of bacteria indicate their potentiality for food spoilage (Braun, Fehlhaber, Klug, & Kopp, 1999). Hence, our second objective was to evaluate the production of these enzymes so that their role in spoilage can be predicted.

When foods containing spores of *B. cereus* are cooked, the spores often survive and may be heat-shocked into germination. If these foods are then left to ambient temperature, germination and growth may take place, leading to achieve a competition-free favourable condition causing spoilage of the food and/ or producing emetic toxins. To understand the hazardous potential of the sporeformers which can survive cooking processes, quantification of thermal inactivation of spores of *B. cereus* isolates from these foods was our third objective.

Nowadays, there is strong interest in the use of natural antimicrobials for preservation of minimally processed foods. The addition of appropriate antimicrobial preservatives is used to reduce the growth of microbial contaminants in foods. Benzoic acid is widely used chiefly on account of its low price, whereas sorbic acid is preferred to others because of its physiological harmlessness and organoleptic neutrality (Lueck, 1980). Since nisin does not persist in the body or the environment, nor it is associated with the bacterial resistance to itself, it has the potentiality for widespread use as a food preservative. Hence, our fourth objective was to find out the minimum inhibitory concentrations (MICs) of sodium chloride, benzoic acid, sorbic acid and nisin individually to prevent this potent pathogen. The microbial stability and safety of most foods are based on a combination of several preservative factors (hurdles), which microorganisms present in the food are unable to overcome. Using an intelligent combination of hurdles it is possible to improve not only the microbial stability and safety but also the sensory and nutritive quality as well as economic aspects of a food (Leistner, 1994). Therefore, an attempt was also made to determine the combined effect of pH, salt, one weak acid preservative and nisin on the growth of an isolate.

Molecular typing methods, including both genotypic and phenotypic analyses, could be employed to find out differences among subspecies or strains of *B. cereus*. Among phenotypic methods whole-cell protein fingerprinting (WCPF) using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) may be employed as a useful tool to study the diversity. Therefore, WCPF is considered here as a discriminatory tool for measuring diversity among the isolates at the subspecies level.

2. Materials and methods

2.1. Organisms

The 48 strains of *B. cereus* used were isolated from 105 random samples of 6 different legume-based fermented foods (Table 1), collected from retail outlets in India (Roy et al., 2006). The organisms were maintained on nutrient agar (HiMedia M561) slants at 4 °C with subculturing after every 6 months.

2.2. Susceptibility to antibiotics

An antibiogram was developed using the disc agar diffusion method. Three colonies, grown on tryptone soya agar (HiMedia M290) at 37 °C for 24 h, were transferred to about 5 ml tryptone soya broth (HiMedia M011) and incubated at the same temperature for 6–8 h until the broth became moderately turbid. A sterile cotton swab (HiMedia PW005) was dipped into the inoculum and applied evenly onto Mueller–Hinton agar (HiMedia M173) plate (4 mm thick). After drying for 15 min, various antibiotic susceptibility test discs (HiMedia) were applied aseptically keeping a distance of at least 3 cm between their centres. The plates were incubated at 37 °C for 14–19 h. The zones showing complete inhibition were measured.

Table 1
Food sources of the *B. cereus* strains used

Source	Nature of the marketed product	Isolate no.
Amriti	Deep-fried (~5 min) and syrup-filled (by dipping into warm sugar syrup for ~5 min) ring-shaped confectionery	104-B1, 104-B2, 104-B3, 105-B1, 105-B2, 105-B3
Dhokla	Steamed (in an open cooker for ~10 min), spongy cake	34-B1, 35-B1, 37-B1
Dosa	Seasoned, griddled (for ~5 min) pancake	16-B1, 55-B1, 98-B1, 98-B2, 98-B3
Idli	Steamed (in an open cooker for ~10 min), spongy cake	94-B1, 94-B2, 94-B3
Papad	Shade dried (to 12–17% moisture content), thin, circular wafer	18-B2, 18-B3, 18-B5, 52-B1, 52-B2, 57-B2, 57-B3, 57-B4, 57-B5, 70-B1, 70-B2, 93-B1, 93-B2, 93-B3, 113-B1, 113-B2, 113-B3
Wadi	Sun-dried (for 4–8 days), hollow, brittle cones	2-B1, 2-B3, 6-B2, 46-B2, 49-B1, 49-B2, 66-B1, 66-B2, 66-B3, 66-B4, 66-B5, 111-B1, 111-B2, 111-B3

2.3. Production of extracellular enzymes

Each of the *B. cereus* isolates was grown in 20 ml brain heart infusion broth (HiMedia M210) at 37 °C for 20 h, and centrifuged (model R-24; Remi Instruments, Mumbai, India) 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 (HiMedia M163), tributyrin agar base (HiMedia M157) added with 1.0% $v v^{-1}$ tributyrin (Fluka 91,012), and starch agar (HiMedia M107), respectively. The incubated starch agar plates were flooded with Lugol's iodine solution. The results were expressed as clear zone diameter (including well diameter of 5 mm).

2.4. Thermal inactivation of sporeformers

The method followed was based on the one described by Johnson, Nelson, and Busta (1982). Overnight growth of *B. cereus* in tryptone soya broth at 30 °C on a rotary shaker (200 rpm) was spread on dried plates of nutrient agar fortified with different minerals and salts (fortified nutrient agar; Kim & Goepfert, 1971) which were kept upright 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 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.

Tubes of 9 ml brain heart infusion broth with 10 g glucose l^{-1} (BHIG) 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.

2.5. Influence of pH on growth

The pH of sterile nutrient broth (HiMedia M002) was adjusted to different levels using 2 N NaOH/ HCl and a pH meter (model 335; Systronics, Ahmedabad, India). The broth (10 ml) in an Erlenmeyer flask was inoculated with 0.1 ml of a 24 h-old culture. The flasks were incubated on a shaker (200 rpm) at 35 °C for 24 h. The growths were measured turbidimetrically at 580 nm using a spectrophotometer (Systronics type 103).

2.6. Influence of food preservatives on growth

For the determination of MIC, plates of nutrient agar supplemented with different concentrations of sodium chloride, or filter-sterilized (0.2 μm cellulose acetate) benzoic acid (1.00134.0005, E. Merck (India) Ltd., Mumbai, India) (stock solution, 3 mg ml^{-1}) or sorbic acid (1,947,109; Sisco Research Laboratories, Mumbai, India) (stock solution, 2 mg ml^{-1}) were spotted (5 spots per plate) with 18 h-old cultures using a 2 mm-diameter loop (Banerjee & Sarkar, 2004).

A sterile stock solution containing 1 mg nisin ml^{-1} was prepared by dissolving 0.4 g Nisaplin (Aplin & Barret Ltd., Beaminster, Dorset, England) 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 sterilized Whatman No. 1 paper (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).

To study combined effects, nutrient broth supplemented with different concentrations of sodium chloride was sterilized by autoclaving. Filter-sterilized 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) nisin (stock solution of 1 mg ml^{-1}) were added separately to sterile nutrient broth to get desired concentrations of those. 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 NaOH/ HCl. Inoculation and incubation of different sets and monitoring of growth were the same as described above.

2.7. 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 solution of 30% (w v^{-1}) acrylamide (SRL 014,022) and 0.8% (w v^{-1}) *N*,*N*-methylene bisacrylamide (SRL 134,985) (solution A) was prepared. While 3.0 M Tris (hydroxymethyl)aminomethane (Tris-HCl; SRL RM262), pH 8.9 was used as resolving gel buffer, 0.5 M Tris-HCl, pH 6.8 served as stacking gel buffer. A 10% (w v^{-1}) solution of running gel was prepared by taking appropriate volume of solution A, resolving gel buffer, 10% (w v^{-1}) sodium lauryl/dodecyl sulphate (SDS; SRL 1948101) and distilled 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 solution A, stacking gel buffer, 10% SDS, distilled 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-saline (0.2 M, pH 7.0) by centrifugation at 3000*g* for 15 min each. The pellet was suspended in stacking gel buffer and boiled in a boiling water-bath for 10 min after adding equal volume of 2x sample buffer containing 20% (v v^{-1}) β-mercaptoethanol (SRL 1327198). A discontinuous buffer system was used. The tank buffer was made up of 0.025 M Tris, 0.192 M glycine (Merck India 4201), 0.1% SDS (pH 8.3). 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 (Merck 60006325001046) for 30 min, stained with coomasie brilliant blue R250 (SRL 024018) solution for 12 h and washed in a destaining solution (methanol (SRL 132977) : acetic acid : water :: 4:1:5) 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).

2.8. Statistical analysis

Experimental data were analysed statistically using Microsoft Excel and SPSS v. 12.0.

3. Results

3.1. Susceptibility to antibiotics

The results for susceptibility of the 48 strains *B. cereus* to 18 different antibiotics, including β-lactams (5), benzene derivative (1), aminoglycosides (2), macrolides (2), peptides (2), glycopeptide (1), quinolones (2), nitro-imidazole (1), tetracycline and trimethoprim, are shown in Table 2. Peptide, glycopeptide, trimethoprim, metronidazole and most of the β-lactam antibiotics had no inhibitory action on the growth of the isolates. Most of the isolates were resistant to antibiotics inhibiting prokaryotic cell wall synthesis, however were sensitive to those inhibiting protein synthesis.

3.2. Production of extracellular enzymes

The results on the production of three extracellular enzymes, viz. protease, lipase and amylase are presented in Table 3. Proteolytic and amylolytic activities were found in 33% and 46%, respectively, of the isolates. However, lipolytic activity was found in only 27% of the isolates. Eleven

Table 2
Antibiogram of *B. cereus* strains ($n = 48$) isolated from cereal based Indian fermented foods

Mechanism of action	Antibiotics (disc^{-1})	Percent score[a]		
		Sensitive	Intermediate	Resistant
Inhibition of cell wall synthesis	Ampicillin (10 μg)			100
	Bacitracin (10 U)		29	71
	Carbenicillin (100 μg)			100
	Cephalothin (30 μg)	2		98
	Cloxacillin (10 μg)			100
	Penicillin G (10 U)			100
	Vancomycin (10 μg)			100
Inhibition of protein synthesis	Chloramphenicol (30 μg)	96	4	
	Erythromycin (15 μg)		60	40
	Kanamycin (30 μg)	58	40	2
	Streptomycin (10 μg)	67	29	4
	Tetracycline (30 μg)	83	17	
Damage to cell membrane	Polymyxin B (300 U)			100
Inhibition of nucleic acid synthesis	Ciprofloxacin (10 μg)	98		2
	Nalidixic acid (30 μg)	38	58	4
	Rifampicin (15 μg)	6	25	69
	Metronidazole (5 μg)			100
Inhibition of folic acid synthesis	Trimethoprim (10 μg)			100

[a] The inhibition zone size (diameter in mm) interpretation was according to Banerjee and Sarkar (2004).

Table 3
Production of extracellular enzymes by *B. cereus* isolates ($n = 48$) from different food sources

Source	Isolate no.[a]	Zone diameter (mm)[b]		
		Protease	Lipase	Amylase
Amriti	104-B3	27		30
	105-B2	26		11
	105-B3	32		33
Dhokla	34-B1	42	25	31
	37-B1			24
Dosa	55-B1		32	
Idli	94-B1			22
	94-B2			14
	94-B3	41	19	39
Papad	18-B2		22	47
	52-B2	41	36	55
	57-B2	41	26	39
	57-B3	42	30	41
	57-B5			23
	70-B1	42	28	41
	93-B2			12
	93-B3	37		44
	113-B2			12
	113-B3	40	22	35
Wadi	2-B1	40	26	35
	6-B2	45	26	38
	49-B1	32		
	66-B3	42	30	49
	111-B1	37	13	18

[a] Others had no activity.
[b] Includes diameter of the well (5 mm).

(23%) isolates produced all the three enzymes, while 10% isolates did not produce any of the enzymes.

3.3. Thermal inactivation of sporeformers

The *D*-values were calculated from the regression analysis best-fit plot of the linear portion of the survivor curve (Fig. 1). In glucose-supplemented brain–heart infusion broth, the correlation coefficient (R^2) values of decimal

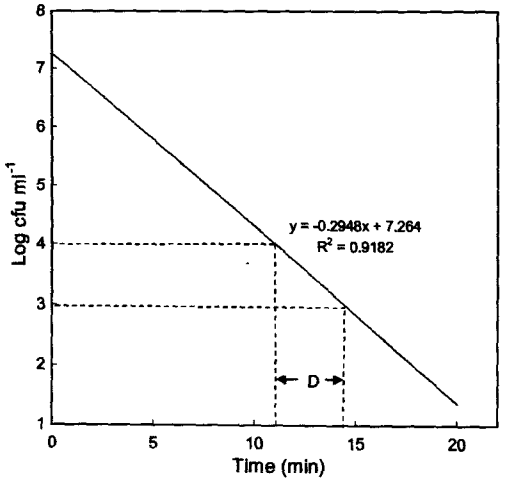

Fig. 1. Determination of *D*-value of *B. cereus* 94-B1 at 100 °C.

Table 4
Thermal inactivation of spores of *B. cereus* isolates from different food sources

Source	Isolate no.	$D_{100\,°C}$ (min)[a]
Amriti	104-B1	7.0 ± 0
	105-B1	5.2 ± 0.2
Dhokla	35-B1	5.3 ± 0.1
	37-B1	7.4 ± 0.1
Dosa	55-B1	5.6 ± 0.1
	98-B1	8.0 ± 0
Idli	94-B1	3.0 ± 0
	94-B2	4.8 ± 0.1
Papad	93-B1	9.2 ± 0.2
	1[illegible]-B1	6.2 ± 0.2
Wadi	66-B1	6.0 ± 0.1
	111-B1	6.8 ± 0.2

[a] Values are mean ± SE of triplicate determinations.

reduction plot curves for spore suspensions of 12 different isolates of *B. cereus* were at least 0.91. The mean $D_{100\,°C}$-values of 48 strains of *B. cereus* spores ranged from 3.0 to 9.2 min (Table 4).

3.4. Influence of food preservatives on growth

The effect of pH on the growth of 6 isolates, one each from the 6 different kinds of foods, is shown in Table 5. In nutrient broth, the minimum and maximum pHs permitting growth of *B. cereus* were 5.3 and 11.6, respectively.

The MICs of different preservatives on the growth of 48 strains of *B. cereus* are shown in Table 5. The growth was inhibited at 65–85 mg sodium chloride ml^{-1}. The MICs of benzoic acid and sorbic acid for growth were 0.4–0.7 mg ml^{-1} (pH 5.0–4.2) and 0.5–0.6 mg ml^{-1} (pH 5.0–4.8), respectively. Most (80%) of the tested 10 strains were resistant to 300 μg nisin ml^{-1} nutrient agar (pH 5.0).

The effect of sodium chloride, benzoic acid and nisin on the growth of *B. cereus* 37-B1 is presented in Fig. 2. In each of these cases, the growth declined with the corresponding increase in concentration of the preservatives. These three effects along with the effect of pH were subjected to Hoke's experimental design. The selected three points were on the trend lines of growth. While the lowest limits were zero in all the cases (excepting pH), the highest limits were judiciously chosen considering the sub-inhibitory concentration levels of the preservatives along with the recommended concentrations of them. Growth of the strain against 19 different combinations of the four types of hurdles is shown in Table 6. While there was no growth in eight sets, the growth reached a maximum level in sets D and E.

3.5. Whole-cell protein fingerprinting

The WCPF of the 48 isolates yielded distinctly different band patterns (Fig. 3). Majority of the strains isolated from the same kind of food could be distinguished by their

Table 5
pH and MIC[a] of food preservatives against the growth of *B. cereus* isolates from different food sources

Source	Target strain	pH range for growth[b]	Preservative in nutrient agar[c]			
			NaCl ($mg\,ml^{-1}$)	Benzoic acid ($\mu g\,ml^{-1}$)	Sorbic acid ($\mu g\,ml^{-1}$)	Nisin ($\mu g\,ml^{-1}$)
Amriti	104-B1	5.3–11.6	65	400 (5.0)	500 (5.0)	175
	105-B1/B2	nd	80	650 (4.3)	500	nd
	104-B3, 105-B3	nd	85	550 (4.5)	500	nd
	104-B2	nd	85	650	500	nd
Dhokla	37-B1	5.4–11.1	50	400	500	>300
	34-B1, 35-B1	nd	85	550	500	>300
Dosa	98-B1	5.3–11.6	65	450 (4.8)	500	>300
	98-B2/B3	nd	80	650	500	nd
	16-B1, 55-B1	nd	85	600 (4.4)	500	nd
Idli	94-B1	5.3–11.6	65	450	600 (4.8)	>300
	94-B2	nd	85	450	600	nd
	94-B3	nd	85	600	500	nd
Papad	113-B1	5.3–11.6	70	400	500	>300
	57-B5	nd	80	450	500	nd
	113-B3	nd	85	550	500	nd
	18-B2/B3/B5, 52-B2, 57-B2/B3/B4, 70-B1/B2, 93-B1/B2/B3, 113-B2	nd	85	600	500	nd
	52-B1	nd	85	650	500	>300
Wadi	111-B1	5.3–11.6	70	450	500	175
	111-B2	nd	70	450	500	nd
	111-B3	nd	80	550	500	nd
	6-B2, 49-B2	nd	85	550	500	nd
	49-B1	nd	85	550	500	>300
	2-B3, 66-B2/B3/B4/B5	nd	85	600	500	nd
	46-B2	nd	85	650	500	>300
	66-B1	nd	85	650	500	nd
	2-B1	nd	85	700 (4.2)	500	nd

[a] MIC (minimum inhibitory concentration) signified minimum concentration of the preservative at which growth was completely inhibited.
[b] nd, not determined.
[c] Values within parentheses indicate pHs of media after the additions.

WCPF patterns. Fig. 4 shows a simplified version of the dendrogram obtained. Basically, the profiles could be grouped into four major clusters emerging at a similarity level of 60%. These clusters, designated A through D, represented 40%, 50%, 6% and 2%, respectively, of the total strains.

4. Discussion

Antibiotic sensitivity studies shows that all the *B. cereus* isolates were multi-drug resistant; each of these was resistant to at least nine different antibiotics. Most of the antibiotics against which the isolates showed resistance belonged to different groups, including β-lactam (ampicillin, carbenicillin, cephalothin, cloxacillin and penicillin G), glycopeptide (vancomycin), peptide (bacitracin and polymyxin B) and trmethoprim. Most of these antibiotics inhibit synthesis of prokaryotic cell wall. As expected, metronidazole, an antiprotozoal drug, had no action on any of the isolates. All these isolates, enriched on *B. cereus* selective medium (which contained 100 U polymyxin B ml^{-1}), were resistant against even a higher concentration (300 U $disc^{-1}$) of polymyxin B. However, an earlier study (Banerjee & Sarkar, 2004) reported susceptibility of only 8% of the 84 *B. cereus* isolates from spices to this higher concentration of polymyxin B. Out of the 48 isolates, 10% were resistant against 9 antibiotics, 21% against 10 antibiotics, 38% against 11 antibiotics, 29% against 12 antibiotics, and 2% against 13 antibiotics. The presence of such a high number of multiple-antibiotic resistant strains of *B. cereus* in foods is a matter of concern. Although use of antibiotics is not the rule of treating gastroenteritis, it is a common therapeutic measure taken (e.g., vancomycin) to combat acute necrotizing gastritis caused by *B. cereus*, particularly in immunocompromised patients (Le Scanff et al., 2006). Genes for resistance and molecular transfer mechanisms have been shown to be the same in bacteria from food and from pathogenic (animal and human) samples. This situation led to scientific and political efforts to handle the problems of antibiotic resistance in food (Teuber, Meile, & Schwarz, 1999).

Microbial enzymes are major causes of quality deterioration and food spoilage (Braun et al., 1999). Activity of the enzymes, like protease, lipase and amylase indicates spoilage potentiality of the producing organisms. While 50% of the 48 isolates were capable of producing at least

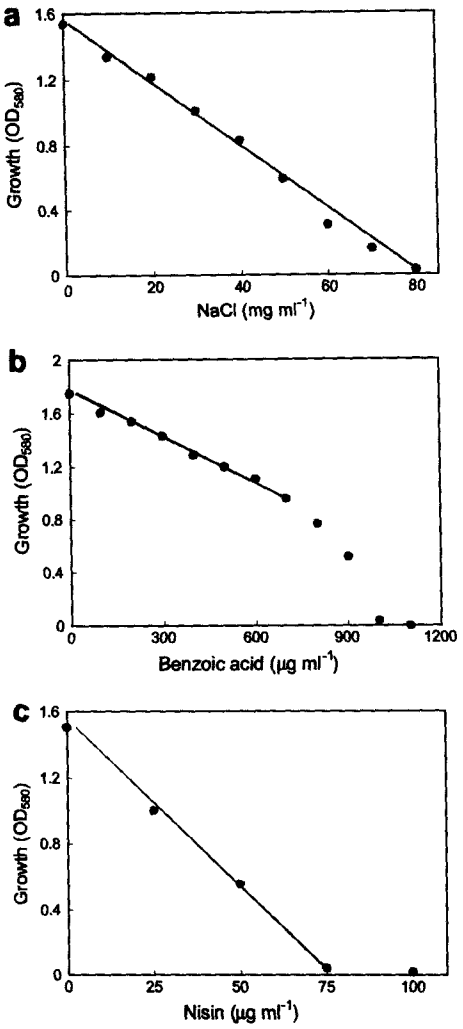

Fig. 2. The effect of sodium chloride (a), benzoic acid (b) and nisin (c) concentration on the growth of *B. cereus* 37-B1.

one of these three enzymes, 23% could produce all these enzymes in vitro. From the results obtained, it can be concluded that many of the strains of *B. cereus* present in fermented foods have the potentiality of causing food spoilage also.

The $D_{100\,°C}$-values of the spore suspensions of 12 different *B. cereus* isolates (two from each of the 6 kinds of foods) were 3.0–9.2 min, which suggest that time–temperature exposure at an appropriate level during cooking may destroy heat-sensitive spores, but not the heat-resistant ones. However, the response of *B. cereus* spores to heating is strain-dependent and is influenced by medium composition (Chung & Sun, 1986). $D_{100\,°C}$-values of 2.7–3.1 min in skimmed milk (Mikolajcik, 1970), 0.6–27.0 min in demineralized water (Rajkowski & Mikolajcik, 1987) and 3.5–5.9 min in BHIG (Banerjee & Sarkar, 2004) were reported for *B. cereus* spores. Spores of *B. cereus*, which may survive heat treatment (e.g., cooking, steaming and frying) during final preparation of these foods, germinate when kept at room temperature before consumption. The data represented here can be used as an aid to predict the time required at 100 °C to achieve a certain number of log-cycle reductions of this potentially dreadful sporeformer.

Now-a-days the use of natural antimicrobial compounds to preserve foods are widely used because of the consumers' demand for additive-free, fresher and more natural tasting food products, while maintaining microbiological safety (Gould, 1996). The addition of salt to foods has been known for centuries. As the common salt acts mainly by reducing water activity (a_w) of foods, its spectrum of action is governed by the demands imposed on a_w by the various microorganisms (Lueck, 1980). In this study, 73% of the strains tolerated a high salt concentration (>80 mg ml^{-1}), which supports halotolerant character of the bacterium.

In most cases weak organic acids, like benzoic acid, have been permitted for food preservation for many years. Benzoic acid is used as an acid or sodium salt at a concentration of 0.5–2.0 mg ml^{-1} in many low pH products (Ray, 2001). In this study, all the isolates were inhibited by benzoic acid concentration within the permissible range. Sorbic acid and sorbates are permitted in all countries of the world for the preservation of many foods, like margarine, cheese, dried and bakery foods using 0.5–2 mg g^{-1} (Ray, 2001). Sorbic acid is a GRAS (generally recognized as safe) substance and its use is permitted in any food product to which preservatives may be added (Lueck, 1980). In this study all the tested strains were inhibited at 0.6 mg sorbic acid ml^{-1}, the level which is lower than the one (0.9 mg ml^{-1}) reported by Del Torre, Della Corte, and Stecchini (2001). As the undissociated form of benzoic and sorbic acids is primarily responsible for antimicrobial activity, and it is highly pH dependent (Jay, 1996), they would be much effective in controlling pathogenic bacteria in lactic fermented foods. As all the *B. cereus* strains tested were inhibited by the acids in their permissible concentration, these acids can be used effectively in controlling the pathogen. Although nisin exhibits a wide range of inhibitory effects against Gram positive sporeformers and pathogens (Hurst, 1981), in the present study it had a low level of inhibitory activity ($\geqslant$175 μg ml^{-1}) against the strains tested. This result was consistent with the finding of Banerjee and Sarkar (2004) who have reported MICs of $\geqslant$125 μg ml^{-1} for the *B. cereus* strains tested. The use of nisin as the sole preservative for a product would probably be unwise, as multiple exposures of a pathogen to nisin would greatly increase the probability of generating stable resistant mutants. However, coupling nisin with several other common food preservation strategies greatly reduces the frequency at which resistance arises (De Martinis, Crandall, Mazzotta, & Montville, 1997). In fact, in most applications, nisin serves as one part of a multiple-barrier inhibitory system.

Prior to undertaking the in vitro multiple-hurdle preservation strategy, the effects of individual hurdles, namely pH, sodium chloride, benzoic acid and nisin on the growth of one isolate were studied separately. Out of 19 different combinations, there was no growth in eight sets. The

Table 6
Growth of *B. cereus* 37-B1 in nutrient broth as influenced by a combination of four independent variables (hurdles) following Hoke's response surface design

Hurdle combination no.	Independent variables				Growth (OD_{580}) Mean ± SE[a]
	pH	Sodium chloride (mg ml^{-1})	Benzoic acid (μg ml^{-1})	Nisin (μg ml^{-1})	
A	5.6	20	300	25	0
B	6.4	0	300	25	0.32b ± 0.03
C	6.4	20	0	25	0.18d ± 0.02
D	6.4	20	300	0	.43a ± 0.02
E	5.6	0	0	0	.46a ± 0.04
F	5.6	40	600	50	0
G	7.2	0	600	50	0.18d ± 0.01
H	7.2	40	0	50	0
I	7.2	40	600	0	0.25c ± 0.02
J	7.2	40	0	0	0.26c ± 0.01
K	7.2	0	600	0	0.30b ± 0.02
L	7.2	0	0	50	0.24c ± 0.03
M	5.6	40	600	0	0.21d ± 0.01
N	5.6	40	0	50	0
O	5.6	0	600	50	0.26c ± 0.02
P	6.4	40	600	50	0
Q	7.2	40	600	50	0
R	7.2	40	300	50	0
S	7.2	40	600	25	0

[a] Values with standard error (SE) were obtained from three replicates. Means within the column sharing a common letter are not significantly different ($P < 0.05$).

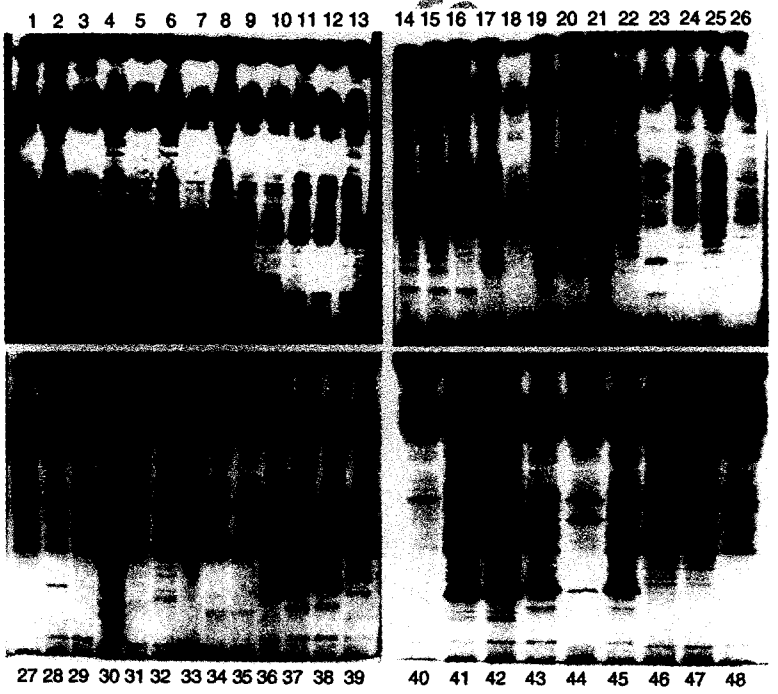

Fig. 3. SDS–PAGE profiles of whole-cell *B. cereus* strains. Lanes: 1, 111-B3 (A11); 2, 111-B2 (A11); 3, 111-B1 (A11); 4, 66-B4 (A9); 5, 66-B3 (C2); 6, 66-B2 (A9); 7, 66-B1 (C1); 8, 49-B2 (A5); 9, 49-B1 (A4); 10, 46-B2 (A4); 11, 6-B2 (A2); 12, 2-B3 (A3); 13, 2-B1 (A1); 14, 18-B2 (B1); 15, 18-B3 (B2); 16, 18-B5 (B3); 17, 52-B1 (B5); 18, 52-B2 (B14); 19, 57-B2 (B6); 20, 57-B3 (B6); 21, 57-B4 (B6); 22, 57-B5 (B15); 23, 70-B1 (B17); 24, 70-B2 (B7); 25, 93-B1 (B16); 26, 93-B2 (B16); 27, 93-B3 (B10); 28, 113-B1 (A12); 29, 113-B2 (B11); 30, 113-B3 (B4); 31, 34-B1 (B11); 32, 35-B1 (C3); 33, 37-B1 (B12); 34, 16-B1 (D); 35, 55-B1 (D); 36, 98-B1 (A7); 37, 98-B2 (A10); 38, 98-B3 (A10); 39, 66-B5 (A8); 40, 104-B1 (B8); 41, 104-B2 (B8); 42, 104-B3 (B8); 43, 105-B1 (A6); 44, 105-B2 (A13); 45, 105-B3 (A5); 46, 94-B1 (B9); 47, 94-B2 (B9); 48, 94-B3 (B13). Cluster/ subcluster numbers are shown within parentheses.

judicious combination found for the cessation of growth of *B. cereus* 37-B1 was 20 mg sodium chloride ml^{-1}, 300 μg benzoic acid and 25 μg nisin ml^{-1} at pH 5.6 (set A), in which all the three preservatives were in moderate concentrations. The same effect was achieved by the omission of benzoic acid, however only at higher concentrations of the

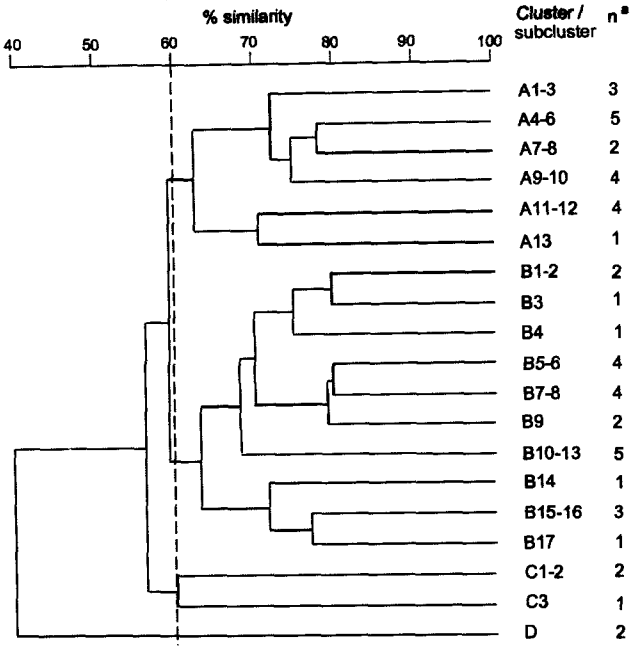

Fig. 4. Simplified dendrogram based on the UPGMA clustering of similarity coefficients (S_D) of whole-cell protein profiles of the 48 strains of *B. cereus* (as shown in Fig. 3). The fingerprint patterns were grouped into four major clusters, designated A through D, on the basis of ⩾60% similarity (arbitrarily chosen) among the strains used. [a] n, number of strains in cluster/subcluster.

other two preservatives (sets H and N). Harris, Fleming, and Klaenhammer (1991) found that the effectiveness of nisin was slightly enhanced by supplementing 25 mg sodium chloride ml^{-1} in a nisin solution. The results of this study will be the basis for an efficient application of hurdle technology in preserving such legume-based fermented foods.

On the basis of WCPF analysis, the 48 isolates of *B. cereus* were found to belong to 34 subclusters. All these subclusters, excepting two (A5 and B11) were source (food)-wise homogeneous. The isolates from wadi were restricted to clusters A (12 isolates out of 14 grouped under 'A') and C (2/14) only, and those from papad were confined to clusters A (1/17) and B (16/17). Cluster D contained isolates from dosa only. All the three isolates from idli were confined to cluster B. This study reveals a diversity of the organism at subspecies level and a relative preference of the WCPF subclusters to particular types of legume-based fermented foods.

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