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.