5

Discussion

5.1. Occurrence of foodborne bacterial pathogens in Indian retail spices

Spices have the capability of transforming a regular diet into romance. Unwillingly and silently they can transform this romance into a disaster by acting as a vehicle of dreaded foodborne bacterial pathogens. They are classed as important vectors for various micro-organisms implicating possible health problems for consumers as well as quality and shelf-life problems for foods (Kneifel and Berger 1994). Uncleaned spices may be grossly contaminated, and contamination of foods by spice carriers has been reported to lead to food spoilage and can even lead to food poisoning (Krishnaswamy *et al.* 1971; Goepfert *et al.* 1972; Powers *et al.* 1976). Sporeformers may lead to food spoilage when they survive the cooking process and

multiply under favourable conditions (Powers *et al.* 1976; Beuchat *et al.* 1980). It is evident that the degree and frequency of contamination of spices are influenced mainly by the hygienic conditions at their origin and at the processing plant as well as by their chemical nature (De Boer and Boot 1983; Lewis 1984; Gerhardt 1990).

The moisture profile of Indian retail spices (Table 9) conformed well with the specifications of European Spice Association (ESA), British Standards Institute (BSI) and International Organization for Standardization (ISO) for the maximum limit of moisture content in spices (http://www.indianspices.com/html/s1490qua.htm)

As per ICMSF specifications (Table 2), a spice containing total aerobic mesophilic bacteria (TAMB) count of <log 4 g^{-1} is of acceptable quality and log 4-6 g^{-1} is of marginal quality. The results of this study indicate a high level of contamination (Table 9); 51% (78/154) of the samples were in the unacceptable range (>log 6 g^{-1}). The mean load of TAMB was found maximum (log 8.7 g^{-1}) in black pepper and minimum (log 3.3 g^{-1}) in garlic. Occurrence of the maximum population of TAMB in black pepper, amongst a number of spices, was demonstrated in several findings (Gecan *et al.* 1983; Pafumi 1986; Kneifel and Berger 1994). Krishnaswamy *et al.* (1974) observed that in black pepper the counts of TAMB ranged between log 4 and log 8 g^{-1}. Samples of black pepper, collected from warehouses in selected spice-trading areas of India, contained log 4-7 TAMB g^{-1} (Seenappa and Kempton 1981). The incidence of TAMB counts in whole black pepper and turmeric powder samples, collected from retail shops in the city of Mumbai in India, were log 8.0-9.9 g^{-1} and log 7.6-9.8 g^{-1}, respectively (Geeta and Kulkarni 1987). The TAMB may have no overt public health significance, but it may contribute to some degree towards spoilage of a food product in which a spice is used. The TAMB may also indicate to what extent the finished spice has been cleaned and whether or not it has been mishandled (Powers *et al* 1975). Mesophilic bacterial spores (MBS) constituted 0.3-90% of the TAMB population. Baxter and Holzapfel (1982) observed that MBS accounted for 50-95% of TAMB in spices. The presence of MBS is important since their survival or the presence of their toxins may result in food poisoning or deterioration of the product in which spices have been added (Baxter and Holzapfel 1982). The findings of the present investigation are not unexpected because in India the spices are routinely marketed without sterilization. On the basis of these results, especially in view of the presence of high numbers of MBS in the samples, the need for clean or preferably commercially sterile spices is emphasized.

The frequency of *B. cereus* was also high (55% of the samples analyzed); except four, all the kinds of spices contained this aerobic sporeformer (Table 10). It occurred in 100% of the samples of ajmud, small cardamom and cumin powder. The results agree well with the findings of Powers *et al.* (1976) where *B. cereus* was found in 53% (58/110) of the spices analyzed. Compared to *B. cereus*, *Cl. perfringens* was less frequent, found in 17% of the total samples analyzed (Table 10). This anaerobic sporeformer was found in only one out of 160

retailed samples of spices in Austrian market (Kneifer and Berger 1994), but in 27 out of 380 samples in Mexico (Rodríguez-Romo *et al.* 1998). Spices harbouring *Cl. perfringens* must be considered a potential health hazard because it may grow in foods that are seasoned with spice if the food is not adequately cooked or properly refrigerated (Powers *et al.* 1975). The frequency of occurrence of *Staph. aureus* was relatively low; it was found in only a few samples of asafoetida (1), small cardamom (2) and garlic (1). It would be of interest to understand the reason for its low incidence when the frequency of associated microflora is significanly higher.

The members of Enterobacteriaceae occurred in 85% of the kinds and 33% of the samples of spices (Table 11). This is in contrast with the reports (Julseth and Deibel 1974; Powers *et al.* 1975) where relatively low numbers of Enterobacteriaceae were mentioned. When the counts of different shops were compared, the most unhygienic shops showed the highest counts of TAMB and Enterobacteriaceae. The counts decreased proportionally with an increase in hygienic condition of the environment. Coliforms occurred in 12 samples representing 10 kinds. Faecal coliforms were found in four kinds. The presence of *E. coli*, a convenient indicative of faecal contamination and the possible presence of enteric pathogens, in only one sample (of garlic) at a load of 233 cfu g^{-1} may be due to contamination by rodents and unhygienic handling methods. Indeed, the level of sanitization in the sampling site of this garlic was poorer than the other places of collection. The low incidence of *E. coli* may be due to the fact that this bacterium was not isolated after enrichment. Following enrichment, *Salmonella* and *Shigella* were isolated from only two samples. Although Enterobacteriaceae could not be detected (detection limit being 10 cfu g^{-1}) in any of the six tested samples of ginger, *Salmonella* could be enriched and isolated from one of those. Such a low contamination frequency agrees with those of others (Julseth and Deibel 1974; Baxter and Holzapfel 1982; Schwab *et al.* 1982; Kneifel and Berger 1994) who reported that *E. coli*, salmonella and shigella in spices were apparently rare and sporadic. Powers *et al.* (1975) detected coliforms in three samples, but no faecal coliforms, in a total of 114 samples of spices tested.

Considerable variations were observed in the microbial counts, even between samples of the same kind (Fig. 28). Hence, the distribution was contagious (Jarvis 1989).

To determine significance of the differences, analysis of variance was computed using log cfu values of TAMB, MBS, *B. cereus* and Enterobacteriaceae. There was no significant ($P<0.05$) correlation between TAMB, *B. cereus* and Enterobacteriaceae. Results of *Cl. perfringens*, *Staph. aureus*, *E. coli*, *Salmonella* and *Shigella* were not considered because of their low incidence in spices. Black cumin was found loaded with significantly ($P< 0.05$) high doses of all these contaminants. On the contrary, asafoetida and garlic were significantly ($P<0.05$) less contaminated. No significant ($P<0.05$) difference was found between the levels of contaminants in spices collected from the eastern and western zones or during the summer and winter seasons of India.

The number of packaged kinds of spices containing TAMB was 54% higher than that of non-packaged ones. On the other hand, the respective numbers of non-packaged kinds containing *B. cereus* and Enterobacteriaceae were 69% and 62% higher than the packaged kinds.

Considering the ICMSF specifications as a guide, the results of this investigation indicate a high level of micro-organisms in spices that may be a source of contamination in the kitchen. Also considering the widespread use of spices, there is a need to stress the importance of correct handling of the food that incorporates spices as an ingredient, at the both domestic and commercial levels. There is a need to establish standards compliance for spices to provide the user with a reliable safe product (Christensen *et al.* 1967). However, it is difficult to select a single microbial index for quality determination of these food additives because they are used as ingredients in a variety of products prepared in different ways. The desired index selected should reflect the use and method of preparation of the food product. Knowledge on the behaviour of these spice isolates can throw a new light to understand and minimize the risk associated with food manufacturing operations.

A high incidence of TAMB, MBS, *B. cereus*, *Cl. perfringens* and Enterobacteriaceae is to be reviewed with serious concern, as many of these are known to cause not only deterioration of keeping quality of spices but also serious health hazards in human population. These data can be the basis in establishing good manufacturing practices (GMP) and good hygienic practices (GHP) and in formulating Indian standards for microbiological quality of spices to ensure retailing and exporting safe quality spices.

5.2. Behaviour of the spice isolates

5.2.1. Susceptibility to antimicrobials

Antibiotic resistance in foodborne pathogens is a reality, though substantial qualitative and quantitative differences exist (Teuber 1999). Strains of foodborne bacterial pathogens that are resistant to a variety of antimicrobial agents have become a major health concern (Kiessling *et al.* 2002). Drug resistance of pathogenic bacteria diminishes the effectiveness of antimicrobial treatment and can lead to the use of less safe, ineffective or expensive alternatives (Morell 1997; Tollefson *et al.* 1999). Fifty years of increasing application of antimicrobial agents have created a situation leading to an ecological imbalance (Levy 1997), the enrichment of multiple antibiotic resistant pathogenic bacteria. 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 agriculture (Teuber *et al.* 1996, 1999).

The present study was undertaken to estimate the extent of prevalence of antimicrobial resistance patterns in the spice isolates (Table 12). A total of 84 strains of *B. cereus*, one from

each of the positive samples, were tested for susceptibility to each of 20 antimicrobial compounds. Maximum number (>50%) of the strains tested showed multiple resistance against a combination of ampicillin, carbenicillin, cephalothin, cloxacillin, metronidazole, penicillin G, polymyxin B, rifampicin and trimethoprim. Eight percent of the *B. cereus* strains, which were enriched on *Bacillus cereus* selective medium (which contained 100 U polymyxin B ml^{-1}), were found sensitive to a higher concentration (300 units $disc^{-1}$) of polymyxin B.

Twenty-six strains of *Cl. perfringens*, one from each positive sample, were tested for determining susceptibility to antimicrobials. Maximum number (>50%) of the strains were resistant to a combination of ampicillin, cephalothin, cloxacillin, erythromycin, gentamicin, kanamycin, metronidazole, nalidixic acid, polymyxin B, rifampicin and streptomycin. Though metronidazole is the drug of choice for anaerobes, all the strains of *Cl. perfringens* were found resistant at its concentration of 5 μg $disc^{-1}$. Gentamicin, kanamycin and streptomycin resistance of *Cl. perfringens* supports the fact that these aminoglycosides have no effect on anaerobes (Egorov 1985; Tripathi 1999). All the four isolates of *Staph. aureus* were resistant to a combination of seven antimicrobials, namely ampicillin, carbenicillin, cephalothin, cloxacillin, metronidazole, penicillin G and rifampicin.

Maximum number (>50%) of the 51 strains of Enterobacteriaceae, one from each positive sample, were resistant to a combination of ampicillin, bacitracin, carbenicillin, cloxacillin, erythromycin, metronidazole, penicillin G, rifampicin and vancomycin.

Both the isolates of *Salmonella* were resistant to a combination of ampicillin, bacitracin, cephalothin, cloxacillin, erythromycin, kanamycin, metronidazole, penicillin G, rifampicin and vancomycin, whereas *Shigella* strains were resistant to ampicillin, carbenicillin, cephalothin, cloxacillin, erythromycin, metronidazole, penicillin G, rifampicin and vancomycin. These results indicate that Gram-negative bacteria were resistant to cloxacillin, erythromycin, metronidazole, penicillin G, rifampicin and vancomycin. The finding supports the observation of Egorov (1985) and Tripathi (1999). These data will help in tackling the problem of treating food poisoning resulting from multiple antimicrobial-resistant spice isolates and their potential role in transmitting antimicrobial resistance to other pathogenic micro-organisms.

The results presented here extend a overwhelming support for the control of antimicrobials in agriculture with resulting benefits to human health. 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 process.

In brain heart infusion broth supplemented with 10 g glucose l^{-1} (BHIG), the correlation coefficient (R^2) values of decimal reduction time curves were in all cases al least 0.98. The $D_{100°C}$-values for spore suspensions of 23 different *B. cereus* isolates (one from each spice type which showed the highest count) ranged from 3.5 min to 5.9 min (mean, 4.4 min), whereas for *Cl. perfringens* it ranged from 10.5 min to 18.5 min (mean, 15.8 min) in fluid thioglycolate medium (Table 13). Thus, compared to *B. cereus*, the spores of *Cl. perfringens* were found more resistant to heat, and for this, in many cases, the clostridial spores are considered as good indicators of the effectiveness of the disinfection routines in food, food production environment and in water. These data suggest that time-temperature exposure at an appropriate level during cooking may destroy heat-sensitive spores, but not the heat-resistant ones. Spices can act as carrier of these survivors which can germinate and multiply rapidly in foods stored at room temperature. The findings resemble the reports of Ingram (1969); Mikolajcik (1970); Chung and Sun (1986) and Adams and Moss (1995).

The *z*-values, used to plot a thermal resistance curve, of two strains of *B. cereus* were 17 and 18°C (Fig. 30). These values are much higher than those obtained (7.4-14.5°C in demineralized water and 6.5-11°C in phosphate buffer) by Rajkowshi and Mikolajcik (1987). The response of *B. cereus* spores to heating is strain-dependent and is influenced by the medium composition (Kim and Goepfert 1971b; Chung and Sun 1986). The unusual degree of heat resistance exhibited by spores accounts for the frequent association of this organism with food spoilage and food poisoning.

The data presented here can be used as an aid to predict the time required at specified temperatures to achieve a certain number of log-cycle reductions of these potentially dreadful sporeformers when heated in precooked and seasoned foods.

5.2.3. Growth and production of enterotoxins

Bacillus cereus was found as the most frequent organism in spices. To define the extent of dreadfulness of this organism, an analysis of *Bacillus cereus* enterotoxin (BCET) was undertaken. Since the stimulatory effect of glucose on growth and enterotoxin production by *B. cereus* is well documented (Carpenter et al. 1975; Spira and Silverman 1979; Kramer 1984; Garcia-Arribas and Kramer 1990), BHIG was the medium of choice for this purpose. Qualitative test showed that 74% of the 23 tested strains (one from each spice kind which showed the highest count among all the samples of that particular kind) produced BCET in BHIG; 15 strains showed the maximum agglutination reaction (+++). While one strain (17-B1)

produced BCET that went beyond the dilution range (2-256 ng ml^{-1}) selected for the test, another (120-B1) produced the highest level of enterotoxin content (256 ng ml^{-1}) within the test range (Table 14). From these data, it is concluded that, in principle, *B. cereus* isolates from spices are able to produce enterotoxins. It should, however, be borne in mind that the BCET-RPLA test does not specifically react with the diarrhoeal toxin, but with an indicator protein. In addition, this test can not detect the *B. cereus* emetic toxin. Consequently, *in vivo* toxicity tests should be carried out in case of doubt of toxicity of samples having high BCET levels (Nout *et al.* 1998).

While selecting for the BCET level in spices, the spice kinds where all the samples of that kind contained *B. cereus* were chosen (Fig. 33). Cumin powder had the highest content of BCET (64 ng g^{-1}) however with the lowest cell count (log 2.6 g^{-1}) which indicates the production of a considerably high level of BCET by the strain 120-B1 (a cumin powder isolate). The results suggest a potential health hazard of foods containing spices with this bacterium.

The most recently developed and only commercially available serological assay for *Clostridium perfringens* enterotoxin (PET) is reversed passive latex agglutination (RPLA) test (Labbé 1989). Out of 16 strains (one from each positive sample) tested, only three strains showed positive response towards PET-RPLA test (Table 15). Hence, those three isolates were subjected to quantitative assay. While the strain 16-C2 produced the maximum quantity (32 ng ml^{-1}), the strains 20-C2 and 135-C3 and produced 2 ng and 8 ng PET ml^{-1}, respectively. However, this culture method is less reliable due to problems in encouraging *Cl. perfringens* to produce sufficient toxin in artificial media. The direct detection of PET in faeces should be carried out parallelly as much larger amount of toxin is formed *in vivo* (Labbé 1989).

To understand how much the environment of a spice is congenial for growth of *B. cereus* and its enterotoxin production, black pepper powder was taken as a support spice since none of the five samples of black pepper powder tested contained *B. cereus*. After intentional inoculation of the spice with *B. cereus* 120-B1 (the most potent BCET producer within the test limit), there was no significant ($P<0.05$) changes in the cell count as well as BCET production (Fig. 34) even after 14 d incubation at room temperature (as spices are conventionally stored). This indicates that any spice can support at least survival (if not growth) of pathogenic organisms and act as a vector for contaminating foods.

One is usually fond of spicy foods, but the issue of their safety needs to be assessed. *Aloo dam*, a potato-based ethnic and popular side dish, was taken as a model food for the growth of *B. cereus*. The rationale behind selecting this starchy food was the findings of Garcia-Arribas and Kramer (1990) that the starch-supplemented BHI supported near-optimum growth of *B. cereus* and production of enterotoxin at levels comparable to those produced in BHIG. Freshly prepared *aloo dam* did not contain *B. cereus*, but small cardamom (where 100% of the samples were positive for *B. cereus*) acted as a carrier of *B. cereus* when the food was

seasoned with it (as is practiced routinely). The inoculum, whether through the spice vector or as a pure culture intruder, multiplied rapidly in this starch-based food at room temperature and produced enterotoxin (Fig. 36).

In a similar study, goat meat gravy was taken as a test food for the growth of a meat-loving organism, *Cl. perfringens* and its enterotoxin (PET) production. This anaerobic sporeforming pathogen occurs in 30-80% of raw and frozen meat and also poultry (Labbé 1989). In this study, it was found that the freshly prepared meat gravy was free from this bacterium (Fig. 37). When the gravy was inoculated with *Cl. perfringens* 16-C2 (@3.4 cfu g^{-1} gravy), it multiplied rapidly, however the enterotoxin level remained unchanged (2 ng PET g^{-1}). Although low levels of production have been observed in vegetative cultures, the PET is synthesized by the sporulating cells (Adams and Moss 1995; Brynestad and Granum 2002). For this, it has been recommended by Juneja and Marmer (1998) that control measures for *Cl. perfringens* food-poisoning must ensure that large numbers of vegetative cells are not consumed. Possibly the gravy and the duration of incubation were not conducive for sporulation. After boiling the 19 h-long incubated gravy (containing log 7.7 cfu g^{-1} and 2 ng PET g^{-1}), the cell count decreased by more than 4 log cycles (to log 3.1 cfu g^{-1}), possibly due to the destruction of vegetative cells and enterotoxin count went below the detection limit (to <2 ng PET g^{-1}). The enterotoxin is heat labile, and heating in saline at 60°C for 5 min destroys 80% of its serological activity (Naik and Duncan 1978). It is likely the reason for non-detection of PET after boiling for 20 min.

Since the frequency of *Staph. aureus* and *E. coli* was remarkably low in spices, attempts were not made to assay enterotoxins produced by these nonsporeforming bacteria.

It was confirmed that spices, when added to foods, are able to support outgrowth of the microflora and production of enterotoxins. The spice microflora can shorten market life of the products through spoilage and/or conceivably contribute to consumer illness. The application of knowledge of the factors inhibiting the growth of different pathogenic bacteria can contribute to minimize the risk of food manufacturing operations.

5.2.4. The influence of hurdles on growth

During the course of study on the incidence of bacterial pathogens in spices, it was found that garlic samples were free from any *B. cereus* and *Cl. perfringens*, and had a relatively low count of TAMB, MBS and Enterobacteriaceae. This observation indicated the presence of a potent antibacterial component in garlic.

The primary screening of spice isolates using garlic slices (Fig. 38), a novel technique developed, showed a high sensitivity (71% of the strains tested) towards *B. cereus*. All the strains of *Cl. perfringens*, *Staph. aureus*, faecal coliforms, *E. coli*, salmonella and shigella were found resistant to garlic (Table 16). As dimension of the inhibition zone varied from

garlic to garlic (Fig. 39), the results of this primary screening can not be considered as absolute. However, it showed a relative degree of sensitivity among the test organisms can be discerned using slices from one garlic bulb.

The minimum inhibitory concentrations (MICs) of crude garlic extract for randomly selected spice isolates as well as a few reference strains suggest that primary screening using garlic slice was sensitive only when the MIC of garlic for an organism was ≤ 8 mg g^{-1} extract (Table 17). The MIC for *B. cereus* was 6-10 mg garlic g^{-1} extract, the value which is much less than what was obtained (100 mg g^{-1}) by Saleem and Al-Delaimy (1982). The MIC for all other organisms were more than 8 mg g^{-1}, and that is why several *B. cereus* strains and all non-*B. cereus* isolates were found resistant during primary screening. The garlic isolates were more tolerant than the strains isolated from other spices. Al-Delaimy and Ali (1970) reported that for the inhibition of the growth of *Staph. aureus*, *E. coli*, *Salmonella typhi* and *Shigella dysenteriae* there required 40 mg garlic ml^{-1} extract.

Interestingly, all the *B. cereus* isolates tested for sensitivity to garlic were resistant to a number of antimicrobials, namely ampicillin (10 μg $disc^{-1}$), cloxacillin (10 μg $disc^{-1}$), metronidazole (5 μg $disc^{-1}$) and penicillin G (10 units $disc^{-1}$). Similarly, each of the tested strains of *Cl. perfringens*, *Staph. aureus*, *E. coli*, salmonella and shigella from spices was found resistant to at least six commonly used antimicrobials.

The growth of *B. cereus* was completely inhibited at 10 mg garlic g^{-1} extract (Fig. 40), but when this totally inhibited culture was returned to a garlic-free broth, the growth resumed. This indicated that the effect of garlic was bacteriostatic (not bactericidal), as was previously described by Srivastava *et al.* (1982) in their studies on Gram-negative pathogenic bacteria. However, Cavallito and Bailey (1944) reported that the active principle, obtained by steam distillation of ethanolic extract of garlic was bactericidal.

The BCET production was completely inhibited at 10 mg garlic ml^{-1} BHIG (Fig. 40). The findings are similar to those of Kumar and Sharma (1982) where *E. coli* enterotoxin production was inhibited significantly ($P<0.01$) at the sub-inhibitory concentration of garlic extract. Although literatures on the effects of garlic on the growth of *B. cereus* are available, the same on inhibitory effect of garlic on its enterotoxin production was not available. Observations of this study confirm the long-standing concept of using garlic as a means of securing safety and extending shelf-life of food.

The potential for more extensive usage of such antimicrobials would appear to be good, in light of the consumer's desire for so-called "natural" and "organic" foods, i.e. foods to which man-made preservatives are not added. The challenge is to isolate, purify, stabilize and incorporate such natural antimicrobials into foods without adversely affecting sensory, nutritional and safety characteristics.

All the spice isolates tested grew optimally at near-neutral (6.61-7.51) pH (Fig. 41) which support their growth in different foods through spice carrier as most of the foods have

pH between 5.6 and 6.6 (Adams and Moss 1995).

The addition of appropriate antimicrobial preservatives is used to reduce the growth of *B. cereus*. 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 spice isolates (Table 18).

The addition of salt to foods have been known for centuries. As common salt acts chiefly by reducing the water activity of foods, its spectrum of action is governed by the demands imposed on the water activity by the various micro-organisms (Lueck 1980). *Staphylococcus aureus* tolerated highest salt concentration, up to 80 mg ml^{-1} which supports halophilic character of the bacterium. Others tolerated up to 65 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 and 2500 $\mu g\ ml^{-1}$ (Lueck 1980). All the isolates tested were inhibited by benzoic acid concentration within permissible range (Table 18).

Sorbic acid and sorbates are permitted in all countries of the world for the preservation of many foods. The maximum permissible quantity, other than in exceptional situations, is between 1000 and 2000 $\mu g\ ml^{-1}$. 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 (Lueck 1980; Liewen and Marth 1985). All pathogenic bacteria, except some *Cl. perfringens* strains, were inhibited at this concentration (Table 18). Hansen and Appleman (1955) reported that sorbic acid neither inhibited nor stimulated growth of clostridia in laboratory media at pH 6.7.

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). In this study, the MICs of Nisaplin against the sporeformers were found higher than those against *Staph. aureus*. 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). In this investigatin, the effect of Nisaplin was studied in near-neutral pH as most of the foods are at this pH level. 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)

For centuries, foods have been preserved by heating, chilling, drying, salting, conserving, acidification, oxygen-removal, fermenting, adding various preservatives etc. Often these methods were applied in combinations. More recently, the underlying principals 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* 120-B1 (Table 19) was investigated in order to understand the scientific basis for an efficient application of hurdle technology in preservation of food which can be contaminated through the spice carrier.

Prior to undertaking the *in vitro* multiple-hurdle preservation strategy, the effects of individual hurdles, namely sodium chloride, benzoic acid and nisin on the growth of *B. cereus* 120-B1 were studied separately (Figs 42-44). The correlation coefficient (R^2) values of the survival curves were in all cases greater than 0.98. Out of 19 different combinations, no growth was found in seven sets (Table 20). Each of these sets had a combination of sodium chloride and Nisaplin. 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 (40-400 IU ml^{-1}). Another set, which had pH 5.8, benzoic acid 350 μg ml^{-1} and Nisaplin 4000 IU ml^{-1}, but no sodium chloride showed only a little growth. This was due to the combined effect of nisin and benzoic acid at acidic pH. Interestingly, at pH 6.6 and in the presence of 4000 IU Nisaplin ml^{-1} but no sodium chloride and benzoic acid, growth was observed, which indicates nisin acted mainly in combination with sodium chloride or benzoic acid. The best combination found for the cessation of growth of *B. cereus* 120-B1 was pH 6.2, 25 mg sodium chloride ml^{-1}, and 2000 IU Nisaplin ml^{-1}. In this combination set, the three hurdles were used in medium concentrations. Another set having pH 5.8, 25 mg sodium chloride ml^{-1}, 175 μg benzoic acid ml^{-1} and 2000 IU Nisaplin ml^{-1} was also considered, however in this combination pH was acidic. The rest of the combinations which did not support growth, contained maximum levels of Nisaplin and either sodium chloride or benzoic acid or both. So, they can not be considered as intelligent combinations.

Spices not only have their unique sensory appeal, but also possess potential for their nutritional and medicinal applications. Their dominance in India's food export sector has the potentiality of boosting the country's economy. However, Indian spice industry is still awaiting its metamorphosis from traditional to the most advanced technology facets. The endeavours envisaged in this dissertation are footsteps towards serving a safe "spice bowl of India" on every dining table of the world.