IDENTIFICATION OF CONTAMINATION SOURCES OF *BACILLUS CEREUS* IN PASTEURIZED MILK

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

SHIRLEY LIN

In partial fulfilment of requirements

for the degree of

Master of Science

June 1997

© Shirley Lin, 1997

National Library of Canada

Acquisitions and Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada

Acquisitions et services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-24483-0

Canada

ABSTRACT

IDENTIFICATION OF CONTAMINATION SOURCES OF *BACILLUS CEREUS* IN PASTEURIZED MILK

Shirley Lin
University of Guelph, 1997

Advisor:
Dr. M. W. Griffiths

This research investigated the potential of Fourier transform infrared spectroscopy (FTIR) and the Microbial Identification System (MIDI) to rapidly identify *Bacillus cereus* and to trace the sources of *B. cereus* in pasteurized milk. Ten *B. cereus* strains tested produced identical characteristic infrared absorbance peak between wavenumbers 1738-1740 cm^{-1} on brain heart infusion(BHI) and trypticase soy agar(TSA) by using FTIR. The specific infrared absorbance peak can be used to differentiate *B. cereus* from other bacteria.

A total of 232 milk samples and 122 environmental swabs were collected in two dairy plants between March and September 1996. The incidence and the average counts of *B. cereus* in the positive heat-treated raw milk, pasteurized milk and final products were over 80% and 1.1×10^5 CFU/ml, respectively, after enrichment at 8 °C for 14 days. A total of 546 *B. cereus* isolates from 183 milk samples and 3 environmental swabs were classified by their fatty acid profiles using MIDI. The results suggested that the *B. cereus* spores in raw milk were the major source of *B. cereus* in pasteurized milk and the post-pasteurization contamination from the dairy environment was possibly another minor source of *B. cereus* in pasteurized milk.

ACKNOWLEDGEMENTS

I would like to take this opportunity to deeply thank my supervisor, Dr. M. W. Griffiths for his kindness, guidance and support throughout the research and thesis-writing. I am very grateful to Dr. H. Schraft for her patience, advice and encouragement during the research and my thesis-writing. I would also like to thank Dr. J. Odumeru for his kindness, valuable suggestions and support for my research and thesis. I would also like to thank Dr. Art Hill to be my exam chair.

I would like to acknowledge the technical advice of Dr. F. Yuan, Mr. K. Y. Lee, as well as to the other people in the Department of Food Science and OMAFRA lab for their valuable suggestions, scientific discussion and friendship.

Special thanks go to my husband, Yurong Chen, for his love, patience, support and encouragement, and my parents, my daughter (Jessica Chen) and my son (Kevin Chen) for their understanding and encouragement throughout my MSc. studies.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i

TABLE OF CONTENTS ii

LIST OF TABLES v

LIST OF FIGURES vii

CHAPTER 1. LITERATURE REVIEW

1.1. Taxonomy and characteristics of *Bacillus* species 1

1.2. Pathogenicity of *Bacillus* species 3

1.2.1. *Bacillus cereus* 4

1.2.1.1. Diarrhoeal toxin 4

1.2.1.2. Emetic toxin 5

1.2.1.3. Non-gastrointestinal infections 6

1.2.2. Other *Bacillus* species 7

1.3. Importance of *B. cereus* in dairy products 8

1.3.1. Incidence of *B. cereus* in dairy products 8

1.3.2. Sporulation of *B. cereus* 9

1.3.3. Germination of *B. cereus* spores 10

1.3.4. Adhesion of *B. cereus* spores 12

1.3.5. Psychrotrophic characteristic of *B. cereus* 13

1.4. Isolation and enumeration of *B. cereus* 14

1.5. Identification and classification of *B. cereus* using rapid methods 16

1.5.1. Principle of Fourier transform infrared spectroscopy (FTIR) 16

1.5.2. Principle of gas liquid chromatography (GLC) 19

1.5.3. Characteristics of fatty acids of bacteria 23

CHAPTER 2. IDENTIFICATION OF *BACILLUS CEREUS* USING FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

2.1. Introduction 26

2.2. Materials and methods 28

2.2.1. Strains and growth conditions 28

2.2.2. Sample preparation 30

2.3. Results 30

2.4. Discussion 31

CHAPTER 3. IDENTIFICATION OF THE SOURCES OF *BACILLUS CEREUS* IN PASTEURIZED MILK USING MICROBIAL IDENTIFICATION SYSTEM (MIDI)

3.1. Introduction 39

3.2. Materials and methods 41

3.2.1. Sample collection 41

3.2.2. Preparation of milk samples and environmental swabs 43

3.2.3. Enumeration and collection of *B. cereus* isolated from milk samples and environmental swabs 43

3.2.4. Analysis of fatty acid profiles of *B. cereus* isolates using MIDI 44

3.2.4.1. Preparation of fatty acid methyl esters (FAMEs) 44

3.2.4.2. Microbial Identification System 46

3.2.5. Identification and further confirmation of *B. cereus* isolates 47

3.2.6. Construction of the 'BCERMK' library 48

3.2.7. Allocation of *B. cereus* isolates to the 'BCERMK' library 51

3.2.8. Allocation of *B. cereus* isolates to the 'MI' library 51

3.3. Results 51

3.3.1. Incidence and distribution of *B. cereus* among milk samples and environmental swabs 51

3.3.2. Identification and further confirmation of *B. cereus* isolates 53

3.3.3. Characteristics of the cellular fatty acid composition of *B. cereus* isolates in each subgroup of the 'BCERMK' library 54

3.3.4. Relationship between *B. cereus* isolates from milk samples at each sampling point and environmental swabs 61

3.3.5. Relationship between *B. cereus* isolates from different dairy plants 63

3.3.6. Relationship between *B. cereus* isolates from different sampling months 65

3.3.7. Relationship between *B. cereus* isolates based on the 'MI' library 69

3.4. Discussion 69

CHAPTER 4. GENERAL DISCUSSION AND FUTURE WORK

4.1. General discussion 74

4.2. Intervention strategies to minimize the sources of contamination of pasteurized milk with *B. cereus* 76

REFERENCES 78

APPENDICES 96

LIST OF TABLES

2.2.1 Table 1. Bacterial strains used and their major infrared absorbance peaks between 1800-1500 cm^{-1} wavenumbers after growth on TSA and BHI media 29

3.2.1. Table 2. The number of milk samples from each sampling point of the milk processing lines and environmental swabs in plant I and plant II 42

3.2.6. Table 3. The number of *B. cereus* isolates from the milk samples of each sampling point of the milk processing lines and environmental swabs in plant I and plant II used for the construction of the 'BCERMK' library 50

3.3.1. Table 4. The incidence and the average counts of *B. cereus* in milk samples from each sampling point of the milk processing lines and environmental swabs in plant I and plan II after enrichment at 8 °C for 14 day 52

3.3.2. Table 5. The number of *B. cereus* isolates and samples from each sampling point of the milk processing lines and environmental swabs in plant I and plant II . 55

3.3.3. Table 6. Main fatty acid composition of *B. cereus* isolates in each subgroup of the 'BCERMK' library 57

3.3.4. Table 7. The number of *B. cereus* isolates from each sampling point of the milk processing lines and environmental swabs allocated to each subgroup of the 'BCERMK' library 62

3.3.5. Table 8. The number of *B. cereus* isolates from plant I and plant II allocated to each subgroup of the 'BCERMK' library 66

3.3.6. Table 9. The number of *B. cereus* isolates from each sampling month allocated to each subgroup of the 'BCERMK' library 68

3.3.7. Table 10. The number of *B. cereus* isolates from each sampling point of the milk processing lines and environmental swabs in plant I and plant II allocated to each subgroup of the 'MI' library 70

LIST OF FIGURES

2.3. Figure 1 (A). Absorbance spectra between wavenumbers 2000-1000 cm^{-1} of the bacterial cultures grown on Brain Heart Infusion agar at 30°C 32

2.3. Figure 1 (B). Absorbance spectra between wavenumbers 2000-1000 cm^{-1} of the bacterial cultures grown on Trypticase Soy agar at 30°C 33

2.3. Figure 2 (A). Absorbance spectra between wavenumbers 2000-1000 cm^{-1} of various isolates belonging to the *B. cereus* group grown on Brain Heart Infusion agar at 30°C 34

2.3. Figure 2 (B). Absorbance spectra between wavenumbers 2000-1000 cm^{-1} of various isolates belonging to the *B. cereus* group grown on Trypticase Soy agar at 35

3.3.3. Figure 3. Dendrogram of *B. cereus* isolates generated by cluster analysis of FAME profiles showing subgroups in the 'BCERMK' library 56

3.3.4. Figure 4. The number of *B. cereus* isolates from heat-treated milk (Heat), pasteurized milk (Past.), final products (F) and environmental swabs (Swab) allocated into each subgroup of the 'BCERMK' library 64

3.3.5. Figure 5. The number of *B. cereus* isolates from two dairy plants allocated into each subgroup of the 'BCERMK' library 67

CHAPTER 1. LITERATURE REVIEW

1.1. Taxonomy and characteristics of *Bacillus* species

The genus of *Bacillus,* established in 1872 by Ferdinand Cohn, belongs to the family BACILLACEAE (Claus and Berkeley, 1986). The characteristics of species and strains in this genus are very variable and they exhibit a wide range of growth properties, from psychrophilic to thermophilic and from acidophilic to alkaliphilic. The optimum growth temperature is from 30°C to 37°C for most *Bacillus* strains, from 45°C to 75°C for thermophilic strains and from -5°C to 25°C for some psychrophilic strains (Gordon, 1989). *Bacillus* species can survive a wide range of pH conditions, from 2 to 11, and grow under either aerobic or facultatively anaerobic conditions (Claus and Berkeley, 1986). Their ability to form spores is one of the most important characteristics of members of the genus *Bacillus* (Gordon *et al.*, 1973).

Bacillus species can be divided into three broad groups based on the morphology of the spore and sporangium swelling (Smith *et al.*, 1952; Gordon *et al.*, 1973). Group 1 mainly consists of *B. cereus*, *B. mycoides*, *B. thuringiensis* and *B. anthracis*. This group produces central or terminally-sited ellipsoid or cylindrical spores that do not distend the sporangia. The vegetative cell is ≤ 1 μm long and Gram-positive (Kramer and Gilbert 1989; Turnbull *et al.*, 1990). *B. cereus*, *B. mycoides*. *B. thuringiensis and B. anthracis* together are usually refereed to as *B. cereus* group. Group 2 includes *B. circulans*, *B. macerans*, *B. polymyxa*, *B. popillae, B. larvae, B. lentimorbus, B. alvei, B. stearothermophilus* and *B. brevis.* Members of this group are Gram-variable and have swollen sporangia with central or terminal ellipsoid

spores. Group 3 is the most fastidious and the morphology is easier to distinguish from other *Bacillus* species. Their spores are located within swollen sporangia (Gordon, *et al*, 1973; Smith, *et al*, 1952; Turnbull and Kramer, 1991; Turnbull *et al.*, 1990).

B. cereus is the best known species of the *B. cereus* group. It can grow over a temperature range from 5 to 55°C, with an optimum around 28°C-35°C, a pH range 5-9 and a water activity (aw) greater than 0.94 (Van Netten and Kramer, 1992). Differentiation of *B. cereus* from other species in the *B. cereus* group using biochemical and molecular characteristics is difficult. The fatty acid composition of *B. cereus*, *B. mycoides* and *B. thuringiensis* is similar and it can not be used to differentiate them (Väisänen *et al.*, 1991). Serological studies have indicated significant cross-agglutination between spore antigens of *B. cereus*, *B. anthracis* and *B. thuringiensis* (Norris and Wolf, 1961; Kramer and Gilbert, 1989) and between the flagella antigens of *B. cereus* and *B. thuringiensis* (Turnbull *et al.*, 1990). The close relationship between these species has also been emphasized by enzyme electrophoretic patterns and numerical phonetic analysis (Baptist *et al.*, 1978; Priest, 1988; Zaher, *et al.*, 1989; Carlson *et al.*, 1994). Neither pulsed-field gel electrophoresis (PFGE) nor multi locus enzyme electrophoresis (MEE) analysis can distinguish between *B. cereus* and *B. thuringiensis* (Carlson *et al.*, 1994). DNA-DNA hybridization studies suggest that considerable chromosomal similarity exists between *B. anthracis* and some non-anthrax *Bacillus* species (Somerville and Jones, 1972; Seki *et al.*, 1978). Sequence analysis has shown that 94% of 16S rRNA gene sequences between *B. anthracis sterne* and *B. cereus* emetic strain NCTC 1143 are identical (Ash and Collins, 1992). Analysis of a 23S rRNA gene sequences derived from PCR amplification of chromosomal DNA of *B. anthracis* and an

emetic strain of *B. cereus* have also indicated that they are almost identical (Ash *et al.*, 1991). Therefore, it has been proposed that *B. mycoides, B. thuringiensis and B. anthracis* are variants of *B. cereus* by some taxonomists since members of *B. cereus* group are so closely related and are difficult to differentiate by their physiological properties (Sommerville and Jones, 1972; Turnbull and Kramer, 1991; Drobniewski, 1994). However, *B. cereus*, *B. anthracis*, *B. mycoides* and *B. thuringiensis* are still classified as distinct species because their pathogenicity to humans and animals and some phenotypic features are different from each other.

B. cereus is actively motile, strongly haemolytic and does not produce rhizoid growth or endotoxin crystals. *B. anthracis* can be differentiated from the other species in the *B. cereus* group by its sensitivity to penicillin and its lysis by a gamma bacteriophage. Furthermore, *B. anthracis* can also be differentiated from *B. cereus* and *B. thuringiensis* by fatty acid analysis (Kaneda, 1967 & 1968; Lawrence *et al.*, 1991). *B. mycoides* shows mycoidal growth and weak motility, whereas *B. cereus*, *B. thuringiensis*, and *B. anthracis* do not produce mycoidal growth. The crystalline parasporal inclusion bodies and the insecticidal-endotoxins produced exclusively by *B. thuringiensis* can be used to differentiate it from the other species in the *B. cereus* group.

1.2. Pathogenicity of *Bacillus* species

B. cereus and *B. anthracis* have long been considered as the most important pathogenic bacteria among *Bacillus* species. Other species, including *B. thuringiensis, B. licheniformis* and *B. subtilis* have also been recognized as pathogens in recent years due to

increasing cases of related food-borne disease (Turnbull and Kramer, 1991; Drobniewski, 1994; Jackson *et al.*, 1995).

1.2.1. *Bacillus cereus*

B. cereus has been implicated or incriminated to an increasing extent with food poisoning (toxin) and other human or animal infections, e.g., post-traumatic wound infections, ophthalmitis, septicaemia, bovine mastitis and bovine abortion (Turnbull and Kramer, 1991). Two different types of enterotoxins are produced by *B. cereus*: diarrhoeal toxin and emetic toxin (Melling *et al.*,1976).

1.2.1.1. Diarrhoeal toxin

The diarrhoeal enterotoxin is a heat-labile protein with a molecular mass of around 50 KD and an iso-electric point of 4.9. The toxin is produced at the late exponential growth phase of cells at an optimum temperature between 32 to 37°C (Gilbert, 1979; Adams and Moss, 1995). The diarrhoeal toxin may possess multicomponents or subunit structures. Thompson *et al.* (1984) found that the toxin consisted of three proteins with molecular weights of 43 KD, 39KD and 38 KD, respectively. The largest and the smallest proteins had essentially no toxic activity. However, these three proteins are not always produced and the molecular weights vary slightly when analysed using different experimental methods. The enterotoxicity has been shown to be associated with a 45 KD protein determined by SDS gel electrophoresis (Shinagawa *et al.*, 1991). A more recent study has revealed that the diarrhoeal toxin consists of three proteins with molecular weights of 48, 40 and 34 KD. Only the protein with the

molecular weight of 40 KD has been considered as enterotoxigenic (Granum *et al.*, 1993). The diarrhoeal enterotoxin can be destroyed by normal cooking temperatures at 56°C for 5 minutes or be inactivated at pH value lower than 4 and higher than 11. The toxin can also be degraded by pepsin, trypsin and chymotrypsin (Kramer and Gilbert, 1989).

The onset of illness caused by diarrhoeal toxin is about 8 to 16 hours after consumption of the food contaminated with *B. cereus*, and lasts for between 12 to 24 hours. Symptoms include predominantly diarrhoea and abdominal pain and, occasionally, vomiting. The symptoms generally resemble those of *Clostridium perfringens*.

The enterotoxin has been studied extensively and a number of methods have been developed for its detection. The first successfully used method for the study of *B. cereus* toxicology was the ligated rabbit ileal loop (RIL) test (Kramer and Gilbert, 1989). Recently, a reversed passive latex agglutination immunoassay (RPLA) (Griffiths, 1990) and an enzyme-linked immunosorbent assay (ELISA)(Burrell *et al.*, 1991) have become commercially available for detection of the enterotoxin.

1.2.1.2. Emetic toxin

The earliest documented emetic outbreak of the organism was in Great Britain in 1971. However, the emetic toxin has not been well characterized until recently. The toxin is produced at the late exponential or stationary growth phase of cells at the optimum temperature of 25 to 30°C (Adams and Moss, 1995). The emetic toxin is probably a peptide, termed "cereulide" (Agata, *et al.*, 1994). The molecular mass of this toxin is less than 10 KD. The cereulide is stable in the digestive tract and induces emesis (Shinagawa *et al.*, 1994, 1996;

Mikami *et al.*, 1995). The toxin is highly heat-stable at 126°C for 90 minutes. The toxin also can withstand extremes of pH from 2 to 11 and the proteolytic enzymes trypsin and pepsin (Hughes *et al.*, 1988; Turnbull *et al.*, 1990).

The emetic syndrome has a much shorter incubation period (1-5 h) than that caused by diarrhoeal toxin (8-16 h) (Turnbull, *et al.*, 1990; Drobniewski, 1993). The predominant symptoms are nausea, vomiting, abdominal, cramps and diarrhea, which are similar to those of *Staphylococcus aureus* intoxication (Turnbull *et al.*, 1990; Adams and Moss, 1995).

The cereulide produces vacuoles in HEp-2 cells (human carcinoma of the larynx) and combination of the vacuolation factor assay with plasmid banding patterns may be a valuable tool for the epidemiological investigation of emetic syndrome outbreaks (Nishikawa, *et al.*, 1996). However, there is no commercial kit or rapid method available for detection of the emetic toxin. The only reliable assay is the monkey feeding test (Kramer and Gilbert, 1989; Melling and Capel, 1978; Turnbull, *et al.*, 1990).

Emetic and diarrhoeal *B. cereus* strains have similar antibiotic sensitivities and fatty acid compositions (Raevuori *et al.*, 1977).

1.2.1.3. Non-gastrointestinal infections

B. cereus infections have been documented since the beginning of this century and the incidence of non-food-poisoning-related opportunistic infections has increased recently, especially in neonates, intravenous drug users, organ transplant patients and immunologically compromised persons (Doyle *et al.*, 1985; Drobniewski, 1993; Kramer and Gilbert, 1992). Local infections, respiratory infections, central nervous system infections, ophthalmitis and

septicaemia caused by *B. cereus* have all been described (Johnson, 1984; Turnbull and Kramer, 1991; Drobniewski, 1993). These infections by *B. cereus* are associated with a variety of extracellular membrane-active enzymes which include phospholipase C, cereolysin and a heat stable cytolysin (Sliman *et al.*, 1987; Beecher and Macmillan, 1991; Granum, *et al.*, 1993). The phospholipase C gene of *B. cereus* has been cloned and well characterized (Granum, 1994). The phospholipase C and sphingomyelinase function synergistically to exhibit cytolysis. The enzyme complex (cereolysin AB) might be responsible for necrotic and lethal toxin activities of *B. cereus* (Gilmore *et al.*, 1989). Cereolysin is also known as haemolytic I or mouse lethal factor I (Beecher and Macmillan, 1991; Granum, 1994).

1. 2. 2. Other *Bacillus* species

B. anthracis is a well-characterized pathogen and anthrax has been a scourge of animals and humans throughout history (Turnbull and Kramer, 1991). It caused worldwide mortality of herbivores for cattle, sheep, goats and horse before an effective veterinary vaccine became available in the late 1930s. The disease, anthrax, has been recognized as a zoonotic infection and humans almost invariably contract anthrax directly or indirectly from infected animals. The symptoms are cutaneous lesions, intestinal infection and meningitis (Turnbull and Kramer, 1991; Ezzell and Wilhelmsen, 1993). However, the incidence of anthrax in both humans and animals has been very low through vaccination and improvements in animal husbandry over the past decades (Ezzell and Wilhelmsen, 1993).

B. licheniformis, B. subtilis, B. pumilus and *B. brevis* have been isolated from milk and milk products and identified as being associated with human illness in recent years (Turnbull

and Kramer, 1991). Cases of *B. licheniformis* food poisoning present a clinical picture similar to the diarrhoeal syndrome caused by *B. cereus*. *B. subtilis* produces an acute-onset emetic syndrome (Kramer and Gilbert, 1989; Drobniewski, 1993). *B. thuringiensis*, *B. popilliae*, *B. sphaericus*, *B. larvae*, and *B. lentimorbus* are considered as insect pathogens (Turnbull and Kramer, 1991). *B. thuringiensis* (BT) is the most widely used and intensively studied microbial insecticide. Many *in vivo* and *in vitro* tests have been conducted to ensure the safety of spraying crop fields with these organisms, but recent observations indicate that both *B. thuringiensis* and *B. sphaericus* can cause human disease (Ray, 1991; Drobniewski, 1994). An outbreak of foodborne illness associated with *B. thuringiensis* has been reported and certain strains of *B. thuringiensis* have been found to produce a diarrhegenic enterotoxin similar to that of *B. cereus* (Ray, 1991; Jackson *et al.*, 1995; Mikami, 1995).

1.3. Importance of *B. cereus* in dairy products

The presence of *B. cereus* in pasteurized milk and other dairy products is a major concern of the dairy industry since contamination of milk by this organism can lead to both spoilage and safety problem. Its spore-forming and psychrotrophic properties enable *B. cereus* to grow and produce toxins in pasteurized milk at refrigerator temperature (Wong *et al.*, 1988a; Christiansson *et al.*, 1989).

1.3.1 Incidence of *B. cereus* in dairy products

The incidence of *B. cereus* in dairy products is fairly high. Thirty-five percent of pasteurized milk, 63% of milk powder, 50% of infant food (based on milk) and 14% of ice-

cream samples have been found to be contaminated with *B. cereus*, with the counts being from 0.3 to 800 CFU/g or ml (Ahmed *et al.*, 1983; Wong, *et al.*, 1988a; Becker, *et al.*, 1994). More recently, seventy-one percent of commercial pasteurized milk samples were reported to contain *B. cereus* after pre-incubation at 30°C for 6 hours (Te Giffel *et al.*, 1996). A large outbreak associated with pasteurized milk in the Netherlands involved 280 cases with patients' age ranging from 55 to 75. The count of *B. cereus* isolated from the milk was 4 x 10^5 CFU/g (Van Netten *et al.*, 1990). Another large outbreak of emetic *B. cereus* due to consumption of ultrahigh temperature (UHT) sterilized milk (120°C for 2 s) has also been reported in Japan in 1991 (Shingawa, 1993).

Besides causing foodborne illness, *B. cereus* is also responsible for the spoilage of pasteurized milk and its products resulting in off-flavours, sweet curdling and bitty cream (Stone and Rowlands, 1952; Billing and Cuthbert, 1958; Overcast and Atmaram, 1974). The initial count of the organism in these products determine their keeping quality. In Sweden, a legal limit of *B. cereus* in milk at the best before day is 10^3 CFU/ml and the ultimate limit is 10^4 CFU/ml. Therefore, monitoring and control of *B. cereus* is very important for the dairy industry from both public health and economic points of view.

1.3.2. Sporulation of *B. cereus*

B. cereus is a sporeforming bacterium. Spores are formed intra-cellularly in round, oval and occasionally cylindrical shapes at the end of exponential cell growth or when vegetative cells are transferred from a rich to a poor medium. The spores are designated as endospores. Sporulation is a multi-phasic, ordered, sequential process of spore formation,

which results in the changes of cellular components such as DNA, membranes, peptidoglycan and dipicolinic acid (DPA). The characteristics of spores have been well documented. The spores are highly refractive (Busta and Foegeding, 1986). Under the electron microscope, a spore consists of a thin outer spore coat, a thick spore cortex and an inner spore membrane. The spores are more resistant to heat, desiccation, radiation, and chemicals than vegetative cells and can survive in a dormant state for long periods in nature, up to centuries in some instances (Sneath, 1986; Doi and McGloughlin, 1992a). The mechanism of the resistance of spores is not fully understood since cytological composition and DPA content of most bacterial spores are similar, but the level of heat resistance of spores differs by as much as 100,000 times. It has been shown that the $D_{90°C}$-value of *B. cereus* spores in phosphate buffer (pH 7.0) is variable within the range of 4.6 to 200 min (Dufrenne, *et al.*, 1994).

1.3.3. Germination of *B. cereus* spores

Under suitable conditions, germination of *B. cereus* spores may take place within minutes. Germination is a process by which spores are activated into growing vegetative cells. The process usually involves several phases, ie., activation, triggering, initiation and outgrowth (Bergère, 1992). Activation means a treatment responsible for breaking dormancy and heat treatment at 70 - 80°C for 10 - 30 min is normally employed. The triggering of germination involves the interaction of a specific compound, called germinant, within the spore that irreversibly commits the spore to lose its dormant properties. The initiation stage of germination involves metabolic reactions that occur as a result of the triggering reaction. Outgrowth is defined as the development of the new vegetative cell from the germinated

spores and comprises synthesis of new macromolecules (RNA, proteins and DNA). The structural, physiological and biochemical changes of spores during germination mainly include the release of DPA and calcium, loss of refractivity or phase darkening, onset of stain ability, increase in permeability and loss of heat resistance (Claus and Berkeley, 1986; Bergère and Cerf, 1992).

The rate and the extent of spore germination is dependent on strains, treatments, and environmental factors. *B. cereus* spores can be classified into fast germinating and slow germinating spores. The slow germinating spores appear more heat-stable than fast germinating spores. The fast germinating spores, which mainly come from soil, dung and fodder, can germinate at 20°C within 24 h and the slow germinating ones, which are largely from the dairy environment, usually do not germinate at 20°C within 24 h (Labots and Hup, 1964). *B. cereus* spores are able to germinate without preliminary heat treatment, however, the rate of germination and the proportion of germinated spores are higher with heat treatment. Heat treatment at 75°C for 5 min for heat-sensitive strains and at 80°C for 10 min for heat-stable ones has been found to be sufficient to activate *B. cereus* spores to germinate (Stadhouders *et al.*, 1980). The spore counts of three strains of *B. cereus* have been reported to decrease to the range of 18.75 to 86.25% of the original population in the pasteurized milk following high temperature short time (HTST) treatment (at 74°C for 15s) (Griffiths and Phillips, 1990). The heat resistance of *B. cereus* spores is affected by milk fat concentration and is higher in high-fat dairy products such as double cream (48% fat) (Champagne, *et al.*, 1994). Besides heat treatment, various agents including L-alanine, inosine, adenosine, and combination of these are usually used to further stimulate the germination of *B. cereus* spores.

L-alanine combined with heat shock has been found to cause almost complete germination of spores (Bergère, 1992). Greater germination of *B. cereus* spores has been observed in pasteurized milk than in raw and ultra-high temperature (UHT) sterilized milk due to the production of a germinating initiator (milk constituents alone or produced due to the interaction between milk constituents and somatic cells) in the pasteurized milk (Bergère, 1992).

The counts of *B. cereus* germinated from spores in pasteurized milk have been found to reach 1 to 7 x 10^6 CFU / ml at 20°C for 24 hours and cause spoilage of milk (off-flavour and sweet curdling) (Stadhouders, *et al.*,1980). Therefore, in order to effectively control the organism, germination and outgrowth of *B. cereus* spores in pasteurized milk must be prevented. An alternative strategy is to promote the germination of the spores before the pasteurization process and, consequently, vegetative cells germinated from the spores can be destroyed more easily by the pasteurization process. Since the D-values of *B. cereus* spores vary considerably ($D_{90°C}$ = 4.6 to 200 min, Dufrenne *et al.*, 1994), at least a 4D reduction must be adopted for the pasteurization process to assure its adequacy (Rönner and Husmark, 1992).

Several new methods, including high voltage pulsed electric field (HVPEF), ultraviolet light, and a combination of hydrogen peroxide and ultraviolet light have been recently developed to destroy *B. cereus* spores, however they are not available for use in milk yet (Waites *et al.*, 1988; Marquez *et al.*, 1997).

1.3.4. Adhesion of *B. cereus* spores

Spores of *B. cereus* are very adhesive to surfaces of dairy equipment used in dairy

plants. This may be another reason for their presence in pasteurized milk and contribute to the difficulty encountered in controlling this organism. The strong adhesive capacity of spores is mainly due to three characteristics: 1) their relatively high hydrophobicity; 2) low spore surface charge; and 3) unique morphology (Rönner and Husmark, 1992). Spore adhesion is especially high on hydrophobic materials due to its relatively high hydrophobicity. The *B. cereus* spore population along the process line of equipment from tanker to packaging (tanker-storage-pasteuriser-packaging) gradually decreases since the hydrophobicity of these materials decreases. Current cleaning procedures (alkali 1%; 7 min, 75°C→water→acid 0.8%; 4 min, 70°C-water) result in only a 40% reduction of the spore counts along the milk process line (Mosteller and Bishop, 1993). Therefore, the spores are apparently very difficult to detach from process lines with current cleaning procedures. A minimum of 15 min at 85°C for sanitizing milk pasteurization plants and 15 min at 140°C to 150°C for ultra-high temperature (UHT) plants are recommended by the International Dairy Federation (International Dairy Federation, 1980).

1.3.5. Psychrotrophic characteristic of *B. cereus*

Psychrotrophic strains of *B. cereus* were first isolated from milk in 1969 (Grosskopf and Harper, 1969). Psychrotrophic *B. cereus* strains can grow at commercial refrigeration temperatures (≤ 7°C). Fifty percent of the enterotoxigenic strains are able to grow at 5°C, and 86.6% at 7°C within 7 days (Rusul and Yaacob, 1995). Studies on psychrotrophic strains of *B. cereus* have indicated that the average generation time of these organisms in milk is 17 h at 6°C (Griffiths and Phillips, 1990). Most strains in milk can produce heat-resistant lipases

and proteases which are associated with a variety of off-flavours as well as physical defects in milk (Coghill & Juffs, 1979; Ahmed *et al.*, 1983 ; Gilliland *et al.*, 1984) and produce toxins during growth at low temperature (6-10°C) (Wong, *et al.*, 1988a & b; Griffiths, 1990). Toxin production is increased with increasing temperature from 6 to 21°C (Griffiths, 1990). Most psychrotrophs can be destroyed by pasteurization and normally will not be a serious problem (Gilliland et al., 1984). However, their extracellular enzymes (lipases and proteases) are, in most cases, heat-resistant and are not inactivated by the pasteurization process (Champagne *et al.*, 1994). The presence of psychrotrophic *B. cereus* strains in pasteurized milk may imply post-processing contamination or germination from spores (Gilliland et al., 1984).

1.4. Isolation and enumeration of *B. cereus*

A standard plate counting method is usually used to enumerate the number of *B. cereus* in food and environmental samples. In general, the direct plating technique gives better reproducibility than the most probable number (MPN) technique at both high and low levels of *B. cereus* contamination (Lancette and Harmon, 1980). For direct plating, blood agar is usually used for the isolation of large numbers of *B. cereus* from foodborne illness outbreaks. Other selective and various differential media are preferably used for the enumeration of *B. cereus* in the presence of high levels of other organisms in food, environmental samples and faecal specimens. Donovan (1958) first developed a selective agar (citrate-egg yolk-lithium chloride-polymyxin B (CELP) agar for the enumeration of *B. cereus* endospores and vegetative cells in milk and on dairy equipment. Other selective media developed later include mannitol-egg yolk-polymyxin B (MYP) agar, KG (Kim and Goepfert) agar, polymyxin B-

pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA) and polymyxin-pyruvate-egg yolk-mannitol-bromocresol purple agar (PEMPA). The rank order of the efficiency of isolation is PEMPA>PEMBA>MYP (Szabo *et al.*, 1984). However, other research also reported that no significant difference existed between PEMBA, KG and MYP in the recovery of *B. cereus* from pasteurized milk and cream, and dried products such as rice, potato powder, beans, soup powders (Holbrook and Anderson, 1980; Harmon *et al.*, 1984). In the selective and differential media, polymyxin is used as a selective agent and egg yolk and mannitol as differential agents. The principal diagnostic features of *B. cereus* are: 1) the characteristic colonial appearance , such as irregular shape (crenate or fimbriate to slightly rhizoid, rough in texture); 2) the production of a lecithinase resulting in colonies surrounded by greyish zones of egg yolk precipitate and; 3) the inability of the organism to utilize mannitol.

MYP is usually the medium of choice for the enumeration of *B. cereus* in foods in North America and other ISO-affiliated (International Standards Organization) countries, and also recommended by the International Commission on Microbiological Specifications for Foods (Anon. 1978) and the Association of Official Analytical Chemists (Lancette and Harmon, 1980). Mossel *et al.* (1967) first designed the mannitol egg yolk polymyxin agar (MYP) to isolate *B. cereus* from food. PEMBA is a widely used alternative, particularly in the UK (van Netten and Kramer, 1992). *B. cereus* produces typical crenated colonies, retaining the turquoise-blue of the pH indicator (bromothymol blue), surrounded by a zone of egg-yolk precipitation caused by lecithinase activity on PEMBA (Bouwer-Hertzberger, 1982; Adams and Moss, 1995).

The most probable number (MPN) and preincubation techniques have been

recommended for estimating low numbers of *B. cereus* in foods. Trypticase soy-polymyxin B (100 IU/ml) broth for MPN techniques is commonly used to enumerate the number of *B. cereus* (less than 10 CFU/g) in foods or dehydrated starchy foods which are inappropriate for the plate count (Lancette and Harmon, 1980). Preincubation is often used to enumerate low numbers of *B. cereus* in milk. Preincubation of milk at 7°C for 10 days is usually required, especially for psychrotrophic *B. cereus* strains (Gilliland, *et al.*, 1984). More rapid modified preincubation techniques, such as 25 h at 21°C, 6 h at 30°C or 24 h at 20°C for detecting *B. cereus* in milk have also been developed (Stadhouders *et al.*, 1980).

1.5. Identification and classification of *B. cereus* using rapid methods

Although *B. cereus* has been recognized as a cause of food poisoning for more than 30 years, it is still difficult to control, especially in the dairy industry. Morphological, biochemical, serological and toxigenic characteristics are commonly used to identify and classify *B. cereus*. However, the conventional methods and procedures are time-consuming or require extended periods of incubation before final identification. Moreover, it is often difficult to assign *Bacillus* species to their proper group on the basis of these properties alone (Debuono, *et al.*, 1988). Fourier Transform Infrared Spectroscopy (FTIR) and fatty acid analysis of bacteria by gas liquid chromatography (GLC), *ie.*, Microbial Identification System (MIDI) can be used as simple, rapid and sensitive methods to identify and classify *B. cereus* in foods.

1.5.1. Principle of Fourier transform infrared spectroscopy (FTIR)

The existence of infrared radiation was first recognized in 1800 by Sir William Herscher. Infrared radiation is an invisible part of the electromagnetic spectrum between visible light and radio waves, i.e., frequencies at 4000-500 cm^{-1} (Cross, 1969, Ferraro and Basile, 1978). The infrared spectrum of any compound expresses a unique "fingerprint" that enables IR-spectroscopy to be used for the identification of unknown samples in forensic medicine, the pharmaceutical industry, and microorganisms (Naumann *et al.*, 1994). The use of IR- spectroscopy as a means of differentiating and identifying bacteria was extensively reported between 1950s and 1960s (Naumann, *et al.*, 1991). The characteristics of IR spectra were summarized at that time as: (1) high reproducibility of IR spectra of intact bacteria provided that each sample preparation was controlled rigidly; (2) unique IR spectra for individual strains; (3) instrumental limitation with low resolution of Michelson interferometers and low sensitivity (Naumann, *et al.*, 1991). Due to the limitation of IR-spectroscopy, the applications of the technique to identify microorganisms were not frequently reported in the late 1960s and eventually ended in the mid-1970s. However, since the advent of modern interferometric and Fourier-transform techniques, there has been renewed interest in IR-spectroscopy for use in many fields of microbiological research. A Fourier transform comes from a complex mathematical computation named after the French mathematician and physicist, J. Fourier. A Fourier transform infrared spectrophotometer (FTIR) uses infrared light and interferometer to yield a wealth of information regarding the vibrational and rotational motions of atoms and molecules in a substance.

A FTIR spectrophotometer mainly consists of infrared light, interferometer, detector and computer system. Infrared light can travel through the optical system of the spectrometer

and the interferometer splits the beam of light into two rays: one beam is reflected to a fixed mirror and the other beam goes through to a moving mirror. The two beams recombine at the beam-splitter and cause interference due to the path difference between the two mirrors, which can be used to measure properties of a substance (Van de Voort and Ismail, 1991). Fluctuations in the intensity of the energy reaching the detector are digitized and an interferogram is produced. The interferogram is converted into a conventional spectrum by the use of a fast Fourier transform algorithm, which requires substantial computing power: a computer is an integral component of FTIR instruments (van de Voort and Ismail, 1991; Kumosinski and Farrell, Jr. 1993). The infrared absorbance spectrum is a graphic representation of a sample in terms of the amount of light absorbed at various frequencies and the size and position of the peak to identify the composition of the sample. After background subtraction, the spectrum can be viewed as absorbance (the amount of IR light absorbed) or transmittance (the amount of IR light passed or reflected from the sample surface). The frequency or energy level of infrared absorptions can be expressed as wavenumbers (cm^{-1}) or wavelength (μm).

FTIR is a nondestructive technique. Complicated and time-consuming procedures for decomposing cells into single chemical or structural components are avoided (Helm *et al.*, 1991b; Naumann *et al.*, 1990). The technique essentially takes advantage of the spectral fingerprints of intact bacteria since the spectra result from the vibrational and rotational features of all cell constituents (nucleic acids, protein, fatty acids, carbohydrates and other components). Therefore, the selectivity of the technique is very high, allowing differentiation down to the strain and/or serogroup / serotype level (Helm, *et al.*, 1991a & b).

1.5.2. Principle of gas liquid chromatography (GLC)

Chromatography was invented by the Russian botanist, Tswett and he first separated plant pigments in 1903. Since then, many chromatographic techniques such as column chromatography, paper chromatography, thin layer chromatography and gas chromatography have been developed. Chromatography is based on certain physical properties of the individual substance and the separation of the volatilized components of the mixture is carried out by selective partitioning between solid and liquid phases (Mitruka, 1975). All chromatography systems basically consist of two phases, the stationary and the mobile phase. In conventional column and thin-layer chromatography (TLC), the stationary phase is a solid adsorbent and the mobile phase is a liquid. However, in gas chromatography (GC), the stationary phase may be a solid adsorbent (gas-solid chromatography) or an involatile liquid adsorbed on an inert support (gas-liquid chromatography). Gas-liquid chromatography (GLC) has had much greater application than gas solid chromatography. Gas liquid chromatography can be traced back to 1941. Martin and Synge (1941) first suggested using gas as the mobile or flowing phase in the column, and this was further developed by James and Martin in 1952 (Mitruka, 1975).

The basic components of a GLC system are the injector, column, and detector, each of which has separate temperature controls. The injector is the location where the sample components are heated and thereby converted to the vapour state. The column is the "heart" of GLC since samples are introduced into the column in the vapour state and separated within the column. The volatilized components of the mixture are distributed between the mobile and stationary phases, and the separation is carried out according to the relative affinity of the

components for the liquid stationary phase coated on the inert support. The compound with the least affinity for the liquid phase will elute first from the column and the highest affinity compound will be the last out of the column. The detector is located at the end of the column. Flame ionization detectors (FID) are most widely used in GLC and have a high sensitivity (10^{-12} g/s) for essentially all organic compounds (Mitruka, 1975; Moss, *et al.*, 1980; Moss, 1985). James and Martin (1952) first successfully used GLC to separate milligram quantities of long chain (C_1 - C_{20}) saturated and unsaturated fatty acids by using silicone oil and stearic acid as the stationary phase and nitrogen as the mobile phase (Mitruka, 1975)

The first commercial GLC instrument became available in 1955 and then many companies rapidly increased interest in GLC. The technique was initially used for the separation of volatile compounds, i.e., alcohols, fatty acids and as an excellent method for separating compounds of low or medium molecular weight. With the advent of chemical reagents for volatilization of large molecular weight compounds, the technique may quantitatively and qualitatively analyse almost all groups and sizes of biochemicals. In the late 1950s, gas chromatography was employed to detect and identify microorganisms and control the end products of fermentation processes, for example, alcohols, aldehydes, and volatile acids in the brewing industry (Jellum, 1975). In the mid 1960s, GLC was advanced to identify microorganisms by three major approaches: (1) analysis of chemical components of microorganisms by pyrolysis or hydrolysis and extraction; (2) characterization of unique or specific metabolites in a growth environment; (3) analysis of degradation products of a metabolite (Mitruka, 1975; Lechevalier and Lechevalier, 1988).

Abel *et al.*(1963) were the first to present evidence that cellular fatty acid (CFA)

analysis by GLC could identify bacteria (Welch, 1991). However, the technique was not used for identification of many microorganisms until open tubular capillary column were introduced (Moss, 1981; Miller and Berger, 1985). The introduction of these columns was a major breakthrough for routine laboratory work and capillary injectors increased the reproducibility. Packed columns consist of an inert supporting surface such as silica or Celite on which the liquid phase (carbowax, methyl silicone, etc.) is coated. Open tubular and fused-silica capillary columns have improved and extended GLC applications. Capillary columns (typically 25 or 50 m in length and with inner diameters of 0.2 or 0.35 mm) possess superior separation and resolution qualities to packed columns (typically 2 m in length and 2 to 4 mm inner diameter) and fused silica open tubular (FSOT) columns can be coated with a stationary phase of either low or high polarity, having more flexibility and durability than glass capillaries. The column also allows reproducible recovery of hydroxy fatty acids and can distinguish several isomers of fatty acids with the same carbon chain length (Lambert and Moss, 1983). The accuracy, speed, sensitivity, and simplicity of GLC resulted in a major expansion of chromatography and therefore, GLC can be used for the analysis of microbial biochemistry and for differentiating microbial species, or even strains within a species (O'Donnell, 1985; Moss, 1995a & b; Haack, *et al.*, 1994).

Certain enzymes, DNA, membrane, lipopolysaccharides, carbohydrates, proteins, and fatty acids of bacteria are most often analysed to identify bacteria by GLC (O'Donnell, 1985). Fatty acid analysis by GLC seems to be one of the best methods. A fatty acid profile of bacterium is a very stable phenotypic expression of a bacterial genotype when the analysis conditions, from bacterial growth to the fatty acid extraction procedure, are rigorously

controlled (Moss, 1981; Stayer and Davis, 1992; Wauthoz, *et al.*, 1995). Moreover, fatty acid metabolism is directed by chromosomal DNA and the presence of certain fatty acids in bacteria has been shown to correlate with chemotaxonomic characteristics (O'Donnell, 1985; Mukwaga and Welch, 1989; Moss, 1995b). The patterns of fatty acids produce "fingerprints" of bacteria (Eerola *et al.*, 1988; Sasser, 1990; Larsson *et al.*, 1991). Hence, GLC of cellular fatty acid (CFA) analysis has emerged as a method in bacterial taxonomy and also has been used for epidemiological typing of microorganisms (Mukwaya and Welch, 1989; Birnbaum *et al.*, 1994).

The Microbial Identification System (MIS) is the only commercially available GLC system dedicated to the identification of bacteria according to their fatty acid profiles and allows automated quantitative analysis of over 300 fatty acid methyl esters ranging in length from 9 to 20 carbons (Welch, 1991). The system was co-developed by Hewlett-Packard Co. and Microbial ID. Inc., Newark, Del., and is now marketed by Microbial ID Inc. The original data base for identification of aerobic bacteria was developed and has been expanded to include 15 genera and 65 species or subspecies of the family *Enterobacteriaceae*; 45 *Pseudomonas* species, subspecies or biovars; 18 *Staphylococcus* species or subspecies; 19 *Bacillus* species; and 53 additional genera of aerobic bacteria containing 197 species, subspecies, or biovars; 32 species, subgroups, or complexes of mycobacteria; 31 genera of anaerobes containing 254 species, types or groups; and 23 genera of yeasts including 195 species or subspecies. In addition, updated software libraries have subsequently been developed and are provided at no cost to users of the system (Stager and Davis, 1992; Stead *et al.*, 1992; Stoakes *et al.*, 1994).

1.5.3. Characteristics of fatty acids of bacteria

The chemical composition of microbial cells generally consists of protein, lipid, polysaccharides and water (Mirtruls, 1975; Ratledge and Wilkinson, 1988 a & b). Fatty acids, amino acids and carbohydrates are the major cellular constituents for study. Fatty acids have been predominantly used for diagnostic purposes since the derivatization procedures for amino acids and carbohydrates are complicated and time-consuming at the present time and sometimes these derivatives are not chemically stable. The fatty acids of bacteria are found in the cell wall/cell membrane fraction and are linked to other cellular components, of which glycerol is the most common (O'Leary and Wilkinson, 1988; Lawrence *et al.*, 1991). Fatty acids can be approximately classified into five major types as their methyl esters: (a) straight chain saturated, (b) mono-unsaturated, (c) cyclopropane, (d) methylbranched, and (e) hydroxy-substituted fatty acid methyl esters (FAMEs). The presence of iso- and anteiso-branched fatty acids and saturated straight chain of carbon atoms is often characteristic for Gram-positive bacteria, such as *Bacillus* species and *Staphylococci*. Cyclopropane type, monounsaturated and hydroxy FAMEs are usually characteristic of Gram-negative bacteria, e.g., *Campylobacter, Pseudomonas* (Lechevalier and Lechevalier, 1988; Welch, 1991). Fatty acids with chain lengths of 10-19 carbon atoms are synthesized by most bacteria, and the most prevalent fatty acids are those with C_{16} or C_{18} (O'Leary and Wilkinson, 1988; Lawrence *et al.*, 1991).

The fatty acid composition of 19 *Bacillus* species has been studied and these organisms are classified into six groups (A-F) (Kaneda, 1967 & 1977). The composition of

Bacillus species generally exhibits the most complexity in terms of branched-chain cellular fatty acid (CFA). An unusual richness in lipid chemistry is not surprising, since the genus *Bacillus* contains a diversity of species (including thermophiles, psychrophiles, acidophiles and alkalophiles) (Kaneda, 1977; Welch, 1991). Characteristics of fatty acids of *Bacillus* spp. are summarized as follows: 1) a marked predominance of branched-chain fatty acids (up to 90% of the total, C_8 to C_{17}, iso and anteiso), 2) a high proportion (up to 60%) of the phospholipids as phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) in most species; 3) unusual positional isomers among the monoenoic acids; and 4) the production of extracellular amphophilic lipoconjugates with surfactant and antimicrobial activities (Kaneda, 1977; O'Leary and Wilkinson, 1988; Welch, 1991).

Fatty acids are rarely found naturally in the free form in the bacterial membrane. To determine total cellular fatty acids, the cells must be hydrolysed with acid or base, even though most lipids are removed by extraction with organic solvents, such as chloroform, hydrocarbons, alcohols, ethers and esters (Moss, 1995b). The hydrolysis procedure is critical to the final results, as acid hydrolysis destroys cyclopropane acids, and base hydrolysis fails to liberate or cleave all the amide-linked hydroxy acids but does not destroy cyclopropane acids (Lambert and Moss, 1983). The combination of base and acid (first base hydrolysis, then acid hydrolysis) is useful to recover most of the fatty acids in bacteria. After treatment, the fatty acids are converted to methyl ester derivatives and then analysed by GLC (Welch, 1991). Meanwhile, there is a large body of literature on physiological and environmental effects on the fatty acid composition of bacteria including: 1) growth phase of bacteria; 2) growth

medium; and 3) incubation temperature (Miller and Berger, 1985; O'Leary and Wilkinson, 1988). Therefore, it is important to establish a standardized procedure for bacterial fatty acid analysis. Currently, the most used procedure for fatty acid analysis of bacteria by GLC is that described by Miller and Berger (1985). The value of data on cellular fatty acids for the identification and classification of microorganisms is now firmly established (Welch, 1991; Stager and Davis, 1992).

CHAPTER 2. IDENTIFICATION OF *BACILLUS CEREUS* USING FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

ABSTRACT: *B. cereus* is widely distributed in food and has been recognized as an important foodborne microbial pathogen. Traditional detection methods are typically insensitive and time-consuming. The objective of this study was to evaluate the potential of Fourier transform infrared spectroscopy (FTIR) for rapid identification of *B. cereus* isolates. Ten *B. cereus* group isolates (comprising *B. cereus*, *B. mycoides* and *B. thuringiensis* strains), five other *Bacillus* species and five non-*Bacillus* were used. Two types of media, brain heart infusion (BHI) and trypticase soy agar (TSA) were tested. The results indicated that all *B. cereus* group isolates produced characteristic absorbance peaks between wavenumbers 1738-1740 cm^{-1}. These peaks were not affected by the growth medium. All the other bacteria tested did not show that peak after growth on BHI or TSA. Therefore, the specific absorbance peak can be used to differentiate *B. cereus* group from other bacteria species.

2.1. INTRODUCTION

B. cereus is widely distributed in rice, spices, meat, eggs and dairy products (Kramer and Gilbert 1989). As well as being a potential spoilage organism, it has been recognized as an important foodborne microbial pathogen (Wong, *et al.* 1988b; Kramer and Gilbert 1989). The gastrointestinal diseases caused by *B. cereus* may occur more frequently in the future, with the increased percentage of elderly and immuno-compromised people in the population and the trend to consumption of refrigerated foods with extended shelf-life. This latter trend

is particularly evident in the dairy industry where psychrotrophic strains of *B. cereus* are commonly isolated from milk and milk products (Meer *et al.* 1991). This has created a need for rapid, sensitive and reliable methods to identify *B. cereus*. Traditional methods are typically time-consuming, requiring a week or even longer to get results (Wolcott 1991). Also, these methods are often incapable of discriminating between strains or between related species.

Characterization of microorganisms by physical techniques promises to be of great value. Several modern physical methods, such as gas chromatography (GC), high performance liquid chromatography (HPLC), gel electrophoresis, flow cytometry, impedance measurements and microcalorimetry have been studied (Naumann *et al.*, 1994), but few of these methods provide all of the desired requirements, namely: 1) rapid and reliable identification of all pathogenic bacteria; 2) simple and cheap operation; 3) identification down to the strain level; and 4) capable of automation (Giesbrecht *et al.* 1984; Naumann *et al.* 1988). Fourier transform infrared spectroscopy (FTIR) may be capable of fulfilling many of these conditions and might provide a future alternative to existing procedures for bacterial identification (Giesbrecht *et al.* 1984).

Infrared spectroscopy (IR) has been developed and widely used in many disciplines. Since compounds having different structures do not have the same IR spectra, except for optical isomers (Cross 1969; Kumosinski and Farrell Jr. 1993), unique molecular fingerprints that can be easily distinguished from the absorption patterns produced by other molecules (Byler and Susi 1986; Helm *et al.* 1991a,b). Moreover, FTIR is a nondestructive technique and allows the rapid characterization of structural features of biological molecules and

complex materials like bacteria. Organisms can be probed by FTIR in a single experiment using simple, uniform procedures that are applicable to all bacteria (Helm *et al.* 1991a,b; Naumann *et al.* 1991).

When FTIR was used to investigate the characteristics of *Bradyrhyzobium japonicum* strains, it was concluded that FTIR could probe the structural changes resulting from growth on different media (Zeroual *et al.* 1994). FTIR has also been used as an easy and safe method to type, identify and classify several bacteria, including *Listeria* (Holt *et al.* 1995), *Staphylococcus*, *Clostridium*, *Streptococcus*, *Legionella* and *Escherichia coli* (Naumann *et al.* 1994).

The objectives of this study were 1) to evaluate the potential of the FTIR technique for classification and identification of bacterial species with special reference to *B. cereus* isolates; 2) and to compare the effect of medium components on the expression of characteristic FTIR absorbance spectra of bacteria.

2.2. MATERIALS AND METHODS

2.2.1. Strains and growth conditions

The bacterial strains used in this study were obtained from the culture collection of the Department of Food Science, University of Guelph (Table 1).

To test the influence of the growth medium on the expression of absorption spectra, selected strains were grown on two different media: i) brain heart infusion (BHI) agar and

Table 1. Bacterial strains used and their major infrared absorbance peaks between 1800-1500 cm^{-1} wave numbers after growth on TSA and BHI media.

Organisms	Average wavenumber of peak (cm^{-1})†					
	grown on TSA			grown on BHI		
	Peak I	Peak II	Peak III	Peak I	Peak II	Peak III
***B. cereus* group**						
Bacillus cereus ATCC 7064	1739.9	1655.1	1545.3	1739.8	1655.4	1545.6
B. mycoides ATCC 6462	1739.9	1655.3	1545.2	1739.9	1655.3	1545
B. thuringiensis ATCC 10792	1739.8	1654.2	1545.6	1739.7	1654.1	1545.1
B. cereus F6-D31-1	1739.9	1653.1	1545.1	1739.9	1653.1	1545.1
B. cereus RT1-D37-3	1739.9	1653.1	1543.1	1739.9	1653.1	1543.1
B. cereus 4810/72E (emetic)	1739.9	1655.1	1545.1	1739.9	1654.2	1545.1
B. cereus Mp38	1739.9	1658.9	1547	1739.9	1657.8	1547.1
B. cereus Mp41	1739.9	1658.9	1547	1739.8	1658.4	1545
B. cereus Mp33	1738	1655	1543.2	1738.3	1655.2	1546
B. cereus F53, ATCC 49064 (diarrhoeal)	1738	1657	1545.1	1738.2	1657.2	1545.5
Other *Bacillus* spp.						
B. licheniformis ATCC 14580	ND*	1653.1	1549.9	ND	1653	1545.6
B. sphaericus ATCC 4525	ND	1653.1	1545.1	ND	1653.2	1543.1
B. pasteurii HRM 55	ND	1653.4	1541.2	ND	1653.2	1545.2
B. psychrosaccharolyticus ATCC 23296	ND	1653.3	1543.2	ND	1653.2	1543.1
B. pumilus ATCC 72	ND	1652.7	1543.1	ND	1653.1	1543.1
Other genera						
L. monocytogenes Scott A 382	ND	1653.1	1545.1	ND	1653.2	1545.2
L. lactis ATCC 11454	ND	1653.1	1543.2	ND	1653.1	1543.2
Staphylococcus aureus ATCC 355556	ND	1651.3	1541.8	ND	1653.1	1541.3
E. coli HB101	ND	1651.3	1541.8	ND	1655	1541.2
Pseudomonas putida JA1	ND	1657	1543.2	ND	1657.2	1543.2

† The wavenumber of peak was average of two times. ND*: peak not detected

trypticase soy broth agar (TSA) (Difco Laboratories Inc., Detroit, MI). All strains were streaked onto agar plates using a four-quadrant streak pattern and incubated at 30°C for 24 hours.

2.2.2. Sample preparation

Late-exponential-phase cells (approx. 60 mg) were carefully removed with a platinum loop from confluent colonies in the third quadrant of the agar surface and suspended in 30 μl sterile distilled water. An aliquot (25 μl) of the cell suspension was transferred to 3M disposable IR cards 62 and 61 (3M, St Paul, MN), and then dried in a vacuum oven or at room temperature for at least 20 - 30 min to form a transparent film suitable for absorbance FTIR measurements. On IR cards 62, spectra were recorded in the range of 4000-1300 cm^{-1} (wavenumbers) on an FTIR spectrophotometer (Bio-Rad FTS-40, Bio-Rad Laboratories, Mississauga, ON) equipped with an internal detector. One hundred scans were taken at a spectral resolution of 4 cm^{-1}. The sensitivity factor of pick peaks was 10 and all samples were analysed in duplicate. Following the same procedure for sample preparation and analysis, IR cards 61 were used to examine components of bacteria between 3000 - 2800 cm^{-1}.

2.3. RESULTS

When absorption spectra in the range of 3000 - 2800 cm^{-1} were analysed for four different bacterial strains (*B. cereus* 4810/73E, ATCC 49064, *E. coli* HB101 and *P. putida* JA1), no patterns that would allow a distinction of *B. cereus* from other species were observed. However, results in the spectral range from 1800 - 1500 cm^{-1} indicated that *B.*

cereus group grown on TSA and BHI produced characteristic infrared absorbance peaks in the area of 1700 cm^{-1}. None of the other bacteria tested exhibited absorbance peaks at these wave numbers (Fig. 1 a & b). For further confirmation of the specific absorbance peak, additional strains of the *B. cereus* group were tested on two types of media (TSA & BHI). The results indicated that all strains of the *B. cereus* group maintained the absorbance peak around wave numbers 1700 cm^{-1}, regardless of growth media (Fig. 2 a & b). To confirm that this peak was unique to *B. cereus* group isolates, the pick peak feature of the spectrophotometer software was used with a sensitivity factor of ten and the exact location of the peaks determined. As shown in Table 1, a peak between wave numbers 1738 - 1740 cm^{-1} was detected for all *B. cereus* group isolates

2.4. DISCUSSION

Owing to the multitude of cellular components, broad and superimposed spectral bands are observed within the infrared range (~ 4,000 - 500 cm^{-1} wavenumbers), but some spectral sub-ranges are dominated by particular cell components. A preselection of spectral windows was done considering their specific information content and their discrimination power as follows: i) the window between 3000 and 2800 cm^{-1} wavenumbers (w_1; the fatty acid region I) is dominated by the $-CH_3$, CH_2 and ≡CH stretching vibrations of the functional groups usually present in the fatty acid components of the cell membrane; ii) the window between 1800 and 1500 cm^{-1} wavenumbers (W_2; the amide region) consists mainly of protein and peptides. Spectral bands at 1700 - 1600 cm^{-1} (amide I) and 1600 - 1500 cm^{-1} (amide II)

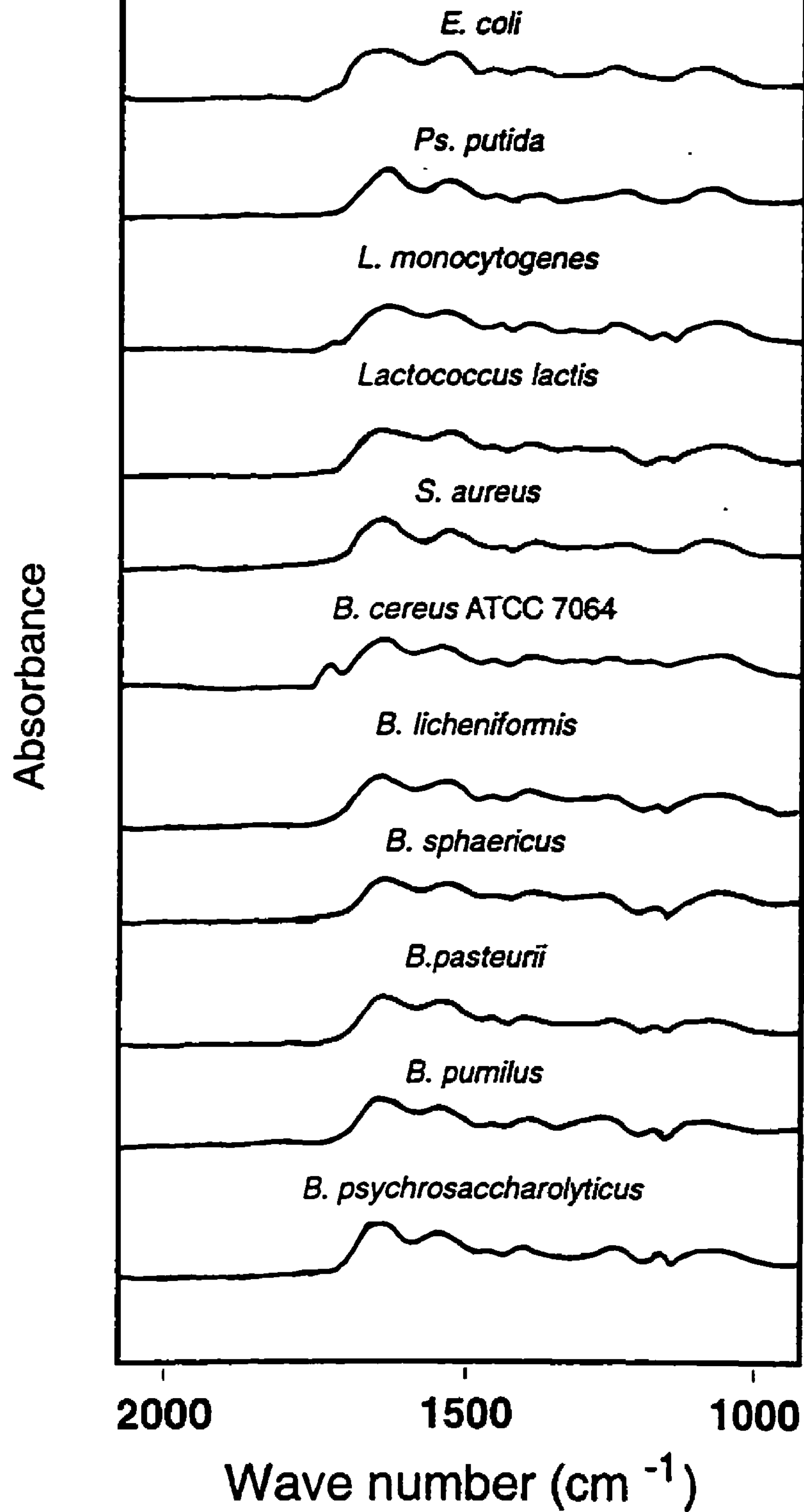


Fig. 1 (A). Absorbance spectra between wavenumbers 2000-1000 cm^{-1} of the bacterial cultures grown on Brain Heart Infusion agar at 30°C

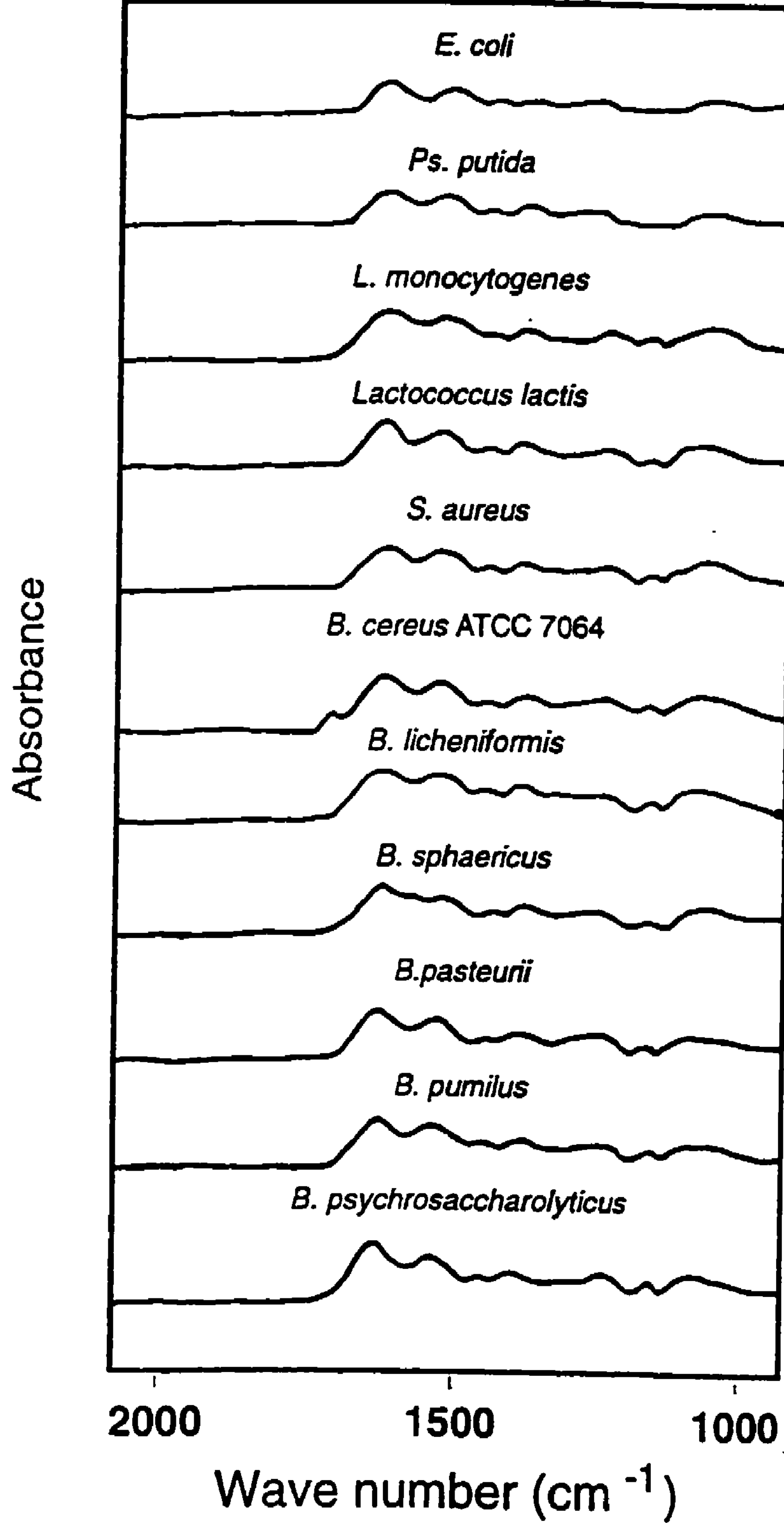


Fig. 1 (B). Absorbance spectra between wavenumbers 2000-1000 cm^{-1} of the bacterial cultures grown on Trypticase Soy agar at 30°C

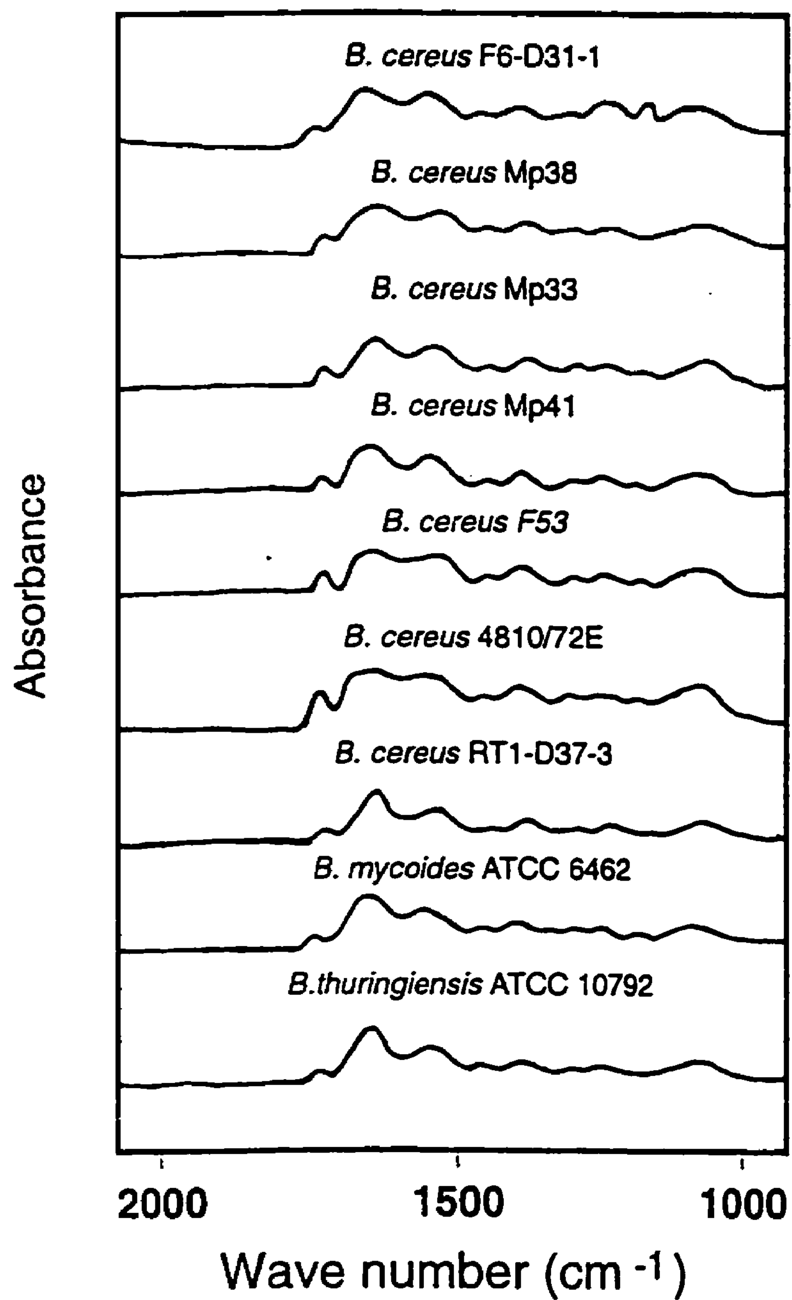


Fig. 2 (A). Absorbance spectra between wavenumbers 2000-1000 cm^{-1} of various isolates belonging to the *B. cereus* group grown on Brain Heart Infusion agar at 30°C

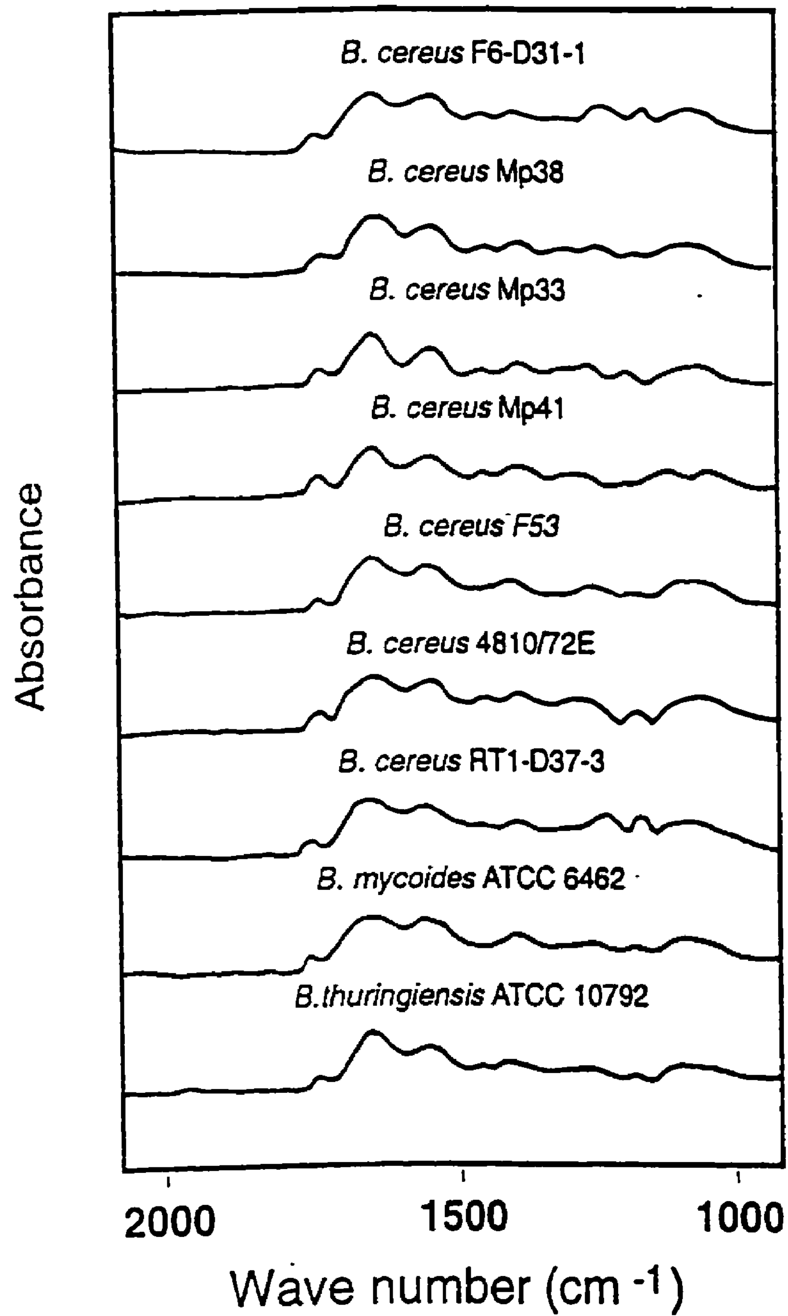


Fig. 2 (B). Absorbance spectra between wavenumbers 2000-1000 cm^{-1} of various isolates belonging to the *B. cereus* group grown on Trypticase Soy agar at 30°C

can be assigned to the C=O, C-N stretching and N-H bending vibrations of proteins; iii) the window between 1500 and 1200 cm^{-1} wavenumbers (W_3; the mixed region) contains information from proteins, fatty acids and phosphate-carrying compounds; iv) the window between 1200 and 900 cm^{-1} wavenumbers (W_4; the polysaccharide region) is dominated by the fingerprint-like absorption spectra of the carbohydrates present within the cell wall; and v) the window between 900 and 700 cm^{-1} wavenumbers (W_5; the true fingerprint) shows some remarkably specific spectral patterns (Naumann *et al.* 1990).

IR cards 61 (3M, St Paul, MN) are recommended for evaluation of the fatty acid region I (3000 - 2800 cm^{-1}). Type 62 IR cards (3M, St Paul, MN) are polyethylene and work especially well for substances requiring interpretation in the fingerprint region at wave numbers from 1800 to 400 cm^{-1}, but they are not suitable for the aliphatic C-H stretching that occurs between 3000 to 2800 cm^{-1}.

Although fatty acid analysis has been successfully used to type *B. cereus* isolates (Schraft *et al.* 1996), analysis of the fatty acid region I (3000 - 2800 cm^{-1}) did not allow differentiation of *B. cereus* from other bacteria. The amide region I (1700 - 1600 cm^{-1}) was more effective in this regard. Since the infrared absorbance peak at wavenumbers between 1738 and 1740 cm^{-1} was present consistently whether *B. cereus* groups were grown on BHI media or TSA media, the peak might represent an important characteristic of *B. cereus* group isolates related to its protein content and be used to distinguish between the *B. cereus* group and other *Bacillus* spp., as well as other genera. Although all *B. cereus* group isolates had similar absorbance spectra in the region of 1800 - 1500 cm^{-1} wavenumbers, the detailed shapes, sizes and positions of their peaks were different. This may have potential for

differentiating species within the *B. cereus* group, but many more isolates need to be analysed to confirm this. Media composition did not have any obvious influence on the characteristic absorbance peak.

Results obtained from this study indicated that FTIR spectroscopy is a useful method to differentiate *B. cereus* group from other bacterial species. It is a simple, rapid and inexpensive alternative to traditional approaches.

CHAPTER 3. IDENTIFICATION OF THE SOURCES OF *BACILLUS CEREUS* IN PASTEURIZED MILK USING MICROBIAL IDENTIFICATION SYSTEM (MIDI)

ABSTRACT: In order to determine the sources of *Bacillus cereus* in pasteurized milk, a total of 232 milk samples from various sampling points of milk processing lines and 122 environmental swabs were collected in two dairy plants between March and September 1996. The incidence and distribution of *B. cereus* among milk samples and environmental swabs was investigated. A library of *B. cereus* fatty acid profiles, named 'BCERMK', comprising 229 *B. cereus* isolates from milk samples and environmental swabs was constructed using a critical Euclidian distance of 6.0 units as the cut-off value. A total of 546 *B. cereus* isolates isolated from 183 milk samples and 3 environmental swabs were classified by MIDI. The relationship between *B. cereus* isolates isolated from the different sampling points along the milk processing lines and the environmental swabs was determined and the sources of *B. cereus* in pasteurized milk were identified. The results showed that the incidence of *B. cereus* vegetative cells in raw milk was low. However, the incidence and the average counts of *B. cereus* spores in the positive raw milk were very high and similar to those of *B. cereus* vegetative cells in pasteurized milk or final products after enrichment (> 80% and 1.1×10^5 CFU/ml). The incidence and average count of both vegetative cells and spores of *B. cereus* in the environmental swabs was low. Analysis of cellular fatty acid composition of *B. cereus* using MIDI showed that most *B. cereus* isolates isolated from the pasteurized milk and final products belonged to the same subgroups as the *B. cereus* strains

germinated from the spores in raw milk. Furthermore, specific subgroups were found in pasteurized milk, different dairy plants and at different sampling times. The results suggested that *B. cereus* spores in raw milk were the major source of *B. cereus* in pasteurized milk and that post-pasteurization contamination along the milk processing lines was possibly another minor source of *B. cereus* in pasteurized milk.

3.1. INTRODUCTION

The presence of *B. cereus* in pasteurized milk is a major concern for the dairy industry since the organism is associated with milk defects such as off-flavours, sweet curdling and bitty cream and also causes outbreaks of food poisoning (Overcast and Atmaram, 1974; Johnson, 1984; Christiansson *et al.*, 1989). *B. cereus* in pasteurized milk may originate from spores that are present in the raw milk and germinate after the pasteurization process or from the dairy environment (Griffiths, 1992; Crielly *et al.*, 1994; Davies, 1975; Coghill, 1982). Results from the different labs were not consistent, probably due to the different sources of raw milk, dairy plant environment and experimental procedures used in their studies. Coghill (1982) found that thermoduric psychrotrophic bacteria could enter pasteurized products as post-heat treatment contaminants, and large numbers of *B. cereus* spores in pasteurized milk were derived from the dairy (Davies, 1975). On the other hand, Griffiths (1992) suggested that raw milk was the main source of psychrotrophic *Bacillus* species in pasteurized milk. Crielly *et al.* (1994) supported the contention that post-pasteurization contamination may not be the main source of *B. cereus*

in pasteurized milk. However, little information is available about the relative contribution made by each source. Furthermore, little research has been conducted in milk samples collected from the processing lines (from the raw milk in the truck to the final products in bags or cartons).

Many traditional and advanced microbiological identification techniques, such as numerical phenotypic analysis, phage typing, measurement of minimum growth temperature, plasmid typing and restriction fragment length polymorphisms of PCR products (RFLP-PCR) have been applied to identify and type the sources of *B. cereus* in dairy products (Väisänen *et al.*, 1991; Ternsträm *et al.*, 1993, Te Giffel *et al.*, 1996; Schraft *et al.*, 1996). Conventional phenotypic methods often lack discriminatory power and results are not reproducible. Genotypic methods offer greater discriminatory power, but they are very expensive and labour-intensive. The gas-liquid chromatographic analysis of cellular fatty acids of bacteria is a relatively easy and simple typing method and has highly discriminatory properties up to the species as well as strain level (Tornabene, 1985; Eerola and Lehtonen, 1988; Huys, *et al.*, 1994). Fatty acid analysis has been successfully applied for epidemiological typing of microorganisms, e.g., *B. cereus, Staphylococci, Listera* and *Pseudomonas* (Schraft et al., 1996; Mukwaya and Welch, 1989; Geoffrey and David, 1989; Ninet, *et al.*, 1992; Birnbaum *et al.*, 1994; Wauthoz *et al.*, 1995).

The objectives of this research were 1) to determine the incidence and distribution of *B. cereus* among sampling points of milk processing lines and environmental swabs; 2) to analyse the fatty acid composition of *B. cereus* isolates isolated from dairy plants; 3) to establish a specific library of fatty acid profiles of *B. cereus* isolates from dairy plants; 4) to

identify the sources of *B. cereus* in pasteurized milk using the microbial identification system (MIDI).

3.2. MATERIALS AND METHODS

3.2.1. Sample collection

The number of milk samples obtained from each sampling point along the milk processing lines and environmental swabs in plant I and plant II is listed in Table 2. Milk samples of about 200 ml each were removed aseptically into sterile bottles from various sampling points, including raw milk in trucks (Truck), tanks (RT), balance tanks (ProHTST), pasteurized milk in high temperature short time pipes (HTST), holding tanks (PT) and filler pipes (Filler). Final products (F) were collected in 125 ml, 500 ml, 1 L, 2 L cartons or 1 L plastic bags. The milk samples were taken at hourly intervals three to five times during each sampling day and collected in March, April, May and September for plant I and in July, August and September for plant II.

Environmental swabs were collected before the start of the milk processing. In plant I, the environmental swabs were aseptically collected from the filler surfaces in March, April and September and suspended in 10 ml brain heart infusion (BHI) broth. In plant II, the environmental swabs were aseptically taken from the surface of frame, floor, pouch track, carton track, case track, bowl, package exterior, lines' exterior, cold plates, drain, cream cooler, silo apron, air eliminator, mix area, blender, hose interior, floor fridge, benches in lab, hall way walls and mandrels in July and August and suspended in 10 ml sterile saline

Table 2. The number of milk samples from each sampling point of the milk processing lines and environmental swabs in plant I and plant II

Sampling locations †	The number of samples		
	Plant I	Plant II	Total
Truck	3	3	6
RT	17	13	30
ProHTST	21	14	35
HTST	20	17	37
PT	46	16	62
Filler	1	12	13
F	31	18	49
Swab	66	56	122
Total	205	149	354

† Truck, RT, ProHTST, HTST, PT, Filler, F and swab indicate milk samples from truck, raw milk tanks, balance tanks (before high temperature short time (HTST), HTST pipes, pasteurizer tanks, fillers, final products and environmental swabs respectively.

solution (0.85 %).

3.2.2. Preparation of milk samples and environmental swabs

Each raw milk sample from trucks, RTs and ProHTST was aseptically divided into two bottles. One bottle was for the *B. cereus* vegetative cell test and the other one was for the spore test. The raw milk samples for a spore test were treated and held at 75°C for 20 min in a water-bath (Labline, Inc., Chicago) and designated as heat-treated milk.

Ten ml brain heart infusion (BHI) broth was added to each tube of environmental swabs and mixed well by vortexing for 2 min. The BHI broth of a swab tube was further split into 2 tubes. The test for *B. cereus* vegetative cells and spores in the environmental swabs was carried out as described for those of the raw milk above.

3.2.3. Enumeration and collection of *B. cereus* isolated from milk samples and environmental swabs

Counts of *B. cereus* vegetative cells in raw milk and environmental swabs were determined after enrichment at 8°C for 3 days (raw milk) and 14 days (environmental swabs) whereas the counts of *B. cereus* vegetative cells in heat-treated milk, heat-treated environmental swabs (measuring the *B. cereus* spores in raw milk and environmental swabs) and pasteurized milk were determined after enrichment at 8°C for 14 days.

Samples (0.1 ml) of raw milk, heat-treated milk, pasteurized milk, final product and BHI broth of environmental swabs were surface-plated in duplicate on polymyxin, egg yolk, mannitol, bromothymol blue agar (PEMBA, Oxoid) using a sterile glass spreading rod

or samples were diluted with Butterfields' phosphate buffer and surface plated in duplicate on PEMBA agar using a spiral plater (Spiral Systems, Inc.). The PEMBA plates were incubated at 30°C for 24 to 48 h and the counts of *B. cereus* were enumerated.

Three to five typical *B. cereus* colonies which were turquoise blue and crenate in shape or mycoidal and surrounded by a zone of egg-yolk precipitation on the media were picked from 1 to 2 plates of each sample and inoculated on Brain Heart Infusion (BHI, Difco) agar. The BHI plates were incubated at 30°C for 24 h to obtain a pure culture for further confirmation by Gram stain and catalase reaction. Sterile cryogenic vials (2 ml) containing 0.8 ml BHI broth were inoculated with one colony from each pure culture and incubated at 30°C for 24 hrs. Another 0.8 ml medium containing glycerol (10% V/V), peptone (1% W/V) and $MgSO_4$ (5% V/V) was added to the vials and the vials were stored at -20°C. A total of 587 presumptive isolates were collected.

3.2.4. Analysis of fatty acids profiles of *B. cereus* isolates using MIDI

Preparation of the samples and analysis of fatty acid profiles of *B. cereus* isolates were carried out as described in Microbial Identification System Operating Manual Version 4, 1993 (MIDI operation manual version 4, 1993).

3.2.4.1. Preparation of fatty acid methyl esters (FAMEs)

The preparation procedure included streaking plates, harvesting cells, saponification, methylation, extraction and base wash.

Streaking plates: Each *B. cereus* isolate from a 2 ml cryogenic vial was streaked on

trypticase soy agar (TSA) plates in a 4-quadrant dilution by a wire loop with flame sterilization. The plates were incubated at 28 ± 1°C for 24 ± 2 h and a well-isolated *B. cereus* colony from the TSA plate was subcultured on two to three TSA plates at the same incubation conditions. Cells from the second subculture plates were harvested for further analysis.

Harvesting cells: Cells of *B. cereus* (50±1 mg fresh weight) were harvested from confluent growth in the third quadrant using a 4 mm disposable loop and transferred into a test tube.

Saponification: One ml of saponification reagent (strong methanolic base used to liberate the fatty acids from the cellular lipids of cells and convert the fatty acids to sodium salts) was added to the culture tube. The tubes were vortexed for 5 to 10 sec and placed into a boiling water bath (Model 184, Precision Scientific Incorporated) at 100°C for 5 min. Each tube was then vortexed for 5-10 seconds, returned to the water bath at 100°C for an additional 25 min and then removed from the boiling water after a total of 30 minutes of saponification at 100°C.

Methylation: To each tube, 2 ml of methylation reagent was added to convert fatty acid sodium salts to fatty acid methyl esters, which increased the volatility of the fatty acids for the GLC analysis. After vortexing for 5-10 seconds, all tubes were placed in a circulating water bath (M20 Lauda) at 80°C for 10 min and then cooled in cold tap water. The step had to be carried out carefully since excess time or temperature of heating could degrade cyclopropane compounds and alter fatty acid profiles.

Extraction: an extraction solvent of hexane/ MTBE (1.25 ml) was added to each tube

and the tubes were placed in a laboratory rotator (Hematology/Chemistry mixer, Fisher Scientific) and mixed end-over-end for 10 min. In this step, fatty acid methyl esters were extracted from the acidic aqueous phase to the upper organic phase. The acidic aqueous phase was removed using a clean Pasteur pipette.

Base wash: Three ml of base solution was added to each tube and the tubes were rotated end-over-end for 5 minutes. The mild base solution was used to remove free fatty acids and residual reagents from the organic extract since they can damage the chromatographic system and produce tailing of peaks or cause loss of the hydroxy fatty acid methyl esters. Only two thirds of the organic extract in the upper phase of a tube was removed using a clean Pasteur pipette for each sample and transferred to a clean sample vial.

3.2.4.2. Microbial Identification System

The syringe was prewashed twice with extraction solvent (hexane/MTBE) and twice with the sample before injection of each sample. Calibration standards including (MIDI) mix 1, a blank control of extraction solvent, and a positive control of *Stenotrophomonas maltophilia* ATCC 13637 were analysed in each batch of the sample. Calibration standard (MIDI) mix 1 was reanalysed again at every 11th injection.

The Microbial Identification System (MIS) consists of a chromatographic unit and a computer system. The MIS chromatographic unit comprises a Gas Chromatograph (Hewlett Packard 5890 series II), an Automatic Sampler (Hewlett Packard 7673) with the controller and communications Module (HP 18594B), Injector Module (HP 18593B), tray Module (HP 18596B) and integrator (Hewlett Packward 3365 ChemStation software). Hydrogen was used

as a carrier gas (30 ml/min FID, 55 ml/min split vent, 5 ml/min septum purge), nitrogen (30 ml/min FID, 40 ml/min trap purge) as auxiliary gas of air (400 ml/min FID). 2 μl of fatty acid methyl ester (FAME) preparation was injected into the column with 1:100 split injection into a splitless-type column liner. The GLC was equipped with a 25 m fused silica capillary column lined with 5% methyl phenyl silicone (Ultra 2, HP 19091 B-102) to separate the fatty acid methyl esters as they passed through the column to the flame ionization detector (FID). The FID burned the carbon in each ester creating a signal that was plotted in the ChemStation. The plotted signal, called the chromatogram, was followed by an Area Percent Report, listing retention times and area percentages for each peak in the chromatogram. The fatty acid profile was then compared to a library of reference organisms stored in the computer to determine the identity of the unknown microorganisms. The column temperature was increased by 5°C/min from 170°C to 270°C. The injector temperature was 250°C and detector temperature was 300°C.

The system of Hewlett-Packard Vectra VL2, 486 CPU was automated with Hewlett-Packard 3365 gas chromatography ChemStation Software, a Microsoft Windows based program. The software allowed two-dimensional principal component plots and cluster analysis dendrograms to be obtained.

3.2.5. Identification and further confirmation of *B. cereus* isolates

The identity of the presumptive *B. cereus* isolates initially identified by Gram stain and catalase reaction was searched in a MIDI standard library (TSBA library) by comparing their fatty acid composition to the mean fatty acid composition of the isolates used to create

the library entry. If the search resulted in more than one possible match, the suggested identities were listed in decreasing similarity index values. The similarity index of the MIS is a numerical value which expresses how closely the fatty acid compositions of the test *B. cereus* isolate compared to those which are listed in the MIS standard library. It is recommended by MIDI that isolates with a similarity index (SI) of 0.500 or higher and with a separation of 0.100 between the first and second choice were considered a good library comparison. If the SI was between 0.300 and 0.500 and well separated from the second choice (> 0.100 separation), it would also be a good match. Values lower than 0.300 suggested that the tested isolates or species did not exist in the database, but it indicated that the tested species might be closely related to the species of the library. Isolates with a SI of 0.300 or higher of only one choice were also considered as good library comparisons (MIDI operating manual version 4, 1993). An SI of 0.3000 was used as cutoff value without consideration of separation of the second choice to identify the *B. cereus* isolates in this study.

3.2.6. Construction of the 'BCERMK' library

The MIDI standard library is good for identification of bacteria, and dendrogram analysis is one of most useful methods to analyse the relationship among small and medium numbers of organisms (MIDI operating manual version 4, 1993). The dendrogram analysis has been used successfully to find the relationship among *Pseudomonas cepacia* isolates obtained from different sources (Mukwaya and Welch, 1989). To avoid analysing the large number of *B. cereus* isolates and losing the resolution of dendrogram, representative *B.*

cereus isolates collected from each sampling month were chosen for the library construction. Seven to fourteen different subgroups of all *B. cereus* isolates from plant I and plant II were obtained in a monthly dendrogram when the Euclidian distance of 6.0 units was used as a cutoff value. The *B. cereus* isolates used for the construction of the library were obtained from each subgroup at each sampling point for each month. All isolates were sampled to establish the library if the number of *B. cereus* isolates isolated from a sampling point was less than three in a subgroup. Three to five *B. cereus* isolates were taken from each subgroup of monthly cluster analysis if the number of isolates from a sampling point was more than five in a subgroup. The library was constructed based on the fatty acid profiles of a total of 229 *B. cereus* isolates (120 isolates from plant I and 109 isolates from plant II) obtained from each sampling point along the milk processing lines and environmental swabs in two dairy plants. The *B. cereus* isolates used to establish the library are shown in Table 3. The standard values used to establish the 'BCERMK' library were similar to those of the MIDI standard library. The critical distance of 6 ED units was used as a cut-off value to allocate *B. cereus* isolates in this study. It is recommended by MIDI that any isolates joining at less than 6.0 ED were considered to be the same biotypes whereas isolates joining at more than 6.0 ED were assigned to different biotypes. The library was named as 'BCERMK', standing for *B. cereus* in milk. The relationships of the FAME profiles of the *B. cereus* isolates to each other were depicted in a dendrogram according to an unweighted pair matching method (UPGMA) using arithmetic averages (Romesburg, 1984; Kotilainen *et al.*, 1991; Birnbaum *et al.*, 1994).

Table 3. The number of *B. cereus* isolates from the milk samples of each sampling point of the milk processing lines and environmental swabs in plant I and plant II used for the construction of 'BCERMK' library

Sampling locations †	The number of *B. cereus* isolates		
	Plant I	Plant II	Total
Truck	3	2	5
RT	20	15	35
ProHTST	15	21	36
HTST	21	17	38
PT	26	18	44
Filler	3	16	19
F	28	20	48
Swab	4	0	4
Total	120	109	229

† notations are the same as in Table 2.

3.2.7. Allocation of *B. cereus* isolates to the 'BCERMK' library

A total of 546 *B. cereus* isolates from each sampling point along the milk processing lines and environmental swabs in two dairy plants were allocated into the 'BCERMK' library. The SI of ≥ 0.3000 was used as a critical value to classify the *B. cereus* isolates into different groups of the 'BCERMK' library.

3.2.8. Allocation of *B. cereus* isolates to the 'MI' library

A data library previously established (Schraft *et al.*, 1996) consisted of 137 '*Bacillus cereus'* isolates obtained from various sources and had 11 subgroups, ie., Mi 1, Mi 2, Mi 3, Mi 4, Mi 5, Mi 6, Mi 7, Mi 8, Mi 9, Mi 10, Mi 11. This library was named 'MI' and used to allocate 546 *B. cereus* isolates from each sampling point along the milk-processing lines and environmental swabs in two dairy plants. The SI of ≥ 0.3000 was used as a critical value to classify the *B. cereus* isolates into different groups of the 'MI' library.

3.3. RESULTS

3.3.1. Incidence and distribution of *B. cereus* among milk samples and environmental swabs

The incidence and distribution patterns of *B. cereus* among the sampling points along the milk processing lines and environmental swabs in the two dairy plants are summarized in Table 4. The incidence of *B. cereus* vegetative cells in raw milk samples (trucks, RTs and ProHTSTs) was low. Positive samples were less than 10% and the average counts of positive samples were less than 50 CFU/ml after enrichment at 8°C for 3 days in

Table 4. The incidence and the average counts of *B. cereus* in milk samples from each sampling point of the milk processing lines and environmental swabs in plant I and plant II after enrichment at 8°C for 14 days

Samples locations †	The positive percentage and average counts			
	Plant I		Plant II	
	%	CFU/ml	%	CFU/ml
Raw Milk ‡	10	50	7	30
Heat-treated milk:				
RT	80	$1.1x10^5$	82	$2.4x10^6$
ProHTST	85	$2.9x10^5$	93	$1.3x10^6$
Pasteurized milk:				
HTST	85	$7.1x10^6$	94	$1.5x10^6$
PT	76	$3.7x10^5$	94	$1.5x10^6$
Final products:				
F	90	$5.5x10^6$	96	$5.0x10^6$
Environmental swab				
Swab	ND*	< 10	ND	< 10
Heat-treated swab	5	30	ND	< 10

† notations are the same as in Table 2;

‡ The average counts of vegetative *B. cereus* in raw milk was estimated after enrichment at 8 °C for 3 days;

ND* = not detected at the sensitivity of 10 CFU/ml.

both plant I and II. However, the incidence of *B. cereus* spores in raw milk samples as measured by the *B. cereus* vegetative cells in heat-treated milk was very high. More than 80% of heat-treated milk samples were found to contain *B. cereus* and the average counts in positive samples were more than 1.1 x 10^5 CFU/ml after enrichment at 8°C for 14 days in both plant I and plant II (Table 4).

The incidence of *B. cereus* in pasteurized milk samples (HTSTs and PTs) and final products was similar to that in heat-treated raw milk. Seventy-six to ninety-four percent of the pasteurized milk samples were contaminated with *B. cereus* and the average counts of positive samples reached up to 3.7 x 10^5 CFU/ml after enrichment at 8°C for 14 days in milk from both plant I and plant II. More than 90% of the final products contained *B. cereus* and the average counts reached 5.5 x 10^6 CFU/ml for plant I and 5.0 x 10^6 CFU/ml for plant II after enrichment at 8°C for 14 days (Table 4).

None of the environmental swabs obtained from either dairy plant contained *B. cereus* vegetative cells after enrichment at 8°C for 14 days. The counts of *B. cereus* in heat-treated environmental swabs were also low. However, five percent of the heat-treated environmental swabs from plant I were positive for *B. cereus*. The average count of *B. cereus* in these swabs was 30 CFU/ml after enrichment at 8°C for 14 days (Table 4).

3.3.2. Identification and further confirmation of *B. cereus* isolates

The reproducibility of each sample run was determined by repeated analyses of the FAME profiles of *Stenotrophomonas maltophilia* ATCC 13637. The mean ED from 15 runs was 0.95 units with a minimum 0.26 and maximum 2.28. The change in ED was less than

2.5 ED for this strain through the entire experiment, and therefore the reproducibility was high and the instrument was stable.

A total of 587 presumptive *B. cereus* isolates initially identified by Gram stain and catalase reaction were further tested by the MIDI standard library. The results showed 343 *B. cereus* isolates with a similarity index (SI) above 0.5000, 203 *B. cereus* isolates with SI between 0.5000 and 0.3000, and 41 *B. cereus* isolates with SI below 0.300. A total of 539 *B. cereus* isolates from milk samples in plant I and plant II and a total of 7 *B. cereus* isolates from the environmental swabs in plant I with similarity index ≥ 0.3000 were used for further studies. The number of *B. cereus* isolates obtained from each sampling point among the milk processing lines and environmental swabs is summarized in Table 5.

3.3.3. Characteristics of the cellular fatty acid composition of *B. cereus* isolates in each subgroup of the 'BCERMK' library

The 'BCERMK' library constructed with 229 *B. cereus* isolates consisted of 18 different subgroups (A-R). The relationship of the FAME profiles of these 229 isolates to each other are depicted by the dendrogram shown in Fig. 3. Based on the principle of cluster analysis, highly related isolates are clustered at relatively short distances from the origin on the dendrogram whereas less closely related isolates join at progressively greater distances. As a result, all isolates can be displayed graphically in a tree diagram that depicts the relatedness of pairs of entries expressed in Euclidian distances (ED).

The characteristics of the cellular fatty acid composition of *B. cereus* isolates in each subgroup of the 'BCERMK' library is shown in Table 6. Under the standardized growth

Table 5. The number of *B. cereus* isolates and samples from each sampling point of the milk processing lines and environmental swabs in plant I and II

Sampling locations†	The number of *B. cereus* isolates and samples					
	Plant I		Plant II		Total	
	Isolates	Samples	Isolates	Samples	Isolates	Samples
Truck	8	3	10	3	18	6
RT	34	11	36	10	70	21
ProHTST	52	17	41	12	93	29
HTST	46	16	41	14	87	30
PT	88	32	38	12	126	44
Filler	3	1	27	10	30	11
F	74	27	41	15	115	42
Swab	7	3	0	0	7	3
Total	312	110	234	76	546	186

† notations are the same as in Table 2.

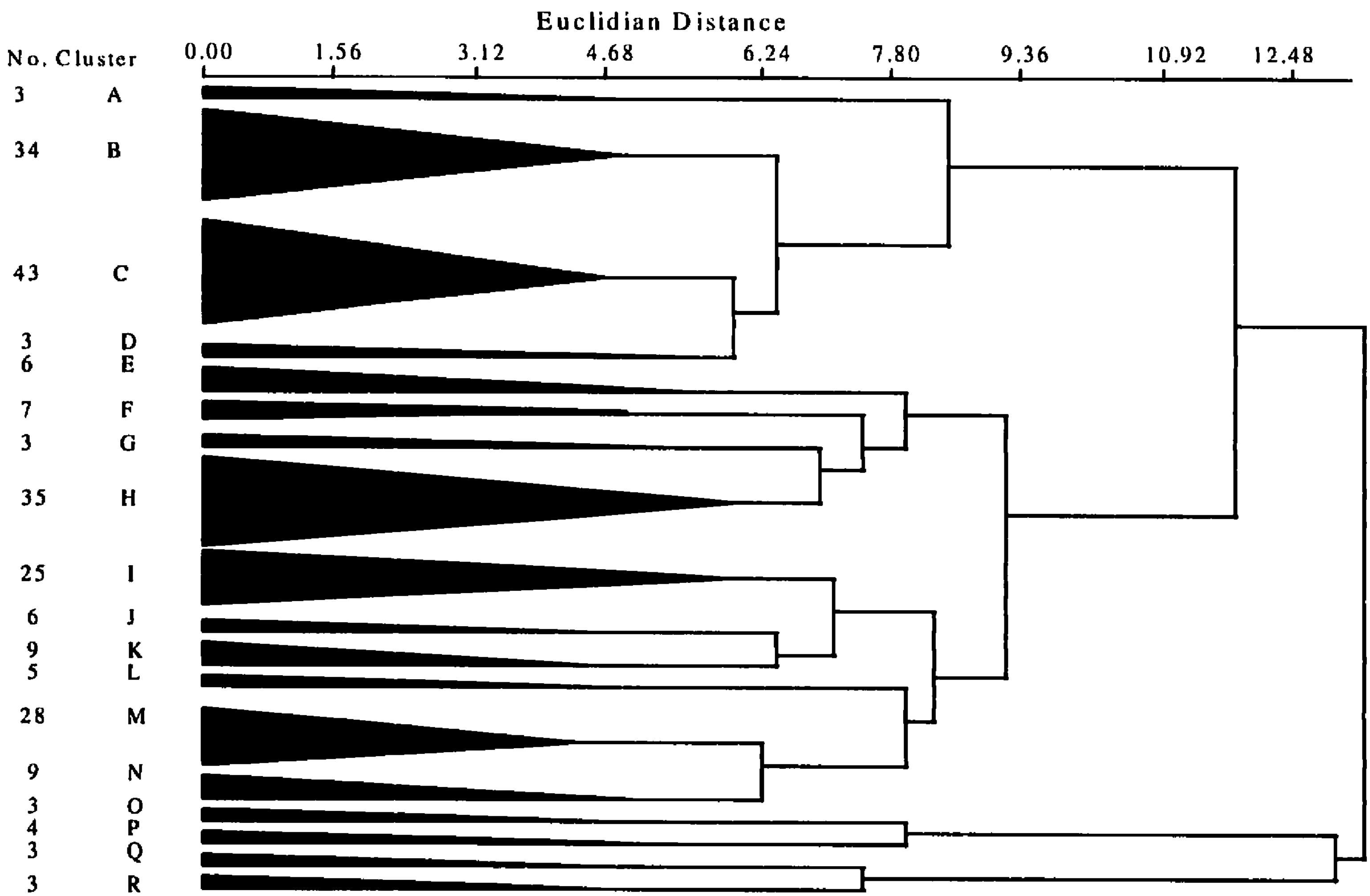


Fig.3 Dendrogram of *B. cereus* strains generated by cluster analysis of FAME profiles showing subgroups in BCERMK library

Table 6. Main fatty acid composition of *B. cereus* isolates in each subgroup of the 'BCERMK' library

Sub-group	No. of isolates	% mean ± SD (Standard Deviation)							
		i-12:0	a-13:0	i-13:0	i-14:0	14:0	i-15:0	a-15:0	i-16:0
A	3	1.68±0.23	1.18±0.22	11.48±0.75	4.15±0.42	4.47±0.47	23.23±1.35	2.24±0.49	7.67±0.13
B	34	1.37±0.27	1.87±0.41	9.04±0.91	4.10±0.60	2.80±0.24	20.41±3.05	4.13±0.60	8.35±0.78
C	43	1.16±0.23	1.48±0.22	10.12±0.89	3.49±0.58	2.92±0.19	23.39±1.89	3.22±0.50	7.24±0.49
D	3	1.15±0.33	1.62±0.47	10.12±1.83	3.78±0.67	3.32±0.13	25.17±0.21	3.60±0.56	7.23±0.28
E	6	1.31±0.33	1.81±0.39	13.66±1.37	3.76±0.37	3.69±0.23	27.26±1.83	3.46±0.40	6.12±0.32
F	7	0.90±0.08	1.13±0.19	10.27±1.02	3.33±0.35	3.42±0.32	29.42±2.50	2.73±0.39	7.05±0.34
G	3	0.93±0.20	1.39±0.24	8.69±0.30	3.59±0.36	2.16±0.04	29.58±1.85	4.03±0.68	7.62±0.50
H	35	0.83±0.29	1.03±0.17	8.53±0.99	3.30±0.61	3.02±0.27	27.40±1.62	2.76±0.46	7.41±0.90
I	25	0.44±0.42*	0.97±0.31	7.94±0.75	3.71±0.46	3.10±0.49	31.31±2.67	3.69±0.45	6.92±0.69
J	6	0.76±0.10	0.90±0.14	9.69±1.09	3.94±0.57	3.84±0.29	32.64±1.28	3.30±0.52	5.26±0.62
K	9	0.88±0.20	1.24±0.17	10.01±0.61	4.77±0.62	4.20±0.52	32.50±1.30	4.20±0.42	6.58±0.63
L	5	1.07±0.19	1.00±0.06	8.88±0.99	5.59±0.68	3.60±0.17	26.96±2.03	3.57±0.34	7.21±0.49
M	28	0.94±0.31	1.29±0.25	7.61±0.70	4.70±0.75	2.99±0.41	26.45±2.98	4.59±0.97	8.47±1.31
N	9	1.46±0.25	1.72±0.35	11.18±1.68	5.10±1.17	3.50±0.23	25.42±2.15	4.00±1.12	7.78±0.92
O	3	0.61±0.42	1.16±0.11	9.17±1.16	3.88±0.23	3.27±0.59	32.36±1.51	4.06±0.20	6.84±0.34

Table 6. Main fatty acid composition of *B. cereus* isolates in each subgroup of the 'BCERMK' library

Sub-group	No. of isolates	% mean ± SD (Standard Deviation)							
		i-12:0	a-13:0	i-13:0	i-14:0	14:0	i-15:0	a-15:0	i-16:0
P	4	0.42±0.51*	1.05±0.28	7.96±1.00	3.31±0.89	3.75±0.82	32.43±3.33	4.21±0.37	5.71±1.06
Q	3	1.23±0.32	1.39±0.48	9.28±0.37	3.54±0.51	2.81±0.17	21.39±2.33	3.11±0.78	7.72±0.93
R	3	1.38±0.42	1.66±0.49	8.82±1.49	5.54±1.35	3.03±0.58	22.05±1.15	4.67±0.79	8.65±1.11
Range		0.42~1.68	0.90~1.87	7.61~13.66	3.30~5.54	2.16~4.47	20.41~32.64	2.24~4.67	5.26~8.65

Table 6. Main fatty acid composition of *B. cereus* isolates in each subgroup of the 'BCERMK' library (continued)

Sub-group	No. of isolates	% mean ± SD (Standard Deviation)							
		16:1 w7c	16:1 w11c	16:0	i-17:0	i-17:1 w10c	a-17:0	summed 4	%
A	3	2.23±0.38	4.15±0.82	15.38±0.62	7.84±0.94	5.75±0.50	1.13±0.74	6.35±0.31	98.93
B	34	2.08±0.20	2.69±0.48	11.66±1.89	10.23±0.68	6.98±0.92	2.26±0.37	6.92±0.62	94.89
C	43	2.16±0.19	2.64±0.45	9.90±1.10	10.19±1.01	9.00±0.89	1.62±0.36	6.93±0.54	95.46
D	3	2.89±0.18	3.71±0.60	9.19±1.32	6.91±0.58	8.59±1.10	1.26±0.09	7.19±0.85	95.73
E	6	2.20±0.18	2.66±0.24	6.43±0.63	6.19±0.64	8.68±0.97	0.81±0.41	7.63±0.67	95.67
F	7	2.80±0.57	3.23±0.51	5.38±2.71	5.79±2.88	9.74±1.45	0.46±0.62*	9.21±0.83	94.86
G	3	3.42±0.13	1.79±0.05	6.03±0.88	7.73±0.39	9.27±0.87	1.86±0.26	5.02±0.02	93.11
H	35	2.91±0.54	3.36±0.82	5.95±0.86	7.93±1.55	11.27±1.65	1.01±0.36	8.26±0.95	94.97
I	25	1.53±0.54	1.12±0.88	6.85±1.25	10.02±1.79	5.70±2.00	1.52±0.38	8.59±0.88	93.41
J	6	0.74±0.43	0.26±0.41*	4.60±0.53	8.40±1.17	3.42±0.44	1.05±0.23	10.94±0.24	89.74
K	9	1.92±0.48	1.25±0.70	5.16±0.90	5.44±0.66	4.91±1.41	0.96±0.12	9.50±0.90	93.52
L	5	1.04±0.14	0.50±0.20*	6.14±0.45	8.73±0.58	2.87±0.49	1.22±0.11	12.44±0.99	90.82
M	28	1.87±0.42	1.45±0.70	8.17±1.35	9.57±0.83	5.29±0.98	2.03±0.50	8.16±1.20	93.58
N	9	2.27±0.56	2.04±0.57	8.12±0.76	7.40±1.05	5.71±1.13	1.27±0.53	8.40±0.85	95.37
O	3	2.36±0.18	2.20±0.33	5.60±1.29	7.13±1.48	7.09±0.87	1.41±0.23	6.73±0.47	93.87

Table 6. Main fatty acid composition of *B. cereus* isolates in each subgroup of the 'BCERMK' library (continued)

Sub-group	No. of isolates	% mean ± SD (Standard Deviation)							
		16:1 w7c	16:1 w11c	16:0	i-17:0	i-17:1 w10c	a-17:0	summed 4	%
P	4	1.18±0.32	0.41±0.48	5.32±1.67	8.13±1.96	4.21±0.57	1.25±0.64	10.70±1.03	90.04
Q	3	2.35±0.42	2.61±0.28	10.43±2.71	10.37±0.65	9.08±2.68	1.56±0.64	6.88±0.33	93.75
R	3	2.50±0.72	2.29±0.73	7.64±0.47	7.64±1.12	5.38±0.66	1.82±0.26	7.46±0.57	90.53
Range		0.74~3.42	0.26~4.15	4.60~15.38	5.44~10.37	2.87~11.27	0.46~2.26	5.02~10.94	89.74~98.93

* : these fatty acids were not used in the library search.

conditions, *B. cereus* isolates of each cluster in the 'BCERMK' library displayed qualitatively similar fatty acid profiles. Fatty acids common to all of the isolates were 10-methyldecanoic acid (i-12:0), 10-methyldodecanoic acid (a-13:0), 11-methyldodecanoic acid (i-13:0), 12-methyltridecanoic acid (i-14:0), tetradecanoic acid (14:0), hexadecanoic acid (16:0), 13-methyltetradecanoic acid (i-15:0), 12-methyltetradecanoic acid (a-15:0), 14-methylpentadecanoic acid (i-16:0), 15-methylhexadecanoic acid (i-17:0), 14-hexadecanoic acid (a-17:0), unsaturated fatty acids with cis-9- hexadecanoic acid (16:1 w7c), cis-5-hexadecanoic acid (16:1 w11c) and cis-6-15-methylhexadecanoic acid (i-17:1 w10c) and summed feature 4. These common fatty acids accounted for 89.74 to 98.93% of total composition used for the library search. There were six predominant fatty acids accounting for 46.19 ~ 91.97% of the total fatty acid composition. They were 13-methyltetradecanoic acid (15:0 iso) (20.41-32.64%), 11-methyldodecanoic acid (13:0 iso) (7.61-13.66%), 14-methylpentadecanoic acid (16:0 iso) (5.26-8.65%), exadecanoic acid (16:0) (4.60-15.38%), 15-methylhexadecanoic acid (17:0 iso) (5.44-10.37%) and cis-6-15-methylhexadecanoic acid (i-17:1 w10c) (2.87- 11.27%) (Table 6).

3.3.4. Relationship between *B. cereus* isolates from milk samples collected at each sampling point and the environmental swabs

The number of *B. cereus* isolates isolated from the milk samples collected at each sampling point (raw milk, pasteurized milk and final products) and the environmental swabs allocated into each subgroup of the 'BCERMK' library is summarized in Table 7. Five hundred forty-five *B. cereus* isolates were allocated into 18 different subgroups of the

Table 7. The number of *B. cereus* isolates from each sampling point of the milk processing lines and environmental swabs allocated to each subgroup of the 'BCERMK' library

Sub-group	The number of *B. cereus* isolates from each sampling point †								
	Heat-treated milk			Pasteurized milk			F	Swab	Total
	Truck	RT	ProHTST	HTST	PT	Filler			
A	0	0	0	0	2	0	3	0	5
B	2	6	3	6	15	3	14	0	49
C	2	13	26	33	49	16	41	2	182
D	0	0	0	0	1	0	2	0	3
E	0	0	4	5	2	0	2	0	13
F	0	3	3	2	1	0	1	0	10
G	0	0	0	0	1	0	2	0	3
H	7	18	11	14	18	3	4	0	75
I	3	6	14	5	13	2	9	4	56
J	0	1	2	2	1	0	1	0	7
K	0	2	5	3	3	3	1	0	17
L	3	0	3	0	0	0	2	0	8
M	1	15	11	8	9	1	15	1	61
N	0	0	1	2	2	1	3	0	9
O	0	0	0	1	2	0	3	0	6
P	0	4	10	3	6	0	4	0	27
Q	0	2	0	2	1	0	6	0	11
R	0	0	0	1	0	0	2	0	3
Total	18	70	93	87	125	30	115	7	545

† notations are the same as in Table 2.

'BCERMK' library (Table 7). The common subgroups in the 'BCERMK' library for *B. cereus* isolates germinated from the spores in raw milk, from pasteurized milk and the final products were B, C, E, F, H, I, J, K, M, N, P, and Q (Fig. 4). The number of the *B. cereus* isolates in those common subgroups of the 'BCERMK' library accounted for 94.8% of all isolates tested. Predominant subgroups of *B. cereus* isolates in this category were B, C, H, I, and M, accounting for 77.3% of all isolates tested. A lot of *B. cereus* isolates in subgroups B, C, and M were found from RT through F while the distribution of isolates was more uniform for groups H and J. The results may indicate that a selection for certain psychrotrophic *B. cereus* isolates existed in the dairy plants since only a small number of isolates isolated from Truck samples were distributed into subgroups B, C and M. Specific subgroups of *B. cereus* isolates only found in pasteurized milk and final products (A, D, G, O, R) accounted for 3.7% of all isolates, and a specific subgroup (L) of *B. cereus* isolates only found in heat-treated milk and final products accounted for 1.5% of all isolates tested. *B. cereus* isolates from environmental swabs were allocated into C, I and M and no specific groups were found. These results showed that a greater variety of subgroups was found for *B. cereus* isolated from pasteurized milk than for those isolated from germinated spores in raw milk and more different *B. cereus* subgroups were isolated from final products than from in-line samples of pasteurized milk, indicating that post-pasteurization contamination with *B. cereus* in pasteurized milk occurred and that the HTST pipes, pasteurized milk tanks, and fillers might be potential reservoirs for *B. cereus* within a dairy plant (Table 7).

3.3.5. Relationship between *B. cereus* isolates from different dairy plants

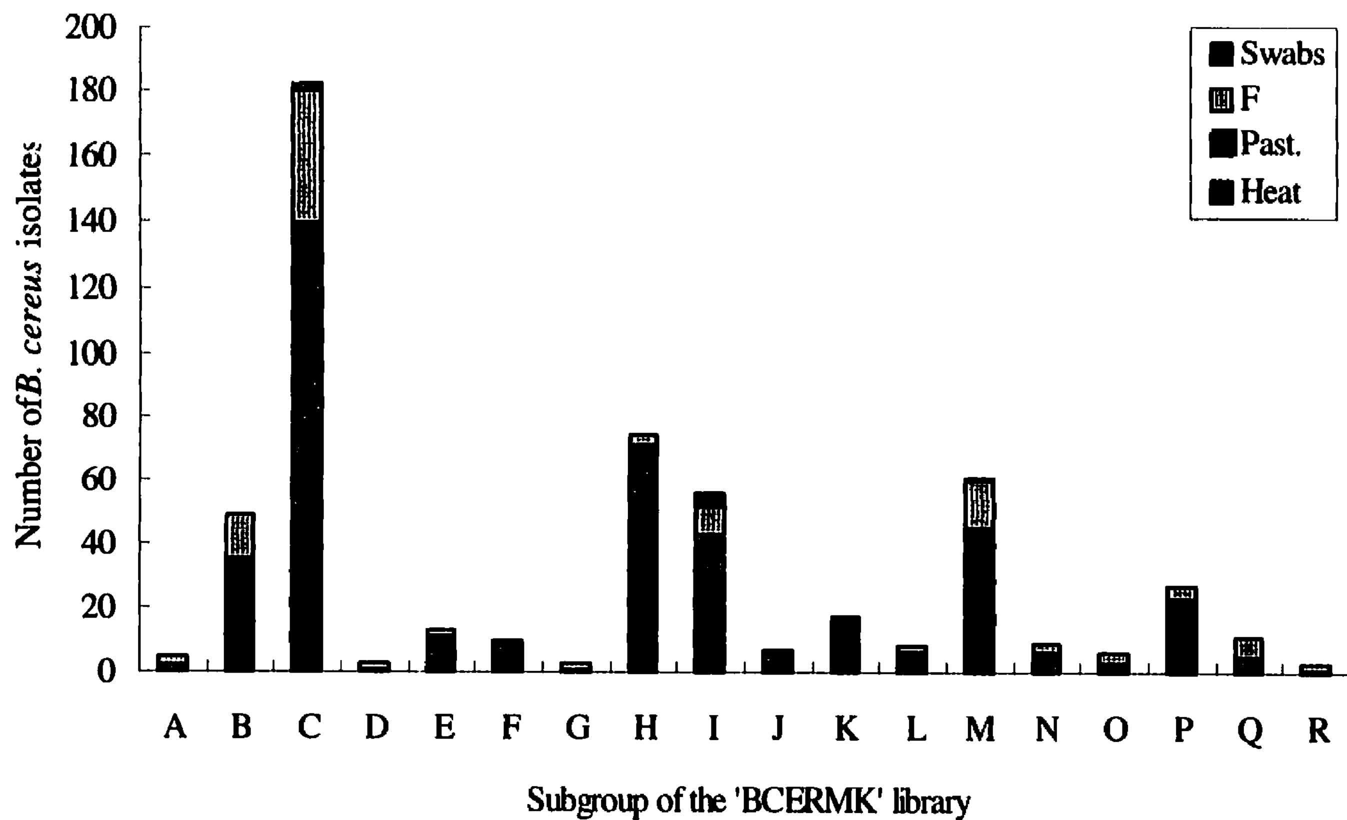


Fig. 4. The number of *B. cereus* isolates from heat-treated milk (Heat), pasteurized milk (Past.), final products (F) and environmental swabs (Swab) allocated into each subgroup of the 'BCERMK' library

The number of *B. cereus* isolates from two dairy plants allocated into each subgroup of the 'BCERMK' library is summarized in Table 8. The common subgroups for *B. cereus* isolates from plant I and plant II were A, B, C, D, E, F, G, H, I, J, L, M, N, and Q (Fig. 5). The number of *B. cereus* isolates in those common subgroups of the 'BCERMK' library accounted for 90.3% of all isolates tested. Predominant subgroups of *B. cereus* isolates in this category were B, C, H, I, and M, accounting for 77.6% of all isolates. However, specific subgroups of *B. cereus* isolates found only in plant I were K, O, P and R. The number of *B. cereus* isolates in these subgroups accounted for 9.7% of all isolates. One *B. cereus* isolate isolated from a filler in plant II was not clustered into any subgroups of the 'BCERMK' library since the similarity index was less than 0.300. The isolate was identified as *B. mycoides* subgroup B by the MIDI library.

3.3.6. Relationship between *B. cereus* isolates isolated from different sampling months

The number of *B. cereus* isolates from different sampling months allocated into each subgroup of the 'BCERMK' library is summarized in Table 9. The common subgroups for *B. cereus* isolates from all sampling months were B, C, I and M. The number of the *B. cereus* isolates in those common subgroups of the 'BCERMK' library accounted for 63% of all isolates tested. In the subgroups of B, C and H, a greater number of *B. cereus* isolates were found in the later sampling season (from May to September) than in the early sampling season (March and April). The *B. cereus* isolates obtained from May were most diversified and classified into 14 groups. The 'plant specific' subgroups K, O and P in plant I were also

Table 8. The number of *B. cereus* isolates from plant I and plant II allocated to each subgroup of the 'BCERMK' library

Sub-group	The number of *B. cereus* isolates								
	Plant I					Plant II			
	Swab	Heat†	Past.†	F †	Total	Heat†	Past.†	F†	Total
A	0	0	1	3	4	0	1	0	1
B	0	2	10	2	14	9	14	12	35
C	2	13	47	27	89	27	52	14	93
D	0	0	0	2	2	0	1	0	1
E	0	2	5	2	9	2	2	0	4
F	0	3	2	1	6	3	1	0	4
G	0	0	1	0	1	0	0	2	2
H	0	17	20	2	39	19	15	2	36
I	4	18	11	7	40	6	8	2	16
J	0	2	3	1	6	1	0	0	1
K	0	7	9	1	17	0	0	0	0
L	0	2	0	1	3	4	0	1	5
M	1	11	9	11	32	16	9	4	29
N	0	1	3	1	5	0	2	2	4
O	0	0	3	3	6	0	0	0	0
P	0	14	9	4	27	0	0	0	0
Q	0	2	3	4	9	0	0	2	2
R	0	0	1	2	3	0	0	0	0
Total ‡	7	94	137	74	312	87	105	41	233

† Heat, Past. and F = heat-treated milk, pasteurized milk and final products;

‡One *B. cereus* isolate from a filler in plant II was not clustered into any subgroup of the 'BCERK' library.

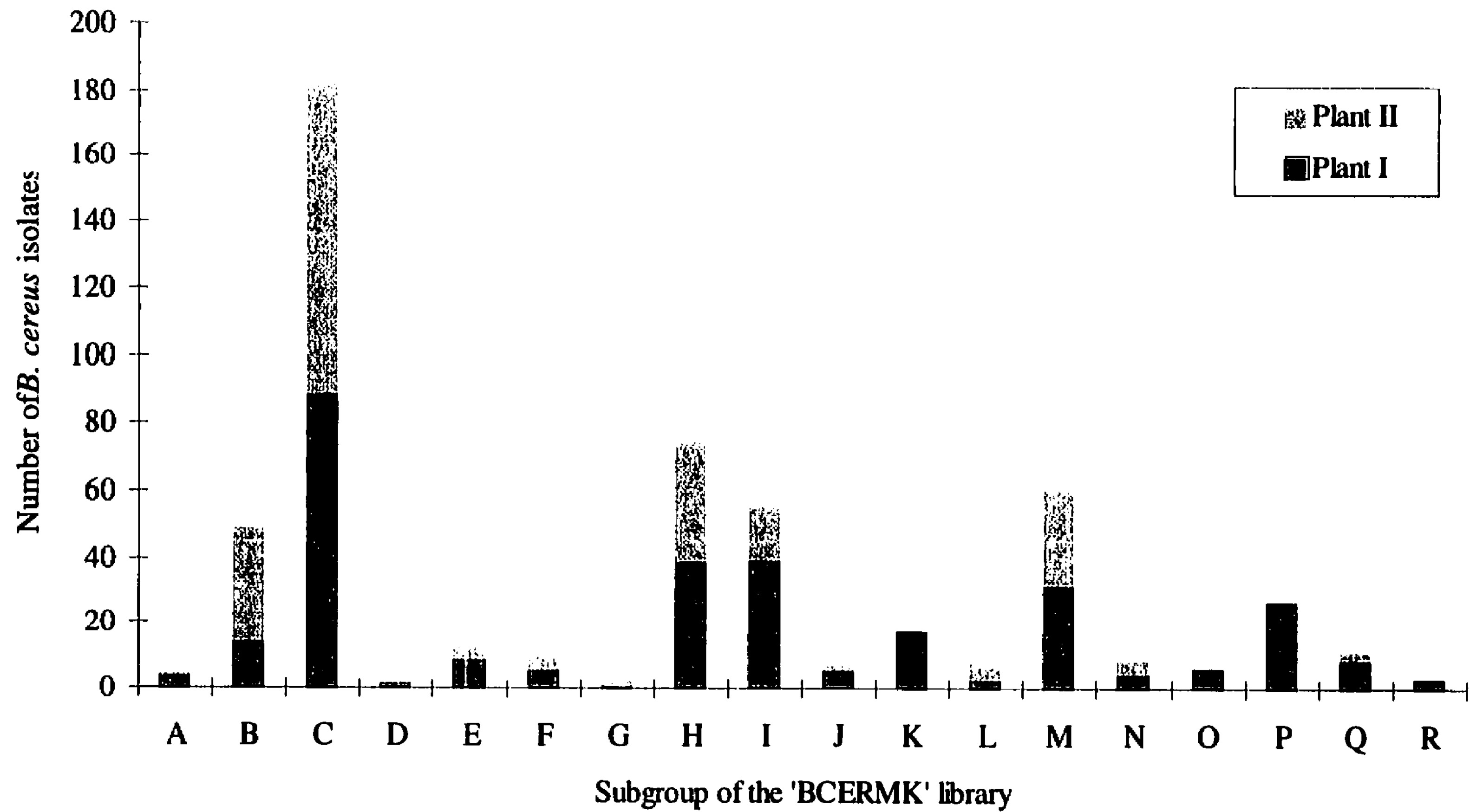


Fig. 5. The number of *B. cereus* isolates from two dairy plants allocated into each subgroup of the 'BCERMK' library

Table 9. The number of *B. cereus* isolates from each sampling month allocated to each subgroup of the 'BCERMK' library

Sub-groups	The number of *B. cereus* isolates						
	March	April	May	July	August	Sept. I†	Sept. II†
A	1	3	0	1	0	0	0
B	3	5	2	13	11	4	11
C	6	18	30	22	42	34	28
D	0	1	1	0	0	1	0
E	0	1	3	0	2	5	2
F	0	0	1	0	3	4	2
G	0	0	0	3	0	0	0
H	0	6	19	15	9	11	15
I	12	1	19	6	4	6	4
J	0	0	6	0	1	0	0
K	1	0	7	2	0	7	0
L	3	1	0	0	4	0	0
M	5	12	8	1	20	8	6
N	1	0	2	0	0	2	4
O	0	0	5	0	0	1	0
P	0	2	25	0	0	0	0
Q	0	0	8	0	2	1	0
R	0	3	0	0	0	0	0

†: Stands for samples collected in plant I and plant II.

mainly isolated in May. Specific subgroups of *B. cereus* isolates were found for April (R) and July (G). The subgroup P mainly consisted of *B. cereus* isolates collected from May..

3.3.7. Relationship between *B. cereus* isolates based on the 'MI' library

The results of the allocation of 546 *B. cereus* isolates are shown in Table 10. Three hundred and forty-three *B. cereus* isolates (63%) were allocated to 'MI' library and classified into Mi 4, Mi 5, Mi 6, Mi 8, Mi 9, Mi 10 and Mi 11 subgroups. No isolates were clustered into Mi 1, Mi 2, Mi 3 and Mi 7 subgroups of the 'MI' library. The common subgroups for *B. cereus* isolates germinated from the spores in raw milk and *B. cereus* isolates in pasteurized milk and the final products were Mi 4, Mi 5, Mi 6, Mi 9 and Mi 11. However, a greater number of *B. cereus* isolates was found in the pasteurized milk and final products than in heat-treated milk in the subgroup Mi 4 in plant I. *B. cereus* isolates in the subgroup Mi 8 were obtained only from plant I.

3.4. DISCUSSION

The incidence of *B. cereus* in milk samples and distribution of *B. cereus* among the sampling points along milk processing lines (from raw milk in the truck to the final products in the bags or cartons) and from environmental swabs in two dairy plants were investigated in this study. The incidence of *B. cereus* in milk samples and the average counts of *B. cereus* in the positive samples of heat-treated milk, pasteurized milk and final products were similar, suggesting that *B. cereus* found in the pasteurized milk and final products were likely to be derived from the germination of spores present in the raw milk. *B. cereus* present in the

Table 10.The number of *B. cereus* isolates from each sampling point of the milk processing lines and environmental swabs in plant I and plant II allocated to each subgroup of the 'MI' library

Sampling locations	The number of *B. cereus* isolates in each subgroup															
	Plant I								Plant II							
	Mi4	Mi5	Mi6	Mi8	Mi9	Mi10	Mi11	other	Mi4	Mi5	Mi6	Mi8	Mi9	Mi10	Mi11	other
Truck	0	0	0	0	2	3	3	0	0	1	6	0	0	0	0	3
RT	5	2	5	1	2	0	9	10	3	1	7	0	2	0	10	13
ProHTST	2	2	4	1	8	0	19	16	10	5	1	0	1	1	7	16
HTST	4	4	5	2	0	0	7	24	6	7	7	0	1	0	10	10
PT	24	6	7	1	9	1	18	22	4	4	2	0	2	0	3	23
Filler	0	0	0	0	0	0	1	2	5	1	3	0	1	0	3	14
F	13	9	2	2	2	1	19	26	8	4	2	0	1	1	4	21
Swab	0	1	0	0	4	0	0	2	0	0	0	0	0	0	0	0
Total	48	24	23	7	27	5	76	102	36	23	28	0	8	2	37	100

† notations are the same as in Table 1

environmental swabs indicated that the dairy plant environment was another source of *B. cereus* in the pasteurized milk. Alternatively, environmental contamination of dairy plants could be a result of contaminated milk. The incidence and the average counts of *B. cereus* in raw milk that was incubated at 8 °C for 3 days could not be compared with those obtained for pasteurized milk stored 8 °C for 14 days. However, determination of *B. cereus* in raw milk at 8 °C for more than 3 days was difficult due to the overgrowth of the spoilage microorganisms. The incidence of *B. cereus* in milk samples and distribution patterns of *B. cereus* among the sampling points along the milk processing lines and environmental swabs were similar in the two dairy plants. The incidence found in this study was higher than in previous reports in Scotland (Griffiths and Phillips, 1990) and Netherlands (Te Giffel *et al.*, 1996). In Scotland, spores of psychrotrophic *Bacillus* species have been isolated from 58% of raw milk samples tested and 94% of the positive milk samples had counts above 1 x 10^5 CFU/ml after the milk was stored at 6°C for 14 days (Griffiths and Phillips, 1990). In the Netherlands, thirty five percent of heat-treated milk samples, 61 to 71% of pasteurized milk samples and 71% of final products have been found to contain *B. cereus* when milk samples were pre-incubated at 30°C for 6 hours, however, the authors did not report the counts of *B. cereus* in the positive milk samples (Te Giffel *et al.*, 1996). In addition to the differences of milk sources and dairy environment, the enrichment conditions could be one of the factors accounting for the differences between the previous studies and this investigation. The results obtained in this study could be more useful to the dairy industry since the enrichment condition used was 8°C for 14 days. The temperature of 8°C for milk storage and transportation is not unusual and the storage duration of 14 days is around the best before

consumption day. The average counts of *B. cereus* in the positive milk samples were similar to a previous report in which *B. cereus* counts of $10^7 \sim 10^8$ CFU/ml in pasteurized milk (HTST at 72 to 80°C for 15 s) were found when milk samples were stored at 7.5°C for 12 to 14 days (Langeveld and Cuperus 1993).

Most *B. cereus* isolates from both heat-treated milk and pasteurized milk samples or the final products were allocated into the common subgroups of the BCERMK library, indicating that *B. cereus* spores in raw milk were a major source of *B. cereus* contamination in pasteurized milk. This finding is similar to previous reports (Griffiths, 1992; Crielly *et al.*, 1994). However, the presence of a specific subgroup of *B. cereus* isolates (A, D, G, O and R) and significant increase in the number of *B. cereus* isolates in a specific subgroup in pasteurized milk (C) and final products (Q) in comparison with raw milk suggested that post-pasteurization contamination, including from equipment along the milk-processing lines and packing materials was possibly another source of *B. cereus* in pasteurized milk. It has been reported that a large number of *B. cereus* in pasteurized milk was derived from the dairy (Davies, 1975; Coghill, 1982). Te Giffel *et al.* (1996) revealed that some biotypes of *B. cereus* were only detected after the pasteurization process. However, more environmental swab samples should be analysed to further confirm the results. The fact that a large number of *B. cereus* isolates were found in raw milk tanks through to final products while only a small number of isolates were isolated from Truck samples might indicate selection for psychrotrophic *B. cereus* isolates in dairy plants. However, more samples of truck milk should be analysed to verify the results.

The presence of specific subgroups of *B. cereus* isolates in different dairy plants may

suggest that the plants themselves are the post-pasteurization contamination sources. Alternatively, different sources of raw milk for each plant may explain the presence of plant-specific *B. cereus* isolates. Collecting samples in two dairy plants at the same time of year could reduce the seasonal changes and would help locate the 'plant-specific' *B. cereus* isolates more easily. Existence of plant-specific isolates has been previously reported (Schraft, *et al.*, 1996).

An increase in the number of *B. cereus* isolates in the subgroup C and H in the summer season, and the presence of the specific *B. cereus* isolates in April, May and July, indicated that seasonal changes of *B. cereus* in milk was evident. However, no attempt was made to accurately investigate the seasonal changes in the *B. cereus* counts. The higher counts of *B. cereus* found in milk or milk products in summer seasons (Christianson et al., 1989; Phillips and Griffiths, 1986) could be due to the increase of the number of the same *B. cereus* isolates and/or the presence of new sources of *B. cereus* isolates.

The results of allocation of the *B. cereus* isolates isolated from the milk samples along the milk processing lines and from environmental swabs in two dairy plants to 'MI' library confirmed the results obtained by allocating *B. cereus* isolates into the 'BCERMK' library. However, 36% of the *B. cereus* isolates could not be allocated into any subgroups of the 'MI' library. The 'BCERMK' library is, therefore, more appropriate for allocating *B. cereus* isolates from dairy plants.

CHAPTER 4. GENERAL DISCUSSION AND FUTURE WORK

4.1. General discussion

The ubiquitous presence of *B. cereus* in nature makes contamination of foodstuffs with this organism a common phenomenon. Gastrointestinal disease and non-gastrointestinal infections caused by this organism are more widespread than once thought and the incidences are also probably higher than reported.

The incidence of *B. cereus* and the average counts of positive milk samples collected from various sampling points of the milk-processing lines and environmental swabs in two independent dairy plants were investigated in detail in this research. The incidence and the average counts of *B. cereus* in heat-treated milk, pasteurized milk and final products were similar, indicating that *B. cereus* present in heat-treated milk, pasteurized milk and final products were possibly derived from its spores in raw milk.

Traditional microbial identification and classification methods for *B. cereus*, such as biochemical properties, serological typing and detection of its toxin are time-consuming and rapid methods, such as RAPD-PCR and PCR-RFLP are expensive. Fourier Transform infrared spectroscopy (FTIR) and Microbial Identification System (MIDI) methods are simple, fast and relatively inexpensive to perform. The FTIR technique was successfully employed in this study to identify and classify *B. cereus* strains based on the specific absorbance peak between wavenumbers 1738 - 1740 cm^{-1}. However, FTIR was not able to differentiate *B. cereus* from *B. thuringiensis* and *B. mycoides* based solely on the specific absorbance peak. Furthermore, a wider range of *B. cereus* strains and mixed cultures of *B.*

cereus with other bacteria should be investigated in order to establish a standard database for further identification and classification.

Analysis of fatty acid profiles of *B. cereus* using the MIDI-system showed that *B. cereus* spores in raw milk were the major source of *B. cereus* in pasteurized milk and that post-contamination along the milk-processing lines or from packing materials was possibly another source.

Since bacteria can alter the fatty acid composition of cells to maintain membrane fluidity with varying environmental conditions, and the MIDI libraries rely on qualitative (which compounds are present) and quantitative (area percentages) analysis, it is essential to keep the culture medium, the time and temperature of incubation and sample preparation procedures the same as the MIS standard libraries to ensure reproducibility of results. The reproducibility of each sample run was determined by repeated analyses of the FAME profiles of *Stenotrophomonas maltophilia* ATCC 13637. The change in ED was less than 2.5 ED for this strain through the entire experiment, indicating that the reproducibility was high and the instrument was stable, since the cut-off value of 2.5 ED was considered critical for identification of the same strains (MIDI operating manual version 4, 1993). The reproducibility of *B. cereus* fatty acid profile was also tested in 32 isolates randomly taken from 546 isolates. The 32 isolates were analysed twice by MIDI and allocated to the subgroups of BCERMK. The reproducibility of the same isolate classified into the same subgroup of these libraries was 72%. The low reproducibility could be due to the isolates used for the repeat experiment, which were from sequential subcultures, i.e., fourth subculture. It has been found that sequential subcultures can cause great variation in MIDI

analysis (Schraft, *et al.*, 1996).

Various factors including medium, reagent preparation, growth temperature, growth rate and instrument could affect the phenotypic expression of relative amounts of fatty acids within bacterial cells even though standardized protocols were used in this research. The findings obtained in this research should be confirmed with molecular methods. RAPD-PCR and PCR-RFLP are increasingly exploited for epidemiological typing of microorganisms, *eg*. *Listeria* spp., *Campylobater* spp., *Staphylococcus aureus*, *B. cereus* (Mazurier and Wernars, 1992; Mazurier *et al.*, 1992; Schraft *et al.*, 1996).

As discussed above, data have been presented to demonstrate that *B. cereus* spores in raw milk are the major source of *B. cereus* in pasteurized milk, however, more detailed analyses should be conducted to prove whether post-pasteurization contamination is another source. The new and specific groups of isolates isolated from pasteurized milk in this research were not always conclusively clustered into the same subgroups of *B. cereus* isolated from the environmental swabs. This could be due to the inadequate sampling because environmental swabs were only collected from fillers in plant I and *B. cereus* in pasteurized milk could be from other sampling points such as valves, flanges or shafts which would be difficult to sample. Components with seals have been found as the critical points of microbial re-contamination of food (Pfeifer and Kessler, 1995). Therefore, more environmental swabs should be collected to confirm the results.

4.2. Intervention strategies to minimize the sources of contamination of pasteurized milk with *B. cereus*

Since spores in raw milk have been found to be a major contamination source of *B. cereus* in pasteurized milk, special care to reduce the *B. cereus* spores in raw milk needs to be taken on the farm. The contamination sources of *B. cereus* at the farm may come from the udder of cows, the milkers, the milking utensils and environment. The udder and teats may become contaminated by soil, fodder and faeces. The milker, utensils and environment during milking can also play a part in the contamination of the raw milk when the cleaning and handling system is not effectively under control. It is therefore very important to clean the udder and the teats and keep equipment and the environment clean. Sanitization system for the milking system and cow sheds that are effective to control *B. cereus* have to be established on the farm. Transportation and storage of the raw milk should be performed under strict sanitary conditions.

Another contamination source of the raw milk may come from transport and storage in the dairy factory, eg., *B. cereus* spores already present in the tanks or pipelines. Thus, effective cleaning procedures to remove *B. cereus* along the process lines have to be developed. Stimulating the germination of *B. cereus* spores by low pasteurization temperature or a combination of heat treatment with various agents, such as L-alanine, or inosine may be used to effectively kill all spores in the milk-processing lines. A minimum of 15 min at 85°C for sanitizing milk pasteurization plants or 15 min at 140°C to 150°C for ultra-high temperature (UHT) plants recommended by the International Dairy Federation (International Dairy Federation, 1980) should be enforced.

REFERENCES

Abel, K., H. DeSchmirtzing, and J. I. Peterson. 1963. Classification of microorganisms by analysis of chemical composition. I. Feasibility of utilizing gas chromatography. J. Bacteriol. 85:1039-1044.

Adams, M. R. and M. O. Moss. 1995. *Bacillus cereus* and other *Bacillus* species. Food microbiology, pp.160-164. The Royal Society of Chemistry.

Agata, N., M. Mori, M. Ohta, S. Suwan, and I. Ohtanil. 1994. A novel dodecadepsipeptide,cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. FEMS Microbiol. Lett. 121:31-34.

Ahmed, A. A.-H., M. K. Moustafa, and E. H. Marth. 1983. Incidence of *Bacillus cereus* in milk and some milk products. J. Food Protect. 46:126-128

Anon. 1978. Microorganisms in foods. 1. Their significance and methods of enumeration. International Commission on Microbiological Specifications for Foods (ICMSF), 2nd. Toronto: University of Toronto Press.

Ash, C. and M. D. Collins. 1992. Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. FEMS Microbiol. Lett. 73:75-80.

Ash, C., J. A. E. Farrow, S. Wallbanks, and M. D. Collins. 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA. Lett. Appl. Microbiol. 13:202-206.

Baptist, J. N., M. Mandel, and R. L. Gherna. 1978. Comparative zone electrophoresis of enzymes in the genus *Bacillus*. Int. J. Syst. Bacteriol. 28:229-244.

Becker, H., G. Schaller, W. von Wiese, and G. Terplan. 1994. *Bacillus cereus* in infant foods and dried milk products. Int. J. Food Microbiol. 23:1-15.

Beecher, D. J. and J. D. MacMillan. 1991. Characterization of the components of haemolysin BL from *Bacillus cereus*. Infect. Immun.. 59:1778-1784.

Bergère, J. -L. 1992. Spore formation and germination of *Bacillus cereus*: The spore cycle. Int. Dairy Food Bull. 275:9-14.

Bergère, J. -L. and O. Cerf. 1992. Heat resistance of *Bacillus cereus* spores. Int. Dairy Food Bull.275:30

Billing, E. and W. A. Cuthbert. 1958. "Bitty" cream: The occurrence and significance of *Bacillus cereus* spores in raw milk. J. Appl. Bact. 21:65-78.

Birnbaum, D., L. Herwaldt, D. E. Low, M. Noble, M. Pfaller, R. Sherertz, and A. W. Chow. 1994. Efficacy of microbial identification system for epidemiologic typing of coagulase-negative *Staphylococci*. J. Clin. Microbiol. 32(9):2113-2119.

Bouwer-Hertzberger, S. A. and D. A. A. Mossel. 1982. Quantitative isolation and identification of *Bacillus cereus*. In: J. E. L. Corry, D. Roberts and F. A. Skinner (eds.), Isolation and Identification Methods for Food Poisoning Organisms. pp.255-259. London and New York: Academic Press.

Burrell, P., L. Farr, J. Bates, and A. Tan. 1991. A rapid enzyme immunoassay for detection of *Bacillus cereus* diarrhoeal enterotoxin in food and culture supernatant. J. Appl. Bacteriol. 71:xxii.

Busta, F. F. and P. M. Foegeding. 1986. Sporeforming bacteria. In: A. M. Pearson and T. R. Duston (ed.), Advances in Meat Research, vol. 2, pp.165-189. AVI Publishing

Company, Inc., Westport, Connecticut.

Byler, D. M., and H. Susi. 1986. Examination of the secondary structure of protein by deconvolved FTIR spectra. Biopolymers, 25:469-487.

Carlson, C. R., D. A. Caugant, A. B. Kolsto. 1994. Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. 60(6):1719-1725.

Champagne, C. P., R. R. Laing, D. Roy, A. A. Mafu, and M. W. Griffiths. 1994. Psychrotrophs in dairy products: their effects and their control. Crit. Rev. Food Sci. Nutri. 34(1):1-30.

Christiansson, A., A. S. Naidu, I. Nilsson, T. Wadsträm, H. E. Pettersson. 1989. Toxin production by *Bacillus cereus* dairy isolates in milk at low temperature. Appl. Environ. Microbiol. 55:2595-2600.

Claus, D. and R. C. W. Berkeley. 1986. Genus *Bacillus* Cohn 1872, 174. In: P. H. A. Sneath, N. S.Mair, M. E. Sharpe, and J. G. Hot (ed.), Bergey's Manual of Systematic Bacteriology, vol. 2, pp.1105-1139. The Willians & Wilkins Co., Baltimore.

Coghill, D. 1982. Studies on thermoduric psychrotrophic bacteria in South East Queensland dairy products. Aust. J. Dairy Technol. 37:147-148.

Coghill, D., and H. S. Juffs. 1979. Incidence of psychrotrophic spore-forming bacteria in pasteurized milk and cream products and effect of temperature on their growth. Aust. J. Dairy Technol. 34:150-153.

Crielly, E. M., N. A. Logan, and A. Anderton. 1994. Studies on the *Bacillus* flora of milk and milk products. J. of Applied Bacteriol. 77:256-263.

Cross, A. D. 1969. An introduction to practical infra-red spectroscopy. Page Bros. (Norwich)

Ltd., Great Britain.

Davies, F. L. 1975. Discussion of papers presented at symposium on bitty cream and related problems. J. Society Dairy Technol. 28:85-90.

Debuono, B. A., J. Brondrum, J. M. Kramer, R. J. Gilbert, and S. M. Opal. 1988. Plasmid, serotypic, and enterotoxin analysis of *Bacillus cereus* in an outbreak setting. J. Clin. Microbiol. 26:1571-1574.

Doi, R. H. and M. McGloughlin, 1992a. Biology of *Bacilli*: Applications to industry, pp.1-105. Butterworth-Heinemann, a division of Reed Publishing Inc., USA.

Doi, R. H. and M. McGloughlin, 1992b. Biology of *Bacilli*: Applications to industry, pp.352. Butterworth-Heinemann, a division of Reed Publishing Inc., USA.

Donovan, K. O. 1958. A selective medium for *Bacillus cereus* in milk. J. Appl. Bacteriol. 21:100-103.

Doyle, R. J., K. F. Keller, and J. W. Ezzell. 1985. *Bacillus*. In: E. H. Lennette, A. Balows, W. J. Hausler Jr., and H. J. Shadomy (eds.), Manual of Clinical Microbiology, 4th, pp.211-215. American Society for Microbiology.

Drobniewski, F. A. 1994. The safety of *Bacillus* species as insect vector control agents. J. Appl. Bacteriol. 76:101-109.

Drobniewski, F. 1993. *Bacillus cereus* and related species. Clin. Microbiol. Rev. 6 (4):324-38.

Dufrenne, J., P. Soentoro, S. Tatini, T. Day, and S. Notermans. 1994. Characteristics of *Bacillus cereus* related to safe food production. Int. J. Food Microbiol. 23:99-109.

Eerola, E and O. Lehtonen. 1988. Optimal data processing procedure for automatic bacterial

identification by gas-liquid chromatography of cellular fatty acids. J. Clin. Microbiol. 26:1745-1753.

Ezzell, J. W. and C. L. Wilhelmsen. 1993. *Bacillus anthracis*. In: C. L. Gyles and C. O. Thoen (eds.), Pathogenesis of Bacterial Infections in Animals, 2nd, pp.36-43. Iowa State University Press, Iowa.

Ferraro, J. R. and Basile L. 1978. Fourier transform infrared spectroscopy: Applications to chemical systems, vol. 1. Academic Press, New York.

Geoffrey, M. M. and David, F. W. 1989. Subgrouping of *Pseudomonas cepacia* by cellular fatty acid composition. J. Clin. Microbial. 27:2640-2646.

Gilbert, R. J. 1979. *Bacillus cereus* gastroenteritis. In: H. Riemann and F. L. Bryan (eds.), Food-borne Infections and Intoxications, 2nd, pp. 495-518. Academic Press, New York.

Gilliland, S. E., Michener, H. D., and Kraft, A. A. 1984. Psychrotrophic Microorganisms. In: M. Speck (ed.). Compendium of methods for the microbiological examination of foods (2ed.). pp. 135-138.

Gilmore, M. S., A. L. Cruz-Rodz, M. Leimeister-Waechter, J. Kreft and W.Goebel. 1989. A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. J. Bacteriol. 171:744-753.

Gordon, R. E., W. C. Haynes, and C. H. Pang. 1973. The genus *Bacillus*. In: A. I. Laskin and H. A. Lechevalier (eds.). Handbook of Microbiology, vol. 1, pp:71-88. CRC Press Cleveland, Ohio.

Gordon, R. E. 1989. The genus *Bacillus*. In W. M. O'Leary (ed.), Practical Handbook of Microbiology, pp.109-126. CRC Press, Boca Raton, Florida.

Granum, P. E. 1994. *Bacillus cereus* and its toxins. J. Appl. Bact. Symp. Supp. 76:61S-66S.

Granum, P. E., S. Brynestad, K. O'Sullivan, and H. Nissen. 1993. Enterotoxin from *Bacillus cereus*: production and biochemical characterization. Neth. Milk Dairy J. 47:63-70.

Griffiths, M. W. and J. D. Phillips. 1990. Incidence, source and some properties of psychrotrophic *Bacillus* found in raw and pasteurized milk. J. Soc. Dairy Technol. 43:62

Griffiths, M. W. 1992. *Bacillus cereus* in liquid milk and other dairy products. Liquid milk, Int. Dairy Food Bull. 275:36.

Griffiths, M. W. 1990. A research note: Toxin production by psychrotrophic *Bacillus spp.* present in milk. J. Food Protect. 53:790-792.

Giesbrecht, P., D. Naumann, H. Labischinski, and G. Barnickel. 1984. A new method for the rapid identification and differentiation of pathogenic microorganisms using Fourier transform infrared spectroscopy. In: K. O. Habermehl, V. Springer, and B. Heidelbery, Rapid Methods and Automation in Microbiology and Immunology, pp.198.

Grosskopf, J. C. and W. J. Harper. 1969. Isolation and identification of psychrotrophic sporeformers in milk. Milchwissenschaft. 29:467.

Haack, S. K., H. Garchow, D. A. Odelson, L. J. Forney, and M. J. Klug. 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. Appl. Environ. Microbiol. 60:2483-2493.

Harmon, S. M., D. A. Kautter, D. A. Golden, and E. J. Rhodehamel. 1992. *Bacillus cereus*.

Bacteriological Analytical Manual, 7th., pp.191-206. AOAC international.

Harmon, S. M.; D. A. Kautter and F. D. McClure. 1984. Comparison of selective plating media for enumeration of Bacillus cereus in foods. J. Food Protect. 47:65-67.

Holbrook, R. and J. M. Anderson. 1980. An improved selective and diagnostic medium for the isolation and enumeration of Bacillus cereus in foods. Can. J. Microbiol. 26:753-759.

Helm, D., H. Labischinski, G. Schallehn, and D. Naumann. 1991a. Classification and identification of bacteria by Fourier-Transform infrared spectroscopy. J. of General Microbiol. 137:69-79.

Helm, D., H. Labischinski, and D. Naumann. 1991b. Elaboration of a procedure for identification of bacteria using Fourier-Transform IR spectral libraries: a stepwise correlation approach. J. of Microbiol. Methods. 14:127-142.

Holt, C., Hirst, D., Sutherland, A. and MacDonald, F. (1995) Discrimination of species in the genus *Listeria* by Fourier transform infrared spectroscopy and canonical variate analysis. Applied and Environmental Microbiol. 61:377-378.

Hughes, S., B. Bartholomew, J. C. Hordy and J. M. Kramer. 1988. Potential application of a HEp-2 assaying in the investigation of *Bacillus cereus* emetic syndrome food poisoning. FEMS Microbiol. Lett. 52:7-12.

Huys, G., M. Vancanney, R. Coopman, P. Janssen, E. Falsen, M. Altwegg, and K. Kersters. 1994. Cellular fatty acid composition as a chemotaxonomic marker for the differentiation of phenospecies and hybridization groups in the genus *Aeromonas*. Internal. J. Syst. Bacteriol. 44:651-658.

International Dairy Federation. 1980. General code of hygienic practice for the dairy industry. Bull. No. 123.

Jackson, S. G., R. B. Goodbrand, R. Ahmed, S. Kasatiya. 1995. *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. Lett. Appl. Microbiol. 21:103-105.

Jellum, E. 1975. Past, present, and future application of gas chromatography. In: B. M. Mitruka, Gas chromatographic Applications in Microbiology and Medicine, pp.448-461. A Wiley Biomedical-Health Publication.

Johnson, K. M. 1984. *Bacillus cereus* foodborne illness: an update. J. Food Protect. 47:145-153.

Kaneda, T. 1977. Fatty acids of the genus *Bacillus*: an example of branched-chain preference. Bacteriol. Rev. 41:391-418.

Kaneda, T. 1968. Fatty acids in the genus *Bacillus*. II. Similarity in the fatty acid composition of *Bacillus thuringiensis*, *Bacillus anthracis*, and *Bacillus cereus*. J. Bacteriol. 95:2210-2216.

Kaneda, T. 1967. Fatty acids in the genus *Bacillus*. I. Iso- and anteiso-fatty acids as characteristic constituents of lipids of 10 species. J. Bacteriol. 93:894-903.

Kotilainen, P., P. Huovinen, and E. Eerola. 1991. Application of gas-liquid chromatographic analysis of cellular fatty acids for species identification and typing of coagulase-negative *staphylococci*. J. Clin. Microbiol. 29:315-322.

Kramer, J. M. and R. J. Gilbert. 1992. *Bacillus cereus* gastroenteritis. In A. T. Tu (ed.), Handbook of Natural Toxins, Food Poisoning, vol.7, . Marcel Dekker Inc., New York.

Kramer, J. M. and R. J. Gilbert. 1989. *Bacillus cereus* and other *Bacillus species*. In: M. P. Doyles (ed.), Foodborne Bacterial Pathogens, pp.21-70. Marcel Dekker, New York.

Kumosinski, T. F., and H. M. Farrell, Jr. 1993. Determination of the global secondary structure of proteins by Fourier transform infrared (FTIR) spectroscopy. Trends in Food Sci. & Technol. 6(4):169-175.

Labots, H., and G. Hup. 1964. *Bacillus cereus* in raw and pasteurized milk. II. The occurrence of slow and fast germinating spores in milk and their significance in the enumeration of *B. cereus* spores. Neth. Milk Dairy J. 18:167-176.

Lambert, M. A. and C. W. Moss. 1983. Comparison of the effects of acid and base hydrolyses on hydroxy and cyclopropane fatty acids in bacteria. J. Clin. Microbiol. 18:1370-1377.

Lancette, G. A. and S. M. Harmon. 1980. Enumeration and confirmation of *Bacillus cereus* in foods: collaborative study. J. Assoc. Off. Anal. Chem. 63:581-586.

Langeveld, L. P. M., and F. Cuperus. 1993. Aspects regarding the application of a predictive model for growth of *Bacillus cereus* in pasteurized milk. Int. Dairy Fed. Bull. 287:6-10.

Larsson, L. 1991. Gas chromatography and mass spectrometry. In: J. H. Jorgensen, Automation in Clinical Microbiology, pp.153-166. CRC Press Inc. Boca Raton, Florida.

Lawrence, D. , S. Heitefuss, and H. S. H. Seifert. 1991. Differentiation of *Bacillus anthracis* from *Bacillus cereus* by gas chromatographic whole-cell fatty acid analysis. J. Clin. Microbial. 29(7):1508-1512.

Lechevalier, H. and M. P. Lechevalier. 1988. Chemotaxonomic use lipids-an overview. In:

C. Ratledge and S. G. Wilkins (eds.), Microbial lipids, vol. 1, pp.869-902. Academic Press, London.

Marquez, V. O, G. S. Mittal and W. M. Griffiths. 1997. Destruction and inhibition of bacterial spores by high voltage pulsed electric field. J. Food Sci. 62(2): 39-401.

Mazurier, S and K. Wernars. 1992. Typing of *Listeria* strains by random amplification of polymorphic DNA. Res. Microbiol. 143: 499-505.

Mazurier, S., A. Van de Giessen, K. Heuvelman and K. Wernars. 1992. RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. Lett. Appl. Microbiol. 14:260-262.

Meer, R.R., Baker, J., Bodyfelt, F.W. and Griffiths, M.W. (1991) Psychrotrophic *Bacillus* spp. in fluid milk products: A review. J. Food Protect. 54 : 969-979.

Melling, J., B. J. Capel, P. C. B. Turnbull, and R. J. Gilbert. 1976. Identification of a novel enterotoxigenic activity associated with *Bacillus cereus*. J. Clin. Pathol. 29:938-940.

Melling, J., and B. J. Capel. 1978. Characteristics of *Bacillus cereus* emetic toxin. FEMS Microbial Lett. 4:133-135.

MIDI. 1993. Microbial identification system operating manual version 4. Microbial ID Inc., Newark, Delaware.

Mikami, T., T. Horikawa, T. Murakami, N. Sato, Y. Ono, T. Matsumoto, A. Yamakawa, S. Murayama, S. Katagiri, and M. Suzuki. 1995. Examination of toxin production from environmental *Bacillus cereus* and *Bacillus thuringiesis*. J. Pharmaceutical Society of Japan. 115(9):742-748.

Mosteller, T. M. and J. R. Bishop. 1993. Sanitizer efficacy against attached bacteria in a milk

biofilm. J. Food Protect. 56(1):34-41.

Miller, L. and T. Berger. 1985. Bacteria identification by gas chromatography of whole cell fatty acids. Hewlett-Packard application note 228-41. Hewlett-Packard Co., Avondale, Pa.

Mitruka, B. M. 1975. Historical development of gas chromatography. In: B. M. Mitruka, Gas Chromatographic Applications in Microbiology and Medicine, pp.4-19. A Wiley Biomedical-Health Publication.

Moss, C. W. 1995a. Chromatographic analysis for identification of microorganisms and diagnosis of infection: An introduction. In: K. -O. Habermehl (ed.), Rapid Methods and Automation in Microbiology and Immunology, pp.1. Springer-Verlag, Berlin Heidelberg New York Tokyo.

Moss, C. W. 1995b. Chromatographic analysis of bacterial cellular components. In: K. -O. Habermehl (ed.), Rapid Methods and Automation in Microbiology and Immunology, pp.233-238. Springer-Verlag, Berlin Heidelberg New York Tokyo.

Moss, C. W. 1985. Uses of gas-liquid chromatography and high-pressure liquid chromatography in clinical microbiology. In: E. H. Lennette, A. Balows, W. J. Hausler Jr., and H. J. Shadomy (eds.), Manual of Clinical Microbiology, 4th, pp.1029-1036. American Society for Microbiology.

Moss, C. W. 1981. Gas-liquid chromatography as an analytical tool in microbiology. J. Chromatogr. 203:337-347.

Moss, C. W., S. B. Dees, and G. O. Guerrant. 1980. Gas-liquid chromatography of bacterial fatty acids with a fused-silica capillary column. J. Clin. Microbiol. 12(1):127-130.

Mossel, D. A. A., Koopman, M. J. and Jongerius, E. 1967. Enumeration of *Bacillus cereus* in foods. Appl. Microbiol. 15:650-653.

Mukwaya, G. M. and D. F. Welch. 1989. Subgrouping of *Pseudomonas cepacia* by cellular fatty acid composition. J. Clin. Microbiol. 27(12):2640-2646.

Naumann, D., D. Helm, and C. Schultz. 1994. Characterization and identification of micro-organisms by FT-IR spectroscopy and FT-IR microscopy. In: F. G. Priest(ed.), Bacterial Diversity and Systematics, pp.67-85. Plenum press, New York and London.

Naumann, D., D. Helm, and H. Labischinski. 1991. Microbiological characterizations by FT-IR spectroscopy. Nature. 351:81-82.

Naumann, D., D. Helm, H. Labischinski, and P. Giesbrecht. 1990. The characterization of microorganisms by Fourier-Transform infrared spectroscopy (FT-IR). In: W. H. Nelson (ed.), Modern Techniques for Rapid Microbiological Analysis, pp.43-96. Weinheim & New York: VCH Verlay Chemie.

Naumann, H., V. Fijala, H. Labischinski, P. Giesbrecht. 1988. The rapid differentiation and identification of pathogenic bacteria using Fourier transform infrared spectroscopic and multivariate statistical analysis. J. of Molecular structure. 174:165-170.

Ninet, B., H. Traitler, J. M. Aeschlimann, and I. Horman. 1992. Quantitative analysis of cellular fatty acids (CFAs) composition of the seven species of *Listeria*. Syst. Appl. Microbiol. 15:76-81.

Nishikawa, Y., J. M. Kramer, M. Hanaoka, and A. Yasukawa. 1996. Evaluation of serotyping, biotyping, plasmid banding pattern analysis, and HEp-2 vacuolation factor assay in the epidemiological investigation of *Bacillus cereus* emetic-syndrome food

poisoning. Int. J. Food Microbiol. 31(1-3):149-159.

Norris, J. R. and J. Wolf. 1961. A study of the antigens of the aerobic spore-forming bacteria. J. Appl. Bacteriol. 24:42-56.

Overcast, W.W. and K. Atmaram. 1974. The role of *Bacillus cereus* in sweet curdling of fluid milk. J. Milk Food Technol. 37:233-236.

O'Donnell, A. G. 1985. Numerical analysis of chemotaxonomic data. In: M. Goodfellow, D. Jones, and F. G. Priest (eds.), pp.403-414. Computer-assisted bacterial systematics. Academic Press, Inc. (London), Ltd., London.

O'Leary, W. M. And S. G. Wilkinson. 1988. The family *Bacillaceae*. In: C. Ratledge and S. G. Wilkinson (eds.), Microbial Lipids, vol.1, pp.155-164. Academic Press, Harcourt Brace Jovanovich, Publishers.

Pfeifer, J. and H. G. Kessler. 1995. Heat resistance of *Bacillus cereus* spores located between seals and seal surfaces. J. Food Protect. 58(11):1206-1210.

Phillips, J. D. and M. W. Griffiths. 1986. Factors contributing to the seasonal variation of *Bacillus* spp. in pasteurized dairy products. J. Appl. Bacteriol. 61:275-285.

Priest, F. G., M. Goodfellow, and C. Todd. 1988. A numerical classification of the genus *Bacillus*. J. Gen. Microbiol. 134:1847-1882.

Raevuori, M., T. Kiutamo, and A. Niskanes. 1977. Comparative studies of *Bacillus cereus* strains isolated from various foods and food poisoning outbreaks. Acta Vet. Scand. 18:397-407.

Ratledge, C. and S. G. Wilkinson. 1988a. Fatty acids, related and derived lipids. In: C. Ratledge and S. G. Wilkinson, Microbial lipids, vol. 1, pp.22-53. Academic Press.

Ratledge, C. and S. G. Wilkinson. 1988b. An overview of microbial lipids. In: C. Ratledge and S. G. Wilkinson, Microbial lipids, vol. 1, pp.1-22. Academic Press.

Ray, D. E. 1991. Pesticides derived from plants and other organisms. In: W. J. Hayes and E. R. Laws (eds), Handbook of pesticide toxicology, vol. 2, pp. 585-627. San Diego: Academic Press.

Romesburg, H. C. 1984. Cluster analysis for researchers. Belmont, California: Lifetime Learning Publications.

Rönner, U. and U. Husmark. 1992. Adhesion of *Bacillus cereus* spores - a hazard to the dairy industry. In: L. F. Melo *et al.* (ed.), Biofilms, pp.403-406. Science and Technology, Kluwer Academic Publishers, The Netherlands.

Rusul, G. and N. H. Yaacob. 1995. Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA. Int. J. Food Microbiol. 25:131-139.

Sasser, M. 1990. Identification of bacterial by gas chromatography of cellular fatty acids. Technical note 101. Microbial ID, Inc., Newark, Del.

Schraft, H., M. Steele, B. McNab, J. Odumeru, and M. W. Griffiths. 1996. Epidemiological typing of *Bacillus* spp. isolated from food. Appl. Environ. Microbiol. 62(11):4229-4232.

Seki, T., C. Chung, H. Mikami, and Y. Oshima. 1978. Deoxyribonucleic acid homology and taxonomy of the genus *Bacillus*. Int. J. Syst. Bacteriol. 28:182-189.

Shinagawa, K. 1993. Serology and characterization of toxigenic *Bacillus cereus*. Neth. Milk Dairy J. 47:89-103.

Shinagawa, K., S. Ueno, H. Konuma, N. Matsusaka, S. Suggi. 1991. Purification and characterization of the vascular permeability factor produced by *Bacillus cereus*. J. Vet. Med. Sci. 53: 281-286.

Shinagawa, K., Y. Ueno, D. L. Hu, S. Ueda, and S. Suggi. 1996. Mouse lethal activity of a Hep-2 vacuolation factor, cereulide, produced by *Bacillus cereus* isolated from vomiting-type food poisoning. J. Vet. Med. Sci. 58(10):1027-1029.

Shinagawa, K. and M. Suzuki. 1994. An improved method for detecting cytostatic toxin (emetic toxin) of *Bacillus cereus* and its application to food samples. FEMS Microbiol. Lett. 119:53-58.

Sliman, R., S. Rehm, and D. M. Shlaes. 1987. Serious infection caused by *Bacillus cereus*. Medicine. 66:218-223.

Smith, N. R., R. E. Gordon, and F. E. Clark. 1952. Aerobic spore-forming bacteria. US Dept. Agric. Monograph No. 16. The United States Dept. of Agriculture, Washington, D. C.

Sneath, P. H. A. 1986. Endospore-forming gram-positive rod and cocci. In: P. H. A. Sneath, N. S. Mair, N. E. Sharpe and J. G. Holt (eds), Bergey's Manual of Systematic Bacteriology, vol. 2, pp.1104-1139. Williams and Wilkins, Baltimore

Somerville, H. J. and M. L. Jones. 1972. DNA competition studies within the *Bacillus cereus* group of *Bacilli*. J. General. Microbiol. 73:257-265.

Stadhouders, J., G. Hup, L. P. M. Langeveld. 1980. Some observations on the germination, heat resistance and outgrowth of fast-germinating and slow-germinating spores of *Bacillus cereus* in pasteurized milk. Neth. Milk Dairy J. 34:215-228.

Stager, C. E. and J. R. Davis. 1992. Automated systems for identification of microorganisms.

Clin. Microbiol. Rev. 5(3):302-327.

Stead, D. E., J. E. Sellwood, J. Wilson, and I. Viney. 1992. Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. J. Appl. Bacteriol. 72:315-321.

Stoakes, L., M. A. John, R. Lannigan, B. C. Schieven, M. Ramos, D. Harley, and Z. Hussain. 1994. Gas-liquid chromatography of cellular fatty acids for identification of *Staphylococci*. J. Clin. Microbiol. 32:1908-1910.

Stone, M. J. and A. Rowlands. 1952. "Broken" or "bitty" cream in raw and pasteurized milk. J. Dairy Res. 19:51-62.

Szabo, R. A., E. C. D. Todd, and M. K. Rayman. 1984. Twenty-four hour isolation and confirmation of *Bacillus cereus* in foods. J. Food Protect. 47:856-860.

Te Giffel, M. C., R. R. Beumer, M. H. Bonestroo, and F. M. Rombouts. 1996. Incidence and characterization of *Bacillus cereus* in two dairy processing plants. Neth. Milk Dairy J. 50:479-492.

Ternsträm, A. D., M. Lindberg, and G. Molin. 1993. Classification of the spoilage flora of raw and pasteurized bovine milk, with special reference to *Pseudomonas* and *Bacillus*. J. Appl. Bacteriol. 75:25-34.

Thompson, N. E., M. J. Ketterhagen, M. S. Bergdoll, E. J. Schantz. 1984. Isolation and some of an enterotoxin produced by *Bacillus cereus*. Infect. Immun.. 43:887-894.

Tornabene, T. G. 1985. Lipid analysis and the relationship to chemotaxonomy. In: J. Norris (ed.), Methods in Microbiology, vol. 18, pp.209-234. Academic Press, London.

Turnbull, P. C. B., J. Kramer, and J. Melling. 1990. *Bacillus*. In: M. T. Parker and B. I.

Duerden (eds.), Topley and Wilson's principles of bacteriology, virology and immunity, 8th. ed., vol. 2, pp.188-210. Edward Arnold, London.

Turnbull, P. C. B., and J. M. Kramer. 1991. *Bacillus*. In: A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (eds.), Manual of Clinical Microbiolgy, 5th., pp.296-303. American Society for microbiology. Washington, D. C.

Väisänen, O. M., N. J. Mwaisumo, and M. S. Salkionja-Saonen. 1991. Differentiation of dairy strains of the *Bacillus cereus* group by phage typing, minimum growth temperature, and fatty acid analysis. J. Appl. Bacteriol. 70:315-324.

Van Netten, P., A. Van de Moosdijk, P. Van Hoensel, D. A. A.Mossel, I. Perales. 1990. Chrotrophic strains of *Bacillus cereus* producing enterotoxin. J. Appl. Bacteriol. 69:73-79.

Van Netten, P., and J. M. Kramer. 1992. Media for the detection and enumeration of *Bacillus cereus* in foods: a review. Int. J. Food Microbiol. 17:85-99.

Van de Voort, F. R., and A. A. Ismail. 1991. Proximate analysis of foods by mid-FTIR spectroscopy. Trends in Food Sci. & Technol. Jan. 13-17.

Waites, W. M., S. E. Harding, D. R. Fowler, S. H. Jones, D. Shaw and M. Martin. 1988. The destruction of spores of Bacillus subtilis by the combined effects of hydrogen peroxide and ultraviolet light. Lett. Appl. Microbiol. 7:139-140.

Wauthoz, P., M. E. Lioui, and J. Decallonne. 1995. Gas chromatographic analysis of cellular fatty acids in the identification of foodborne bacteria. J. Food Protect. 58(11):1234-1240.

Welch, D. F. 1991. Applications of cellular fatty acid analysis. Clin. Microbiol. Rev. 4 (4):422-438.

Wirtanen, G., U. Husmark, and T. Mattila-Sandholm. 1996. Microbial evaluation of the biotransfer potential from surfaces with *Bacillus* biofilms after rinsing and cleaning procedures in closed food-processing systems. J. Food Protect. 59(7):727-733.

Wolcott, M. J. 1991. DNA-based rapid methods for the detection of foodborne pathogens. J. Food Protect. 54(5):387-401.

Wong, H. C., Chen, Y. L. and Chen, C. L. F. 1988a. Growth, germination and toxigenic activity of *Bacillus cereus* in milk products. J. Food Protect. 51(9):707-710.

Wong, H. C., M. H. Chang, and J. Y. Fan. 1988b. Incidence and characterization of *Bacillus cereus* isolated contaminating dairy products. Appl. Environ. Microbiol. 45(3):699-702.

Zahner, V., H. Momen, C. A. Salles, and L. Rabinovitch. 1989. A comparative study of enzyme variation in *Bacillus cereus* and *Bacillus thuringiensis*. J. Appl. Bacteriol. 67:275-282.

Zeroual, W., C. Choisy, S. M. Doglia, H. Bobichon, J. F. Angiboust, and M. Manfait. 1994. Monitoring of bacterial growth and structural analysis as probed by FT-IR spectroscopy. Biochimica et Biophysica Acta 1222:171-178.

APPENDICES

Polymyxin B-egg-Yolk- Mannitol bromo thymol blue agar (PEMBA): *B. cereus* selective agar base (20.5g; Oxoid) was suspended in 475 ml distilled water in a 1000 ml flask and heated to dissolve the agar completely. The agar solution was then autoclaved at 121°C for 20 min and cooled to until 50°C in the water bath. One vial polymyxin B (SR99; reconstituted with 2 ml sterilized water) and 25 ml egg yolk emulsion (SR45) were aseptically added and mixed well with the media. The media was poured into sterile petri dishes.

Broth heart infusion (BHI) agar/ broth: BHI agar and BHI broth were prepared following the instructions of the manufacturer and autoclaved at 121°C for 20 min. The BHI agar was aseptically dispensed into petri dishes and BHI broth was stored in bottle for use.

Butterfield's Phosphate-Buffered Dilution Water (Harmon, *et al.*, 1992): Thirty-four gram KH_2PO_4 was dissolved in 500 ml distilled water and the pH was adjusted to 7.2 with 1N NaOH. The final volume of the solution was adjusted to 1 litre with distilled water. The solution was autoclaved at 121°C for 20 min and stored it in the refrigerator for use.

Medium for frozen strain stock (per 100 ml) (Doi and McGloughlin, 1992b): One gram of peptone, 5 ml 1M Mg SO_4 and 10 ml of glycerol were dissolved in 50 ml distilled water in a 200 bottle. The final volume of the solution was adjusted to 100 ml and autoclaved at

121°C for 20 min.

Hydrogen peroxide solution (3%): Three ml of 30% H_2O_2 solution was made of 30 ml with distilled water in a 100 ml bottle.

Gram staining: One colony was suspended in a drop of 0.85% NaCl on a glass slide by adding a drop of 0.85% sodium chloride and the colony was fixed over a flame. The slide was immersed in crystal voilet for 2 min, and washed in tap water. After drying, the slide was immersed in iodine (mordant) for 3 min, rinsed and dried again. The treated slide was then washed in a solution containing alcohol and acetone (3:1), followed by wash in tap water then dried. Finally, the slide was immersed in safranine solution for one minute, washed in tap water and dried. The slide was examined under a microscope

Trypticase soy agar: Trypticase soy agar (TSA) was selected to be a standard medium for aerobes growth in MIS analysis. Thirty grams of Trypticase Soy Broth (BBL # 4311771) and 15 grams granulated agar (BBL # 4311850) were dissolved in 1 litre distilled water and prepared in a automatic media preparation machine. The medium were autoclaved for 15 min at 121°C under 15 psi, then cooled down to 55°C. Around 20 ml of medium solution was aseptically dispensed into sterile petri dishes. When the agars was solidified at room temperature and stood for 24 hrs prior to packing in sterile sleeves and stored at cool room at 4°C for use. The storage duration of the media for MIDI use was not longer than 30 days.

Preparation of reagents: During the reagent preparation and fatty acids extraction, the

culture tubes, screw caps, reagent bottles etc contacted with reagents were only made of Teflon or glass materials.

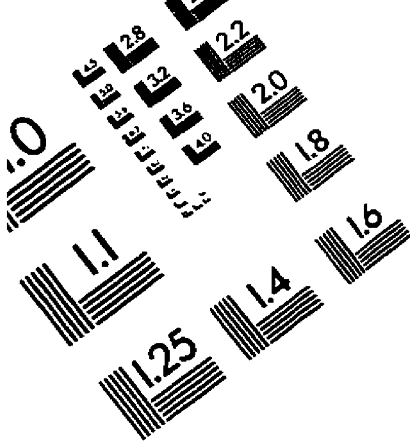
Reagent 1 (saponification reagent): Forty-five grams of NaOH pellets (Certified CAS, Fisher) was added to 150 ml deionized distilled water (Millique) and 150 ml methanol (Reagent grade, Caledon) was also added while the solution was stirring until the sodium hydroxide pellets were dissolved completely.

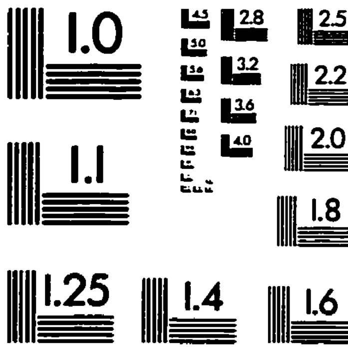
Reagent 2 (Methylation reagent): Two hundred seventy-five ml methanol (Reagent grade, Caledon) was added to 325 ml 6.00N hydrochloric acid (Baker) and mixed well.

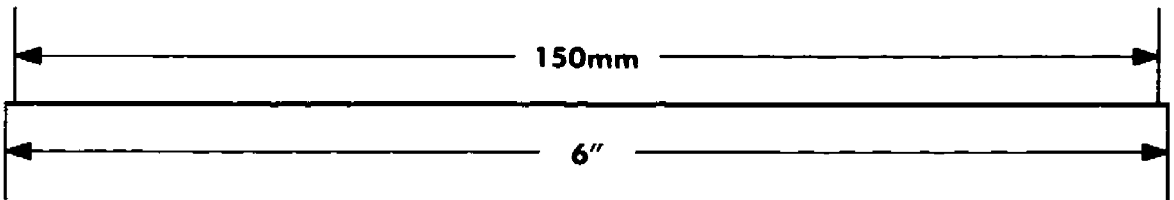
Reagent 3 (Extraction solvent (hexane/MTBE)): Two hundred ml of methyl-tert Butyl Ether(MTBE) (HPLC grade, Fisher) was added to 200 ml hexane while the solution was stirring until mixed well.

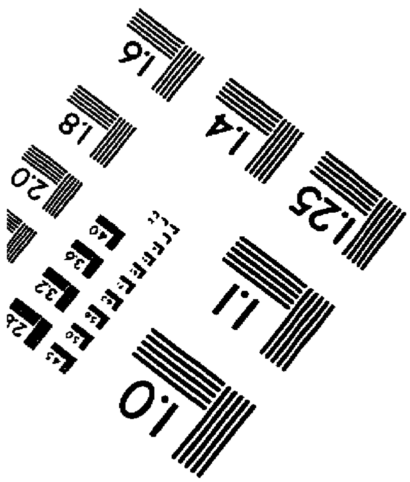
Reagent 4 (Base wash): Ten point eight grams sodium hydroxide (certified ASC, Fisher) pellets and 15 grams sodium chloride (Anachemia) were dissolved completely in 900 ml deionized distilled water (Millique).

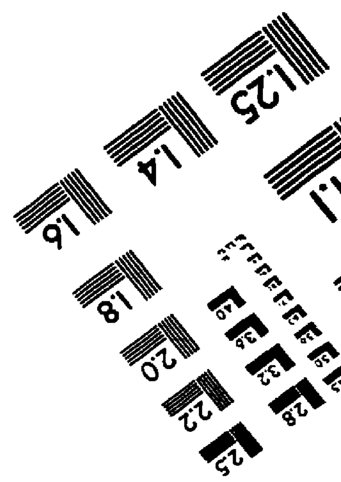
TEST TARGET (QA-3)











APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved