Isolation, identification and characterization of *Bacillus cereus* from the dairy environment

Promotor: dr. ir. F.M. Rombouts
hoogleraar in de levensmiddelenhygiëne en -microbiologie

Copromotor: dr. R.R. Beumer
universitair docent in de levensmiddelenmicrobiologie

Meike te Giffel

Isolation, identification and characterization of *Bacillus cereus* from the dairy environment

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen
dr. C.M. Karssen,
in het openbaar te verdedigen
op 20 mei 1997
des namiddags te vier uur in de Aula.

The research described in this thesis was financially supported by NESTEC (Lausanne, Switzerland), the Dutch Ministry of Agriculture, Nature Conservation and Fisheries and the AAIR Concerted Action PL 920630.

Meike te Giffel

Isolation, identification and characterization of *Bacillus cereus* from the dairy environment
Thesis Landbouwuniversiteit Wageningen. -160 p -With ref.- With summary in Dutch
ISBN 90-5485-694-7
Subject headings: *Bacillus cereus*/occurrence/identification/characterization/dairy.

Cover design: Van den Berg's Drukkerij, Maarn

STELLINGEN

1

Het aantal bacteriesporen in een monster wordt vaak onderschat doordat de tijd tussen het maken van een primaire verdunning en de hitte-activatie stap te lang is.

Dit proefschrift
Anonymous (1996) ISO Standard Procedure 7218; Microbiology- General guidance for microbiological examinations, International Organization for Standardization, Switzerland.

2

Bij het bevestigen van verdachte *Bacillus cereus* isolaten volgens de ISO methode blijft altijd de vraag: To *Be cereus* or not to *Be cereus* ?

Dit proefschrift

3

Het gebruik van *Bacillus thuringiensis* als insecticide kan leiden tot voedselvergiftigingen.

Dit proefschrift
Damgaard PH, Larsen HD, Hansen BM, Bresciani J and K Jørgensen (1996) Enterotoxin-producing strains of *Bacillus thuringiensis* isolated from food. *Letters in Applied Microbiology* **23** 146-150.

4

De speurtocht naar sporevormende micro-organismen is niet moeilijk; ze laten overal hun sporen achter.

5

Om verspreiding van resistente micro-organismen in het milieu te voorkomen is het gebruik van antibiotica in microbiologische media af te raden.

6

De feitelijke houdbaarheid van veel (verse) levensmiddelen wordt aanzienlijk bekort door de bewaaromstandigheden bij de consument.

7

Hygiëne codes, HACCP en risk assessment hebben pas dan effect als de consument ook hiernaar handelt.

8

A couple of months in the laboratory can frequently save a couple of hours in the library.

Westheimer's Discovery

9

In plaats van rehydratatiesportdrankjes kan een sporter net zo goed water drinken.

Koops H (1995) Een oppepper in de strijd. *Chemisch magazine* 117-120.

10

Een kwaliteitsgarantie garandeert niets.

11

Een goed uitgevoerd experiment kan niet mislukken.

12

Het toenemend aantal verkeersrotondes is tekenend voor de toenemende individualisering in de maatschappij.

13

Weinig woorden zeggen vaak meer dan veel.

14

Er blijven altijd fouten onopgemerkt totdat een proefschrift gedrukt is.

Stellingen behorende bij het proefschrift
"Isolation, identification and characterization of *Bacillus cereus*
from the dairy environment"

Meike te Giffel
Wageningen, 20 mei 1997

VOORWOORD

Waar een afstudeervak microbiologie; groei van *Listeria* op rauw vlees en vleeswaren, een stage bij NESTEC in Zwitserland; gedrag van *Listeria* op gerookte zalm en een jaar als toegevoegd onderzoeker bij Levensmiddelenmicrobiologie; de rol van proline, betaine en carnitine in de osmoregulatie van *Listeria* al niet toe kunnen leiden..... een proefschrift over *Bacillus*.

Ik heb de afgelopen vier jaar met veel plezier gewerkt aan *Bacillus cereus*, de resultaten van dit project zijn beschreven in dit proefschrift. Ik heb al het werk natuurlijk niet alleen gedaan en ik wil graag een aantal mensen bedanken voor hun bijdrage.

Allereerst mijn co-promotor, Rijkelt Beumer, en mijn promotor, Frans Rombouts, voor de goede samenwerking, de grote vrijheid die ik gekregen heb bij de opzet en uitvoering van het onderzoek en de snelle correctie van concept publicaties.

Alle andere medewerkers van de sectie, voor de prettige sfeer waarin ik met jullie heb mogen samenwerken.

Alle studenten die in het kader van een afstudeervak aan het *B. cereus* project hebben meegewerkt; Annelies Postma, Sandra Tap, Heidi van Gennip, Hermien Tolboom, Sandra van Kippersluis, Frank Oudshoorn, Roelina Dijk, Niek Oosterkamp, Willem van Dam, Jeroen Pol, Isabel Ratão, Carolien van Bloemendal, Suzanne Leijendekkers, Esther Poelwijk, Saskia Schulten, Marloes de Koning en Monique Brugmans voor hun enthousiasme en inzet.

De medewerkers van het Praktijkonderzoek Rundvee, Schapen en Paarden, Coberco en het NIZO voor de hulp bij het boerderijonderzoek, fabrieksonderzoek en het sequencen, ontwikkelen van een DNA probe en de buizenpasteurexperimenten.

Per Einar Granum and all the others of the Department of Pharmacology, Microbiology and Food Hygiene of the Norwegian College of Veterinary Medicine in Oslo for the opportunity to work on enterotoxin production of *B. cereus* within the framework of the AAIR Concerted Action PL920630 and the kind hospitality. It has been a valuable experience to work in your lab.

Iedereen die niet bij naam genoemd is, voor o.a gezellige carpoolritjes, lunches, klaverjasavonden en hardlooptrainingen.

Wim, ik weet wel dat leven meer is dan werken alleen, maar ik vergeet het wel eens. Bedankt voor je steun bij het afronden van mijn proefschrift, bedankt voor alles !!

Meike

ABSTRACT

In this thesis the occurrence of *Bacillus cereus* in the milk production and processing environment was investigated. Isolates were identified biochemically and by DNA probes based on the variable regions of 16S rRNA. Further characterization was carried out using biochemical and molecular typing, in order to determine the major contamination sources of milk. Furthermore, properties in relation to carbohydrate utilization, growth at low temperatures and enterotoxin production were examined.

B. cereus is important as food spoilage organism. In the present study the micro-organism was isolated from food ingredients such as yeast, flour, cacoa, herbs and spices. *B. cereus* was also found in a wide variety of processed food products including bakery products, Chinese meals, pasta products, chocolate and meat products. In pasteurized milk and dairy products, *B. cereus* was frequently present and it is well-known that it can be responsible for spoilage when post-heat-treatment contamination is absent.

To enumerate spores in a sample, the most common procedure is to carry out a heat-activation treatment of 10 min at 80 °C, followed by plating on a (selective) agar medium. To prevent germination of spores during sample preparation, the time between the preparation of the primary dilution and heat-activation step should be less than 10 min and the temperature during the analysis should be as low as possible (e.g. by keeping dilutions in melting ice). After isolation, presumptive *B. cereus* are confirmed by biochemical tests, however, this may lead to incorrect identification. Several isolates, involved in food poisoning incidents, were shown to be *B. thuringiensis*, by sequencing part of the 16S rRNA. These results suggest that use of *B. thuringiensis* as insecticide may lead to foodborne infection or intoxication. To improve the confirmation procedure, we developed a specific and sensitive method, using DNA probes based on variable regions of the 16S rRNA, to differentiate between *B. cereus* and *B. thuringiensis*.

On farms, *B. cereus* is introduced into raw milk by contamination of the udder with faeces, soil and, in winter, used bedding. In the dairy processing plants, additional contamination takes place via the equipment. Biochemical and growth characterization and molecular typing of isolates confirmed this and also showed that selection of strains occurs in the milk production and processing chain. Cleaning and disinfection will not eliminate all *B. cereus* in milking installations or heat exchangers, particularly not those adhering to surfaces of the equipment.

Although only a few cases of milkborne infection and intoxication by *B. cereus* have been reported, most isolates were able to produce enterotoxin as determined by immunoblotting, cytotoxicity tests and PCR. However, if pasteurized milk is stored at 7 °C and consumed within the "best before" date, this will not cause any problems for healthy adults.

CONTENTS

	Voorwoord	v
	Abstract	vii
CHAPTER		
1	General introduction	1
2	Germination of bacterial spores during sample preparation	21
3	Discrimination between *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based on variable regions of 16S rRNA	31
4	Incidence of *Bacillus cereus* and *Bacillus subtilis* in foods in the Netherlands	41
5	Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands	51
6	Sporicidal effect of disinfectants on *Bacillus cereus* isolated from the milk processing environment	67
7	Incidence and characterization of *Bacillus cereus* in two dairy processing plants	77
8	The role of heat exchangers in the contamination of milk with *Bacillus cereus* in dairy processing plants	97
9	Isolation and characterization of *Bacillus cereus* from pasteurized milk in household refrigerators in the Netherlands	111
10	General discussion	127
	Summary	139
	Samenvatting	143
	Curriculum vitae	147
	List of publications	148

CHAPTER 1

GENERAL INTRODUCTION

HISTORY

Bacillus cereus was first isolated and described by Frankland and Frankland in 1887 as a common inhabitant of the soil (Hutchinson and Taplin 1978, Kramer and Gilbert 1989).

From the beginning of this century, cases of food poisoning caused by *B. cereus* or *B. cereus*-like organisms have been reported in literature. One of the earliest recorded episodes was described in 1906 by Lubenau; a hospital outbreak involving 300 patients and staff who developed symptoms of acute gastroenteritis shortly after eating dinner. It was shown that the meat balls were highly contaminated with an aerobic spore-forming bacterium, most probably a *B. cereus*. In 1913, Seitz reported the isolation of large numbers of *B. cereus*-like micro-organisms from the faeces of a young man suffering from severe enteritis and diarrhoea. Brekenfeld (1926) implicated aerobic sporogenic bacilli as the cause of two separate outbreaks involving vanilla sauce and a jellied meat dish (Kramer and Gilbert 1989).

Early reports of food poisoning associated with *Bacillus* species were somewhat sketchy; quantitative bacteriology was rarely undertaken either on suspect foods or clinical specimens. Moreover, the disorder in *Bacillus* taxonomy led to imprecise classification of isolates. This contributed to the slow recognition of pathogenicity of *B. cereus*. Hauge succeeded in establishing the organism as a recognized food poisoning organism, following publication of four large outbreaks involving 600 people that occured in Norway in 1947-1949. He found that the vanilla sauce consumed with chocolate pudding as dessert, contained high levels (10^7 - 10^9 per ml) of *B. cereus*; nevertheless it was not much changed in odour, taste or consistency. Spores of *B. cereus* (up to 10^4 per g) were found in corn starch, one of the constituents of the vanilla sauce powder. In order to prove the toxicity of the *B. cereus* isolated from the outbreak, a vanilla sauce was prepared from a dry sterilized sauce powder and inoculated with about 10^4 cells per ml. After incubation for 24 h at room temperature, Hauge consumed 200 ml of the product containing approximately 10^9 *B. cereus* ml^{-1}. After 13 h of consumption, the symptoms started with severe abdominal pains, diarrhoea and rectal tenusmus, lasting more or less continuously for 8 h (Hauge 1955).

Since 1950, increasing awareness and recognition of *B. cereus*-associated illness has resulted in a substantial increase in the number of reports of this type of food poisoning.

The importance of *B. cereus* in the dairy industry has been recognized since 1938, when the occurrence of bitty cream was first recorded. It was demonstrated that this defect could be attributed to *B. cereus* (Stone and Rowlands 1952). In addition, spore-formers, including *B. cereus*, have been associated with sweet curdling of pasteurized milk stored at refrigeration temperatures by Hammer and Babel (1957) and Jayne-Williams and Franklin (1960) (Overcast and Atmaram 1974).

THE GENUS *BACILLUS*

The genus *Bacillus* is the largest of the *Bacillaceae*, encompassing more than 60 species (Priest 1993). Members of the genus *Bacillus* are aerobic or facultatively anaerobic, gram-positive spore-forming rods (Claus and Berkeley, 1986). However, a great genetic diversity exists in this genus. That is why a subdivision of the genus into six smaller genera, based on studies of phenotypic similarities and range of genome base composition (GC content), is suggested (Priest 1981, Priest 1993).

The *B. cereus* group comprises the closely related species *B. cereus*, *B. anthracis*, *B. mycoides* and *B. thuringiensis*. All four species belong to the larger *B. subtilis* group (Priest 1993). *B. anthracis* has been recognized as non-haemolytic, non-motile, penicillin- and gamma phage-sensitive, encapsulated bacilli causing anthrax in man and animals (Claus and Berkeley 1986). *B. mycoides* can be differentiated from *B. cereus* by its lack of motility and rhizoid colonies. *B. thuringiensis* can be distinguished from *B. cereus* by its ability to produce a crystal protein toxin, toxic to various insects, inside the cell during sporulation (Priest 1993).

In spite of extensive similarities (Priest 1981, Ash *et al.* 1991), the four species are still considered to be distinct species (Claus and Berkeley 1986, Priest 1993, Nakamura and Jackson 1995).

THE LIFE CYCLE OF ENDOSPORE-FORMING BACTERIA

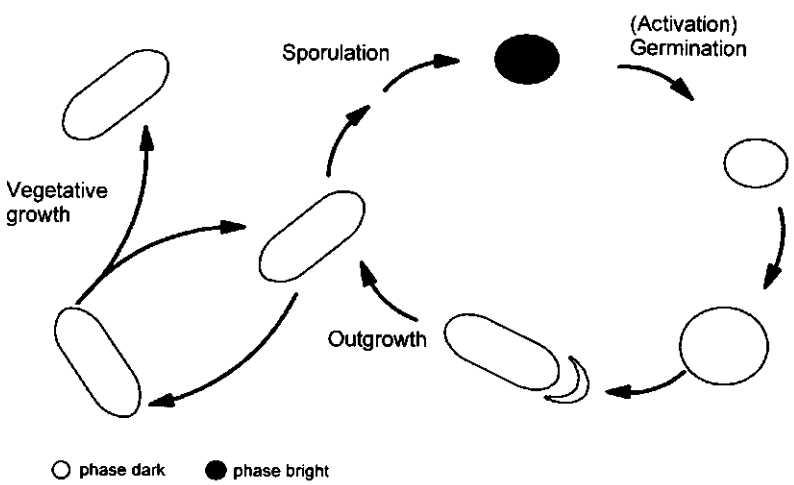


Figure 1.1 The life cycle of endospore-forming bacteria (Cook and Pierson 1983)

The relationship of vegetative growth, spore formation, germination and outgrowth of the spore to multiplication of the vegetative cells, the spore life cycle, is represented in Figure 1.1. Vegetative cells of spore-forming micro-organisms will grow and divide until the organism becomes nutritionally deprived, then the cells may go on to sporulate. Sporulation is an irreversible process, affected by factors as growth temperature, pH of the medium, aeration, presence of minerals (especially Mn^{2+} and Ca^{2+}), the presence of certain carbon and nitrogen compounds, and their concentrations (Claus and Berkeley 1986, Bergère 1993). The morphology of an endospore is given in Figure 1.2.

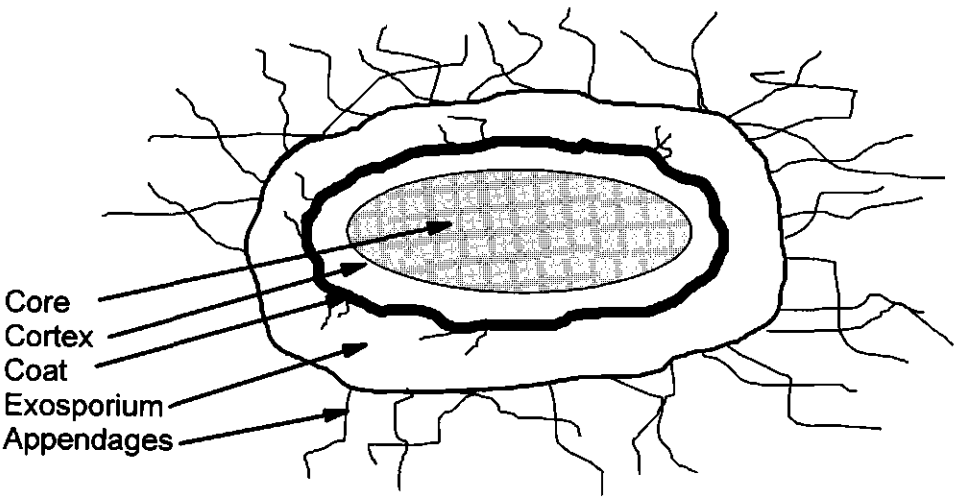


Figure 1.2 The different layers and structures of *Bacillus cereus* spores (Andersson *et al.* 1995)

Endospores are characterized by their extreme dormancy as shown by their dehydration, lack of metabolism (most of the high-energy compounds found in growing cells such as ATP and NADH are not present (Setlow 1994)), and resistance to many treatments including heat, UV light and chemicals (Foster 1994). The coat is important in determining resistance by limiting penetration to the underlying cortex and protoplast. The spore cortex is responsible for maintainance of dormancy and thus important for the survival of the organism in this quiescent state (Foster 1994). There is increasing evidence that the cortex also plays a vital role in the resistance of spores to heat and chemical agents by limiting diffusion into the protoplast (Bloomfield and Arthur 1994). *B. cereus* spores are covered with long appendages that promote adhesion to various materials (Andersson *et al.* 1995).

Germination is characterized by the change from a phase-bright dormant endospore to a phase-dark germinated spore due to the release of spore components and the uptake of

water. In the process of germination four phases can be recognized. Often spores are first activated, followed by interaction of specific compounds with the spore, triggering. Triggering irreversibly commits the spore to lose its dormant properties. As a result a sequence of degradative reactions occurs, metabolic reactions take place and finally the germinated spore develops into a new vegetative cell. This outgrowth comprises synthesis of macromolecules (RNA, proteins and DNA) (Cook and Pierson 1983, Claus and Berkeley 1986).

Activation is not really considered as a stage of germination because it is not always required and it does not lead to an easily measurable transformation. Moreover, it can be reversible. However, the rate of germination and the proportion of germinated spores increases upon activation. Activation can be caused by heat treatment, ageing of spores, γ-radiation, reducing agents such as thioglycolate or mercaptoethanol or oxidizing agents as perborate. The efficiency of activation depends on environmental conditions such as temperature, pH, water activity and composition of the medium (Bergère 1993). Subsequently, various chemical and physical stimuli can initiate germination by triggering. In *B. cereus* the usual germinants are amino acids (L-alanine), ribosides (inosine, adenosine), sugars (glucose, fructose), lactate, bicarbonate and enzymes (lysozyme). However, germination is often achieved most rapidly and completely by using combinations of germinants or complex culture media (Claus and Berkeley 1986, Gould 1992). Physical initiators of germination such as mechanical germination by shaking with glass beads, by abrasion or by high pressure (4-5 kbar) have also been described (Bergère 1993, Gould 1992). Germination is strain dependent and influenced by conditions in which spores were prepared, stored and activated. Furthermore, germination is influenced by environmental factors (temperature, pH, water activity, ionic strength, presence of inhibitors) (Stewart 1975, Bergère 1993).

Further outgrowth to a vegetative cell is only possible in a medium that supports cell growth. Nutritional, chemical and physical requirements for outgrowth are the same as for growth and multiplication of vegetative cells (Cook and Pierson 1983). If germinated spores are held under conditions that do not allow growth, they will become inactivated.

Between various *B. cereus* spores clear differences were detected in rapidity and extent of germination. It was shown that two types of spores exist, i.e. slow-germinating and fast-germinating spores. Slow-germinating spores do not germinate in milk within 24 h at 20 °C after a HTST pasteurization of the milk for 10 s at 72 °C, whereas fast-germinating spores do (Labots and Hup 1964).

CHARACTERISTICS OF *B. CEREUS*

Microscopically, cells of *B. cereus* are seen as large rods that are motile by means of peritrichous flagella. There is a wide strain variation in properties of *B. cereus*. Germination of endospores in laboratory media is observed in a range of -1 to 59 °C, with an optimum at 30 °C (Goepfert *et al.* 1972).

Growth of vegetative cells occurs within the temperature range of 10-50 °C, with an optimum between 28 and 35 °C (Johnson 1984). However, psychrotrophic variants of *B. cereus*, capable of growth at temperatures as low as 5 °C have been identified (Christiansson *et al.* 1989, Väisänen *et al.* 1990). Growth is possible under aerobic or facultative anaerobic conditions (Claus and Berkeley 1986). *B. cereus* can multiply in a pH range from 4.9 to 9.3 (Goepfert *et al.* 1972, Johnson 1984). The minimum water activity for growth is 0.91-0.95 (Raevuori and Genigeorgis 1975, Bryan *et al.* 1981, Johnson 1984). The organism is able to metabolize glucose, fructose and trehalose but no pentoses nor many of the sugar alcohols. Certain strains utilize sucrose, salicin, maltose, mannose, glycerol, m-inositol and lactose. Most actively hydrolyze starch, casein and gelatin (Goepfert *et al.* 1972, Kramer and Gilbert 1989).

Factors that have been shown to have an inhibitory effect on growth of *B. cereus* include high levels of spoilage micro-organisms, nisin, sorbic acid and potassium sorbate. Effective antibiotics against *B. cereus* are aureomycin, dihydrostreptomycin, terramycin, bacitracin, oxytetracycline, chloramphenicol and gentamycin (Johnson 1984).

The heat resistance of *B. cereus* spores is a factor of primary concern to the food and pharmaceutical industries. Vegetative cells are not very heat-resistant and are destroyed at time-temperature combinations which inactivate non spore-forming micro-organisms. Endospores of *B. cereus* show considerable variation in thermal resistance. D-values determined in skim-milk at 90 °C were 4.4 - 6.6 min (Shehata and Collins 1972). D_{100} values of 2.7 to 3.1 min and 5 min have been reported for spores in skim-milk (Mikolajcik 1970) and in low-acid foods (pH > 4.5) (Ingram 1969), respectively. Using aqueous spore suspensions or phosphate-buffered suspensions z-values ranging from 6.1 to 9.2 °C were obtained (Kramer and Gilbert 1989). It was demonstrated that lipid material had a protective effect on the thermal resistance; in milk the D_{95} is 0.5 - 3.5 min (Shehata and Collins 1972, Bergère and Cerf 1993). In products containing high levels of fat, such as cream, the D-value is much higher (Meer *et al.* 1991, Champagne *et al.* 1994). The occurrence of very heat resistant *B. cereus* spores (in underprocessed canned soups) that had D_{95} values of more than 250 min has also been reported (Kramer and Gilbert 1989).

ISOLATION AND IDENTIFICATION OF *B. CEREUS*

Procedures for the isolation, detection and enumeration of *B. cereus* almost all involve direct agar plating techniques (Van Netten and Kramer 1992). To obtain an indication of the proportion of bacteria present as spores in a sample, is to conduct a viable count of a sample in which the vegetative cells are killed by heat or some other treatment (e.g. air drying, solvent treatment, selective germination followed by pasteurization for specific types of endospore-formers). The most common procedure for *B. cereus* spores is the application of a heating step of 10-15 min at 80 °C. By this heat treatment, vegetative cells will be killed, but it will not harm most endospores (Priest and Grigorova 1990).

Blood agar has been used for isolation of *B. cereus* from foodborne illness outbreaks, but a selective and differential medium is required for detection of low levels of the organism in the presence of other bacteria. Various plating media have been developed based on characteristic features of the organism such as hemolysin production, lecithinase activity, fermentation properties or morphological features. Polymyxin B sulfate is normally incorporated as selective agent to inhibit competitive micro-organisms (Donovan 1958, Mossel *et al.* 1967, Kim and Goepfert 1971a, Holbrook and Anderson 1980). The Mannitol Egg Yolk Polymyxin B sulfate agar (MEYP) is widely used in Europe and the United States to isolate *B. cereus* from food products. MEYP is based on lecithin hydrolysis and mannitol assimilation for differentiation of *B. cereus* from most other commonly occurring *Bacillus* species. Polymyxin B sulfate is added as selective agent to inhibit gram-negative bacteria (Mossel *et al.* 1967).

According to the ISO Standard Procedure presumptive *B. cereus* colonies should be confirmed by glucose fermentation, reduction of nitrate to nitrite and Voges Proskauer reaction, i.e. production of acetylmethylcarbinol (acetoin) (Anonymous 1992).

TYPING OF *BACILLUS* SPECIES

To differentiate *Bacillus* strains several methods have been applied including biotyping, serological methods, phage typing, gas-liquid chromatography of whole-cell fatty acids, Pyrolysis Gas-liquid Chromatography (Py-GC) or Pyrolysis Mass Spectrometry (Py-MS), plasmid profiling, and Polymerase Chain Reaction (PCR) based methods.

Biotyping

There has been considerable interest concerning biochemical differentiation between strains. For that purpose, the API system (a standardized, miniaturized version of conventional biochemical tests) has been used in several studies (Logan and Berkeley 1981,

Logan and Berkeley 1984). It was found possible to discriminate between *B. cereus* strains causing the diarrhoeal and emetic types of food poisoning on the basis of failure to ferment salicin, dextrin, starch and glycogen (Logan and Berkeley 1981, Shinagawa 1993). In contrast, some workers have found no consistent differences between diarrhoeal and emetic strains (Major *et al.* 1979).

Serotyping

Serotyping can be based on flagellar, vegetative cell somatic or spore antigens. The most successful method for serotyping of *B. cereus* is based on the flagellar (H) antigens. A typing scheme was developed in the UK which employs more than 20 agglutinating sera (Kramer *et al.* 1982). The scheme was proven of value in the epidemiological investigation of food poisoning (Gilbert and Parry 1977, Kramer *et al.* 1982, DeBuono *et al.* 1988) and in the investigation into the source of a *B. cereus* meningitis (Barrie *et al.* 1992). However, the antisera are not commercially available. Moreover, as many as 54 to 61 % of isolates from food sources may be untypable (Gilbert and Parry 1977, Gilbert and Kramer 1986).

Phage typing

Phage typing is based on the sensitivity of strains to bacteriophages. Bacteriophage typing has not been extensively used for *Bacillus* species. Where use of bacteriophages has been made, identification tends to be based upon combinations of bacteriophage sensitivities with other types of data, rather than on sensitivity patterns alone. Phage typing has good discrimination, but leaves many strains untypable. Strains of the *B. cereus* group originating from dairy products could be differentiated using phage typing. However, *B. cereus*, *B. mycoides* and *B. thuringiensis* cross-reacted with their respective phages. Even though phage typing could not be used for accurate species identification, it was useful to cluster isolates of the *B. cereus* group, permitting to track routes of contamination (Väisänen *et al.* 1991). The use of phage typing of isolates associated with outbreaks of food poisoning has also been reported (Ahmed *et al.* 1995, Jackson *et al.* 1995)

Gas chromatographic analysis of cellular fatty acids

This method is based on the extraction of fatty acids (by saponification and methylation) followed by chemical analysis by gas chromatography. It was shown that this was a reproducible method and that it was possible to discriminate between species of the heterogeneous genus *Bacillus*. The technique was, however, not able to distinguish between closely related species, e.g. *B. cereus*, *B. mycoides* and *B. thuringiensis* (Väisänen *et al.* 1991, Wauthoz *et al.* 1995).

Pyrolysis gas-liquid chromatography (Py-GC) or pyrolysis mass spectrometry (Py-MS)

In these techniques, pyrolysis (the thermal degradation of matter in an inert atmosphere to produce a series of volatile low molecular weight substances characteristic of the original matter) is combined with analytical techniques of gas-liquid chromatography or mass spectrometry. Although both techniques have been used for the identification of *Bacillus* species, there are problems concerning reproducibility and standardization of the technique (Berkeley *et al.* 1984). Moreover, the critical levels at which isolates are distinguished are not known (Pitt 1992). The application of Py-MS to the investigation of outbreaks of food poisoning or non-gastrointestinal infection associated with *Bacillus* species showed that all epidemiologically related isolates were clustered correctly, and that all the single, epidemiologically unrelated isolates were distinguished (Sisson *et al.* 1992)

Plasmid profiling

Plasmid profiling is based on the extraction of extrachromosomal DNA, which is a variable part of the genome of a bacterium. The sizes and number of plasmids that will be carried by the strains will vary depending on a range of influencing environmental factors. These strain to strain variations can be used as a way of separating the micro-organisms into different types.

Plasmid profiling has been applied in an investigation of an outbreak among patients and staff in a nursing home (DeBuono *et al.* 1988), to differentiate between *B. cereus* strains in an egg pasteurization plant (Ellison *et al.* 1989) and in an epidemiological investigation of *B. cereus* emetic-syndrome food poisoning (Nishikawa *et al.* 1996). The results show that plasmid profiling is a convenient method to differentiate between *B. cereus* strains. Of the strains isolated from the environment 92 % contained plasmid DNA (Ellison *et al.* 1989). The possibility of plasmid loss or gain by a strain is a legitimate concern, but unless there are some strong selective pressures acting, bacterial plasmid DNA is generally stably maintained within the cell by specific mechanisms.

Polymerase Chain Reaction (PCR) methods

PCR-based methods rely on the heterogeneity present in the chromosome of a bacterial species due to such factors as small deletions, base changes, rearrangements and randomly distributed insertion sequences.

PCR/RAPD

The randomly amplified polymorphic DNA (RAPD) technique provides a new means for characterization of bacteria. Primers are chosen arbitrarily and the PCR amplifies sets of short sequences, which can be visualised by agarose gel electrophoresis to give multiple banding patterns. The more closely related the genomic DNA of two strains, the more

similar the banding patterns. RAPD typing has been used for epidemiological subtyping of *B. licheniformis* (Stephan *et al.* 1994) and *B. thuringiensis* (Brousseau *et al.* 1993). Recently, successful application of the method for the differentiation of *B. cereus* strains in milk processing lines (Nilsson and Christiansson 1996) and baby food (Stephan 1996) has been described. The RAPD-PCR technique provides good discrimination, especially if multiple primers are used (Williams *et al.* 1990).

A major advantage of the technique is its speed. In addition, the reaction is so sensitive that with many species there is no necessity to extract the DNA and cells can be used directly from a plate. However, there are still questions about the reproducibility of the technique (Dodd 1994).

Ribotyping

Ribotyping, in which a Southern blot of restriction-enzyme-digested chromosomal DNA is hybridized to a probe based on the prokaryotic 16S rRNA gene has provided a useful approach to bacterial typing of *B. thuringiensis* and related bacteria at the species and subspecies level (Priest *et al.* 1994).

This approach offers an advantage over the direct visualization of restriction fragments because the number of bands and, hence the complexity of the fingerprint pattern are reduced and easier to resolve. The length of the procedure makes this method slower than plasmid profiling and the PCR/RAPD methods described above. However, recently, a commercial instrument, the Qualicon L.L.C RiboPrinter™ has become available. This allows automated, simple and convenient typing of bacteria (Banks 1996).

ECOLOGY OF *B. CEREUS*

The ability to sporulate coupled with adaptation of many species to environmental extremes has enabled endospore-formers to become amongst the most widespread of all bacteria (Claus and Berkeley 1986, Priest and Grigorova 1990, Priest 1993).

Bacillus species are ubiquitous and can be isolated from a wide variety of environments, including soil, sediments, dust, natural waters and many food types as listed in Table 1.1, notably milk and dairy products, cereals, dried foods, spices, meat products and vegetables (Goepfert *et al.* 1972, Johnson 1984, Kramer and Gilbert 1989, Priest 1993). Direct contamination via soil is important for some food products e.g rice. *Bacillus* species are reported to account for about 90 % of the microflora of soil in rice paddies (Asanuma *et al.* 1979). From its natural habitats the organism is easily spread to foods.

Table 1.1 Incidence of *Bacillus cereus* in raw and processed foods

Food product	*% B. cereus* positive	Range (cfu g^{-1} or ml^{-1})	Reference
Rice and rice products			
Raw rice	40 -100	10^2 - 10^3	1,8,13
Boiled rice	10 - 93	10^1 - 10^7	1,8,13
Fried rice	12 - 86	10^1 - 10^5	1,8,13
Rice dishes	3 - 40	10^1 - 10^5	1,8,10,13
Milk and dairy products			
Raw milk	7 - 35	10^1 - 10^2	2,6,7
Pasteurized milk	2 - 35	10^1 - 10^3	2,8,9,10,11
Sterilized milk	40	nd	1
UHT milk	48	nd	1
Milk powder	15 - 75	10^1 - 10^3	3,4,7,8,11,12
Cream	5 - 11	10^1 - 10^5	8,10
Cheese	2 - 40	10^1 - 10^3	2,10,12
Ice cream	40 - 52	10^1 - 10^4	2,8,11,12
Dried products			
Herbs, spices and seasonings	10 - 75	10^1 - 10^6	3,5,8,10
Pulses and cereals	56	10^2 - 10^5	1
Raw barley	62 - 100	10^2 - 10^4	1,8
Wheat flour	17 - 90	nd	1,8,9
Dried potatoes	40	10^2 - 10^4	1
Soup	4 - 50	10^2 - 10^4	8,9
Meat and meat products			
Raw meat	2 - 34	10^1 - 10^3	1
Cooked meat	22	10^1 - 10^3	1
Sausage	4 - 12	10^1 - 10^3	1
Miscellaneous			
Vegetables	0 - 100	10^1 - 10^4	1,10,13
Pastries	6 - 41	10^1 - 10^4	1
Fish products	4 - 9	10^1 - 10^4	1,10

References: 1 Kramer and Gilbert 1989, 2 Ahmed *et al.* 1983, 3 Kim and Goepfert 1971b, 4 Walthew and Lück 1978, 5 Powers *et al.* 1976, 6 Shehata and Collins 1971, 7 Shinagawa 1993, 8 Johnson 1984, 9 Mosso *et al.* 1989, 10 Van Netten *et al.* 1990, 11 Wong *et al.* 1988, 12 Rangasamy *et al.* 1993, 13 Harmon and Kautter 1991

Spores can survive during food processing and after germination and outgrowth cause spoilage and even outbreaks of foodborne illness (Johnson 1984). The ingestion of *B. cereus* may be regarded as an inevitable consequence of the widespread contamination of our food supply with this organism. In healthy individuals, spores surviving passage through the upper alimentary tract eventually become part of the transitory intestinal flora. The prevalence of *B. cereus* in 14-15 % of faecal specimens from healthy individuals has been reported. Excretion of *B. cereus* probably reflects the dietary intake of the organism, human carriage is not considered to be of any significance in food poisoning (Johnson 1984, Kramer and Gilbert 1989).

FOODBORNE ILLNESS DUE TO *B. CEREUS*

The first description of the diarrhoeal syndrome was provided by Hauge (1955). He investigated four outbreaks in Norway caused by vanilla sauce, as described before. Since then, there have been several outbreaks in which various products were implicated. *B. cereus* is now well-established as a significant cause of foodborne illness in humans, accounting for 1-23 % of reported outbreaks of known bacterial cause (Hutchinson and Taplin 1978, Kramer and Gilbert 1989, Drobniewski 1993). The WHO Surveillance Program for Control of Foodborne Infections and Intoxications in Europe combined with data on surveillance of foodborne diseases showed that an average of less than 1 to 10 % of reported cases with known aetiology were caused by *B. cereus* (WHO 1992, Todd 1996). In the Netherlands 19 % of the outbreaks was attributed to *B. cereus* in the period 1983-1990 (Notermans and Van de Giessen 1993). Other *Bacillus* species, including *B. licheniformis*, *B. pumilus* and *B. subtilis* have also been associated with incidents of foodborne illness (Kramer and Gilbert 1989, Meer *et al.* 1991).

There are two types of *B. cereus* food poisoning. The characteristics of the two types of food poisoning are listed in Table 1.2.

The first type, having symptoms which are similar to those of *Staphylococcus aureus* food intoxication, is caused by enterotoxin(s), produced during vegetative growth of the organism in the small intestine. The enterotoxin(s) can probably also be preformed in foods, but then the number of cells is at least two orders of magnitude higher than that necessary for food poisoning (Christiansson 1993, Granum 1994). The total infective doses seems to vary between about 10^5 and 10^{11} viable cells or spores, partly due to the large differences in the toxin production by different strains (Hauge 1955, Kramer and Gilbert 1989, Granum 1994). The second type, resembling *Clostridium perfringens* food poisoning, is caused by an emetic toxin, preformed in the food.

Table 1.2 Characteristics of the two types of disease caused by *Bacillus cereus*

	Diarrhoeal syndrome	Emetic syndrome
Infective dose	10^5-10^7 (total)	10^5-10^8 (cells g^{-1})
Toxin produced	small intestine	preformed in food
Type of toxin	protein	cyclic peptide
Incubation period	8-16 h	0.5-5 h
Duration of illness	12-24 h	6-24 h
Symptoms	abdominal pain, watery diarrhoea, occasionally nausea	nausea, vomiting and malaise, (diarrhoea)
Principal food vehicles	meat products, soups, vegetables, puddings, sauces, milk(products)	rice (fried and cooked), pasta, pastry and noodles

(Kramer and Gilbert 1989)

The diarrhoeal type of food poisoning is associated with a wide range of foods including cooked meat dishes, soups, vegetable puddings, milk based products and sauces (Johnson 1984, Kramer and Gilbert 1989, Meer *et al.* 1991). There is still confusion regarding how many different enterotoxins are produced by *B. cereus*. At least two have been characterized, so far (Beecher *et al.* 1995, Agata *et al.* 1995). One is a three component enterotoxin with haemolytic and necrotic activities (Beecher and Wong 1994, Beecher *et al.* 1995), while the other is a single component enterotoxin (Agata *et al.* 1995). Recently, it has been shown that there is at least one enterotoxin complex in addition to the haemolysin BL toxin and enterotoxin T (Granum *et al.* 1996).

There are two commercially available kits for the detection of enterotoxin: a Reversed Passive Latex Agglutination kit (RPLA) and an Enzyme-Linked ImmunoSorbent Assay (ELISA). The problem is that the antibodies used in both kits are not specific for the enterotoxin and detect two different non-toxic proteins (Beecher and Wong 1994).

The emetic toxin, cereulide, consists of a ring structure (dodecapsipeptide) of three repeats of four amino- and/or oxy-acids; [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$ (Agata *et al.* 1994). The biosynthetic pathway and mechanism of action of the emetic toxin still have to be elucidated. Approximately 95 % of all emetic syndrome episodes have been associated with the consumption of rice. It appears that the two syndromes cannot be related to specific serovars, although most strains (63.5 %) involved in emetic syndrome outbreaks belong to

serotype H.1 (Kramer and Gilbert 1989). Strains causing the emetic syndrome have been found to be unable to hydrolyze starch or ferment salicin. However, some studies have observed no differences between diarrhoeal and emetic strains (Johnson 1984).

In addition, *B. cereus* produces several haemolysins and phospholipases. The in vivo role of these enzymes is not entirely clear, but they could be significant virulence determinants in infections (Johnson 1984, Drobniewski 1993)

OBJECTIVE OF THIS THESIS

Psychrotrophic *B. cereus* determine the keeping quality of pasteurized milk. The importance of *B. cereus* in the dairy industry has been recognized for many years. The presence of this organism in the milk production environment has been described, however, the major contamination sources are not yet known.

It is unlikely that the occurrence of *B. cereus* in milk can be completely avoided. Spores will survive the pasteurization process and due to lack of competition by other bacteria, they can, after germination, grow to high levels and cause spoilage of pasteurized milk(products) such as off-flavours, bitty cream and sweet curdling. In addition, some cases of food poisoning attributed to *B. cereus* in milk and milk products have been reported. However, it is still not clear if dairy isolates can produce enterotoxin(s) and if milk plays an important role in foodborne illness caused by *B. cereus*.

OUTLINE OF THIS THESIS

In Chapter 2 of this thesis the effect of germination of bacterial spores during sample preparation is described. This phenomenon is important in the microbiological examination of food and environmental samples and should be taken into account when the proportion of spore-forming bacteria present as spores is determined.

Identification of *B. cereus* and differentiation of *B. cereus* and closely related species is currently based on biochemical tests. However, one of the major difficulties associated with studying *B. cereus* is the heterogeneity in response of reactions shown by the organism. Therefore, the identification and discrimination between *B. cereus* and *B. thuringiensis* using probes based on the V1 region of the 16S rRNA is described in Chapter 3.

To estimate the incidence of *B. cereus* and *B. subtilis* in foods in the Netherlands, various food products including milk, yeast, flour, pasta products, Chinese meals, cocoa, chocolate, bakery products, meat products, herbs and spices were examined [Chapter 4].

In order to determine the major contamination sources of milk with *B. cereus*, the occurrence of *B. cereus* on farms [Chapter 5], two dairy processing plants [Chapter 7] and

households [Chapter 9] was investigated. To differentiate between strains, confirmed isolates were characterized biochemically and upon growth characteristics [Chapters 5, 6, and 7] and by the PCR/RAPD technique and plasmid profiling [Chapter 7].

The initial contamination level of milk can be influenced by cleaning and disinfection. Chapter 6 describes the sporicidal efficacy of disinfectants on *B. cereus* spores, isolated from the milk processing environment, as evaluated by the European Suspension Test, surface tests and field trials in a milking installation.

In Chapter 8 the possibility of contamination via the equipment in dairy processing plants and the effect of cleaning and disinfection on the numbers of *B. cereus* present, was investigated in detail.

The ability of the dairy strains, isolated from pasteurized milk in household refrigerators, to produce enterotoxin was determined and compared with strains isolated from various food products [Chapter 9].

Finally, in Chapter 10 the results are discussed and the human exposure to *B. cereus* via milk is evaluated.

REFERENCES

Agata N, Mori M, Ohta M, Suwan S, Ohtani I and M Isobe (1994) A novel dodecapsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. *FEMS Microbiology Letters* **121** 31-34.

Agata N, Ohta M, Arakawa Y and M Mori (1995) The *bce*T gene of *Bacillus cereus* encodes an enterotoxic protein. *Microbiology* **141** 983-988.

Ahmed AA-H, Moustafa MK and EH Marth (1983) Incidence of *Bacillus cereus* in milk and some milk products. *Journal of Food Protection* **46** 126-128.

Ahmed R, Sankarmistry P, Jackson S, Ackermann HW and SS Kasatiya (1995) *Bacillus cereus* phage typing as an epidemiological tool in outbreaks of food poisoning. *Journal of Clinical Microbiology* **33** 636-640.

Andersson A, Rönner U and PE Granum (1995) What problems does the food industry have with the sporeforming pathogens *Bacillus cereus* and *Clostridium perfringens* ? *International Journal of Food Microbiology* **28** 145-155.

Anonymous (1992) ISO/DIS 7932 Microbiology-general guidance for enumeration of *Bacillus cereus*-colony count technique at 30 °C, the International Organization for Standardization, Switzerland.

Asanuma S, Tanaka H and M Yatazawa (1979) Rhizoplane microorganisms of rice seedlings as examined by scanning electron microscopy. *Soil Science and Plant Nutrition* **25** 539-551.

Ash C, Farrow JA, Dorsch M, Stackebrandt E and MD Collins (1991) Comparative analysis of *Bacillus anthracis*, *Bacillus cereus* and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *International Journal of Systematic Bacteriology* **41** 343-346.

Banks JG (1996) DNA- and RNA based methods. In *Proceedings of the Symposium on Food Associated Pathogens*, 6-8 May 1996, Uppsala, Sweden, 86-93.

Barrie D, Wilson JA, Hoffman PN and JM Kramer (1992) *Bacillus cereus* meningitis in two neurosurgical patients: an investigation into the source of the organism. *Journal of Infection* **25** 291-297.

Beecher DJ and ACL Wong (1994) Identification of hemolysin BL-producing *Bacillus cereus* isolates by a discontinuous hemolytic pattern in blood agar. *Applied and Environmental Microbiology* **60** 1646-1651.

Beecher DJ, Schoeni JL and ACL Wong (1995) Enterotoxin activity of hemolysin BL from *Bacillus cereus*. *Infection and Immunity* **63** 632-639.

Bergère JL (1993) Spore formation and germination of *Bacillus cereus*: the spore cycle. *IDF Bulletin* **275** 9-14.

Bergère JL and O Cerf (1993) Heat resistance of *Bacillus cereus* spores. *IDF bulletin* **275** 23-25.

Berkeley RCW, Logan NA, Shute LA and AG Capey (1984) Identification of *Bacillus* species. In T Bergan (ed.) *Methods in Microbiology* **16**, Academic Press, London, 291-328.

Bloomfield SF and M Arthur (1994) Mechanisms of inactivation and resistance of spores to chemical biocides. *Journal of Applied Bacteriology Symposium Supplement* **76** 91S-104S.

Brousseau R, Saint-Onge A, Préfontaine G, Masson L and J Cabana (1993) Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. *Applied and Environmental Microbiology* **59** 114-119.

Bryan FL, Bartleson CA and N Christopherson (1981) Hazard analysis, in references to *Bacillus cereus*, of boiled and fried rice in Cantonese-style restaurants. *Journal of Food Protection* **44** 500-512.

Champagne CP, Laing RR, Roy D, Mafu AA and MW Griffiths (1994) Psychrotrophs in dairy products: their effects and their control. *Critical Reviews in Food Science and Nutrition* **34** 1-30.

Christiansson A (1993) The toxicology of *Bacillus cereus*. *IDF bulletin* **275** 30-35.

Christiansson A, Naidu AS, Nilsson I, Wadstrom T and HE Petterson (1989) Toxin production by *Bacillus cereus* isolates in milk at low temperatures. *Applied and Environmental Microbiology* **55** 2595-2600.

Claus D and RCW Berkeley (1986) Genus *Bacillus*. In Sneath PHA, Mair NS, Sharpe ME and JG Holt (eds.) *Bergey's Manual of Systematic Bacteriology* **2**, Williams and Wilkins, Baltimore, 1104-1139.

Cook FK and MD Pierson (1983) Inhibition of bacterial spores by antimicrobials. *Food Technology* **37** 115-126.

DeBuono BA, Brondum J, Kramer JM, Gilbert RJ and SM Opal (1988) Plasmid, serotypic and enterotoxin analysis of *Bacillus cereus* in an outbreak setting. *Journal of Clinical Microbiology* **26** 1571-1574.

Dodd CER (1994) The application of molecular typing techniques to HACCP. *Trends in Food Science and Technology* **5** 160-164.

Donovan KO (1958) A selective medium for *Bacillus cereus* in milk. *Journal of Applied Bacteriology* **21** 100-103.

Drobniewski FA (1993) *Bacillus cereus* and related species. *Clinical Microbiology Reviews* **6** 324-338.

Ellison A, Dodd CER and WM Waites (1989) Use of plasmid profiles to differentiate between strains of *Bacillus cereus*. *Food Microbiology* **6** 93-98.

Foster SJ (1994) The role and regulation of cell wall structural dynamics during differentiation of endospore-forming bacteria. *Journal of Applied Bacteriology Symposium Supplement* **76** 25S-39S.

Gilbert RJ and JM Parry (1977) Serotypes of *Bacillus cereus* from outbreaks of food poisoning and from routine foods. *Journal of Hygiene* **78** 69-74.

Gilbert RJ and JM Kramer (1986) *Bacillus cereus* food poisoning. In Cliver DO and BA Cochrane (eds.) *Progress in Food Safety*, University of Wisconsin, Madison, 85-93.

Goepfert JM, Spira WM and HU Kim (1972) *Bacillus cereus*: food poisoning organism. A review. *Journal of Milk and Food Technology* **35** 213-225.

Gould GW (1992) Formation, germination and enumeration of *Bacillus cereus* spores. IDF Symposium *Bacillus cereus*, 13-14 October 1992, Ede, The Netherlands.

Granum PE (1994) The toxins of *Bacillus cereus*. *Journal of Applied Bacteriology Symposium Supplement* **76** S61-S66.

Granum PE, Andersson A, Gayther C, te Giffel M, Larsen H, Lund T and K O'Sullivan (1996) Evidence for a further enterotoxin complex produced by *Bacillus cereus*. *FEMS Microbiology Letters* **141** 145-149.

Hauge S (1955) Food poisoning caused by aerobic spore forming bacilli. *Journal of Applied Bacteriology* **18** 591-595.

Harmon SM and DA Kautter (1991) Incidence and growth potential of *Bacillus cereus* in ready-to-serve foods. *Journal of Food Protection* **54** 372-374.

Holbrook R and JM Anderson (1980) An improved selective and diagnostic medium for the isolation and enumeration of *Bacillus cereus* in foods. *Canadian Journal of Microbiology* **26** 753-759.

Hutchinson EMS and J Taplin (1978) *Bacillus cereus* in food. *Food Technology in Australia* **30** 329-333.

Ingram M (1969) In Gould GW and A Hurst (eds.) *The Bacterial Spore*, Academic Press, London, 549-610.

Jackson SG, Goodbrand RB, Ahmed R and S Kasatiya (1995) *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. *Letters in Applied Microbiology* **21** 103-105.

Johnson KM (1984) *Bacillus cereus* foodborne illness-An update. *Journal of Food Protection* **47** 145-153.

Kim HU and JM Goepfert (1971a) Enumeration and identification of *Bacillus cereus* in foods. I. 24-hour presumptive test medium. *Applied Microbiology* **22** 581-587.

Kim HU and JM Goepfert (1971b) Occurrence of *Bacillus cereus* in selected dry food products. *Journal of Milk and Food Technology* **34** 12-15.

Kramer JM, Turnbull PCB, Munshi G and RJ Gilbert (1982) Identification and characterization of *Bacillus cereus* and other *Bacillus* species associated with food poisoning. In Corry JEL, Roberts D and FA Skinner (eds.) *Isolation and Identification Methods for Food Poisoning Organisms*, Academic Press, London and New York, 261-287.

Kramer JM and RJ Gilbert (1989) *Bacillus cereus* and other *Bacillus* species. In Doyle MP (ed.) *Foodborne Bacterial Pathogens*, Marcel Dekker, New York and Basel, 21-70.

Labots H and G Hup (1964) *Bacillus cereus* in raw and pasteurized milk. II. Occurrence of slow and fast germinating *Bacillus cereus* in milk and their significance in the enumeration of *Bacillus cereus* spores. *Netherlands Milk and Dairy Journal* **18** 167-176.

Logan NA and RCW Berkeley (1981) Classification and identification of members of the genus *Bacillus* using API tests. In Berkeley RCW and M Goodfellow (eds.) *The Aerobic Endospore-forming Bacteria: Classification and Identification*, Academic Press, London and New York, 104-140.

Logan NA and RCW Berkeley (1984) Identification of *Bacillus* strains using the API system. *Journal of General Microbiology* **130** 1871-1882.

Major P, Rimanoczi I, Ormay L and A Belteky (1979) Characteristics of *Bacillus cereus* strains isolated from various foods. *Elelmezesi Ipar* **33** 314-315.

Meer RR, Baker J, Bodyfelt FW and MW Griffiths (1991) Psychrotrophic *Bacillus* spp. in fluid milk products: A review. *Journal of Food Protection* **54** 969-979.

Mikolajcik EM (1970) Thermodestruction of *Bacillus* spores in milk. *Journal of Milk and Food Technology* **33** 61-63.

Mossel DAA, Koopman MJ and E Jongerius (1967) Enumeration of *Bacillus cereus* in foods. *Applied Microbiology* **15** 650-653.

Mosso MA, Garcia Arribas ML, Cuena JA and ML De La Rosa (1989) Enumeration of *Bacillus* and *Bacillus cereus* spores in food from Spain. *Journal of Food Protection* **52** 184-188.

Nakamura LK and MA Jackson (1995) Clarification of the taxonomy of *Bacillus mycoides*. *International Journal of Systematic Bacteriology* **45** 46-49.

Nilsson J and A Christiansson (1996) A RAPD-PCR fingerprinting method for differentiation of *Bacillus cereus* strains with application to dairy milk processing lines. In *Proceedings of the Symposium on Food Associated Pathogens*, 6-8 May 1996, Uppsala, Sweden, 207.

Nishikawa Y, Kramer JM, Hanaoka M 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. *International Journal of Food Microbiology* **31** 149-159.

Notermans SHW and A Van de Giessen (1993) Foodborne diseases in the 1980s and 1990s. *Food Control* **4** 122-125.

Overcast WW and K Atmaram (1974) The role of *Bacillus cereus* in sweet curdling of fluid milk. *Journal of Milk and Food Technology* **37** 233-236.

Pitt TL (1992) Recent developments in typing methods. *PHLS Microbiology Digest* **9** 160-165.

Powers EM, Latt TG and T Brown (1976) Incidence and levels of *Bacillus cereus* in processed spices. *Journal of Milk and Food Technology* **39** 668-670.

Priest FG (1981) DNA homology in the genus *Bacillus* In Berkeley RCW and M Goodfellow (eds.) *The aerobic endospore-forming bacteria: classification and identification*, Academic Press New York, 33-57.

Priest FG (1993) Systematics and ecology of *Bacillus*. In Sonenshein AL, Hoch JA and R Losick (eds.) *Bacillus subtilis and other gram-positive bacteria. Biochemistry, physiology and molecular genetics*, American Society for Microbiology, Washington, 3-16.

Priest FG and R Grigorova (1990) Methods for studying the ecology of endospore-forming bacteria. In Grigorova R and JR Norris (eds.) *Methods in Microbiology* **22**, Academic Press, London, 566-591.

Priest FG, Kaji DA, Rosato YB and VP Canhos (1994) Characterization of *Bacillus thuringiensis* and related bacteria by ribosomal RNA gene restriction fragment length polymorphisms. *Microbiology* **140** 1015-1022.

Raevuori M and C Genigeorgis (1975) Effect of pH and sodium chloride on growth of *Bacillus cereus* in laboratory media and certain foods. *Applied Microbiology* **29** 68-73.

Rangasamy PN, Iyer M and H Roginski (1993) Isolation and characterisation of *Bacillus cereus* in milk and dairy products manufactured in Victoria. *Australian Journal of Dairy Technology* **48** 93-95.

Setlow P (1994) Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. *Journal of Applied Bacteriology Symposium Supplement* **76** 49S-60S.

Shehata TE and EB Collins (1971) Isolation and identification of psychrophilic species of *Bacillus* from milk. *Applied Microbiology* **21** 466-473.

Shehata TE and EB Collins (1972) Sporulation and heat-resistance of psychrophilic strains of *Bacillus*. *Journal of Dairy Science* **55** 1405-1409.

Shinagawa K (1993) Serology and characterization of toxigenic *Bacillus cereus*. *Netherlands Milk and Dairy Journal* **47** 89-103.

Sisson PR, Kramer JM, Brett MM, Freeman R, Gilbert RJ and NF Lightfoot (1992) Application of pyrolysis mass spectrometry to the investigation of outbreaks of food poisoning and non-gastrointestinal infection associated with *Bacillus* species and *Clostridium perfringens*. *International Journal of Food Microbiology* **17** 57-66.

Stephan R (1996) Randomly amplified polymorphic DNA (RAPD) assay for genomic fingerprinting of *Bacillus cereus* isolates. *International Journal of Food Microbiology* **31** 311-316.

Stephan R, Schraft H and F Untermann (1994) Characterization of *Bacillus licheniformis* with the RAPD technique (Randomly Amplified Polymorphic DNA). *Letters in Applied Microbiology* **18** 260-263.

Stewart DB (1975) Factors influencing the incidence of *Bacillus cereus* spores in milk. *Journal of the Society of Dairy Technology* **28** 80-85.

Stone JM and A Rowlands (1952) 'Broken' or 'bitty' cream in raw and pasteurized milk. *Journal of Dairy Research* **19** 51-62.

Todd ECD (1996) Worldwide surveillance of foodborne disease the need to improve. *Journal of Food Protection* **59** 82-92.

Van Netten P, van Moosdijk AM and DAA Mossel (1990) Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *Journal of Applied Bacteriology* **69** 73-79.

Van Netten P and JM Kramer (1992) Media for the detection and enumeration of *Bacillus cereus* in foods: a review. *International Journal of Food Microbiology* **17** 85-99.

Väisänen OM, Mwaisumo NJ and MS Salkinoja-Salonen (1990) Differentiation of dairy strains of the *Bacillus cereus* group by phage typing, minimum growth temperature and fatty acid analysis. *Journal of Applied Bacteriology* **70** 315-324.

Walthew J and H Lück (1978) Incidence of *Bacillus cereus* in milk powder. *South African Journal of Dairy Technology* **10** 47-50.

Wauthoz P, El Lioui M and J Decallonne (1995) Gas chromatographic analysis of cellular fatty-acids in the identification of foodborne bacteria. *Journal of Food Protection* **58** 1234-1240.

WHO Surveillance Programme (1992) Fifth report of WHO Surveillance Programme for control of foodborne infections and intoxications in Europe, 1985-1989. Institute of Veterinary Medicine, Robert von Ostertag Institute, Berlin, Germany, 235 p.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and SV Tingey (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18** 6531-6535.

Wong HC, Chang MH and JY Fan (1988) Incidence and characterisation of *Bacillus cereus* isolates contaminating dairy products. *Applied and Environmental Microbiology* **54** 699-702.

CHAPTER 2

GERMINATION OF BACTERIAL SPORES DURING SAMPLE PREPARATION

ABSTRACT

The effect of environmental conditions on the germination of *Bacillus cereus* was investigated. Germination was affected to varying extent by strain type, complexity of the medium, presence of germinants and temperature.

In the microbiological examination of food(ingredients) it was shown that germination during sample preparation is observed for all bacterial spores present in these products. In standard procedures for microbiological examination it is mentioned that the time between preparation of the initial suspension and the mixing of dilutions and media shall not exceed 45 min. However, in this study it was shown that in 45 min significant numbers of spores have already germinated. Therefore, too much time between preparation of the primary dilution and the heat activation step led to an underestimation of the numbers of spores.

In order to determine the proportion of spore-forming bacteria present as spores, the time between preparation of the primary dilution and the heat-activation step should be as short as possible ($<$ 10 min) and the temperature of diluents during the analysis should be as low as possible (e.g. by keeping dilutions in melting ice).

This chapter has been published as:
Germination of bacterial spores during sample preparation
MC Te Giffel, RR Beumer, J Hoekstra and FM Rombouts
Food Microbiology (1995) **12** 327-332.

INTRODUCTION

The ability to sporulate coupled with adaptation of many species to environmental extremes has enabled endospore-formers to become amongst the most widespread of all bacteria (Priest and Grigorova 1990).

To obtain an indication of the proportion of bacteria present as spores, viable counts are conducted on a sample in which vegetative cells are killed by some treatment (e.g. heat treatment, air drying, solvent treatment). The most common procedure is heating primary suspensions in buffer, saline solution or water in a thermostatically controlled water-bath for 5 to 10 minutes at 80 or 85°C (Anonymous 1996, Priest and Grigorova 1990). Most vegetative cells (except some extreme thermophiles) will be killed at these temperatures but most endospores will be unaffected or activated. Activation is often required, since dormant spores will not necessarily germinate under favourable conditions. Once a spore is activated it will germinate in a suitable environment (Cook and Pierson 1983). Environmental factors necessary for optimal germination, like temperature, pH and ionic strength, vary widely and depend on the organism (Stewart 1975). Germination can be induced by certain amino acids (e.g. L-alanine), ribosides and sugars, but is often achieved most rapidly and completely by using combinations of germinants or complex culture media (Bergère 1992).

Much experimental work has been carried out on the germination in simple media (Harrell and Halvorson 1955, Dring and Gould 1971). Relatively little is known of the factors influencing the germination of *B. cereus* in complex media such as food products. This study was initiated as a result of the effects observed during the analysis of thermo-resistant micro-organisms in whey powder. The thermoresistant, spore-forming micro-organisms in whey powder may consist, for a great part, of *B. cereus*, due to unhygienic processing conditions. Spore counts were influenced by germination during the microbial investigation. It was shown that upon heating 55 % of the thermoresistant organisms had germinated after 30 min at 25°C (Te Giffel *et al.* 1993).

First, the effect of different factors that could influence germination of *B. cereus* in foods was examined. Next, in order to study the germination during sample preparation in general, the germination of aerobic spores in naturally contaminated food ingredients, tea and dried soups was examined.

MATERIALS AND METHODS

Strains

The four *B. cereus* strains used in this study were obtained from the Netherlands

Institute for Dairy Research (NIZO, Ede, The Netherlands). Strain Lb2 (slow germinating, i.e. no germination within 24 h at 20°C in milk, after a heat activation of 10 s at 72°C (Stadhouders *et al.* 1980)), strain Lb5 (slow germinating), strain VC2 (fast germinating, i.e. germination within 24 h at 20°C in milk, after a heat activation of 10 s at 72°C) and strain P5 (germination type unknown). These strains were all isolated from milk and milk products. The strains were stored at - 80°C in Brain Heart Infusion broth (BHI, Difco 0037-01-6) + 20% glycerol.

Preparation of spore suspensions

Sporulation of the *B. cereus* was achieved on Nutrient Agar medium (NA, Oxoid CM3). After incubation for 3 days at 30°C spores were washed from the surface of the agar with sterile distilled water and centrifuged at 2000 *g* for 20 min. The pellets were washed three times and resuspended in sterile distilled water. To destroy the vegetative cells the spore suspension was given a heat shock of 60 $\pm$ 5 sec at 80°C. The number of spores was determined after plate counting on Mannitol Egg Yolk Polymyxin agar (MEYP, Cereus selective agar, Merck 5267) and by microscopy. Spore suspensions prepared were used immediately.

Factors influencing germination of *B. cereus*

Spore suspensions (10^8 ml^{-1}) were diluted in sterile distilled water. The experiments were conducted with tubes containing 10 ml of the various media which were inoculated with 0.1 ml of a 10^5 spore suspension resulting in an inoculum level of 10^3 spores ml^{-1}. This level was checked by surface streaking on MEYP agar. After 10, 20, 30 and 60 min the number of heat resistant *B. cereus* spores was determined after a heat shock (10 min 80°C) by plating on MEYP. The MEYP plates were incubated at 30°C for 24 h. Spore germination was calculated as (1 - residual spores ml^{-1}/original spores ml^{-1}) 100 % (Johnson *et al.* 1982, Wong *et al.* 1988).

Type of strain

The influence of germination type (fast or slow) in peptone saline solution (PSS; composition: NaCl (8.5 g l^{-1}) and Neutralized Bacteriological Peptone (Oxoid L34, 1 g l^{-1})) was determined using four different *B. cereus* strains.

Medium

In addition to PSS the germination of *B. cereus* spores was determined in saline solution (SS; composition: NaCl (8.5 g l^{-1}) and in more complex media i.e. raw, pasteurized and UHT sterilized milk.

Presence of germinants

The influence of germinants was examined by addition of L-alanine (Sigma A7627) to PSS, raw and pasteurized milk. L-alanine was added as filter sterilized solution to a final concentration of 10 mmol l^{-1}.

Temperature

To investigate the influence of temperature on the germination of *B. cereus* spores the experiments were performed in PSS and pasteurized milk at room temperature and at 0°C (melting ice).

Germination in food

Inoculated food suspensions

Ten-fold dilutions (in PSS; composition: NaCl (8.5 g l^{-1}) and Neutralized Bacteriological Peptone (Oxoid L34, 1 g l^{-1}) or SS; composition: NaCl (8.5 g l^{-1})) of four pasteurized milk products and two pasta/rice products, were inoculated with 1 ml 10^5 *B. cereus* Lb5 (slow germinating) or *B. cereus* VC2 (fast germinating), resulting in an inoculum level of 10^3 spores ml^{-1}. After 10, 20, 30 and 60 min at room temperature, 5 ml of the suspensions was heated (10 min, 80°C) before enumeration by plate counting on MEYP agar. Colony counts after 24 h at 30°C gave a measure of the numbers of ungerminated, heat-resistant spores remaining at each sampling time.

Naturally contaminated food ingredients

Sixty-five samples of herbs, spices, seasonings, tea and dried soups were examined. The samples were diluted in PSS and within 5 min, 5 ml of this primary dilution was heated at 80°C for 10 min. After leaving the diluted food ingredients 30 min at room temperature the remaining numbers of spores were determined by heating 5 ml of the primary dilution (10 min, 80°C). The total number of heat-resistant spores was determined after plate counting Tryptone Soya Agar (TSA; Oxoid CM131) with an overlayer of 1.5 % agar (Oxoid L13). Furthermore, for 18 samples, *B. cereus* spores were enumerated on MEYP agar. Plates were incubated for 24 h at 30°C and counted. Confirmation was carried out by testing glucose fermentation (+), Voges-Proskauer reaction (+) and nitrate reduction (+).

RESULTS

Factors influencing germination of *B. cereus* spores

Type of strain

The results in Table 2.1 show that there was a difference in germination between slow

and fast germinating strains. After 30 min at room temperature 6 and 9 % of the spores of slow germinating strains (Lb2 and Lb5) had germinated in PSS compared to 41 % of the spores of the fast germinating strain (VC2).

Table 2.1. Influence of temperature on the germination of *Bacillus cereus* spores (%) within 30 min in peptone saline solution (PSS) and pasteurized milk

Strain	Room temperature		0°C (melting ice)	
	PSS	Pasteurized milk	PSS	Pasteurized milk
Lb2	6±2[a]	39±5	2±2	2±2
Lb5	9±3	32±4	6±3	-[b]
VC2	41±5	92±3	58±3	48±6
P5	26±8	40±5	13±5	7±8

[a] mean ± s.d. of three experiments; [b] no germination

Table 2.2. Influence of medium on the germination of *Bacillus cereus* spores (%) within 30 min at room temperature

Strain	SS	PSS	Raw milk	Pasteurized milk	UHT-sterilized milk
Lb2	-[a]	6±2[b]	-	39±5	6 ±4
Lb5	3±1	9±3	4±2	32±4	30±10
VC2	23±3	41±5	9±8	92±3	49±13
P5	18±4	26±8	1±2	40±5	41±5

[a] no germination; [b] mean ± s.d. of three experiments; SS, saline solution

Medium

The extent of germination is dependent on the type of medium in which the spores

are present. Most germination was observed in pasteurized milk followed by UHT-treated milk, dilution fluid (PSS and SS) and raw milk (Table 2.2). This effect was observed for all four strains. In raw milk all strains showed less than 10 % germination within 30 min. For the dilution fluids it was shown that in SS the extent of germination was reduced compared to PSS for all strains.

Presence of germinants

Addition of the germinant L-alanine (10 mmol l^{-1}) stimulated, in most cases, the germination of *B. cereus* spores (Table 2.3 versus Table 2.2). This stimulation was strain and medium dependent. The slow germinating strain Lb2 was most affected upon addition of L-alanine, in PSS and pasteurized milk germination increased to 90 %.

In general, the germination of spores was increased in presence of L-alanine in all three media tested. In raw milk without germinants spores were unable to germinate, however, addition of L-alanine led to an increase in germination of 10 % (strain VC2) to 38 % (strain P5). In pasteurized milk the addition of L-alanine was less effective and did not lead to further increase in germination of the strain VC2 and P5.

Table 2.3. Influence of L-alanine (10 mM) on the germination of *Bacillus cereus* spores (%) within 30 min in peptone saline solution (PSS), raw and pasteurized milk at room temperature

Strain	PSS	Raw milk	Pasteurized milk
Lb2	90±2[a]	28±6	88±3
Lb5	16±7	28±10	40±1
VC2	60±4	19±3	88±7
P5	14±8	39±3	33±3

[a] mean ± s.d. of three experiments

Temperature

As the temperature during the analysis is decreased from room temperature to 0°C the germination of three of the four strains was reduced (Table 2.1). However, the extent of this reduction was dependent on the medium. Strain VC2 showed 58 % germination within 30 min in PSS at 0°C compared to 41% in PSS at room temperature. In pasteurized milk the

germination was 92 % after 30 min at room temperature and 48 % in pasteurized milk at 0°C.

Germination in food

Inoculated foods

In food suspensions inoculated with *B. cereus* spores, germination of spores during the analysis also occurred. Most germination was observed for the fast germinating strain. Germination was dependent on the product and varied from 34 % in diluted semolina pudding to 72 % in diluted macaroni within 30 min at room temperature compared to 3-69 % for the slow germinating strain. In general, in diluted pasta and rice products the germination rate was higher than in diluted pasteurized milk products. In Table 2.4 the results for germination in 1:10 dilutions of rice porridge and fried rice are presented. Using SS as diluent decreased the germination rate of *B. cereus* spores considerably, especially in the dairy products.

Table 2.4. Germination (%) of *Bacillus cereus* Lb5 (slow germinating) and *Bacillus cereus* VC2 (fast geminating) in 1:10 dilutions of rice porridge and fried rice in peptone saline solution (PSS) or saline solution (SS) at room temperature

Time (min)	Rice porridge				Fried rice			
	PSS		SS		PSS		SS	
	Lb5	VC2	Lb5	VC2	Lb5	VC2	Lb5	VC2
10	-[a]	19±5[b]	-	-	2±2	46±5	-	34±4
20	-	22±4	10±3	14±2	15±3	53±4	20±3	55±3
30	7±5	53±7	3±2	20±3	27±6	63±5	17±3	55±7
60	26±7	84±6	-	25±2	38±4	69±7	17±3	50±4

[a] no germination; [b] mean ± s.d. of three experiments
PSS, peptone saline solution; SS, saline solution

Naturally contaminated foods

The results in Figure 2.1 show that germination during microbial analysis of foods could play a role for certain products. For the total aerobic spore counts, a reduction within

30 min at room temperature of more than 20 % was noticed in 33 (50 %) of the samples. However, in nine (14 %) samples more than 60 % germination was observed.

Similar effects were shown for *B. cereus* spores (Figure 2.1). In most samples less than 20% germination was observed but in three samples a considerable germination (more than 60 %) was observed.

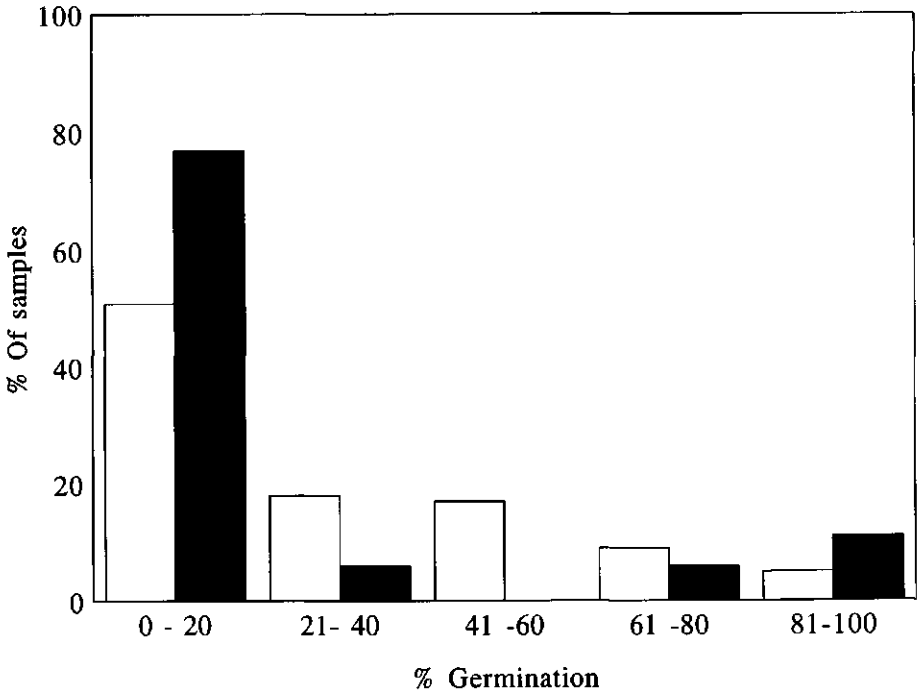


Figure 2.1 Germination of (□) total aerobic (n = 65) and (■) *Bacillus cereus* (n= 18) spores in 1:10 dilutions of naturally contaminated food products in 30 min at room temperature

DISCUSSION

In this study the effect of environmental factors on germination of *B. cereus* spores was investigated. For all four strains most germination occurred during the first 10 min (data not shown). For the fast germinating strain, germination occurred at a higher rate and was more extensive than for the slow germinating strains. The results obtained in the experiments with naturally contaminated food products agree with this, i.e. more extensive germination was observed for the fast germinating strain. The germination observed in the different

products varied considerably. This may be explained (in part) by the distribution of slow and fast germinating strains. The composition of the medium in which the spores are present, substantially influences the extent of germination. It was shown that in PSS germination could take place. However, in complex media the number of non-germinated *B. cereus* spores was lower. Johnson *et al.* (1983) also showed more extensive germination in rice than in Trypticase Soy Broth. In pasteurized milk more germination was observed compared with raw and UHT sterilized milk. This is also reported by Mikolajcik and Koka (1968). During pasteurization of milk, a germinant is formed that allows the spores to germinate in pasteurized milk (Wilkinson and Davies 1973, Davies 1977). This germinant is probably still (partly) active in UHT-sterilized milk since more germination was observed in this type of milk than in raw milk. In most cases the germination was stimulated upon addition of 10 mM L-alanine. This stimulation was strain and medium dependent. The concentrations of germinants (e.g. amino acids, sugars) present in different food products may also have had an effect on the results observed in the experiments with (naturally contaminated) food products.

In general, as the temperature of the analysis was decreased the extent of germination was reduced. These results are consistent with the literature since the optimum temperature for germination of *Bacillus* spores is 30 °C (minimum -1 °C, maximum 59 °C) (Gould 1969).

Although in the ISO Standard Procedure 7218, "Microbiology- General guidance for microbiological examinations" (Anonymous 1996), it is mentioned that the time between preparation of the initial suspension, the mixing of dilutions and media shall not exceed 45 min (unless specially mentioned in the specific international standard), the results in this study indicate that a large number of spores may have already germinated within 30 min. Thus, too much time between preparation of the primary dilution and the heat-activation step will result in an underestimation of the (total) numbers of endospore-forming bacteria present as spores in a product. To improve the procedure to determine spores of spore-forming bacteria it should be recommended to use a saline solution as diluent, to keep the time between preparation of the primary dilution and the heat-activation step as short as possible ($<$ 10 min) and maintain the temperature during the analysis as low as possible (e.g. by keeping dilutions in melting ice).

REFERENCES

Anonymous (1996) ISO Standard Procedure 7218; Microbiology- General guidance for microbiological examinations, International Organization for Standardization, Switzerland.

Bergère J-L (1992) Spore formation and germination of *Bacillus cereus*: the spore cycle. *IDF Bulletin* **275** 9-14.

Cook FK and MD Pierson (1983) Inhibition of bacterial spores by antimicrobials. *Food Technology* **11** 115-126.

Davies FL (1977) The role of various milk fractions and the importance of somatic cells in the formation of germinant(s) for *Bacillus cereus* when milk is pasteurized. *Journal of Dairy Research* **44** 555-568.

Dring GJ and GW Gould (1971) Sequence of events during rapid germination of spores of *Bacillus cereus*. *Journal of General Microbiology* **65** 101-104.

Gould GW (1969) Germination. In: Gould GW and A Hurst (eds.) *The bacterial spore* Academic Press, New York, 397-444.

Harrell WK and H Halvorson (1955) Studies on the role of L-alanine in the germination of spores of *Bacillus terminalis*. *Journal of Bacteriology* **69** 275-279.

Johnson KM, Nelson CL and FF Busta (1982) Germination and heat resistance of *Bacillus cereus* spores from strains associated with diarrheal and emetic foodborne illnesses. *Journal of Food Science* **47** 1268-1271.

Johnson KM, Nelson CL and FF Busta (1983) Influence of temperature on germination and growth of spores of emetic and diarrheal strains of *Bacillus cereus* in a broth medium and in rice. *Journal of Food Science* **48** 286-287.

Mikolajcik EM and M Koka (1968) Bacilli in milk. I. Spore germination and growth. *Journal of Dairy Science* **51** 1579-1582.

Priest FG and R Grigorova (1990) Methods for studying the ecology of endospore-forming bacteria. In Grigorova R and JR Norris (eds.) *Methods in Microbiology* **22**, Academic Press, London, 566-591.

Stadhouders J, Hup G and LPM Langeveld (1980) Some observations on the germination, heat resistance and outgrowth of fast-germinating and slow-germinating spores of *Bacillus cereus* in pasteurized milk. *Netherlands Milk and Dairy Journal* **34** 215-228.

Stewart DB (1975) Factors influencing the incidence of *Bacillus cereus* spores in milk. *Journal of the Society of Dairy Technology* **28** 80-85.

Te Giffel MC, Beumer RR and J Hoekstra (1993) Detection of *Bacillus cereus* in environmental samples and milk on farms. *Abstracts of the 62nd Annual Meeting and Summer Conference* University of Nottingham 13-16 July 1993 xiv.

Wilkinson G and FL Davies (1973) Germination of spores of *Bacillus cereus* in milk and milk dialysates: effect of heat treatment. *Journal of Applied Bacteriology* **36** 485-496.

Wong HC, Chen YL and CLF Chen (1988) Growth, germination and toxigenic activity of *Bacillus cereus* in milk products. *Journal of Food Protection* **51** 707-710.

CHAPTER 3

DISCRIMINATION BETWEEN *BACILLUS CEREUS* AND *BACILLUS THURINGIENSIS* USING SPECIFIC DNA PROBES BASED ON VARIABLE REGIONS OF 16S rRNA

ABSTRACT

Identification of *Bacillus cereus* and differentiation between *B. cereus* and closely related species are currently based on biochemical tests. The main problem is to discriminate between *B. cereus* and *B. thuringiensis*. Sequencing part of the 16S rRNA showed that several *B. cereus* isolates present in food and involved in food poisoning, confirmed according to the classical biochemical methods, were in fact *B. thuringiensis*. As this organism is the most commonly used microbial insecticide worldwide, the results of this study emphasize the need for accurate identification methods and for careful screening of strains for use as insecticides.

Therefore, specific DNA probes based on the variable region V1 of 16S rRNA of *B. cereus* and *B. thuringiensis* were designed. The probes were used in hybridization experiments with the variable region amplified using the polymerase chain reaction. In this way, a rapid and sensitive method was developed to distinguish *B. cereus* and *B. thuringiensis*.

This chapter has been published as:

Discrimination between *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based on variable regions of 16S rRNA

MC Te Giffel, RR Beumer, N Klijn, A Wagendorp and FM Rombouts

FEMS Microbiology Letters (1997) **146** 47-51.

INTRODUCTION

Spore-forming micro-organisms are widely distributed in nature due to the resistance of their endospores to various stresses and their long term survival under unfavourable conditions. Therefore, most aerobic spore-formers can be isolated from a wide variety of sources including foods (Claus and Berkeley 1986).

Bacillus species are important as food spoilage organisms and can be isolated from fruits, vegetable products, nuts, cereals, milk and dairy products, meat, dried foods and spices (Kramer and Gilbert 1989).

The current classification of *Bacillus* is based on the work of Smith *et al.* (1952) and Gordon *et al.* (1973) (Drobniewski 1993). This scheme includes a primary subdivision on the basis of endospore and sporangium morphology and further subdivision to species level on the basis of biochemical tests. Differentiation between *Bacillus* species can be difficult, and a large number of phenotypic tests are used to distinguish between them, although sometimes only a single feature separates species (Logan and Berkeley 1984, Drobniewski 1993). Although in DNA/DNA hybridization studies high homologies have been found with *B. anthracis*, *B. mycoides* and *B. thuringiensis* and *B. cereus* and *B. thuringiensis* were indistinguishable by pulsed-field gel electrophoresis and multilocus enzyme electrophoresis, they are still considered separate species (Claus and Berkeley 1986, Drobniewski 1993, Carlson *et al.* 1994, Damgaard 1995). It has been shown recently that *B. thuringiensis* strains, used as a biological control agent for insects, can produce *B. cereus*-diarrhoeal-type of enterotoxin (Abdel-Hameed and Landén 1994, Damgaard 1995, Jackson *et al.* 1995). This emphasizes the need for re-evaluation of the taxonomy of these organisms. Moreover, the use of *B. thuringiensis* strains as insecticides should be evaluated carefully.

In recent years, molecular methods have been described for the sensitive and specific identification of micro-organisms. DNA-probes, based on variable regions of the secondary structure of 16S rRNA have been successfully applied for identification of or discrimination between various micro-organisms (Barry *et al.* 1990, Klijn *et al.* 1991, Wang *et al.* 1992, Klijn *et al.* 1994).

This paper decribes the development and the evaluation of DNA probes based on the V1 region of the 16S rRNA for the identification of *B. cereus* and *B. thuringiensis*. To this end, *B. cereus* isolates were first grouped according to the profiles obtained using the PCR/RAPD technique. Then, part of the 16S rRNA of a set of strains with different phenotypic properties was sequenced and hybridization with DNA probes based on specific 16S rRNA sequences was performed.

MATERIALS AND METHODS

Strains

Sixty-five *B. cereus* isolates obtained from farms, dairy processing plants, households, various food products and different culture collections (Netherlands Institute for Dairy Research (NIZO), National Institute of Public Health and the Environment (RIVM), Norwegian College of Veterinary Medicine, American Type Culture Collection (ATCC) and the Dutch Inspectorate for Health Protection (IGB Leeuwarden) were used. All isolates were identified as *B. cereus* according to biochemical tests described in the ISO Standard Procedure for enumeration of *B. cereus*, i.e. glucose fermentation, nitrate reduction and Voges Proskauer (VP) reaction. *B. cereus* should be positive for all three confirmation reactions (Anonymous 1992). In adddition, carbohydrate utilization patterns (API 50 CHB, BioMérieux) were determined for identification of the isolates. Twenty-three of the strains used in this study were unable to reduce nitrate and/or were negative for the VP reaction, however, in the API 50 CHB typical *B. cereus* carbohydrate utilization patterns were observed.

Isolation of DNA

For DNA isolation the strains were grown on Tryptone Soya Agar (TSA; Oxoid CM 131) for 18-24 h at 30 °C. A single bacterial colony (± 1.5 mm) was picked and the DNA was isolated using InstaGene Matrix (Biorad 732-6030) according to the manufacturer's protocol with minor adjustments.

PCR/RAPD amplification

PCR amplifications using the random primers R1: [GAAGCAGCGTGG] and R2: [GTCGTTATGCGGTA] were performed by using a Thermocycler 480 (Perkin-Elmer, Gouda, The Netherlands). The reactions were carried out in 0.5-ml tubes which contained 50 μl of the following buffer: 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM NaCl, 2.5 mM (each) deoxynucleoside triphosphates, 1 U of *Taq* polymerase (GibcoBRL 18038-026) and 250 ng of primer R1 or R2. Then, 5 μl (500- 1000 ng) of template DNA was added. Amplification was done in 55 cycles of 1 min at 94 °C (denaturation), 1 min at 25 °C (annealing), and 8 min at 72 °C (extension-polymerization). PCR products were analyzed by agarose gel electrophoresis.

Sequencing of variable regions of 16S rRNA

The V1, V2 and V3 regions of the 16S rRNA were amplified using the following primers based on conserved sequences; P1: [GCGGCGTGCCTAATACATGC] (position 41 to 60 in the *E. coli* numbering system) and P2: [ATCTACGCATTTCACCGCTAC] (complementary to position 685 to 705 in the *E. coli* numbering system).

The PCR amplifications were carried out as described above in 0.5-ml sterile tubes containing 50 μl of the following buffer: 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM NaCl, 2.5 mM (each) deoxynucleoside triphosphates, 1 U of *Taq* polymerase (GibcoBRL 18038-026) and 250 ng of each primer. After being heated to 95 °C to eliminate all protease activity, 5 μl (500- 1000 ng) of template DNA was added. Amplification was performed for 30 cycles of melting DNA at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and elongation at 72 °C for 2.5 min.

After PCR amplification, the presence of the PCR product was checked by gel electrophoresis and the product was subsequently purified using the Easy Prep™ PCR Product Prep Kit (Pharmacia Biotech 17-1170-01). The purified DNA was then sequenced; sequencing reactions were performed using the Thermosequenase kit (Amersham Life Sciences RPN 2436) according to the manufacturer's instructions with minor adjustments. Fluorescence-based DNA sequence analyses were performed with a Pharmacia Automated Laser Fluorescent DNA Sequencer. The obtained sequences of 16S rRNA were compared with those from the RPD Database (Maidak *et al.* 1994).

Hybridization with specific DNA probes

After amplification of the V1 region of the 16S rRNA (90 bp), as described for sequencing, using primer P1: [GCGGCGTGCCTAATACATGC] and P3: [TTCCCCACGCGTTACTCACC] (antisense to position 111 to 130 in the *E. coli* numbering system) hybridizations were performed. For hybridization with a general probe for 16S rRNA (P1; [GCGGCGTGCCTAATACATGC]) and specific probes for *B. cereus* [TTAAGAGCTTGCTCTTAT] and for *B. thuringiensis* [TTGAGAGCTTGCTCTCAA], the PCR-amplified fragment (the amplified V1 region) was, after agarose gel electrophoresis, transferred to GeneScreen Plus (DuPont, Boston, MA) via Southern blotting. After blotting, the membranes were neutralized in 0.2 M Tris.HCl (pH 7.5) in 0.3 M NaCl-0.03 M sodium citrate and air dried. Prehybridization and hybridization were performed in 0.5 M sodium phosphate buffer (pH 7.2) containing 3 % sodium dodecyl sulfate and 1 % bovine serum albumin. After 30 min of prehybridization at 45 °C, the probe, which had been 5'-end labeled with [γ-^{32}P]ATP (Amersham, Buckinghamshire, United Kingdom), was added and

the incubation was continued for 4 h. The blots were washed with 0.9 M NaCl-0.09 M sodium citrate folllowed by washing in 0.3 M NaCl-0.03 M sodium citrate at 40 °C until a clear signal was found and then exposed to Kodak X-ray films.

RESULTS AND DISCUSSION

In this study, *B. cereus* isolates obtained from different environments were identified and characterized by biochemical tests and PCR profiles using two random primers (data not shown). PCR profiles allowed differentiation of the strains in seven groups. Twenty isolates, representing the various groups, were selected for sequencing of variable regions of the 16S rRNA. The results of the sequencing in Table 3.1 show that 6 strains were confirmed to be *B. cereus* and thus agree with the classical identification tests. Also three isolates that were not identified as *B. cereus* using the confirmation tests described in the ISO Standard Procedure, i.e. unable to reduce nitrate or VP negative, were shown to belong to the *B. cereus* group by sequencing. Five isolates were considered to be *B. mycoides*, two of these showed rhizoid growth on agar plates, for the other three strains this was not observed. It has been reported that rhizoid growth may be lost (Claus and Berkeley 1986, Nakamura and Jackson 1995); however *B. mycoides* can be distinguished from *B. cereus* by differences in fatty acid profiles and acetanilide production (Nakamura and Jackson 1995). Six of the 20 isolates identified as *B. cereus* by classical, biochemical tests were in fact *B. thuringiensis* and thus wrongly classified.

B. thuringiensis can phenotypically only be distinguished from *B. cereus* by production of toxin crystals. All isolates identified as *B. thuringiensis* by sequencing reacted with the primers for the three known *B. thuringiensis* delta-toxin gene sequences (data not shown, Bourque *et al.* 1993). However, this character is plasmid encoded and transmissible to *B. cereus* by conjugation (Gonzalez *et al.* 1982, Damgaard 1995). *B. thuringiensis* preparations for use in controlling both agricultural pests and vectors of human and animal disease are commercially available. The results presented in this study emphasize the need to screen strains carefully for use as insecticides, as some of the strains identified as *B. thuringiensis* were involved in food poisoning, e.g. strain L3 and 1230. These strains produced enterotoxin determined by Western immunoblot technique, PCR and Vero cell assays (Granum *et al.* 1996, [Chapter 9]). It has already been reported in previous studies that strains of *B. thuringiensis*, including strains isolated from commercial insecticides, produced a *B. cereus*-diarrhoeal-type enterotoxin (Claus and Berkeley 1986, Damgaard 1995, Jackson *et al.* 1995) and some incidents linking *B. thuringiensis* to human infection have been described (Kramer and Gilbert 1989, Jackson *et al.* 1995). Cases of *B. cereus* diarrhoeal outbreaks from ingestion of raw contaminated vegetable sprouts and from improperly cooked green beans

have been reported (Damgaard 1995). These cases may in fact have been caused by *B. thuringiensis*, due to misclassification of isolates. This could suggest that the role of *B. thuringiensis* in food poisoning may be underestimated.

Table 3.1 Results of biochemical testing, sequencing and hybridization with specific probes based on the V1 region of the 16S rRNA

Strain	Source	ISO[a]	Sequencing	Hybridization with specific probes	
				Bc	Bt
9308	faeces cow	+	*B. thuringiensis*		+
9367[b]	concentrate	+	*B. mycoides*	+	
9383	faeces cow	N-	*B. mycoides*	+	
94453[b]	milk	+	*B. cereus*	+	
95008	milk	+	*B. thuringiensis*		+
16	milk	N-	*B. cereus*	+	
20	milk	N-	*B. thuringiensis*		+
36b[b]	milk	+	*B. mycoides*	+	
38b	milk	+	*B. mycoides*	+	
46	milk	+	*B. cereus*	+	
143	milk	+	*B. mycoides*	+	
164	milk	+	*B. cereus*	+	
9c	baker's yeast	VP-	*B. cereus*	+	
11a	flour	VP-	*B. cereus*	+	
12826	ATCC	+	*B. cereus*	+	
9139	ATCC	+	*B. cereus*	+	
Lb5	NIZO	+	*B. thuringiensis*		+
1230	Norway	+	*B. thuringiensis*		+
L3	RIVM	+	*B. thuringiensis*		+
L21	RIVM	+	*B. cereus*	+	

[a]ISO confirmation tests: nitrate reduction (N) and Voges Proskauer reaction (VP); [b]shows rhizoid growth on TSA agar; Bc, *B.cereus*; Bt, *B. thuringiensis*

Based on these observations and the results presented in this study, it seems necessary to re-evaluate the taxonomy of these organisms. Therefore, a specific and sensitive identification method to discriminate between the species, using DNA probes based on the variable regions of the 16S rRNA, was developed. To this end, the 16S rRNA sequences of *B. cereus*

and *B. thuringiensis* were compared and, it was observed that the V1 region contained sufficient variability (Figure 3.1) to enable the design of DNA probes (see the underlined sequence in the V1 region in Figure 3.1) allowing differentiation between these species.

V1-region

Bc TCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGT

Bm TCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGT

Bt TCGAGCGAATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGGTGAGTAACACGT

Figure 3.1 Alignment of the sequence of the V1 region of the 16S rRNA of *Bacillus cereus* (*Bc*), *Bacillus mycoides* (*Bm*) and *Bacillus thuringiensis* (*Bt*)

Figure 3.2 shows that it is possible to identify and discriminate *B. cereus* and *B. thuringiensis* with DNA probes that are based on the variable V1 region. The *B. cereus* probe did not give a signal with the *B. thuringiensis* strains that differ in only three nucleotides from *B. cereus*. By using the *B. thuringiensis* probe only these strains were detected. These results indicate that specific DNA probes based on the V1 region the 16S rRNA are suitable to differentiate *Bacillus* species that are difficult to identify based on their phenotypic properties.

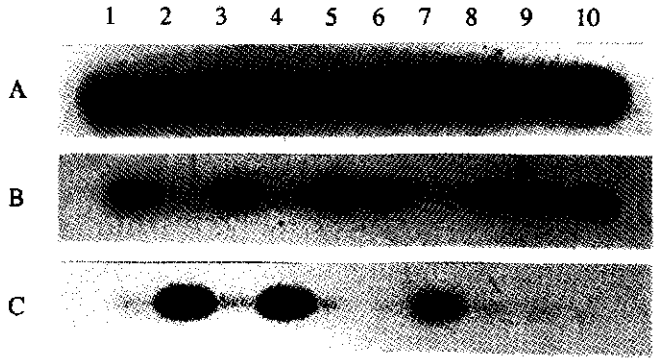


Figure 3.2 Identification of *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based on the V1 region (90 bp) of the 16S rRNA. Lane 1, strain 9c; lane 2, 20; lane 3, 143; lane 4, 1230; lane 5, 11a; lane 6, 38b; lane 7, L3; lane 8, 36b; lane 9, 46; lane 10, L21. Hybridization with panel A: control hybridization; general probe for 16S rRNA (P1) to check the blotting, panel B: *B. cereus* probe and panel C: *B. thuringiensis* probe

REFERENCES

Abdel-Hameed A and R Landén (1994) Studies on *Bacillus thuringiensis* strains isolated from Swedish soils: insect toxicity and production of *Bacillus cereus*-diarrhoeal-type enterotoxin. *World Journal of Microbiology and Biotechnology* **10** 406-409.

Anonymous (1992) ISO/DIS 7932 Microbiology- General guidance for enumeration of *Bacillus cereus* colony-count technique at 30 °C, International Organization for Standardization, Switzerland.

Barry T, Powell R and F Gannon (1990) A general method to generate DNA probes for micro-organisms. *Biotechnology* **8** 233-236.

Bourque SN, Valéro JR, Mercier J, Lavoie MC and RC Levesque (1993) Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis*. *Applied and Environmental Microbiology* **59** 523-527.

Carlson CR, Caugant DA and A-B Kolstø (1994) Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. *Applied and Environmental Microbiology* **60** 1719-1725.

Claus D and RCW Berkeley (1986) Genus *Bacillus*. In Sneath PHA, Mair NS, Sharpe ME and JG Holt (eds.) *Bergey's Manual of Systematic Bacteriology* **2**, Williams and Wilkinson, Baltimore, 1104-1139.

Damgaard PH (1995) Diarrhoeal enterotoxin production by strains of *Bacillus thuringiensis* isolated from commercial *Bacillus thuringiensis*-based insecticides. *FEMS Immunology and Medical Microbiology* **12** 245-250.

Drobniewski FA (1993) *Bacillus cereus* and related species. *Clinical Microbiology Reviews* **6** 324-338.

Gonzalez JMJr, Brown BJ and BC Carlton (1982) Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *Bacillus thuringiensis* and *Bacillus cereus*. *Proceedings of the National Academy of Sciences USA* **79** 6951-6955.

Granum PE, Andersson A, Gayther C, Te Giffel MC, Larsen H, Lund T and K O'Sullivan (1996) Evidence for a further enterotoxin complex produced by *Bacillus cereus*. *FEMS Microbiology Letters* **141** 145-149.

Jackson SG, Goodbrand RB, Ahmed R and S Kasatiya (1995) *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. *Letters in Applied Microbiology* **21** 103-105.

Klijn N, Weerkamp AH and WM de Vos (1991) Identification of mesophilic lactic acid bacteria using PCR-amplified variable regions of 16S rRNA and specific DNA probes. *Applied and Environmental Microbiology* **57** 3390-3393.

Klijn N, Bovie C, Dommes J, Hoolwerf JD, Van der Waals CB, Weerkamp AH and FFJ Nieuwenhof (1994) Identification of *Clostridium tyrobutyricum* and related species using sugar fermentation, organic acid formation and DNA probes based on specific 16S rRNA sequences. *Systematic and Applied Microbiology* **17** 249-256.

Kramer JM and RJ Gilbert (1989) *Bacillus cereus* and other *Bacillus* species. In Doyle MP (ed.) *Foodborne bacterial pathogens*, Marcel Dekker, New York, 21-77.

Logan NA and RCW Berkeley (1984) Identification of *Bacillus* strains using the API system. *Journal of General Microbiology* **130** 1871-1882.

Maidak BL, Larsen M, McCaughey MJ, Overbeek R, Olson GJ, Fogel K, Blandy J and CR Woese (1994) The ribosomal database. *Nucleic Acids Research* **22** 3485-3487.

Nakamura LK and MA Jackson (1995) Clarification of the taxonomy of *Bacillus mycoides*. *International Journal of Systematic Bacteriology* **45** 46-49.

Wang R-F, Cao W-W and MG Johnson (1992) 16S rRNA-based probes and polymerase chain reaction method to detect *Listeria monocytogenes* cells added to foods. *Applied and Environmental Microbiology* **58** 2827-2831.

CHAPTER 4

INCIDENCE OF *BACILLUS CEREUS* AND *BACILLUS SUBTILIS* IN FOODS IN THE NETHERLANDS

ABSTRACT

The incidence of *B. cereus* and *B. subtilis* in various food products in the Netherlands was investigated. In total, 229 samples (milk, yeast, flour, pasta products, Chinese meals, cocoa, chocolate, bakery products, meat products, herbs and spices) were analysed. Of these 109 (48 %) contained *B. cereus*. The contamination level ranged from 10^2 - 10^6 bacteria g^{-1} or ml^{-1}. *B. subtilis* was present in 18 of 72 (25 %) samples examined, the levels varied from 10^2 - 10^5 bacteria g^{-1}. From 12 of these 72 (17 %) samples, both *B. cereus* and *B. subtilis* could be isolated.

In total, 91 presumptive *B. cereus* colonies were isolated from the various samples. According to the ISO confirmation tests and the carbohydrate patterns (API 50 CHB) 81 (89 %) of these isolates were confirmed to be *B. cereus*. Of the 50 suspect *B. subtilis* colonies, 10 isolates were shown to be *B. pumilus* based on inability to ferment sorbitol and carbohydrate utilization pattern in the API 50 CHB. Of the remaining 40 isolates 30 (75 %) were determined to be *B. subtilis* according to the confirmation tests and the carbohydrate patterns, 4 (10 %) were *B. licheniformis* and 6 (15 %) were shown to be *B. amyloliquefaciens*. These *Bacillus* species, belonging to the '*B. subtilis* group', are also suspected as food-poisoning agents.

Numbers of *B. subtilis* appeared to decrease more readily by processing and storage of food products than those of *B. cereus*. This phenomenon, and the fact that direct isolation methods for *B. subtilis* are not available, indicate that the role of *B. subtilis* in food spoilage and foodborne infections and intoxication may be underestimated.

This chapter has been published as:
Incidence of *Bacillus cereus* and *Bacillus subtilis* in foods in the Netherlands
MC Te Giffel, RR Beumer, S Leijendekkers and FM Rombouts
Food Microbiology (1996) **13** 53-58.

INTRODUCTION

Spore-forming micro-organisms are widely distributed in nature because of the resistance of their endospores to various stresses and their long term survival under unfavourable conditions. Therefore, most aerobic sporeformers can be isolated from a wide variety of sources including foods (Claus and Berkeley 1986).

Bacillus species are important as food-spoilage organisms, and can be isolated from fruits, vegetable products, nuts, cereals, milk and dairy products, meat, dried foods and spices (Goepfert *et al.* 1972, Johnson 1984). *B. cereus* is the predominant organism that determines the keeping quality of pasteurized milk(products) (Stewart 1975). The organism is associated with defects as off-flavours, sweet curdling and bitty cream caused by proteinase, lipase and phospholipase enzymes produced (Davies and Wilkinson 1973, Overcast and Atmaram 1974). *B. subtilis* is the causative agent of ropy (slimy) bread. The degradation of the bread-crumb is caused by the combined effects of microbial proteolytic and amylolytic enzymes, and the slime produced by certain rope-inducing *Bacillus* strains (Bailey and von Holy 1993). The prevalence of *B. subtilis* in other food products has received little attention, and only limited information on this is available.

Several *Bacillus* species have often been found to be responsible for outbreaks of food poisoning (Goepfert *et al.* 1972, Johnson 1984). Since 1950, there has been an increasing number of reports which have established *B. cereus* as a food-poisoning organism. Food poisoning caused by *B. cereus* is mostly associated with the consumption of rice and other starchy products (Johnson 1984, Kramer and Gilbert 1989). *Bacillus brevis*, *Bacillus licheniformis*, *B. subtilis* and *Bacillus sphaericus* have also been implicated in food poisoning (Johnson 1984, Claus and Berkeley 1986, Kramer and Gilbert 1989). Foods involved in incidents of foodborne illness where *B. subtilis* was considered the causative organism were pastry products, meat, seafood and rice (Kramer and Gilbert 1989).

This paper reports the incidence of *B. cereus* and *B. subtilis* in various food products in the Netherlands. The effect of storage conditions and preparation of food products on both organisms was also investigated.

MATERIALS AND METHODS

Samples

Samples were purchased from retail stores, restaurants and households. In total 229 foods were analysed for the presence of *B. cereus* and *B. subtilis*.

Enumeration and confirmation of *B. cereus* and *B. subtilis*

Samples were diluted 1: 10 in peptone saline solution (PSS; composition: NaCl (8.5 g l^{-1}) and Neutralized Bacteriological Peptone (Oxoid L34, 1 g l^{-1})) and homogenized using a Stomacher (Lab-Blender 400) for 2 min. Powder samples were rehydrated by soaking 10 g separately in 90 ml 0.85 % saline solution followed by homogenization using a Stomacher. Serial dilutions of the samples were prepared in PSS and surface plated on Tryptone Soya Agar (TSA; Oxoid CM131) and Mannitol Egg Yolk Polymyxin (MEYP) agar (Cereus Selective Agar, Merck 5267). To enumerate spores 10 ml of the primary dilution was heated at 80 °C for 10 min and cooled in melting ice before further serial dilution, followed by surface plating on TSA and MEYP. The heat activation step was applied within 5 min after homogenization in order to prevent germination of spores during the sample preparation [Chapter 2]. The plates were incubated at 30 °C for 24 h and examined for typical colonies.

From each MEYP plate, presumptive *B. cereus* colonies were selected for confirmation as specified in the ISO procedure (Anonymous 1992). Suspect colonies, pink colonies surrounded by a zone of precipitation, were counted and subsequently plated out on a non-selective medium i.e. TSA. Confirmation was carried out testing colonies for glucose fermentation, Voges-Proskauer (VP) reaction (production of acetylmethylcarbinol) and nitrate reduction. *B. cereus* strains should be positive for all three reactions (Anonymous, 1992). Furthermore, the isolates were identified using the API 50 CHB (BioMérieux Sa, France). Isolates were grown on TSA plates (18-24 h at 30 °C). Growth was harvested in 2 ml sterile 0.85 % saline solution to correspond with tube No. 2 of McFarland scale of standard opacities and 0.1 ml of this suspension was diluted in 10 ml of API 50 CHB medium. The strips were inoculated, incubated for 48 h at 30 °C and read after both 24 and 48 h. The results were scored according to the manufacturers instructions and the emerging biochemical profile was identified by means of APILAB software V 2.1, 1990.

Presumptive *B. subtilis*, large smooth or slimy colonies yellow on MEYP and white on TSA, were counted and streaked on TSA for purification. Strains were confirmed by glucose and sorbitol fermentation, VP reaction, nitrate reduction (*B. subtilis* should be positive for all four reactions) and carbohydrate metabolism using the API 50 CHB according to the procedure described above for *B. cereus*.

Effect of storage and preparation on *B. cereus* and *B. subtilis* in food products

Ten *B. cereus* and ten *B. subtilis* strains, isolated from various products, were used to investigate the effect of processing of food products on these organisms. The isolates were grown in Brain Heart Infusion broth (BHI; Difco 0037-01-6) for 24 h at 30 °C and the cell

suspensions (10^8 ml^{-1}) were diluted in 0.85 % saline solution. Vanilla pudding and fried rice, 100 g samples, were inoculated with 1 ml of 10^6 *B. cereus* and *B. subtilis*, resulting in an inoculum level of 10^4 cfu ml^{-1} for both organisms. The inoculum level was checked by plating on TSA. The vanilla pudding was stored at 7 °C for 24 h, the fried rice was stored at 7 °C for 24 h and -20 °C for 48 h. The numbers of *B. cereus* and *B. subtilis* were determined by plate counting on MEYP agar. Plates were incubated for 24 h at 30 °C and counted. The fried rice samples stored at 7 °C were also heated before enumeration for 20 s in a 1600 Watt microwave-oven without rotating plate (Panasonic NE-1670) reaching temperatures of 70 - 80 °C.

RESULTS AND DISCUSSION

The incidence of *B. cereus* and *B. subtilis* in various food products is listed in Table 4.1.

Table 4.1 Incidence and contamination levels (vegetative cells and/or spores) of *Bacillus cereus* and *Bacillus subtilis* in various food products in the Netherlands.

Sample	Number of samples	Number of samples positive for *B. cereus*	*B. subtilis*	*B. subtilis* + *B. cereus*
Pasteurized milk	157	56(10^0-10^4)[a]	ND(ND)	ND
Yeast	5	3 (ND)	-(-)	-
Flour	9	5 (10^3)	2(10^2-10^4)	-
Cocoa/chocolate	6	2 (10^3-10^5)	2(10^2-10^4)	-
Bakery products	10	9 (10^3-10^4)	1(10^3-10^4)	-
Herbs/spices/ seasonings[b]	6	6 (10^2-10^6)	6(10^3-10^5)	6
Meat(products)	10	9 (10^2-10^4)	2(10^2-10^4)	1
Pasta products	8	4 (10^4)	2(10^3)	2
Cinese meals	18	15(10^3-10^5)	3(10^2-10^4)	3
Total	229	109	18	12
Percentage		48 %	25 %	17 %

[a] contamination range of positive samples in cfu per g or ml
[b] garlic powder, pepper, cinnamon, chili powder, oregano, thyme
ND, not determined; -, not present

In total, 229 samples (milk, yeast, flour, pasta products, Chinese meals, cocoa, chocolate, bakery products, meat products, herbs and spices) were analyzed. Of these 109

(48 %) contained *B. cereus*, the contamination level ranged from 10^2 - 10^6 bacteria g^{-1} or ml^{-1}. *B. subtilis* was present in 18 of 72 (25 %) samples examined, the levels varied from 10^2 - 10^5 bacteria g^{-1}. From 12 of these 72 (17 %) samples both *B. cereus* and *B. subtilis* could be isolated. In almost all samples in which *Bacillus* species were present, the organisms were detected in both the unheated and the heated samples (data not shown). The fact that, at present, direct isolation methods for *B. subtilis* are not available may have partly contributed to the low incidence of this organism in food products compared with *B. cereus*.

In total, 91 presumptive *B. cereus* colonies were isolated from the various samples on MEYP agar. Of these, 81 (89 %) were determined to be *B. cereus* through use of both the ISO confirmation tests and the carbohydrate patterns (API 50 CHB). This percentage is comparable with the 90 % noted earlier for *B. cereus* isolated from various sources at farms (Te Giffel *et al.* 1995b [Chapter 5]). Other *Bacillus* species identified were *B. circulans* (3), *B. brevis* (2) and *B. laterosporus* (5). Of the 50 *B. subtilis* suspect colonies isolated 10 strains were shown to be *B. pumilus*, on the basis of inability to ferment sorbitol and by the carbohydrate pattern in the API 50 CHB. Of the remaining 40 isolates, 30 (75 %) were confirmed to *B. subtilis*, according to both the confirmation tests and the carbohydrate patterns, 4 (10 %) were *B. licheniformis* and 6 (15 %) were shown to be *B. amyloliquefaciens*. These organisms, that are closely related to *B. subtilis*, are important because they can also cause food-poisoning.

There are several studies describing the incidence of *B. cereus* in food ingredients and food products. In contrast, there is little information available on the occurrence of *B. subtilis* in food. In general, the contamination levels found in this study confirm those observed in similar studies, however, for some products the levels observed were somewhat higher compared with levels reported by others (Kim and Goepfert 1971, Kaur 1986, Kramer and Gilbert 1989). This can be explained by the fact that the time between preparation of the primary solution and the application of a heat-activation step should be as short as possible in order to prevent germination during sample preparation. This will lead to underestimation of the actual level of spores of spore-forming micro-organisms present in a sample (Te Giffel *et al.* 1995a [Chapter 2]).

The frequency of *B. cereus* in pasteurized milk was comparable to those reported by others, two to 35 % of the samples analyzed are reported to contain *B. cereus*; numbers ranging from less than 10 to 1000 per ml (Ahmed *et al.* 1983, Johnson 1984, Wong *et al.* 1988, Kramer and Gilbert 1989, Mosso *et al.* 1989, Van Netten *et al.* 1990).

Both *Bacillus* species were isolated from raw bakery materials, *B. cereus* was present in 55 and 60 % of the flour and yeast samples, respectively. *B. subtilis* was only found in flour samples (22 %). The percentage of wheat flours reported to contain *B. cereus* ranged from 16 to 90 %. The numbers present were low and ocassionally more than 10 per g

(Johnson 1984, Kaur 1986, Kramer and Gilbert 1989, Mosso *et al.* 1989). In a similar study, *B. subtilis* occurred in 38 % of raw bakery ingredients (flour (10^2- 10^3), yeast (10^5-10^6), crumbs, premix and water). *B. cereus* could not be detected in any of the samples (Bailey and von Holy 1993). Because these organisms survive as spores during baking they may, depending on storage conditions, germinate and multiply to high levels and cause spoilage of these products or even lead to food poisoning or foodborne infections (Kramer and Gilbert 1989).

According to literature, the incidence of *B. cereus* in selected dried products (herbs, spices, seasonings, dried soups) is 4-100 %. Most of those dried products are highly contaminated with sporeforming micro-organisms, up to 10^5 per g (Johnson 1984, Konuma *et al.* 1988, Kramer and Gilbert 1989, Mosso *et al.* 1989, Van Netten *et al.* 1990, Harmon and Kautter 1991, Rusul and Yaacob 1995). *B. subtilis* was isolated from 60 % of Nigerian spices (Antai 1988). In this study all herbs, spices and seasonings were found to contain *B. cereus* and *B. subtilis*, in levels up to 10^6 or 10^5 per g, respectively.

In pasta products and Chinese meals, *B. cereus* was observed in 50 and 83 % of the samples analysed, respectively. The contamination level was 10^3 - 10^5 per g. The incidence of *B. subtilis* in these products was lower, the organism occurred in 25 % of the pasta products, and in 17 % of the Chinese meals. The contamination levels were comparable with those of *B. cereus*. In similar studies *B. cereus* was found in 3-12 % of such products, numbers varying from less than 100 up to 10^5 per g (Johnson 1984, Van Netten *et al.* 1990). Rice and pasta may be contaminated, because *Bacillus* was found in 40-100 % of samples of these raw materials (Johnson 1984, Kramer and Gilbert 1989, Harmon and Kautter 1991, Da Silva *et al.* 1993). However, *Bacillus* species are most likely introduced in these products via the additives used, e.g. herbs, spices and seasonings.

The high prevalence of *Bacillus* species in meat products compared with raw meat (Konuma *et al.* 1988, Kramer and Gilbert 1989), confirms the role played by meat additives such as spices, seasonings, proteins, starch, salt, sugar and colorants as a source of contamination.

All of the herbs, spices and seasonings tested contained both *B. cereus* and *B. subtilis*. However, in the products in which these agents are used as additives, e.g. meat products, Chinese meals and pasta products, *B. cereus* was the predominant organism. A possible explanation is that *B. subtilis* could be more affected by processing, storage conditions and preparation of food than *B. cereus*. Both organisms were influenced to the same extent by storage, i.e. cooling and freezing, resulting in limited decreases in the numbers present as listed in Table 4.2. However, *B. subtilis* was more affected by cooking, and the numbers decreased almost with 3 log units to below the detection level in all samples. This effect can not be explained by differences in heat-resistance because, in general, the D-values reported

for *B. cereus* vegetative cells are lower than for *B. subtilis* cells: in phosphate buffer the D-value reported for *B. cereus* was 10 min at 47-48 °C compared with 10 min at 55-57 °C for *B. subtilis* (Mitscherlich and Marth 1984). Heat-resistance profiles for *Bacillus* spores determined in quarter-strength Ringer's solution also indicated that *B. cereus* was less resistant than *B. subtilis*: 6 % of the *B. cereus* spores survived steaming for 10 min at 110 °C compared with 80 % of the *B. subtilis* spores (Davies 1975).

Table 4.2 Effect of storage (7 or -20 °C) and preparation (micro-wave cooking) on *Bacillus cereus* and *Bacillus subtilis* in food products.

Sample	Storage temperature (°C)	Storage time (h)	Reduction[a] of *B. cereus*	Reduction[a] of *B. subtilis*
Vanilla pudding	7	24	-0.10 ± 0.27	-0.26 ± 0.43
Fried rice	7	24	0.12 ± 0.44	-0.02 ± 0.32
Fried rice[b]	7	24	-1.07 ± 0.85	-2.68 ± 0.22
Fried rice	-20	48	-0.24 ± 0.40	-0.02 ± 0.28

[a] log N (after storage i.e. t= 24 or 48 h) - log N (t = 0); mean and standard deviation for 10 experiments;
[b] after storage at 7 °C these samples were heated for 20 s in a microwave-oven

Because it is still not known whether foodborne infections/intoxications caused by *Bacillus* species are a result of preformed toxins or of ingestion of cells and/or spores, the findings in this study suggest that in outbreaks in which *B. cereus* is isolated from the food available for analysis and is mentioned as the causative micro-organism, the outbreak may in fact have been caused by *B. subtilis*. Therefore, the number of outbreaks in which *B. subtilis* is involved may be under-reported.

The results in this study suggest that there may be two reasons why the role of *B. subtilis* in food spoilage and foodborne infections and intoxications may be underestimated up until now. First, there is no selective isolation medium for *B. subtilis* available and, second, the organism is more affected during storage and preparation of food than *B. cereus*. The potential hazard of the presence of this organism in food or food ingredients should therefore not be ignored.

REFERENCES

Ahmed AA-H, Moustafa MK and EH Marth (1983) Incidence of *Bacillus cereus* in milk and some milk products. *Journal of Food Protection* **46** 126-128.

Anonymous (1992) ISO/DIS 7932 Microbiology- General guidance for enumeration of *Bacillus cereus*- colony-count technique at 30 °C, International Organization for Standardization, Switzerland.

Antai SP (1988) Study of the *Bacillus* flora of Nigerian spices. *International Journal of Food Microbiology* **6** 259-261.

Bailey CP and A von Holy (1993) *Bacillus* spore contamination associated with commercial bread manufacture. *Food Microbiology* **10** 287-294.

Claus D and RCW Berkeley (1986) Genus *Bacillus*. In Sneath PHA, Mair NS, Sharpe ME and JG Holt (eds.) *Bergey's Manual of Systematic Bacteriology* 2, Williams and Wilkinson, Baltimore, 1104-1139.

Da Silva SM, Rabinovitch, L and PG Robbs (1993) Quantification and behavioural characterization of *Bacillus cereus* in formulated infant foods. I. Generation time. *Revista Microbiologica* **24** 125-131.

Davies FL (1975) Discussion of papers presented at symposium on bitty cream and related problems. *Journal of the Society of Dairy Technology* **28** 85-90.

Davies FL and G. Wilkinson (1973) *Bacillus cereus* in milk and dairy products In BC Hobbs and JHB Christian (eds.) *The Microbiological Safety of Foods*, Academic Press, London, 57-67.

Goepfert JM, Spira WM and HU Kim (1972) *Bacillus cereus*: food poisoning organism. A review. *Journal of Milk and Food Technology* **35** 213-225.

Harmon SM and DA Kautter (1991) Incidence and growth potential of *Bacillus cereus* in ready-to-serve foods. *Journal of Food Protection* **54** 372-374.

Johnson KM (1984) *Bacillus cereus* in food-borne illness-An update. *Journal of Food Protection* **47** 145-153.

Kaur P (1986) Survival and growth of *Bacillus cereus* in bread. *Journal of Applied Bacteriology* **60** 513-516.

Kim HU and JM Goepfert (1971) Occurrence of *Bacillus cereus* in selected dry food products. *Journal of Milk and Food Technology* **34** 12-15.

Konuma H, Shinagawa K, Tokumaru M, Onoue Y, Konno S, Fujino N, Shigehisa T, Kurata H, Kuwabara Y and CAM Lopes (1988) Occurrence of *Bacillus cereus* in meat products, raw meat and meat product additives. *Journal of Food Protection* **51** 324-326.

Kramer JM and RJ Gilbert (1989) *Bacillus cereus* and other *Bacillus* species. In MP Doyle (ed.) *Foodborne bacterial pathogens*, Marcel Dekker, New York, 21-77.

Mitscherlich E and EH Marth (1984) *Microbial Survival in the Environment*, Springer-Verlag, Berlin Heidelberg New York Tokyo 802p.

for *B. cereus* vegetative cells are lower than for *B. subtilis* cells: in phosphate buffer the D-value reported for *B. cereus* was 10 min at 47-48 °C compared with 10 min at 55-57 °C for *B. subtilis* (Mitscherlich and Marth 1984). Heat-resistance profiles for *Bacillus* spores determined in quarter-strength Ringer's solution also indicated that *B. cereus* was less resistant than *B. subtilis*: 6 % of the *B. cereus* spores survived steaming for 10 min at 110 °C compared with 80 % of the *B. subtilis* spores (Davies 1975).

Table 4.2 Effect of storage (7 or -20 °C) and preparation (micro-wave cooking) on *Bacillus cereus* and *Bacillus subtilis* in food products.

Sample	Storage temperature (°C)	Storage time (h)	Reduction[a] of *B. cereus*	Reduction[a] of *B. subtilis*
Vanilla pudding	7	24	-0.10 ± 0.27	-0.26 ± 0.43
Fried rice	7	24	0.12 ± 0.44	-0.02 ± 0.32
Fried rice[b]	7	24	-1.07 ± 0.85	-2.68 ± 0.22
Fried rice	-20	48	-0.24 ± 0.40	-0.02 ± 0.28

[a] log N (after storage i.e. t= 24 or 48 h) - log N (t = 0); mean and standard deviation for 10 experiments;
[b] after storage at 7 °C these samples were heated for 20 s in a microwave-oven

Because it is still not known whether foodborne infections/intoxications caused by *Bacillus* species are a result of preformed toxins or of ingestion of cells and/or spores, the findings in this study suggest that in outbreaks in which *B. cereus* is isolated from the food available for analysis and is mentioned as the causative micro-organism, the outbreak may in fact have been caused by *B. subtilis*. Therefore, the number of outbreaks in which *B. subtilis* is involved may be under-reported.

The results in this study suggest that there may be two reasons why the role of *B. subtilis* in food spoilage and foodborne infections and intoxications may be underestimated up until now. First, there is no selective isolation medium for *B. subtilis* available and, second, the organism is more affected during storage and preparation of food than *B. cereus*. The potential hazard of the presence of this organism in food or food ingredients should therefore not be ignored.

REFERENCES

Ahmed AA-H, Moustafa MK and EH Marth (1983) Incidence of *Bacillus cereus* in milk and some milk products. *Journal of Food Protection* **46** 126-128.

Anonymous (1992) ISO/DIS 7932 Microbiology- General guidance for enumeration of *Bacillus cereus*- colony-count technique at 30 °C, International Organization for Standardization, Switzerland.

Antai SP (1988) Study of the *Bacillus* flora of Nigerian spices. *International Journal of Food Microbiology* **6** 259-261.

Bailey CP and A von Holy (1993) *Bacillus* spore contamination associated with commercial bread manufacture. *Food Microbiology* **10** 287-294.

Claus D and RCW Berkeley (1986) Genus *Bacillus*. In Sneath PHA, Mair NS, Sharpe ME and JG Holt (eds.) *Bergey's Manual of Systematic Bacteriology* **2**, Williams and Wilkinson, Baltimore, 1104-1139.

Da Silva SM, Rabinovitch, L and PG Robbs (1993) Quantification and behavioural characterization of *Bacillus cereus* in formulated infant foods. I. Generation time. *Revista Microbiologica* **24** 125-131.

Davies FL (1975) Discussion of papers presented at symposium on bitty cream and related problems. *Journal of the Society of Dairy Technology* **28** 85-90.

Davies FL and G. Wilkinson (1973) *Bacillus cereus* in milk and dairy products In BC Hobbs and JHB Christian (eds.) *The Microbiological Safety of Foods*, Academic Press, London, 57-67.

Goepfert JM, Spira WM and HU Kim (1972) *Bacillus cereus*: food poisoning organism. A review. *Journal of Milk and Food Technology* **35** 213-225.

Harmon SM and DA Kautter (1991) Incidence and growth potential of *Bacillus cereus* in ready-to-serve foods. *Journal of Food Protection* **54** 372-374.

Johnson KM (1984) *Bacillus cereus* in food-borne illness-An update. *Journal of Food Protection* **47** 145-153.

Kaur P (1986) Survival and growth of *Bacillus cereus* in bread. *Journal of Applied Bacteriology* **60** 513-516.

Kim HU and JM Goepfert (1971) Occurrence of *Bacillus cereus* in selected dry food products. *Journal of Milk and Food Technology* **34** 12-15.

Konuma H, Shinagawa K, Tokumaru M, Onoue Y, Konno S, Fujino N, Shigehisa T, Kurata H, Kuwabara Y and CAM Lopes (1988) Occurrence of *Bacillus cereus* in meat products, raw meat and meat product additives. *Journal of Food Protection* **51** 324-326.

Kramer JM and RJ Gilbert (1989) *Bacillus cereus* and other *Bacillus* species. In MP Doyle (ed.) *Foodborne bacterial pathogens*, Marcel Dekker, New York, 21-77.

Mitscherlich E and EH Marth (1984) *Microbial Survival in the Environment*, Springer-Verlag, Berlin Heidelberg New York Tokyo 802p.

Mosso MA, Garcia Arribas ML, Cuena JA and MC De La Rosa (1989) Enumeration of *Bacillus* and *Bacillus cereus* spores in food from Spain. *Journal of Food Protection* **52** 184-188.

Overcast WW and K Atmaram (1974) The role of *Bacillus cereus* in sweet-curdling of fluid milk. *Journal of Milk and Food Technology* **37** 233-236.

Rusul G and NH Yaacob (1995) Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA. *International Journal of Food Microbiology* **25** 131-139.

Stewart DB (1975) Factors influencing the incidence of *Bacillus cereus* spores in milk. *Journal of the Society of Dairy Technology* **28** 80-85.

Te Giffel MC, Beumer RR, Hoekstra J, and FM Rombouts (1995a) Germination of bacterial spores during sample preparation. *Food Microbiology* **12** 327-332.

Te Giffel MC, Beumer RR, Slaghuis BA and FM Rombouts (1995b) Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands. *Netherlands Milk and Dairy Journal* **49** 125-138.

Van Netten P, van Moosdijk AM and DAA Mossel (1990) Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *Journal of Applied Bacteriology* **69** 73-79.

Wong HC, Chang MH and JY Fan (1988) Incidence and characterisation of *Bacillus cereus* isolates contaminating dairy products. *Applied and Environmental Microbiology* **54** 699-702.

CHAPTER 5

OCCURRENCE AND CHARACTERIZATION OF (PSYCHROTROPHIC) *BACILLUS CEREUS* ON FARMS IN THE NETHERLANDS

ABSTRACT

In order to determine the major contamination sources of raw milk with (psychrotrophic) *Bacillus cereus*, the incidence of *B. cereus* spores and vegetative cells on farms in the Netherlands was investigated. Samples were taken from air, soil, grass, bedding (used and unused), feed (hay, silage, concentrate, beet), drinking water, faeces, udders and milk. Some biochemical and growth characteristics of the strains isolated were examined.

In total 847 presumptive *B. cereus* colonies were isolated from all sources examined. The levels present in the various samples ranged from < 10 up to 10^7 per g or ml for vegetative cells and < 10 to 10^5 per g or ml for spores. According to the ISO confirmation tests and/or the carbohydrate patterns (API 50 CHB), 766 (90 %) of these isolates were confirmed to be *B. cereus*. The carbohydrate patterns revealed more than 30 different *B. cereus* types. No distinct relation between the biochemical characteristics and the contamination source was observed. The major contamination sources of *B. cereus* were soil and faeces. In winter when cows are housed, used bedding probably also participates in this contamination route. The udder will be contaminated, finally resulting in the presence of these organisms in raw milk.

About 40 % of the isolated strains showed growth at 7 °C and were considered to be psychrotrophic. Of the strains isolated from raw milk 30 % were able to grow at 7 °C. The presence of psychrotrophic spores in raw milk will lead to their presence in pasteurized milk due to heat-resistance properties. Since at low storage temperatures these psychrotrophs will germinate, grow and subsequently spoil the product faster than mesophilic types, the psychrotrophic strains will have a more significant impact on the keeping quality of milk.

This chapter has been published as:

Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands

MC Te Giffel, RR Beumer, BA Slaghuis and FM Rombouts

Netherlands Milk and Dairy Journal (1995) **49** 125-138.

INTRODUCTION

The presence of thermoduric, spore-forming bacteria in milk and milk products has been well documented and extensively investigated over several decades. The most important heat-resistant species belong to the genus *Bacillus* (Collins 1981). In various surveys, *Bacillus cereus* has been found to occur in 9 to 37 % of raw milk samples, the numbers present varying from < 10 to a maximum of 100 per ml (Donovan 1959, Martin *et al.* 1962, Ahmed *et al.* 1983, Rangasamy *et al.* 1993).

It has been shown that *B. cereus* is the predominant organism that determines the keeping quality of pasteurized milk (Stewart 1975). In previous studies *B. cereus* was isolated from 2 to 35 % of pasteurized milk samples; levels of contamination ranging from < 10 to 1000 per ml have been quoted (Ahmed *et al.* 1983, Krusch 1986, Wong *et al.* 1988, Rangasamy *et al.* 1993). The organism is associated with defects such as off-flavours, sweet curdling and bitty cream caused by proteinase, lipase and phospholipases produced by the bacteria (Davies and Wilkinson 1973, Overcast and Atmaram 1974). In addition to causing these effects in dairy products, *B. cereus* has also been associated with outbreaks of food poisoning (Johnson 1984). Despite the worldwide occurrence of this bacterium in milk, surprisingly few reports on food poisoning caused by *B. cereus* in milk and cream have been reported (Christiansson *et al.* 1989).

Trends in the dairy industry such as the extended refrigerated storage of raw milk prior to processing, the application of higher pasteurization temperatures and prolonged shelf-life requirements have enhanced the importance of thermoduric psychrotrophs (Meer *et al.* 1991). The combination of heat-resistance and psychrotrophic properties represents substantial potential for causing spoilage of perishable milk products. The International Dairy Federation (IDF) defined psychrotrophs as micro-organisms that can grow at 7 °C or less, irrespective of their optimum growth temperature (Collins 1981). Psychrotrophs are ubiquitous; their natural habitats are soil, water, plants and animals (Cousin 1982). Psychrotrophic *Bacillus* species are the predominant organisms in (pasteurized) milk and milk products, although psychrotrophs also include heat-tolerant members of the genera *Clostridium*, *Arthrobacter*, *Microbacterium*, *Streptococcus* and *Corynebacterium* (Thomas *et al.* 1962, Credit *et al.* 1972, Washam *et al.* 1977, Johnston and Bruce 1982). The incidence of psychrotrophic *B. cereus* in raw milk varies from 23 to 83 % of the total psychrotrophic spore count, levels ranging from 1 to 1600 spores per l (Chung and Cannon 1971, Shehata and Collins 1971, Cannon 1972, Grosskopf and Harper 1974, Coghill and Juffs 1979, Johnston and Bruce 1982, McKinnon and Pettipher 1983, Griffiths and Phillips 1990, Griffiths 1993). In one study 70 % of the heat-tolerant psychrotrophic organisms in freshly pasteurized milk belonged to the genus *Bacillus*, of which more than 75 % was *B. cereus*

(Griffiths and Phillips 1990). In other studies *B. cereus* was present in about 30 % of pasteurized milk samples (Overcast and Atmaram 1974, Coghill and Juffs 1979, Griffiths 1993).

Milk obtained from cows with a healthy udder contains relatively low numbers of (pathogenic) micro-organisms (Walker 1988). Since *B. cereus* is widely distributed in the environment, the organism can be introduced into raw milk from soil, air, water, bedding, feeds, pasture, the udder, excreta from the cows and milking equipment (Walker 1988, Van Heddeghem and Vlaemynck 1993).

This paper presents a study on the contamination sources and the occurrence of (psychrotrophic) *B. cereus* in raw milk on six farms, geographically spread in the Netherlands. The isolated strains were used to investigate some biochemical and growth characteristics.

MATERIALS AND METHODS

Sample collection

Samples were collected over one year, from six experimental farms geographically spread in the Netherlands, in collaboration with the Research Station for Cattle, Sheep and Horse Husbandry, Lelystad, The Netherlands. The farms were visited three or four times during this period. Samples were taken from air (using a surface air system method, sampling 0.12 m^3 in 2 min (pool bioanalysis italiana (pbi), Milano, Italy)), soil, grass, bedding (used and unused; taken from 10 % of the cubicles), feed (hay, silage, concentrate, beet), drinking water and faeces. At each visit, faeces and udder samples were taken from 10 individual cows using swabs; these were stored in peptone saline solution (PSS; NaCl 8.5 g l^{-1} and Neutralized Bacteriological Peptone (Oxoid L34) 1 g l^{-1}). Milk samples were taken from 10 individual cows (100 ml) and from the bulk tank (250 ml) and kept refrigerated until examination.

Isolation and enumeration of *B. cereus*

Samples of soil, grass, bedding, feedstuffs and faeces were diluted 1: 10 in PSS and homogenized using a Stomacher (Lab-Blender 400) for 2 min. Serial dilutions of the samples were prepared in PSS and surface-plated on Mannitol Egg Yolk Polymyxin (MEYP) agar (Cereus Selective Agar, Merck 5267). To enumerate spores 10 ml of the primary dilution was heated at 80 °C for 10 min and cooled in melting ice prior to further serial dilution, followed by surface plating on MEYP. The heat-activation step was applied within 5 min

after homogenization in order to prevent germination of spores during the sample preparation [Chapter 2].

The swabs with samples from the faeces and the teats of the individual cows were streaked on MEYP agar.

Since low numbers were expected in the milk samples, 100 ml of milk from the individual cows or 250 ml of the bulk tank milk was incubated shaken at 30 °C for about 6 h. Then, 10 ml was centrifuged for 30 min at 2000 *g*. Pellets were resuspended in 0.1 ml PSS and plated on MEYP agar.

The MEYP plates were incubated at 30 °C for 24 h and examined for typical colonies. From each plate presumptive colonies were selected for confirmation as specified in the ISO procedure (ISO/DIS 7932 General guidance for enumeration of *Bacillus cereus* colony count technique at 30 °C). Suspect colonies (pink colonies surrounded by a zone of precipitation) were counted and subsequently plated out on a non-selective medium i.e. Tryptone Soya Agar (TSA; Oxoid CM 131). The identity of colonies was confirmed by testing them for glucose fermentation, Voges-Proskauer (VP) reaction (production of acetylmethylcarbinol) and nitrate reduction. *B. cereus* strains should be positive for all three reactions (Anonymous 1992).

Growth at 7 °C

To determine whether the isolated strains were psychrotrophic they were grown in Brain Heart Infusion broth (BHI; Difco 0037-01-6) for 24 h at 30 °C. The cell suspensions (10^8 ml^{-1}) were diluted in PSS. Tubes containing 10 ml of BHI or UHT sterilized milk, precooled to 4 °C, were inoculated to a final level of 1-10 cfu per ml. This level was checked by surface streaking on MEYP agar. After 7 days of incubation at 7 °C, 0.1 ml of 1000-fold dilutions of BHI and milk were plated on MEYP agar. These plates were incubated at 7 °C and counted after 7 days; more than 100 colonies ($> 10^5$ cfu ml^{-1}) was considered to be positive i.e. psychrotrophic.

Carbohydrate metabolism

The carbohydrate metabolism of the isolated strains was examined using API 50 CHB strips (BioMérieux Sa, France). Isolates were grown on TSA plates (18-24 h at 30 °C). Growth was harvested in 2 ml sterile 0.85 % saline solution to correspond with tube No. 2 of the McFarland scale of standard opacities and 0.1 ml of this suspension was diluted in 10 ml of API 50 CHB medium. The strips were inoculated, incubated for 48 h at 30 °C and read after both 24 and 48 h. The results were scored according to the manufacturer's

instructions and the emerging biochemical profile was identified by means of APILAB software V 2.1, 1990.

RESULTS

Identity of the isolates

In total, 847 suspected colonies were isolated from the different samples on MEYP agar. When these presumptive colonies were checked by the ISO standard procedure, 152 (17.9 %) strains were not confirmed to be *B. cereus*: 3 strains (0.4 %) were negative for glucose fermentation, 81 (9.6 %) were VP negative and 41 (4.8 %) were unable to reduce nitrate. Furthermore, 24 strains (2.8 %) gave negative reactions on both VP reaction and nitrate reduction and 3 strains (0.4 %) were negative on all three confirmation tests.

Subsequent identification by API 50 CHB showed that 65 strains (7.7 % of the original isolates), confirmed by the ISO tests, were not considered to be *B. cereus*. However, 71 strains (8.4 %) that were negative following the ISO confirmation gave a typical *B. cereus* pattern in the API 50 CHB. Of these strains one strain (0.1 %) was glucose negative, 46 (5.4 %) were negative for the VP reaction and 30 (3.5 %) were unable to reduce nitrate. Moreover, 81 strains (9.6 %) were not regarded as *B. cereus* according to both the ISO confirmation tests and their carbohydrate metabolism. Thus, in total, 766 (90.4 %) of the 847 suspected colonies isolated on MEYP agar were shown to be *B. cereus* following ISO confirmation tests and/or API patterns.

Several other *Bacillus* species were found using the API 50 CHB system following identification of strains confirmed by the ISO method. The *Bacillus* species isolated were *B. alvei*, *B. brevis*, *B. circulans*, *B. megaterium*, *B. laterosporus*, *B. licheniformis*, *B. macerans*, *B. mycoides*, *B. pumilus*, *B. sphaericus* and *B. thuringiensis*. Several species of the genus *Bacillus*, i.e. *B. mycoides*, *B. anthracis*, *B. thuringiensis* and *B. laterosporus*, are egg yolk positive. In addition, the precipitation might also be caused by factors other than lecithinase production (Kim and Goepfert 1971). Especially *B. laterosporus* and *B. mycoides* were frequently isolated in this study. *B. mycoides* can be differentiated from *B. cereus* by the rhizoid growth shown by this organism on agar plates. In contrast to *B. cereus*, *B. laterosporus* is able to ferment mannitol. However, the reaction of colonies on mannitol is often impossible to ascertain due to masking of non-fermentative colonies by the acid generated by proximal actively fermenting colonies. This is mainly observed in samples containing high numbers of competitive organisms.

Carbohydrate utilization patterns

Table 5.1 Carbohydrate utilization patterns of *Bacillus cereus* strains (in %), as determined with API 50 CHB.
Isolates from this study compared to those from other reports.

	Origin of strains			Other studies		
	API[a]	Farms	Culture collection	Logan and Berkeley[b] all	emetic	Various products[c]
Carbohydrate						
Glycerol	93	100	26	92	70	
Ribose	99	100	97	97	93	92
Galactose	1	2	2	6	6	23
Glucose	100	100	100	100	100	100
Fructose	100	99	100	98	100	100
Mannose	14	24	19	0	3	46
Inositol	16	24	0	4	0	0
α-methyl-D-glucoside	4	2	0	2	0	0
N-acetyl-glucosamine	100	100	97	99	100	100
Amygdalin	50	40	2	8	0	19
Arbutin	99	100	85	91	60	92
Esculin	100	100	82	100	100	100
Salicin	99	98	65	87	0	92
Cellobiose	94	96	53	84	43	27
Maltose	100	100	100	98	100	100
Lactose	< 1	1	0	8	0	15
Saccharose	34	21	63	47	83	50
Trehalose	100	100	100	98	100	100
Starch	98	98	96	96	6	89
Glycogen	99	100	95	92	10	89
β- Gentiobiose	6	3	0	18	0	0
Turanose	< 1	0	0	15	6	0
Fucose	0	0	0	0	3	0
Gluconate	25	71	0	29	40	0
Number of strains		766	90	119	30	26

[a] *B. cereus* results according to API (BioMérieux Sa, France)
[b] Logan and Berkeley (1984)
[c] Rangasamy *et al.* (1993)

The results of carbohydrate metabolism of 766 strains revealed that there were many different types of *B. cereus* strains present: more than 30 patterns were observed. In Table 5.1 the results of the API 50 CHB of this study are compared to results obtained in other investigations. Generally, the percentage of positive results for the biochemical reactions of *B. cereus* obtained in this study are in line with those reported by others (Logan and Berkeley 1984, Rangasamy *et al.* 1993). There was however a higher percentage of strains utilizing inositol, amygdalin, salicin and cellobiose and a lower percentage of strains utilizing saccharose.

Several substrates were negative for all *B. cereus* strains tested: erythritol, arabinose, xylose, adonitol, β-methyl-D xyloside, sorbose, rhamnose, dulcitol, mannitol, sorbitol, α-methyl-D mannoside, melibiose, inuline, melizitose, raffinose, xylitol, lyxose, tagatose, fucose, arabitol, 2-ketogluconate, 5-ketogluconate. More than 90 % of the strains were able to utilise glycerol, ribose, glucose, fructose, N-acetylglucosamine, arbutin, aesculin, salicin, cellobiose, maltose, trehalose, starch and glycogen. For the other substrates the utilization as carbon source varied between < 10 and 50 %.

Distribution of isolates among sampling sites

B. cereus strains were isolated from all contamination sources examined (Table 5.2). No major differences in contamination levels between the six farms were observed (data not shown). There were however considerable differences in the numbers found in the samples. Highest counts for both vegetative cells and spores were observed in soil (10^3-10^7 per g for vegetative cells and 10^2-10^5 per g for spores), grass, silage, concentrate and faeces (up to 10^4 per g for vegetative cells and up to 10^4 per g for spores). Used bedding occasionally contained high numbers of spores (10^4 per g). Low numbers (< 10 - 10/ ml or g) of vegetative cells and/or spores were present in drinking water and unused bedding. The frequency diagram (Figure 5.1) shows the number of samples of each type with different levels of *B. cereus* spores. High spore counts were most frequently observed in soil and faeces. All unused bedding, drinking water and air samples could be placed in the first two intervals: numbers of *B. cereus* spores present varied from less than 10 to less than 100 per g, ml or m^3. *B. cereus* was observed in 0 to 7 and 1 to 10 of the udder and faeces samples, respectively, of individual cows per farm per sampling.

There was no significant variation between farms in the percentage of positive faeces samples (Table 5.3). For farms II and V the percentage of positive udder and faeces samples was somewhat higher; this is probably due to the fact that on these farms the cows were housed on only one of three or four visits compared to two of three or four visits on other farms. However, this did not result in substantial differences in milk contamination levels.

The results for the milk from individual cows showed that *B. cereus* was present in 51 (27 %) of the 190 samples analyzed. The organism was isolated from only one of 19 equipment samples taken by means of a rinse water sample. Seven of the 19 (36 %) bulk tank milk samples were found to contain *B. cereus* (data not shown).

Table 5.2 Presence of *Bacillus cereus* vegetative cells and spores in environmental samples on farms

Sample	Vegetative cells (N/ g, ml or m^3)	Spores (N/ g, ml or m^3)
Environmental samples		
Air	< 10 - 28	
Soil	10^3 - 10^7	10^2 - 10^5
Grass	10^2 - 10^4	10^1 - 10^3
Unused bedding	< 10 - 10	< 10 - 10
Used bedding	< 10 - 10^3	10^1 - 10^4
Silage	10^1 - 10^4	< 10 - 10^4
Concentrate	< 10 - 10^4	< 10 - 10^4
Drinking water	< 10	< 10
Faeces	< 10 - 10^4	10^2 - 10^4
Samples from individual cows		
	Number of cows positive for *B.cereus*[a]	
Udder	0 - 7	
Faeces	1 -10	
Milk	0 - 7	

[a] number of cows positive for *B. cereus* of the 10 cows sampled per visit

Table 5.3 Percentage of *Bacillus cereus* positive individual cow samples per farm

Farm	Sample		
	Udder	Faeces	Milk
I	27[a]	52	20
II	50	70	20
III	27	57	40
IV	17	67	27
V	40	80	40
VI	27	40	13

[a] percentage of samples positive for *B. cereus*

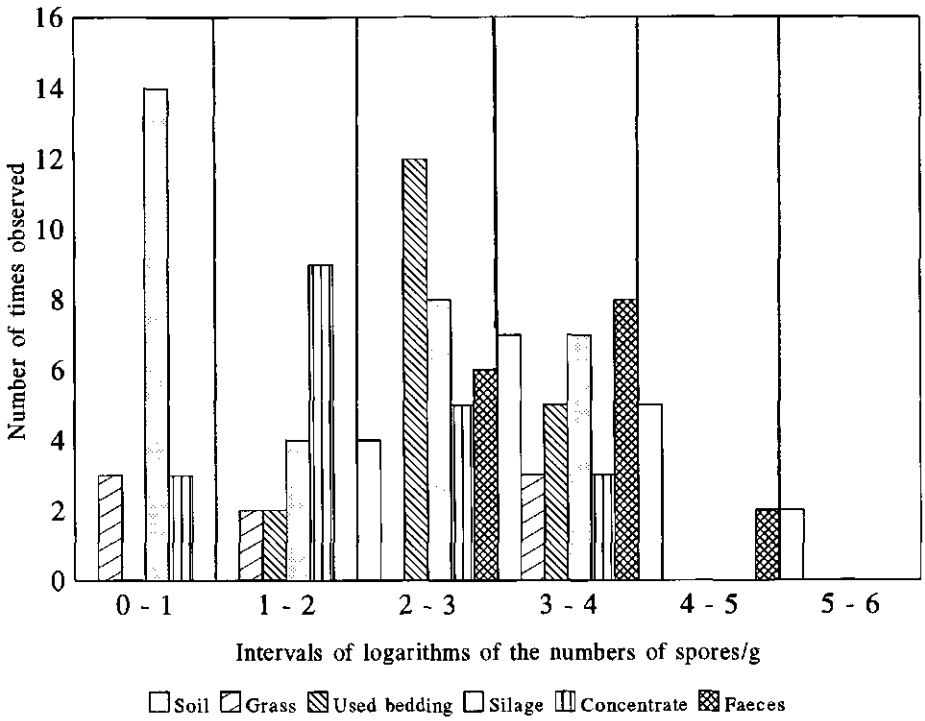


Figure 5.1 Numbers of *Bacillus cereus* spores in environmental samples on farms

Psychrotrophic growth

In total, 329 (43 %) of the 757 strains tested were able to grow at 7 °C (Table 5.4). For the environmental samples highest levels were found in soil, grass and faeces, in which 66, 63 and 66 % of the isolates were psychrotrophic, respectively. None of the strains found in concentrate was able to grow at 7 °C. Of the strains isolated from other sources the percentage of psychrotrophs varied between 24 and 42 %. For the individual cow samples 66 % of the faeces strains and 55 % of the udder strains appeared to be psychrotrophic. In milk about 30 % of the isolated strains were able to grow at 7 °C.

Table 5.4 The presence of psychrotrophic *Bacillus cereus*

	Number of strains isolated	Psychrotrophic strains	Percentage of psychrotrophs (%)
Environmental samples			
Air	31	13	42
Soil	59	39	66
Grass	19	12	63
Hay	15	5	33
Unused bedding	29	7	24
Used bedding	38	12	32
Silage	84	33	39
Concentrate	66	-	0
Drinking water	14	5	36
Faeces	59	23	39
Bulk tank milk	14	5	36
Individual cow samples			
Udder	87	48	55
Faeces	154	102	66
Milk	88	25	28
Total	757	329	43

DISCUSSION

The use of MEYP agar for the detection of *B. cereus* is based on fermentation of mannitol and lecithinase production as the differential principles. Polymyxin B sulfate is added to inhibit Gram-negative organisms (Mossel *et al.* 1967). Although this medium is selective for *B. cereus*, other micro-organisms, including other bacilli, may be found. Therefore, suspected colonies should be confirmed. For the confirmation of *B. cereus* glucose fermentation, VP reaction and nitrate reduction were applied in this study. According to the ISO procedure *B. cereus* should be positive for all three reactions; however up to 5 % of the strains might be VP negative. Among the API 50 CHB *B. cereus*-positive strains there were 5.4 % VP negative strains and 3.5 % unable to reduce nitrate. This deviation is acceptable since according to numerical taxonomy in bacterial classification the average difference in replicate tests on the same strain is commonly about 5 % (Sneath 1984). In total, 766 (90 %) of the 847 suspected colonies isolated on MEYP were determined to be *B. cereus* through use of both confirmatory tests and carbohydrate patterns. This is a higher percentage than

noted by Ahmed *et al.* 1983, who found 71.5 % of their suspected colonies isolated on Kim and Goepfert agar medium, a comparable egg yolk polymyxin medium, were *B. cereus*. Heterogeneity of response has also been observed in other studies; in general glucose was fermented by all strains, 0 to 42 % of the *B. cereus* strains were VP negative while 0 to 13 % did not reduce nitrate (Lancette and Harmon 1980, Logan and Berkeley 1984, Rajkowski and Mikolajcik 1987, Wong *et al.* 1988, Mosso *et al.* 1989, Rangasamy *et al.* 1993).

The percentage of positive results for the carbohydrate tests (Table 5.1) of our *B. cereus* isolates was within the range reported by others (Logan and Berkeley 1984, Rangasamy *et al.* 1993). There was no distinct relation between the carbohydrate pattern and the contamination source. However, certain *B. cereus* types could be isolated from different sources in the contamination route (data not shown). It can be observed that < 1 % of the strains were able to ferment lactose, the main carbohydrate in milk. Only two lactose-fermenting strains were isolated from udders and faeces. In earlier studies with *B. cereus* strains isolated from raw milk and pasteurized dairy products, 8 and 15 % could ferment lactose (Logan and Berkeley 1984, Rangasamy *et al.* 1993). The majority of these strains probably do not originate from sources at the farm. This might indicate that in the milk production chain a selection of strains or adaptation of strains to the environment may take place; further research is needed to confirm this.

It has been suggested that *B. cereus* strains producing emetic toxin are unable to hydrolyze starch or ferment salicin (Shinagawa 1990). Very few of such isolates were found in the present study: 98 % of the strains were able to hydrolyze starch and 99 % could ferment salicin. The diarrhoeal food-poisoning syndrome is caused by an unstable enterotoxin. This type of food poisoning is caused by ingestion of the cells rather than by ready-formed toxin (Granum *et al.* 1993). Therefore strains that can grow at low temperatures and at 37 °C should be regarded as potential food-poisoning organisms, since they can grow both in the product and in the ileum. In this study, most of the strains that were able to grow at 7 °C grew only poorly at 37 °C (data not shown); this might indicate that these strains are unable to grow in the ileum and may probably not be enterotoxigenic. This suggests that dairy products do not play an important role in outbreaks of diarrhoeal type of food poisoning. However, it is reported that *B. cereus* was able to produce toxin in milk at 8 °C under certain conditions (Christiansson *et al.* 1989). The findings in this study suggest that both production of emetic and diarrhoeal toxins is probably not caused by strains isolated on farms.

Generally, the contamination levels of the analyzed samples were comparable to other investigations. The numbers in soil were estimated to be 10^3-10^7 per g (Labots *et al.* 1965) and the levels in grass 10^3-10^5 per g (Labots *et al.* 1965, McKinnon and Pettipher 1983). The presence of high numbers of (psychrotrophic) *B. cereus* on grass can be explained by the

contamination from the soil and faeces from the cows, since on grass without earth and not to close to faeces levels of < 10 per g were observed. In this study the highest numbers of *B. cereus* were most frequently found in soil and faeces. However, the levels found in faeces were somewhat lower than obtained by others (Labots *et al.* 1965). There is supposed to be a positive correlation between the numbers in faeces and the feedstuffs used (Van Heddeghem and Vlaemynck 1993). Since the numbers present in the feedstuffs sampled were lower than those in earlier investigations (Labots *et al.* 1965) this would result in lower levels in faeces. Used bedding occasionally contains high levels of spores, most likely caused by increased contamination by faeces and soil. Individual udder and faeces samples indicated that the contamination of these samples is higher when the cows are at pasture, this confirms previous findings (Labots *et al.* 1965). As also observed in other studies (Stewart 1975, McKinnon and Pettipher 1983, Phillips and Griffiths 1986) there was seasonal variation in levels of *B. cereus* (data not shown). Numbers were highest in the summer/autumn months (June to October), when cows were at pasture. The higher levels may partly be explained by the fact that in late summer the contamination by soil is higher. A positive correlation between the water content of the soil and the numbers of *B. cereus* present was shown to exist (Labots *et al.* 1965). Another explanation might be that the level of spore-laden dust particles in the atmosphere increases due to harvesting operations during summer (Stewart 1975).

Isolation of psychrotrophic strains at 30 °C and subsequent testing for growth within 7 days at 7 °C will not influence the levels of psychrotrophs found since these strains can grow well at both 7 and 30 °C. Normally psychrotrophs are isolated by incubation at low temperatures (5-7 °C) for 7 to 10 days (Cousin 1982). It was shown that the psychrotrophic strains were able to grow at temperatures ranging from 7 to 37 °C although slowly at 37 °C compared to strains unable to grow at 7 °C. These non-psychrotrophic strains grew from 10-15 °C to 42 °C (data not shown). In total 329 (43 %) of the 757 strains tested for growth at 7 °C were able to grow at this temperature. Highest levels of psychrotrophs were observed in soil and faeces (66 %), indicating that these are the main contamination sources of these organisms. None of the strains isolated from concentrate (a dried, pelleted feed) was able to grow at 7 °C, which is probably due to the high temperatures used in the production process of this type of feed. Spores of psychrotrophic isolates are more easily destroyed by heat-processing than mesophilic types (Cousin 1982). About 30 % of the strains isolated from milk were found to be psychrotrophic, 28 % of the strains isolated from milk of individual cows and 36 % of the strains found in the bulktank milk. These results are in agreement with other studies (Shehata and Collins 1971, Johnston and Bruce 1982, Griffiths 1993). The seasonal variation was also observed for psychrotrophs, the contamination being higher in summer than in winter. This effect might be caused by the high levels found in soil (66 %) and grass (63 %). Therefore, higher levels of psychrotrophs will be present on the udder in

summer, resulting in an increased contamination of the raw milk.

CONCLUSION

B. cereus could be isolated from all environmental sources sampled on farms. The heterogeneity of response to the confirmation tests, the variability in carbohydrate utilization patterns and temperature ranges of growth indicate that many different *B. cereus* types can be isolated from the farm environment.

Vegetative cells of *B. cereus* are destroyed by heat, therefore the spore content of raw milk immediately prior to pasteurization is important. For the keeping quality of pasteurized milk and milk products the presence of heat-resistant psychrotrophic spore-formers is most important. These spores are able to survive the pasteurization process, germinate at temperatures as low as 7 °C followed by outgrowth and subsequent spoilage of the milk faster than mesophilic types. Furthermore, at low temperatures there will be no or limited interaction of psychrotrophs with mesophilic *Bacillus* spp. present (Sutherland and Murdoch 1994).

About 40 % of the isolated strains were able to grow at 7 °C. At the farm, the major contamination with psychrotrophs will originate from soil and faeces. These will contaminate the udder and bedding, resulting in contamination of raw milk with *B. cereus* spores. Milk may also be contaminated during transport to the factory or at the dairy. In addition, selection of e.g. lactose-positive or toxin-producing strains may occur further in the milk production chain. Another possibility is that strains can adapt to the environment. Therefore, it is important to determine additional contamination sources by examination of milk collection tankers and dairy factories and to study the behaviour of *B. cereus* under different environmental conditions.

To indicate the major vectors of contamination with greater certainty, the development of other tests for identification and differentiation is necessary. Using DNA typing methods alone or in combination with classical, biochemical techniques might be useful in future. The RAPD technique (randomly amplified polymorphic DNA) has been used for epidemiological subtyping of e.g. *Listeria* spp. and *Campylobacter* spp. (Mazurier and Wernars 1992, Mazurier *et al.* 1992) and also proved to be a promising tool for identification and discrimination of *B. licheniformis* (Stephan *et al.* 1994) and *B. thuringiensis* (Brousseau *et al.* 1993).

ACKNOWLEDGEMENTS

The authors would like to thank AE Postma, SHM Tap and HAM van Gennip for their technical support.

REFERENCES

Ahmed AA-H, Moustafa MK and EH Marth (1983) Incidence of *Bacillus cereus* in milk and some milk products. *Journal of Food Protection* **46** 126-128.

Anonymous (1992) ISO/DIS 7932 Microbiology- General guidance for enumeration of *Bacillus cereus*- colony-count technique at 30 °C, International Organization for Standardization, Switzerland.

Brousseau R, Saint-Onge A, Préfontaine G, Masson L and J Cabana (1993) Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. *Applied and Environmental Microbiology* **59** 114-119.

Cannon RY (1972) Contamination of raw milk with psychrotrophic sporeformers. *Journal of Dairy Science* **55** 669.

Christiansson A, Satyanarayan Naidu A, Nilsson I, Wadström T and H-E Petterson (1989) Toxin production by *Bacillus cereus* isolates in milk at low temperatures. *Applied and Environmental Microbiology* **55** 2595-2600.

Chung BH and RY Cannon (1971) Psychrotrophic sporeforming bacteria in raw milk supplies. *Journal of Dairy Science* **54** 448.

Coghill D and HS Juffs (1979) Incidence of psychrotrophic sporeforming bacteria in pasteurised milk and cream products and effect of temperature on their growth. *Australian Journal of Dairy Technology* **34** 150-153.

Collins EB (1981) Heat-resistant psychrotrophic organisms. *Journal of Dairy Science* **64** 157-160.

Cousin MA (1982) Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. *Journal of Food Protection* **45** 172-207.

Credit C, Hedeman R, Heywood P and D Westhoff (1972) Identification of bacteria isolated from pasteurized milk following refrigerated storage. *Journal of Milk and Food Technololgy* **35** 708-709.

Davies FL and G Wilkinson (1973) *Bacillus cereus* in milk and dairy products. In Hobbs BC and JHB Christian (eds.) *The Microbiological Safety of Foods*, Academic Press, London, 57-67.

Donovan KO (1959) The occurrence of *Bacillus cereus* in milk and on dairy equipment. *Journal of Applied Bacteriology* **22** 131-137.

Granum PE, Brynestad S, O'Sullivan K and H Nissen (1993) Enterotoxin from *Bacillus cereus*: production and characterization. *Netherlands Milk and Dairy Journal* **47** 63-70.

Griffiths MW (1993) *Bacillus cereus* in liquid milk and other milk products. *IDF bulletin* **275** 36-39.

Griffiths MW and JD Phillips (1990) Incidence, sources and some properties of psychrotrophic *Bacillus* spp. found in raw and pasteurized milk. *Journal of the Society of Dairy Technology* **43** 62-66.

Grosskopf JC and WJ Harper (1974) Isolation and identification of psychrotrophic sporeformers in milk. *Milchwissenschaft* **29** 467-470.

Johnson KM (1984) *Bacillus cereus* in food-borne illness- An update. *Journal of Food Protection* **47** 145-153.

Johnston DW and J Bruce (1982) Incidence of thermoduric psychrotrophs in milk produced in the west of Scotland. *Journal of Applied Bacteriology* **52** 333-337.

Kim HU and JM Goepfert (1971) Occurrence of *Bacillus cereus* in selected dry food products. *Journal of Milk and Food Technology* **34** 12-15.

Krusch U (1986) *Bacillus cereus* und die Haltbarkeit von pasteurisierter Milch. *Chemie, Mikrobiologie, Technologie der Lebensmittel* **10** 96-98.

Labots H, Hup G and ThE Galesloot (1965) *Bacillus cereus* in raw and pasteurized milk. III. The contamination of raw milk with *Bacillus cereus* spores during its production. *Netherlands Milk and Dairy Journal* **19** 191-221.

Lancette GA and SM Harmon (1980) Enumeration and confirmation of *Bacillus cereus* in foods: collaborative study. *Journal of the Association of Official Analytical Chemists* **63** 581-586.

Logan NA and RC Berkeley (1984) Identification of *Bacillus* strains using the API system. *Journal of General Microbiology* **130** 1871-1882.

Martin JH, Stahly DP, Harper WJ and IA Gould (1962) Spore-forming micro-organisms in selected milk supplies. *XVI International Dairy Congress C (VIII:1)* 295-304.

Mazurier S and K Wernars (1992) Typing of *Listeria* strains by random amplification of polymorphic DNA. *Research in Microbiology* **143** 499-505.

Mazurier S, Van de Giessen A, Heuvelman K and K Wernars (1992) RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. *Letters in Applied Microbiology* **14** 260-262.

McKinnon CH and GL Pettipher (1983) A survey of sources of heat-resistant bacteria in milk with particular reference to psychrotrophic spore-froming bacteria. *Journal of Dairy Research* **50** 163-170.

Meer RR, Baker J, Bodyfelt FW and MW Griffiths (1991) Psychrotrophic *Bacillus* spp. in fluid milk products: A review. *Journal of Food Protection* **54** 969-979.

Mossel DAA, Koopman MJ and E Jongerius (1967) Enumeration of *Bacillus cereus* in foods. *Applied Microbiology* **15** 650-653.

Mosso MA, Garcia Arribas ML, Cuena JA and MC De La Rosa (1989) Enumeration of *Bacillus* and *Bacillus cereus* spores in food from Spain. *Journal of Food Protection* **52** 184-188.

Overcast WW and K Atmaram (1974) The role of *Bacillus cereus* in sweet-curdling of fluid milk. *Journal of Milk and Food Technology* **37** 233-236.

Phillips JD and MW Griffiths (1986) Factors contributing to the seasonal variation of *Bacillus* spp. in pasteurised dairy products. *Journal of Applied Bacteriology* **61** 275-285.

Rajkowski KT and EM Mikolajcik (1987) Characteristics of selected strains of *Bacillus cereus*. *Journal of Food Protection* **50** 199-205.

Rangasamy PN, Iyer M and H Roginski (1993) Isolation and characterisation of *Bacillus cereus* in milk and dairy products manufactured in Victoria. *Australian Journal of Dairy Technology* **48** 93-95.

Shehata TE and EB Collins (1971) Isolation and identification of psychrophilic species of *Bacillus* from milk. *Applied Bacteriology* **21** 466-469.

Shinagawa K (1990) Analytical methods for *Bacillus cereus* and other *Bacillus* species. *International Journal of Food Microbiology* **10** 125-142.

Sneath PHA (1984) Numerical Taxonomy. In Krieg NR and JG Holt (eds.) *Bergey's Manual of Systematic Bacteriology* **1**, Williams and Wilkins, Baltimore, 5-11.

Stephan R, Schraft H and F Untermann (1994) Characterization of *Bacillus licheniformis* with RAPD technique (randomly amplified polymorphic DNA). *Letters in Applied Microbiology* **18** 260-263.

Stewart DB (1975) Factors influencing the incidence of *Bacillus cereus* spores in milk. *Journal of the Society of Dairy Technology* **28** 80-85.

Sutherland AD and R Murdoch (1994) Seasonal occurence of psychrotrophic *Bacillus* species in raw milk, and studies on the interactions with mesophilic *Bacillus* sp. *International Journal of Food Microbiology* **21** 279-292.

Thomas SB, Druce RG, Peters GJ and DG Griffiths (1967) Incidence and significance of thermoduric bacteria in farm milk supplies: a reappraisal and review. *Journal of Applied Bacteriology* **30** 265-298.

Van Heddeghem A and G Vlaemynck (1993) Sources of contamination of milk with *Bacillus cereus* on the farm and in the factory. *IDF Bulletin* **275** 19-22.

Walker SJ (1988) Major spoilage micro-organisms in milk and dairy products. *Journal of the Society of Dairy Technology* **41** 91-92.

Washam CJ, Olson HC and ER Vedamuthu (1977) Heat-resistant psychrotrophic bacteria isolated from pasteurized milk. *Journal of Food Protection* **40** 101-108.

Wong HC, Chang MH and JY Fan (1988) Incidence and characterisation of *Bacillus cereus* isolates contaminating dairy products. *Applied and Environmental Microbiology* **54** 699-702.

CHAPTER 6

SPORICIDAL EFFECT OF DISINFECTANTS ON *BACILLUS CEREUS* ISOLATED FROM THE MILK PROCESSING ENVIRONMENT

ABSTRACT

The sporicidal efficacy of sodium hypochlorite and a combination of peracetic acid and hydrogen peroxide on *Bacillus cereus* spores isolated from the milk processing environment was examined using the European Suspension Test and by a surface disinfection test on stainless steel and rubber. The results of the laboratory tests were compared to field trials in a milking installation. In general, it was difficult to obtain consistent results, the repeatability and reproducibility of the tests was found to vary according to the test strain, spore suspension preparation, disinfectant test solution, organic load, contact time and temperature. The sporulation medium used to obtain spores influenced the sporicidal effect considerably. To overcome this problem a standard method for preparation of spore suspensions should be prescribed.

The various disinfectants were more effective in suspensions than on surfaces and in field trials. For the suspension tests SE values ranging from 1.0 to 3.0 were reached within 10 min at 50 °C, depending on the disinfectant used. Sodium hypochlorite based products were most effective. The activity on spores on surfaces and in field trials was limited. In surface tests reductions of 0.4 to 0.8 were observed within 10 min at 50 °C, depending on the type of surface. The SE values obtained for rubber were lower compared to stainless steel. The decrease in spore levels found in the milking installation was comparable to the surface experiments, i.e. 0.4 to 1.0.

It is important to develop standard test procedures to assess the sporicidal efficacy of disinfectants used in food hygiene. Surface tests should be included to reflect the in-use conditions more closely and minimum standards should be determined for both suspension tests and surface tests.

This chapter has been published as:

Sporicidal effect of disinfectants on *Bacillus cereus* isolated from the milk processing environment

MC Te Giffel, RR Beumer, WF Van Dam, BA Slaghuis and FM Rombouts

International Biodeterioration and Biodegradation (1995) **36** 421-430.

INTRODUCTION

Spore-forming bacteria can survive as spores during food processing due to their resistance properties and, after germination and outgrowth, cause spoilage and outbreaks of foodborne illness. In dairy industry for instance, spore-forming micro-organisms, especially *Bacillus cereus*, are important as they determine the keeping quality of pasteurized milk and milk products (Credit *et al.* 1972, Stewart 1975, Washam *et al.* 1977, Meer *et al.* 1991). It is unlikely that the presence of spore-forming organisms can be prevented. Therefore, it is important to keep the initial contamination low and to prevent recontamination during processing by maintaining good manufacturing practices (Collins 1981, Meer *et al.* 1991, Champagne *et al.* 1994). In addition, the initial level can be influenced by cleaning and disinfection.

For the evaluation of disinfectants standard tests which are robust, relevant to use conditions and internationally acceptable are required to verify and compare activity. As field trials under use conditions are difficult and expensive to perform, the approval of disinfectants, for the most part, is based on results of laboratory tests (Bloomfield *et al.* 1994). Suspension tests are based on the 'Method of test for the antimicrobial activity of disinfectants in food hygiene', the European Suspension Test (EST, Anonymous 1988). Although suspension tests can be used to assess the activity under a range of conditions, they give no information about how products actually perform on contaminated surfaces. Surface tests involve quantitative determination of viable organisms recovered from a contaminated dried surface before and after application of a disinfectant (Bloomfield *et al.* 1994).

A considerable amount of information is available about the effect of disinfectants used in food industry on vegetative bacterial cells. A number of chemical compounds effective as bactericidal agents are also sporicidal, however much higher concentrations and longer contact times are required for sporicidal action. The sporicidal activity of disinfectants used in food industry is incompletely investigated and the precise mechanisms of sporicidal action are still unknown (Bloomfield and Arthur 1994).

In this study, the sporicidal efficacy of sodium hypochlorite and a combination of peracetic acid and hydrogen peroxide on *B. cereus* spores isolated from the milk processing environment was evaluated using the European Suspension Test and by a surface disinfection test on surfaces found commonly in a food processing plant i.e. stainless steel and rubber. The repeatability and reproducibility of these tests were evaluated. The results of the laboratory tests were also compared to field trials using a milking installation.

MATERIALS AND METHODS

Media and reagents

Media and reagents were prepared as described in the EST (Anonymous 1988). Chemicals were obtained from BDH, Merck and Sigma. Diluent containing 8.5 g l^{-1} NaCl was used.

Disinfectants and test solutions

Disinfectants used in this study were: Alfablink (3.6 % w/v available chlorine, Alfa Laval Agri Ltd., Groningen, The Netherlands), Puremel (3.7 % w/v available chlorine, Laporte Esd Ltd., Ambt Delden, The Netherlands) and P3 Oxonia BK (2.8 % w/v peracetic acid and 32 % w/v hydrogen peroxide, Henkel Ecolab Ltd., Nieuwegein, The Netherlands). The concentrations used in this study were 0.5 % v/v for Alfablink and Puremel and 0.5 and 0.7 % v/v for P3 Oxonia BK. Test solutions were freshly prepared on each day of testing.

Test organisms and test suspensions

The test organism in the disinfection tests was *B. cereus*, five strains were isolated from dairy factories and *B. cereus* ATCC strains 9139 and 12826, were used. The strains were stored at - 80°C in Brain Heart Infusion broth (BHI; Difco 0037-01-6) + 20% glycerol.

Sporulation of the *B. cereus* was achieved on Tryptone Soya Agar (TSA; Oxoid CM 131) or on Sporulation Agar (SA; composition (g l^{-1}): Peptone 15.0, Yeast extract 3.0, NaCl 6.0, D(+)-Glucose 1.0, Manganese sulphate 0.1, Agar 12.0). After incubation (3 or 28 days at 30°C for TSA and 3 to 8 weeks at 30 °C for SA), spores were washed from the surface of the agar with sterile distilled water and centrifuged at 2000 g for 20 min. The pellets were washed three times and resuspended in sterile distilled water. To destroy the vegetative cells the spore suspension (5 ml portions in 200 × 18 mm tubes) was given a heat shock of 60 ± 5 sec at 80°C in a thermostatically controlled water-bath. The number of spores was determined after plate counting on Mannitol Egg Yolk Polymyxin agar (MEYP, Cereus selective agar, Merck 5267) and by microscopy. Freshly prepared suspensions were used in each experiment.

Sporicidal activity

The sporicidal effect (SE value) was calculated according to the formula:

$$SE\ value = \log Nc - \log Nd,\ \text{in which}$$

Nc = the number of spores per ml in the control test without the disinfectant;
Nd = the number of spores per ml after the action of the disinfectant.

Disinfectant testing method

European Suspension Test

The sporicidal activity of the disinfectants in suspensions was evaluated using the EST (Anonymous 1988). To represent the in-use situation in the dairy industry, a contact time of 10 min and a temperature of 50 °C was used. Tests were carried out in the presence of 4 % milk as organic soil instead of bovine albumin. All tests were performed by an experienced microbiologist.

Surface tests

The surface test for comparative testing of bactericidal activity of disinfectants described by Bloomfield *et al.* 1993 and Bloomfield *et al.* 1994 was slightly modified, the following procedure was used in the experiments.

Test surfaces were 20 mm diameter stainless steel and rubber discs. These surfaces were chosen because they relate to the practical application in the dairy industry. The stainless steel discs were stored in 70 % alcohol until required and then flamed and placed in a petridish. Rubber discs were swabbed with 70 % alcohol and placed in a petridish overnight to dry. Before each use the surfaces were washed in detergent and rinsed in sterile distilled water.

Contaminated surfaces were prepared by inoculating the carriers with 0.1 ml drops of test suspension containing 10^8 spores/ml. As organic soil 4 % milk was added. Surfaces were dried at 42 °C for 1 h. Samples of disinfectant test solution or water of standard hardness (WSH) (0.1 ml) were dropped onto the surface to cover the test film. After a contact time of 10 min at 50 °C in a thermostatically controlled water-bath, the discs were placed in a 100-ml sterile flask containing 10 ml neutralization medium together with 6 g of glass beads (3 mm diameter) and placed in a shaking incubator for 5 min at 50 °C. The neutralization medium and 10-folds dilutions were plated on TSA. Plates were incubated at 30 °C for 24 h, counted and the sporicidal effect was calculated.

Field trials

The field trials were carried out in a milking installation on an experimental farm at the Research Station for Cattle, Sheep and Horse Husbandry (Lelystad, The Netherlands). The installation was contaminated by circulating 15 l spore suspension for 2 min. The number of spores was determined in the suspension before and after circulation to estimate the number of spores that remained in the installation. The installation was rinsed by circulating 20 l of water (control) or 20 l of disinfectant test solution for 10 min. The temperature regime during the procedure was 70 °C start temperature, ± 30 °C end temperature. Next, 1 ml and 100 ml of the rinsing water was added to a flask containing 9 and 900 ml neutralization medium, respectively. After 5 min the number of surviving spores was determined by plating on TSA. Plates were incubated at 30 °C for 24 h and counted.

Furthermore, survival of spores in the installation was investigated after 20 h. To this end, the installation was rinsed with 16 l of water and 4 l of UHT sterilized milk. The number of *B. cereus* spores was determined by a Most Probable Number (MPN) procedure in BHI.

RESULTS AND DISCUSSION

The sporicidal efficacy of various disinfectants on *B. cereus* spores was assessed using the EST and surface disinfection tests. The results were compared to field trials under use conditions. A disinfectant is considered to be sporicidal if a three decimal reduction in 30 min at 20 °C in the viable spore count is reached (Pullen and van Klingeren 1989).

In general, it was difficult to obtain consistent results, the repeatability and reproducibility of the tests were found to vary according to the test strain, spore suspension preparation, disinfectant test solution, organic load, contact time and temperature. These results confirm previous studies on vegetative bacterial cells (e.g. Bloomfield and Looney 1992, Bloomfield *et al.* 1993, Bloomfield *et al.* 1994).

Table 6.1 Mean sporicidal effect (SE values) of disinfectants against *Bacillus cereus* spores in suspension tests.

	Puremel	Alfablink	P3 Oxonia BK	
Strain	0.5 %[a]	0.5 %	0.5 %	0.7 %
A	3.1 ± 0.3[b]	3.0 ± 0.1	1.2 ± 0.2	1.8 ± 0.2
B	3.1 ± 0.1	3.0 ± 0.1	ND	ND
C	3.1 ± 0.2	3.1 ± 0.3	1.1 ± 0.1	2.0 ± 0.1
D	3.3 ± 0.1	3.2 ± 0.2	ND	ND
E	3.2 ± 0.1	3.2 ± 0.1	ND	ND
ATCC 9139	1.6 ± 0.1	1.6 ± 0.1	ND	ND
ATCC 12826	1.6 ± 0.1	1.6 ± 0.2	ND	ND

[a] disinfectant concentration in % v/v;

[b] mean and standard deviation of three experiments;

ND, not determined

In the suspension tests (Table 6.1) SE values up to 3 could be reached. The extent of

the sporicidal efficiency varied with the test strain used and disinfectant solution. The ATCC strains were relatively more resistant to disinfectants than the strains isolated at the dairy. This was in contrast to what was expected i.e. the dairy strains could have undergone some selection in resistance and therefore increased resistance of these strains was expected. This was observed in a study by Alasri *et al.* 1993 in which *B. subtilis* ATCC strains were less resistant than strains isolated under practical conditions. The chlorine-releasing agents, Puremel and Alfablink, showed the highest sporicidal activity. Both compounds had similar activity on *B. cereus* spores, which confirms earlier studies (Bloomfield and Arthur 1989). Between spores variation in resistance to the chlorine disinfectants was observed, this has also been reported in literature: differences in resistance may exist within species, among species and among genera (Foegeding 1983).

Table 6.2 Mean sporicidal effect (SE values) of disinfectants against *Bacillus cereus* spores grown on different media using the EST.

	TSA[a]		SA[b]	
Strain	3 days	28 days	21 days	59 days
C	2.6 ± 0.2[c]	2.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.2
D	2.7 ± 0.1	2.8 ± 0.1	1.6 ± 0.1	1.7 ± 0.1

[a] TSA; Tryptone Soya Agar;

[b] SA; Sporulation Agar;

[c] mean and standard deviation of four experiments

It can be observed in Table 6.2 that spore suspensions prepared on TSA and SA differ in resistance to disinfectants using the EST. This may be partly explained by the difference in germination rate of the spores. TSA spores germinate somewhat faster in WSH than SA spores. Of the spores grown on TSA 0 to 23 % germinated in WSH within 30 min at room temperature compared to 0 to 16 % of the spores grown on SA. Germinated spores will be killed more easily and the actual effect of the disinfectants is probably not directed towards spores but to the germinated spores. Bacterial spores will germinate even faster in complex media such as milk than in simple media e.g. water or diluent (Te Giffel *et al.* 1995 [Chapter 2]). So, addition of organic matter will further increase the germination of spores and lead to higher SE values. For strain ATCC 9139 the mean SE value reached in three replicate tests was 1.1 ± 0.1 in the absence of organic soil compared to 1.6 ± 0.1 in

presence of 4 % milk. Increasing the contact time and temperature will lead to higher SE values (data not shown). However, these factors also affect the germination of spores (Te Giffel *et al.* 1995 [Chapter 2]) and influence the results obtained in the disinfectant tests. To overcome these problems, a standard method for preparation of spore suspensions should be described and no organic matter should be incorporated when disinfectants are tested.

Spores were more resistant to disinfection when adhered to surfaces compared to the same spores in suspension (Table 6.3). There was significant difference between stainless steel and rubber. The SE values ranged from 0.4 for rubber to 0.7 to 0.8 for stainless steel compared to SE values up to 3 in the EST. The enhanced resistance of attached spores may be related to the environment surrounding the bacteria and the nature of the surface. The rubber discs were obtained from in-use rubber of a milking installation. Spores attached to the pits and crevices may be very difficult to be reached, giving them protection due to poor penetration of the disinfectant.

Table 6.3 Mean sporicidal effect (SE values) of disinfectants against *Bacillus cereus* strain C spores inoculated onto stainless steel and rubber.

Surface	Puremel 0.5 % v/v	Alfablink 0.5 % v/v
Stainless steel	0.8 ± 0.0[a]	0.7 ± 0.1
Rubber	0.4 ± 0.1	ND

[a] mean and standard deviation of three experiments
ND, not determined

The effect of bacterial attachment on disinfectant resistance has also been observed for vegetative bacterial cells by Holah *et al.* (1990) using a conductance based disinfection test for biofilms on stainless steel in parallel with the EST, and bacteria were shown to be 10 to 100 times more resistant to biocides when surface attached. Studies by Matilla-Sandholm and Wirtanen (1992) showed that *Listeria monocytogenes*, *Pseudomonas fragi*, *Enterococcus hirae* and *Bacillus subtilis* had increased resistance towards various disinfectants on surfaces compared to the values found in suspension tests. An impedimetric evaluation of disinfectant efficacy has shown that biofilms of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis* and *L. monocytogenes* attached to polyvinyl chloride (PVC), teflon, plexiglass, wood, rubber and stainless steel were more resistant than the same bacteria in suspension (Dhaliwal *et al.* 1992).

The major problems in surface testing are achieving consistent recovery of survivors from surfaces and substantial loss of viability in drying the inoculum onto the surface (Bloomfield *et al.* 1994). However, the results described in their paper and the results obtained in this study indicate that the variability of the surface tests was comparable to that associated with suspension tests.

Table 6.4 shows that there was little or no difference in mean SE values obtained in field trials, i.e. 0.4 to 1.0 and surface tests, suggesting that surface tests could be chosen and represent in-use conditions. The limited sporicidal effect observed in the installation indicates that spores will be present after the disinfection step. These remaining spores were shown to survive in the installation as determined by MPN technique; after 20 h the MPN count was more than 2.3 per ml in all experiments.

Table 6.4 Mean sporicidal effect (SE values) of disinfectants against *Bacillus cereus* strain E in a milking installation.

	Puremel 0.5 % v/v	Alfablink 0.5 % v/v	P3 Oxonia BK 0.7 % v/v
SE value	0.8 ± 0.1[a]	1.0 ± 0.2[b]	0.4 ± 0.1[b]

[a] mean and standard deviation of four experiments;

[b] mean and standard deviation of two experiments

The variability between strains and limited sporicidal effect of the various disinfectants obtained in the surface test and in the milking installation suggest that to achieve enhanced sporicidal action combinations of sporicidal agents or combinations of potentiators with sporicidal agents should be investigated.

Summarizing, it has been shown that a standard method for spore suspension preparation is necessary to obtain consistent results in disinfectant testing. This study illustrates that a simple medium such as TSA is suitable for this. Suspension tests should only be used for disinfectant screening and should not be used solely to determine recommended in-use conditions. A standard protocol for the evaluation of disinfectants in suspension and on surfaces should be described which simulates in-use conditions more closely. In this standard test procedure no organic matter should be incorporated. The contact time and temperature should reflect practical conditions; in this case an exposure time of 10 min at a temperature of 50 °C represents conditions in the dairy industry. The 30 min contact time at 20 °C proposed for testing sporicidal activity of disinfectants (Pullen and van Klingeren

1989) is too long. It must be kept in mind that the factors time and temperature affect germination and thus the values obtained as "sporicidal" efficacy of a disinfectant.

REFERENCES

Alasri A, Valverde M, Roques C, Michel G, Cabassud C and P Aptel (1993) Sporocidal properties of peracetic acid and hydrogen peroxide, alone and in combination, in comparison with chlorine and formaldehyde for ultrafiltration membrane disinfection. *Canadian Journal of Microbiology* **39** 52-60.

Anonymous (1988) Method of test for the antimicrobial activity of disinfectants in food hygiene. DD177, British Standards Institution, London.

Bloomfield SF and M Arthur (1989) Effect of chlorine-releasing agents on *Bacillus subtilis* vegetative cells and spores. *Letters in Applied Microbiology* **8** 101-104.

Bloomfield SF and M Arthur (1994) Mechanisms of inactivation and resistance of spores to chemical biocides. *Journal of Applied Bacteriology Symposium Supplement* **76** 91S-104S.

Bloomfield SF and E Looney (1992) Evaluation of the repeatability and reproducibility of European suspension test methods for antimicrobial activity of disinfectants and antiseptics. *Journal of Applied Bacteriology* **73** 87-93.

Bloomfield SF, Arthur M, Begun K and H Patel (1993) Comparative testing of disinfectants using proposed European surface test methods. *Letters in Applied Microbiology* **17** 119-125.

Bloomfield SF, Arthur M, van Klingeren B, Pullen W, Holah JT and R Elton (1994) An evaluation of the repeatability and reproducibility of a surface test for the activity of disinfectants. *Journal of Applied Bacteriology* **76** 86-94.

Champagne CP, Laing RR, Roy D, Mafu AA and MW Griffiths (1994) Psychrotrophs in dairy products: their effects and their control. *Critical Reviews in Food Science and Technology* **34** 1-30.

Collins EB (1981) Heat resistant psychrotrophic micro-organisms. *Journal of Dairy Science* **64** 157-160.

Credit C, Hedeman R, Heywood P and D Westhoff (1972) Identification of bacteria isolated from pasteurized milk following refrigerated storage. *Journal of Milk and Food Technology* **35** 708-709.

Dhaliwal DS, Cordier JL and LJ Cox (1992) Impedimetric evaluation of the efficiency of disinfectants against biofilms. *Letters in Applied Microbiology* **15** 217-221.

Foegeding PM (1983) Bacterial resistance to chlorine compounds. *Food Technology* **11** 100-104.

Holah JT, Higgs C, Robinson S, Worthington D and H Spenceley (1990) A conductance-based surface disinfection test for food hygiene. *Letters in Applied Microbiology* **11** 255-259.

Matilla-Sandholm T and G Wirtanen (1992) Biofilm formation in the food industry: A review. *Food Reviews International* **8** 573-603.

Meer RR, Baker J, Bodyfelt FW and MW Griffiths (1991) Psychrotrophic *Bacillus* spp. in fluid milk

products: A review. *Journal of Food Protection* **54** 969-979.

Pullen W and B van Klingeren (1989) Report. no. 358704001, National Institute for Public Health and Environmental Hygiene, Bilthoven, The Netherlands.

Stewart DB (1975) Factors influencing the incidence of *Bacillus cereus* spores in milk. *Journal of the Society of Dairy Technology* **28** 80-85.

Te Giffel MC, Beumer RR, Hoekstra J and FM Rombouts (1995) Germination of bacterial spores during sample preparation. *Food Microbiology* **12** 327-332.

Washam CJ, Olson HC and ER Vedamuthu (1977) Heat-resistant psychrotrophic bacteria isolated from pasteurized milk. *Journal of Food Protection* **40** 101-108.

CHAPTER 7

INCIDENCE AND CHARACTERIZATION OF *BACILLUS CEREUS* IN TWO DAIRY PROCESSING PLANTS

ABSTRACT

In order to determine the contamination of raw and pasteurized milk with (psychrotrophic) *Bacillus cereus*, samples were taken at two dairy processing plants, from raw milk (milk collection tankers and raw milk storage tanks at both plants) and at various stages of processing from raw milk to pasteurized milk (dairy I) or milk powder (dairy II). The incidence of *B. cereus* in the different samples was investigated, and carbohydrate metabolism and the ability to grow at 7 °C were assessed for the strains isolated.

In total, 507 presumptive *B. cereus* strains were isolated. The levels present in the various samples were low; the organism could only be isolated after a pre-incubation step of 6 h at 30 °C. According to the ISO confirmation tests and/or carbohydrate utilization patterns (API 50 CHB), 443 (87 %) of the isolates were confirmed to be *B. cereus*. The carbohydrate patterns revealed more than 20 different *B. cereus* types in raw milk and the various points in the pasteurized milk production process. Strikingly, only 10 different types were identified in the milk powder processing plant. These results indicate that selection or adaptation of strains takes place in the milk production chain, because 27 different carbohydrate utilization profiles were observed for the raw milk isolates at dairy I and II and in a previous study more than 30 different types were identified on farms. No distinct relation between the biochemical characteristics and the contamination source was observed. However, it was demonstrated that some biotypes were found in the raw milk, during processing and in the endproducts whereas other biotypes were only detected after the pasteurization process. This indicates that post-pasteurization contamination with *B. cereus* occurs. This assumption was supported by subtyping of strains using molecular typing i.e. Randomly Amplified Polymorphic DNA (RAPD) and plasmid profiling.

The presence of *B. cereus* was demonstrated in 35 % of the raw milk samples. When the contamination level of the raw milk was low, due to seasonal effects, *B. cereus* could still be detected at the various processing sites and in the final products. The percentage of positive samples increased during processing up to 71 % for the pasteurized milk. The levels were generally low; the MPN counts in freshly pasteurized milk ranged from 0.9 to more than 110 per 100 ml. In dairy II the percentage of positive samples increased up to 60 % for the milk powder. This is partly due to concentration effects. However, these results, in addition to the outcome of the carbohydrate utilization profiles and molecular characterization of isolates, also indicate that *B. cereus* can be introduced

into the milk via sources other than raw milk. In this respect, the equipment is probably an important source of contamination.

Only a few strains (6 %) isolated at dairy I were able to grow at 7 °C. Since psychrotrophs have a significant impact on the keeping quality of pasteurized milk(products) the findings in this study suggest that adaptation or more likely selection of strains to growth at low temperatures occurs during storage and distribution. In the milk powder processing plant, no psychrotrophs were observed; this is due to the type of process.

This chapter has been (partly) published as:
Incidence and characterization of *Bacillus cereus* in two dairy processing plants
MC Te Giffel, RR Beumer, MH Bonestroo and FM Rombouts
Netherlands Milk and Dairy Journal (1996) **50** 479-492.

INTRODUCTION

Bacillus species represent a group of bacteria commercially important for the dairy industry. Due to the resistance of their spores and the capacity of the vegetative cells to produce extracellular enzymes they can cause spoilage of a variety of processed dairy products (Collins 1981, McGuiggan *et al.* 1994).

It has been shown that the predominant organism that determines the keeping quality of pasteurized milk(products) is *Bacillus cereus* (Phillips and Griffiths 1986, Meer *et al.* 1991). The organism is associated with defects such as off-flavours, sweet curdling and bitty cream caused by the production of proteinase, lipase and phospholipase enzymes (Overcast and Atmaram 1974, Washam *et al.* 1977, Meer *et al.* 1991). In addition to causing these effects in dairy products, *B. cereus* has also been associated with outbreaks of food poisoning (Johnson 1984, Christiansson *et al.* 1989). This underlines the significance of this organism to the dairy industry.

Trends such as the extended refrigerated storage of raw milk prior to processing, the application of higher pasteurization temperatures and prolonged shelf-life requirements have enhanced the importance of thermoduric psychrotrophs (Meer *et al.* 1991). The combination of heat-resistance and psychrotrophic properties represents substantial potential for causing spoilage of perishable milk products.

Psychrotrophic *Bacillus* species are the predominant organisms in (pasteurized) milk and milk products, although heat-tolerant psychrotrophs also include members of the genera *Clostridium*, *Arthrobacter*, *Microbacterium*, *Streptococcus* and *Corynebacterium* (Credit *et al.* 1972, Washam *et al.* 1977, Johnston and Bruce 1982, Meer *et al.* 1991). In various surveys, the incidence of psychrotrophic *B. cereus* in raw milk varies from 23 to 83 % of the total psychrotrophic spore count with levels ranging from 1 to 1600 spores per l (Shehata and Collins 1971, Cannon 1972, Grosskopf and Harper 1974, Coghill and Juffs 1979, Johnston and Bruce 1982, McKinnon and Pettipher 1983, Griffiths and Phillips 1990, Griffiths 1993, Te Giffel *et al.* 1995). In pasteurized milk *B. cereus* has been found to occur in about 30 % of the samples (Overcast and Atmaram 1974, Coghill and Juffs 1979, Griffiths 1993).

Dried milk products are known to be frequently contaminated with *B. cereus*, the incidence was reported to be 4 to 100 %; numbers are generally low ($< 10\ g^{-1}$), but levels of more than 1000 per g have also been quoted (Walthew and Lück 1978, Wong *et al.* 1988, Stadhouders and Driessen 1992, Rangasamy *et al.* 1993, Becker *et al.* 1994, Meira De Vasconcellos and Rabinovitch 1995).

Since *Bacillus* species are widely distributed in the environment, they can be introduced into milk from a variety of sources during production, handling and processing

(Griffiths and Phillips 1990, Walker 1988, Van Heddeghem and Vlaemynck 1993, Te Giffel *et al.* 1995).

This paper presents a study on the incidence of (psychrotrophic) *B. cereus* in raw and pasteurized milk and milk products at two dairy processing plants in the Netherlands. Some biochemical and growth characteristics of the strains isolated were investigated. Furthermore, the Randomly Amplified Polymorphic DNA (RAPD) technique and plasmid profiling were used as tools for epidemiological subtyping of strains isolated at the two processing plants.

MATERIALS AND METHODS

Sample collection

Samples were collected over one year, from two different processing plants in the Netherlands: a fluid milk processing plant (dairy I) and a milk powder processing plant (dairy II). During this period, the factories were visited 24 and 20 times, respectively. Raw milk samples were taken from the milk collection tankers (100 ml or 1 l) and from discharging sites where the milk is conveyed from the tanker to the dairy by means of a hose. At the discharging sites samples were taken of the hose that is coupled to the tanker using swabs; these were stored in peptone saline solution (PSS; NaCl 8.5 g l^{-1} and Neutralized Bacteriological Peptone (Oxoid L34) 1 g l^{-1}).

The raw milk from the collection tankers was followed through the whole process; in this way it was ascertained that the samples taken at the various sampling points were from the same batch of milk.

At dairy I, samples (100 ml) were taken at various production stages from raw milk to pasteurized milk: raw milk storage tanks, outlet of the pasteurizer, (inlets of) the pasteurized milk bulk tanks, during the filling operation of the package and retail packs of freshly pasteurized milk. All samples were kept refrigerated until examination.

At the milk powder plant, samples were taken from additives (e.g. amino acids, lactose, fat, lecithin, citrate). Furthermore, samples were taken at various points in the processing from raw milk to powder: standardization tank; halfway through the evaporation process; after evaporation of the milk, just before the spray drying and the startup powder.

Isolation and enumeration of *B. cereus*

The swabs taken from the hoses at discharging sites were streaked on Mannitol

Egg Yolk Polymyxin (MEYP) agar (Cereus Selective Agar, Merck 5267).

Since low numbers were expected in the milk samples, the milk was incubated shaken at 30 °C for about 6 h. Then, 10 ml was centrifuged for 30 min at 2000 *g*. Pellets were resuspended in 0.1 ml PSS and surface-plated on MEYP agar. To enumerate spores 10 ml of the milk was heated at 80 °C for 10 min in a thermostatically controlled water bath and cooled in melting ice prior to further serial dilution, followed by centrifugation (30 min at 2000 *g*), resuspension in 0.1 ml PSS and surface plating on MEYP.

For 38 samples of freshly pasteurized milk, just after packaging, the numbers of *B. cereus* were determined using a MPN procedure. The milk samples were divided into portions of 3 × 100 ml; 3 × 10 ml and 3 × 1 ml. After incubation at 30 °C for 18 ± 2 h, 0.1 ml of each portion was surface plated on MEYP agar. The total aerobic counts of these samples were also ascertained by plating on Plate Count Agar (PCA; Oxoid CM 325). The PCA plates were incubated at 30 °C for 72 h and counted.

Milk powder samples and additives were reconstituted by soaking 10 g samples separately in 90 ml of sterile distilled water. The samples were then treated as described above for the milk samples.

The MEYP plates were incubated at 30 °C for 24 h and examined for typical colonies. From each plate, presumptive colonies were selected for confirmation as specified in the ISO/DIS 7932: General guidance for enumeration of *Bacillus cereus*- Colony count technique at 30 °C. Suspect colonies (pink colonies surrounded by a zone of precipitation) were counted and subsequently plated out on a non-selective medium i.e. Tryptone Soya Agar (TSA; Oxoid CM 131). The identity of colonies was confirmed by testing them for glucose fermentation, Voges-Proskauer (VP) reaction (production of acetylmethylcarbinol) and nitrate reduction. *B. cereus* strains should be positive for all three reactions (Anonymous 1992).

Growth at 7 °C

To determine whether the isolated strains were psychrotrophic they were grown in Brain Heart Infusion broth (BHI; Difco 0037-01-6) for 24 h at 30 °C. The cell suspensions (10^8 ml^{-1}) were diluted in PSS. Tubes containing 10 ml of BHI or UHT sterilized milk precooled to 4 °C were inoculated to a final level of 1-10 cfu ml^{-1}. This level was checked by surface streaking on MEYP agar. After 7 days of incubation at 7 °C, 0.1 ml samples of 1000-fold dilutions of BHI and milk in PSS were plated on MEYP agar. These plates were incubated at 7 °C and counted after 7 days; more than 100 colonies (> 10^5 cfu ml^{-1}) was considered to be positive i.e. psychrotrophic.

Carbohydrate metabolism

The carbohydrate metabolism of the isolated strains was examined using API 50 CHB strips (BioMérieux Sa, France). Isolates were grown on TSA plates (18-24 h at 30 °C). Growth was harvested in 2 ml sterile 0.85 % saline solution to correspond with tube No. 2 of the McFarland scale of standard opacities and 0.1 ml of this suspension was diluted in 10 ml of API 50 CHB medium. The strips were inoculated, incubated for 48 h at 30 °C and read after both 24 and 48 h. The results were scored according to the manufacturer's instructions and the emerging biochemical profile was identified by means of APILAB software V 2.1, 1990. If the probability of the APILAB result was more than 90 % the isolate was considered to be *B. cereus*.

Molecular typing of *B. cereus* isolates

In total, 219 strains were characterized by molecular typing using PCR/RAPD and plasmid profiling. At dairy I, 11 series of isolates (142 strains) obtained at the various sampling sites, from raw milk to the end product, on the same day were selected. In a similar way, 8 series (77 strains) were chosen at dairy II.

Polymerase Chain Reaction/Randomly Amplified Polymorphic DNA (PCR/RAPD)

B. cereus isolates were grown on TSA for 18-24 h at 30 °C. A single bacterial colony (± 1.5 mm) was picked and the DNA was isolated using InstaGene Matrix (Biorad 732-6030) according to the manufacturer's protocol with minor adjustments.

PCR amplifications using the random primers R1: [5' CGGCCTCTGGGC 3'] and R2: [5' TGACTGACGC 3'] were performed by using a Thermocycler 480 (Perkin Elmer, Gouda, The Netherlands). The reactions were carried out in 0.5-ml tubes which contained 50 µl of the following buffer: 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM NaCl, 2.5 mM (each) deoxynucleoside triphosphates, 1 U of *Taq* polymerase (GibcoBRL 18038-026) and 250 ng of primer R1 or R2. Then, 5 µl (500 - 1000 ng) of template DNA was added. Amplification was done in 55 cycles of 1 min at 94 °C (denaturation), 1 min at 25 °C (annealing), and 8 min at 72 °C (extension-polymerization). PCR products were analyzed by agarose gel electrophoresis.

Plasmid profiling

Strains were grown overnight (16-20 h) at 30 °C in BHI with an additional 10 g l^{-1} of glucose. For screening for plasmid DNA the QIAprep Spin Plasmid kit (QIAgen, Westburg, The Netherlands) was used according to the manufacturer's instructions. Plasmid profiles were analyzed by agarose gel electrophoresis.

RESULTS

Identity of the isolates

Table 7.1 Confirmation of *Bacillus cereus* strains isolated on MEYP agar by the ISO standard procedure and carbohydrate utilization patterns (API 50 CHB)

	Number of isolates			G-	VP-	N-	2-	3-
Raw milk	185	ISO+	147	1	11	5	4	17
		API+	131					
		ISO+/API+	101					
		ISO+/API-	54					
		ISO-/API+	8		8			
		ISO-/API-	30	1	3	5	4	17
Dairy I								
Pasteurized milk	221	ISO+	182		5	21	4	14
		API+	167					
		ISO+/API+	140					
		ISO+/API-	54					
		ISO-/API+	12		2	9	1	
		ISO-/API-	32		3	12	3	14
Dairy II								
Milk powder	101	ISO+	88		8	7		
		API+	86					
		ISO+/API+	87					
		ISO+/API-	14					
		ISO-/API+	13		7	6		
		ISO-/API-	2		1	1		
Total	507	ISO+	417	1	24	33	8	31
		API+	384					
		ISO+/API+	328					
		ISO+/API-	122					
		ISO-/API+	33		17	15	1	
		ISO-/API-	64	1	7	18	7	31

G-, glucose negative; VP-, Voges Proskauer negative; N-, nitrate negative;
2-, both VP and N-; 3-, negative for all three confirmation reactions

The results of confirmation of *B. cereus* by the ISO standard procedure and carbohydrate utilization patterns (API 50 CHB) are listed in Table 7.1.

Raw milk

From the raw milk samples obtained from milk collection tankers, discharging sites and the bulk storage tanks at both processing plants, 185 presumptive *B. cereus* were isolated on MEYP agar. Confirmation of these colonies showed that 147 (79 %) were considered to be *B. cereus* according to the ISO standard procedure. Of the isolates that could not be confirmed, one strain was unable to ferment glucose, 11 strains were VP negative and 5 strains could not reduce nitrate. Moreover, 4 isolates were negative on two of the three reactions and 17 isolates were negative for all three reactions. Identification by API 50 CHB resulted in 131 (71 %) positive isolates. Of the 11 VP negative strains in the ISO confirmation tests, 8 (5.4 %) gave a typical *B. cereus* reaction pattern in the API 50 CHB. In total, 155 (84 %) of the 185 strains isolated on MEYP were shown to be *B. cereus* following ISO confirmation tests and/or carbohydrate utilization pattern.

Dairy I Pasteurized milk processing plant

Of the 221 suspect colonies isolated, 182 (82 %) were identified as *B. cereus* following the ISO confirmation tests. However, 5 strains were VP negative and 21 were unable to reduce nitrate. In addition, 4 strains gave negative results on two of the three reactions and 14 reacted negative on all three confirmation tests. Using the API 50 CHB for further identification showed that 167 (76 %) were *B. cereus*. Of the isolates that were not confirmed to be *B. cereus* according to the ISO standard procedure, 2 VP negative strains (1.1 %), 9 nitrate negative (4.9 %) and 1 (0.5 %) strain negative on both VP and nitrate, gave typical *B. cereus* carbohydrate utilization patterns. Of the strains isolated 32 were not considered to be *B. cereus*, following both the ISO standard and the API 50 CHB identification, resulting in 189 (86 %) strains regarded as *B. cereus* by the ISO confirmation tests and/or API 50 CHB reaction patterns.

Dairy II Milk powder processing plant

At the various points in the production of milk powder 101 presumptive *B. cereus* were isolated. Subsequent confirmation by the tests prescribed in the ISO standard procedure showed that 88 (87 %) isolates were regarded as *B. cereus*. However, 8 strains were VP negative and 7 strains could not reduce nitrate. Identification of the isolates by carbohydrate utilization pattern resulted in 86 (85 %) *B. cereus*. Seven (7.8 %) VP negative and 6 (6.6 %) nitrate negative isolates were identified as *B. cereus* using API 50 CHB. Only 2 isolates were not considered to be *B. cereus* following both the standard confirmation tests and API reaction patterns. 99 (98 %) of the isolates were regarded as *B. cereus* by the ISO standard procedure and/or carbohydrate utilization pattern.

Carbohydrate utilization patterns

Table 7.2 Carbohydrate utilization patterns of *Bacillus cereus* strains (in %), as determined with API 50 CHB.
Isolates from this study compared to those from other reports.

	Origin of isolates					
	API[a]	Farms[b]	Raw milk	DairyI	DairyII	Various products
Carbohydrate						
Glycerol	26	93	51	32	53	88
Ribose	97	99	81	78	84	100
Galactose	2	1	11	1	20	1
Glucose	100	100	100	99	100	100
Fructose	100	100	96	96	88	100
Mannose	19	14	18	10	15	15
Inositol	0	16	3	5	1	13
α-methyl-D-glucoside	0	4	0	0	0	2
N-acetyl-glucosamine	97	100	91	98	100	100
Amygdalin	2	50	18	15	21	23
Arbutin	85	99	68	75	71	99
Esculin	82	100	87	93	94	100
Salicin	65	99	60	69	64	87
Cellobiose	53	94	43	40	50	62
Maltose	100	100	100	99	100	100
Lactose	0	< 1	11	2	23	< 1
Saccharose	63	34	46	25	41	51
Trehalose	100	100	100	99	100	100
Starch	96	98	64	90	59	80
Glycogen	95	99	64	90	58	82
ß-Gentiobiose	0	6	2	< 1	1	2
Gluconate	0	25	22	16	31	53
Number of strains		766	131	167	86	171

[a] *B. cereus* results according to API (BioMérieux Sa, France)
[b] farm isolates (Te Giffel *et al.* 1995)

The results of carbohydrate metabolism tests revealed that there were many different types of *B. cereus* strains present: more than 20 types were observed in the raw milk and during the processing of pasteurized milk. Strikingly, only about 10 different

patterns were observed in the milk powder processing plant.

In Table 7.2 the results of the API 50 CHB tests of this study are compared to results obtained in other investigations. Generally, the percentage of positive results for the biochemical reactions of *B. cereus* obtained in this study are in line with those reported in the literature (Logan and Berkeley 1984, Rangasamy *et al.* 1993, Te Giffel *et al.* 1995 [Chapter 5]).

Several substrates were negative for all *B. cereus* strains tested: erythritol, arabinose, xylose, adonitol, β-methyl-D xyloside, sorbose, rhamnose, dulcitol, mannitol, sorbitol, α-methyl-D mannoside, melibiose, inulin, melizitose, raffinose, xylitol, turanose, lyxose, tagatose, fucose, arabitol, 2-ketogluconate, 5-ketogluconate. More than 90 % of the strains were able to utilise glucose, fructose, N-acetylglucosamine, aesculin, maltose and trehalose. For the other substrates the utilization as carbon source varied between < 10 and 90 %.

The main carbohydrate in milk, lactose, was fermented by 11 % of the raw milk isolates. Of the *B. cereus* strains isolated during processing at dairy I and II, 2 % and 23 %, respectively, were able to utilize lactose as carbon source.

Distribution of isolates among sampling sites

B. cereus strains could be isolated at all stages of processing of pasteurized milk and milk powder (Table 7.3). *B. cereus* was present in low numbers since in most cases the organism could only be isolated after incubation at 30 °C. Only from the standardization tank in the milk powder plant was it possible to isolate the organism without pre-incubation. It was shown that both vegetative cells and spores (isolated after a heat-activation step of 10 min at 80 °C) were present at the various sampling sites in the processing plants.

The contamination of raw milk with *B. cereus* was 35 % both in the samples of the milk collection tankers and in the storage tanks at the plants. As also observed in other studies (McKinnon and Pettipher 1983, Phillips and Griffiths 1986) there was seasonal variation in levels of *B. cereus*. Numbers were highest in the summer/autumn months (June to October), when cows were at pasture. In these months the contamination of the milk collection tankers increased to 50 % compared with 25 % during the rest of the year. Of the samples taken at the discharging sites of the milk collection tankers 13 % contained *B. cereus*. During processing the percentage of positive samples increased to 71 % after the pasteurization process. The MPN counts of freshly pasteurized milk varied from 0.9 to > 110 per 100 ml. The higher contamination level of raw milk in the summer/autumn months also resulted in higher numbers present in the final product: the

MPN counts ranged from 2 to > 110 in summer/autumn and from 0.9 to 9 in the winter months. The total aerobic counts of these samples were between 240 and 4600 ml^{-1}.

During the production of milk powder the percentage of positive samples increased up to 60 %. The ingredients added for some powder applications frequently contained *B. cereus*; 68 % of the samples were shown to be contaminated.

Some biotypes, including the strains able to ferment lactose, could be isolated from the raw milk, at all stages of processing and from the final products, whereas others were only present after the pasteurization process. At dairy II several *B. cereus* biotypes were introduced by the additives.

Table 7.3 Incidence of *Bacillus cereus* in samples (after pre-incubation for 6 h at 30 °C) taken from milk collection tankers and at various stages of processing in two dairy plants

	Number of samples	Positive samples (%)	
Milk collection			
Milk collection tankers (r)[a]	185	65	(35)
Hose unloading site (r)	45	6	(13)
Dairy I Pasteurized milk			
Storage bulk tanks (r)	26	9	(35)
Pasteurizer	33	20	(61)
Inlet tank	32	22	(69)
Tank	35	25	(71)
Filling process	26	17	(65)
Package	31	22	(71)
Dairy II Milk powder			
Storage bulk tank (r)	20	7	(35)
Additives	60	41	(68)
Standardization tank	20	12	(60)
Evaporation	20	9	(45)
Start spray drying	20	8	(40)
Powder	20	12	(60)

[a] (r) raw milk

Psychrotrophic growth

In total, 9 of the 155 strains (6 %) isolated from raw milk of the milk collection

tankers and the storage tanks at dairy I and II were able to grow at 7 °C. Further, 11 of 189 strains (6 %) originating from pasteurized milk at dairy I were found to be psychrotrophic. None of the isolates obtained from ingredients or from the milk powder production process showed growth at 7 °C.

Molecular typing of *B. cereus* isolates

In Figure 7.1 an example of RAPD fingerprinting of *B. cereus* strains isolated, on one day, at various stages of processing of milk powder in dairy II is presented. In Figure 7.2 an example of results obtained with plasmid profiling of strains, isolated on one day, at various sampling sites at dairy I is shown.

The molecular typing of *B. cereus* isolates showed that some types could be isolated from the raw milk and throughout the whole process, whereas other strains could only be isolated after the pasteurization step in the process.

1 2 3 4 5 6 7 8 9 10

Figure 7.1 PCR/RAPD fingerprinting of *Bacillus cereus* strains isolated at dairy II using the random primer R1: [5' CGGCCTCTGGGC 3']. Lane 1, raw milk; lane 2, raw milk; lane 3, standardization tank; lane 4, standardization tank; lane 5, after evaporation; lane 6, milk powder; lane 7, milk powder; lane 8, additives; lane 9, additives; lane 10, additives.

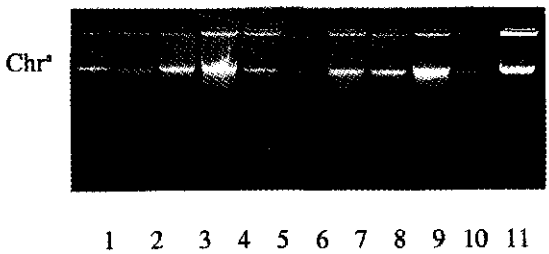


Figure 7.2 Plasmid profiles of *Bacillus cereus* strains isolated at dairy I. Lane 1, raw milk; lane 2, raw milk; lane 3, raw milk; lane 4 pasteurizer; lane 5, pasteurizer; lane 6, inlet pasteurized milk tank; lane 7, inlet pasteurized milk tank; lane 8, filling; lane 9, filling; lane 10, package; lane 11, package.
[a] Chr=chromosomal DNA

DISCUSSION

The use of MEYP agar for the detection of *B. cereus* is based on a double diagnostic system: fermentation of mannitol and lecithinase production. Polymyxin B sulfate is included as a selective agent to inhibit Gram-negative organisms (Mossel *et al.* 1967). Although this medium is selective for *B. cereus*, other bacilli may be found, and it is therefore necessary to confirm suspected colonies. For the confirmation of *B. cereus* glucose fermentation, VP reaction and nitrate reduction were applied in this study. According to the ISO procedure *B. cereus* should be positive for all three reactions; however up to 5 % of the strains might be VP negative. Among the API 50 CHB *B. cereus*-positive strains there were 4.4 % VP negative strains, 3.9 % could not reduce nitrate and 0.3 % were negative for both these confirmation tests. This deviation is acceptable since according to numerical taxonomy in bacterial classification the average difference in replicate tests on the same strain is commonly about 5 % (Sneath 1984).

In total, 443 (87 %) of the 507 suspected colonies isolated on MEYP were determined to be *B. cereus* through use of both confirmatory tests and carbohydrate patterns. This is comparable to the percentage (90.4 %) observed by Te Giffel *et al.* (1995 [Chapter 5]) but higher than the percentage noted by others; Walthew and Lück (1978) showed that 74.2 % of the strains isolated from milk powder on MEYP agar were

B. cereus, Ahmed *et al.* (1983) found 71.5 % of their suspected colonies isolated from milk and milk products on Kim and Goepfert agar medium, a comparable egg yolk polymyxin medium, were *B. cereus*, and Rusul and Yaacob (1995) confirmed 42.3 % of the presumptive *B. cereus* isolated from various products on Polymyxin B Egg yolk Mannitol Bromothymolblue Agar (PEMBA), a modification of MEYP agar in which the phenol red is replaced by bromothymol blue. These studies suggest that in samples containing high levels of competitive organisms it is more difficult to isolate *B. cereus*. This was also demonstrated in this study; from highly contaminated samples suspected *B. cereus* could not always be confirmed and other bacilli were frequently isolated. Especially *B. laterosporus* and *B. mycoides* were present. *B. mycoides* can be differentiated from *B. cereus* by the rhizoid growth shown by this organism on agar plates. In contrast to *B. cereus*, *B. laterosporus* is able to ferment mannitol. However, the reaction of colonies on mannitol is often impossible to ascertain due to masking of non-fermentative colonies by the acid generated by proximal actively fermenting colonies. Heterogeneity of response has also been observed in other studies; in general glucose was fermented by all strains, 0 to 42 % of the *B. cereus* strains were VP negative while 0 to 13 % did not reduce nitrate (Lancette and Harmon 1980, Logan and Berkeley 1984, Rajkowski and Mikolajcik 1987, Wong *et al.* 1988, Mosso *et al.* 1989, Rangasamy *et al.* 1993, Te Giffel *et al.* 1995 [Chapter 5]).

The percentage of positive results for the carbohydrate tests (Table 7.2) of our *B. cereus* isolates was within the range reported by others (Logan and Berkeley 1984, Rangasamy *et al.* 1993, Te Giffel *et al.* 1995 [Chapter 5]). There was no distinct relation between the carbohydrate pattern and the contamination source. However, certain *B. cereus* types could be isolated from different sources in the contamination route from raw milk to final products. Several biotypes were introduced after the pasteurization process, indicating post-pasteurization contamination. To indicate the major vectors of contamination with greater certainty, the use of other tests for identification and differentiation is necessary. The RAPD technique (randomly amplified polymorphic DNA) has been used for epidemiological subtyping of e.g. *Listeria* spp. and *Campylobacter* spp. (Mazurier and Wernars 1992, Mazurier *et al.* 1992). Other methods that can be utilized to differentiate strains on species level include plasmid profiling (Ellison *et al.* 1989) and gas chromatographic analysis of cellular fatty-acids (Väisänen *et al.* 1991, Wauthoz *et al.* 1995). Epidemiological subtyping of strains by PCR/RAPD and plasmid profiling of strains isolated in the dairy processing plants confirmed the results of biochemical characterization; some types could be isolated from raw milk and throughout the whole process, indicating that raw milk is an important source of *B. cereus* contamination. Other types could only be isolated after the pasteurization step in the process, indicating that *B.*

cereus can also be introduced into milk by sources other than the raw milk.

There was considerable variation among strains isolated from different sources in the ability to ferment lactose, the main carbohydrate in milk. In earlier studies with *B. cereus* strains isolated from raw milk and pasteurized dairy products, 8 to 15 % could ferment lactose (Johnston and Bruce 1982, Logan and Berkeley 1984, Rangasamy *et al.* 1993). For strains isolated from various, non-dairy foods it was shown that less than 1 % were able to use lactose as carbon source. In similar studies a percentage of 0 to 8 was observed for *B. cereus* found in various food products (Hutchinson and Taplin 1978, Logan and Berkeley 1984, Nikodémusz 1979). Of the *B. cereus* strains isolated on farms less than 1 % utilized lactose (Te Giffel *et al.* 1995 [Chapter 5]). Of the raw milk isolates 11 % fermented lactose; this might suggest a selection or an adaptation of strains to the environment during storage and transport of raw milk. Of the strains isolated during the processing of pasteurized milk only 2 % were able to utilize lactose as carbon source, indicating that some of the *B. cereus* strains isolated may originate from other, additional sources in the processing plant. In the milk powder factory, 23 % of the isolates could utilize lactose; this high percentage can be explained by the fact that for some applications lactose is added during the production process. In 8 of the 11 samples of lactose analyzed *B. cereus* was present (data not shown). A similar effect was observed for *Listeria* in a saw mill and a potato processing plant; xylose-fermenting strains were most frequently isolated. This reflects the nature of the substrates available in environments where organic matter is common (Cox *et al.* 1989).

It has been proposed that *B. cereus* strains producing emetic toxin are unable to hydrolyze starch or ferment salicin (Shinagawa 1990). On farms very few such isolates were found: 98 % of the strains were able to hydrolyze starch and 99 % could ferment salicin. However, furtheron in the production process of pasteurized milk and milk powder the percentage of isolates unable to use salicin or starch as carbon source increased, suggesting that strains producing emetic toxin could be present in dairy products.

The diarrhoeal food-poisoning syndrome is caused by an unstable enterotoxin. This type of food poisoning is caused by ingestion of the cells rather than by ready-formed toxin (Granum *et al.* 1993). Therefore strains that can grow at low temperatures and at 37 °C should be regarded as potential food-poisoning organisms, since they can grow both in the product and in the ileum. Only a few strains were able to multiply at 7 °C, these strains grew only poorly at 37 °C (data not shown) suggesting that they are unable to grow in the ileum and may not be very enterotoxigenic.

Generally, the contamination levels of the analysed samples were comparable to those found in other investigations. For raw milk from milk collection tankers and storage

tanks at the processing plants 35 % of the samples contained *B. cereus*. In a previous study similar levels (36 %) were observed in raw milk in storage tanks on farms (Te Giffel *et al.* 1995 [Chapter 5]). In the winter months, when the numbers of *B. cereus* in raw milk were very low, it was still possible to detect the organism at all stages in the production of milk or powder, suggesting that the organism may originate from additional sources. In this respect the equipment may be important; *B. cereus* is able to strongly adhere to the surfaces of milk processing equipment (Rönner *et al.* 1990, Stadhouders *et al.* 1982 [Chapter 8]). Therefore it may colonize a processing line and remain there to continually contaminate milk. During processing an increase in contamination was observed; up to 71 % in the fluid milk processing plant and up to 60 % in the milk powder processing plant. These levels were higher than those reported in other studies (Overcast and Atmaram 1974, Walthew and Lück 1978, Coghill and Juffs 1979, Wong *et al.* 1988, Stadhouders and Driessen 1992, Griffiths 1993, Rangasamy *et al.* 1993, Becker *et al.* 1994, Meira De Vasconcellos and Rabinovitch 1995) and might be partly due to the pre-incubation step (6 h at 30 °C) used for the samples. Due to concentration effects in the production process of milk powder the numbers of micro-organisms in the powder are generally 10 times higher compared with raw milk (Stadhouders *et al.* 1982); this may result in a higher percentage of contamination, as observed in the powder samples. In addition, the organism was isolated from 68 % of the additives, indicating that these might be a source of contamination and contribute to the increase in contamination levels during processing. Furthermore, all powder samples were taken from the startup powder, which may have affected the results. It was reported in another study that the first powder was more often positive than after some time of operation, due to contamination via the equipment (Stadhouders *et al.* 1982).

Both vegetative cells and spores could be isolated at the various sampling points. From most of the raw milk samples *B. cereus* could only be isolated after a heat-activation step, indicating that *B. cereus* was present in raw milk as spores. These are slow-germinating spores since they do not germinate during pre-incubation (6 h at 30 °C). This was confirmed by assessment of the germination rate as described in a previous study (Phillips and Griffiths 1986). However, after pasteurization the organism could be isolated from the majority of the samples without a heat-activation step. The *B. cereus* strains isolated from pasteurized milk were of the fast-germinating type. These observations are consistent with those reported earlier (McKinnon and Pettipher 1983, Phillips and Griffiths 1986).

Only 6 % of the raw milk isolates were found to be psychrotrophic, compared with 30 % of the strains isolated from milk of individual cows and in the bulk tank milk on farms (Griffiths 1993, Johnston and Bruce 1982, Shehata and Collins 1971, Te Giffel

et al. 1995 [Chapter 5]). In dairy I, 6 % of the isolates showed growth at 7 °C. Because psychrotrophic strains will have a significant impact on the keeping quality of pasteurized milk(products), if the low temperatures during storage and distribution are maintained in the right manner, the results in this study indicate that adaptation, or more likely selection, to low growth temperatures occurs during further storage and distribution. None of the strains isolated from the milk powder production process was able to grow at 7 °C; this is probably due to the type of process. Spores of psychrotrophic isolates are more easily destroyed by heat-processing than mesophilic types (Cousin 1982). Mesophilic *B. cereus* are more important for the spoilage, after reconstitution, of this type of product.

In conclusion, *B. cereus* could be isolated from milk collection tankers and all stages of production from raw milk to pasteurized milk or powder. The heterogeneity in response to the confirmation tests, the variability in carbohydrate utilization patterns, temperature ranges of growth and PCR/RAPD fingerprinting show that many different *B. cereus* types can be isolated. At the dairy plants fewer differences between strains were observed than for the farm isolates, which may indicate selection or adaptation to the environment. Biochemical and molecular characterization demonstrated that raw milk is an important source of contamination, but it was also suggested that the organism can be introduced into milk via additional sources, e.g. equipment.

ACKNOWLEDGEMENTS

The authors would like to thank W.C. Tolboom, M.C. van Kippersluis and F. Oudshoorn for their technical support.

REFERENCES

Ahmed AA-H, Moustafa MK and EH Marth (1983) Incidence of *Bacillus cereus* in milk and some milk products. *Journal of Food Protection* **46** 126-128.

Anonymous (1992) ISO/DIS 7932 Microbiology-General guidance for enumeration of *Bacillus cereus* colony-count technique at 30 °C, International Organization for Standardization, Switzerland.

Becker H, Schaller G, von Wiese W and G Terplan (1994) *Bacillus cereus* in infant foods and dried milk products. *International Journal of Food Microbiology* **23** 1-15.

Cannon RY (1972) Contamination of raw milk with psychrotrophic sporeformers. *Journal of Dairy Science* **55** 669.

Christiansson A, Satyanarayan Naidu A, Nilsson I, Wadström T and H-E. Petterson (1989) Toxin production by *Bacillus cereus* isolates in milk at low temperatures. *Applied and Environmental Microbiology* **55** 2595-2600.

Coghill D and H.S. Juffs (1979) Incidence of psychrotrophic sporeforming bacteria in pasteurised milk and cream products and effect of temperature on their growth. *Australian Journal of Dairy Technology* **34** 150-153.

Collins EB (1981) Heat-resistant psychrotrophic organisms. *Journal of Dairy Science* **64** 157-160.

Cousin MA (1982) Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. *Journal of Food Protection* **45** 172-207.

Cox LJ, Kleiss T, Cordier JL, Cordellana C, Konkel P, Pedrazzini C, Beumer R and A Siebenga (1989) *Listeria* spp. in food processing, non-food and domestic environments. *Food Microbiology* **6** 49-61.

Credit C, Hedeman R, Heywood P and D Westhoff (1972) Identification of bacteria isolated from pasteurized milk following refrigerated storage. *Journal of Milk and Food Technology* **35** 708-709.

Ellison A, Dodd CER and WM Waites (1989) Use of plasmid profiles to differentiate between strains of *Bacillus cereus*. *Food Microbiology* **6** 93-98.

Granum PE, Brynestad S, O'Sullivan K and H Nissen (1993) Enterotoxin from *Bacillus cereus*: production and characterization. *Netherlands Milk and Dairy Journal* **47** 63-70.

Griffiths MW (1993) *Bacillus cereus* in liquid milk and other milk products. *IDF bulletin* **275** 36-39.

Griffiths MW and JD Phillips (1990) Incidence, sources and some properties of psychrotrophic *Bacillus* spp. found in raw and pasteurized milk. *Journal of the Society of Dairy Technology* **43** 62-66.

Grosskopf JC and WJ Harper (1974) Isolation and identification of psychrotrophic sporeformers in milk. *Milchwissenschaft* **29** 467-470.

Hutchinson EMS and J Taplin (1978) *Bacillus cereus* in food. *Food Technology in Australia* **30** 329-333.

Johnson KM (1984) *Bacillus cereus* in food-borne illness- An update. *Journal of Food Protection* **47** 145-153.

Johnston DW and J Bruce (1982) Incidence of thermoduric psychrotrophs in milk produced in the west of Scotland. *Journal of Applied Bacteriology* **52** 333-337.

Lancette GA and SM Harmon (1980) Enumeration and confirmation of *Bacillus cereus* in foods: collaborative study. *Journal of the Association of Official Analytical Chemists* **63** 581-586.

Logan NA and RC Berkeley (1984) Identification of *Bacillus* strains using the API system. *Journal of General Microbiology* **130** 1871-1882.

Mazurier S and K Wernars (1992) Typing of *Listeria* strains by random amplification of polymorphic DNA. *Research in Microbiology* **143** 499-505.

Mazurier S, Van de Giessen A, Heuvelman K and K Wernars (1992) RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. *Letters in Applied Microbiology* **14** 260-262.

McGuiggan JTM, Gilmour A and LM Lawrence (1994) Factors influencing the recovery of psychrotrophic, mesophilic and thermophilic *Bacillus* spp. from bulk raw milk. *Journal of the Society of Dairy Technology* **47** 111-116.

McKinnon CH and GL Pettipher (1983) A survey of sources of heat-resistant bacteria in milk with particular reference to psychrotrophic spore-forming bacteria. *Journal of Dairy Research* **50** 163-170.

Meer RR, Baker J, Bodyfelt FW and MW Griffiths (1991) Psychrotrophic *Bacillus* spp. in fluid milk products: A review. *Journal of Food Protection* **54** 969-979.

Meira De Vasconcellos FJ and L Rabinovitch (1995) A new formula for an alternative culture medium, without antibiotics, for isolation and presumptive quantification of *Bacillus cereus* in foods. *Journal of Food Protection* **58** 235-238.

Mossel DAA, Koopman MJ and E Jongerius (1967) Enumeration of *Bacillus cereus* in foods. *Applied Microbiology* **15** 650-653.

Mosso MA, Garcia Arribas ML, Cuena JA and MC De La Rosa (1989) Enumeration of *Bacillus* and *Bacillus cereus* spores in food from Spain. *Journal of Food Protection* **52** 184-188.

Nikodémusz I (1979) Occurrence of *Bacillus cereus* in foods. *Acta Alimentaria* **8** 111-116.

Overcast WW and K Atmaram (1974) The role of *Bacillus cereus* in sweet-curdling of fluid milk. *Journal of Milk and Food Technology* **37** 233-236.

Phillips JD and MW Griffiths (1986) Factors contributing to the seasonal variation of *Bacillus* spp. in pasteurised dairy products. *Journal of Applied Bacteriology* **61** 275-285.

Rajkowski KT and EM Mikolajcik (1987) Characteristics of selected strains of *Bacillus cereus*. *Journal of Food Protection* **50** 199-205.

Rangasamy PN, Iyer M and H Roginski (1993) Isolation and characterisation of *Bacillus cereus* in milk and dairy products manufactured in Victoria. *Australian Journal of Dairy Technology* **48** 93-95.

Rönner U, Husmark U and A Henriksson (1990) Adhesion of *Bacillus* spores in relation to hydrophobicity. *Journal of Applied Bacteriology* **69** 550-556.

Rusul G and NH Yaacob (1995) Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA. *International Journal of Food Microbiology* **25** 131-139.

Shehata TE and EB Collins (1971) Isolation and identification of psychrophilic species of *Bacillus* from milk. *Applied Bacteriology* **21** 466-469.

Shinagawa K (1990) Analytical methods for *Bacillus cereus* and other *Bacillus* species. *International Journal of Food Microbiology* **10** 125-142.

Sneath PHA (1984) Numerical Taxonomy. In Krieg NR and JG Holt (eds.) *Bergey's Manual of Systematic Bacteriology* **1**, Williams and Wilkins, Baltimore, 5-11.

Stadhouders J and FM Driessen (1992) Other milk products. *IDF bulletin* **275** 40-45.

Stadhouders J, Hup G and F Hassing (1982) The conceptions index and indicator organisms discussed on the basis of the bacteriology of spray-dried milk powder. *Netherlands Milk and Dairy Journal* **36** 231-260.

Te Giffel MC, Beumer RR, Slaghuis BA and FM Rombouts (1995) Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands. *Netherlands Milk and Dairy Journal* **49** 125-138.

Van Heddeghem A and G Vlaemynck (1993) Sources of contamination of milk with *Bacillus cereus* on the farm and in the factory. *IDF Bulletin* **275** 19-22.

Väisänen OM, Mwaisumo NJ and MS Salkinoja-Salonen (1991) Differentiation of dairy strains of the *Bacillus cereus* group by phage typing, minimum growth temperature, and fatty acid analysis. *Journal of Applied Bacteriology* **70** 315-324.

Walker SJ (1988) Major spoilage micro-organisms in milk and dairy products. *Journal of the Society of Dairy Technology* **41** 91-92.

Walthew J and H Lück (1978) Incidence of *Bacillus cereus* in milk powder. *South African Journal of Dairy Technology* **10** 47-50.

Washam CJ, Olson HC and ER Vedamuthu (1977) Heat-resistant psychrotrophic bacteria isolated from pasteurized milk. *Journal of Food Protection* **40** 101-108.

Wauthoz P, Ellioui M and J Decallonne (1995) Gas chromatographic analysis of cellular fatty acids in the identification of foodborne bacteria. *Journal of Food Protection* **58** 1234-1240.

Wong HC, Chang MH and JY Fan (1988) Incidence and characterisation of *Bacillus cereus* isolates contaminating dairy products. *Applied and Environmental Microbiology* **54** 699-702.

CHAPTER 8

THE ROLE OF HEAT EXCHANGERS IN THE CONTAMINATION OF MILK WITH *BACILLUS CEREUS* IN DAIRY PROCESSING PLANTS

ABSTRACT

In the dairy industry, microbiological contamination may arise from equipment used for handling or processing. Bacterial attachment to and colonization of milk contact surfaces is likely to be of considerable importance in subsequent contamination of products. Milk components are simply deposited on contact surfaces, forming residue build-ups which serve to protect the associated bacteria from cleaning and disinfectants and at the same time provide a source of nutrients which allows bacterial reproduction.

In this study, it was demonstrated that *B. cereus* spores could adhere to stainless steel, germinate and/or multiply, depending on the temperature, in a tube heat exchanger. The levels of *B. cereus* detected in the tubes, after 24 h at 20 °C, varied from less than 1 to 2600 cm^{-2}. Adhered cells and/or spores were more resistant to cleaning with K500 and sodium hydroxide, determined by laboratory suspension tests and surface tests on stainless steel. This was also shown in a field trial with a tube heat exchanger; after cleaning *B. cereus* could still be isolated from all tubes determined by swab samples of individual tubes.

PCR/RAPD typing of isolates revealed that the organisms found on the surfaces were identical to those in the ingoing milk.

This chapter has been accepted for publication as:

The role of heat exchangers in the contamination of milk with *Bacillus cereus* in dairy processing plants

MC Te Giffel, RR Beumer, LPM Langeveld and FM Rombouts

International Journal of Dairy Technology (1997) (in press)

INTRODUCTION

In the dairy industry spore-forming micro-organisms, especially *Bacillus cereus*, are important as they determine the keeping quality of pasteurized milk and milk products (Meer *et al.* 1991).

Since *Bacillus* species are widely distributed in the environment, they can be introduced into milk from a variety of sources during production, handling and processing (Meer *et al.* 1991, Van Heddeghem and Vlaemynck 1993, Te Giffel *et al.* 1995a, Andersson *et al.* 1995). A recent study on the incidence and characterization of *B. cereus* in two dairy processing plants in the Netherlands indicated that the organism can be introduced into the milk via sources other than raw milk (Te Giffel *et al.* 1996 [Chapter 7]). In this respect, the equipment is probably an important source of contamination. Contact surfaces of milk pipelines and processing equipment have been suggested to be a direct source of post pasteurization contamination (Austin and Bergeron 1995). *B. cereus* spores are very hydrophobic and will attach to equipment surfaces more readily than vegetative cells, where they might multiply and resporulate (Andersson *et al.* 1995, Austin and Bergeron 1995). The attached organisms, if left undisturbed, will form biofilms (Hood and Zottola 1995).

Attachment of micro-organisms to food contact surfaces is a concern in the food industry because previous studies have shown that these cells appear to be more resistant to antibiotics, chemical sanitizers and heat (Hood and Zottola 1995, Wirtanen *et al.* 1996). In the dairy industry, milk residue that remains on inadequately cleaned surfaces may provide a nutrient pool for any micro-organisms that do attach (Hood and Zottola 1995). Even with acceptable Cleaning-In-Place (CIP) systems in milking and dairy plant operations, micro-organisms have been shown to accumulate on equipment surfaces (Austin and Bergeron 1995). Micro-organisms including streptococci, *Thermus thermophilus*, *Bacillus stearothermophilus*, *Escherichia coli* and *Acinetobacter* were able to adhere to and grow on internal surfaces at various places in preheaters and evaporators, resulting in release of large numbers of cells into the milk (Langeveld *et al.* 1995).

The aim of this study was to confirm that *B. cereus* spores can be retained in a (tube) heat exchanger, germinate and grow out resulting in contamination of product in a later stage in the process. A second aim was to evaluate the effect of cleaning on *B. cereus* spores present in the heat exchangers. Therefore, suspension tests, surface tests on stainless steel and field trials in the heat exchanger were used.

To distinguish between inoculated strains and other bacteria with similar colony appearances and growth characteristics, the identity of the isolates throughout the experiments was confirmed by DNA/RAPD typing.

MATERIALS AND METHODS

Strains and preparation of spore suspensions

Two *B. cereus* isolates, one psychrotrophic strain 95003 (able to grow at 7 °C) and one mesophilic strain 94495, obtained from a pasteurizer in a dairy processing plant were used. The isolates were identified as *B. cereus* according to biochemical tests described in the ISO Standard Procedure for enumeration of *B. cereus*, i.e. glucose fermentation, nitrate reduction and Voges Proskauer (VP) reaction. *B. cereus* should be positive for all three confirmation reactions (Anonymous 1992). In addition, carbohydrate utilization patterns (API 50 CHB, BioMérieux Sa, France) were determined for identification of the isolates. Both isolates were slow-germinating, determined by assessment of the germination rate described by Phillips and Griffiths (1986).

Vegetative cells of *B. cereus* were grown in Brain Heart Infusion broth (BHI; Difco 0037-01-6) for 16-20 h at 30 °C.

Sporulation of *B. cereus* was achieved on Tryptone Soya Agar (TSA; Oxoid CM 131). TSA plates were inoculated with 0.1 ml of a culture (10^8 cfu ml^{-1}) grown in BHI for 16-20 h. After incubation for 7 days at 30 °C, spores were washed from the surface of the agar with sterile distilled water and centrifuged at 2000 *g* for 20 min. The pellets were washed three times and resuspended in sterile distilled water. To destroy the vegetative cells the spore suspension was given a heat shock of 60 $\pm$ 5 s at 80 °C in a thermostatically controlled water-bath. The number of spores was determined after plate counting on TSA and by microscopy. Freshly prepared suspensions were used in each experiment.

The behaviour of *B. cereus* spores in a tube heat exchanger

A pilot tube heat exchanger composed of 20 stainless steel tubes (length 1 m; internal diameter 0.01 m) in which the milk could be heated from 2 °C to 80 °C was used. The walls of these tubes had temperatures increasing from 15 to 85 °C. The system was described in detail by Langeveld *et al.* (1995).

Experimental protocol

About 100 l of raw whole milk, directly collected from farms, was sterilized for 5 s at 140 °C, in order to destroy any micro-organism and spores that could interfere with the experimental results, cooled to 4 °C and stored in a supply tank which had been steamed just before. The milk was inoculated with spores to obtain a level of 10^3 spores per ml, this was checked by plate counting. In experiment I and II the isolates were used seperately, in

experiment III both strains were added at the same time.

The heat exchanger was circulated for 60 min with 1 % sodium hydroxide (NaOH) at 80 °C, followed by rinsing with water and circulation for 60 min with 0.5 % nitric acid (HNO_3) at 80 °C. The HNO_3 solution was flushed out by cold water containing 2 mg available chlorine per l. In each experiment the numbers of micro-organisms were determined in this chlorinated water by plate counting on TSA. The experimental temperatures were adjusted during flushing with chlorinated water. When the desired temperatures were reached, the chlorinated water was replaced by the milk coming from the supply tank. The flow of the milk was 85 l h^{-1}, corresponding to a velocity in the tubes of 0.3 m s^{-1}.

The milk entering tube 1 had a temperature of 3-4 °C, the milk leaving tube 20 had a temperature of 79-80 °C. During the experiment samples of the outcoming milk were taken after 20, 40 and 60 min. The samples were cooled immediately in melting ice and analyzed within 2 h. After 1 h the heating water flow was stopped, the milk was replaced by fresh mains water, running for 10 min. The water flow was stopped, followed by loosening the couplings of the water tubes, in order to drain the water. The bends were loosened in such a sequence that backflow of the water was prevented, to exclude cross-contamination of tubes. Then, the system was left at 20 °C for 24 h.

Subsequently, water was pumped through the system for 10 min. Samples of this water were taken of the first water coming out. After that the bends were loosened as described above and swab samples were taken from the milk tubes. In experiment III the system was cleaned first by flowing a solution of K500/NaOH for 15 min at 80 °C. A sample was taken of the first solution coming out of the heat exchanger. Subsequently, the cleaning solution was displaced by fresh mains water and the same procedure as in experiments I and II was followed.

To take swab samples, the couplings between the milk tubes could be detached and the interior of the tubes swabbed with a squeegee. This squeegee was composed of a round teflon disk mounted on the end of a 1.2 m long stainless steel stem. The diameter of the disk was slightly larger than the internal diameter of the milk tube. After introducing the squeegee, 10 ml of Peptone Saline Solution (PSS; NaCl 8.5 g l^{-1} and Neutralized Bacteriological Peptone (Oxoid L34), 1 g l^{-1}) in experiment I and II and 10 ml inactivation fluid in experiment III (composition as described in the EST (Anonymous 1988)), was poured into the tube and the squeegee was moved three times back and forth. The swab fluid was poured out and placed in melting ice and examined within 2 h.

Serial dilutions of the samples were prepared in PSS and surface plated on TSA and Mannitol Egg Yolk Polymyxin (MEYP) agar (Cereus Selective Agar; Merck 5267) or, in experiment III, an agar medium comparable with MEYP containing saccharose instead of mannitol to differentiate between the isolates as strain 94495 was unable to ferment this

carbohydrate. To enumerate spores, 5 ml of the swab fluid was heated at 80 °C for 10 min and cooled in melting ice before further serial dilution, followed by surface plating on TSA and MEYP. Plates were incubated at 30 °C for 24 h and counted. From each plate a colony was plated out on a TSA for confirmation of its identity by PCR/RAPD.

The effect of cleaning on *B. cereus*, vegetative cells and spores, determined by suspension tests and surface tests on stainless steel

Media and reagents

Media and reagents were prepared as described in the European Suspension Test (EST; Anonymous, 1988). Chemicals were obtained from BDH, Merck and Sigma. Diluent containing 8.5 g l^{-1} NaCl was used.

A combined solution of 1 % K500, containing amongst others Ethylenediaminetetraacetic acid (EDTA) tetrasodium salt (Cooperatieve Stremsel- en Kleurselfabriek, Leeuwarden, The Netherlands) and 1 % NaOH, used as a cleaning agent in the dairy industry, instead of an alkali/acid procedure for sanitation, was tested. Cleaning solutions were freshly prepared on each day of testing. All tests were performed in quintuplicate by an experienced microbiologist.

The effect of treatment on vegetative cells (ME) and on spores (SE), was calculated according to the formula:

ME or SE value = log Nc - log Nd, in which

Nc= the number of vegetative cells/spores per ml in the control test;
Nd= the number of vegetative cells/spores per ml after treatment (e.g heat or cleaning solution (K500/NaOH)).

European Suspension Test

The activity of the agent in suspensions was evaluated using the EST (Anonymous 1988). To represent the in-use situation in the dairy industry, a contact time of 15 min and a temperature of 80 °C was used. Tests were carried out in the presence of 4 % milk as organic soil instead of bovine albumin.

Surface test

For the surface tests for comparative testing of bactericidal or sporicidal activity, the following procedure was used.

Test surfaces were 20 mm diameter stainless steel discs. This surface was chosen because it relates to the practical application in the heat exchanger. The stainless steel discs

were stored in 70 % alcohol until required and then flamed and placed in a petridish. Before each use the discs were washed in detergent and rinsed in sterile distilled water. Contaminated surfaces were prepared by inoculating the carriers with 0.1 ml drops of test suspension containing 10^7 vegetative cells or spores ml^{-1}. As organic soil 4 % milk was added. Surfaces were dried at 42 °C for 1 h. Samples of cleaning test solution or water of standard hardness (WSH) (0.1 ml) were dropped onto the surface to cover the test film. After a contact time of 15 min at 80 °C in a thermostatically controlled water-bath, the discs were placed in a 100-ml sterile flask containing 10 ml neutralization medium (Anonymous 1988) together with 6 g of glass beads (3 mm diameter) and placed in a shaking incubator for 5 min at 50 °C. The neutralization medium and 10-folds dilutions were plated on TSA. Plates were incubated at 30 °C for 24 h, counted and the sporicidal effect was calculated.

Field trials

The field trials were carried out in a tube heat exchanger, this experiment was described above as experiment III, and in a pilot plate heat exchanger.

Confirmation of the identity of the *B. cereus* isolates by DNA typing

Isolation of DNA

For DNA isolation the strains were grown on TSA for 18-24 h at 30 °C. A single bacterial colony (± 1.5 mm) was picked and the DNA was isolated using InstaGene Matrix (Biorad 732-6030) according to the manufacturer's protocol with minor adjustments.

PCR/RAPD amplification

PCR amplifications using the random primer R1: [5' CGGCCTCTGC 3'] were performed by using a Thermocycler 480 (Perkin-Elmer, Gouda, The Netherlands). The reactions were carried out in 0.5-ml tubes which contained 50 µl of the following buffer: 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM NaCl, 2.5 mM (each) deoxynucleoside triphosphates, 1 U of *Taq* polymerase (GibcoBRL 18038-026) and 250 ng of primer R1. Then, 5 µl (500- 1000 ng) of template DNA was added. Amplification was done in 55 cycles of 1 min at 94 °C (denaturation), 1 min at 25 °C (annealing), and 8 min at 72 °C (extension-polymerization). PCR products were analyzed by agarose gel electrophoresis.

RESULTS AND DISCUSSION

This study was carried out to prove that equipment plays an important role in the contamination of milk with *B. cereus* at dairy processing plants. Therefore, pilot plant scale

experiments were carried out in a tube heat exchanger.

None of the chlorinated water samples, taken before starting the experiment, contained detectable levels of micro-organisms. This illustrates that the pretreatment of the heat exchanger was effective.

Milk was inoculated with about 10^3 spores per ml; comparable with levels ranging from 1 to 1600 *B. cereus* spores per ml in raw milk that have been reported in literature (Te Giffel *et al.* 1996 [Chapter 7]). During the experiment the numbers of spores in the outcoming milk decreased, while the total counts (vegetative cells and spores) remained unchanged as shown in Table 8.1, indicating that germination occurred. Spores were activated by heating the milk from 2 °C to 80 °C, resulting in 60 to 87 % germination within 60 min. In a previous study, comparisons of spore counts in unheated milk and pasteurized milk suggested activation of more than 95 % of the spores present (Griffiths and Phillips 1990).

Table 8.1 Numbers of *Bacillus cereus*, vegetative cells and spores, in the outcoming milk, samples taken during the experiment at t= 0, 20, 40 and 60 min.

Time (min)	Exp I	Exp II	Exp III	
	95003[a]	94495	95003	94495
0	3.1[b](3.1)[c]	3.5 (3.4)	3.0 (2.8)	3.5 (3.3)
20	3.0 (2.4)	3.4 (2.3)	3.0 (2.6)	3.4 (3.0)
40	3.0 (2.1)	3.4 (2.6)	2.8 (2.3)	3.3 (2.7)
60	3.0 (2.4)	3.5 (2.6)	2.9 (2.3)	3.4 (3.0)

[a] strain identity number; [b] numbers of vegetative cells and spores (log N ml^{-1}); [c] numbers of spores, determined after a heat-activation step of 10 min at 80 °C (log N ml^{-1})

After running the experiment for 60 min, the *B. cereus* counts, vegetative cells and spores, in the milk varied from 0.8×10^3 (log value 2.9) to 3.2×10^3 (log value 3.5) per ml in the different experiments. After 24 h at 20 °C, the levels of *B. cereus* in the first rinse water samples were 1.2×10^5 ml^{-1} in experiment I and 1.7×10^4 ml^{-1} in experiment II. The spore counts were low in both experiments: 1.8×10^1 and 2.5×10^1 ml^{-1}, respectively. Thus, the organisms were mainly present as vegetative cells, indicating germination of spores and subsequent outgrowth of vegetative cells in the equipment. The swab samples taken after loosening of the bends showed that presence of *B. cereus* could be demonstrated in all 20 tubes, in levels ranging from 16 to 1.6×10^5 per ml of swab fluid, corresponding to less

than 1 to about 2600 bacteria cm^{-2} (Table 8.2). Growth of other bacteria was not detected. In a study in which numbers of bacteria attaching were enumerated by epifluorescence microscopy, levels of 10^4 to 10^5 organisms per cm^2, depending on the type of micro-organism, were detected on stainless steel after 1 h at 25 °C in presence of milk. The suspensions used to inoculate contained about 10^6 cfu ml^{-1} (Speers and Gilmour 1985).

Although a psychrotrophic strain was used in experiment I and a mesophilic strain in experiment II no significant differences between both experiments were observed. Psychrotrophic strains produce spores that are less resistant to heat than those produced by mesophiles. However, these spores produced by psychrotrophs are also able to survive the pasteurization process (Griffiths and Phillips 1990, Meer *et al.* 1991), and even a more severe heat treatment as applied here.

Table 8.2 Levels of *Bacillus cereus* on tube walls in a tube heat exchanger after 24 h at 20 °C determined by swab samples calculated as bacterial density per cm^2

	Bacterial density on wall (cm^{-2})	
	Exp I	Exp II
Tube	95003[a]	94495
1	2324	2662
2	2578	1866
3	891	107
4	509	14
5	13	< 1
6	68	1
7	57	< 1
8	8	5
9	3	< 1
10	< 1	5
11	< 1	< 1
12	< 1	9
13	< 1	1
14	3	3
15	2	1
16	< 1	< 1
17	< 1	< 1
18	2	< 1
19	3	< 1
20	4	< 1

[a] strain identity number

In the tubes that had the lowest temperatures growth could be noted clearly. In experiment I, up to tube 7, levels of more than the 10^3 per ml, corresponding to about 10 per cm^{-2}, were found. The numbers of spores present were low; less than 1 per cm^{-2}. In experiment II levels of more than 10 per cm^2 were observed up to tube 4. The numbers of spores were between less than 1 and 7 per cm^2.

In tube 1 to 4 most milk deposit was present in both experiments as shown by turbidity of the swab samples. Attachment and growth of *B. cereus* are likely to be influenced by this. The deposit may enhance the absorption of spores or bacteria to the surface and by encapsulation in the deposit these micro-organisms may become protected against e.g. cleaning agents (Hood and Zottola 1995, Wirtanen *et al.* 1996).

In experiment III the system was cleaned first by means of pumping a mixture of a combination of K500 and NaOH. As expected the levels of *B. cereus* decreased, the vegetative cells were mostly affected. Both the total counts and the spore counts in the cleaning fluid were less than 5 per ml supporting previous studies. For *B. cereus* isolated from sugarcane bagasse, exposure of spores for less than 1 min to 80 °C with 1 % NaOH in the heating menstruum was required to reduce the spore population by 90 % (Han *et al.* 1971). Stadhouders (1964) observed for three strains of *B. cereus* a decrease in numbers from 10^5 to less than 10 per ml within 10 min in a 1 % NaOH solution at 80 °C. However, in all swab samples *B. cereus* was shown to be present, mainly as spores determined after a heat activation step of 10 min at 80 °C, in levels of less than 1 cm^{-2}. In an additional experiment in a plate heat exchanger, in which we applied the traditional two-stage alkali (sodium hydroxide)-acid (nitric acid) procedure (Timperley and Smeulders 1987) for cleaning, *B. cereus* could still be detected on the separate plates by means of swab samples (data not shown). This was also demonstrated previously by others; Rönner and Husmark (1992) showed in an evaluation of the cleaning process of stainless steel plates, inserted into the pipeline before the pasteurizer, that there were many *B. cereus* spores surviving and still attached to the stainless steel surfaces after cleaning. For vegetative cells of *B. cereus*, a scanning electron microscopic (SEM) study on some effects of sodium hypochlorite on attachment of bacteria to stainless steel indicated that treatment with a water rinse followed by a sanitizer was not sufficient to remove the attached organisms (Schwach and Zottola 1984). However, the authors did not determine if the organisms were viable after treatment. Stone and Zottola (1985) used a model CIP system to determine the effectiveness of cleaners on *Pseudomonas fragi*. SEM revealed attached cells remaining on the surfaces after the CIP procedure.

The results obtained in the laboratory experiments, i.e. suspension tests and surface tests, confirm those of the field trial. These results are presented in Tables 8.3 and 8.4.

Table 8.3 Mean effect of time/temperature (15 min/80 °C) and cleaning (K500/NaOH) against *Bacillus cereus* vegetative cells and spores in suspension tests

	Initial level	Control	Effect[b]	After cleaning	Effect[b]
Vegetative cells					
95003[a]	6.3	1.4 ± 0.1[c]	4.9 ± 0.2	< 0.7	> 5.6
94495	6.3	2.2 ± 0.2	4.1 ± 0.2	< 0.7	> 5.6
Spores					
95003	5.5	5.5 ± 0.1	0.0 ± 0.1	1.8 ± 0.2	3.7 ± 0.2
94495	6.8	6.6 ± 0.1	0.2 ± 0.1	4.1 ± 0.1	2.7 ± 0.1

[a] strain identity number; [b] number of vegetative cells/spores intially - number of vegetative cells/spores in control test (control) or after treatment with cleaning solution (after cleaning); [c] mean and standard deviation of five experiments given in log values

Table 8.4 Mean effect of time/temperature (15 min/80 °C) and cleaning (K500/NaOH) against *Bacillus cereus* vegetative cells and spores in surface tests on stainless steel

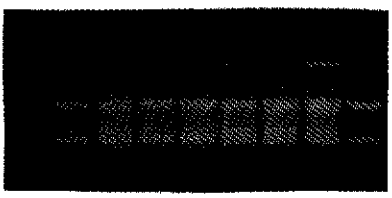
	Initial level	Control	Effect[b]	After disinfection	Effect[b]
Vegetative cells					
95003[a]	6.2	2.2 ± 0.1[c]	4.0 ± 0.1	< 0.7	> 5.5
94495	6.4	2.4 ± 0.1	4.0 ± 0.1	< 0.7	> 5.7
Spores					
95003	4.7	4.6 ± 0.1	0.1 ± 0.1	2.9 ± 0.3	1.8 ± 0.3
94495	5.6	5.3 ± 0.1	0.3 ± 0.1	5.2 ± 0.2	0.4 ± 0.2

[a] strain identity number; [b] number of vegetative cells/spores intially - number of vegetative cells/spores in control test (control) or after treatment with cleaning solution (after cleaning); [c] mean and standard deviation of five experiments given in log values

For the vegetative cells, the time (15 min) and temperature (80 °C) combination had already considerable effect, leading to a reduction of more than 4 log units for both isolates. After the treatment with the cleaning agent, the numbers decreased to below the detection level. Vegetative cells seemed somewhat more resistant in surface tests on stainless steel, however the experiments resulted in reductions comparable with the suspension tests.

For the spores, hardly any effect of the time and temperature combination could be observed, while with K500/NaOH a sporicidal effect of 2.7 and 3.7 was observed for strains 94495 and 95003, respectively. Spores were more resistant to cleaning when adhered to surfaces compared with the same spores in suspension as also observed by others (Te Giffel *et al.* 1995b [Chapter 6], Austin and Bergeron 1995, Wirtanen *et al.* 1996). For the surface tests SE values of 0.4 for strain 94495, and 1.8 for strain 95003, were reached within 15 min at 80 °C. Between-spores variation in resistance to cleaning and disinfection agents has also been reported in literature: differences may exist within species, among species and among genera (Te Giffel *et al.* 1995b [Chapter 6]).

The identity of the isolates was checked by PCR/RAPD (Figure 8.1) to confirm that the bacteria found on the surfaces were the same as the bacteria in the ingoing milk. The use of a selective agar, i.e. MEYP, combined with PCR, allowed discrimination between the inoculated strain and other bacteria with similar colony appearances and growth characteristics. No other bacteria were found, neither on the MEYP agar plates nor by PCR.



1 2 3 4 5 6 7 8 9

Figure 8.1 PCR fingerprinting of *Bacillus cereus* strains isolated in experiment I using the random primer R1: [5' CGGCCTCTGC 3']. Lane 1, ingoing milk; lane 2, outgoing milk (t= 40 min); lane 3, rinse water after 24 h; lane 4, rinse water after 24 h; lane 5, tube 3; lane 6, tube 9; lane 7, tube 13; lane 8, tube 18; lane 9, initial spore suspension

In this study, it has been shown that the *B. cereus* present in milk can remain in the heat exchanging equipment, germinate and/or grow out and possibly contaminate the product. The final concentration of spores that will adhere on the surface depends on the running time, fraction of the passing spores that really attach, wall temperature and cleaning conditions; e.g. proper chemical concentration of sanitizers, contact time and temperature, pH of the water and type of surface.

Contamination of milk via the equipment has to be taken into account as cleaning and disinfection will not eliminate all *B. cereus*. However, at present, producing pasteurized milk and milk products under good manufacturing practices and application of proper cleaning and disinfection, improving the cleaning and disinfection procedures will not result in significant prolongation of the shelf-life of this type of products. In future, development of new sanitizers or combinations of agents and/or systems may be successful in further reducing numbers of *B. cereus* in the milk processing equipment. For example, the efficacy of sterilizing agents on *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* biofilms could be increased by addition of a relatively small direct current electric field (Wellman *et al.* 1996). The use of weak electric fields might also have applications in the food industry.

REFERENCES

Andersson A, Rönner U and PE Granum (1995) What problems does the food industry have with the sporeforming pathogens *Bacillus cereus* and *Clostridium perfringens* ? *International Journal of Food Microbiology* **28** 145-155.

Anonymous (1988) Method of test for the antimicrobial activity of disinfectants in food hygiene. DD177, British Standards Institution, London.

Anonymous (1992) ISO/DIS 7932 Microbiology- General guidance for enumeration of *Bacillus cereus* colony-count technique at 30 °C, International Organization for Standardization, Switzerland.

Austin JW and G Bergeron (1995) Development of bacterial biofilms in dairy processing lines. *Journal of Dairy Research* **62** 509-519.

Griffiths MW and JD Phillips (1990) Incidence, source and some properties of psychrotrophic *Bacillus* spp. found in raw and pasteurized milk. *Journal of the Society of Dairy Technology* **43** 62-66.

Han YW, Schuyten Jr HA and CD Callihan (1971) Combined effect of heat and alkali in sterilizing sugarcane bagasse. *Journal of Food Science* **36** 335-337.

Hood SK and EA Zottola (1995) Biofilms in food processing. *Food Control* **6** 9-18.

Langeveld LPM, Van Montfort-Quasig RMGE, Weerkamp AH, Waalewijn R and JS Wever (1995) Adherence, growth and release of bacteria in a tube heat exchanger for milk. *Netherlands Milk and Dairy Journal* **49** 207-220.

Meer RR, Baker J, Bodyfelt FW and MW Griffiths (1991) Psychrotrophic *Bacillus* spp. in fluid milk products. *Journal of Food Protection* **54** 969-979.

Phillips JD and MW Griffiths (1986) Factors contributing to the seasonal variation of *Bacillus* spp. in pasteurised dairy products. *Journal of Applied Bacteriology* **62** 275-285.

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

Schwach TS and EA Zottola (1984) Scanning electron microscopic study on some effects of sodium hypochlorite on attachment of bacteria to stainless steel. *Journal of Food Protection* **47** 756-759.

Speers JGS and A Gilmour (1985) The influence of milk and milk components on the attachment of bacteria to farm dairy equipment surfaces. *Journal of Applied Bacteriology* **59** 325-332.

Stadhouders J (1964) The destruction of spores of aerobic sporing bacilli in hot caustic solutions. *Netherlands Milk and Dairy Journal* **18** 182-203.

Stone LS and EA Zottola (1985) Relationship between growth phase of *Pseudomonas fragi* and its attachment to stainless steel. *Journal of Food Science* **50** 957-960.

Te Giffel MC, Beumer RR, Slaghuis BA and FM Rombouts (1995a) Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands. *Netherlands Milk and Dairy Journal* **49** 125-138.

Te Giffel MC, Beumer RR, Van Dam WF, Slaghuis BA and FM Rombouts (1995b) Sporicidal effect of disinfectants on *Bacillus cereus* isolated from the milk processing environment. *International Biodeterioration and Biodegradation* **36** 421-430.

Te Giffel MC, Beumer RR, Bonestroo MH and FM Rombouts (1996) Incidence and characterization of *Bacillus cereus* in two dairy processing plants. *Netherlands Milk and Dairy Journal* **50** 479-492.

Timperley DA and CNM Smeulders (1987) Cleaning of dairy HTST plate heat exchangers: comparison of single- and two stage procedures. *Journal of the Society of Dairy Technology* **40** 4-7.

Van Heddeghem A and G Vlaemynck (1993) Sources of contamination of milk with *Bacillus cereus* on the farm and in the factory. *IDF Bulletin* **275** 19-22.

Wellman N, Fortun SM and BR McLeod (1996) Bacterial biofilms and the bioelectric effect. *Antimicrobial Agents and Chemotherapy* **40** 2012-2014.

Wirtanen G, Husmark U 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. *Journal of Food Protection* **59** 727-733.

CHAPTER 9

ISOLATION AND CHARACTERIZATION OF *BACILLUS CEREUS* FROM PASTEURIZED MILK IN HOUSEHOLD REFRIGERATORS IN THE NETHERLANDS

ABSTRACT

The incidence and some characteristics (carbohydrate metabolism, growth profiles, haemolysin production and enterotoxin production) of *Bacillus cereus*, in pasteurized, low-fat (1.5 %) milk, in household refrigerators in the Netherlands was investigated. In 247 (74 %) of the 334 milk samples analyzed, the mesophilic aerobic counts were between 50 and 5000 ml^{-1}. *B. cereus* could be isolated from 133 (40 %) of the samples. In general, the *B. cereus* counts were low; numbers of less than 5 per ml were observed in 258 (77 %) of the samples. As expected, both the mesophilic aerobic counts and levels of *B. cereus* increased with increasing storage temperatures in the refrigerator and prolonged storage times.

In total, 143 presumptive *B. cereus* colonies were isolated. According to the ISO confirmation tests and the carbohydrate patterns (API 50 CHB) 134 (94 %) of these isolates were confirmed to be *B. cereus*. Of these 134 isolates 20 % fermented lactose and 53 % of the 106 strains tested were able to grow at 7 °C. These percentages are much higher than expected for strains isolated from non-dairy products, suggesting that environmental conditions in pasteurized refrigerated milk are selective for these strains. All 106 strains tested, produced haemolysin, 27 % showed the discontinuous haemolytic pattern characteristic for haemolysin BL, possibly a virulence factor. Of the 37 *B. cereus* isolates tested for enterotoxin production 27 (73 %), 28 (76 %) and 26 (70 %) were found to be enterotoxigenic as determined by Western immunoblot technique, PCR and Vero cell assays, respectively. Isolates unable to ferment lactose, produced less enterotoxin in comparison with those able to utilize lactose. Although only a few outbreaks of food poisoning caused by *B. cereus* in milk(products) have been reported, most strains isolated from these products are able to produce enterotoxin and may represent a health hazard.

This chapter has been published as:

Isolation and characterization of *Bacillus cereus* from pasteurized milk in household refrigerators in the Netherlands

MC Te Giffel, RR Beumer, PE Granum and FM Rombouts

International Journal of Food Microbiology (1997) **34** 307-318.

INTRODUCTION

Bacillus species are common contaminants of many food ingredients and can cause spoilage of a variety of processed food products (Johnson 1984, Kramer and Gilbert 1989, McGuiggan *et al.* 1994). *Bacillus cereus* is important as it affects the shelf-life of pasteurized milk and milk products. The organism is associated with defects as off-flavours, sweet curdling and bitty cream caused by proteinase, lipase and phospholipase enzymes produced (Meer *et al.* 1991). In addition to causing these effects in dairy products, *B. cereus* has also been associated in outbreaks of food poisoning (Johnson 1984, Kramer and Gilbert 1989). Despite the worldwide occurrence of the bacterium in milk, surprisingly few reports of food poisoning caused by *B. cereus* from milk and cream have been reported (Christiansson *et al.* 1989).

B. cereus causes two different types of food poisoning: a diarrhoeal type and an emetic type. Various foods including meat products, vegetables, soups, puddings and sauces have been implicated in outbreaks of the diarrhoeal food poisoning, while the emetic type is almost exclusively associated with the consumption of rice (Kramer and Gilbert 1989). The diarrhoeal type is caused by enterotoxins, however, the number of enterotoxins and their properties have been debated for a long time (Kramer and Gilbert 1989, Granum 1994). Several reports on proteins with enterotoxic activity have been published (Kramer and Gilbert 1989, Heinrichs *et al.* 1993, Agata *et al.* 1995, Beecher *et al.* 1995). This study was undertaken to determine the level of *B. cereus* in pasteurized milk at the time of consumption in relation to the mesophilic aerobic counts, by sampling and testing milk stored in household refrigerators. The *B. cereus* isolates were used to investigate some biochemical and growth characteristics. For 37 strains, selected to represent different types in respect to carbohydrate metabolism and growth temperatures the ability to produce enterotoxin, by immunoblotting using antiserum against the purified enterotoxin (Granum *et al.* 1993a, Granum *et al.* 1996), in vivo cell-assays (using a Vero cell assay (Sandvig and Olsnes 1982) and PCR (using a set of primers for the B component of the haemolysin BL (Granum *et al.* 1996)), was also examined and compared with enterotoxin production by isolates obtained from various other food products.

MATERIALS AND METHODS

Samples

Samples were collected over five months (March till August), from 334 households in the Netherlands. The samples of pasteurized, low-fat (1.5 %) milk (about 100 ml) were

taken from opened cartons stored in the door of the refrigerator and the temperature of the milk samples was measured using a digital thermometer. The expiry dates on the packages were also noted. All samples were kept at 4 °C until analyzed within 2 h.

Determination of mesophilic aerobic bacteria and *B. cereus*

The milk obtained from the households was analyzed without pre-incubation at 30 °C and without a heat-activation step. The mesophilic aerobic count was determined by plating 0.1 ml of the undiluted and 0.1 ml of a 100-fold dilution in Peptone Saline Solution (PSS; NaCl 8.5 g l^{-1} and Neutralized Bacteriological Peptone (Oxoid L34), 1 g l^{-1}) on Plate Count Agar (PCA; Oxoid CM 325). The PCA plates were incubated at 30 °C for 72 h and counted. For *B. cereus*, 1 ml of the undiluted milk was spread over three Mannitol Egg Yolk Polymyxin (MEYP) agar plates (Cereus Selective Agar, Merck 5267) and 0.1 ml of a 10-fold dilution in PSS was surface plated on MEYP agar.

The MEYP plates were incubated at 30 °C for 24 h and examined for typical colonies. From each plate presumptive colonies were selected for confirmation as specified in the ISO/DIS 7932 method (Anonymous 1992). Suspect colonies (pink colonies surrounded by a zone of precipitation) were counted and subsequently plated out on a non-selective medium i.e. Tryptone Soya Agar (TSA; Oxoid CM 131). Gram stains were made and the isolates were examined microscopically. Then, the identity of colonies was confirmed by testing them for glucose fermentation, Voges-Proskauer (VP) reaction (production of acetylmethylcarbinol) and nitrate reduction. *B. cereus* strains should be positive for all three reactions (Anonymous 1992).

Carbohydrate metabolism

The carbohydrate metabolism of all presumptive *B. cereus* isolates was determined using the API 50 strips (BioMérieux Sa, France). Isolates were grown on TSA plates (18-24 h at 30 °C). Colonies were suspended in 2 ml sterile 0.85 % saline solution to correspond with tube No. 2 of the McFarland scale of standard opacities and 0.1 ml of this suspension was diluted in 10 ml of API 50 CHB medium. The strips were inoculated, incubated for 48 h at 30 °C and read after both 24 and 48 h. The results were scored according to the manufacturer's instructions and the emerging biochemical profile was identified by means of APILAB software V 2.1, 1990.

Determination of growth temperatures

To investigate growth at different temperatures, 106 *B. cereus* strains were grown in Brain Heart Infusion broth (BHI; Difco 0037-01-6) for 24 h at 30 °C. Then, a loopful of culture (10^8 cfu ml^{-1}) was streaked on to TSA plates, and incubated at 7, 10, 15, 20, 25, 30, 37, 42 and 50 °C ± 0.5 °C. Growth was monitored daily by visual examination of the plates for 1 week.

Haemolysin production

Culture supernatants of 106 *B. cereus* isolates grown in BHI (24 h at 30 °C) were used to detect production of the tripartite haemolysin: haemolysin BL (Beecher and Wong 1994, Beecher *et al.* 1995). The haemolysin activity was assayed by a well diffusion technique on sheep blood agar plates with 30 μl of the supernatants (Beecher and Wong 1994). Plates were incubated for 16 - 18 h at 30 °C and activity was recorded in terms of diameter of the cleared zone.

Enterotoxin production

Of the 134 *B. cereus* isolates, 37 were selected to represent the different types in respect to carbohydrate metabolism and growth temperatures as observed in the API 50 CHB and growth experiments. In addition, 49 strains isolated from various other food products including yeast, flour, pasta products, Chinese meals, cocoa, chocolate, bakery products, meat products, herbs and spices were selected [Chapter 2].

The strains were cultured in BHI with an additional 10 g l^{-1} of glucose (BHIG) for 6 h at 32 °C with moderate agitation (200 cycles min^{-1}) in a thermostatically controlled water-bath. The cells were harvested by centrifugation (5000 g for 20 min at 4 °C). The supernatants were used for immunoblotting and in the cell-assays. If necessary the supernatants were concentrated 20-fold using 70 % ammonium sulfate precipitation (Granum *et al.* 1996).

Electrophoresis and immunoblotting were carried out as described by Towbin *et al.* (1979) using the Mini-Protean II Dual Slab Cell (Biorad). The test antiserum against the purified enterotoxin was provided by the PHLS Food Hygiene Laboratory, London, UK.

Cytotoxicity was determined using a Vero cell assay according to Sandvig and Olsnes (1982). The anti-enterotoxin neutralized the cytotoxic activity of the culture supernatants. Supernatants (20-fold concentrated) that gave at least 40 % inhibition of protein synthesis of the Vero cells were recorded as cytotoxic.

Polymerase chain reaction (PCR) was performed using the primers described by Agata *et al.* (1995) for the *bce*T gene (forward: 5'-TTACATTACCAGGACGTGCTT-3' and reverse: 5'-TGTTTGTGATTGTAATTCAGG-3') and a set of primers for the B component of the hemolysin BL described by Granum *et al.* (1996) (primer sequence (5' to 3') 1F: ACGAACAATGGAGATACGGC and 7R: ATTTTTGTGGAGTAACAGTTTCTAC; 3F: ATAACTATTAATGGAAATACA and 4R: CTCCTTGTAAATCTGTAATCCCT). The target DNA was from 1 μl of a *B. cereus* culture (10^8 ml^{-1}) grown in BHIG as described above or DNA ($\pm$ 5 ng) purified according to Granum *et al.* 1996.

Amplification was carried out in a DNA thermal cycler (Perkin-Elmer Cetus 4800) for 30 cycles of 1 min at 94 °C (denaturation), 1 min at 50 °C (annealing), and 1 min at 72 °C (extension-polymerization). PCR products were analyzed by agarose gel electrophoresis.

RESULTS

The mesophilic aerobic counts in the milk samples varied from less than 50 to more than 5 $\times$ 10^4 cfu ml^{-1} (Table 9.1). In 247 (74 %) of the samples the contamination level ranged from 50 - 5000 cfu per ml. Levels of more than 5 $\times$ 10^4 cfu ml^{-1} were found in 43 (13 %) of the samples.

Table 9.1. Mesophilic aerobic count (log N ml^{-1}) and *Bacillus cereus* (log N ml^{-1}) in pasteurized milk in households (334 samples)

	< 0.7	0.7-1.7	1.7-2.7	2.7-3.7	3.7-4.7	> 4.7
Mesophilic count[a]		15	92	155	29	43
B. cereus[b]	258	24	21	14	16	1

[a] detection limit for mesophilic aerobic count: log 1.7 ml^{-1}; [b] for *B. cereus*: log 0.7 ml^{-1}

B. cereus could be isolated, without pre-incubation, from 133 of 334 (40 %) pasteurized milk samples obtained from household refrigerators. The counts were usually low: less than 5 cfu per ml. These low contamination levels were observed in 258 (77 %) of the 334 samples. However, about 5 % of the samples contained levels above 5000 cfu ml^{-1}. The mean temperature of the milk in the packages was 7.4 °C (minimum - 1 °C, maximum 17.9 °C). In 135 (43 %) of the 313 refrigerators, the temperature of the milk was lower than 7 °C. In Figure 9.1 the percentage of samples containing more than 5 *B. cereus* per ml is given as a function of the temperature measured in the refrigerator and time till

expiry date of the milk. As expected, the level of *B. cereus* present in milk increased with storage temperature and storage time. For example, one day after the expiry date (-1) 67 % of the milk samples stored above 7 °C contained more than 5 *B. cereus* per ml. Of the milks stored below 7 °C contamination levels of more than 5 *B. cereus* per ml were observed in 25 % of the samples. Similar results were observed for the mesophilic aerobic counts.

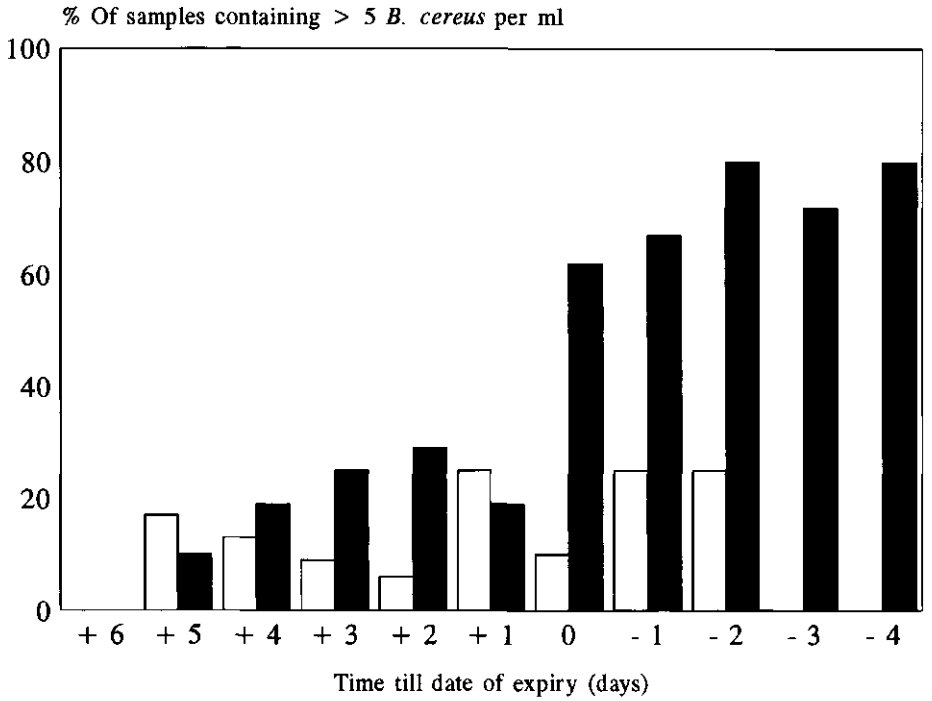


Figure 9.1 The incidence of *Bacillus cereus* in pasteurized milk (100 ml samples) in households in relation to storage time and temperature of the refrigerator (□ < 7 °C; ■ > 7 °C)

Identity of the isolates

On MEYP agar, 143 suspected *B. cereus* colonies were isolated from the milk samples. When these presumptive colonies were checked by the confirmation tests described in the ISO standard procedure, 24 (16.8 %) strains were not confirmed to be *B. cereus*: one strain (0.7 %) was VP negative and 16 (11.2 %) were unable to reduce nitrate. Furthermore,

two strains (1.4 %) gave negative reactions on both VP reaction and nitrate reduction and five strains (3.5 %) were negative on all three confirmation tests.

Subsequent identification by API 50 CHB showed that 30 strains (21 % of the original isolates), confirmed by the ISO tests, were not considered to be *B. cereus*. However, 15 strains (10.4 %) that were negative following the ISO confirmation gave a typical *B. cereus* pattern in the API 50 CHB. Of these strains one strain (0.7 %) was negative for the VP reaction and 14 (9.8 %) were unable to reduce nitrate.
Moreover, 9 strains (6.3 %) were not regarded as *B. cereus* according to both the ISO confirmation tests and their carbohydrate metabolism. Thus, in total, 134 (94 %) of the 143 suspected colonies isolated on MEYP agar were shown to be *B. cereus* following the ISO confirmation tests and/or API patterns.

Carbohydrate utilization patterns

The results of carbohydrate metabolism revealed that there were 10 different types of *B. cereus* strains present. In Table 9.2 the results of the API 50 CHB are compared to results obtained in other investigations. Generally, the percentage of positive results for the biochemical reactions of *B. cereus* obtained in this study are in line with those reported by others (Logan and Berkeley 1984, Rangasamy *et al.* 1993, Te Giffel *et al.* 1995, Te Giffel *et al.* 1996a, Te Giffel *et al.* 1996b).

Several substrates were not fermented by any of the *B. cereus* strains tested: erythritol, arabinose, xylose, adonitol, β-methyl-D xyloside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D mannoside, α-methyl-D glucoside, melibiose, inuline, melizitose, raffinose, xylitol, turanose, lyxose, tagatose, fucose, arabitol, 2-ketogluconate, 5-ketogluconate (results not shown). More than 90 % of the strains were able to utilise ribose, glucose, fructose, N-acetylglucosamine, arbutin, aesculin, salicin, maltose and trehalose. For the other substrates the utilization as carbon source varied between < 10 and 90 %.

Growth profiles

All *B. cereus* isolates tested (106 strains) were able to grow at 10, 15, 20, 25 and 30 °C. In total, 56 (53 %) of the 106 strains were able to grow at 7 °C and were considered to be psychrotrophic. Most strains that grew at 7 °C, grew slowly at 37 °C and 42 °C. At 37 °C growth was observed for 92 (87 %) of the isolates, and only 40 (38 %) were able to grow at 42 °C. Growth at both 7 °C and 37 °C, was observed for 42 (40 %) of the isolates. Three (3 %) of the *B. cereus* isolates grew at both 7 and 42 °C.

Table 9.2 Carbohydrate utilization patterns of *Bacillus cereus* strains (in %), as determined with API 50 CHB.
Isolates from this study compared to those from other reports.

	Origin of strains						
	API[a]	Farms[b]	Raw milk[c]	Dairy I[c]	Dairy II[c]	House holds	Non-dairy products[d]
Carbohydrate							
Glycerol	26	93	51	32	53	27	88
Ribose	97	99	81	78	84	94	100
Galactose	2	1	11	1	20	13	1
Glucose	100	100	100	99	100	100	100
Fructose	100	100	96	96	88	100	100
Mannose	19	14	18	10	15	9	15
Inositol	0	16	3	5	1	0	13
α-methyl-D-glucoside	0	4	0	0	0	0	2
N-acetyl-glucosamine	97	100	91	98	100	98	100
Amygdalin	2	50	18	15	21	37	23
Arbutin	85	99	68	75	71	99	99
Esculin	82	100	87	93	94	100	100
Salicin	65	99	60	69	64	95	87
Cellobiose	53	94	43	40	50	71	62
Maltose	100	100	100	99	100	100	100
Lactose	0	< 1	11	2	23	20	< 1
Saccharose	63	34	46	25	41	47	51
Trehalose	100	100	100	99	100	99	100
Starch	96	98	64	90	59	77	80
Glycogen	95	99	64	90	58	80	82
ß-Gentiobiose	0	6	2	< 1	1	3	2
Gluconate	0	25	22	16	31	4	53
Number of strains		766	131	167	86	106	171

[a] *B. cereus* results according to API (bioMérieux Sa, France); [b] farm isolates (Te Giffel *et al.* 1995); [c] raw milk isolates, pasteurization process (dairy I), powder process (dairy II) (Te Giffel *et al.* 1996b); [d] various non-dairy products (Te Giffel *et al.* 1996a)

Haemolysin production

When culture supernatant fluids of *B. cereus* were added to the wells in sheep blood

agar, all strains exhibited haemolytic activity. Of the 106 isolates tested 29 (27 %) showed the discontinuous haemolytic pattern characteristic for haemolysin BL (Beecher and Wong 1994).

Toxin production

Most of the 37 strains isolated from pasteurized milk (Table 9.3) were found to be enterotoxigenic as revealed by Western blots (27 strains producing enterotoxin), Vero cell assays (26 strains showing enterotoxigenic activity on Vero cells) and PCR (28 isolates positive). In general, most of the lactose negative strains were only weakly positive. Therefore, it was necessary to concentrate the supernatants 20-fold (numbers given in brackets in Table 9.3), using 70 % ammonium sulfate precipitation.

Of the 27 (73 %) strains that produced enterotoxin in the Western immunoblots, 11 produced 3 bands (48, 40 and 34 kDa), using the antiserum produced by the PHLS Food Hygiene Laboratory. Sixteen *B. cereus* strains produced one or two of the proteins, whilst for the strains unable to utilize lactose, 19 (68 %) were found positive. Of those 7 were only positive after concentration of the supernatant. Of the lactose positive strains 8 of the 9 isolates tested were positive on Western immunoblot without 20-fold concentration of the supernatant.

Of the strains isolated from various non-dairy products, all isolates able to utilize starch were enterotoxigenic (Table 9.4), whereas of the starch negative strains 6, 20 and 20 strains were enterotoxigenic as determined by Western blots, Vero cell assays and PCR, respectively.

Of the 49 strains tested, 30 produced enterotoxin as detected by immunoblotting using the antiserum against the enterotoxin; 21 of the enterotoxin-positive strains produced 3 bands (48, 40 and 34 kDa). Nine *B. cereus* strains produced one or two of the proteins. Three starch negative isolates were only positive after concentration of the supernatant.

The cytotoxic activity tested on Vero cells showed that 20 (80 %) of the starch negative strains were enterotoxigenic; the activity of these strains was in general lower than that of the starch positive isolates because 12 of the 20 supernatants tested were only positive after concentration. For all 24 lactose positive strains cytotoxic activity was observed in unconcentrated supernatants.

In total, 44 (90 %) of the 49 *B. cereus* isolated from various food products were PCR-positive for the B component of haemolysin BL and 24 (49 %), which were all starch hydrolyzing strains, gave a band of 428 bp, as expected for the *bce*T gene. Strikingly, the DNA sequence corresponding for the *bce*T gene was not detected in any of the starch negative strains.

Contamination of pasteurized milk with *B. cereus*

and potato processing plant; strains able to ferment xylose occurred most frequently (Cox *et al.* 1989). Although 80 % of the *B. cereus* isolates are unable to ferment lactose, they can grow in the milk upon hydrolysis of casein and other proteins by proteinases (Cousin 1982, Meer *et al.* 1991) or by utilization of glucose following the decomposition of lactose by competitive micro-organisms.

It has been proposed that *B. cereus* strains producing emetic toxin are unable to hydrolyze starch or ferment salicin (Shinagawa 1990). Few of such isolates were found in this study: 77 % of the strains were able to hydrolyze starch and 95 % could ferment salicin (Table 9.2).

The growth profiles of the *B. cereus* isolates showed that 56 (53 %), 92 (87 %) and 40 (38 %) strains of the 106 tested grew at 7, 37 or 42 °C, respectively. The high percentage of psychrotrophs illustrates that these probably have a more significant impact on the keeping quality of milk during storage at low temperatures than mesophilic *B. cereus*.

The diarrhoeal food-poisoning syndrome is caused by a heat labile enterotoxin. This type of food poisoning is caused by ingestion of the cells rather than by pre-formed toxin (Granum *et al.* 1993b). Therefore, strains that can grow at low temperatures and at 37 °C should be regarded as potential food-poisoning organisms, since they can grow both in the product and in the ileum. Of the isolates able to grow at 7 °C, 42 (40 %) also grew at 37 °C; which might suggest that these strains are able to multiply both in dairy products and in the ileum and may probably be enterotoxigenic. Furthermore, it was shown in the haemolysin experiments that 29 (27 %) of the 106 milk isolates produced the discontinuous haemolytic pattern characteristic for the haemolysin BL. Evidence is accumulating that this haemolysin is one of the *B. cereus* virulence factors (Beecher and Wong 1994).

The presence of enterotoxigenic *B. cereus* in pasteurized milk was confirmed by toxin production experiments; most strains were able to produce toxin as revealed by the Western immunoblots, the cytotoxicity tests on Vero-cells and PCR and thus may be able to cause diarrhoeal-type food poisoning. The activity of dairy strains was low compared with the activity of *B. cereus* isolates originating from various non-dairy products; 65 % of the enterotoxigenic supernatants of dairy isolates were only positive after concentration compared with 27 % of the supernatants of other strains.

The percentages of enterotoxin-producing *B. cereus* found in this study were comparable with those reported by others. Granum *et al.* (1993a) reported that 59 % of 85 dairy isolates were positive on Western blots compared with 73 % found in this study. Hostacka *et al.* (1992) reported that 18 out of 26 (69 %) strains isolated from milk showed enterotoxic activity on Vero cells. Christiansson *et al.* (1989) observed that approximately 70 % of dairy isolates produced an extracellular verocytotoxin when grown in milk at 8 °C. In our experiments, 70 % of the *B. cereus* isolates were cytotoxic to Vero cells.

According to Agata *et al.* (1995) the DNA sequence corresponding to the *bce*T gene was detected in all *B. cereus* strains, regardless of source. Interestingly, our results indicate that only 13 (35 %) of dairy isolates and 24 (49 %) of *B. cereus* isolated from other foods were PCR-positive for the *bce*T gene. Strikingly, in none of the starch negative strains the *bce*T gene DNA sequence was present. It remains to be elucidated if this protein plays a role in food poisoning.

The enterotoxigenic activity of the lactose and starch positive isolates was higher than for *B. cereus* strains unable to utilize lactose or starch. It has been reported in literature that the presence of carbohydrates (i.e. glucose, lactose and starch) in amounts up to 10 g l^{-1}, enhanced enterotoxin production (Garcia-Arribas and Kramer 1990, Sutherland and Limond 1993). It is not yet known how this effect is elicited, however, the carbohydrate metabolism of *B. cereus* might play a role in enterotoxin production in milk. Therefore, lactose positive strains, better adapted to the growth conditions in milk, may produce higher levels of enterotoxin. Further physiological research is necessary to confirm this assumption.

Up untill now, it is not clear whether *B. cereus* in pasteurized milk should be considered as hazardous. Only a few cases of food poisoning by *B. cereus* in which milk (products) were involved have been reported. However, most strains isolated from these products can produce enterotoxin under optimal growth conditions. Protein purification, followed by cloning, sequencing and mutation studies are nessecary to characterize the enterotoxin(s) produced by the organism. Then, factors that influence toxin production in food and/or in the small intestine have to be determined.

ACKNOWLEDGEMENTS

The authors would like to thank R. Dijk for her technical support and the AAIR Concerted Action PL 920630 for financial support to perform the enterotoxin experiments at the Norwegian College of Veterinary Medicine.

REFERENCES

Agata N, Ohta M, Arakawa Y and M Mori (1995) The *bce*T gene of *Bacillus cereus* encodes an enterotoxic protein. *Microbiology* **141** 983-988.

Ahmed AA-H, Moustafa MK and EH Marth (1983) Incidence of *Bacillus cereus* in milk and some milk products. *Journal of Food Protection* **46** 126-128.

Anonymous (1992) ISO/DIS 7932 Microbiology- General guidance for enumeration of *Bacillus cereus*- colony-count technique at 30 °C, International Organization for Standardization, Switzerland.

Beecher DJ and ACL Wong (1994) Identification of hemolysin BL-producing *Bacillus cereus* isolates by a discontinuous hemolytic pattern in blood agar. *Applied and Environmental Microbiology* **60** 1646-1651.

Beecher DJ, Schoeni JL and ACL Wong (1995) Enterotoxin activity of hemolysin BL from *Bacillus cereus*. *Infection and Immunity* **63** 632-639.

Christiansson A, Satyanarayan Naidu A, Nilsson I, Wadström T and H-E Petterson (1989) Toxin production by *Bacillus cereus* isolates in milk at low temperatures. *Applied and Environmental Microbiology* **55** 2595-2600.

Craven HM and BJ Macauley (1992) Microorganisms in pasteurised milk after refrigerated storage 1. Identification of types. *Australian Journal of Dairy Technology* **47** 38-45.

Credit C, Hedeman R, Heywood P and D Westhoff (1972) Identification of bacteria isolated from pasteurized milk following refrigerated storage. *Journal of Milk and Food Technology* **35** 708-709.

Cousin MA (1982) Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. *Journal of Food Protection* **45** 172-207.

Cox LJ, Kleiss T, Cordier JL, Cordellana C, Konkel P, Pedrazzini C, Beumer R and A Siebenga (1989) *Listeria* spp. in food processing, non-food and domestic environments. *Food Microbiology* **6** 49-61.

Garcia-Arribas ML and JM Kramer (1990) The effect of glucose, starch and pH on growth and haemolysin production by strains of *Bacillus cereus* associated with food poisoning and non-gastrointestinal infection. *International Journal of Food Microbiology* **11** 21-34.

Granum PE (1994) *Bacillus cereus* and its toxins. *Journal of Applied Bacteriology Symposium Supplement* **76** 61S-66S.

Granum PE, Brynestad S and JM Kramer (1993a) Analysis of enterotoxin production by *Bacillus cereus* from dairy products, food poisoning incidents and non-gastrointestinal infections. *International Journal of Food Microbiology* **17** 269-279.

Granum PE, Brynestad S, O'Sullivan K and H Nissen (1993b) Enterotoxin from *Bacillus cereus*: production and characterization. *Netherlands Milk and Dairy Journal* **47** 63-70.

Granum PE, Andersson A, Gayther C, Te Giffel M, Larsen H, Lund T and K O'Sullivan (1996) Evidence for a further enterotoxin complex produced by *Bacillus cereus*. *FEMS Microbiology Letters* **141** 145-149.

Heinrichs JH, Beecher DJ, MacMillan JM and BA Zilinskas (1993) Molecular cloning and characterization of the hblA gene encoding the B component of hemolysin BL from *Bacillus cereus*. *Journal of Bacteriology* **175** 6760-6766.

Hostacka A, Kosiarova A, Majtan V and S Kohutova (1992) Toxic properties of *Bacillus cereus* strains isolated from different foodstuffs. *Zentralblatt für Bakteriologie* **276** 303-312.

Johnson KM (1984) *Bacillus cereus* in food-borne illness- An update. *Journal of Food Protection* **47** 145-153.

Kramer JM and RJ Gilbert (1989) *Bacillus cereus* and other *Bacillus* species In MP Doyle (ed.) *Foodborne bacterial pathogens*, Marcel Dekker, New York and Basel, 21-70.

Lancette GA and SM Harmon (1980) Enumeration and confirmation of *Bacillus cereus* in foods: collaborative study. *Journal of the Association of Official Analytical Chemists* **63** 581-586.

Logan NA and RCW Berkeley (1984) Identification of *Bacillus* strains using the API system. *Journal of General Microbiology* **130** 1871-1882.

McGuiggan JTM, Gilmour A and LM Lawrence (1994) Factors influencing the recovery of psychrotrophic, mesophilic and thermophilic *Bacillus* spp. from bulk raw milk. *Journal of the Society of Dairy Technology* **47** 111-116.

Meer RR, Baker J, Bodyfelt FW and MW Griffiths (1991) Psychrotrophic *Bacillus* spp. in fluid milk products: A review. *Journal of Food Protection* **54** 969-979.

Mosso MA, Garcia Arribas ML, Cuena JA and MC De La Rosa (1989) Enumeration of *Bacillus* and *Bacillus cereus* spores in food from Spain. *Journal of Food Protection* **52** 184-188.

Rajkowski KT and EM Mikolajcik (1987) Characteristics of selected strains of *Bacillus cereus*. *Journal of Food Protection* **50** 199-205.

Rangasamy PN, Iyer M and H Roginski (1993) Isolation and characterisation of *Bacillus cereus* in milk and dairy products manufactured in Victoria. *Australian Journal of Dairy Technology* **48** 93-95.

Sandvig K and S Olsnes (1982) Entry of toxic proteins abrin, modeccin, ricin and diphtheria toxin into cells. *Journal of Biological Chemistry* **257** 7495-7503.

Shinagawa K (1990) Analytical methods for *Bacillus cereus* and other *Bacillus* species. *International Journal of Food Microbiology* **10** 125-142.

Sutherland AD and AM Limond (1993) Influence of pH and sugars on the growth and production of diarrhoeagenic toxin by *Bacillus cereus*. *Journal of Dairy Research* **60** 575-560.

Te Giffel MC, Beumer RR, Slaghuis BA and FM Rombouts (1995) Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands. *Netherlands Milk and Dairy Journal* **49** 125-138.

Te Giffel MC, Beumer RR, Leijendekkers S and FM Rombouts (1996a) Incidence of *Bacillus cereus* and *Bacillus subtilis* in foods in the Netherlands. *Food Microbiology* **13** 53-58.

Te Giffel MC, Beumer RR, Bonestroo MH and FM Rombouts (1996b) Incidence and characterization of *Bacillus cereus* in two dairy processing plants. *Netherlands Milk and Dairy Journal* **50** 479-492.

Towbin H, Staehlin T and J Gordon (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proceedings. National Academy of Sciences USA* **72** 4350-4354.

Wong HC, Chang MH and JY Fan (1988) Incidence and characterisation of *Bacillus cereus* isolates contaminating dairy products. *Applied and Environmental Microbiology* **54** 699-702.

CHAPTER 10

GENERAL DISCUSSION

ISOLATION OF *BACILLUS CEREUS*

In order to determine the proportion of spore-forming organisms present as spores in a sample, the most common procedure is to conduct a viable count after application of a heat-activation step or some other treatment (Priest and Grigorova 1990). Activation is often required, since dormant spores will not necessarily germinate in a suitable environment. The most common temperature/time combination employed as heat-activation treatment is 80 °C for 10 min. This results in the highest germination rate for slow, intermediate and fast germinating spores when compared with 90 °C/10 min, 100 °C/10 min and 100 °C/1 min (Moran *et al.* 1990). In this way, vegetative cells will be killed but most (dormant) endospores will be unaffected or stimulated to germinate, grow out and multiply to produce colonies on an agar medium or turbidity in a broth.

Germination of spores is to some extent affected by factors as composition of the sporulation medium, preparation of spore suspensions, age of the spore suspensions and environmental conditions as temperature and pH (Stewart 1975, Moran *et al.* 1990). Germination of *B. cereus* spores in various media was influenced by strain type, slow or fast germinating, composition of the medium, presence of germinants and temperature as shown in Chapter 2.

Most germination (up to 80 %) was observed in complex media such as food products, however, considerable germination (up to 40 %) was also noted in peptone saline solution (PSS), prescribed as diluent in standard procedures for microbiological examinations, within 30 min at room temperature. This indicates that the 45 min mentioned in the standard procedures (e.g. Anonymous 1996a) as maximum time allowed between preparation of the primary dilution and mixing of dilutions and media is too long and will lead to an underestimation of the numbers of endospore-forming bacteria present as spores.

Therefore, it is recommended to use saline solution as diluent and to limit the time between preparation of the primary dilution and the heat-activation step to 10 min. Furthermore, the temperature during the analysis has to be maintained as low as possible (e.g. by keeping dilutions in melting ice).

IDENTIFICATION OF *B. CEREUS*

Almost all procedures for the isolation, detection and enumeration of *B. cereus* involve direct agar plating techniques on non-selective media such as blood agar or selective media of which Mannitol Egg Yolk Polymyxin B sulfate (MEYP) agar is most commonly used (Mossel *et al.* 1967). For the confirmation of presumptive colonies and differentiation between *B. cereus* and closely related species biochemical tests are employed. In the studies

described in this thesis 87 % to 94 % of suspect *B. cereus* could be confirmed using biochemical confirmation tests [Chapters 4, 5, 7 and 9]. However, results of sequencing part of the 16S rRNA of '*B. cereus*' isolates indicated that these biochemical tests do not always identify a species correctly. Several isolates, involved in food poisoning, were classified, using sequencing, as *B. thuringiensis* instead of *B. cereus* [Chapter 3]. These two species are very closely related and can only be differentiated by the production of toxin crystals (Bourque *et al.* 1993). However, this character is plasmid encoded and transmissible to *B. cereus* by conjugation. Moreover, it has been shown recently that strains of *B. thuringiensis* produced a *B. cereus*-diarrhoeal-type of enterotoxin and outbreaks of *B. cereus* foodborne illness from ingestion of vegetables have been reported (Damgaard 1995, Damgaard *et al.* 1996). As *B. thuringiensis* is the most frequently used microbial insecticide worldwide, these results emphasize the need to re-evaluate the taxonomy of these organisms and to develop accurate identification methods. Therefore, we developed a specific and sensitive method, using DNA probes based on the variable regions of the 16S rRNA, to discriminate between *B. cereus* and *B. thuringiensis* [Chapter 3].

OCCURRENCE OF *B. CEREUS* IN FOODS

The fact that spore-forming bacteria are widely distibuted in nature and their endopsores are able to survive different environmental stresses makes it very difficult, if not impossible, to exclude these micro-organisms from food products and the food production environment. *Bacillus* species are important as food spoilage organisms and can be involved in foodborne infection or intoxication. In Chapter 4 the incidence of *B. cereus* and *B. subtilis* in various food products, including milk, yeast, flour, pasta products, Chinese meals, cocoa, chocolate, bakery products, meat products, herbs and spices, was investigated.

Of the samples analyzed, 48 % contained *B. cereus* in levels ranging from 10^2 - 10^6 per g or ml. *B. subtilis* was present in 25 % of the samples, numbers varied from 10^2 - 10^5 bacteria per g or ml. From 17 % of the samples both organisms could be isolated.

Of the presumptive *B. cereus* 89 % could be confirmed, while of the suspect *B. subtilis* 75 % were determined to be *B. subtilis* according to the ISO confirmation tests and carbohydrate utilization patterns (API 50 CHB, BioMérieux Sa, France).

Processing and storage of food products affected *B. subtilis* more than *B. cereus*. Combined with the fact that at present direct isolation media for *B. subtilis* are not available, these results indicate that the role of *B. subtilis* in food spoilage and foodborne infection and intoxication may be underestimated.

CONTAMINATION OF MILK WITH *B. CEREUS*

In order to determine the major contamination sources of milk with (psychrotrophic) *B. cereus*, the incidence of vegetative cells and spores of *B. cereus* on farms, at two dairy processing plants and in pasteurized milk in household refrigerators was investigated. The *B. cereus* isolates obtained were further characterized by biochemical and molecular typing and growth characteristics.

Contamination sources

On farms, the major contamination sources were soil (10^3 - 10^7 per g (spores 10^2 - 10^5 g^{-1})) and faeces (< 10 - 10^4 per g (spores 10^2 - 10^4 g^{-1})). In winter, when the cows are housed, used bedding (< 10 - 10^3 per g (spores 10^1 - 10^4 g^{-1})) probably also participates in this contamination route. The udder will be contaminated, finally resulting in the presence of *B. cereus* in raw milk [Chapter 5].

B. cereus could be isolated from 36 % of the raw milk samples [Chapter 5]. At the dairy processing plants, the organism was present in 35 % of the raw milk samples analyzed [Chapter 7]. As also shown in other studies (Stewart 1975, McKinnon and Pettipher 1983, Phillips and Griffiths 1986) there was seasonal variation in levels of *B. cereus* in raw milk; numbers were highest in the summer/autumn months (June to October), when the cows were at pasture.

During processing, an increase in the percentage of positive samples was observed. In dairy I, in which pasteurized milk is produced, a rise up to 71 % was noted while in dairy II, in which milk powder is produced, the percentage of positive samples increased up to 60 % in the end product. The contamination levels were generally low, presence of the organism could only be demonstrated after pre-incubation of the samples for 6 h at 30 °C. These results suggest that *B. cereus* can be introduced via sources other than raw milk; equipment may play an important role in this. It has been shown that *B. cereus* spores, isolated at the dairy processing plant, can remain in a heat exchanger, adhere to the surface, germinate and grow out. If the spores detach, the equipment can be a continuous source of contamination [Chapter 8].

In pasteurized milk stored in household refrigerators *B. cereus* could be isolated from 40 % of the samples, without pre-incubation [Chapter 9]. In general, the contamination levels were low; in 77 % of the positive samples levels of less than 5 per ml were present. Occasionally, in 5 % of the samples containing *B. cereus*, numbers of more than 5000 ml^{-1} were detected.

Confirmation of isolates

Presumptive *B. cereus*, isolated on MEYP agar, were confirmed according to the ISO confirmation tests (glucose fermentation, nitrate reduction and Voges Proskauer (VP) reaction (formation of acetylmethylcarbinol) (Anonymous 1992) and carbohydrate utilization patterns using API 50 strips.

On farms 766 (90 %) of the 847 isolates were confirmed to be *B. cereus* [Chapter 5]. At the dairy processing plants 507 presumptive colonies were isolated. According to the ISO confirmation tests and carbohydrate utilization patterns 443 (87 %) were identified as *B. cereus* [Chapter 7]. Of the 143 suspected colonies obtained from pasteurized milk in households, 134 (94 %) were confirmed to be *B. cereus* [Chapter 9].

These results indicate that MEYP agar can be employed to isolate and enumerate *B. cereus* from foods and environmental samples. The percentages of isolates that could be confirmed are higher than noted by others using MEYP or comparable egg yolk media as Kim and Goepfert agar medium or Polymyxin B Egg yolk Mannitol Bromothymolblue Agar (PEMBA) (Walthew and Lück 1978, Ahmed *et al.* 1983, Rusul and Yaacob 1995). Only from samples containing high levels of competitive organisms it might be more difficult to isolate *B. cereus* as the reaction of colonies on mannitol is often impossible to ascertain due to masking of non-fermentative colonies by the acid generated by proximal actively fermenting colonies.

Biochemical characterization of *B. cereus* isolates

In general, the percentage of positive results for the carbohydrate tests, examined using API 50 strips, was within the range reported by others (Logan and Berkeley 1984, Rangasamy *et al.* 1993). On farms, the results of 766 strains revealed that there were many different types present, representing the wide variety of sources. More than 30 carbohydrate utilization patterns were observed [Chapter 5]. At dairy I, more than 20 types were identified within the isolates obtained from the various sampling points in the pasteurized milk production process [Chapter 7]. Strikingly, the results for the strains isolated from pasteurized milk stored in household refrigerators yielded only 10 different types [Chapter 9]. At dairy II, the milk powder processing plant, there were 10 different *B. cereus* types present [Chapter 7]. These results indicate that selection of strains takes place in the milk production chain. No distinct relation between the biochemical characteristics was observed. However, it was shown that some biotypes were found in the raw milk, during processing and in the end products, whereas other biotypes could only be detected after the pasteurization step in the production process.

Lactose, the main carbohydrate in milk, was fermented by less than 1 % of the strains isolated on farms [Chapter 5]. At dairy I, the percentage of isolates able to ferment lactose appeared to be 2 %; at dairy II, this percentage was 23 %. The high percentage found at dairy II can be explained by the fact that lactose is added for some applications in the milk powder production process [Chapter 7]. Of the *B. cereus* present in pasteurized milk stored in household refrigerators 20 % fermented lactose [Chapter 9]. In previous studies, 8 - 15 % of the *B. cereus* strains isolated from pasteurized milk and milk products were able to utilize lactose as carbon source (Logan and Berkeley 1984, Rangasamy *et al.* 1993). The high percentage of strains fermenting lactose isolated from the pasteurized milk suggests selection on environmental conditions in milk occurs.

Psychrotrophic growth

Thermoduric psychrotrophs, that have both heat resistance and psychrotrophic properties, are most important for spoilage of pasteurized milk and dairy products. To determine whether *B. cereus* strains isolated were able to grow at low temperatures, the ability to grow at 7 °C was tested.

On farms, 43 % of the isolates examined appeared to be psychrotrophic [Chapter 5]. Only a few strains (6 %) isolated in the factory producing pasteurized milk were able to multiply at 7 °C. This suggests that adaptation or more likely selection of strains to growth at low temperature occurs during storage and distribution [Chapter 7]. This hypothesis is supported by the fact that 53 % of the isolates obtained from pasteurized milk in households were psychrotrophic [Chapter 9]. None of *B. cereus* isolated in the milk processing plant grew at low temperature, this is due to the type of process [Chapter 7].

Molecular typing of *B. cereus* isolates

Molecular typing techniques permit rapid and sensitive differentiation between strains of a bacterial species. Plasmid profiling and PCR/RAPD typing of *B. cereus* isolates obtained from the two dairy processing plants confirmed the results of biotyping; some types could be detected throughout the whole process of milk or milk powder production, indicating that the raw milk is an important source of contamination. Other types were only present after the pasteurization step in the process suggesting that there are additional sources of contamination e.g. equipment [Chapter 7].

EFFECT OF CLEANING AND DISINFECTION ON *B. CEREUS*

It is unlikely that the presence of *B. cereus* in the milk production and processing environment can be precluded. Therefore, the initial level should be low and recontamination during processing should be prevented. The initial level of *B. cereus* depends mainly on the season but can be influenced by cleaning and disinfection.

In Chapter 6, the sporicidal effect of sodium hypochlorite and a combination of peracetic acid and hydrogen peroxide on *B. cereus* spores was evaluated using the European Suspension Test (EST), surface tests on stainless steel and rubber and field trials in a milking installation. Factors influencing the sporicidal efficacy were test strain, sporulation conditions, the disinfectant test solution, organic load, contact time and temperature. The various disinfectants were more effective in suspensions than on surfaces and in field trials in the milking installation. The results of the surface tests reflected the in-use conditions more closely than the suspension tests. Based on our results it is advised to include this type of test to assess the sporicidal efficacy of cleaning and disinfecting agents.

The experiments described in Chapter 8 confirm that the effect on spores adhered to stainless steel was limited compared with the effect in suspensions. In the field trials with the tube heat exchanger there was an effect of cleaning, however, *B. cereus* spores could be detected throughout the whole system by means of swab samples using a squeegee. This supports the assumption that equipment can play a role in contamination of milk with *B. cereus* [Chapter 7].

TOXIN PRODUCTION BY DAIRY ISOLATES

Although *B. cereus* is the predominant organism in pasteurized milk and determines the keeping quality of pasteurized milk and milk products, relatively few cases of food poisoning by the organism from dairy products have been reported (Christiansson *et al.* 1989, Meer *et al.* 1991, Christiansson 1993, Sutherland 1993, Langeveld *et al.* 1996).

It has been proposed that strains producing the emetic toxin are unable to hydrolyze starch or ferment salicin (Shinagawa 1990). The majority of the strains isolated on farms, at the dairy processing plants and from pasteurized milk in households were able to use starch and salicin as carbon source suggesting that emetic toxin production is not very important in the dairy environment.

The diarrhoeal food poisoning syndrome is caused by ingestion of cells rather than by pre-formed toxin (Granum *et al.* 1993). Therefore, strains able to grow at both low temperatures (7 °C), in the product, and high temperatures (37 °C), in the ileum are of importance. Most strains, isolated on farms and at the two dairy processing plants, that were

able to multiply at 7 °C, grew only slowly at 37 °C, suggesting that these strains may not be very enterotoxigenic [chapters 5 and 7]. Of the isolates originating from pasteurized milk in households and able to grow at 7 °C (53 %), 40 % also grew at 37 °C. In addition, 27 % of the *B. cereus* isolates obtained from the households produced haemolysin BL, possibly a virulence factor [Chapter 9].

The ability of dairy strains to produce enterotoxin was confirmed by immunoblotting using antiserum against the purified enterotoxin, in-vivo cell assays on Vero-cells and by PCR, using a set of primers for the B component of the haemolysin BL [Chapter 9].

The carbohydrate metabolism of *B. cereus* might play a role in enterotoxin production; lactose and/or starch positive strains showed higher enterotoxigenic activity compared with strains unable to utilize lactose or starch [Chapter 9]. This may be of relevance to dairy products in which starch-based stabilizers are used. The problem may be exacerbated in these products because many are highly flavoured, masking off-flavours produced by high numbers of *B. cereus* (Champagne *et al.* 1994). High counts and the presence of toxin could be detected in the absence of recognizable spoilage in creme caramel (Sutherland 1993). Thus, in milk products or if milk is mixed as an ingredient in other food products and stored incorrectly (Christiansson 1993), food poisoning may occur.

Pasteurized milk kept at refrigeration temperatures (7 °C) will not contain more than 10^5 cfu ml^{-1} within the expiry date and probably cause no problems for healthy adults (Langeveld *et al.* 1996, Notermans *et al.* 1997). Moreover, the low rate of *B. cereus* infection and intoxication from the consumption of milk(products) may be due to a number of reasons. Firstly, visible spoilage occurs before numbers are sufficiently high to cause illness and the milk is not consumed. Secondly, the symptoms of the disease are transient and may go underreported. Finally, the growth conditions in milk are not suitable for toxin production (e.g. nutrient composition (low free amino acid content, lack of glucose), insufficient oxygen supply) (Christiansson *et al.* 1989, Kramer and Gilbert 1989).

CONCLUDING REMARKS

B. cereus spores present in raw milk, originating mainly from soil and faeces, can easily survive during pasteurization of milk. The organism was shown to be present in 70 % of freshly pasteurized milk. *B. cereus* spores can germinate and grow out in pasteurized milk during storage at low temperatures. Most dairy strains were able to produce enterotoxin, determined by immunoblotting, cytotoxicity tests and PCR. Moreover, outbreaks, although relatively few, in which milk or milk related products containing *B. cereus* were suggested to be the cause of the disease, have been reported. As a consequence, *B. cereus* in milk and dairy products should be considered as a hazard.

Exposure calculations, based on storage conditions in households, by inquiries on storage time and temperature, and storage tests of freshly pasteurized milk, revealed that at time of consumption approximately 7 % and 4 % of the total portions of pasteurized milk consumed contain more than 10^5 and more than 10^6 *B. cereus* ml^{-1}, respectively (Notermans *et al.* 1997).

Although no clear dose-response data are available there are distinct indications that 10^4 - 10^5 *B. cereus* per g or ml should be set as a limit (Christiansson *et al.* 1989, Anonymous 1996b, Northolt 1996, PHLS 1996). The results obtained in a study by Notermans *et al.* (1997) showed that about 20 % does not meet these criteria, as the milk is frequently consumed after the expiry date and the storage temperature of the milk in the refrigerators was in 57 % of the cases above 7 °C. This may lead to foodborne infections or intoxications by the organism. However, if stored under proper conditions (maximum storage temperature 7 °C) the levels of *B. cereus* in pasteurized milk will not exceed 10^5 ml^{-1} within the expiry date and will not lead to foodborne infection or intoxication in healthy adults.

REFERENCES

Ahmed AA-H, Moustafa MK and EH Marth (1983) Incidence of *Bacillus cereus* in milk and some milk products. *Journal of Food Protection* **46** 126-128.

Anonymous (1992) ISO/DIS 7932 Microbiology-General guidance for enumeration of *Bacillus cereus* colony count technique at 30 °C, International Organization for Standardization, Switzerland.

Anonymous (1996a) ISO Standard Procedure 7218; Microbiology-General guidance for microbiological examinations, International Organization for Standardization, Switzerland.

Anonymous (1996b) Warenwetbesluit Bereiding en behandeling van levensmiddelen, artikel 4, *Warenwet*, Koninklijke Vermande BV, Lelystad.

Bourque SN, Valéro JR, Mercier J, Lavoie MC and RC Levesque (1993) Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis* strains. *Applied and Environmental Microbiology* **59** 523-527.

Champagne CP, Laing RR, Roy D, Mafu AA and MW Griffiths (1994) Psychrotrophs in dairy products: their effects and their control. *Critical Reviews in Food Science and Nutrition* **34** 1-30.

Christiansson A (1993) The toxicology of *Bacillus cereus*. *IDF Bulletin* **275** 30-33.

Christiansson A, Satyanarayan Naidu A, Nilsson I, Wadström T and H-E Petterson (1989) Toxin production by *Bacillus cereus* isolates in milk at low temperatures. *Applied and Environmental Microbiology* **55** 2595-2600.

Damgaard PH (1995) Diarrhoeal enterotoxin production by strains of *Bacillus thuringiensis* isolated from commercial *Bacillus thuringiensis*-based insecticides. *FEMS Immunology and Medical Microbiology* **12** 245-250.

Damgaard PH, Larsen HD, Hansen BM, Bresciani J and K Jørgensen (1996) Enterotoxin-producing strains of *Bacillus thuringiensis* isolated from food. *Letters in Applied Microbiology* **23** 146-150.

Granum PE, Brynestad S, O'Sullivan K and H Nissen (1993) Enterotoxin production from *Bacillus cereus*: production and characterization. *Netherlands Milk and Dairy Journal* **47** 63-70.

Kramer JM and RJ Gilbert (1989) *Bacillus cereus* and other *Bacillus* species. In MP Doyle (ed.) *Foodborne pathogens*, Marcel Dekker, New York, 21-70.

Langeveld LPM, Van Spronsen WA, Van Beresteijn ECH and SHW Notermans (1996) Consumption by healthy adults of pasteurized milk with a high concentration of *Bacillus cereus*: A double-blind study. *Journal of Food Protection* **59** 723-726.

Logan NA and RCW Berkeley (1984) Identification of *Bacillus* strains using the API system. *Journal of General Microbiology* **130** 1871-1882.

McKinnon CH and GL Pettipher (1983) A survey of sources of heat-resistant bacteria in milk with particular reference to psychrotrophic spore-forming bacteria. *Journal of Dairy Research* **50** 163-170.

Meer RR, Baker J, Bodyfelt FW and MW Griffiths (1991) Psychrotrophic *Bacillus* spp. in fluid milk products: A review. *Journal of Food Protection* **54** 969-979.

Moran L, Rowe MT and JA Hagan (1990) The effect of various heat activation treatments on fast, intermediate and slow germinating spores of *Bacillus* spp. *Letters in Applied Microbiology* **10** 43-46.

Mossel DAA, Koopman MJ and E Jongerius (1967) Enumeration of *Bacillus cereus* in foods. *Applied Microbiology* **15** 650-653.

Northolt MD (1996) Opstellen van microbiologische normen voor HACCP. *Voedingsmiddelen-technologie* **29-12** 26-29.

Notermans S, Dufrenne J, Teunis P, Beumer R, Te Giffel M and P Peeters Weem (1997) A risk assessment study of *Bacillus cereus* present in pasteurised milk. *Food Microbiology* (in press).

Phillips JD and MW Griffiths (1986) Factors contributing to the seasonal variation of *Bacillus* spp. in pasteurised dairy products. *Journal of Applied Bacteriology* **61** 275-285.

PHLS (1996) Microbiological guidelines for some ready-to-eat foods sampled at the point of sale. *PHLS Microbiology Digest* **13** 41-43.

Priest FG and R Grigorova (1990) Methods for studying the ecology of endospore-forming bacteria. In *Methods of Microbiology* **22** 566-591.

Rangasamy PN, Iyer M and H Roginski (1993) Isolation and characterisation of *Bacillus cereus* in milk and dairy products manufactured in Victoria. *Australian Journal of Dairy Technology* **48** 93-95.

Rusul G and NH Yaacob (1995) Prevalence of *Bacillus cereus* in selected foods and the detection of enterotoxin using TECRA-VIA and BCET-RPLA. *International Journal of Food Microbiology* **25** 131-139.

Shinagawa K (1990) Analytical methods for *Bacillus cereus* and other *Bacillus* species. *International Journal of Food Microbiology* **10** 125-142.

Stewart DB (1975) Factors influencing the incidence of *Bacillus cereus* spores in milk. *Journal of the Society of Dairy Technology* **28** 80-85.

Sutherland AD (1993) Toxin production by *Bacillus cereus* in dairy products. *Journal of Dairy Research* **60** 569-574.

Walthew J and H Lück (1978) Incidence of *Bacillus cereus* in milk powder. *South African Journal of Dairy Technology* **10** 47-50.

SUMMARY

Bacterial spores are of special interest and importance to the food industry, since they are capable of surviving food processing treatments. If conditions such as temperature and pH are suitable and the food provides the proper nutritive environment, spores may germinate, grow out and multiply in the food, causing spoilage. Outgrowth of some spore-formers, e.g *Clostridium botulinum*, *Cl. perfringens* and *Bacillus cereus*, may lead to foodborne infections and/or intoxications. *Bacillus* species are natural contaminants of many food ingredients and can be isolated from a wide variety of processed food products including milk, yeast, flour, pasta products, Chinese meals, cocoa, chocolate, bakery products, meat products, herbs and spices. In the dairy industry, (psychrotrophic) *B. cereus* are important as they determine the shelf-life of pasteurized milk and milk products, if the level of post-pasteurization contamination is low.

The most common procedure used to determine the proportion of spore-forming organisms present as spores is to conduct a viable count after a heat-activation treatment of 10 min at 80 °C. In the standard procedures for microbiological examinations the maximum time allowed between preparation of the primary dilution and mixing of dilutions and media is 45 min. Strikingly, it was shown that considerable germination (up to 40 %) occurs within 30 min at room temperature in diluents prescribed in the standard procedures resulting in underestimation of the numbers of endospore-forming bacteria present as spores. Therefore, the time between preparation of the primary dilution and the heat-activation step should be limited (< 10 min) and the temperature during the analysis should be as low as possible (e.g. by keeping dilutions in melting ice).

To enumerate *B. cereus* from food and environmental samples Mannitol Egg Yolk Polymyxin B sulfate (MEYP) agar is the most frequently employed selective medium. The percentage of presumptive isolates that could be confirmed by biochemical tests was 87 to 94 %. However, it was noted that biochemical tests do not always identify a species correctly. By sequencing part of the 16S rRNA several *B. cereus* isolates, involved in food poisoning incidents, were identified as *B. thuringiensis*, which is applied as insecticide worldwide. Recently, it has been demonstrated that *B. thuringiensis* produced a *B. cereus*-diarrhoeal-type of enterotoxin. In addition, several cases of '*B. cereus*' food poisoning in which vegetable products were involved have been reported. This emphasized the need to re-evaluate taxonomy of these closely related species and led us to the development of a method, using DNA probes based on variable regions of 16S rRNA, to discriminate between *B. cereus* and *B. thuringiensis*.

B. cereus can be introduced into milk from a variety of sources during production, handling and processing. To determine the major contamination sources, the incidence of *B. cereus* on farms, in two dairy processing plants and in pasteurized milk in household refrigerators was investigated. On farms, the major contamination sources were soil and

faeces. In winter, when the cows are housed, used bedding probably also participates in this contamination route. The udder will be contaminated, resulting in the presence of the organism in raw milk. *B. cereus* could be detected in 35 % of the raw milk samples analyzed. During processing of raw milk to pasteurized milk or milk powder an increase in the percentage of positive samples was observed, suggesting that *B. cereus* can also be introduced via sources other than raw milk, e.g. equipment. *B. cereus* spores can remain in a heat exchanger, adhere to the surface, germinate and grow out. If these spores detach, the equipment can be a continuous source of contamination. The contamination levels were generally low; the organism could only be isolated after pre-incubation for 6 h at 30 °C. *B. cereus* could be isolated from 40 % of the pasteurized milk samples obtained from household refrigerators without pre-incubation. In 77 % of the positive samples levels of less than 5 per ml were detected. However, in 5 % of the samples numbers were higher than 5000 ml^{-1}.

Biochemical characterization, by carbohydrate utilization patterns (API 50 CHB; BioMérieux), and molecular typing, by PCR/RAPD fingerprinting and plasmid profiling of strains, demonstrated that selection of strains occurs in the milk production chain and during storage. This is illustrated by the high percentage of strains present in pasteurized milk in household refrigerators able to utilize lactose as carbon source (20 %) and able to grow at low temperatures (53 %). It was also observed that some *B. cereus* types could be isolated from raw milk, at the various stages of processing and in the end products, indicating that raw milk is an important source of contamination. Other types could only be detected after the pasteurization step in the process supporting the assumption that there are additional sources of contamination during processing.

It is unlikely that the presence of *B. cereus* in the milk production and processing environment can be prevented. The initial level should be low and this can be influenced by cleaning and disinfection. The assessment of sanitizer efficacy tested in the laboratory with non-adherent bacteria can lead to false assumptions as to the true effectiveness under in-use conditions. The results of surface tests reflected these conditions more closely than the suspension tests. It is recommended to include this type of test to evaluate sporicidal efficacy of cleaning and disinfection agents.

Despite a world-wide occurrence of *B. cereus* in milk and milk products relatively few reports on food poisoning caused by these products have been reported. The emetic toxin production by dairy isolates is probably not very significant as very few strains unable to hydrolyze starch or to ferment salicin, properties which have been reported to be related to production of this type of toxin, were found. The diarrhoeal type of food poisoning is caused by ingestion of cells. Thus, strains able to grow at both low temperatures, in the product, and high temperatures, in the human intestine, are of importance. Most *B. cereus* strains isolated on farms and at the two processing plants, that were able to multiply at 7 °C, grew

only slowly at 37 °C, suggesting that these strains may not be very enterotoxigenic. Of the isolates originating from pasteurized milk stored in household refrigerators 53 % was able to grow at 7 °C, 40 % of these strains could also grow at 37 °C. In addition, 27 % of the *B. cereus* obtained from households produced haemolysin BL, possibly a virulence factor. The ability of dairy strains to produce enterotoxin was confirmed by immunoblotting, using antiserum against the purified enterotoxin, in-vivo cell assays on Vero-cells and by PCR, using a set of primers for the B component of the haemolysin BL. *B. cereus* strains able to utilize lactose as carbon source may have a growth advantage in milk, moreover they showed higher enterotoxigenic activity compared with strains unable to ferment this substrate. This may be of significance in highly flavoured dairy products in which spoilage and/or presence of enterotoxin may be masked. However, the role of the carbohydrate metabolism in enterotoxin production remains to be further elucidated. If milk is mixed as an ingredient in other food products and stored incorrectly foodborne infections or intoxications may also occur.

If stored under proper conditions, maximum storage temperature 7 °C, and consumed within the expiry date, the levels of *B. cereus* in pasteurized milk will, in general, not exceed 10^5 ml^{-1} and cause no problems for healthy adults. Factors that may contribute to the low rate of *B. cereus* infection and intoxication are: visible spoilage occurs before numbers are sufficiently high to cause problems, duration of disease symptoms, unsuitable conditions for growth and toxin production in milk.

SAMENVATTING

Sporevormende bacteriën zijn van groot belang voor de levensmiddelenindustrie omdat ze vaak als primaire besmetting op grondstoffen voorkomen, bovendien kunnen sporen tijdens het produktieproces overleven omdat ze resistent zijn tegen bijvoorbeeld hittebehandelingen en reinigings- en desinfectieprocedures. Onder gunstige omstandigheden kunnen deze sporen, na ontkieming, in levensmiddelen uitgroeien tot hoge aantallen en bederf veroorzaken. Sommige sporevormers, zoals *Clostridium botulinum*, *Cl. perfringens* en *Bacillus cereus* kunnen zelfs tot voedselinfectie of -vergiftiging leiden.

Bacillus species zijn ubiquitair in de natuur en komen voor in allerlei ingrediënten van levensmiddelen bijvoorbeeld bakkerijgrondstoffen (onder andere gist en meel), cacao, kruiden en specerijen en zijn belangrijke bederfveroorzakers in verschillende producten zoals pasta producten, Chinese maaltijden, chocola, vlees en vleeswaren, cake en gebak. Regelmatig worden ziektegevallen geregistreerd na consumptie van pasta- en rijstproducten met hoge aantallen *B. cereus*. In de zuivelindustrie is met name *B. cereus* belangrijk omdat deze de houdbaarheid van gepasteuriseerde melk en melkproducten bepaalt wanneer nabesmetting met gramnegatieve bacteriën laag is.

Om het aantal endosporevormers aanwezig in de vorm van sporen te bepalen wordt meestal een telling gedaan na een hitte-activeringsstap van 10 min bij 80 °C. Hierdoor worden vegetatieve cellen gedood en worden sporen geactiveerd tot ontkieming en verdere uitgroei tot kolonies op agarmedia. In de standaardprocedures voor de microbiologische analyse is maximaal 45 min toegestaan tussen bereiding van de primaire verdunning en het maken van verdere verdunningen en het uitplaten op agarmedia. *B. cereus* sporen bleken in staat zeer snel te ontkiemen, wat tot een onderschatting van het aantal aanwezige sporen leidt. Zelfs in Pepton Fysiologische Zout (PFZ) oplossing, voorgeschreven als verdunningsvloeistof in veel standaard procedures, ontkiemde tot 40 % van de sporen in 30 min bij kamertemperatuur. Dit effect wordt niet alleen bij *B. cereus* maar ook bij andere sporevormers waargenomen; daarom moet bij microbiologische analyses op bacteriesporen de tijd tussen het maken van een primaire verdunning en het verder verwerken van monsters zo kort mogelijk zijn (< 10 min) en moet de temperatuur tijdens de analyse zo laag mogelijk gehouden worden, bijvoorbeeld door gebruik van (smeltend) ijs.

Om aantallen *B. cereus* in levensmiddelen en omgevingsmonsters te bepalen wordt meestal gebruik gemaakt van het selectieve Mannitol Eidooier Polymyxine B sulfaat (MEYP) agar medium. Na isolatie worden verdachte kolonies bevestigd met biochemische testen volgens de ISO standaard procedure. In deze procedure worden drie bevestigingsreacties voorgeschreven: glucose fermentatie, nitraat reductie en vorming van acetylmethylcarbinol (Voges Proskauer reactie). In de studies beschreven in dit proefschrift kon 87 tot 94 % van de verdachte *B. cereus* bevestigd worden. Door sequencen van een deel van het 16S rRNA bleek biochemisch testen niet altijd in staat tot correcte identificatie van isolaten.

Verscheidene *B. cereus* stammen, betrokken bij voedselvergiftigingen, werden als *B. thuringiensis*, wereldwijd als insecticide gebruikt, geïdentificeerd. Recent is aangetoond dat *B. thuringiensis* stammen ook een op *B. cereus* enterotoxine lijkend toxine produceren. Bovendien zijn er verschillende uitbraken van voedselinfectie door '*B. cereus*' in groente beschreven. Daarom is in dit proefschrift een specifieke en gevoelige methode, gebruik makend van DNA probes gebaseerd op variabele gebieden van het 16S rRNA, ontwikkeld om *B. cereus* en *B. thuringiensis* te onderscheiden.

Melk kan besmet worden met (psychrotrofe) *B. cereus* op boerderijen, tijdens transport en in fabrieken. Om de belangrijkste besmettingsbronnen van melk met *B. cereus* te bepalen werden monsters genomen op boerderijen, in twee zuivelfabrieken (één fabriek waar gepasteuriseerde melk geproduceerd wordt en één melkpoederfabriek) en in huishoudens. Op de boerderij is het organisme voornamelijk afkomstig uit grond en faeces. In de winter wanneer de koeien op stal staan, vindt ook besmetting plaats via gebruikt strooisel. Zo worden de spenen besmet en uiteindelijk de rauwe melk; ongeveer 35 % van de rauwe melk monsters was besmet met *B. cereus*. In de fabrieken nam het percentage besmette monsters toe, dit geeft aan dat er extra besmetting optreedt via apparatuur. *B. cereus* sporen kunnen achterblijven in de pasteur, hechten, ontkiemen en uitgroeien. Wanneer de sporen van de apparatuur loslaten kan een continue bron van besmetting ontstaan. In het algemeen waren de aantallen laag; *B. cereus* kon alleen na ophoping (6 uur bij 30 °C) aangetoond worden. In 40 % van de gepasteuriseerde melkmonsters genomen in koelkasten in huishoudens werd *B. cereus* zonder ophoping aangetroffen. In 77 % van de positieve monsters werden aantallen van minder dan 5 per ml gevonden, in 5 % van de positieve monsters werden echter aantallen van meer dan 5000 per ml aangetoond.

Uit biochemische karakterisering, door onderzoek van het koolhydraatmetabolisme van stammen, en moleculaire typering van isolaten, met PCR/RAPD en analyse van plasmide profielen bleek dat selectie van stammen plaatsvindt in de melkproductieketen en tijdens distributie en opslag. Dit wordt geïllustreerd door het toenemende percentage stammen dat lactose als koolstofbron kan gebruiken, van minder dan 1 % op boerderijen tot 20 % van de isolaten uit gepasteuriseerde melk in huishoudens, en het grote aantal psychrotrofe stammen in deze melk (53 %). Er werd ook geconstateerd dat sommige typen in de rauwe melk en tijdens het productieproces en in de eindproducten aanwezig waren, dit geeft aan dat rauwe melk een belangrijke besmettingsbron is. Andere typen konden alleen na de pasteurisatiestap in het proces geïsoleerd worden, dit ondersteunt de veronderstelling dat tijdens het proces extra besmetting met *B. cereus* optreedt.

De aanwezigheid van sporevormers in melk(producten) is nauwelijks te voorkomen omdat de kiemen ubiquitair zijn. Het is daarom van belang om de beginbesmetting zo laag mogelijk te houden en besmetting tijdens verdere verwerking te voorkomen door toepassen

van hygiënische werkwijzen en goede reiniging en desinfectie. Het bepalen van de effectiviteit van reinigingsmiddelen en desinfectantia in laboratoriumtesten met suspensietesten met niet gehechte cellen, leidt tot onderschatting van het effect onder praktijkomstandigheden. Resultaten van oppervlaktetesten benaderden de werkelijke situatie meer dan suspensietesten en deze zouden dan ook gebruikt moeten worden om het sporicide effect van reinigings- en desinfectiemiddelen te testen.

Hoewel *B. cereus* de belangrijkste bederfveroorzaker van melk is zijn er weinig gevallen van voedselinfectie en/of -vergiftiging, waarbij melk of melkproducten betrokken waren, gerapporteerd. Het braaktoxine is waarschijnlijk niet zo belangrijk omdat weinig stammen aangetroffen zijn die niet in staat waren zetmeel te hydrolyseren of salicine te fermenteren. Volgens literatuurgegevens kunnen stammen van het braak-type deze substraten niet omzetten. Het diarree-type wordt veroorzaakt door opname van cellen. Stammen die zowel bij lage temperatuur, tijdens distributie en opslag, als bij hoge temperatuur, in het lichaam kunnen groeien zijn daarom belangrijk. De meeste stammen geïsoleerd op boerderijen en in fabrieken die in staat waren om zich te vermeerderen bij lage temperatuur groeiden maar langzaam bij 37 °C. Dit geeft aan dat deze isolaten waarschijnlijk geen grote hoeveelheden toxine produceren. Van de stammen afkomstig uit gepasteuriseerde melk in huishoudens daarentegen kon 53 % groeien bij 7 °C, waarvan 40 % ook bij 37 °C. Verder produceerde 27 % van de isolaten uit huishoudens het haemolysine BL, wat zeer waarschijnlijk een virulentiefactor is. De mogelijkheid van deze *B. cereus* stammen om enterotoxine te produceren werd bevestigd door immunoblotting met antiserum tegen gezuiverd enterotoxine, *in-vivo* celtoxiciteitstesten met Verocellen en PCR met behulp van primers tegen de B component van het haemolysine BL. Lactose-metaboliserende stammen produceerden meer toxine dan andere stammen. Dit kan van belang zijn omdat deze biotypen een groeivoordeel hebben in melk en melkproducten en meer toxine produceren. Het koolhydraatmetabolisme van *B. cereus* zou dus een rol kunnen spelen bij enterotoxine-productie. In zuivelproducten waarin op zetmeel gebaseerde stabilisatoren toegepast worden, of die grote hoeveelheden suikers bevatten kunnen bederf en/of toxineproductie onopgemerkt blijven. Ook wanneer melk als ingrediënt in andere producten gebruikt wordt kan dit, bij onjuiste bewaaromstandingheden, tot voedselinfectie en/of -vergiftiging leiden.

Indien melk onder normale omstandigheden bewaard wordt en binnen de uiterste houdbaarheidsdatum geconsumeerd wordt zal het aantal *B. cereus* in het algemeen niet boven 10^5 cellen per ml uitkomen en geen problemen veroorzaken voor gezonde volwassenen. Andere factoren die kunnen bijdragen aan het lage aantal voedselinfecties en -vergiftigingen door *B. cereus* via melkproducten zijn: de beperkte duur van de ziekte-symptomen en de ongunstige omstandigheden in melk voor groei en toxineproductie. Bovendien treedt zichtbaar bederf eerder op dan dat aantallen die voor problemen kunnen zorgen bereikt worden.

CURRICULUM VITAE

Meike te Giffel werd op 16 juli 1968 geboren in Groningen. In 1986 behaalde zij het gymnasium β diploma aan de katholieke scholengemeenschap 'De Breul' in Zeist. In datzelfde jaar begon zij met de studie Levensmiddelentechnologie aan de Landbouwuniversiteit in Wageningen. In januari 1992 studeerde zij af met als richting levensmiddelenmicrobiologie. Haar stage-periodes bracht zij door bij Nestlé; Nestec, Central Quality Assurance Department, in Vevey, Zwitserland en bij Grace Cocoa, Cacao de Zaan in Koog aan de Zaan.

Vanaf februari 1992 werkt zij als toegevoegd onderzoeker bij de Vakgroep Levensmiddelentechnologie, sectie levensmiddelenchemie en -microbiologie en verrichtte het onderzoek wat leidde tot dit proefschrift. Het onderzoek werd financieel ondersteund door NESTEC, AAIR Concerted Action PL 920630 en het Ministerie van Landbouw, Natuurbeheer en Visserij in het kader van de bijdrageregeling "Kwaliteitsprojecten agrarische produkten en productieprocessen".

Sinds 1 april 1997 werkt zij ook bij de sectie proceskunde aan het uitbreiden van databases ten behoeve van kwantitatieve modellen voor risico-management van levensmiddelen en het valideren van modellen.

LIST OF PUBLICATIONS

Beumer RR, Te Giffel MC, Doornik PC and LJ Cox (1992) The isolation of *Listeria monocytogenes* from food and environmental samples using enhanced haemolysis agar. Proceedings of the XI International Symposium on Problems of Listeriosis, Copenhagen, Denmark, 11-14 May 1992.

Beumer RR, Te Giffel MC, Cox LJ, Rombouts FM and T Abee (1994) Effect of exogenous proline, betaine and carnitine on growth of *Listeria monocytogenes* in a minimal medium. *Applied and Environmental Microbiology* **60** 1359-1363.

Beumer RR, Te Giffel MC, Kok MTC and FM Rombouts (1995) A comparison of (rapid) methods for the detection of *Listeria monocytogenes*. Proceedings of the XII International Symposium on Problems of Listeriosis, Perth, Australia, 2-6 October 1995, 53-56.

Beumer RR and MC Te Giffel (1996) Op zoek naar *Listeria monocytogenes*. *Voedingsmiddelentechnologie* **29-21** 45-48.

Beumer RR, Te Giffel MC, Kok MTC and FM Rombouts (1996) Confirmation and identification of *Listeria* spp. *Letters in Applied Microbiology* **22** 448-452.

Beumer RR, Te Giffel MC, Anthonie SVR and LJ Cox (1996) The effect of acriflavine and nalidixic acid on the growth of *Listeria* spp. in enrichment media. *Food Microbiology* **13** 137-148.

Beumer RR, Te Giffel MC, De Boer E and FM Rombouts (1996) Growth of *Listeria monocytogenes* on sliced cooked meat products. *Food Microbiology* **13** 333-340.

Beumer RR, Te Giffel MC and LJ Cox (1996) Optimization of haemolysis in Enhanced Haemolysis Agar (EHA)- A selective medium for the isolation of *Listeria monocytogenes*. *Letters in Applied Microbiology* (in press).

Beumer RR, Te Giffel MC, Spoorenberg E and FM Rombouts (1996) *Listeria* spp. in domestic environments. *Epidemiology and Infection* **117** 437-442.

Beumer RR, Te Giffel MC and FM Rombouts (1997) Comparison of rapid methods for the detection of *Listeria* spp. and *Listeria monocytogenes* (submitted for publication).

Beumer RR, Te Giffel MC, Van Asselt A and FM Rombouts (1997) Growth of *Listeria monocytogenes* on raw meat (submitted for publication).

De Boer E, Beumer RR, Van Asselt A and MC Te Giffel (1995) Growth of *Listeria monocytogenes* on sliced cooked meat products. Proceedings of the XII International Symposium on Problems of Listeriosis, Perth, Australia, 2-6 October 1995, 253-256.

Dufrenne J, Bijwaard M, Te Giffel M, Beumer R and S Notermans (1995) Characteristics of some psychrotrophic *Bacillus cereus* isolates. *International Journal of Food Microbiology* **27** 175-183.

Granum PE, Andersson A, Gayther C, Te Giffel M, Larsen H, Lund T and K O'Sullivan (1996) Evidence for a further enterotoxin complex produced by *Bacillus cereus*. *FEMS Microbiology Letters* **141** 145-149.

Notermans S, Dufrenne J, Teunis P, Beumer R, Te Giffel M and P Peeters Weem (1997) A risk assessment study of *Bacillus cereus* present in pasteurised milk. *Food Microbiology* (in press).

Slaghuis BA, Te Giffel MC, Beumer RR and G André (1997) Effect of pasturing on the incidence of *Bacillus cereus* spores in raw milk. *International Journal of Dairy Technology* (in press).

Te Giffel MC, Beumer RR and J Hoekstra (1993) Detection of *Bacillus cereus* in environmental samples and milk on farms. Abstracts of the 62nd Annual Meeting and Summer Conference University of Nottingham, 13-16 July 1993, xiv.

Te Giffel MC (1995) Toepassing van moleculaire technieken voor de detectie en typering van micro-organismen in de levensmiddelenmicrobiologie. EFFI Symposium HACCP- een stap verder, Wageningen, 15 Juni 1995.

Te Giffel MC, Beumer RR en FM Rombouts (1995) Voorkomen en gedrag van *Bacillus cereus* in levensmiddelen. *De Ware(n) Chemicus* **25** 17-28.

Te Giffel MC, Beumer RR, Hoekstra J en FM Rombouts (1995) Germination of bacterial spores during sample preparation. *Food Microbiology* **12** 327-332.

Te Giffel MC, Beumer RR, Slaghuis BA en FM Rombouts (1995) Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands. *Netherlands Milk and Dairy Journal* **49** 125-138.

Te Giffel MC, Beumer RR, van Dam W and FM Rombouts (1995) Sporicidal effect of disinfectants on *Bacillus cereus* isolated from the milk processing environment. Abstracts of the Symposium on Disinfection, Sterilization and Preservation: Problems and Practices, Society for Applied Bacteriology and the Biodeterioration Society, University of Surrey, Guilford, 23-24 March 1995, xi-xii.

Te Giffel MC, Beumer RR, van Dam W, Slaghuis BA and FM Rombouts (1995) Sporicidal effect of disinfectants on *Bacillus cereus* isolated from the milk processing environment. *International Biodeterioration and Biodegradation* **36** 412-430.

Te Giffel MC, Beumer RR, Cox LJ, Abee T and FM Rombouts (1995) Effects of osmoprotectants on growth of *Listeria* under osmotic stress conditions. Proceedings of the XII International Symposium on Problems of Listeriosis, Perth, Australia, 2-6 October 1995, 123-127.

Te Giffel MC, Beumer RR, Leijendekkers S and FM Rombouts (1996) Incidence of *Bacillus cereus* and *Bacillus subtilis* in foods in the Netherlands. *Food Microbiology* **13** 53-58.

Te Giffel MC, Beumer RR, Slaghuis BA en FM Rombouts (1996) Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands. Abstracts of the Symposium on Bacteriological Quality of Raw Milk, International Dairy Federation, Wolfpassing, Austria, 13-15 March 1996, 40-45.

Te Giffel MC, Beumer RR, van Dam W and FM Rombouts (1996) Sporicidal effect of disinfectants on *Bacillus cereus* isolated from the milk processing environment. Abstracts of the Symposium on Bacteriological Quality of Raw Milk, International Dairy Federation, Wolfpassing, Austria, 13-15 March 1996, 167-168.

Te Giffel MC, Beumer RR, Bonestroo MH and FM Rombouts (1996) Incidence and characterization of *Bacillus cereus* in two dairy processing plants. Abstracts of the 16th IUMS-ICFMH Symposium Food Micro '96, Budapest, Hungary, 27-30 August 1996, 178.

Te Giffel MC, Beumer RR, Bonestroo MH and FM Rombouts (1996) Incidence and characterization of *Bacillus cereus* in two dairy processing plants. *Netherlands Milk and Dairy Journal* **50** 479-492.

Te Giffel MC, Beumer RR, Granum PE and FM Rombouts (1997) Isolation and characterization of *Bacillus cereus* from pasteurized milk in household refrigerators in the Netherlands. *International Journal of Food Microbiology* **34** 307-318.

Te Giffel MC, Beumer RR, Klijn N, Wagendorp A and FM Rombouts (1997) Discrimination between *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based on variable regions of 16S rRNA. *FEMS Microbiology Letters* **146** 47-51.

Te Giffel MC, Beumer RR, Langeveld LPM and FM Rombouts (1997) The role of heat exchangers in the contamination of milk with *Bacillus cereus* in dairy processing plants. *International Journal of Dairy Technology* (in press).