INCIDENCE AND IMPORTANCE OF *BACILLUS* SPECIES IN RAW MILK AND IN THE DAIRY ENVIRONMENT

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DECLARATION

This work has not been accepted for any other degree and is not currently being submitted in candidature for any other degree. The work submitted here was carried out by the candidate herself and due acknowledgement has been made of any assistance received.

Candidate..... .....

Director of Studies.....

ABSTRACT

The objective of this project has been to further existing knowledge of the incidence, seasonality and toxinogenicity of *Bacillus cereus* and other *Bacillus* species, particularly in milk and the dairy farm environment.

The occurrence of psychrotrophic and mesophilic populations of *Bacillus* spp. in milk were seasonal. The study conducted here demonstrated that the same seasonality trends extend to raw milk, udder washes and faecal samples taken from dairy cattle. Mesophilic and psychrotrophic *Bacillus* spp. populations were not found to be exclusive of the other. Psychrotrophic sporeformers were detected outwith the summer-autumn season when they were anticipated. This indicates that there is a potential problem arising from the presence of psychrotrophic *Bacillus* spp. in milk and dairy products throughout the year. Spores of psychrotrophic strains of *Bacillus* spp. occurred in raw milk in the survey reported here at low levels (<1 cfu/ml). The most commonly identified species of psychrotrophic bacilli were *B.cereus* and *B.mycoides*; in addition another 14 psychrotrophic Bacillus species were also recovered during the course of the survey.

Contamination of raw milk appears to be derived from the udder teat surface. The teat surface may be contaminated by faecal material or soil, contamination may also occur from soiled winter bedding. The extent of udder contamination was affected by husbandry practices. Data for viable and sporeforming psychrotrophic, mesophilic and

thermoduric populations occurring in raw milk and samples from the cow and the immediate farm environment over a 13 month period are presented.

The work presented here describes an improved cell cytotoxicity assay which determines colorimetrically the inhibition of cell metabolism by *Bacillus* toxins. The cell cytotoxicity assay had good correlation with commercial *B.cereus* enterotoxin detection systems. Screening of *B.cereus* isolates for toxin production demonstrated their widespread ability to produce toxins. Although this was the case, there was also a wide spectrum of levels of toxin produced by the isolates. Species of *Bacillus* other than *B.cereus* were shown to be cytotoxic. These species included isolates of *B.mycoides*, *B.thuringiensis*, *B.subtilis*, *B.lentus*, *B.brevis*, *B.circulans*, *B.licheniformis* and *B.polymyxa*.

The effectiveness of Fourier transform infrared spectroscopy (FTIR) for the discrimination between *Bacillus* spp. was investigated, and was found to be a useful tool for this purpose. It was possible to distinguish between the members of the *Bacillus cereus* group tested.

Interactions between mesophilic and psychrotrophic *Bacillus* species were confirmed. The factor produced by *B.subtilis* which inhibits *B.cereus* is released into its growth environment. Strains of *B.subtilis* which caused inhibition also had a cytotoxic effect. It was unclear whether the inhibitor and the toxin were separate compounds, and therefore the potential for exploitation of the inhibitory factor to repress *B.cereus* in milk or other food products is removed.

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PUBLICATIONS

Beattie, S.H. and Williams, A.G. (1996)

Incidence of Psychrotrophic and Mesophilic *Bacillus* Species in Raw Milk and the Farm Environment

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The Seasonal Incidence and Toxigenicity of Psychrotrophic *Bacillus* species in Raw Milk in Spain and Scotland

Bacteriological Quality of Raw Milk, *International Dairy Federation*,

Garcia-Armesto, M.R, Sanz, J.J., Rodriguez-Perez, R., Otero, A., Beattie, S., and Williams, A. (1996)

Patogenicidad de Bacillus spp. Aislados de Embutidos Crudos Curados

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ABBREVIATIONS

Abbreviation (non-standard)	Definition
B	Boiled cell free culture supernatants
BCET-RPLA	*Bacillus cereus* enterotoxin - reverse passive latex agglutination
BDE	*Bacillus* diarrhoeal enterotoxin
BHI	Brain heart infusion broth
cfu	Colony forming unit
CHO	Chinese hamster ovary cells
D	Dialysed cell free culture supernatants
D/B	Dialysed and boiled cell free culture supernatants
FTIR	Fourier transform infrared spectroscopy
g	Gravitational force
HRI	Hannah Research Institute
IR	Infrared
kDa	Kilo Daltons
MSC	Mesophilic spore count
MTT	3-(4,5,-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide
N/B	Not boiled cell free culture supernatants
OD	Optical density
PSC	Psychrotrophic spore count
Shak	*B.cereus* cultured under shaking conditions (200rpm)
SMP	Skim milk powder
spp	Species
Stat	*B.cereus* cultured under static conditions
TMC	Total mesophilic count
TPC	Total psychrotrophic count

Abbreviations table continued.

Abbreviation (non-standard)	Definition
TSC	Thermophilic spore count
TTC	Total thermoduric count
VIA	Visual immunoassay

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CHAPTER 1.

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1:1 The genus Bacillus

The genus *Bacillus* was created in 1872 by Ferdinand Cohn, who changed the name of "*Vibrio subtilis*" (Ehrenberg, 1835) to *Bacillus subtilis*. This strain of Cohn (1872) is still the type species of the genus.

The name *Bacillus* is derived from the Latin meaning "small rod". Members of the genus are aerobic or facultatively anaerobic, endospore forming, Gram positive rods.

The genus *Bacillus* is one of the most diverse groups of micro-organisms. These bacteria are of importance both because they possess many unusual physiological features which have found numerous practical and commercial applications (Table 1:1), and also because of the problems they cause through infection and food spoilage. They have been exploited to man's benefit in fermentation processes such as the preparation of cocoa beans, and the production of natto, a Japanese food derived from the *B.subtilis* fermentation of soybeans (Hara and Veda, 1982). Heat stable enzymes produced by *B.stearothermophilus* have been utilised in a commercial kit which tests for the presence of antibiotic residues in animal products (Turnbull *et al.*, 1990). Amylase and protease enzymes have been used in food processing; purine nucleosides and nucleotides produced by *B.subtilis* have been used as flavour enhancers (Priest,

1989). *Bacillus thuringiensis* produce parasporal protein crystals (-endotoxin) which are used as insecticides. *Bacillus thuringiensis* insecticides, along with those from *B.popilliae,* account for the majority of the world biological insecticide market. *Bacillus popilliae* is used to control the Japanese beetle, which is a problem in the US (Priest, 1989).

In contrast to these positive aspects, some *Bacillus* species can also cause problems. They are able to produce highly heat resistant spores, and because of this *Bacillus* spp. are difficult to inactivate in foodstuffs. Here their subsequent growth can cause spoilage; this is especially a problem in dairy products. In addition to causing spoilage, many members of the genus produce toxins. Although *Bacillus* have been recognised as opportunistic pathogens since the nineteenth century, micro-organisms from the genus are still widely considered as unimportant contaminants by many in medical microbiological laboratories, and are therefore disregarded from samples. There is now, however, an increased likelihood of opportunistic pathogens causing infection, owing to host predisposition caused by compromised or suppressed immunity, metabolic disorders, exposure to clinical or surgical procedures, accidental trauma and drug abuse; in view of this more consideration should be given to the potential pathogenicity of some *Bacillus* spp..

1:2 Classification of Bacillus *species*

According to the classification system used in Bergey's manual (Gordon *et al.*, 1973),

there are 34 species of *Bacillus* and an additional 26 species listed as *species incertia sedis* (Table 1:2).

The classification of *Bacillus* was haphazard until Smith *et al.* (1946) produced the first comprehensive study on the taxonomy of the genus. Before this the only major advances had taken place in 1884 when de Bary proposed that spore formation should be used as a character in the key for classification of *Bacillus*. This was the first time that the genus had been restricted to endospore-forming bacteria.

Smith *et al.* (1952) published further work on the classification of the genus *Bacillus*, and it was this study which Gordon *et al.* (1973) continued. The classification of the genus *Bacillus* in Bergey's Manual of Systemic Bacteriology has been reviewed by Claus and Berkeley (1986), and was based upon the system which was initially proposed by Gordon *et al.* (1973).

Three groups of species are recognised within the genus *Bacillus* (Smith *et al.*, 1952). Group I contains those species that do not form definitely swollen sporangia, and have centrally positioned, ellipsoidal spores. Group II have swollen sporangia and oval spores, while Group III have swollen sporangia and round spores (Goepfert *et al.*, 1972) (Figure 1:1).

The work in this study has been mainly involved with Group I *Bacillus*. Group I consists of 13 species of *Bacillus*, including *B.cereus*, *B.anthracis*, *B.firmus*,

B.mycoides, *B.thuringiensis*, *B.licheniformis*, *B.megaterium*, *B.subtilis* and *B.pumilus* (Priest, 1989) (Table 1:3).

Classification of *Bacillus* spp. has traditionally been based on Gram stain, spore shape and position, morphology of colonies, and biochemical reaction studies including sugar utilisation profiles, haemolytic and lecithinase activity. Other tests include motility studies.

Individual biochemical tests may be carried out conventionally using test tubes, or more conveniently using pre-made miniaturised kits, such as API (bioMérieux (UK) Ltd., Basingstoke) may be utilised. The API system has been shown to be more reproducible than classical tests (Logan and Berkeley, 1984). The test kits API 20E and API 50CHB can be used in combination. The two kits have been shown to be in good agreement with those obtained by other methods (Logan and Berkeley, 1984). The API 20E can be used as a direct substitute for conventional methodology (Varnam and Evans, 1991), and was found to be very reproducible, especially for the Voges Proskauer test (Encinas *et al.*, 1996).

1:3 Bacillus and the dairy industry

1:3:1 Spoilage

Members of the genus *Bacillus*, in particular *B.cereus* have long been recognised by

the dairy industry to represent a problem in terms of product spoilage (Table 1:4). *Bacillus* spp. have the ability to produce heat resistant spores which may withstand pasteurisation temperatures. Many strains are psychrotrophic, which means they have the capacity for growth at refrigeration temperatures. These factors occurring uniquely together in *Bacillus* make this genus a problem in dairy products.

Traditionally milk spoilage has been caused by Gram negative contaminants (Meer *et al.*, 1991), which enter the product at the post-pasteurisation stage of processing. Aseptic packaging of liquid milk has reduced contamination from these traditional spoilage sources, and has extended product shelf life without the need for higher heat treatments (Dommett, 1992). Without competition from the faster growing Gram negative contaminants, psychrotrophic strains of *B.cereus* have become a limiting factor in the keeping quality of pasteurised milk (Labots and Hup, 1964; Andersson *et al.*, 1995; Christiansson *et al.*, 1996). The numbers of *B.cereus* spores in pasteurised milk are low, generally <5/ml (Labots and Galesloot, 1959; Donovan, 1959; Labots and Hup, 1964, te Giffel *et al.*, 1997b), but it has been shown that *B.cereus* is the predominant species at the end point of keeping (Soriano, 1946; Franklin 1969). An estimated 25% of shelf life problems encountered with pasteurised milks are due to the proliferation of *Bacillus* spp. (Griffiths, 1992). About 30% of *Bacillus* spp. strains isolated from raw milk have been found to be psychrotrophic (Coghill and Juffs, 1979; Johnson and Bruce, 1982; te Giffel *et al.*, 1995b), and 70% isolated from pasteurised milks were also psychrotrophic (Griffiths, 1992).

Bacillus cereus acts as a spoilage organism in milk causing sweet curdling and bitty or broken cream. In commercially pasteurised milk samples in Tennesse 28% exhibited sweet curdling caused by *B.cereus* within ten days in refrigerated storage (Overast and Atmaram, 1974). Sweet curdling is caused without any reduction in pH of the product (Andersson *et al.*, 1995). Bitty cream is an aggregation into flocks of the cream layer of milk. Lecithinase positive strains of *B.cereus* cause "bitty cream" (Labots and Hup, 1964); most strains of *B.cereus* are lecithinase positive (Labots and Galesloot, 1959). The lecithinase enzyme attacks the phospholipid component of the fat globule membrane, causing the fat globules to stick together as flocks (Labots and Gailsloot, 1959). It has been shown that heat resistant spores germinate and can reach a colony count of 10^5 /ml without any change in milk composition or sensoric properties occurring (Hammer *et al.*, 1995).

1:3:2 Legal requirements for the quality of milk and dairy products

New Dairy Products (Hygiene) Regulations were put before British Parliament in 1995 which implement the regulations of the EC directive (92/46/EEC) (Anon, 1992). These lay down the requirements for production and marketing of raw milk, heat treated milk and milk based products from cows, sheep, goats and buffaloes.

The microbial criteria for milk-based products on removal from the processing establishment, under the Scottish Dairy Products (Hygiene) (Scotland) Regulations 1995 (Anon, 1995), are that they should not contain pathogenic micro-organisms or

toxins from pathogenic micro-organisms in such a quantity which may affect the health of the ultimate consumer. Immediately after milking, raw milk must be placed in a clean, suitably equipped place, to prevent the milk from suffering from adverse effects. Raw milk is to be cooled to a maximum temperature of 8°C as soon as is practicable after milking if the milk is collected from the farm daily. If the milk is not collected daily, the storage temperature must not exceed a maximum of 6°C. During transportation the temperature must not exceed 10°C (92/46/EEC) (Anon, 1992).

In other EC countries similar measures have been introduced. In 1989 the Milk Ordinance (Milchverodung) was introduced into Germany, to bring their legislation into line with the EEC Milk Hygiene Directive (85/397/EEC) (Anon, 1985), which stated that UHT milk must meet a standard plate count of <10 cfu in 0.1ml milk after 15 days at 30°C. Before the introduction of this legislation *Bacillus* had not been detected in milk in Germany (Hammer *et al.*, 1994).

In Sweden milk may have no more than 10^4 cfu/ml after six days at 8°C. In order to satisfy these requirements there must be no more than 10 spores in 100ml of milk at the packaging stage (Andersson *et al.*, 1995).

1:4 Seasonality

Bacillus species occur in milk and dairy products with seasonality (Ridgway, 1953; Franklin *et al.*, 1956; Billing and Cuthbert, 1958; Stewart, 1970; McKinnon and

Pettipher, 1983; Slaghuis and Wolters, 1992; Griffiths, 1992; Crielly *et al.*, 1994; Sutherland and Murdoch, 1994; Larsen and Jorgensen, 1997). Early work reported that the aerobic spore counts were much higher in the winter than in the summer (Ridgway, 1953; Franklin *et al.*, 1956). These studies looked at the entire population of aerobic sporeformers, and did not attempt to separate this group down into psychrotrophic and mesophilic sporeformers as later work did. When the mesophilic and psychrotrophic populations were distinguished from one another it became evident that they followed different seasonality patterns. The mesophilic populations were highest in the winter, while the psychrotrophic ones were highest during the summer to autumn months (Sutherland and Murdoch, 1994).

Bacillus cereus was the predominant psychrotrophic *Bacillus* isolated at all stages of milk processing (Crielly *et al.*, 1994; Sutherland and Murdoch, 1994). The maximum numbers of *B.cereus* were found in raw milks from August to September (Billing and Cuthbert, 1958). The prevalence of *B.cereus* in pasteurised milk samples collected from Danish dairies occurred during the summer (Larsen and Jorgensen, 1997). Psychrotrophic spore count was found to remain fairly constant throughout the year, while the total spore count decreased in the summer months (McKinnon and Pettipher, 1983). This meant that although the psychrotrophic spore count was consistent, its proportion of the total count increased markedly during the summer months (McKinnon and Pettipher, 1983). This finding was supported by a recent study (Slaghuis and Wolters, 1992), in which 54 different farms were assessed for *B.cereus* contamination in their raw milk. The study showed that the incidence of *B.cereus*

contamination of the milk was similar in the summer and the winter. However, in other work the mesophilic and psychrotrophic populations each appear exclusively at certain times of the year (Sutherland and Murdoch, 1994). It has been proposed that there may be interactions between the mesophilic and psychrotrophic populations, whereby the psychrotrophs are repressed by the mesophilic species (Sutherland and Murdoch, 1994), or that a germination factor promoting the germination of psychrotrophic strains may occur at a higher level in milk during the summer, than in the winter (Phillips and Griffiths, 1986).

1:5 Source of contamination

The source of contamination of raw milk by *Bacillus* spp. has been investigated (Ridgway, 1953; Griffiths and Phillips, 1990b; Slaghuis and Wolters, 1992; te Geiffel *et al.*, 1995). Winter housing of cattle probably contributes to the high levels of spores present in the milk during the winter months, through soiled bedding material (te Giffel *et al.*, 1995b) and aerial contamination from dusty feeds (Ridgeway, 1954). However, although the total aerial spore count during the winter was found to be high (Ridgway, 1954), the *B.cereus* spore content of the air was considered to be too low to be an important contamination source (Christiansson *et al.*, 1996). Soiled bedding material has also been implicated during the winter, as a source of contamination of raw milk by *Bacillus* spp. (te Giffel *et al.*, 1995b). The type of housing conditions experienced by the animals also contributes to the quality of the milk (Herlin *et al.*, 1994).

Amongst the mesophilic isolates *B.licheniformis* (Crielly *et al.*, 1994; Sutherland and Murdoch, 1994), *B.pumilis* and *B.subtilis* (Sutherland and Murdoch, 1994) dominated the populations. *B.licheniformis* was also the most common *Bacillus* isolated from "in-use" teat dips (Bruce, 1981). The dips are used to disinfect the teat and so reduce mastitis, but they may also cause cross contamination between animals. The flora of the "in-use" dips entirely consisted of *Bacillus* species (Bruce, 1981). *Bacillus licheniformis* was found to be ubiquitous in the farm environment (Crielly *et al.*, 1994), and so the origin of the contamination in raw milk could arise from many sources.

1:6 Psychrotrophic sporeformers

The dairy industry is more concerned with the presence of psychrotrophs in the milk than with mesophiles. Under normal storage conditions, without temperature abuse, mesophilic populations are unable to grow because the temperatures are too low. Mesophilic strains isolated from UHT processed milks had optimal growth temperatures of 37-45°C (Westhoff and Dougherty, 1981). Although none of these isolates could grow at 5°C, several could grow at 10°C. This is not far outside the 8°C temperature limits set by legislation for storage of milk and dairy products, and emphasises the need to ensure against temperature abuse. Particularly in light of a recent study in the Netherlands which found that pasteurised milk was stored in households at temperatures up to 13°C (Notermans *et al.*, 1997).

Psychrotrophic *Bacillus* spp. have adapted so that they are able to grow at lower temperatures. Although they have the capacity for growth under refrigeration conditions, the temperatures for optimal growth rates are much higher (21-30°C). It is therefore believed that psychrotrophic *Bacillus* spp. have adapted from mesophilic strains, rather than being truly psychrophilic. The minimum growth temperature for *B.cereus* has been shown to vary between <5-11°C (Dufrenne *et al.*, 1994). Others have found *B.cereus* causing spoilage of milks down to a minimum temperature of 7°C, and have suggested that to avoid growth of *B.cereus* the milk should be stored below 5°C (Ternstrom *et al.*, 1993). The duration of the lag phase at 7°C was found to be strongly influenced by the previous temperature history of the cells (Dufrenne *et al.*, 1995). If a psychrotrophic isolate adapts for growth at higher temperatures, it loses its capacity for growth at lower temperatures (Grosskopf and Harper, 1974).

Some other species of *Bacillus* spp., for instance *B.circulans* (Langefeld *et al.*, 1973; Dommett, 1992) and *B.polymyxa* (Ternstrom *et al.*, 1993) are better adapted to psychrotrophic growth than *B.cereus*, and are therefore able to grow at lower temperatures. However, of all psychrotrophic strains of *Bacillus* spp. isolated from pasteurised milk samples, more than 75% have been *B.cereus* or a related species (Griffiths, 1992).

As has already been indicated, psychrotrophic *B.cereus* enter raw milk from numerous sources. Soil is thought to be an important reservoir of the organism, occurring via

dirty teats (te Giffel *et al.*, 1995b; Christiansson *et al.*, 1996). During the summer *B.cereus* has been found in dung and on teat surfaces, although it was not detected in these samples during the winter (Slaghuis and Wolters, 1992). *Bacillus cereus* spores were detected in silage and bedding samples in both summer and winter (Slaghuis and Wolters, 1992), and another survey also found an association between *B.cereus* and cattle feed throughout the year (Crielly *et al.*, 1992).

Faeces have been linked with contamination of raw milk (te Gieffel, 1995). The contamination of the udder surface appears to be an important source of *Bacillus* spp. spores (Cousins, 1972; McKinnon *et al.*, 1974, Underwood *et al.*, 1974, McKinnon *et al.*, 1983). The teats may become heavily contaminated with a semi-solid mixture of bedding and faecal material when the cattle are housed inside (McKinnon *et al.*, 1983). When the cattle are at pasture contamination of the udder may occur while the animal is grazing (Phillips and Griffiths, 1990; Meer *et al.*, 1991). This wide variety of potential sources for *Bacillus* spp. to gain entry into raw milk illustrates the difficulties faced in trying to firstly determine the true route of entry into milk, and secondly to find a way of preventing that entry. It should also be recognised that contamination from *Bacillus* spp. may not be restricted to the raw milk supply. For instance, cardboard packaging materials used to package pasterurised milk for sale, have been shown to be contaminated with spores of *Bacillus* spp. (Vaisanen *et al.*, 1991; van Heddegham and Vlaemynck, 1992; Christiansson, 1993; Kneifel and Kaser, 1994). The bulk tank on the farm may be a contamination source for psychrotrophic spores (Phillips and Griffiths, 1990), as can poorly cleaned pipes and teat cups in the milking

parlour (Waes, 1992), and the dairy processing plant may also be a source (Kalogridou and Tsiabtas, 1992).

1:7 Spores

The vegetative cells of *Bacillus* spp. are destroyed by pasteurisation treatments, and therefore it is the presence of spores in milk and dairy products which causes particular concern. Environmental conditions during sporulation have a significant influence on the heat resistance and subsequent germination of spores (Davies, 1975). Other sporeformers, for instance *Clostridium* spp., may contaminate milk or milk products. *Clostridium* spp. are anaerobic and so are of greatest importance as spoilage organisms of cheese and canned milk products (Gilmour and Rowe, 1990). Colonisation may be difficult for *Clostridium* spp. in some cheeses because of the low pH, salt and temperature conditions (Christiansson, 1995), and so certain types of cheeses, such as Gruyere, are more susceptible than others (Gilmour and Rowe, 1990). *Clostridium butyricum, C.tyrobutyricum* and *C.sporogenes* cause the "late blowing" (extensive gas production approximately 2-3 months after manufacture) of cheeses such as Gruyere, Grana and other cheeses with high pH values (Gilmour and Rowe, 1990).

Germination of spores is dependent on prevailing environmental conditions (Veld *et al.*, 1993). Germination of spores in milk has been shown to be delayed compared to that in brain heart infusion broth (Notermans and Tatini, 1993; Dufrenne *et al.*, 1995).

Germination can occur even at temperatures below the minimum growth temperature for an isolate (Dufrenne *et al.,* 1994 and 1995). The lowest temperatures for the germination of spores followed by vegetative growth of psychrotrophic strains of *Bacillus* was between 1 to 7°C (Coghill and Juffs, 1979).

Bacillus cereus spores are hydrophobic, have a low surface charge, and are covered with long appendages which make them very adhesive to different surfaces (Husmark and Ronner, 1992; Husmark, 1993). This makes *B.cereus* spores very difficult remove from the surfaces of milking equipment. Also, vegetative cells of *B.cereus* more readily form spores in films of diluted milk, such as would be seen in a poorly washed milk can or tank, than they can in undiluted milk (Donovan, 1959).

The heat activation temperature for *B.cereus* spores is strain dependent (Overast and Atmaram, 1974). Hydrostatic pressure will also activate of *Bacillus* spp. spores (Nishi *et al.*, 1994). It has been proposed that it might be possible to control *Bacillus* spp. in milk and dairy products by taking advantage of such activation properties. In order for spores to resist the heat treatments they must be in a non-germinated dormant state; therefore if milks were treated to activate the spores prior to pasteurisation, the germinated spores would be eliminated by the subsequent pasteurisation treatments (Davies, 1975). Such treatments probably would not be feasible owing to the increased production costs and the requirement for legislative approval for additions, of amino acids for example, to be made to milk (Davies, 1975). In some countries a method of high speed centrifugation called "bactofugation" is used. This method is cheap, simple

and removes 99.9% of bacterial flora from milk. Nevertheless, it has been suggested that the most appropriate measure for controlling *B.cereus* in milk is to keep the temperature as low as possible throughout the chain from milking through to consumption, also entry into the chain should be minimised (Griffiths, 1992).

Nisin inhibits the outgrowth of *Bacillus* spores in cream (Phillips *et al.*, 1983), but for this bacteriocin, which is produced by *Lactococcus lactis* (DeVurst and Vandamme, 1994), to be an effective means of increasing product shelf life there must be absolute certainty that post pasteurisation contamination by Gram negative microorganisms has not occurred, because nisin has also been shown to have a stimulatory effect on their growth (Griffiths *et al.*, 1986).

1:8 Toxins of Bacillus species

Members of the genus *Bacillus* exhibit various degrees of pathogenicity. The most toxic member of the genus is *B.anthracis*, which is the causative organism of anthrax. Anthrax is primarily an infectious disease of wild and domestic herbivores, in which cutaneous, pulmonary and intestinal infections conclude with a fatal septicaemia (Logan, 1988). The infection may be spread to man through contact with infected animals or animal products (Logan, 1988). *Bacillus anthracis* is very closely related to *B.cereus*, and is a member of the *B.cereus* group. Virulent *B.anthracis* strains carry a toxin encoding plasmid (pXO1), and a capsule associated plasmid (pXO2) (Robertson *et al.*, 1990). It has been proposed that *B.anthracis* is a variant of *B.cereus*, rather than

a separate species (Gordon *et al.*, 1973). *Bacillus cereus* is far less pathogenic than *B.anthracis*, but *B.cereus* does produce many different toxins, including haemolysins, phospholipase C and those which cause foodborne disease. *Bacillus cereus* causes two types of foodborne illness. These are the diarrhoeagenic and emetic syndromes.

1:8:1 Foodborne disease

According to the food poisoning statistics of Communicable Disease Review (Djuretic *et al.*, 1996) *B.cereus* accounts for 1% of food poisoning outbreaks of either bacterial or viral origin in the U.K.. Internationally, *B.cereus* has accounted for between 0.8-22% of outbreaks of bacterially caused food poisoning. The worst affected country was the Netherlands, where 22.4% of bacterial food poisoning was caused by *B.cereus* between 1977-1982 (Table 1:5) (Kramer and Gilbert, 1989).

Although these figures appear to be low, the actual infection and intoxication rates caused by *B.cereus* are probably much higher. With the short duration of both the diarrhoeal and the emetic syndromes, many cases will pass unreported or unrecognised. Figures have been calculated to assess the economic implications of food poisoning in the U.S. and Canada. Working with the estimated case figures, and using the following parameters for assessment: costs of medical treatment, welfare payments, loss of productivity, loss of export income, cost of preventative measures and outbreak costs; it has been estimated that the annual cost of *B.cereus* food poisoning in the US was $36 million (Todd, 1989b). Similar figures were calculated

for Canada, where the annual cost was estimated to have been $10 million (Todd, 1989a).

Outbreaks of *B.cereus* food poisoning have been associated with a wide variety of foods (Table 1:6), although the majority of emetic syndrome outbreaks are connected with the consumption of rice. The mishandling of food products is the main factor leading to *B.cereus* food poisoning. In most outbreaks several mishandling errors have occurred (Table 1:7).

1:8:2 *Bacillus cereus* diarrhoeal enterotoxin

1:8:2:1

Enterotoxins are defined in the dictionary as intestinal toxins which cause food poisoning (Kirkpatrick, 1983). They are more specifically protein exotoxins with a site of action on the mucosal cells of the intestinal tract (Granum *et al.*, 1995).

Bacillus cereus diarrhoeal enterotoxin is produced during the logarithmic stage of growth (Glatz *et al.*, 1974; Spira and Goepfert, 1975). It causes a type of food poisoning which is almost identical to that of *Clostridium perfringens* (Table 1:8); both toxins are membrane damaging, although they have different modes of action (Granum *et al.*, 1995). *B.cereus* enterotoxin is about one hundred times more toxic to human epithelial cells than the toxin of *C.perfringens* (Granum *et al.*, 1995).

It is considered that the diarrhoeagenic syndrome may be caused by ingestion of *B.cereus* cells or spores, rather than preformed toxin (Granum *et al.*, 1993a). *Bacillus cereus* can grow (Williams and Withers, 1983) and produce enterotoxin under anaerobic conditions (Granum *et al.*, 1993a). The bacterium itself does not harm the host, but the diarrhoeagenic syndrome is referred to as an "infection" rather than an "intoxication" (Granum *et al.*, 1995).

Nutrient availability appears to be important in the production of diarrhoeagenic toxin. High levels of sugars did not support production (Sutherland, 1993; Sutherland and Limond, 1993); whereas starch enhanced production (Sutherland and Limond, 1993). Water activity has a significant effect on toxin production and also growth of *B.cereus* (Baker and Griffiths, 1993). Low pH prevented toxin production (Sutherland, 1993); and a pH outside the 5-10 pH range results in the rapid loss of activity (Spira and Goepfert, 1975; Granum *et al.*, 1993b). Diarrhoeal enterotoxin is unstable over a wide range of conditions, with ionic strength being especially critical (Spira and Goepfert, 1975). However, stability was greater upon heating in milk than in cell free supernatants (Baker and Griffiths, 1995). Glatz and Goepfert (1976) were able to produce high levels of diarrhoeagenic toxin in semi-defined media in a laboratory size fermenter.

In diarrhoeal strains of *B.cereus* there is considerable variety in the amount of toxin produced (Dufreinne *et al.*, 1995). It has been shown that 60-70% of strains from milk products can produce diarrhoeal toxin, but that there are only a small number which

are able to produce large enough amounts to cause a danger from food poisoning (Ronner and Andersson, 1995). Also it is considered that diarrhoeagenic toxin production is unlikely to occur in dairy products if they are maintained in the cold chain (Sutherland, 1993). Nevertheless, the presence of the organism still constitutes a potential hazard to the consumer.

1:8:2:2 Structure of the enterotoxin

There is considerable debate concerning the structure and molecular mass of *B.cereus* diarrhoeagenic toxin (Table 1.9). Some research suggests it is a single protein (Turnbull *et al.*, 1979; Shinagawa *et al.*, 1991b and 1991c), while others that it is a tripartite complex (Thompson *et al.*, 1984; Beecher and MacMillan, 1991; Granum and Nissen, 1993).

The three components of a proposed enterotoxin complex have been purified and partially sequenced (Granum and Nissen, 1993). The molecular masses of this complex were determined as 34, 40 and 48 kDa (Granum and Nissen, 1993), 38, 39 and 43 kDa (Thompson *et al.*, 1984), 35, 36 and 45 kDa (Beecher and MacMillan, 1991) respectively. Further work of Granum *et al.* (1993) determined the molecular masses to be 40, 50 and 58 kDa, with a larger protein of about 100kDa occurring occasionally (Granum *et al.*, 1993b). The 100kDa protein was found to be non-toxic, and therefore not considered to be of great importance.

The enterotoxin complex purified by Beecher and Macmillan (1991) appears to be the

same tripartite complex described by Thompson *et al.* (1984). Beecher and Wong consider the enterotoxin complex to be haemolysin BL. Haemolysin BL is made up of the components B, L_1 and L_2; with the following molecular masses: B was found to be 37.8kDa, L_1 was 38.5kDa and L_2 was 43.2kDa (Beecher and Wong, 1994a). It is haemolytic, cytotoxic, dermonecrotic and causes vascular permeability changes (Beecher and Wong, 1994a) and has been shown to cause fluid accumulation in the ligated rabbit ileal loop (Beecher *et al.*, 1995). The individual components of haemolysin BL do not possess these activities separately; all three are required for maximal activity (Beecher and Wong, 1994a; Beecher *et al.*, 1995). However, Thompson *et al.* (1984) found the intermediate weight protein of the enterotoxin complex was toxic, as did Granum and Nissen (1993). The molecular weights of the intermediate protein were determined as 40kDa and 50kDa by the respective researchers. When the components of haemolysin BL were tested for *B.cereus* enterotoxin on the OXOID BCET-RPLA test kit, the L2 component interacted with antibody component of the kit (Beecher and Wong, 1994c).

Of the three component enterotoxin complex described by Granum and Nissen (1993), the 34kDa was found to be haemolytic. The sequence of first 14 amino acids of this 34kDa component was identical to residues 28-41 of sphingomylinase. This is the section of sphingomylinase which becomes the N-terminal end after the loss of signal sequence; it is therefore considered that sphingomylinase is a part of the tripartite enterotoxin complex (Granum and Nissen, 1993).

Turnbull *et al.* (1979) and Shinagawa *et al.* (1991c and 1991d) found the toxin to be a single protein rather than a complex, with a molecular weight of 50kDa and 45kDa respectively, and an isoelectric point of 4.9 and 5.5 respectively. These proteins were both shown to exhibit vascular permeability and fluid accumulation factors (Shinagawa *et al.*, 1991e). The protein was purified using chromatography, chromatofocusing and gel filtration techniques (Shinagawa *et al.*, 1991c and 1991d). The single protein described has a similar molecular weight as the intermediate proteins described by Thompson *et al.* (1984) and Granum and Nissen (1993).

Shinagawa's research group raised three monoclonal antibodies (D-8, B-10 and H-1) against the enterotoxin. Each monoclonal reacted with a different competitive binding site on a single 45 kDa protein (Shinagawa *et al.*, 1991b). The B-10 monoclonal was then used to purify *B.cereus* enterotoxin by immunoaffinity chromatography (Shinagawa *et al.*, 1992b). The resulting single protein possessed vascular permeability activity and caused fluid accumulation in ligated mouse ileal loops, but did not show haemolytic activity (Shinagawa *et al.*, 1992b). The enterotoxin complex was haemolytic (Granum and Nissen, 1993).

The bceT gene of *B.cereus* encodes an enterotoxin protein with the characteristics of the diarrhoeal toxin, known as enterotoxin T (Agata *et al.*, 1995a). It is capable of encoding a polypeptide of 336 amino acids with a molecular mass of 41kDa (Agata *et al.*, 1995a).

In a large outbreak of *B.cereus* diarrhoeal food poisoning in Norway in 1995 a strain of *B.cereus* (0075-95) was isolated (Granum *et al.*, 1995) whose toxic effects are caused by a different protein complex than the toxic complex previously purified (Beecher *et al.*, 1995). This complex comprised of 3 proteins (39kDa, 45kDa and 105kDa), which were not toxic individually, but were cytotoxic in combination (Lund and Granum, 1996). The 45kDa and 105kDa proteins reacted with the commercially available TECRA BDE visual immunoassay (VIA), but the 45kDa protein was considerably more reactive than 105kDa (Lund and Granum, 1996). This *B.cereus* strain was negative when tested with the OXOID RPLA kit (Lund and Granum, 1996).

It is possible that *Bacillus cereus* may produce more than one type of diarrhoeal enterotoxin. Evidence has been presented for two distinct enterotoxin complexes (Beecher and Wong, 1994a; Lund and Granum, 1996), and also of a single protein coded for by the *bceT* gene (Agata *et al.*, 1995a). There is also evidence to suggest that more than one enterotoxin may be produced by a single strain of *B.cereus* (Ombui *et al.*, 1997).

1:8:3 Emetic toxin

The emetic syndrome was first characterised in the UK following several incidents associated with the consumption of rice from Chinese restaurants and take away outlets (Public Health Laboratory Service, 1972; Mortimer and McCann, 1974). The

emetic syndrome is an intoxication as opposed to an infection (Szabo *et al.*, 1991); it has a rapid onset of <1-5h after the consumption of the incriminated food stuff. The symptoms of the illness are vomiting and nausea, with accompanying diarrhoea in about 30% of cases (Kramer and Gilbert, 1989) (Table 1:9). The syndrome is not associated with fever. The emetic toxin of *B.cereus* causes similar symptoms to *Staphylococcus aureus* toxin (Johnson, 1984; Bennett *et al.*, 1993) (Table 1:8). Duration of the illness is generally short, recovery occurring within 24 hours without the requirement for medical intervention. However, a recently published incident resulted in the death of a 17 year old boy from fulminant liver failure caused by *B.cereus* (Mahler *et al.*, 1997). The levels of *B.cereus* which have been associated with emetic food poisoning outbreaks vary considerably, but have been as low as $<10^4$ (Table 1:10).

In Japan 93-95% of *B.cereus* food poisoning outbreaks were of the emetic type syndrome (Shinagawa, 1990). There is a tendency for cases to occur between June to September (Kramer and Gilbert, 1989; Shinagawa, 1990). In approximately 95% of cases of *B.cereus* emetic syndrome is associated with the consumption of rice from Chinese restaurants (Kramer and Gilbert, 1989; Drobniewski, 1993). The emetic toxin of *B.cereus* is thought to be of very low molecular weight; it has a molecular weight of <15K (Hughes *et al.*, 1988; Szabo *et al.*, 1991). It is very stable to heat, extremes of pH (pH2; pH11) and proteolysis with trypsin (Shinagawa *et al.*, 1992a; Mikami *et al.*, 1994). It has been proposed that the toxin is a breakdown product of a component in the growth medium of the organism (Granum *et al.*, 1995), possibly from a lipid.

Emetic toxin appeared after spore formation of *B.cereus* (Shinagawa *et al.*, 1992a).

The emetic toxin of *B.cereus* has been shown to be a cyclic dodecadepsipeptide ionophore named cereulide (Isobe *et al.*, 1995; Agata *et al.*, 1995b). Cereulide has been extracted and purified from the culture supernatant of a strain of *B.cereus* which caused emetic food poisoning syndrome (Agata *et al.*, 1994); it induced vomiting in Rhesus monkeys (Shinagawa *et al.*, 1995).

Cereulide causes vacuole formation in HEp2 cells (Agata *et al.*, 1994; Isobe *et al.* 1995). It is thought to cause emesis through the 5-HT3 receptor and stimulation of the vegas afferent (Agata *et al.*, 1995b). The emetic toxin causes swelling of the mitochondria (Sakuri *et al.*, 1994), and uncoupling of mitochondrial oxidative phosphorylation of mitochondria (Sakuri *et al.*, 1994).

Cereulide is very closely related to the potassium ionophore valinomycin (Agata *et al.*, 1994). Cereulide is a rubidium- and potassium-ion selective ionophore, forming a 1:1 complex with the alkali metal ions (Suwan *et al.*, 1995). Using modelling techniques it has been proposed that cereulide has a main chain conformation showing a hexagonal cylinder-like framework (Suwan *et al.*, 1995).

Bacillus cereus isolated from emetic type outbreaks were unable to hydrolyse starch (Shinagawa, 1993). Serotyping has revealed that emetic strains of *B.cereus* are either H-1, H-5 or H-8 (Shinagawa, 1993); and others have found all emetic toxin producing

strains tested to be H-1 serotype (Mikami *et al.*, 1994; Mikami *et al.*, 1995). Emetic isolates of serotype H-1 produced emetic toxin in particularly high concentrations (Shinagawa *et al.*, 1992a; Shinagawa, 1993). Diarrhoeal strains did not belong to any particular serotypes (Shinagawa, 1993).

Culture media appear to be important in emetic toxin production. Milk was found to be a superior media for emetic toxin production (Szabo *et al.*, 1991; Shinagawa, 1993). Rice was also found to be a good medium (Melling *et al.*, 1976; Szabo *et al.*, 1991; Shinagawa, 1993). When emetic strains were grown on brain heart infusion broth (BHI) or on tryptose soya broth media no emetic toxin production was detected (Shinagawa *et al.*, 1992a). Factors controlling the formation of the emetic toxin have not been determined.

1:8:4 Other toxins of *B.cereus*

All of the toxins of *B.cereus*, except for the emetic, are produced during the exponential phase of the life cycle (Drobniewski, 1993). Other toxins of *B.cereus* include phospholipase C and haemolysins. One of the phospholipases, sphingomylinase, is also a haemolysin (Tomita *et al.*, 1991). The enzyme phospholipase C catalyses the hydrolysis of the phosphodiester bonds in phosphatidylcholine and phosphatidylinositol, and play an important role in the cascade of events involved in signal transduction (Hergenrother *et al.*, 1995). Phospholipases, along with proteinases and lipases, are degradative enzymes produced

by *Bacillus* in milk. These enzymatic activities result in the "off-flavours" and defects associated with bad milk (Meer *et al.*, 1991). Phospholipase activity is measured on egg yolk agar plates (Mossel *et al.*, 1967; Rojkowski and Mikolajcik, 1987).

Nutrient availability has an effect on the ability of *B.cereus* to produce haemolysin and phospholipase C, and also upon the stability of these compounds (Ivers and Potter, 1977). Haemolytic and phospholipase activities are easily inactivated by heat (56°C for 5min), digestion by trypsin and extremes of pH (below pH3, or above pH11) (Shinagawa *et al.*, 1991a). Moderately acid or alkaline conditions repressed haemolysin and enterotoxin production (Garcia-Arribas and Kramer, 1990). *Bacillus cereus* can produce haemolysin and phospholipase in similar amounts under anaerobic conditions and aerobic conditions (Granum *et al.*, 1993a).

Diarrhoeal toxin is easily distinguished from phospholipase C, and from heat stable cereolysin, but is less readily differentiated from sphingomylinase (Turnbull *et al.*, 1979). Phospholipase C causes vascular permeability changes in mice. However, unlike the enterotoxin induced reactions, those of phospholipase C are only transient (Glatz *et al.*, 1974).

The haemolysins of *B.cereus* consist of sphingomylinase, cereolysin, cereolysin AB, haemolysin II (Sinev *et al.*, 1993), haemolysin III (Baida and Kuzumin, 1995), haemolysin BL, and a "cereolysin-like" haemolysin (Honda *et al.*, 1991). Several of the extracellular haemolysins, including haemolysin BL (Beecher and Wong, 1994a),

are considered to be virulence factors (Baida and Kuzmin, 1995).

Cereolysin is a thiol-activated haemolysin (Drobniewski, 1993). Its binding site on the eukaryotic cell membrane is cholesterol. Once bound, it results in a pitting and micropuncturing of the cell membrane, which is visible using electron microscopy. Intracellular potassium ions are lost, but the net flow of ions and water is into the cell, which swells and ruptures (Turnbull *et al.*, 1990). Cereolysin AB has been shown, using PCR, to encode for lecithin-hydrolysis and haemolytic activity in *B.cereus* (Schraft and Griffiths, 1995).

1:8:5 Testing for *B.cereus* toxins

Detection of *B.cereus* toxins has traditionally been through the use of *in vivo* studies. For the diarrhoeal toxin these have included the rabbit or guinea-pig ileal loop test (Spira and Goepfert, 1972), vascular permeability testing (Glatz *et al.*, 1974), dermonecrotic tests on guinea pigs (Glatz and Goepfert, 1973), mouse lethality testing and Rhesus monkey feeding trials. Only the Rhesus monkey feeding trials were suitable for determining the presence of the emetic toxin. *In vivo* studies are time consuming and expensive, and so a viable *in vitro* alternative has been required. Also, public pressure has forced a move away from animal testing.

Cell culture techniques have been used for the detection of both the diarrhoeal and the emetic toxins. Cell culture techniques using HeLa cells for the detection of the

diarrhoeagenic toxin had good correlation with the TECRA assay (Baker and Griffiths, 1995). Other cell lines have also been used in the detection of diarrhoeal toxin, including Vero and Chinese hamster ovary cells (Wong *et al.*, 1988; Shinagawa *et al.*, 1991a; Hostiacka *et al.*, 1992).

Hughes *et al.* (1988) were the first to develop a cell culture assay for the detection of the emetic toxin, after they observed that 87% of *B.cereus* strains isolated from emetic syndrome outbreaks caused vacuoles to appear in HEp-2 cells. The emetic toxin affects the proliferation of cells, and this was used to develop another assay for the detection of emetic toxin (Mikami *et al.*, 1994).

Studies of seven different cell lines indicated that the response of the cells to the emetic toxin varied. Of those tested, HEp-2, INT 407 and Chinese hamster ovary (CHO) cells were found to be equally the most sensitive to the emetic toxin (Szabo *et al.*, 1991). HEp-2 cells responded to emetic toxin by the formation of vacuoles; INT 407 cells also produced many prominent vacuoles in the presence of the toxin (Szabo *et al.*, 1991).

Cereulide causes vacuolation in HEp-2 cells (Agata *et al.*, 1994; Agata *et al.*, 1995b; Isobe *et al.*, 1995); the vacuolation factor is thought to be the emetic toxin itself (Shinagawa, 1993). Electron microscopy has revealed that the apparent vacuoles are actually swollen mitochondria (Sakuri *et al.*, 1994).

Chinese hamster ovary cells, although being equally sensitive to emetic toxin, responded in a different way. Vacuolation did not occur, but the cells became spherical with granulation of the cell contents. In all cell lines, cell multiplication was arrested (Szabo *et al.*, 1991).

Development of the cell culture assay has been made to detect the formation of acid by HEp-2 cells induced by *B.cereus* emetic type culture supernatants (Mikami *et al.*, 1995).

There are now two commercial *in vitro* kits, which have been developed to test for *B.cereus* diarrhoeal enterotoxin. These are the BCET-RPLA kit (OXOID, Unipath, Basingstoke), and the TECRA *Bacillus* Diarrhoeal Enterotoxin (BDE) Visual Immunoassay (VIA) (TECRA diagnostics, Batley).

The two test kits are antibody based. OXOID BCET-RPLA is a reverse passive latex agglutination assay. Latex agglutination methods use latex particles to amplify the antibody:antigen agglutination reaction. With a reverse latex agglutination assay, the latex particles are coated with antibody, raised against a component of the diarrhoeal toxin, for the detection of the antigen (Varnam and Evans, 1991) (Figure 1:2). The TECRA kit is a sandwich ELISA assay (Figure 1:3). The antibody is absorbed onto the solid phase, and the sample (antigen) is then added to complex with the antibody. Unbound antigen is removed by washing, and then an enzyme labeled conjugate is added, which binds to the antigen. After the removal of excess conjugate by washing,

an enzyme substrate is added; a coloured reaction product may then be detected (Varnam and Evans, 1991).

The two commercial test kits detect different antigens (Christiansson, 1993; Day *et al.*, 1994; Beecher and Wong, 1994c; Andersson *et al.*, 1995). The OXOID kit detects a component of the enterotoxin complex (Baker and Griffiths, 1995); observations by Beecher and Wong (1994c) suggest that it is the L2 constituent of haemolysin BL. The TECRA antibody was raised against the so called antigenic fraction, antigen 577 (Baker and Griffiths, 1995), which has been shown to cause diarrhoea in monkey feeding trials (Bennet *et al.*, 1993). Using Western blotting the TECRA kit was shown to react with six different proteins, with molecular weights ranging from 40-114 kDa. The strongest reactions occurred at 40 and 41 kDa, which are apparently non-toxic proteins (Beecher and Wong, 1994c). However, enterotoxin T (Agata *et al.*, 1995a) had a molecular weight of 41kDa. The 45 kDa and 105 kDa proteins of the enterotoxin complex described by Lund and Granum (1996) reacted with the TECRA assay.

There has been some variability in the results obtained from the OXOID BCET-RPLA and TECRA. Agreement between the tests has been found in around 80% of isolates (Buchanan and Schultz, 1994; Rusul and Yaacob, 1995), although others have been unable to determine correlation between the results from the two kits (Christiansson, 1993; Notermans and Tatini, 1993). The OXOID BCET-RPLA test has been shown to give a high number of false negative results (Buchanan and Schultz, 1992), including strains which have been isolated from diarrhoeal food

poisoning outbreaks, and were positive against the TECRA assay (Notermans and Tatini, 1993; Day *et al.*, 1994). When the commercial kits were compared with cell cytotoxicity reactions, TECRA was shown to be less sensitive than the cell assay, but gave similar results (Christiansson, 1993; Buchanan and Schultz, 1994). The cell response was generally found to be in agreement with the OXOID kit, but clear differences were noted for particular strains (Buchanan and Schultz, 1992).

The enterotoxin does not have to be active for detection by the OXOID BCET-RPLA method. After heating to destroy toxigenicity, the molecule may be detected by the BCET-RPLA kit, but the TECRA kit and cell cytotoxicity assays loose their reactivity (Buchanan and Schultz, 1992; Buchanan and Schultz, 1994).

The OXOID BCET-RPLA kit has been used to test species of *Bacillus* other than *B.cereus* for enterotoxin production. As well as other members of the *B.cereus* group, strains of *B.circulans, B.lentus, B.pumilus, B.polymyxa* and *B.carotarum* reacted positively to the OXOID kit (Griffiths, 1990). *Bacillus thuringiensis* strains were positive against the TECRA kit (Hydlebrink-Damgaard, 1995).

In addition to the commercial test kits and cell cytotoxicity assays, a microslide immunodiffusion assay has been developed (Bennett, 1992). Strains which were negative for OXOID yet positive for TECRA produced different identity patterns on the immunodiffusion assay (Day *et al.*, 1994).

1:8:6 Other *Bacillus* species associated with foodborne disease

Other *Bacillus* spp. have also been associated with outbreaks of food borne disease. These include *B.subtilis*, *B.licheniformis* and *B.pumilis* (Table 1:11) (Kramer and Gilbert, 1989).

1:9 Objectives

As has been discussed *Bacillus* spp. are of importance in the dairy industry because they cause spoilage problems in milk and dairy products. The first objective of this project was to examine the incidence *Bacillus* spp. in raw milks and the dairy environment of one farm. This was done in order to ascertain the route of entry of *Bacillus* spp. into raw milk. Seasonality in the occurrence of psychrotrophic and mesophilic species was considered. The seasonality of psychrotrophic *Bacillus* spp. occurring in raw milk has been attributed to interactions which exist between psychrotrophic and mesophilic *Bacillus,* and these interactions were therefore investigated.

Bacillus cereus and other *Bacillus* spp. have been identified as causative agents of food poisoning. Examination of the toxigenicity of isolates of *B.cereus* and other *Bacillus* spp. were made to determine the extent of toxigenicity isolates. Many of the isolates tested had originated in raw milk samples or from the farm environment.

The classification of *Bacillus* spp. using traditional methods can be difficult, and may produce inconclusive results. Another objective of this project was therefore to examine alternative methods for the classification of *Bacillus* spp..

TABLES AND FIGURES

FOR GENERAL INTRODUCTION

Table 1:1 Functional uses of various *Bacillus* spp.

Bacillus species	Product produced	Nature of product	Reference
B.subtilis	Natto	Japanese fermention of soy-beans. Carried out for thousands of years	Hara and Veda, 1982
B.subtilis	Lipopeptide surfactin	Surfactant	Cooper *et al.*, 1981
B.coagulans	Glucose isomerase production	Enzyme which converts glucose derived from starch into high-fructose syrups	Bucke, 1983
i. *B.thuringiensis*	Insecticide	**i.** Produces - endtoxin, used worldwide as insecticide	Deacon, 1983
ii. *B.popilliae*	Insecticide	**ii.** Used in the US for control of Japanese beetle	Deacon, 1983
i. *B.licheniformis* **ii.** *B.polymyxa* **iii.** *B.brevis* **iv.** *B.circulans*	Antibiotic producers	**i.** Bacitracin - a cyclic-ogliopeptide. Inhibits cell wall synthesis **ii.** Gramicidins & polymyxin. Interfere with membrane function **iii.** Edeines -Basic peptides.Inhibits the formation of initiation complex on ribosomal subunits **iv.** Aminoglycoside antibiotics. Affect ribosomal function. Usually used for actinomycetes	Katz and Demain, 1977

Table 1.2 Morphology of *Bacillus* species

Characteristics	1. *B. subtilis*	2. *B. acidocaldarius*	3. *B. alcalophilus*	4. *B. alvei*	5. *B. anthracis*	6. *B. azotoformans*	7. *B. badius*	8. *B. brevis*	9. *B. cereus*	10. *B. circulans*	11. *B. coagulans*	12. *B. fastidiosus*	13. *B. firmus*	14. *B. globisporus*	15. *B. insolitus*	16. *B. larvae*	17. *B. laterosporus*
Width of rod (μm)	0.7–0.8	0.9–1.1	0.7–0.9	0.5–0.8	1.0–1.2	0.9–1.0	0.8–1.2	0.6–0.9	1.0–1.2	0.5–0.7	0.6–1.0	1.5–2.5	0.6–0.9	0.6–1.0	1.1–1.5	0.5–0.6	0.5–0.6
Length of rod (μm)	2–3	2–3	3–4	2–5	3–5	3–10	1.5–4	1.5–4	3–5	2–5	2.5–5	3–6	1.2–4	1.5–5	1.1–2.5	1.5–6	1.5–6
Sporangium swollen	–	+	–	+	–	+	–	+	–	+	d	–	–	+	–	+	+
Spore shape	E	E	E	E	E	E	E	E	E	E	E	E	E	S	S	E	E
Spore position	C	T	T	C/T	C	T	C/T	C/T	C	C/T	C/T	C/T	C	T	C/T	C/T	CL

Characteristics	18. *B. lentimorbus*	19. *B. lentus*	20. *B. licheniformis*	21. *B. macerans*	22. *B. masquariensis*	23. *B. marinus*	24. *B. megaterium*	25. *B. mycoides*	26. *B. pantothenticus*	27. *B. pasteurii*	28. *B. polymyxa*	29. *B. popilliae*	30. *B. pumilus*	31. *B. schlegelii*	32. *B. sphaericus*	33. *B. stearothermophilus*	34. *B. thuringiensis*
Width of rod (μm)	0.5–0.7	0.6–0.9	0.6–0.8	0.5–0.7	0.5–0.7	0.9–1.2	1.2–1.5	1.0–1.2	0.5–0.7	0.5–1.2	0.6–0.8	0.5–0.8	0.6–0.7	0.6–0.8	0.6–1	0.6–1	1.0–1.2
Length of rod (μm)	1.3–7	1.2–4	1.5–3	2.5–5	2–6	2–4	2–5	3–5	2–5	1.3–4	2–5	1.3–5.2	2–3	2.5–5.8	1.5–5	2–3.5	3–5
Sporangium swollen	+	–	–	+	+	+	–	–	+	+	+	+	–	+	+	d	–
Spore shape	E	E	E	E	E	S	E	E	E & S	S	E	E	E	S	S	E	E
Spore position	C/T	C	C	T	T	T	C	C	T	T	T	C	C	T	T	T	C

* Symbols: –, 90% or more of strains are negative; +, 90% or more of strains are positive; d, 11–89% of strains are positive; E, ellipsoidal; S, spherical; C, central; T, terminal; and CL, central and lateral.

(Sneath, 1986)

Table 1:3 Differential characteristics of Group I *Bacillus* species

	B. amyloliquefaciens	*B. anthracis*	*B. badius*	*B. cereus*	*B. fastidiosus*	*B. firmus*	*B. lentus*	*B. licheniformis*	*B. megaterium*	*B. mycoides*	*B. pumilus*	*B. subtilis*	*B. thuringiensis*
Cell diameter >1.0 µm	−	+	−	+	+	−	−	−	+	+	−	−	+
Parasporal crystals	−	−	−	−	−	−	−	−	−	−	−	−	d
Anaerobic growth	−	+	−	+	−	−	−	+	−	+	−	−	+
Voges Proskauer test	+	+	−	+	NG	−	−	+	−	+	−	+	d
Egg yolk lecithinase	−	+	−	+	−	−	−	−	−	+	−	−	+
Growth in lysozyme	−	+	−	+	ND	−	−	d	−	+	d	d	+
Acid from													
D-glucose	+	+	−	+	NG	+	+	+	+	+	+	+	+
L-arabinose	d	−	−	−	NG	−	+	+	d	−	+	+	−
D-xylose	d	−	−	−	NG	−	+	+	d	−	+	+	−
D-mannitol	+	−	−	−	NG	+	+	+	d	−	+	+	−
Hydrolysis of		−											
Starch	+	+	−	+	−	+	+	+	+	+	−	+	+
Casein	+	+	+	+	−	+	d	+	+	+	+	+	+
Nitrate reduction	+	+	−	+	−	d	d	+	d	+	−	+	+
Degradation of tyrosine	−	d	+	+	−	d	−	−	d	ND	−	−	ND
Allantoin or urate required	−	−	−	−	+	−	−	−	−	−	−	−	−
Growth in 7% NaCl	+	+	ND	d	−	+	d	+	d	d	+	+	+
Growth at													
10°C	ND	−	−	d	+	d	ND	−	+	d	+	d	d
50°C	d	−	+	−	−	−	−	+	−	−	d	d	−
55°C	ND	−	−	−	−	−	−	+	−	−	−	−	−
Utilization of													
Citrate	d	d	−	+	−	−	−	+	+	d	+	+	+
Proprionate	ND	ND	−	ND	−	−	−	+	ND	ND	−	−	ND

(Priest, 1989)

Table 1:4 Spoilage problems caused by *Bacillus* spp. in dairy products

Species	Spoilage Condition	Growth Temperature
B.cereus	1."Bitty" cream 2. Sweet curdling	5-35 °C
B.subtilis	1. Ropy milk 2. Levan production	20-45 °C
B.licheniformis	Levan production	20-45 °C
B.stearothermophilus	Spoilage of canned dairy products eg. evaporated milk and milk puddings	46-65 °C
B.coagulans	Spoilage of U.H.T., condensed or canned milk products	20-55 °C

Table 1:5 Outbreaks of foodborne illness caused by *B.cereus*

Reporting country (Period covered)	% Total bacterial outbreaks	Mean number of cases per outbreak
Canada (1973-1982)	7.3	2.2
England and Wales (1976-1984)	2.3	0.7
Finland (1975-1984)	11.9	17.8
Hungary (1960-1968)	8.0	15.0
Japan (1976-1982)	0.8	0.7
Netherlands (1977-1982)	22.4	11.5
Scotland (1973-1985)	0.9	0.8
USA (1972-1982)	2.9	1.3

Kramer and Gilbert, 1989

Table 1:6 Origin of *B.cereus* food poisoning outbreaks in United States of America (1973-1987)

Outbreak Source	Number of outbreaks
Chinese food	24
Mexican food	5
Beef	3
Fruit and vegetables	3
Shellfish	2
Chicken	1
Finfish	1
Icecream	1
Turkey	1
Other foods/ Unknown causes	17

Schultz and Smith, 1994

Table 1:7 Causes of *B.cereus* foodborne illness outbreaks in United States of America

Cause	% Outbreaks
Improper holding temperatures	94%
Contaminated equipment	53%
Inadequate cooking	32%
Poor personal hygiene	24%
Unsafe source of food	5%

Schultz and Smith, 1994

Table 1:8 *Bacillus cereus* (diarrhoeal and emetic), *Clostridium perfringens* and *Staphylococcus aureus* food poisoning syndromes compared

	C.perfringens	*B.cereus*		*S.aureus*
		Diarrhoeal	Emetic	
Onset of symptoms (h)	8-22	8-16	1-5	2-6
Duration of illness (h)	12-24	12-24	6-24	6-24
Diarrhoea, abdominal cramps	Predominant	Predominant	Fairly common	Common
Nausea	Rare	Occasional	Predominant	Predominant
Pathogenesis	Toxin-mediated (Sporulation associated toxin release in small intestine)	Toxin-mediated (May be preformed in food, or produced in small intestine)	Toxin-mediated (Preformed in food)	Toxin-mediated (Preformed in food)
Principal food vehicles	Cooked meat and poultry	Meat products, soups, vegetables, puddings, sauces	Cooked rice and pasta	Cold cooked meat and poultry, dairy products

Kramer and Gilbert, 1989

Table 1:9 Toxins of *B.cereus*

Toxin	Molecular mass	Characteristics	Reference
Enterotoxin			
a) Haemolysin BL	37.8 kDa 38.9 kDa 43.2 kDa	Tripartite protein complex Haemolytic Cytotoxic Dermonecrotic Causes vascular permeability changes	Beecher *et al.*, 1995
b) Enterotoxin T	41 kDa	Single protein coded by bceT gene Cytotoxic Causes vascular permeability changes Mouse lethality Fluid accumulation in ligated mouse ileum	Agata *et al.*, 1995
c) Second tripartite protein complex	39 kDa 45 kDa 105 kDa	Non-haemolytic Tripartite protein complex Causes fluid accumulation in rabbit ileal loop Cell cytotoxic Sphingomylinase is part of this complex (Granum and Nissen, 1996)	Lund and Granum, 1996
Cereolysin	~56 kDa	Haemolysin Thiol activated Heat labile Low suscepability to proteolysis Mouse lethality	Cowell *et al.*, 1976
Haemolysin II	~30 kDa	Haemolysin Heat labile Susceptible to proteolysis	Coobaugh and Williams, 1978

Table 1:9 Toxins of *Bacillus cereus* continued

Toxin	Molecular mass	Characteristics	Reference
Sphingomylinase	34 kDa	Haemolysin and phospholipase C Heat stable Metallo-enzyme (Mg^{2+}) Part of the enterotoxin complex described by Lund and Granum (1996)	Johansen *et al.*, 1988 Granum and Nissen, 1992 Granum and Nissen, 1996
Phosphatidyl-inositol hydrolase (PIH)	34 kDa	Phospholipase C Non-metallo enzyme	Kuppe *et al.*, 1989
Phosphatidyl-choline hydrolase (PCH)	27 kDa	Phospholipase C Stable metallo enzyme (Zn^{2+}, Ca^{2+})	Little *et al.*, 1975
Emetic toxin	5-7 kDa	Heat stable to121°C Causes emesis <1-5h after consumption Cyclic dodecadepsipeptide ionophore (cereulide)	Public Health Laboratory Service, 1972 Mortimer and McCann, 1974 Isobe *et al.*, 1995

Table 1:10 Levels of *B.cereus* detected in foods incriminated in emetic syndrome food poisoning outbreaks in the UK (1971-1985)

Colony count of *B.cereus* /g	% of outbreaks
$<10^4$	4
$1x10^4$ - $9.9x10^4$	7
$1x10^5$ - $9.9x10^5$	14
$1x10^6$ - $9.9x10^6$	20
$1x10^7$ - $9.9x10^7$	26
$1x10^8$ - $9.9x10^8$	19
$1x10^9$ - $9.9x10^9$	8
$>10^{10}$	2

(The majority of the foods were fried or boiled rice)

Kramer and Gilbert, 1989

Table 1:11 Outbreaks of foodborne illness derived from *Bacillus* spp. other than *B.cereus*

Species	Incidents UK (1975-1986)	Onset	Symptoms	Duration	Food
B.subtilis	49	10min-14h	Vomiting (80%) Diarrhoea (49%) Abdominal cramps (27%) Nausea (20%)	1.5-8h	Meat pies/pasties/ rolls (25%) Indian meat curries with rice (17%) Chinese meat dishes with rice (12%) Bread/crum -pets (10%)
B.licheniformis	24	8h	Diarrhoea (90%) Vomiting (54%) Abdominal cramps (46%) Nausea (NR)	6-24h	Meat/ poultry pie/ pasties (21%) Meat/ veg. Stews (17%) Curried meat /poultry with rice (13%) Chicken with sauce dishes (8%)
B.pumilis	5	3-11h	Diarrhoea Vomiting	NR	Meat pies/ pasties/ Scotch eggs Cheese Tomato juice

NR = Not reported
Kramer and Gilbert, 1989

Figure 1:1 Morphological groups of the genus *Bacillus*

a) Group I

Sporangia are not swollen or, are only very slightly swollen, by endospores.

Spores are ellipsodal or cylindrical, and are central or terminal.

eg. *B.cereus, B.mycoides, B.subtilis*

b) Group II

Sporangia are definitely swollen by the endospores. Spores are oval, or rarely cylindrical. They are central, subterminal or terminal.

eg. *B.circulans, B.brevis, B.stearothermophilus*

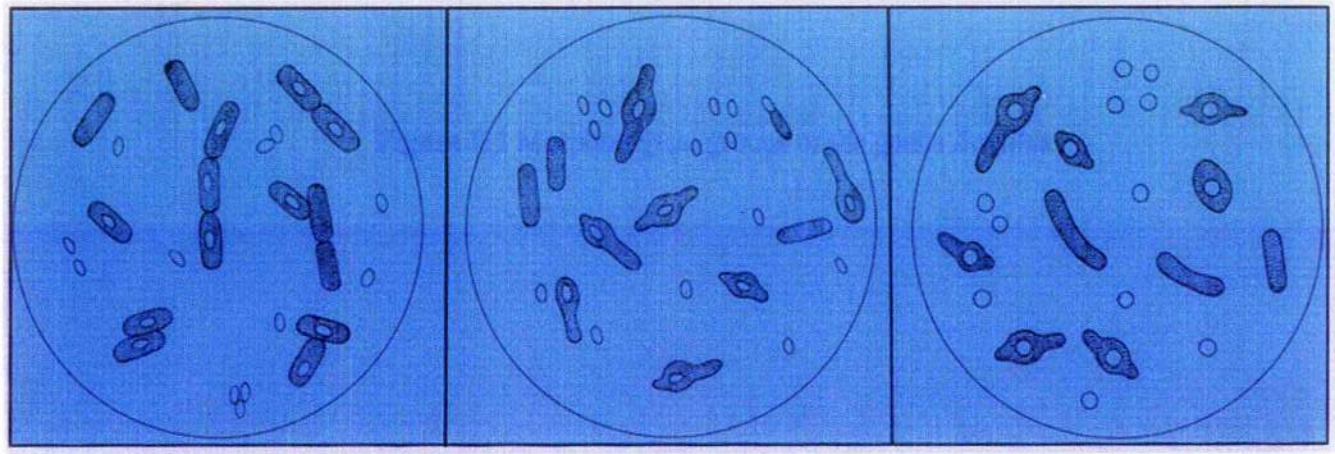
c) Group III

Endospores spherical, subterminal or terminal. Sporangia are swollen.

eg. *B.sphaericus, B.pasteurii*

(Varnam and Evans, 1991)

Figure 1:1 Morphological groups of the genus *Bacillus*



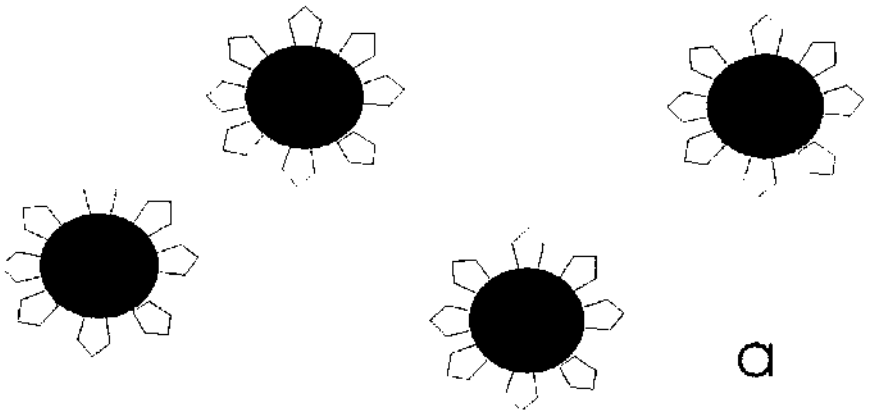
a) Group I b) Group II c) Group III

Figure 1:2 Detection of antibodies by latex co-agglutination, as used in the OXOID BCET RPLA assay to detect *Bacillus cereus* diarrhoeal enterotoxin. Latex coagglutination uses latex particles to amplify antibody: antigen agglutination reactions.

a) Latex particles coated with antigens

b) Antibody

c) Addition of antibody to antigen coated latex particles, causes agglutination of particles which can be visually detected.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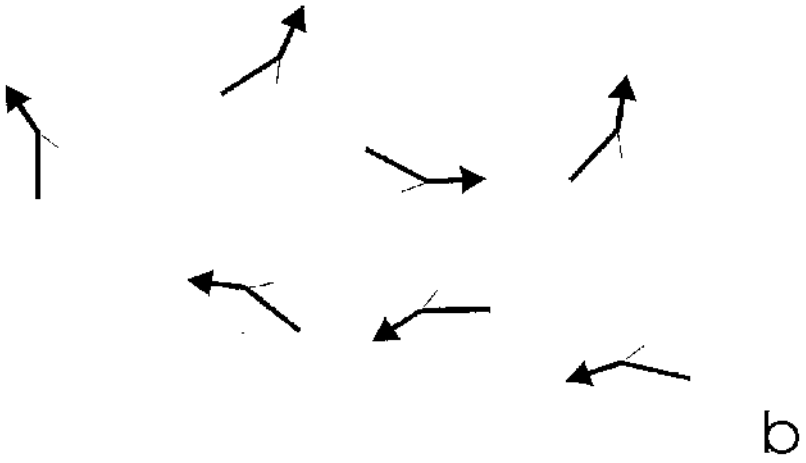

b

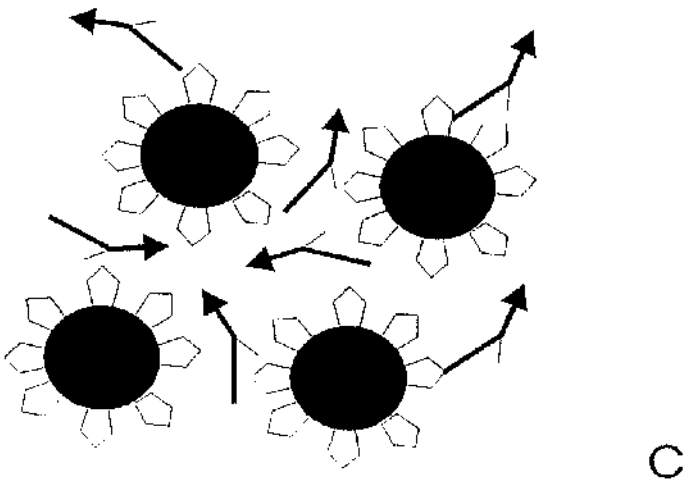

c

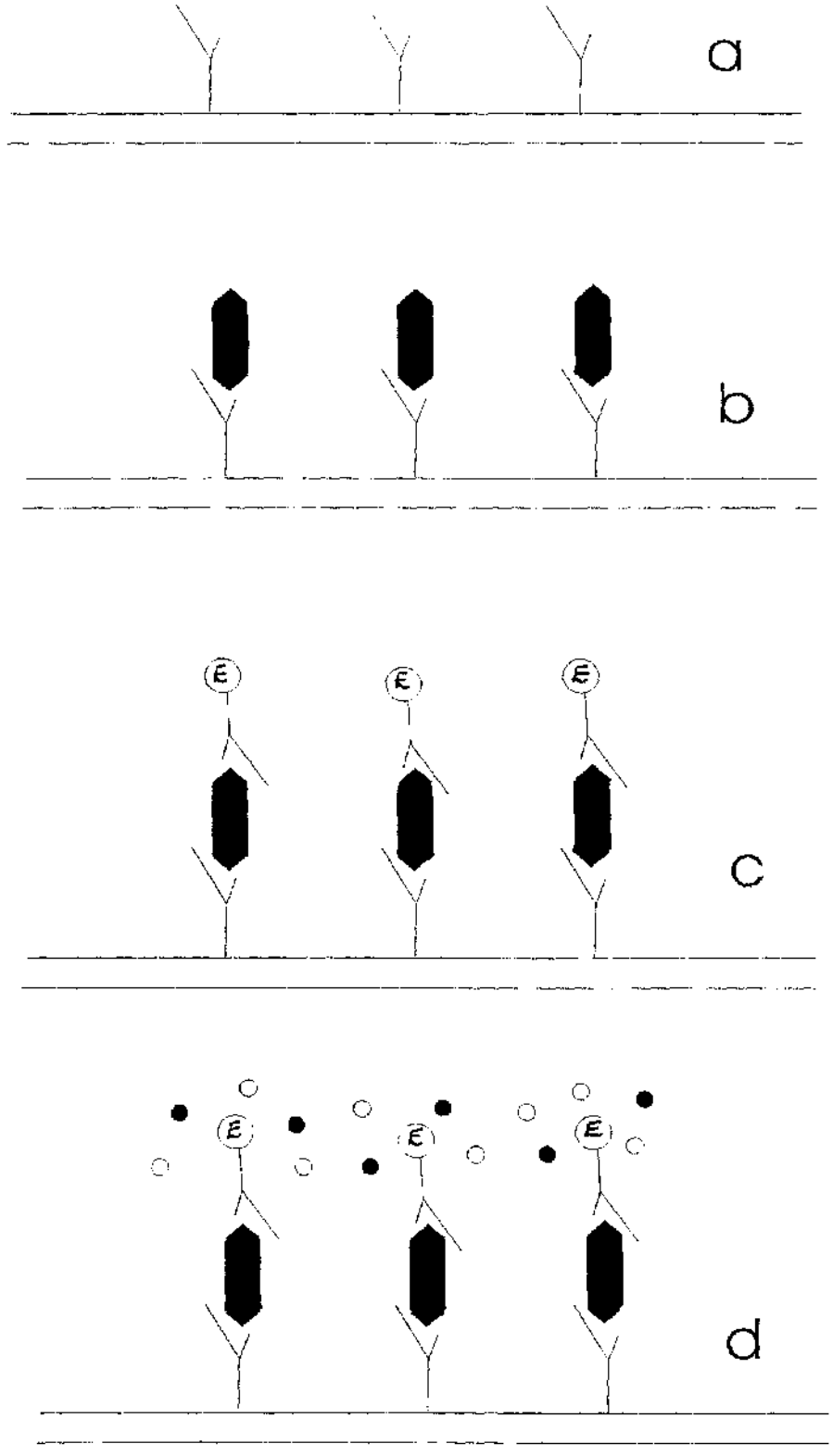
Figure 1:3 Enzyme linked immunosorbent assay: sandwich configuration, as used in TECRA BDE VIA.

a) Antibody to *Bacillus cereus* diarrhoeal enterotoxin is absorbed onto the surface of the TECRA test well.

b) A test solution of *B.cereus* culture supernatant is added to the wells. If the enterotoxin target antigen is present it complexes with the bound antibody. The wells are washed, to remove unbound antigen.

c) An enzyme labelled antibody (conjugate) is added, which binds to the antigen.

d) An enxyme substrate is added, which reacts over a minimum time period of 30min, resulting in a colour change from colourless to green. The amount of colour change can be measured on a plate reader ($Ab_{405nm +/- 10nm}$).


a
b
c
d

CHAPTER 2.

FARM SURVEY TO DETERMINE THE INCIDENCE OF *BACILLUS* SPP. DETECTED ON THE FARM AND IN THE DAIRY ENVIRONMENT

2:1 INTRODUCTION TO INCIDENCE OF *BACILLUS* SPP. ON THE FARM AND IN THE DAIRY ENVIRONMENT

Members of the genus *Bacillus*, and especially *B.cereus* are recognised by the dairy industry because they cause product spoilage. *Bacillus* spp. produce heat resistant spores which may withstand pasteurisation temperatures; many strains also have the capacity to grow at refrigeration temperatures. These attributes occurring in psychrotrophic strains of *Bacillus* make them a problem in dairy products. In addition to product spoilage some species of *Bacillus* also pose a potential public health threat due to their ability to produce food poisoning toxins.

Bacillus species occur in milk and dairy products with seasonality (Ridgway, 1953; Franklin *et al.*, 1956; Billing and Cuthbert, 1958; Stewart, 1970; McKinnon and Pettipher, 1983; Crielly *et al.*, 1994; Sutherland and Murdoch, 1994). Early work reported much higher aerobic spore counts in the winter months than the summer. These studies examined the entire aerobic sporeforming population. When mesophilic and psychrotrophic populations of sporeformers were distinguished from one another it became evident that they followed different seasonality patterns. The mesophilic populations were highest during the winter, and the psychrotrophic populations in the summer to autumn months.

When the milking process was surveyed from the raw milk through to the pasteurised product, a consistent seasonality was observed throughout the samples (Sutherland and Murdoch, 1994).

The objective of this piece of work was to perform a detailed survey of the incidence of *Bacillus* populations associated with cattle, raw milk and the immediate environment of a commercial farm over a period of one year. The aim was to assess where *Bacillus* entered the milk chain, and to observe the seasonality patterns expressed in samples from the cow and farm environment. An understanding of the reasons for seasonal incidence of *Bacillus* species may allow the implementation of measures to decrease the extent of the contamination of raw milk by organisms of this genus.

2:2 METHODS USED TO DETERMINE THE INCIDENCE OF BACILLUS SPP. DETECTED ON THE FARM AND IN THE DAIRY ENVIRONMENT

2:2:1 Collection of samples and processing in the laboratory for enumeration of bacterial populations

The commercial dairy farm at the Hannah Research Institute (HRI) was used in this survey. Samples were taken from 8 cattle, once a month for a thirteen month period. Samples of the individual milks, udder washes and faecal samples were taken from each cow. The same animals were sampled each month, until the animals became dry. In this event the animal was replaced by another to maintain a pool of 8 cows, when other cows were available.

2:2:1:1 Milk

For each animal milk was collected at the p.m. milking, from a front teat and the diagonally opposite hind teat on the udder. Milk (300ml) was collected aseptically in sterile 500ml bottles, attached "in-line", beneath the milking machine. The sample was gathered from milk taken across the whole of the milking process to ensure that a representative sample was collected.

In addition to the individual samples taken from the 8 cows, a bulk tank milk sample (300ml) was also collected using a sterile dipper. The sample was taken after the entire herd had been milked.

At the laboratory, the milk samples were split into four samples, so that suitable treatments could be performed before plating out.

All of the viable count populations were plated out for enumeration using a spiral plater (Don Whitley Scientific Ltd., Shipley) onto milk agar plates (Oxoid, Unipath, Basingstoke). Duplicate plates were made with undiluted sample and serial dilutions to x100. The plates were then incubated according to the population count being assessed. The incubation times and temperatures are given in Table 2:1. Zero counts from spirally plated samples represent counts below the minimum detectable numbers of <20/ml.

Spore samples were heat treated (80°C, 10min) (Moran *et al.,* 1990) to destroy viable cells and activate spores. Mesophilic and thermophilic spore samples (10ml) were heat treated (80°C, 10min) and cooled rapidly on ice. To ensure precise heat treatment a control milk sample (10ml) was placed into the water bath in an identical container at the same time as the milk samples; the 10 minute treatment period commenced when the temperature of the control sample was 80°C. Undiluted sample and serial dilutions were then spiral plated in duplicate onto milk agar. For the psychrotrophic spore count (PSC) 100ml milk was heated (80°C, 10 min), and cooled rapidly on ice. A control sample of 100ml milk was heated in the same water bath, and heat treatment commenced when this control sample had reached 80°C. The samples were then centrifuged for 15 min at 5300*g* at 4°C. The pellet formed by centrifugation was resuspended in approximately 5ml of sterile maximum recovery diluent (MRD), and

the entire suspension spread plated onto milk agar plates, in 500 µl volumes per plate. Minimum detectable number of PSC in milk and udder wash samples was 1/100ml.

2:2:1:2 Udder washes

Samples (200ml) of udder washes were collected by submerging a single teat, in a sterile 300ml sample jar which contained 200ml of phosphate buffered saline solution (pH 7.3) (Oxoid, Unipath, Basingstoke). The jar was large enough to fit over the teat, and submerge the teat entirely without spilling the sample fluid. Once over the teat, the jar was held so that its rim just touched the udder surface. The jar was rotated six times in a clockwise direction, and a further six times in an anti-clockwise direction. After removal of the sample jar from the teat, the jar was held beneath the teat for several seconds to catch any sample residue draining from the teat surface. The process of sample collection was repeated on a second, diagonally opposite, teat. The same procedure was carried out on all eight cows. Udder wash samples were taken from the two teats which were not sampled for milk.

In the laboratory the samples were divided into 4 and subjected to appropriate heat treatments as described for the milk samples (Table 2:1).

2:2:1:3 Faeces

Individual faeces samples were collected internally from the cows. The samples were withdrawn manually using sterile gloves, and then placed into sterile collecting jar (250ml).

In the laboratory a suspension of 1g faeces in 9ml sterile maximum recovery diluent (MRD) (pH 7.0+/-0.2) (Oxoid, Unipath, Basingstoke) was made. These were heat treated (80°C, 10 min). Samples (100µl) were then spread plated on milk agar, and incubated at 30°C for 48h (MSC), or 14d at 6°C (PSC).

2:2:1:4 Drinking water

A sample (200ml) of the cows' drinking water was collected aseptically from the drinking trough, into a sterile container (250ml). For the psychrotrophic spore count 100ml was heated (80°C, 10mins), and then centrifuged for 15 min at 5300*g*, at 4°C. The pellet was resuspended in sterile MRD and spread plated onto milk agar plates, and incubated at 6°C for 14d.

2:2:1:5 Pasture grass

Samples of grass were taken from a fixed site and a variable site. The situation of the fixed site remained constant over the entire 13 month period. The variable site corresponded to the field in which the cattle were at pasture on the day of the survey. Variable site samples were only taken during the months when the cattle were at pasture.

Grass (approximately 30g) was cut aseptically from each site using sterile scissors; sterile gloves were worn. In the laboratory, 10g of grass was homogenised in a stomacher (Don Whitley Scientific Ltd., Shipley) for 10 min with 90ml MRD, to detach the bacteria and spores. The suspensions were heat treated as described for the various populations in Table 2:1. After cooling, undiluted samples and serial dilutions were plated in duplicate onto milk agar using a spiral plater, and incubated under the conditions specified for each population (Table 2:1).

2:2:1:6 Pasture soil

Samples (20g) of soil were taken from the same fixed and variable sites as used for the grass samples. Soil samples were taken from the top 10 cm of soil using a 2cm diameter sterile core borer.

In the laboratory 10g of soil were suspended in 90ml sterile MRD, and homogenised using a stomacher for 10 minutes. The samples were heat treated (Table 2:1), serially diluted to x100, plated onto milk agar using a spiral plater, and incubated for the times and at the temperatures specified for each population group (Table 2:1).

2:2:1:7 Winter bedding

The cattle at the HRI farm are housed indoors from November to April each year. During this period of the survey, samples were taken of the winter bedding and feeds provided to the cattle.

The cattle were bedded on sawdust. The sample of bedding material was taken from where the cattle had been lying. In the laboratory 10g of bedding was homogenised in a stomacher for 10 min with 90ml sterile MRD. Samples were heat treated (Table 2:1), and cooled on ice. Serial dilutions were plated in duplicate onto milk agar using a spiral plater, and incubated under the conditions specified for each population (Table 2:1).

2:2:1:8 Winter feeds

During the winter, the cows are fed on silage and dry food concentrates. Samples (10g) of the winter feeds were homogenised in a stomacher with 90ml MRD. Heat treatments were made in accordance with those listed in Table 2:1, and cooled on ice. Serial dilutions to x100 were plated in duplicate on milk agar using a spiral plater, and the plates were incubated under the conditions specified for each population (Table 2:1).

2:2:2 Identification of Bacillus isolates

Identification to the species level was based on morphological and biochemical characteristics.

2:2:2:1 Morphology

A description of morphology was made for each of the presumptive *Bacillus* colonies.

2:2:2:2 Gram Stain

The Gram staining method used was a modification of the method described by Preston and Morell (1962).

Bacterial cells from a single colony, of a pure culture were suspended in water, and a drop of the suspension heat fixed onto a microscope slide. The cells were stained with ammonium oxalate(2%)-crystal violet (0.8%) solution for 20s. This solution was then washed off, and replaced with an iodine (1% w/v)-potassium iodide (2% w/v) for 1 min. The iodine solution was washed off using iodine-acetone (3.5% liquour iodi fortis in acetone) to decolourise Gram negative cells. Liquor iodi fortis contained

iodine (10% w/v), and potassium iodide (6% w/v). The acetone was left on the slide for only a few seconds, and then washed with water. Finally the slide was counter stained with carbol fuchsin (0.5% basic fuchsin; 2.5% w/v phenol) for 30s. Carbol fuchsin will stain Gram negative cells pink-red, which makes them easier to differentiate from Gram positive cells.

2:2:2:3 Spore stain

The acid-fast stain for spores was used. A thin film of bacteria taken from a colony on a 4 day old agar plate, was fixed onto a microscope slide.

The film was stained with Ziehl-Neesen's carbol fuchsin (5% w/v phenol, 1% w/v basic fuchsin) for 3-5 min, and then washed with water. The bacteria were then decolourised with 5% (w/v) sodium sulphite solution for 1 min. The film was washed again, and counter-stained with 1% (w/v) malachite green. Spores stain red by the carbol fuchsin in this method, and cells counterstain green from the malachite green.

2:2:2:4 Motility

Motility testing was carried out using the hanging drop technique. A drop of liquid bacterial culture (18h) was placed in the centre of a glass cover slip. A small pellet of Vaseline was placed at each corner of the slip, then a microscope slide was adhered to the Vaseline, and the whole slip inverted carefully. The drop of bacterial culture was suspended beneath the cover slip. Motility was observed using bright field microscopy at magnification of x1000.

2:2:2:5 Carbohydrate utilisation

Presumptive *Bacillus* spp. were assigned to the species level on the basis of carbohydrate utilisation studies, which were preformed using API 50 CHB test kits (bioMérieux, Basingstoke). The ability of the test strain to utilise 49 substrates was tested.

The kits were set up in accordance with the instructions of the manufacturer. The concentration of bacterial cells in the inoculum was adjusted until the optical density of the bacterial suspension was approximately that of a McFarlane's number 2 standard.

The prepared strips were incubated at 30°C. The bacterial suspension was prepared in a liquid which contains a pH indicator. If the bacteria being tested were able to ferment the carbohydrate in the test well, acid would be produced, causing the pH to fall. The drop in pH was accompanied by a colour change in the well. The colour change was scored subjectively between 0-5 in accordance with the instructions of the manufacturer. Following scoring, the plates were reincubated for a further 24h, after which time they were reassessed and scored as described for 24h. Identification was determined from the carbohydrate utilisation profile which was compared with a data base for the genus provided by the manufacturer. A typical profile for *B.cereus* is shown in Figure 2:1, and a typical *B.subtilis* profile is illustrated in Figure 2:2.

2:2:2:6 Oxidase test

Strips of filter paper were soaked with 1% aqueous tetramethyl-*p*-phenylenediamine dihydrochloride. A single colony from a pure culture plate (18-24h) was removed using a clean, sterile platinum wire, and was rubbed onto the filter paper. A positive result was recorded if a blue colour developed within 10s.

2:2:2:7 Voges Proskauer test (VP)

The Voges Proskauer test determines the ability of organisms to produce the neutral end product acetyl methyl carbinol (acetoin) from glucose fermentation. Acetoin is oxidised by the reagent to diacetyl, which produces a red colour with guanidine residues in the media.

Glucose phosphate broth (5% w/v glucose; 5% w/v dipotassium phosphate buffer in 0.7% peptone broth) was inoculated with a single colony from an 18-24h pure culture plate, and incubated for 48h at 30°C.

To 2ml of the culture 0.5ml of Reagent 1 (5% w/v α-naphthol in absolute ethanol), followed by 0.5ml of Reagent 2 (0.3% creatine in 40% w/v KOH) were added and mixed. The reagents were supplied by Roche (Welwyn Garden City).

A positive reaction was indicated by a pink/red colouration occurring within 5 min.

2:2:2:8 Selective agar (PEMBA) for the presumptive identification of *B.cereus*

Polymyxin-pyruvate-egg-yolk-mannitol-bromothymol blue agar (PEMBA) (Holbrook and Anderson, 1980) was made according to the manufacturer's instructions (Oxoid, Unipath, Basingstoke) with the addition of polymyxin B (100 IU/ml agar) and egg yolk emulsion.

The inclusion of peptone (0.1% w/v) and the addition of sodium pyruvate improve egg yolk precipitation on the agar, and enhance sporulation. The medium is made selective

by the addition of the antibiotic polymyxin B to a final concentration of 100IU per ml medium. Polymyxin B is a branched cyclic decapeptide antibiotic produced by *B.polymyxa*. It has virtually no effect on Gram positive bacteria, but inhibits the growth of most Gram negative bacteria (Priest, 1989).

A single colony of the isolate was streaked onto a PEMBA plate. The plate was incubated at 21°C for 18-48h.

The selectivity of PEMBA for *B.cereus* is based upon the inability of *B.cereus* to ferment mannitol. PEMBA contains a pH indicator, bromothymol blue, and is bright green when made. The pH of PEMBA is lowered by mannitol fermenting organisms; the drop in pH, however does not cause a colour change of bromothymol blue. *Bacillus cereus* is unable to ferment mannitol and utilises more complex substrates in the medium. The pH of the medium therefore rises, and the bromothymol blue indicator changes colour from green to peacock blue.

PEMBA is also used to detect lecithinase activity. *Bacillus cereus* are lecithinase positive; activity was indicated by a zone of egg yolk precipitation surrounding the *B.cereus* colony. The typical appearance of *B.cereus* grown on PEMBA is indicated in Figure 2:3.

2:2:2:9 Haemolysin production

Haemolytic activity was determined using blood agar plates. A peptone based agar, blood agar base, (Oxoid, Unipath, Basingstoke) was prepared in accordance with the instructions of the manufacturer, and supplemented with 7% (v/v) sterile sheep blood, added to the sterilised base at 45-50°C.

A singly colony of the test isolate was streaked onto a blood agar plate. A haemolysin positive organism would cause the lysis of the red blood cells; a positive reaction was therefore indicated by the development of a clearing zone surrounding the culture, due to haemolysis.

2:2:3 Air sampling

Air sampling was carried out on three separate occasions, to monitor the extent of the aerial bacterial contamination in the milking parlour, and to assess the influence which the cattle themselves had upon the aerial contamination level. Samples were collected when the cattle were being milked and the bacterial load compared in air samples taken after the milking parlour had been vacated for 2h.

Air samples were collected using Castella Mark II Airborne Bacteria Sampler (Castella London Ltd., Bedford). The slit sampler was connected to a vacuum pump, the flow rate of air drawn through the sampler was 30L/min.

Samples were collected for total population counts of mesophiles and psychrotrophs, and also for spore counts. The counts for total counts were made directly onto milk agar plates, with the air sampler positioned at one end of the milking parlour. Sampling was carried out for 2min and 5min on triplicate plates, for total mesophilic and psychrotrophic counts respectively. The plates were incubated for psychrotrophic (6°C/14d) and mesophilic (30°C/48h) growth, after which time colony counts were made.

Samples collected for spore counts were made on gelatin plates, made using 15ml gelatin (10% w/v). Triplicate samples were taken for mesophilic and psychrotrophic sporeformers, using a 5min sampling time for each. In the laboratory the gelatin plates were melted over steam, and poured into glass universal containers. The samples were heated to 80°C for 10min with a separate sample containing a thermometer to ensure accurate timing of temperature treatments, and then mixed with 15ml double strength milk agar, which had previously been sterilised and maintained in a liquid state. The resulting mixture of gelatin and agar was pour plated into several petri dishes, and incubated for psychrotrophic (6°C/14d) and mesophilic (30°C/48h) growth. After incubation colony counts were made.

2:2:4 Examination of bacterial contamination of milking machines and line

Assessment of the milking machine and the milking lines as a potential source of bacterial contamination was made after the normal sterilisation procedure carried out by the dairyman was complete.

To examine the residual bacterial contamination of the teat cup clusters sterile phosphate buffered saline (PBS, 5L) was drawn through one set of milk machine teat-cup clusters, using the suction of the milk machine vacuum pump. The teat-cup clusters are the part of the milking machine which is attached to the teats of the cow when milking takes place. A 200ml sample of the PBS was collected in a sterile in-

line sample bottle, beneath the machine. The bottles used, and the collection procedure was the same as for the raw milk samples (Section 2:2:1:1).

To sample the milking machine line, a further 5L sterile PBS was drawn through the system. The fluid was drawn into the system via the teat-cup cluster that had been previously flushed with sterile PBS (see previously) so as to minimise the introduction of bacteria from the cluster into the line sample. A 200ml sample of the PBS was collected after it had been drawn through the complete length of the milk line, but before it entered the bulk tank.

The bulk tank was sprayed with 5L of sterile PBS, using a sterilised manual pressurised spray pump. A 200ml PBS sample was then collected through the drain port. The contents of the bulk tank are transferred to the milk tanker through this port during milk collections.

In the laboratory the samples from the different collection sites were treated in the same way. The sample heat treatments and incubations were carried out in accordance with those used in the main farm survey (Table 2:1). Results were quoted as colony forming units per ml (cfu/ml) of sample taken.

2:2:5 Effect of husbandry practices on microbial contamination of udder and milk

An investigation of the influences of different husbandry practices on the contamination of udder wash samples and raw milk was carried out.

Cows were kept under three different husbandry regimes. Four animals were kept inside, in an open plan byre, a further 4 cows were kept outside in an area with short pasture, and 4 cows were kept alongside the regular herd, on regular pasture. This study was conducted during the summer.

After two weeks under the various husbandry conditions, udder wash and raw milk samples were taken from the animals. All samples were taken in the manner described for the comparable samples in the main farm survey (Sections 2:2:1:1 and 2:2:1:2). The survey was repeated weekly, using the same cattle during a 4 week period.

In the laboratory the samples were processed using the procedures described for the main survey (Table 2:1).

2:2:6 Effect of pre-milking teat preparation on the number of bacteria remaining on the teat surface

A study of the effect of no treatment, washing, or washing followed by drying the teats of cattle was made to assess the effect of the washing protocol on the cleanliness of the udder and teats prior to milking.

Two diagonally opposite teats of 4 cattle were washed, while two diagonally opposite teats of 4 other cows were washed and dried.

Washing took place using a commercial towel (Genus Animal Health, Worcester). These contain a disinfectant substance. The towels were soaked in sterile water, and used to wipe the teat surface. The teats were each wiped from the top to the tip 5

times. An individual towel was used for each cow. The udder wash sample was then taken, using the method used in the main farm survey (Section 2:2:1:2). An udder wash sample was taken from the two remaining teats without any prior washing procedure.

Cattle which were washed and then dried, underwent the same wash procedure as the animals which were only washed. After the washing, the two teats being cleaned were dried using an individual paper towel, until the teat surface was moisture free. Udder wash samples were taken using the method used in the main survey (Section 2:2:1:2). The 2 uncleaned teats were also sampled.

Milk samples were taken from the two washed teats and two unwashed (control) teats of all eight animals used in the experiment. The survey was repeated on 4 occasions.

The udder wash and milk samples were treated in the laboratory, in the same way as those in the main farm survey (Table 2:1); the mesophilic and psychrotrophic total viable counts (TMC, TPC) and spores (MSC, PSC) were enumerated on milk agar as described.

2:2:7 Statistical Analysis

Statistical analysis of survey data consisted of the students t-test, the F-ratio test, and correlation analysis. Analysis was carried out using Excel (Microsoft) statistical functions. Regression analysis was performed (results not shown) (McNulty, personal communication) to confirm seasonality trends within sample populations.

2:3 RESULTS OF INCIDENCE OF *BACILLUS* SPP. DETECTED ON THE FARM AND IN THE DAIRY ENVIRONMENT

2:3:1 Farm survey of incidence of Bacillus species

A twelve month survey of the occurrence of different groups of *Bacillus* in raw milk; incidence in association with animals, in dairy and farm environment were studied to identify sources of *Bacillus* that may lead to milk contamination.

The data for the survey were the mean of results taken from eight cattle. During February, only six cattle were sampled owing to the high proportion of the animals which had "dried off", or stopped producing milk, because of the stage of their lactation cycle. Between animal variation was evident in the cattle sampled. This was especially evident in the psychrotrophic sporeforming populations in milk and udder wash samples, because the numbers detected were so low. There was also a "within" animal variation source. This is to say that particular animals were not consistently high or low month after month. The raw data for all the animals examined during the course of the survey are included in Appendix.

The information on the incidence of psychrotrophic, mesophilic, thermoduric bacterial and spore populations throughout the course of the calendar is presented in the following sections.

2:3:1:1 Raw Milk

The counts for the mesophilic and psychrotrophic populations in milk (Figures 2:4a-b) remained fairly constant over the period November-April, when the cattle were housed inside. The levels of psychrotrophs were slightly lower than those of the mesophiles while the cattle were housed indoors; the psychrotrophs ranged from $1.7x10^3$-$1.2x10^4$ cfu/ml, and the mesophiles ranged from $9.6x10^3$- $4.3x10^4$ cfu/ml. The counts for mesophiles and psychrotrophs, however, were more variable when the cattle were outside, ranging from $3.9x10^2$ to $1.5x10^5$ for mesophiles, and $2.1x10^2$ to $9.7x10^3$ for psychrotrophs. However, no correlation could be demonstrated between the weather conditions on the day of the survey, or during the week preceeding the survey, and variation in bacterial numbers. The total psychrotrophic populations in milk were lowest in June ($2.1x10^2$ cfu/ml) and September ($2.0x10^2$ cfu/ml); the psychrotrophic counts for these months were significantly lower than the winter counts ($P=<0.05$).

The thermoduric populations were able to withstand a heat treatment of 63°C for 30min, and were then incubated at 30°C, the standard temperature for mesophilic growth. The thermoduric population in raw milk exhibited a distinct seasonality (Figure 2:4c). The thermoduric population reached a maximum ($4x10^4$ cfu/ml) in December; the population then steadily decreased monthly, until in July the thermoduric population was absent from the raw milk samples. However, from August to the end of the survey in October the thermoduric population was again present, although only at numbers of 10^2-10^3 cfu/ml.

The sporeforming populations in raw milks were low (max. 10^3 cfu/ml) and exhibited seasonality patterns which were confirmed using regression analysis. The mesophilic sporeformers (MSC) (Figure 2:5a) were predominately present during the winter months when the cattle were housed inside. They reached a maximum of $9.3x10^2$

cfu/ml in January. The December MSC was unusually low (2.5 cfu/ml), differing significantly from the preceding (November) and following (January) counts (P=<0.05). There were no unusual events which occurred around the December sampling period, such as changes in animal husbandry practices or sample collection techniques. The difference in the mesophilic spore count in udder wash samples in December, compared to the counts in November and January-April (Figure 2:9a), was also found to be significant (P=<0.05).

Psychrotrophic sporeformers (Figure 2:5b) were present in the milk predominantly in the summer-autumn period. They were only detected in five of the monthly samples, whereas the mesophilic sporeformers were always detected. They occurred in very low numbers (<1 cfu/ml). Psychrotrophic sporeformers were also detected in the February raw milk samples. This incidence may have arisen from environmental disturbances due to major building work, or as a result of the presence of the organisms in the feed.

Thermophilic sporeformers (Figure 2:5c) demonstrated seasonality. They predominated during the winter months when the animals were indoors (max. $3.8x10^5$ January; min 0 in June, July and September). The numbers present in milk were significantly different (P=<0.05), when the cows were at pasture or housed in the byre.

2:3:1:2 Bulk Tank Milk

The mesophilic count in the bulk tank milk (Figure 2:6a) was relatively constant during the winter months ranging between $1.7x10^3$ to $9.3x10^3$ cfu/ml, except for November which was $2.1x10^5$ cfu/ml. The counts were more variable when the cattle were outside from May to October '94 ($8.1x10^1$ cfu/ml June to $1.0x10^5$ cfu/ml August). Overall the mesophilic population tended to be higher during the winter, but not significantly so (P>0.05).

The count of mesophilic sporeformers detected in the bulk tank (Figure 2:7a) were much more variable than the counts from the individual milk samples. Seven months recorded zero counts. These months occurred without season throughout the year.

The psychrotrophic counts (range $8.1x10^1$ cfu/ml June, to $6.1x10^4$ cfu/ml September) (Figure 2:6b) were similar to those determined in the individual milk samples (Figure 2:4b). The count was not significantly different when the cattle were either inside or outside.

Psychrotrophic sporeformers (Figure 2:7b) were only detected twice in the bulk tank milk throughout the whole survey. They were detected both times in the summer-autumn period. This was consistent with the individual raw milk results. The counts were very low as they had been for the individual milks (<1 cfu/ml).

Thermodurics predominated (Figure 2:6c) in the winter. They also occurred from August to October. Thermophilic sporeformers (Figure 2:7c) followed a similar pattern to the thermodurics. There was a significant difference (P=<0.05) between the winter and summer months for thermophilic sporeformers.

2:3:1:3 Udder Washings

The mesophilic population (TMC) (Figure 2:8a) in the udder washes ranged from $2.3x10^5$-$1.9x10^8$ cfu/ml. The psychrotrophic population (TPC) (Figure 2:8b) was lower during the winter than the summer months (P=<0.05).

The thermoduric population (TTC) (Figure 2:8c) was more variable than the TMC; it reached a maximum in February-March (10^5 cfu/ml), and again in October '94. The

minimum value occurred in June ($2.1x10^3$ cfu/ml). There was a significant difference ($P=<0.05$) between the maxima and minima detected in the thermoduric populations throughout the year.

Mesophilic sporeformers exhibited similar seasonality trends in milk and in udder washes (Figure 2:5a, 2:9a). The seasonality exhibited was more defined in the udder washes than in the milk samples. Regression analysis confirmed the seasonality. The udder wash peak for MSC came in February-March ($6.3x10^5$ and $7.8x10^5$), as it did in the raw milk samples. In the September udder wash sample no mesophilic sporeformers were detected.

Psychrotrophic sporeformers (PSC) (Figure 2:9b) occurred in the summer to autumn months, as they had done in the raw milks (Figure 2:5b). There was also an incidence of PSC in March. The numbers detected exceeded 1 cfu/ml in October '93 and August only (Figure 2:9b).

Thermophilic sporeformers (Figure 2:9c) were at their maximum levels in February-March and in October '94; the numbers present were reached 1.1-2.4 $x10^5$ cfu/ml. There were no thermophilic or other sporeformers detected in the September sample.

2:3:1:4 Faeces

The mesophilic spore count (Figure 2:10a) in faeces tended to be highest during the winter months, ranging from $2.7x10^5$- $2.5x10^6$ cfu/g. October'94 was at a high level, more comparable with the winter than the summer months. The mesophilic sporeformers in faeces correlated (92%) with the MSC in the winter feed samples.

The psychrotrophic spore count (Figure 2:10b) was highest in the summer-autumn months, just as it had been in the milk and udder wash samples. Spores of psychrotrophs were not detected in the faecal samples collected in December-March or in June. The psychrotrophic sporeformers isolated from faeces demonstrated seasonality. There was also a high occurrence in April ($2.7x10^4$ cfu/g).

2:3:1:5 Winter Bedding

The mesophilic population in winter bedding (Figure 2:11a) ranged from $1x10^6$-$1.3x10^9$ cfu/g. The range from November to March was $1.2x10^8$-$1.2x10^9$ cfu/g. April was the lowest ($1x10^6$ cfu/g); October '94 was high again at $1.3x10^9$ cfu/g.

The psychrotrophic population in winter bedding (Figure 2:11b) was more variable month to month, and spanned a greater range ($1.4x10^5$-$5x10^9$ cfu/g). The thermoduric population (Figure 2:11c) was also very variable, ranging from ($2.8x10^4$-$1.1x10^9$ cfu/g).

The spore-forming populations were variable. Mesophilic sporeformers in winter bedding (Figure 2:12a) ranged from $4.3x10^3$ in December to $1.1x10^7$ cfu/g in Oct 94. Thermophilic sporeformers (Figure 2:12c) ranged from $5.3x10^3$-$8.5x10^6$ cfu/g. Psychrotrophic sporeformers (Figure 2:12b) were only detected in February and October '94.

2:3:1:6 Winter Feed

Winter feed comprised of silage and dried food concentrate pellets; the mean results of the individual components are presented. Mesophilic sporeformers (Figure 2:14a) ranged between $8.2x10^4$-$2.3x10^6$ cfu/g. Only the count for December fell outside this

range ($4x10^2$ cfu/g), but the difference between this and the other counts was not significant (P>0.05). The number of mesophiles detected in the feed ranged from $x10^5$ to $x10^7$cfu/ml (Figure 2:13a).

Psychrotrophic counts (Figure 2:13b) were higher from November to January ($1.8x10^5$-$1.6x10^8$ cfu/g) than from February to March ($1.26x10^4$-$1.9x10^5$ cfu/g), but the difference was not found to be significant. Psychrotrophic sporeformers (Figure 2:14b) were absent from November to January, but were present in the period February to April (Range 0.6-$1x10^6$ cfu/10g).

The thermoduric count (Figure 2:13c) was highest in January to March ($1x10^7$-$3x10^7$ cfu/g). Except for a high peak in January, the thermophilic sporeformers (Figure 2:14c) were relatively constant.

2:3:1:7 Grass

The incidence of mesophiles (Figure 2:15a) on the fixed site grass samples over the whole survey was very similar ($3.7x10^7$- $6.7x10^8$ cfu/g), with the exception of the February sample ($1.1x10^5$ cfu/ml), which was significantly lower than for the other months (P=<0.05). The number of psychrotrophs (Figure 2:15b) was variable across the year ranging from $2.3x10^5$ to $5x10^8$ cfu/g; the size of the thermoduric population (Figure 2:15c) also varied considerably throughout the period (range $6.1x10^2$ to $6.2x10^8$ cfu/g). The thermoduric peaks occurred in the autumn, and early winter. No significant differences between the fixed and variable site grass were determined (P>0.05).

There was no significant difference between the fixed and variable site grass samples for any of the spore forming populations (P>0.05).

Mesophilic sporeformers (Figure 2:16a) were not consistantly at higher or lower levels in either the fixed and variable site samples. Psychrotrophic sporeformers (Figure 2:16b) were present on fixed and variable site grass in October'93, and also on the variable site grass in February and March. The thermophilic sporeformers (Figure 2:16c) had peaks in February (fixed), July (variable), and October '94 (fixed).

2:3:1:8 Soil

The numbers of mesophiles (Figure 2:17a) present in fixed soil site samples varied widely ($9.4x10^3$ to $9.3x10^8$ cfu/g). The samples from grazed sites were almost as variable. The differences between populations in fixed and variable site samples were not significantly different. Psychrotrophic (Figure 2:17b) and thermoduric (Figure 2:17c) populations in the fixed and the variable site samples also varied widely throughout the survey. Psychrotrophs in fixed site and grazed sites samples ranged from $4.1x10^3$ to $2.4x10^8$ cfu/g, and from $1.5x10^4$ to $3.5x10^7$ cfu/g respectively. Thermodurics ranged from $9.4x10^3$ to $9.3x10^8$ cfu/g at the fixed site, and from $2.0x10^3$ to $1.3x10^8$ cfu/g at the grazed site. Neither fixed nor variable site samples gave consistently higher counts in either population.

The mesophilic sporeforming populations (MSC) (Figure 2:18a) ranged from $2.0x10^2$ to $1.4x10^8$ cfu/g in the fixed site samples, and from $4.5x10^3$ to $4.7x10^6$ cfu/g in the grazed site soil samples. The MSC population were not detected with seasonality. Counts from samples of soil taken from where the cattle were grazing, were generally similar to those taken from the fixed site which had no grazing.

In the PSC the population (Figure 2:18b) was not consistently higher at either the fixed or grazed sites. Counts were generally low, ranging from 0 to $4.1x10^2$ cfu/g in the fixed site samples, with the exception of the February and the June count ($2.0x10^4$ and $1.6x10^5$ cfu/g respectively). It was difficult to determine trends in the PSC population in the soil, but overall they tended to predominate in the late summer to autumn period.

The numbers of thermophilic sporeformers detected (Figure 2:18c) were fairly constant in the fixed and variable site soil across the survey ($2x10^3$ to $5x10^4$ cfu/g and $4x10^3$ to $5x10^4$ cfu/g respectively); the numbers in October '94 were outwith this range ($2x10^6$ cfu/g and $6x10^6$ cfu/g respectively).

2:3:1:9 Incidence of *Bacillus* spp. during the farm survey

The principal psychrotrophic species of *Bacillus* identified from samples during the farm survey were *B.cereus* and *B.mycoides*. These species were more prevalent during the summer to autumn months (Figure 2:19a-b). *Bacillus cereus* was identified from widespread sources during the survey, including raw milk, udder wash, faeces, soil and grass, concentrated food pellets and silage, and in water. During the course of the survey *Bacillus mycoides* was also identified in all of these sites, and also in winter bedding samples. Other psychrotrophic *Bacillus* spp. identified during the farm survey were *B.amyloliquefaciens, B.brevis, B.circulans, B.firmus, B.laterosporous, B.lentus, B.licheniformis, B.megaterium, B.pantothenicus, B.polymyxa, B.pumilus, B.sphaericus, B.stearothermophilus* and *B.subtilis* (Table 2:2).

The most prevalent mesophilic *Bacillus* spp. were *B.pumilus, B.subtilis* and *B.mycoides*. Other mesophilic species identified during the farm survey included *B.amyloliquefaciens, B.brevis, B.cereus, B.circulans, B.firmus, B.laterosporous,*

B.lentus, B.licheniformis, B.megaterium, B.pantothenicus, B.polymxa, B.sphaericus and *B.stearothermophilus* (Table 2:3).In the spring sampling (April '94) the greatest number of mesophilic *Bacillus* spp. were detected.

2:3:2 Assessment of bacterial and spore populations in the air of the milking parlour

Air sampling was carried out when the cattle were being milked, and again after the milking parlour had been vacated for 2 h, to monitor the level of aerial contamination occurring, and to assess the effect that the cattle had on the bacterial and spore loading in the air in the milking parlour.

The average air sampling results are illustrated (Figure 2:20a-c). Each value was the average of 3 samplings. Aerial contamination was detected. The contamination level was markedly higher when the cattle were being milked, rather than in the empty parlour. This was the case for both the mesophilic and psychrotrophic populations ($P=<0.05$). Mesophilic sporeformers (Figure 2:20c) were detected in the presence of the cattle on two of the three sampling days. A small number of mesophilic sporeformers were detected on day 1 and day 3 when there were no cows present; on day 1 the highest level (3.33 cfu/L) of sporeformers was detected. No psychrotrophic sporeformers were detected on any of the sampling occasions.

2:3:3 Bacterial contamination of milking machine teat clusters and milk line

The extent of the residual bacterial contamination of the sterilised teat cluster and milk line between milking machine and bulk tank, and the bulk tank itself were assessed. Mesophiles were recovered from all stages of the milking line, namely from the teat clusters, the pipeline joining the machine to the bulk tank, and from the bulk tank itself (Figure 2:21a). Psychrotrophic sporeformers were also recovered from all parts of the milking system (Figure 2:22b), as were thermoduric isolates (Figure 2:21c). No thermophilic sporeformers were isolated at any stage. The milking system thus represents a reservoir of contamination for raw milk.

2:3:4 Bacterial populations in raw milk and udder washes collected at the morning and afternoon milking sessions

The time intervals between the milking sessions were unequal (a.m.-p.m. 8h; p.m.-a.m. 16h). In view of this samples of raw milk and udder washings were taken at both the morning and afternoon sessions from 4 cows on 2 occasions, to establish whether the length of time between milkings was a significant factor influencing microbial contamination of milk and the udder surface. The protocols used were as described for the main survey (Sections 2:2:1 and 2:2:2.)

On both sampling occasions the psychrotrophic sporecount was higher in the morning than the afternoon sampling (Figures 2:23a-b). These differences however, were not found to be significant for either milk or udder washes ($P>0.05$).

2:3:5 Effects of animal husbandry practices on bacterial populations in raw milk and udder washes

The cattle in the farm used in the 12 month survey reported (section 2:3:1) were subjected to different conditions of husbandry, depending on the time of year. From the end of October to the end of April they were housed indoors, while for the remainder of the year they were at pasture. The blade length of the grass varied with season and extent of grazing. As the season progresses, the grass is grazed and regrows, but overall the blade length becomes shorter. A seasonal pattern for the occurrence of *Bacillus* spp. was established by the main survey, but it was unclear whether the changes detected arose from a seasonal effect, or whether they were due to altered husbandry practices throughout the year. In light of such considerations, 12 cattle were exposed to different husbandry conditions (4 byre housed, 4 normal pasture and 4 short pasture conditions) over the same six week period, to determine whether differences were detected in the populations arising from the different conditions.

The husbandry of the animals did not, have a significant effect on the numbers of colony forming units detected in milk and udder wash samples (Figures 2:24a-b). However, the cattle kept on ordinary pasture conditions tended to have the lowest counts in both the milk and udder washing samples. Exceptions were the PSC populations which were similar for cattle housed in the byre produced to those obtained from the ordinary pasture conditions; additionally the TPC populations in milk were the same for cattle grazing ordinary and short pasture ($2.57x10^3$ cfu/ml and $2.44x10^3$cfu/ml respectively).

The sporeforming populations were highest in the short pasture conditions in both the udder washings and the milk samples, although the difference was not significant (Figures 2:24a-b).

Over the 4 week period when the survey into the effects of husbandry practices was conducted there was considerable variation in the numbers recorded for each population. The TPC results from the udder wash samples of the cattle on short pasture on the second sampling day ($1.5x10^6$ cfu/ml) were significantly higher ($P=<0.05$) than the TPC in the udder washes for the other sampling days (mean $3.1x10^3$cfu/ml). Although the PSC for the udder washes of the cattle on short pasture were also higher on the second sampling day the difference between them and the other surveys was not significant. Significant differences were not detected in the milk samples taken on the second sampling occasion and the other sampling days.

2:3:6 Effect of pre-milking teat preparation on the number of bacteria remaining on the teat surface

Before cattle are milked, various teat preparations may be performed by the dairyman. Such preparations are made to improve the quality of the milk by reducing the amount of contamination which is washed from the teat surface into the milk while milking is taking place. A study of the effect of no treatment, washing, or washing followed by drying was made to assess the effectiveness of the washing protocols.

Washing the teats using single sterile moist, disinfectant cloths, one for each teat, had no benefit in reducing the numbers of bacteria detected in any udder wash sample population (Figure 2:25a). The numbers recorded when washing was carried out were very similar to those that had not been cleaned.

When the teats were dried following the wash procedure a significant reduction in the number of bacteria detected in the udder wash populations was detected ($P=<0.05$). The reduction was seen in TMC, TPC, MSC and PSC counts (Figure 2:25b).

No effects from either of the washing procedures were detected in the milk samples (Figure 2:26a-b).

2:4 SURVEY DISCUSSION

2:4:1 Seasonal incidence of sporeformers

2:4:1:1 Raw milk

Ridgway (1953) found in his four year survey of raw milk and the farm environment, that the incidence of aerobic sporeformers was highest in the winter milk. McKinnon and Pettipher (1983) agreed with these findings, as did the survey reported here, where the highest spore counts were detected in the milk in January. Winter housing is thought to contribute to the high levels of spores detected in milk during the winter (Stewart, 1975).

The survey results confirmed a seasonality for mesophilic and psychrotrophic sporeformers (PSC) in raw milk. The mesophiles dominated the sporeforming population in the winter months, whereas the psychrotrophic sporeformers became more prevalent during the summer to autumn period. This has previously been observed (Phillips and Griffiths, 1986; Sutherland and Murdoch, 1994). The seasonality which Sutherland and Murdoch (1994) determined was very exclusive for either mesophilic or psychrotrophic populations, depending on the time of year. Although the same overall trends in the milk were apparent in the survey reported, mesophilic sporeformers were also detected in the late summer-autumn period, and psychrotrophs outwith the summer-autumn period. A seasonal incidence of *B.cereus* has been found in pasteruised milk samples collected from Danish dairies, where *B.cereus* was more prevalent during the summer (Larsen and Jorgensen, 1997)

It has been suggested that one reason for the psychrotrophic sporeforming population only existing in its specific season, is that there is inhibition of the psychrotrophs by mesophilic sporeformers. Only when the mesophilic sporeformers themselves decline in the summer-autumn period, do the psychrotrophs have the opportunity to flourish (Sutherland and Murdoch, 1994). The existence of such interactions will be discussed further in Chapter 5. However, if an inhibitor is a contributory factor in the seasonal occurrence of *Bacillus* species, it cannot be the only factor in play as both groups can coexist in raw milk. Psychrotrophic *Bacillus* spp. were detected in the raw milk in the February sample, although at this time the mesophilic sporeforming population was at its height. Also, during the summer months (July-August) when psychrotrophic *Bacillus* spp. were detected in milk, the mesophilic population was also high. Conversely, in June and September, when the mesophilic sporeforming population was at very low levels, psychrotrophic sporeformers were undetectable. It would therefore seem that inhibitory interactions cannot be the only cause of the seasonalities.

McKinnon and Pettipher (1983) similarly found that the total spore count was higher in the winter than the summer. However, they did not report an actual increase in the psychrotrophic sporeforming population, during the summer-autumn period. They found that although the numbers of psychrotrophic sporeformers was relatively consistent across the year, the proportion of psychrotrophs within the sporeforming population increased in the summer. This indicated that a decline of mesophilic sporeformers had occurred during the summer as compared to the winter. The findings of the study by McKinnon and Pettipher (1983), and the survey reported here are thus consistent, since the mesophilic sporeformers predominated during the winter, with the maximum for the survey of the milk produced by the HRI herd, occurring in

January. However, psychrotrophic sporeformers were not consistent across the year as described by McKinnon and Pettipher (1983) and exhibited a definite seasonality.

The existence during the summer, of a higher concentration of one or more germination factors in the milk, that might enhance the germination of psychrotrophic spores has been proposed (Phillips and Griffiths, 1986). The food which cattle are fed affects the composition of their milk considerably. It is therefore possible that a dietary component, or metabolite may enhance germination of psychrotrophic spores, which could be present in milk in the summer-autumn months. Enhancement of psychrotrophic species with potential germinants, without any repression of mesophilic species would explain why both mesophilic and psychrotrophic populations were detected simultaneously in milk. However, the presence of a germinant in milk would not explain why the distinctive seasonality patterns were seen not only in milk, but also in the udder washes and faecal populations.

2:4:1:2 Udder washings and faeces

The udder wash and faecal samples demonstrated similar seasonality profiles to the raw milk samples for mesophilic and psychrotrophic sporeforming populations. As with the milk samples, there were instances of psychrotrophic sporeformers being detected at times outwith the anticipated season; however the occurrences of psychrotrophic sporeformers during the winter period were at different times for milk, udder washes and faecal samples. In milk they only occurred in February; in udder washes they occurred only in March and in the faecal samples they occurred only in April. The episodes of contamination occurring at different times for the three different sample types, implies that a number of contamination routes are in play. In February psychrotrophic *Bacillus* spp. were detected in the winter bedding sample. This coincided with the contamination of the raw milk. The anticipated route of entry

from the winter bedding into the milk would be via the surface of the udder (Figure 2:27). However the psychrotrophic spore count in the udder wash samples were negative in February. The feed was contaminated during this period, and spores of psychrotrophic *Bacillus* spp. were being ingested by the animals.

These findings indicate that the spoilage problems associated with *B.cereus* contamination and the potential public health risk which such contamination occasions, are not necessarily restricted to certain periods of year.

2:4:1:3 Thermoduric bacteria

The survey of milk produced by the HRI herd showed that thermoduric bacteria and thermophilic sporeformers exhibit seasonalities which are similar, but more defined than the mesophilic sporeformers. There are six species of thermophilic *Bacillus* listed by Sneath (1986) in Bergey's Manual of Systematic Bacteriology that are capable of growth at 55°C. They are *B.stearothermophilus*, *B.acidocalclarius*, *B.licheniformis*, *B.coagulans*, *B.subtilis* and *B.brevis*; only the first two are obligate thermophiles. They can grow at temperatures between 65-75°C, but not below 40°C. The other species will grow at room temperature, but not above 65°C (Norris *et al.*, 1981). The themophiles were not identified in the survey reported here, but *B.stearothermophilus*, *B.licheniformis*, *B.circulans*, *B.coagulans*, *B.macerans* and *B.subtilis* have all been isolated from milk or evaporated milk products (Chopra and Mathur, 1984; Kalogridouvassiliadou, 1992). Thermophilic *Bacillus* spp. are the cause of flat sour spoilage in canned foods, such as evaporated milk (Norris *et al.*, 1981; Kalogridouvassiliadou, 1992).

2:4:2 Routes of contamination

The data gathered in the survey are highly complex. Following a clear route of contamination is complicated by the very low numbers of psychrotrophic *Bacillus* spp. which were detected. In milk and udder wash samples the numbers were <1cfu/ml. A similarly low level of *B.cereus* spores had been detected previously (Labots and Galesloot, 1959). It is probable that there are many different contamination sources and routes involved, which contribute to the contamination of raw milk by *Bacillus* spp.. The survey of the incidence of *Bacillus* spp. in milk produced at the Hannah Research Institute (HRI) farm, indicated several possible points at which *Bacillus* spp. may enter and contaminate the milk chain (Figure 2:27). These events and how they may influence one another will be discussed in the following section.

2:4:2:1 *Bacillus* species contamination arising from the immediate farm environment

When the cattle were housed indoors, the populations of mesophiles and psychrotrophs were relatively constant in milk. However, when the animals were outside they experienced greater differences in climatic conditions, and this was reflected in the bacterial numbers and spores in the milk samples, which were more variable.

In addition to samples taken from the cow, a selection of samples were also taken monthly from the immediate farm environment, the milking parlour and the feed consumed by the cattle.

When the animals were at pasture samples of fixed and variable site soil and grass were taken. The sample from the fixed site was always taken from the same situation, and the variable sample from the field in which the cattle were grazing. Although the

samples from the two sites differed from one another, neither the fixed or variable site was consistently higher than the other, and statistical analysis confirmed that they were not significantly different. This implies that the cattle themselves were not causing an increase or a decrease in the levels of *Bacillus* spp. found in the soil or on the grass. The husbandry practice for the cattle used in the survey involved the rotation of the cattle between different fields on a regular basis. This was to encourage grass recovery following grazing. The information obtained from the variable site sample on the influence of the cattle upon their environment may therefore be different than it would have been if the animals had been kept in one field throughout the duration of the summer-autumn period. In such a situation, the cattle may influence the soil and grass populations to a greater extent.

Bacillus are telluric in origin, and therefore the soil could be a potential originating source for the contamination of raw milk. Grass did not appear to be an important reservoir for contamination, as psychrotrophs were only detected in the variable grass samples once throughout the survey, and three times in the fixed grass sample.

As the season progresses the blade length of grass becomes shorter, the growth rate of the grass decreases, and therefore its recovery following grazing becomes slower. When this happens the grazing cattle become increasingly likely to make contact with the soil while they feed. An increase in the consumption rate of the soil would also increase the consumption of *Bacillus* spores. Also, as the blade length of grass becomes shorter there is greater potential for there to be direct contact between the soil and the udder surface when the animal lies down. It has been considered in the past that psychrotrophic sporeformers could be introduced into the milk chain by udder contamination when the cattle are grazing (Griffiths and Phillips, 1990b). It seems

likely that this may be the case, but that the introduction arises from udder contact with soil, rather than grass.

The condition of the field is influenced not only by the state and quality of the grazing during the season, but also by the climatic conditions experienced. Wet conditions are reported to encourage an increase in the level of *B.cereus* spores in the soil (Labots *et al.*, 1965). Wet conditions also result in greater disruption of the fields by the cattle as they move around and graze. This in turn increases the probability of the animals coming into contact with the soil. Contact may be as has been described, through ingestion, whilst the animals lie down, or through splashing of mud onto the udder surface or the hair of the animals. Some researchers have suggested that periods of warm dry weather may be required for heavy contamination (Nokes, 1965). The disturbance caused by harvesting could lead to an increased aerial contamination level (Nokes, 1965). It is possible that the combined effect of a period of warm, dry weather, followed by one of heavy rain may be ideal for *B.cereus* contamination (Christiansson *et al.*, 1996). Such conditions resulted in a significant increase in spores detected in milk (Christiansson *et al.*, 1996), and would be the type of conditions often experienced in late summer-autumn in south west Scotland.

The importance of the pasture status and animal husbandry was examined in a study in which the cattle were managed under various conditions (normal pasture; very short, sparse pasture; and indoor housing in the byre), and the extent of udder and raw milk contamination was assessed. The different husbandry conditions did not have a significant effect on udder or milk contamination. The long-pasture grazed animals had the cleanest udders by visual assessment, and also had the lowest bacterial counts for all populations in both udder washes and milks, although they were not

significantly lower. The cattle on short pasture had the highest populations of mesophilic and psychrotrophic sporeformers, although again the differences were not statistically significant.

The counts from those cattle on short-pasture were affected most by the weather conditions. The first sample day had been preceded by a long period of hot, dry conditions, whereas the night before the second sample collection there was a heavy thunder storm. This changed the short pasture conditions from a clay-like surface, into a very muddy environment; the psychrotrophic spore count in the udder washes rose ten fold following the wet conditions. Although the increase in the PSC was not found to be significant, the increase in the total psychrotrophic count in the udder washes also rose, from $3.4x10^3$ cfu/ml in the first sample to $1.5x10^6$ cfu/ml in the second sample; this difference was significant (P=<0.05). The increase in psychrotrophs detected in the udder washes from the more heavily contaminated udder, was not apparent in the milk samples, probably because of the effective teat cleaning which the dairy-men performed prior to milking. In the second study of the effects of husbandry the animals kept on short pasture were caked with mud. Before milking, these animals were thoroughly cleaned. Visibly less dirty animals, such as those on ordinary-pasture, were less vigorously cleaned.

The husbandry practices of some farms, especially on the continent, are such that the cattle are housed inside throughout the year, with the animals being provided with an outside yard for exercise. The yards are generally spartan. Given such husbandry conditions coupled with the influence of the weather, it may be possible that when the season favours psychrotrophs, an increased number of *Bacillus* spp. may find their way into the raw milk supplies as a consequence of more extensive udder contamination.

Samples taken on different sampling days for the husbandry survey detected similar for mesophilic spore counts.

Faecal contamination by mesophilic sporeformers seem to be influenced by the housing conditions of the animals. When the animals were housed indoors, the mesophilic spore counts were at a relatively constant level, which was much higher than that seen when the animals were at pasture. The October '94 sampling produced interesting results. The cattle remained outside during the day in October, but following their afternoon milking the animals were retained in the byre over night. This appears to have had an effect upon the mesophilic spore count, which in October was similar to that experienced during the winter months.

From November through to January the animals were housed in individual pens. They were each provided with a separate supply of feed, bedding and water, and defaecated into a gutter which ran behind the back of the stalls. The stalls were separated from one another by partition walls. From February through to their return to pasture, after the April sampling, the cattle moved to a newly refurbished open-plan byre. Here, the animals could move around freely. They were given communal food and water, which was provided in a central area distinct from the bedding section. The bedding was provided on rubber matting, covered in a thin layer of sawdust. Sawdust had also been used in the previous housing scheme. In the open plan byre, no restrictions were imposed on the cows as to where they should lie. Defaecation occurred in the wide walkways which ran around the edge of the bedding area and next to the feeding area. An automatic mechanical scrapping system cleaned the faecal matter away by dragging it to gutters at ends of the barn.

The effect of the different housing regimes upon the winter feed and bedding populations was reflected in changes in the psychrotrophic sporeforming populations. During the period when the animals had been segregated from one another, psychrotrophic sporeformers were not detected in either the feed or bedding samples. From the time when the cattle had been moved into the communal living situation psychrotrophic sporeformers were regularly detected in the feed and bedding samples. The presence in the feed is not related to husbandry conditions, but complicates interpretation of data. During the communal housing period psychrotrophic sporeformers were also found in the milk, udder washes and faecal samples. The presence of psychrotrophic sporeformers in the animal samples could be related to their presence in the feed. It may also be connected to the increased level of inter animal contact brought about by the new regime, which may be responsible for cross-contamination leading to this rise. In comparative studies to examine the effects of different housing systems, the hygienic functioning of the systems was found to vary (Herlin *et al.*, 1994). Cattle in tie-stalls, such as the animals experienced from November-January, contaminate their lying area the most severely. However, in another survey of housing conditions and teat contamination (McKinnon *et al.*, 1983) it was found that animals kept together in a yard often soiled their bedding material, but when kept in cubicles the bedding was rarely soiled. The results of the survey reported here, support the latter findings; contamination of the bedding was only detected in the communal regime. Dry bedding material was not often found to soil the teat and udder surface, but if the bedding became soiled it readily coated the teats with a semi-solid mixture of bedding and faecal material, that formed a dry, adherent crust (McKinnon *et al.*, 1983); this crust can be dislodged by the bathing action of the milking machine.

The mesophilic sporeforming population in the winter feed was found to affect the mesophilic sporeforming population in the faecal population. Previously, no correlation has been detected between numbers of *B.cereus* in faeces and fodder (Labots and Gailsloot, 1959). The nature of the feed, and the level of its contamination, appear to have influenced the resulting faecal populations. In February the silage fed to the animals was heavily contaminated with psychrotrophic sporeformers, yet none were detected the faecal counts. However, in April when the concentrate feed pellets were contaminated with psychrotrophic sporeformers, even though the contamination was less than had previously been detected in silage, psychrotrophs were present in the faeces at their highest level during the survey. Disparity between the spores detected in the faeces, compared to those on different types of feed may be related to digesta flow differences. Feed concentrates are made from starches, proteins and minerals, that are extensively digested and are not retained in the rumen. Silage is fibrous, and so is retained longer in the rumen and the large intestine. Therefore spores from silage have a reduced chance of being passed straight through the animal ungerminated.

Previous studies (Christiansson *et al.*, 1996) have only detected relatively small numbers of spores in winter feed samples; these researchers concluded that the winter feed was not an important source of spores. However, the levels of spores detected in the winter feed here were considerably higher, and because there was also a high correlation between the number of mesophilic spores in faecal and winter feed populations, they should be considered of importance. *Bacillus* spores are able to germinate and vegetative cells survive within the bovine rumen; *Bacillus* spp. maintained a constant population level in the rumen by associating with plant material (Williams and Withers, 1983). This implies that the animal itself potentially contributes to the events which result in the contamination of milk. *Bacillus* spores

ingested with the feed may not behave as inert particles to be transported directly through the intestinal tract, but instead, the cattle may act as a reservoir for *Bacillus* species. In view of this, the implications of the ingestion of contaminated feed are potentially more serious. It is unknown whether the rumen populations of *Bacillus* exhibit the seasonal fluctuations that occur in faeces and milk.

2:4:2:2 Effect of teat washing procedures

The milking of the cattle used in this survey was performed using a highly automated milking-machine system. Such a system involves the use of a teat cluster, which is placed over the teats of the udder. The teat-clusters are held in place by a vacuum. It is the suction of the vacuum pump which produces the milking action which expels the milk from the mammary gland. During milking the teat surface is bathed in milk. Such a bathing action allows ample opportunity for bacteria on the surface of the teat to be washed into the milk. Teat washing greatly reduces the spore content of the milk (Waes, 1992). Therefore it is considered important that for minimum contamination of milk, the teat surface should be clean. Various methods of cleaning may be employed prior to milking. To consider the effectiveness of the various methods, we conducted an experiment to examine the effects of such procedures.

When the teats of the cattle were prepared by washing and drying prior to milking a significant reduction in the contamination of the udder wash samples was detected. However, washing the teats alone without drying had no effect on the contamination level, with no reduction being observed. Washing the udder before milking probably helps to dislodge the bacteria from the teat surface, but if the teats are not then dried, a bacterial solution from the teat surface can be washed into the milk. Washing the teat surface before milking with a variety of disinfectants was found to be beneficial in reducing the total bacterial count of milk, although the differences were not significant

ones (Galton *et al.*, 1986). These authors also found that drying the teats further reduced the bacterial count.

The farm surveyed here is a commercial one, and the bacteriological quality of the milk is a determinant of the sale price. Because of this it was not possible to milk visibly dirty udders in an untreated state. Therefore, all milk samples were taken, after the dairymen had performed their usual cleaning procedures. These consisted of wiping the teats with a cloth rinsed in disinfectant, followed by drying with an individual paper towel. This was not useful in terms of our experiment, because it resulted in all of the milk samples recording approximately the same levels of contamination, in spite of the variation in the initial cleanliness of the udders. The milk samples did however, demonstrate the effectiveness of the cleaning procedures carried out at this commercial farm. The vigilance of their cleaning methods may indicate that the counts of raw milks in the rest of the survey are very low because of the effective intervention of the dairymen. The HRI farm used in the study has modern facilities, with highly efficient equipment and high hygiene standards. The levels of contamination in the survey may potentially therefore be low, and higher contamination levels in milk may be detected where less rigorous practices are in place.

The potential for cross-contamination between animals when their teats are cleaned should be considered. If a single cloth is used to clean several animals, then the risk arises of bacteria being washed from the teats of one animal, only to be inoculated onto those of the next. Similar cross-contamination was demonstrated in post-milking teat dips, used to reduce mastitis (Bruce, 1981). Commercial towels are available for cleaning teats. They contain a disinfectant, and are designed to be used once. Such towels were used in our udder-wash study. They stand to eliminate the problems of

cross-contamination arising between animals, and as was seen here, if they are coupled with a drying stage they may be effective.

2:4:2:3 Aerial contamination of the milking parlour

Aerial contamination drawn into the milking machines forms another possible route of entry for *Bacillus* spp. into the raw milk supply. The results of air sampling tests detected sporeformers in samples of air when the animals were being milked. This illustrates the potential for low levels of contamination to enter the system from the air. The milking machine operates through the use of a vacuum pump. The system in the farm used in our survey has been designed to minimise the intake of air into the system. However, there is a time span of approximately 5-10 seconds per animal, when the vacuum is active and the teat cluster is not connected to the animal. This occurs when the dairyman connects the milking apparatus to the cow; he pulls the teat clusters away from their holder, thus activating the vacuum. From that time until he has connected the clusters, the machine is drawing in air, which is fed directly into the milk line. At the completion of milking the clusters fall automatically away from the teats, when the milk stops flowing. At this point the vacuum is disarmed, and so no air is drawn into the system. Air may however, be drawn in during milking, because the cattle frequently dislodge the clusters from their udders. When this occurs they perhaps only dislodge one or two clusters, and these may continue to draw on the vacuum, thus allowing more air to be drawn into the system.

The high level of spore contamination in raw milk seen during the winter months was attributed to increased aerial contamination arising from dusty fodders (Ridgway, 1953). *Bacillus* spp. were detected during the winter in the concentrate feed which the cattle were fed. These pellets are given to the animals in the milking stall, as well as in the byre. When the pellets are dispensed in the stall, they are released from an open

trough above the stall, and fall through a pipe into a feeding trough below. This action releases dust from the pellets into the atmosphere of the milking parlour, which may contain *Bacillus* spp. spores. Once in the atmosphere the spores may potentially be drawn into the milking system as aerial contamination.

Aerial contamination may be of greater importance in more old fashioned milking parlours. Some parlours are equipped with milking machines which are moved from cow to cow, rather than the cattle coming one at a time to the stationary machines. In movable-systems the vacuum pump is active before the machine is connected to the udder, if the animal dislodges the clusters during milking, and also following removal of clusters at the end of milking. The duration of air intake into the system is therefore longer than in a system such as was used in the survey reported here, and hence the risk from contamination would also be greater. Also, when a milking system is not "closed", the milk is drawn into a collection can which is then emptied into a bulk tank vessel, allowing increased exposure to the environment. A closed system takes the milk straight through a sterile sealed line to the bulk tank. The farm used in the survey reported here is equipped with a closed milking system.

Nokes (1965) considered that the dry conditions and the disruption associated with harvest could also be associated with the contamination in summer-autumn of milk by psychrotrophic *Bacillus* spores. Christiansson (1996) on the other hand felt that the spore count in the air was much too low to be considered important. Nevertheless, the presence of spores in the atmosphere has been demonstrated, and so aerial contamination should not be excluded as a potential source of contamination.

2:4:2:4 Milking machines as a potential source of contamination

Following milking, the milking system used in the survey was cleaned and sterilised using an automated washing procedure; however, psychrotrophic sporeforming bacteria were recovered from the teat-clusters, the milking line and from the bulk tank. Spore formation is promoted in dilute milk (Donovan, 1959) and therefore ineffective cleaning of the milking system could result in a residual inoculum of *Bacillus* spores that could contaminate the next milk in the system. Christiansson (1996) found that the milking equipment did not contribute greatly to the contamination of the milk supply; but it should be noted that in farms which consistently fail to destroy spores, a gradual increase in contamination levels are likely to occur (Donovan, 1959). The level of contribution must vary from farm to farm, depending on the efficiency of the cleaning system employed.

2:4:2:5 Differences between samples taken at the morning milking compared with those taken at the afternoon

The herd of cattle at the HRI farm, which were used in the survey reported here, were milked twice daily. Milking times were at approximately 7am and 2pm respectively. The time difference between the morning and afternoon milking is 7 hours, but between the afternoon milk and that taken the following morning, the time difference was much larger, at 17h. Assessment was therefore made to determine whether the increased time period preceding the morning milking, compared with that before the afternoon milking, had any effect on the contamination of udder wash or milk samples of the cattle. An increase in the bacterial counts was observed in both the milk and udder wash samples collected in the morning session, but the rise was not significant in either case. This showed that the contamination of the udder is not exclusively related to its exposure time before milking. Differences in the behaviour patterns of

the cattle by day and by night, may also have a role in influencing eventual contamination levels.

2:4:3 Species of Bacillus identified

The most prevalent species of psychrotrophic *Bacillus* identified over the course of the survey were members of the *B.cereus* group, namely *B.cereus* and *B.mycoides*. *Bacillus cereus* had been previously found to predominate in other surveys in the west of Scotland (Johnson and Bruce, 1982; Phillips and Griffiths, 1986; Griffiths and Phillips, 1990b; Sutherland and Murdoch, 1994; Crielly *et al.*, 1994). In surveys conducted in other parts of the United Kingdom *B.cereus* has also predominated (McKinnon and Pettipher, 1983), as they have in other parts of the world, such as India (Sharma *et al.*, 1984) and Australia (Coghill and Juffs, 1979).

The predominant mesophilic sporeformers identified in the survey were *B.pumilus, B.subtilis* and *B.mycoides*. The *B.cereus* and *B.mycoides* identified in the mesophilic populations, were not restricted to the summer-autumn time, but also occurred when the cattle were housed indoors.

Bacillus licheniformis was not detected in this survey, whereas this species had been amongst the most prevalent psychrotrophic and mesophilic species isolated in past surveys (Johnson and Bruce, 1982; Phillips and Griffiths, 1986; Sutherland and Murdoch, 1994; Crielly *et al.*, 1994). These surveys used the "pre-incubation" technique in the preparation of their samples, whereby samples are incubated in an enrichment medium before plating takes place. Enrichment procedures are unsuitable for enumeration within samples, although most probable numbers may be determined. Species recovered may reflect methods used.

Very low numbers of psychrotrophic sporeformers were isolated from the milk and udder wash samples in the survey, even though centrifugation had been incorporated into the procedure as a concentration step. The low numbers were comparable to those found in other surveys (Labots and Galesloot, 1959).

A provisional survey examining the species isolated from milk, udder washes and faecal samples, with and without pre-enrichment was undertaken (data not shown). Enrichment proved to be a means of maximising the recovery of organisms. It does not take into account the competitive nature of the inter-species interactions, or the variety of growth rates, which may favour some species to the detriment of others. The survey (Griffiths and Phillips, 1990b) which did not include a pre-enrichment step, also did not detect *B.licheniformis*. The method of sample treatment does therefore appear to alter the species detected.

2:4:4 Conclusions concerning the control of psychrotrophic Bacillus species

In the absence of post-pasteurisation contamination, the keeping quality of pasteurised double cream is mainly limited by the growth of *Bacillus* spp. (Griffiths *et al.*, 1986). Psychrotrophic strains of *Bacillus* exist in raw milk at low levels. They occur with seasonality, but may also arise at times outwith their predominant season. Psychrotrophic *Bacillus* should therefore be considered by the milk and dairy industry to be a year long problem, rather than one restricted to the summer-autumn period alone.

In view of these considerations measures should be in place to control these organisms. However, because of the ubiquitous nature of *Bacillus* spp. in the environment it may prove difficult to entirely prevent them from entering the milk chain. Effective cleaning of the teat surface by washing and drying with individual cloths, may minimise contamination associated with the udder from passing into the milk. Other measures, such as clipping of the udder and flank region to improve cleanliness of the animals (Stewart, 1975), may serve to reduce contamination.

Stringent sanitisation of the milking parlour and milking machines following use is imperative. The efficient and rapid cooling of milk is an important factor. This helps to slow outgrowth of spores. Some farms today have their milk collected every other day, as opposed to every day. In these farms it is even more important to effectively cool the milk, and hold it at 4°C. The effect of instant cooling of milk was found to significantly improve the keeping quality of the milk, compared to systems which delayed cooling of the bulk tank until the end of milking (Slaghuis and Boerekamp, 1996). The extra energy requirements of such procedures could prove to be prohibitive (Slaghuis and Boerekamp, 1996), but this could be compensated by the cost benefit of improved bacteriological quality of the milk, and by increased shelf life of products. The shelf life of milk in the US is expected by the consumer to be approximately two weeks (Zall, 1990). Therefore it is imperative that minimum storage temperatures are maintained throughout the processing and delivery chains. Disruption in the cold chain leads to the bacterial quality of many pasteurised products being disappointing at the point of sale (Griffiths *et al.*, 1986). Public awareness should be heightened in order to improve consumer handling of milk and dairy products. Temperature abuse by the public can occur when, after buying milk or dairy products from supermarkets, the goods are left unrefrigerated for extended periods of time. Similarly temperature abuse can occur when milk delivered to the doorstep is left for extended periods before

refrigeration. Many domestic refrigerators are set at temperatures which are above the 8°C maximum requirement for the storage of milk and dairy products. All of these factors provide psychrotrophic *Bacillus* spp. with opportunities for quicker growth.

Higher pasteurisation temperatures do not provide an answer for the elimination of *Bacillus* spp. from milk. If milk is subjected to higher temperatures, the problem of spore germination is exacerbated, and hence the problem with *Bacillus* spp. may increase (Meer *et al.*, 1991).

In some European countries "bactofugation" is used to remove bacterial spores from milk (Davies, 1975). This is a high speed centrifugation procedure, which claims to remove 99.9% of the bacterial flora from milk (Davies, 1975). Such a method is a cheap and safe way to remove bacterial populations from milk, and it does not actively select for resistant organisms.

Filtration of milk takes place on many farms before the milk enters the bulk tank. The step serves to remove visible extraneous matter, but does not reduce bacterial counts (McKinnon *et al.*, 1983). Therefore although such measures improve the quality of milk in other ways, they do not serve a useful purpose in the reduction of *Bacillus* spp. spores.

Reduction of the population of psychrotrophic *Bacillus* spp. in milk may be made by improving hygiene at the farm level. This would minimise the contamination arising directly from the cattle or from milking equipment. Keeping the temperature of milk down to 4°C would prevent the outgrowth of *B.cereus* spores. Although other species of *Bacillus* may still grow at 4°C, since *B.cereus* presents the greatest problems, a reduction in storage temperature of milk throughout the production chain and into the

home is advised. However, since the EC directive 92/46/EEC (Anon, 1992) has been laid down, the maximum temperature for the storage of milk in the UK has been raised to 8°C. While for other EU member states, 8°C may represent a tightening of the law, in countries like Britain it represents an increase in minimum allowable temperatures, which if adhered to will result in an increase in the spoilage and health problems caused by the presence of psychrotrophic *Bacillus* spp. in milk.

TABLES AND FIGURES

FROM FARM SURVEY

Table 2:1 Sample treatments and media used to isolate and enumerate microbial populations

Sample	Heat treatment	Incubation temperature (°C)	Incubation time (days)
TMC	None	30	3
TPC	None	6	14
TTC	63°C / 30 min	30	3
MSC	80°C / 10 min	30	3
PSC	80°C / 10 min	6	14
TSC	80°C / 10 min	50	2

TMC = Total mesophilic count; TPC = Total psychrotrophic count; TTC = Total thermoduric count; MSC = Mesophilic spore count; TSC = Thermophilic spore count

Table 2:2 Number of psychrotrophic *Bacillus* species isolated as spores during the course of the survey

Oct '93	Nov	Dec	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct '94
bre 1	cer 1	-	-	bre 1	cer 3	cer 2	cer 2	cer 2	cer 7	bre 1	cer 1	cer 1
cer 13	myc 2			cer 1	myc 4	cir 2	cir 2	myc 1	myc 10	cer 11	cer/myc	myc 2
myc 7				myc 5		myc 1	myc 2	pol/cir 1	sph/fir 1	myc 15	1	sub/amy
lat/cer 2				NI 2		sub 1				pol 1	NI 2	1
NI 4						NI 3				sph 1		NI 3
										cir/pol 1		
										NI 4		

The species identified were:
amy = *B.amyloliquefaciens*; bre = *B.brevis*; cer = *B.cereus*; cir = *B.circulans*; fir = *B.firmus*; lat = *B.laterosporus*; len = *B.lentus*; lic = *B.licheniformis*; meg = *B.megaterium*; myc = *B.mycoides*; pan = *B.panothenicus*; pol = *B.polymyxa*; pum = *B.pumilus*; sph = *B.sphaericus*; sub = *B.subtilis*; NI = not identifiable

Table 2:3 Number of isolates of mesophilic *Bacillus* species recovered as spores seasonally during the survey

October '93	December	March	June	September
amy 2	cer 1	amy 8	cer 3	cer 6
cer 2	cir 2	cer 1	lic 2	lat 1
cir 1	len 1	lic 3	myc 3	lic 3
len 2	meg 4	myc 3	pum 5	pum 10
myc 10	myc 4, pan 2	pum 22	sub 1	sub 2
pan 1	pum 1	sub 11	amy/sub 1	cer/myc 2
pum 5, sub 1	cer/myc 1	amy/ste 1	lic/sub 1	sub/amy 1
cer/myc 2	lic/sub 1	lic/sub 7	NI 1	NI 1
lat/ste 1	NI 5	sph/bre 1		
NI 2		sub/amy 7		
		NI 10		

The species identified were:

amy = *B.amyloliquifaciens*; bre = *B.brevis*; cer = *B.cereus*; cir = *B.circulans*;

fir = *B.firmus*; lat = *B.laterosporous*; len = *B.lentus*; lic = *B.licheniformis*;

meg = *B.megaterium*; myc = *B.mycoides*; pan = *B.pantothenicus*; pol = *B.polymyxa*;

pum = *B.pumilus*; sph = *B.sphaericus*; ste = *B.stearothermophilus*; sub = *B.subtilis*;

NI = Not identifiable

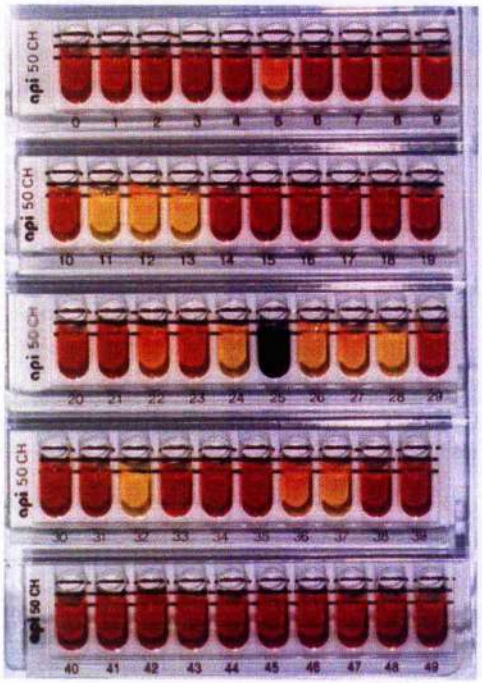


Figure 2:1 Photograph illustrating API 50CHB profile for *Bacillus cereus.*

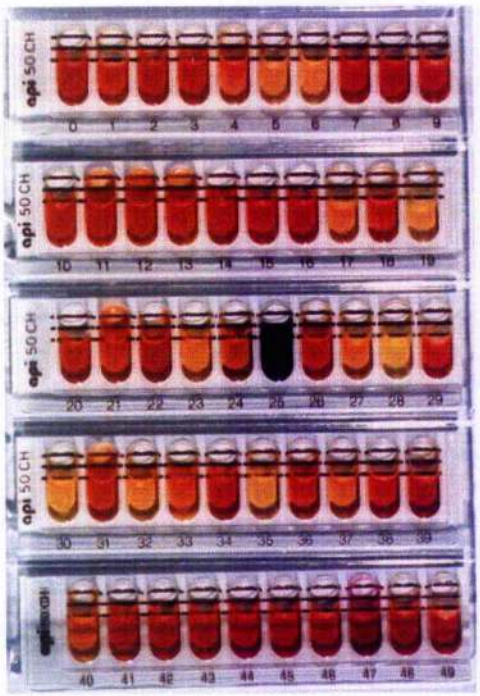
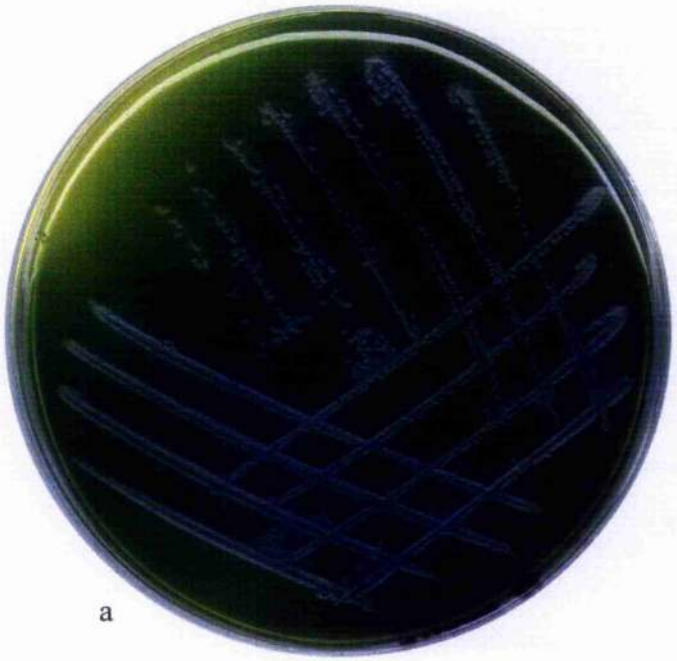
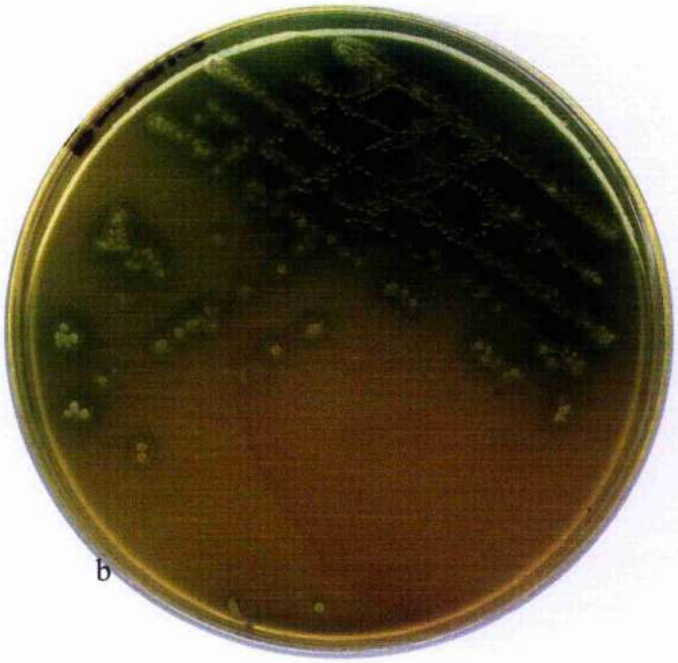


Figure 2:2 Photograph illustrating API 50CHB profiles for *Bacillus subtilis.*

Figure 2:3 Photograph illustrating typical appearance of a) *Bacillus cereus* and b) *B.subtilis* grown on PEMBA selective agar.



a



b

Figure 2:4a Mesophiles in raw milk

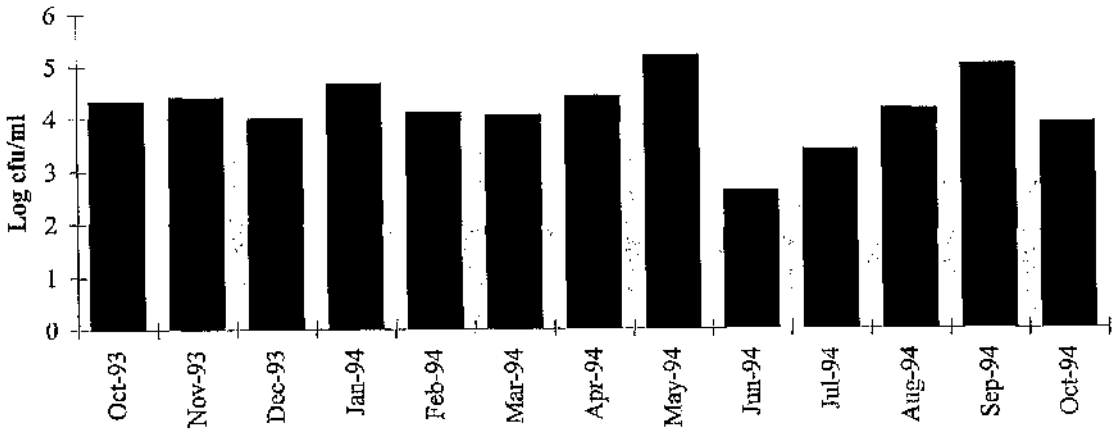


Figure 2:4b Psychrotrophs in raw milk

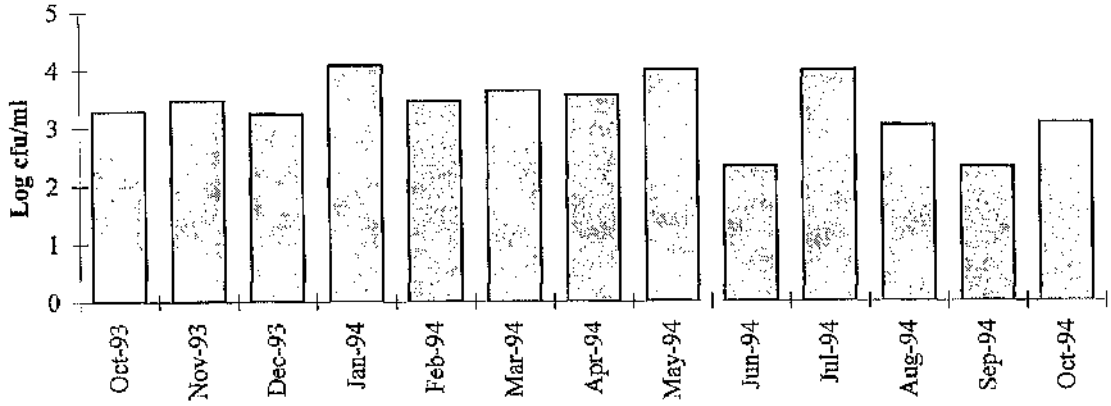
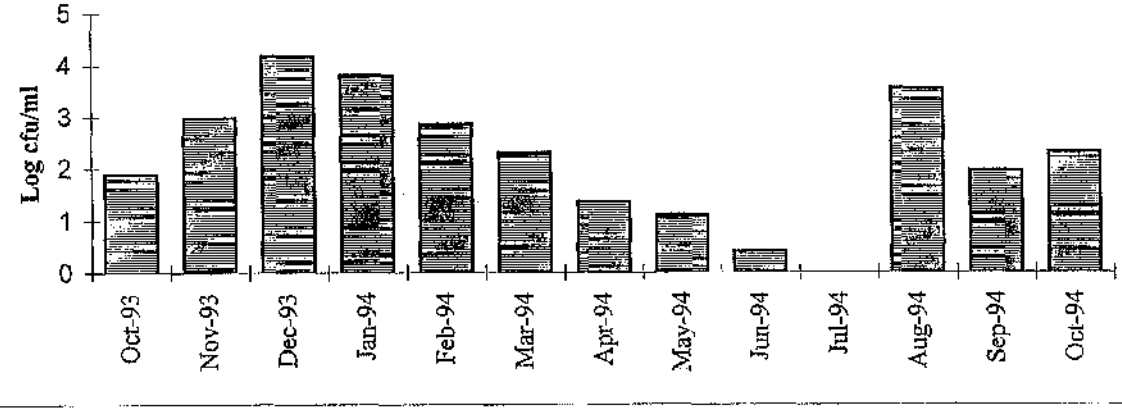


Figure 2:4c Thermodurics in raw milk



Log = Log 10

Zero = < Minimum detectable number for plating method

Figure 2:5a Mesophilic sporeformers in raw milk

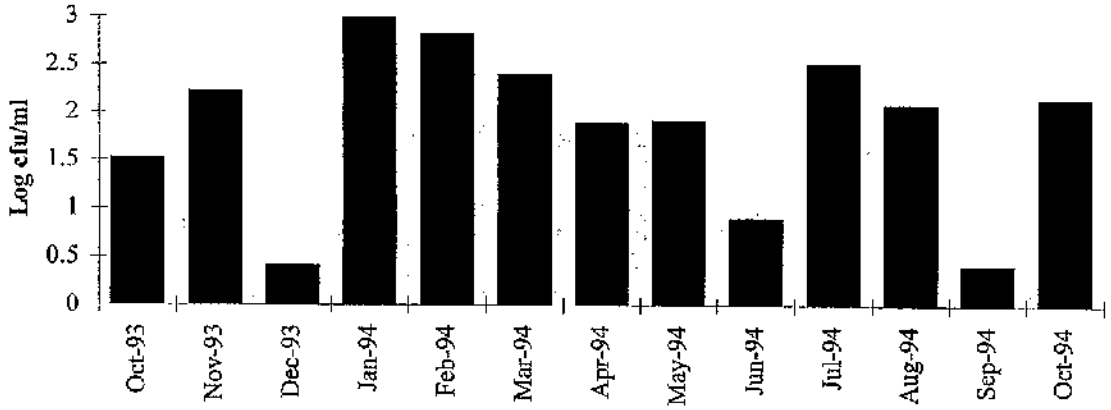


Figure 2:5b Psychrotrophic sporeformers in raw milk

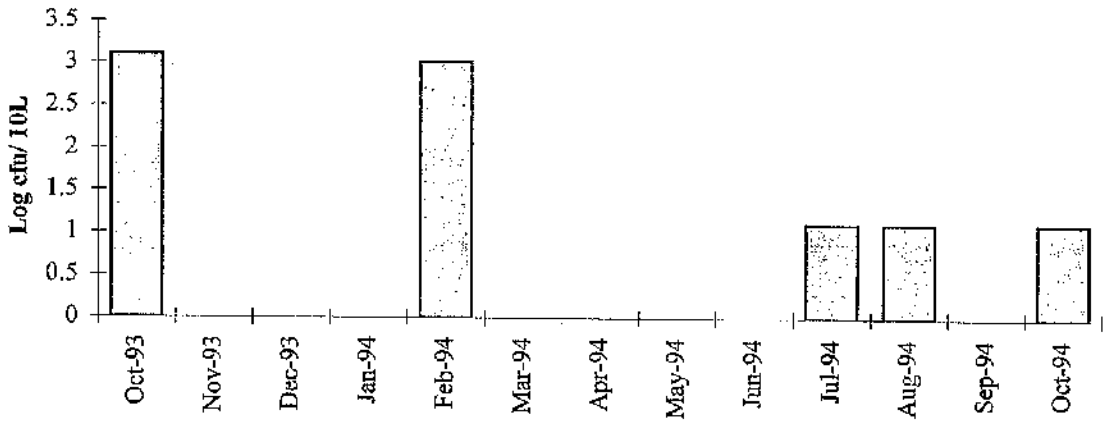


Figure 2:5c Thermophilic sporeformers in raw milk

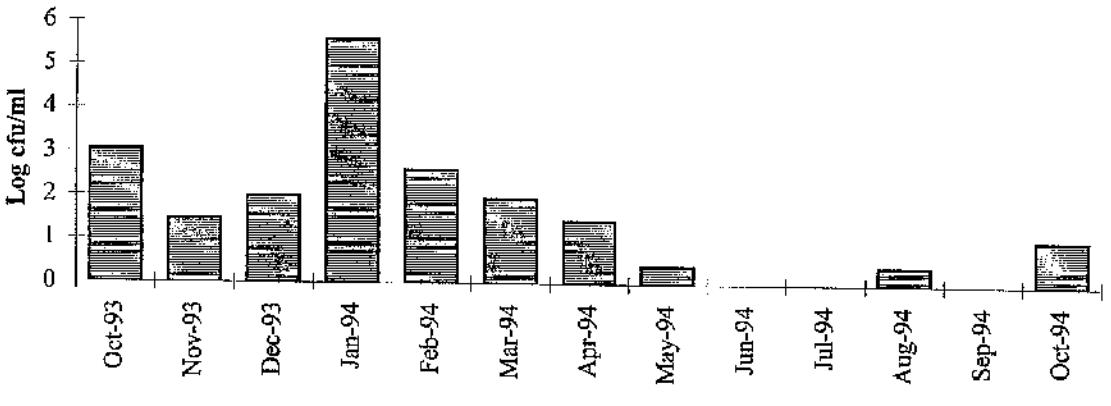


Figure 2:6a Mesophiles in raw bulk tank milk

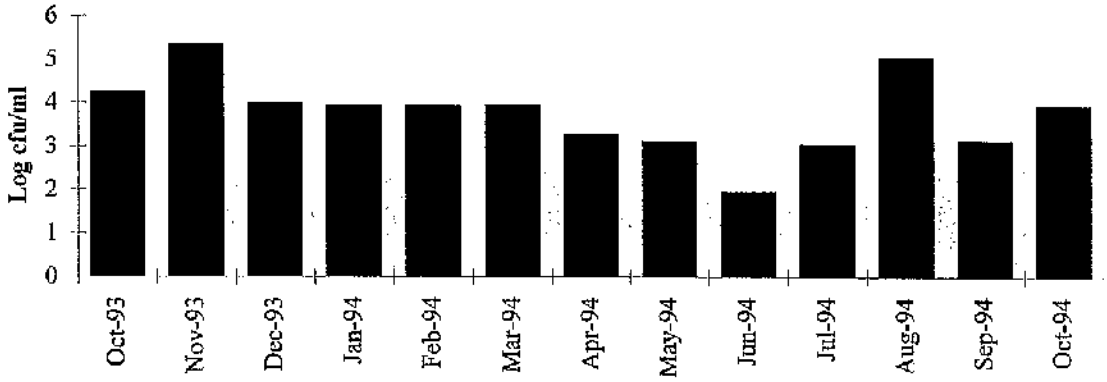


Figure 2:6b Psychrotrophs in raw bulk tank milk

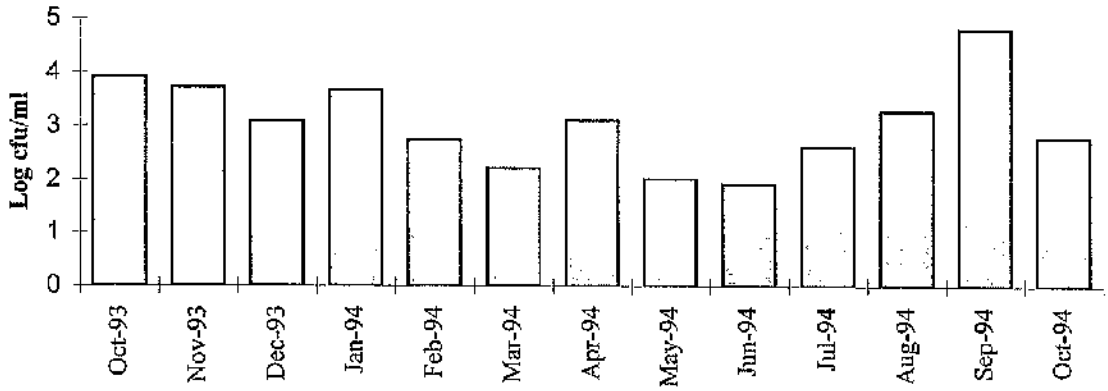


Figure 2:6c Thermodurics in raw bulk tank milk

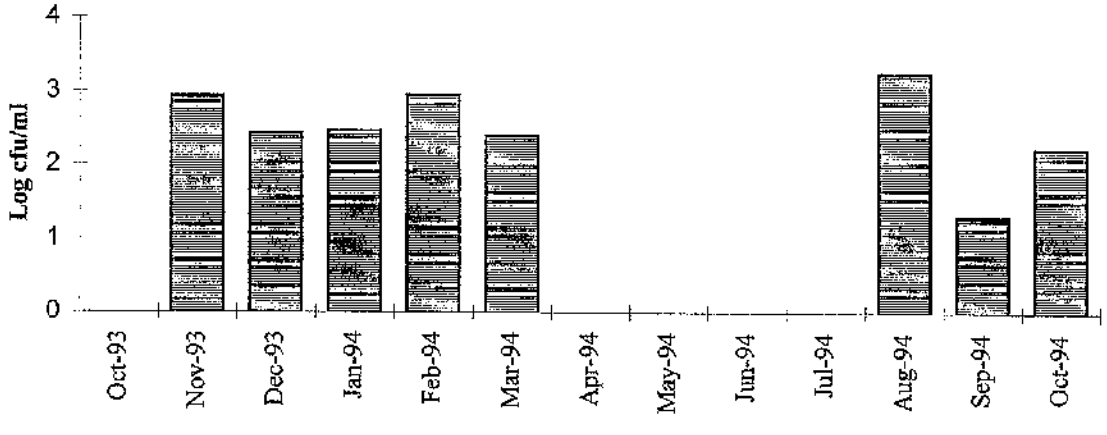


Figure 2:7a Mesophilic sporeformers in raw bulk tank milk

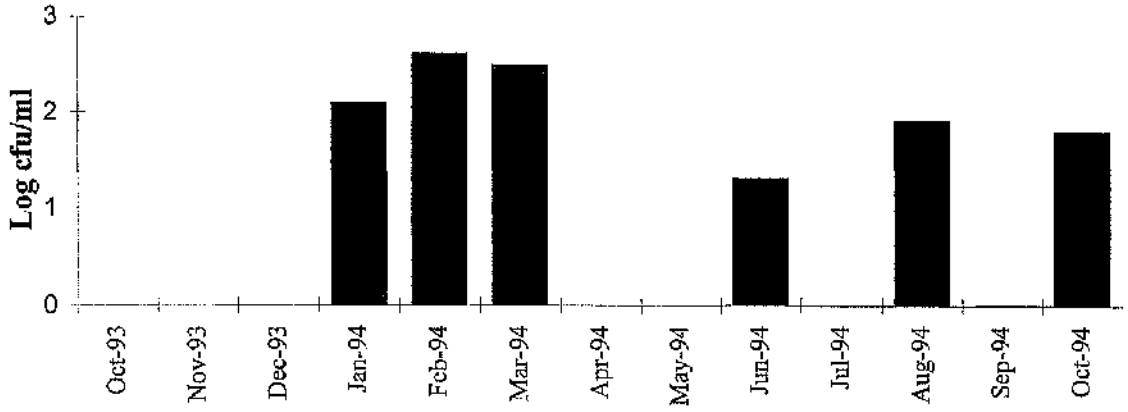


Figure 2:7b Psychrotrophic sporeformers in raw bulk tank milk

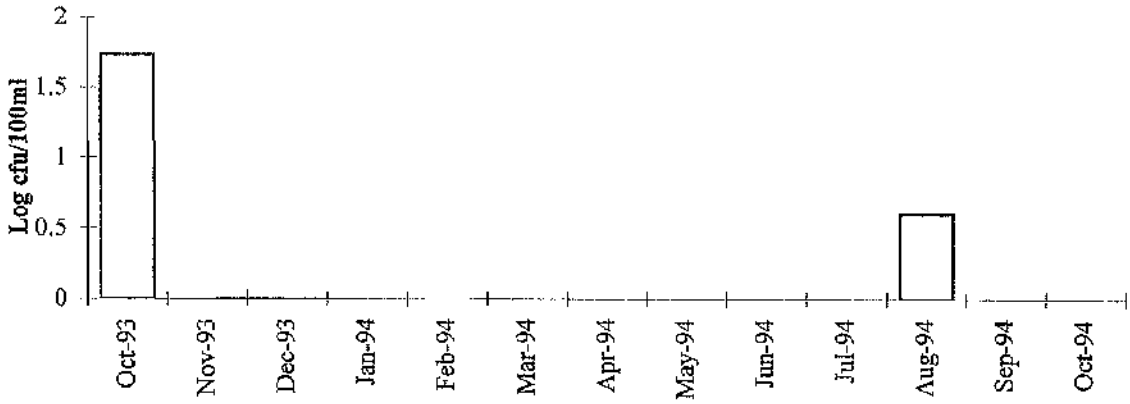


Figure 2:7c Thermophilic sporeformers in raw bulk tank milk

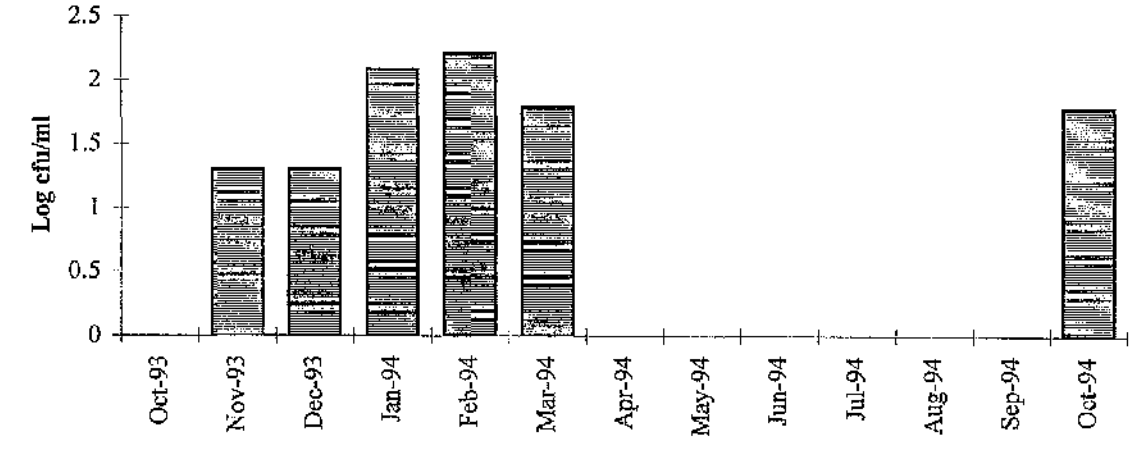


Figure 2:8a Mesophiles in udder washes

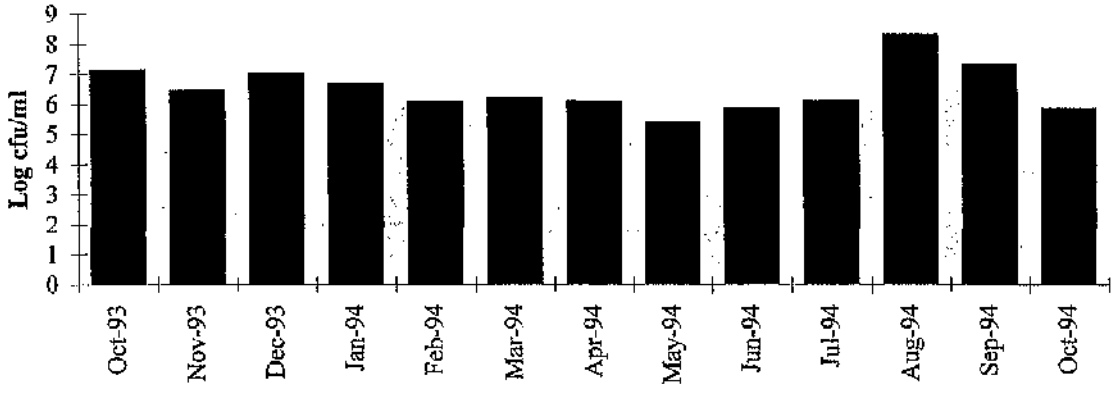


Figure 2:8b Psychrotrophs in udder wash samples

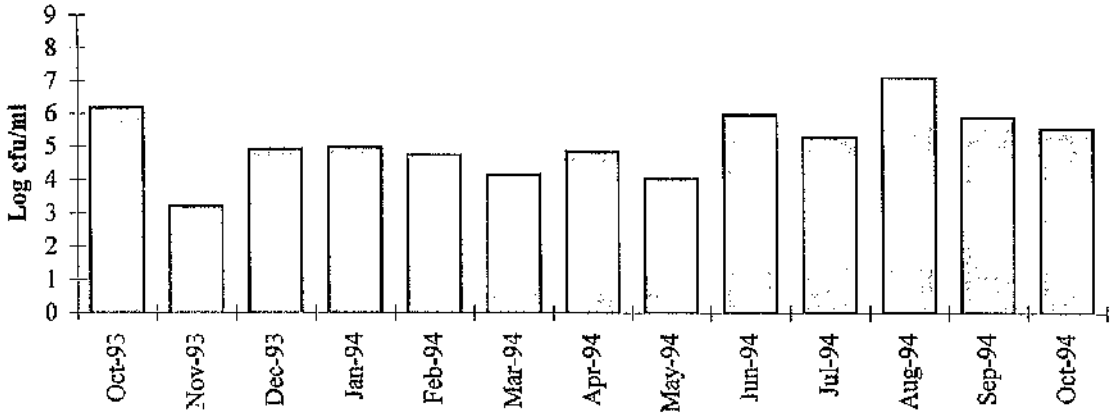


Figure 2:8c Thermoduries in udder wash samples

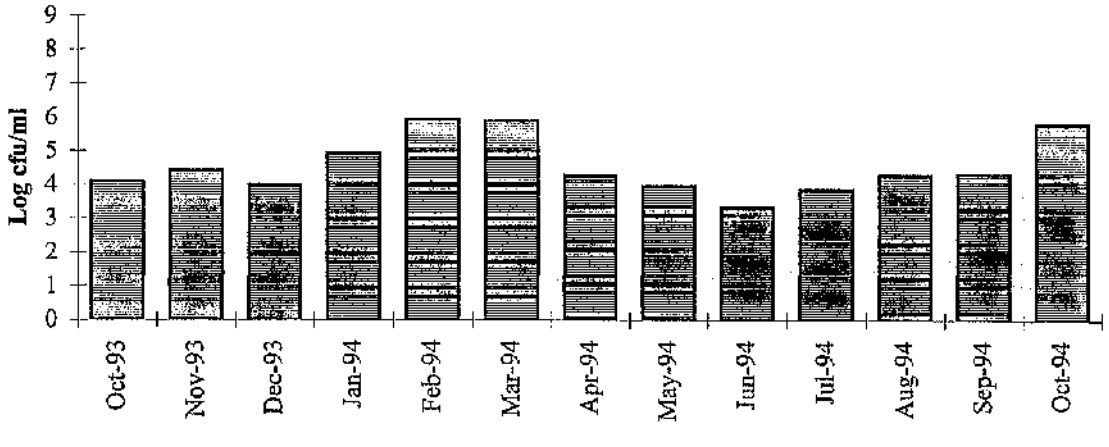


Figure 2:9a Mesophilic sporeformers in udder wash samples

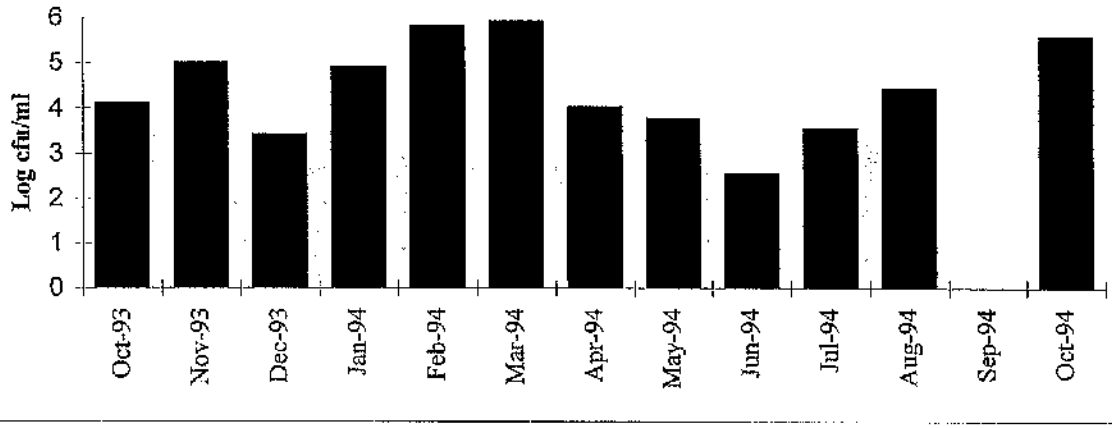


Figure 2:9b Psychrotrophic sporeformers in udder wash samples

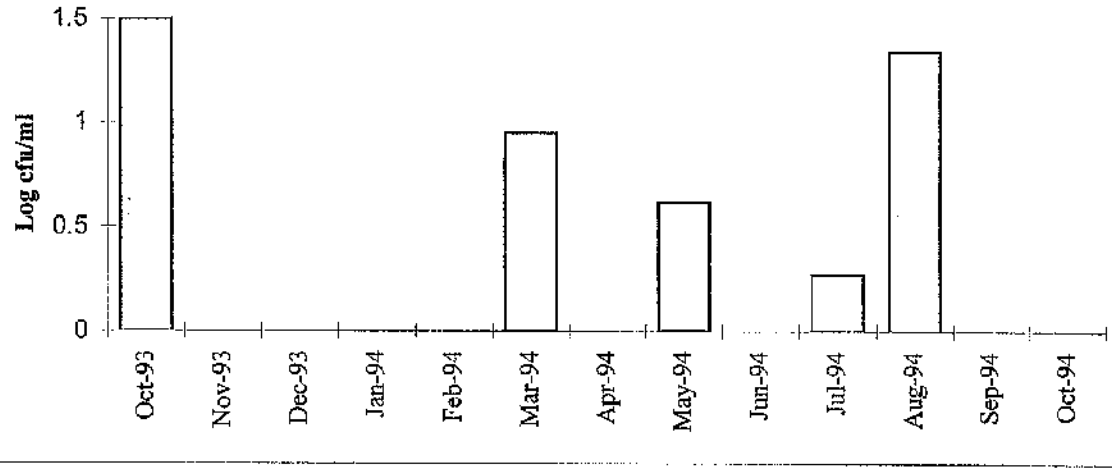


Figure 2:9c Thermophilic sporeformers in udder wash samples

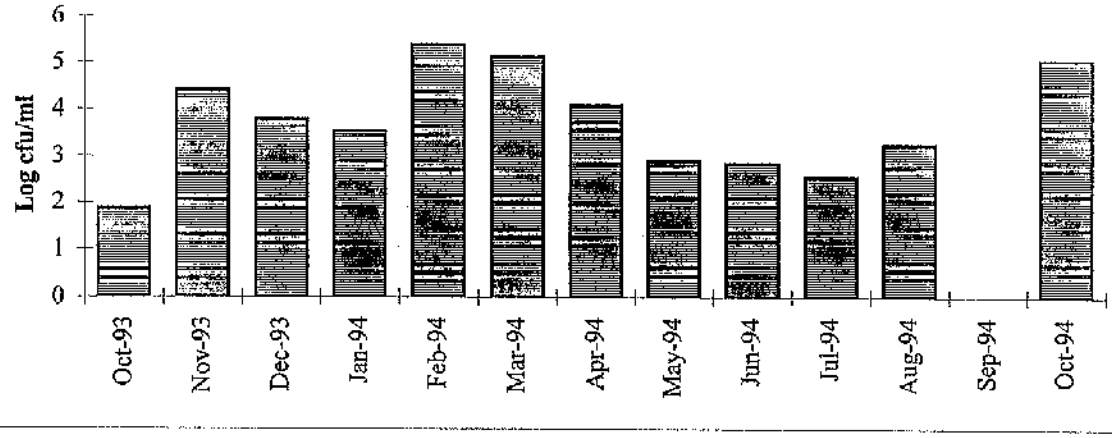


Figure 2:10a Incidence of mesophilic sporeformers in faeces samples

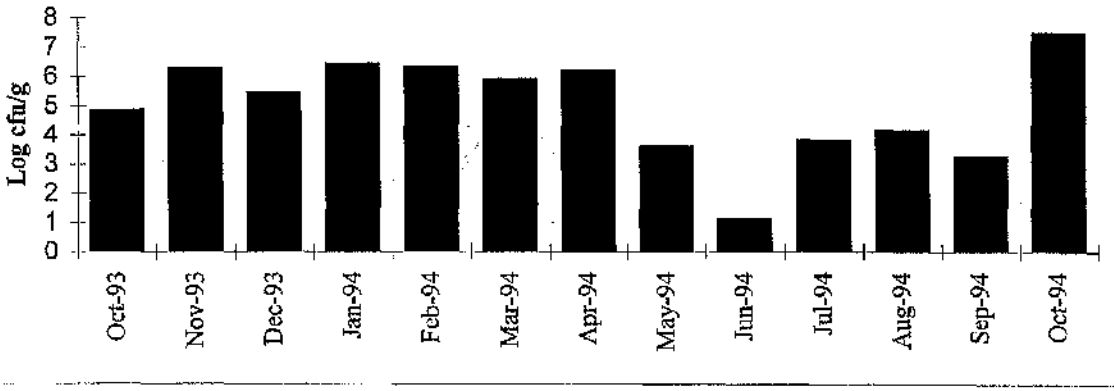
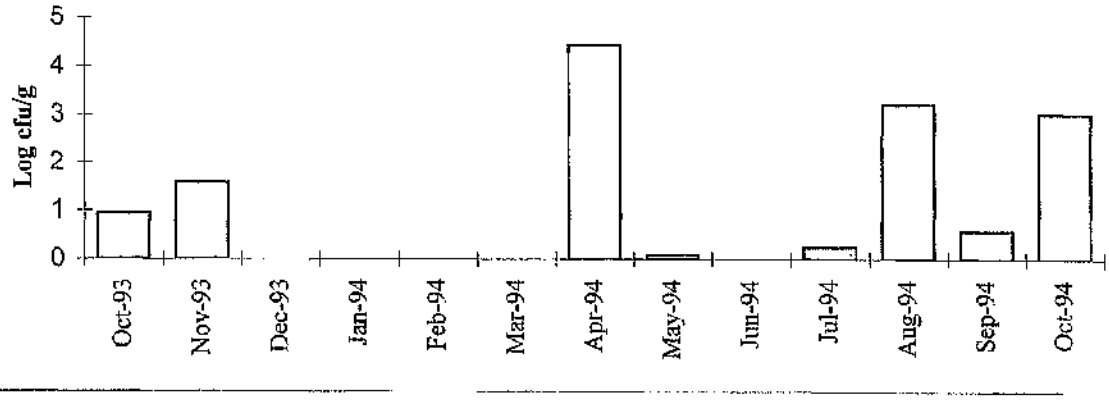


Figure 2:10b Incidence of psychrotrophic sporeformers in faeces samples



Figures 2:11a-c and 2:12a-c Graphs showing incidence of mesophilic, psychrotrophic and thermoduric viable cells or spores respectively, which had been isolated from the sawdust bedding which the cattle were provided with during the period when they were housed indoors.

Figure 2:11a Incidence of mesophiles in winter bedding

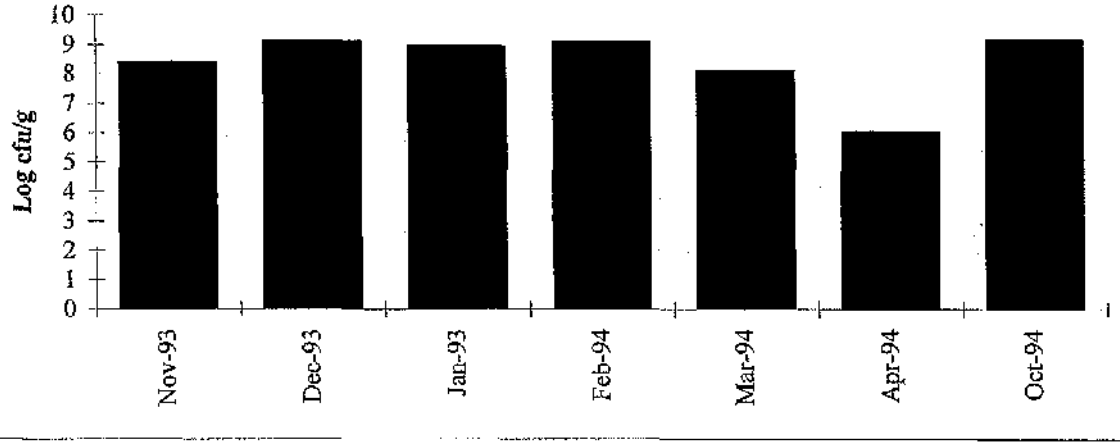


Figure 2:11b Incidence of psychrotrophs in winter bedding

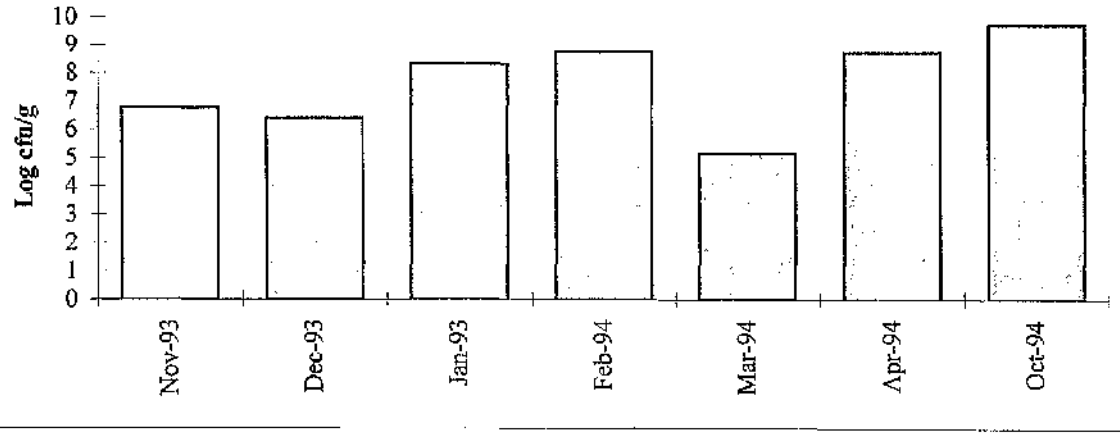


Figure 2:11c Incidence of thermoduries in winter bedding

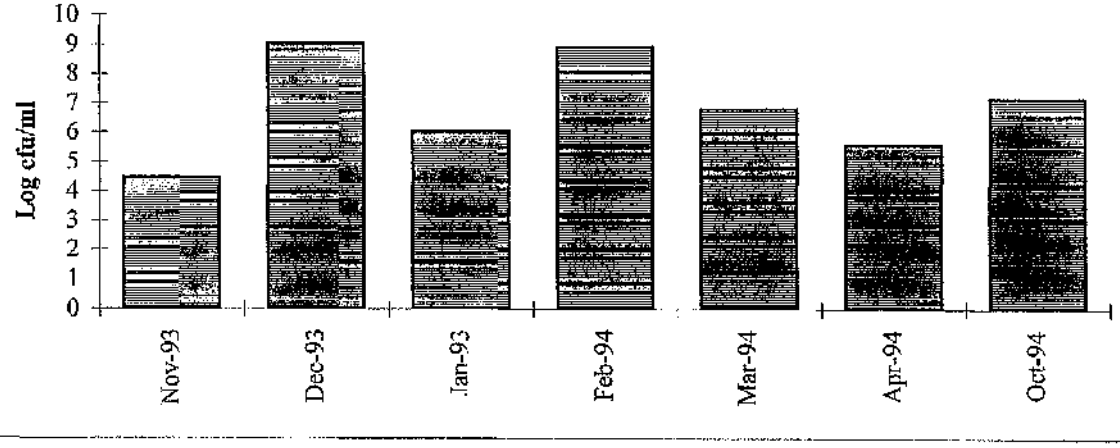


Figure 2:12a Incidence of mesophilic sporeformers in winter bedding

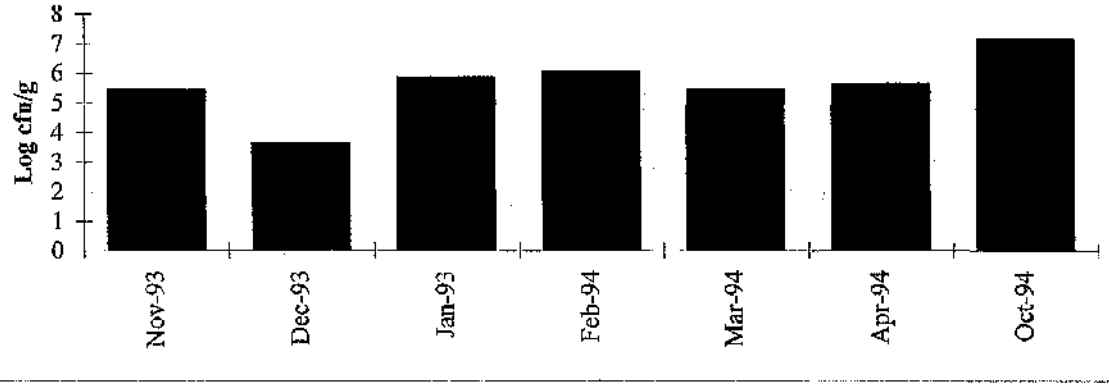


Figure 2:12b Incidence of psychrotrophic sporeformers in winter bedding

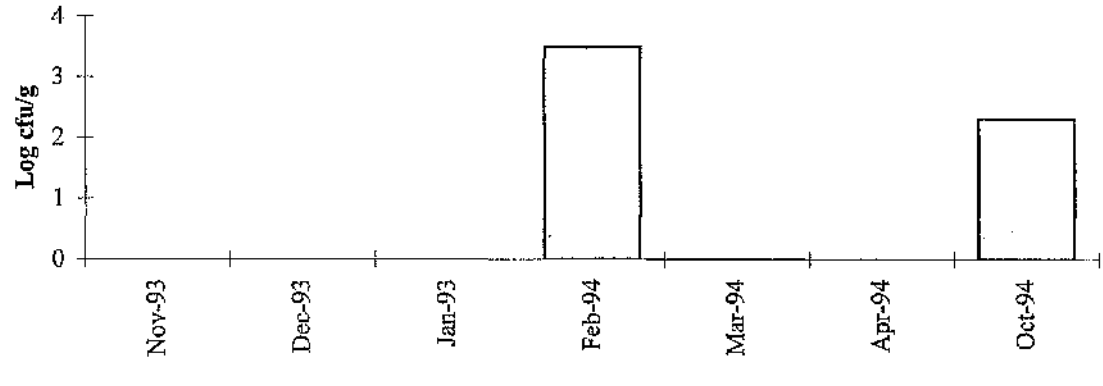
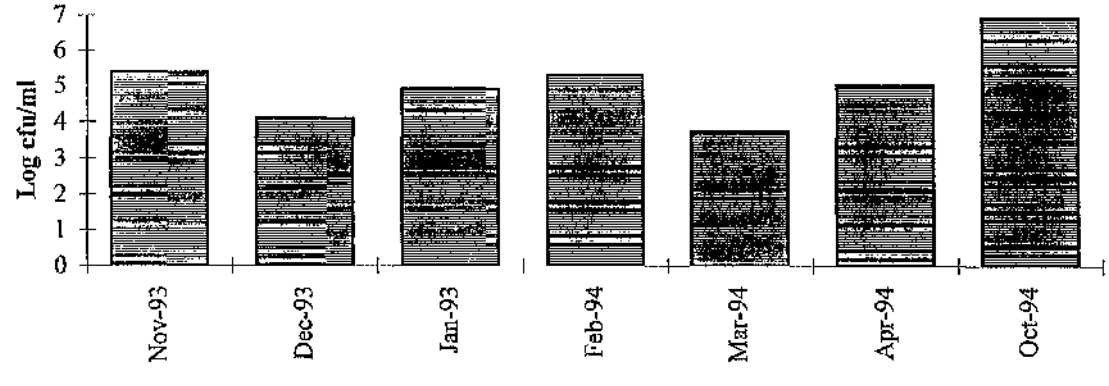


Figure 2:12c Incidence of thermophilic sporeformers in winter bedding



Figures 2:13 a-c and 2:14 a-c Graphs showing the incidence of mesophilic, psychrotrophic and thermoduric viable cells and spores respectively, which were isolated from the feed which the cattle were provided with during the period inwhich they were housed inside.

Figure 2:13a Incidence of mesophiles in winter feed

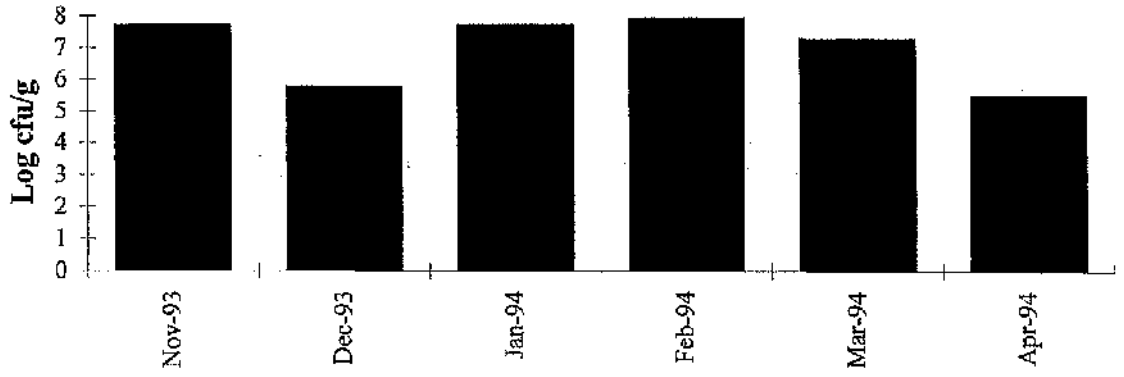


Figure 2:13b Incidence of psychrotrophs in winter feed

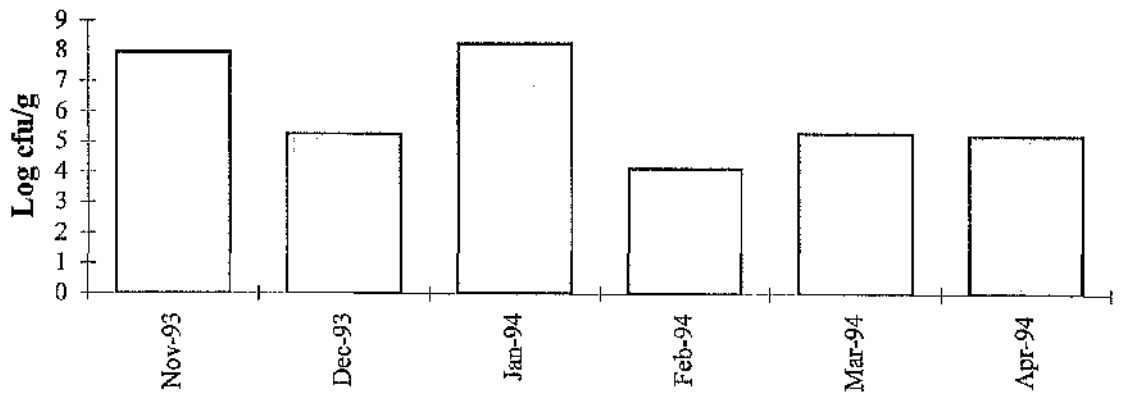


Figure 2:13c Incidence of thermodurics in winter feed

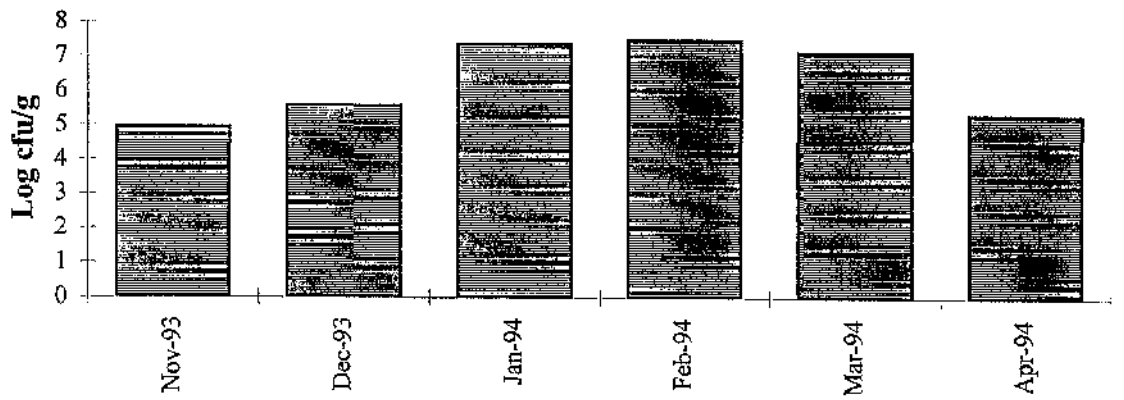


Figure 2:14a Incidence of mesophilic sporeformers in winter feed

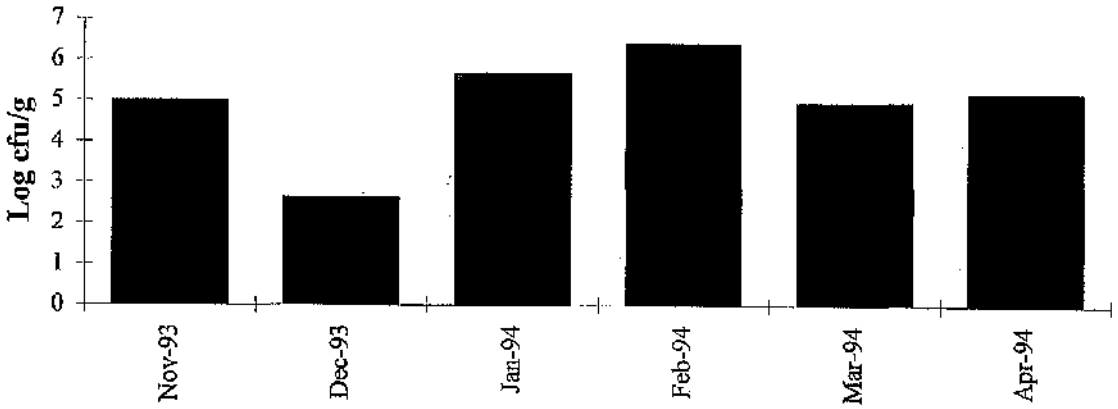


Figure 2:14b Incidence of psychrotrophic sporeformers in winter feed

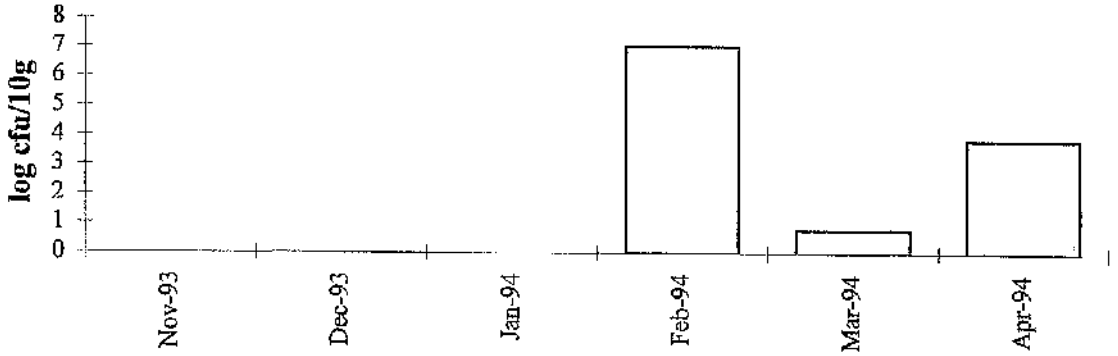
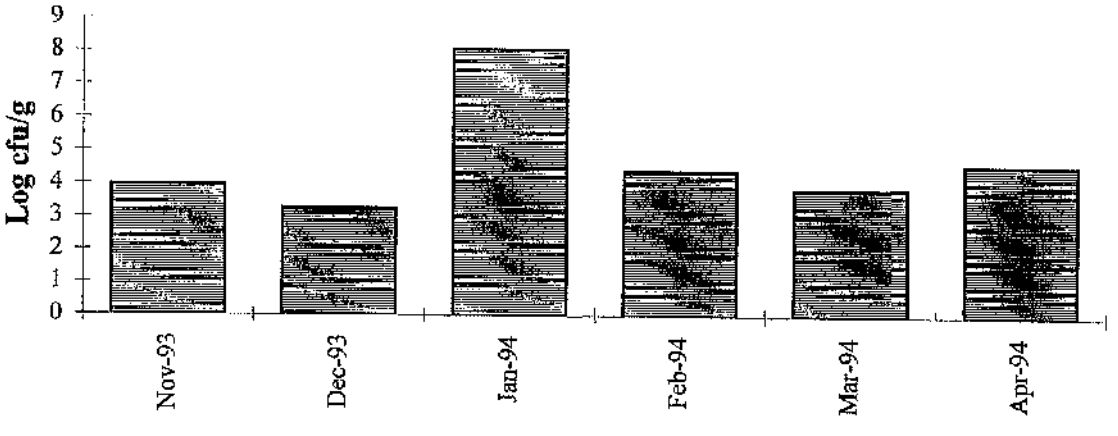


Figure 2:14c Incidence of thermophilic sporeformers in winter feed



Figures 2:15a-c, 2:16a-c, 2:17a-c and 2:18a-c Graphs showing the incidence of mesophilic, psychrotrophic or thermoduric viable cells or spores respectively, which had been isolated from grass or soil samples taken either from a fixed site or a variable site. The fixed site remained constant throughout the survey, and the variable site corresponded to the pasture which the cattle grazed on the day of the survey. From the period November 1993 to April 1994 inclusive, the cattle were housed inside, and therefore only samples from the fixed site were taken.

Figure 2:15a Incidence of mesophiles on fixed and variable site grass

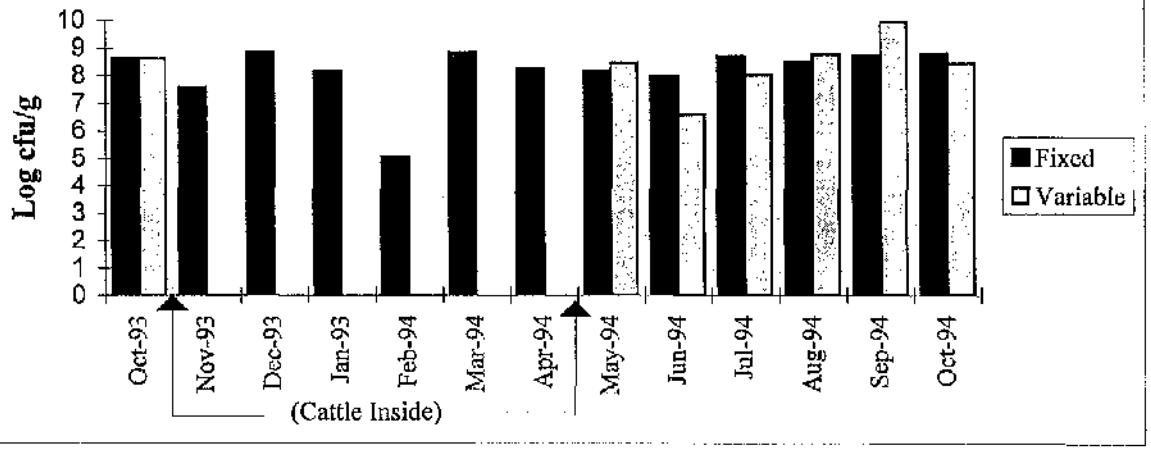


Figure 2:15b Incidence of psychrotrophs on fixed and variable site grass

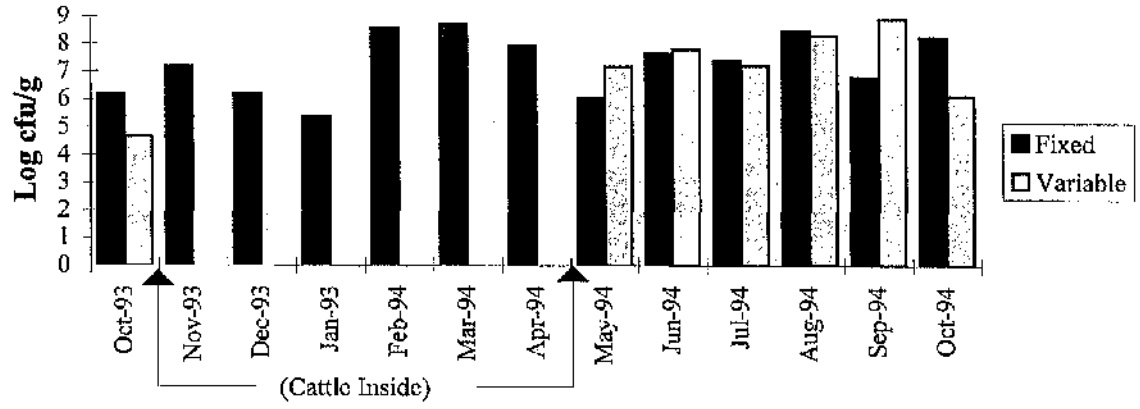


Figure 2:15c Incidence of thermodurics on fixed and variable site grass

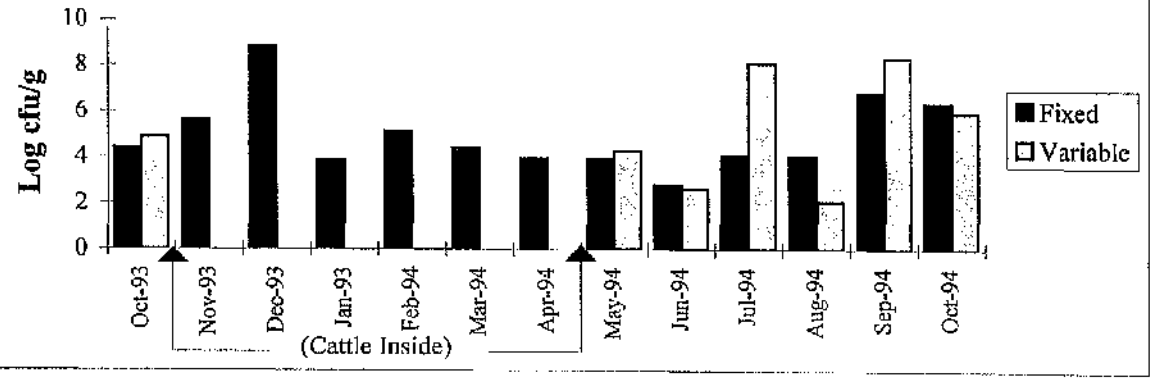


Figure 2:16a Incidence of mesophilic sporeformers on fixed and variable site grass

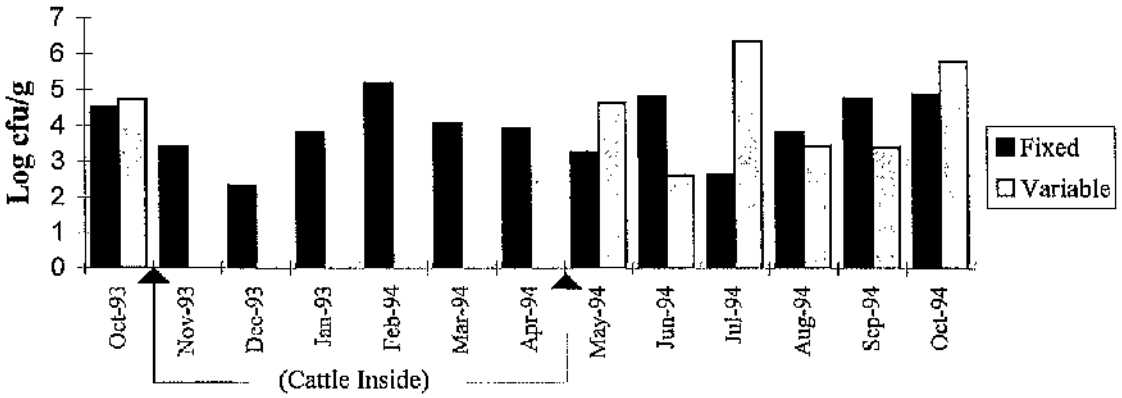


Figure 2:16b Incidence of psychrotrophic sporeformers on fixed and variable site grass samples

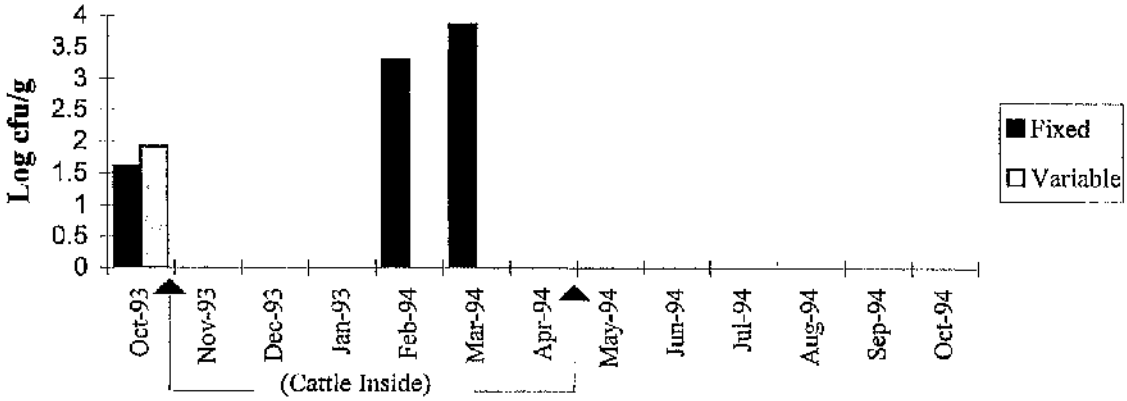


Figure 2:16c Incidence of thermophilic sporeformers on fixed and variable site grass samples

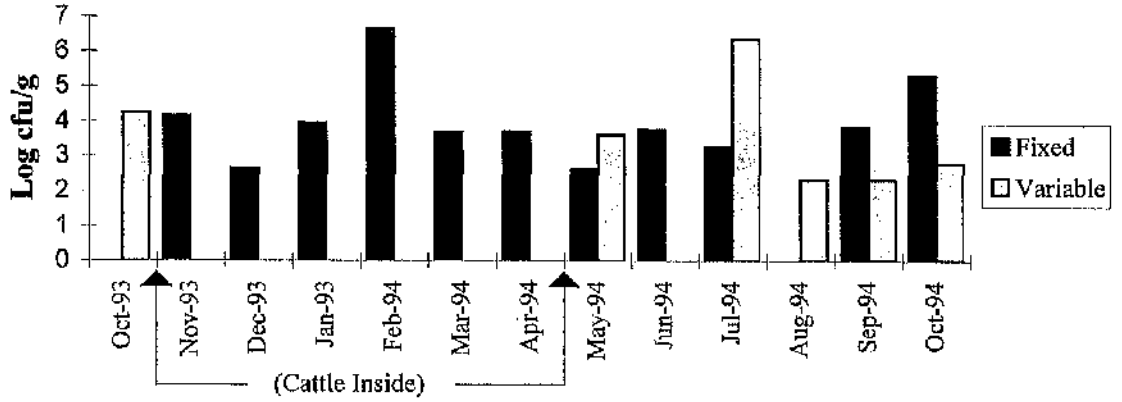


Figure 2:17a Incidence of mesophiles in fixed and variable site soil samples

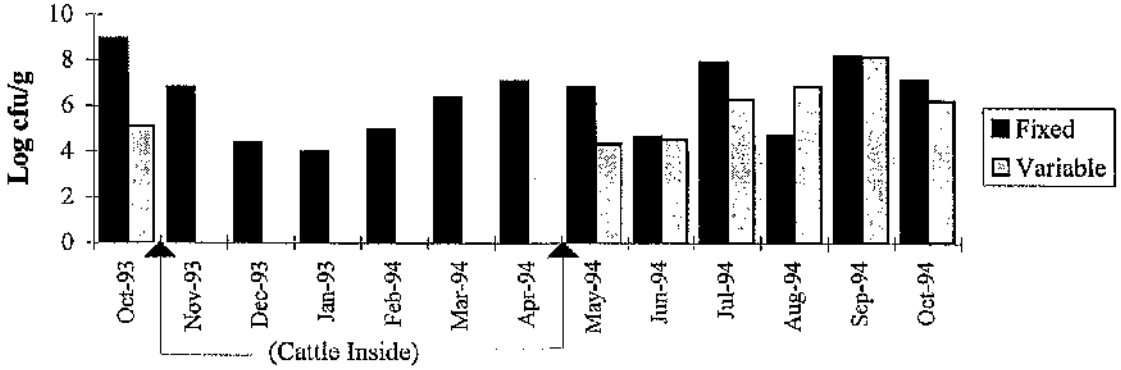


Figure 2:17b Incidence of psychrotrophs in fixed and variable site soil samples

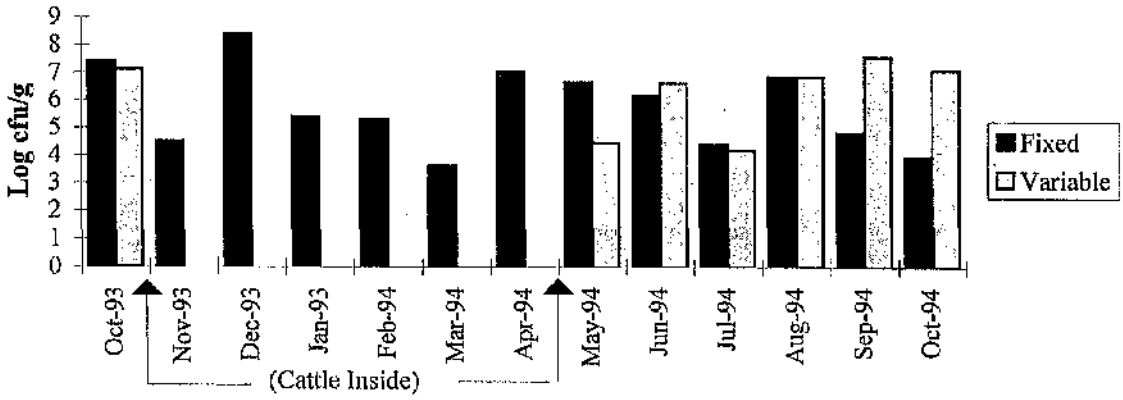


Figure 2:17c Incidence of thermodurics in fixed and variable site soil

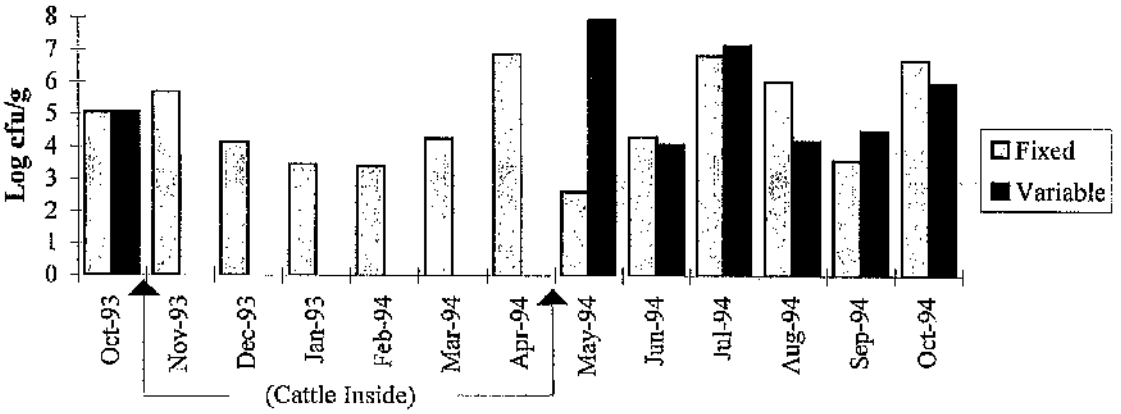


Figure 2:18a Incidence of mesophilic sporeformers in fixed and variable site soil

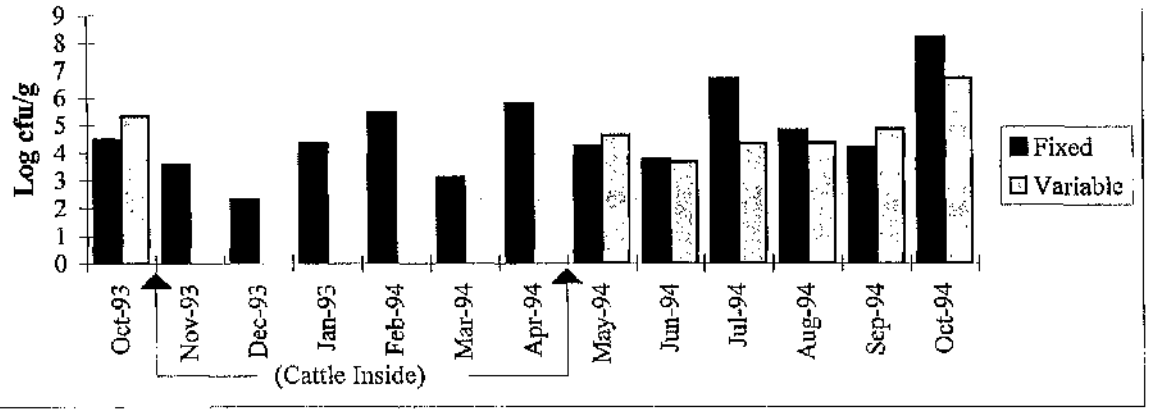


Figure 2:18b Incidence of psychrotrophic sporeformers in fixed and variable site soil

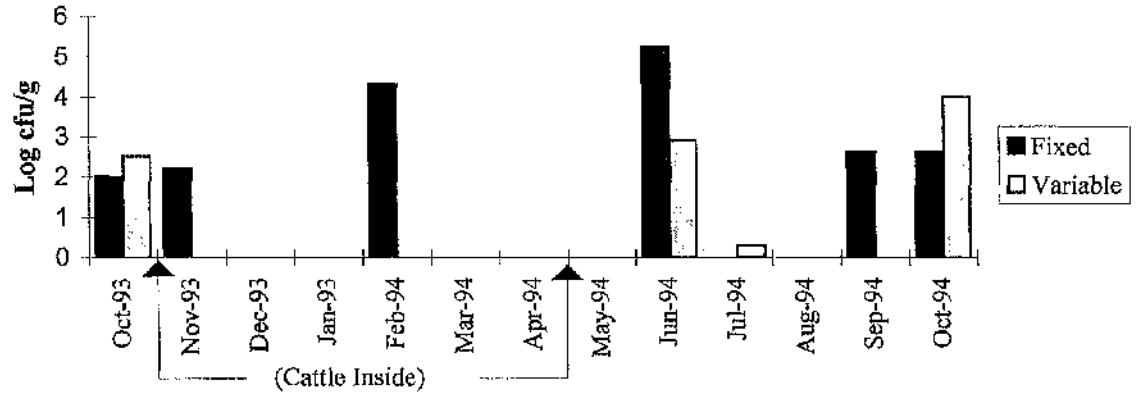
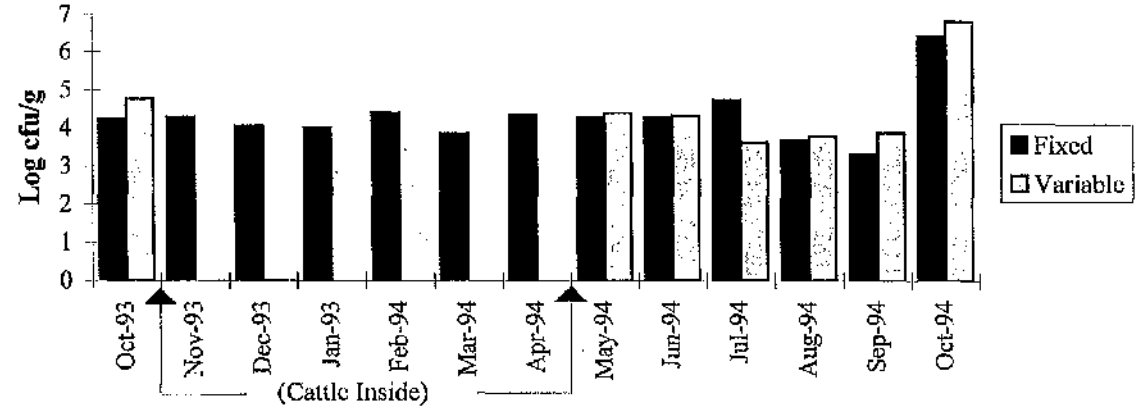


Figure 2:18c Incidence of thermophilic sporeformers in fixed and variable site sporeformers



Figures 2:19a-b Annual incidence of psychrotrophic *Bacillus cereus and Bacillus mycoides* isolated from any source during survey

Figure 2:19a Annual incidence of psychrotrophic *B.cereus*

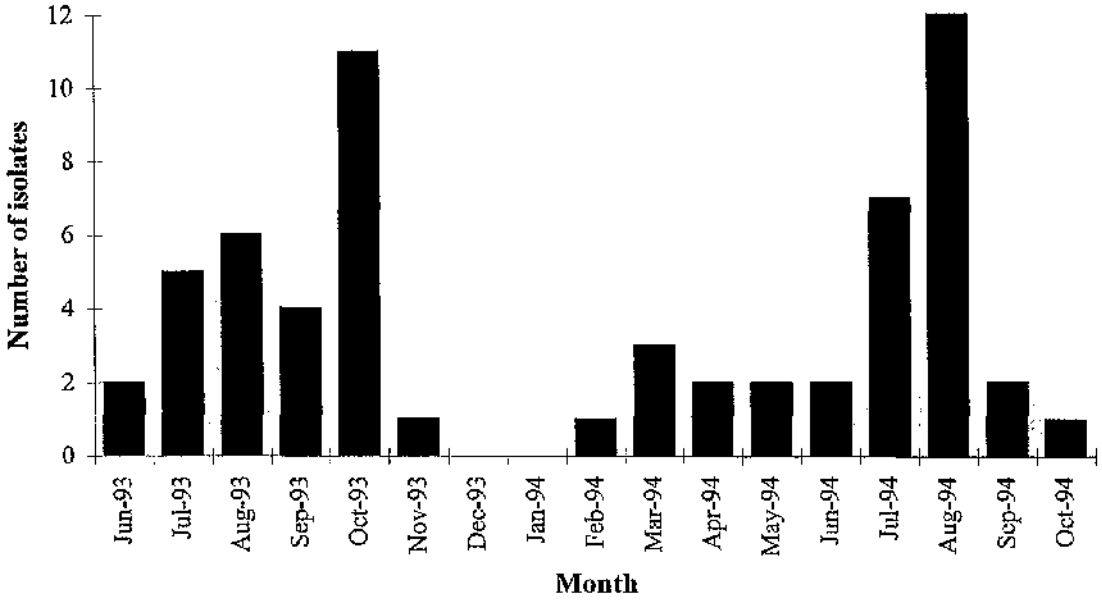


Figure 2:19b Annual incidence of psychrotrophic *B.mycoides*

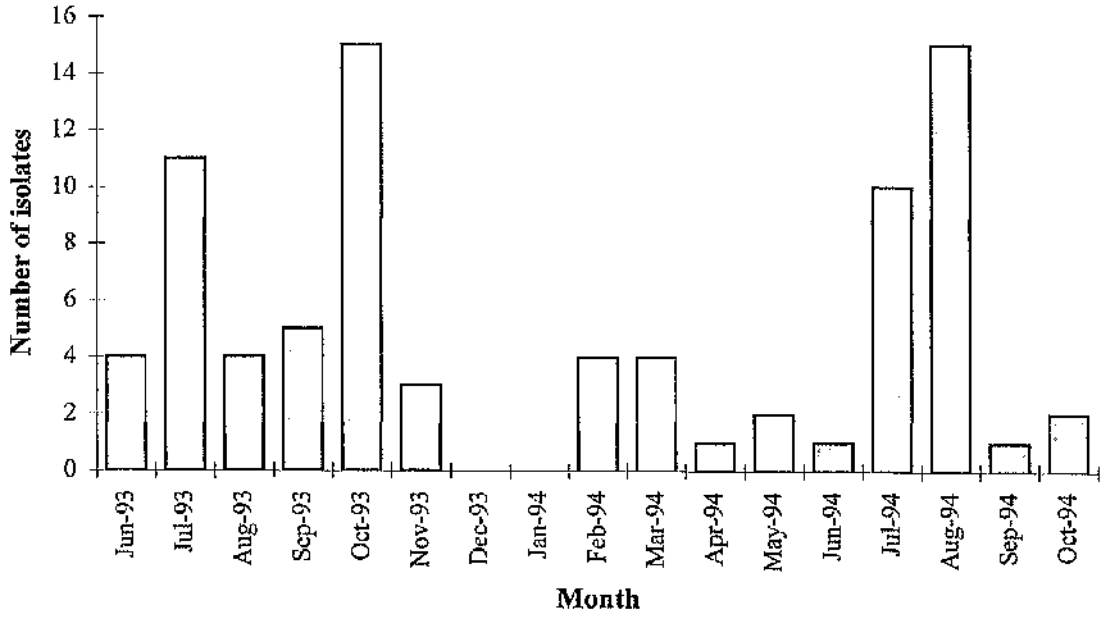


Figure 2:20a Mesophiles in detected in air samples from the milking parlour

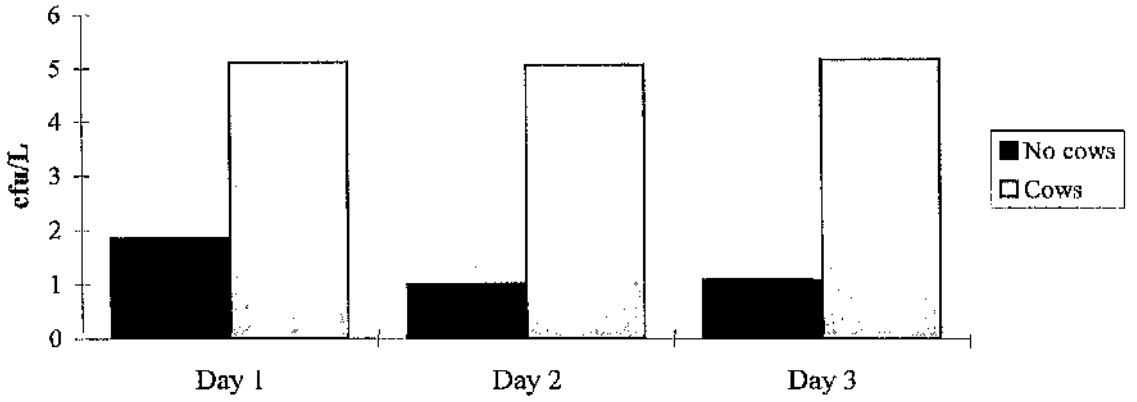


Figure 2:20b Psychrotrophs detected in air sample from the milking parlour

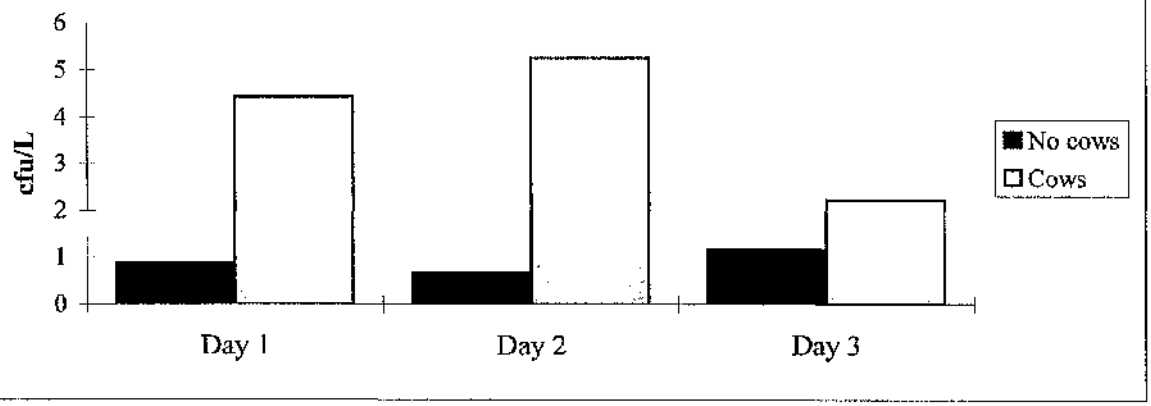
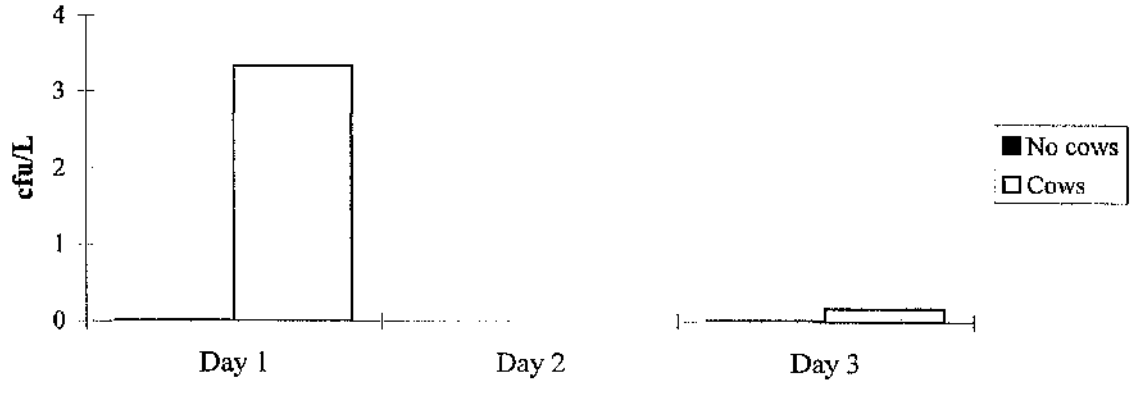


Figure 2:20c Mesophilic sporeformers detected in air sample from the milking parlour



Figures 2:21a-c and 2:22a-b Mesophilic, psychrotrophic and thermoduric viable cells or spores respectively, isolated from various points along the sterilised milking machine lines.

T/C = Teat cup clusters

Line = Pipeline connecting milking machine teat cup clusters with the bulk tank

B/T = Bulk tank

Figure 2:21a Mesophiles isolated from sterilised milking machines

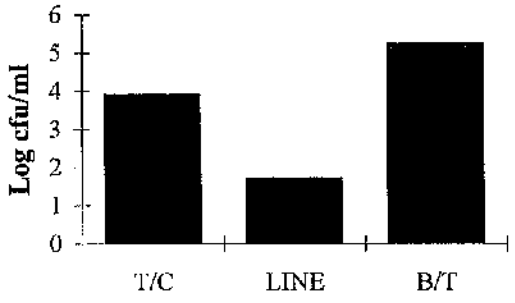


Figure 2:22a Mesophilic sporeformers isolated from sterilised milking machines

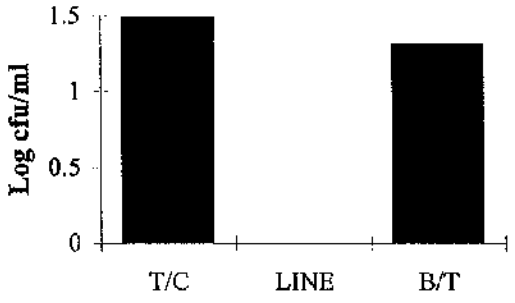


Figure 2:21b Psychrotrophs isolated from sterilised milking machines

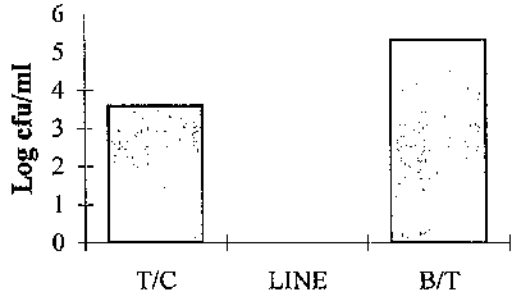


Figure 2:22b Psychrotrophic sporeformers isolated from sterilised milking machines

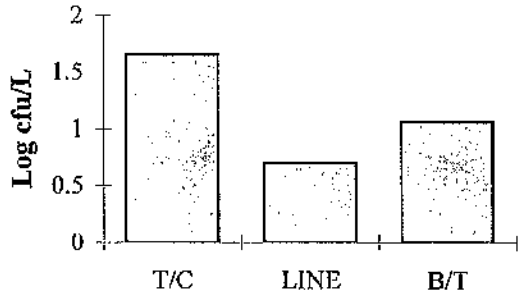


Figure 2:21c Thermodurics isolated from sterilised milking machines

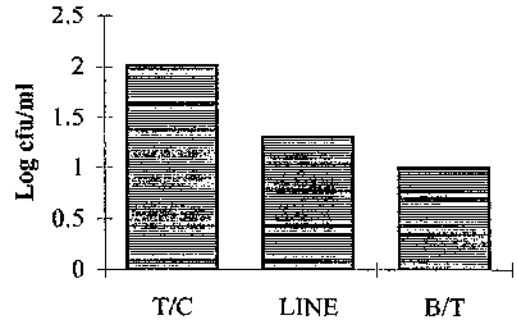


Figure 2:23a-b Comparison of numbers of psychrotrophic sporeformers isolated from raw milk and udder wash samples collected at morning (06:00) and afternoon (14:00) milking sessions

Figure 2:23a Comparison of psychrotrophic sporeformers in morning and afternoon milk samples

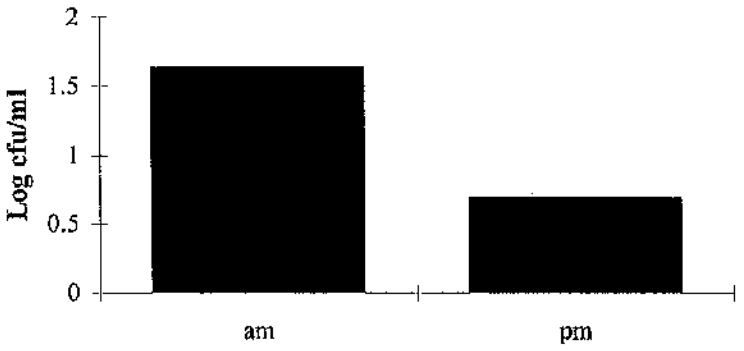
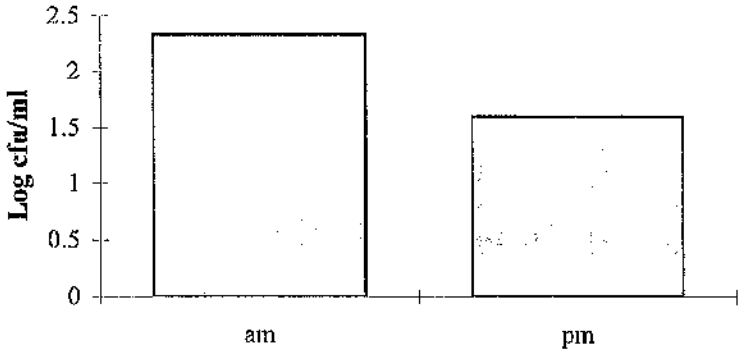


Figure 2:23b Comparison of psychrotrophic sporeformers in morning and afternoon udder wash samples



Figures 2:24a-b Effect of husbandry of cattle on bacterial populations in raw milk udder washings.

O/C = Pasture conditions on which the general herd were grazing

S/P = Short pasture conditions

Byre = Byre housed cattle

Figure 2:24a Effect of husbandry on bacterial populations in raw milk

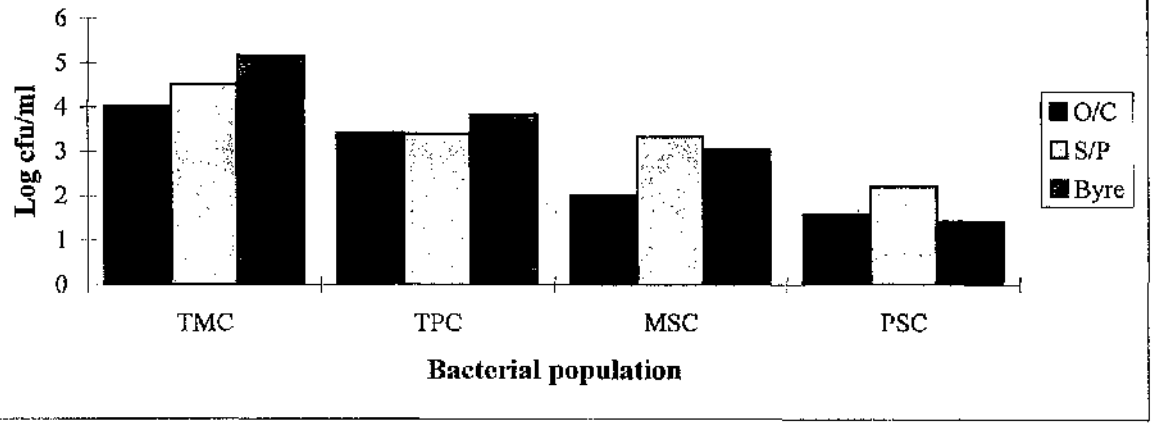
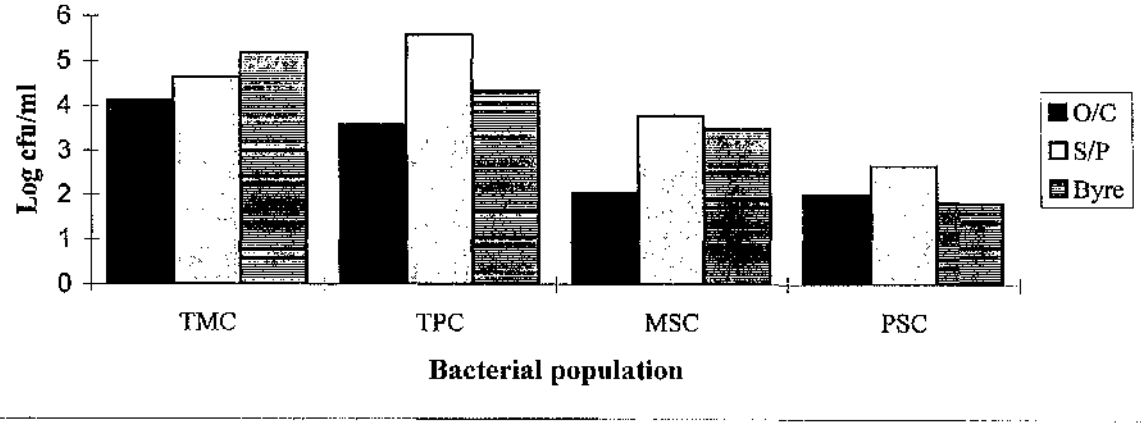


Figure 2:24b Effect of animal husbandry on bacterial populations in udder washings



Figures 2:25a-b and 2:26a-b Effect of pre-milking teat preparation (washing or washing and drying) on udder wash and raw milk samples.

Figure 2:25a Effect of teat washing on bacterial populations of udder washes

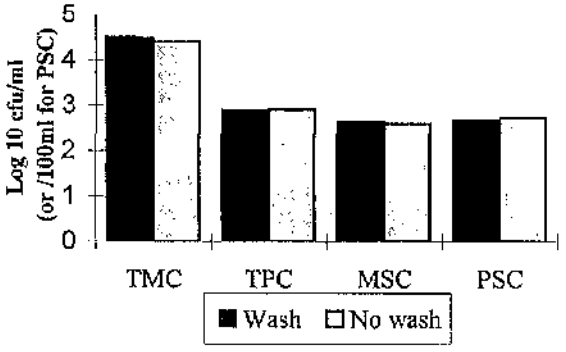


Figure 2:25b Effect of washing & drying teats on udder washes

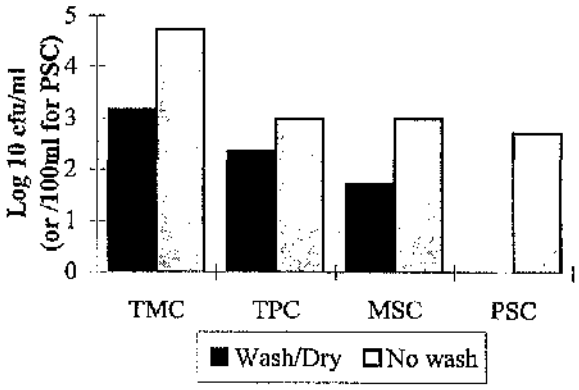


Figure 2:26a Effect of teat washing on milk samples

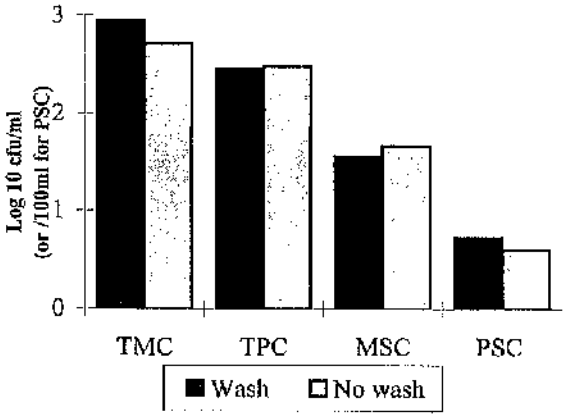


Figure 2:26b Effect of teat washing and drying on milk samples

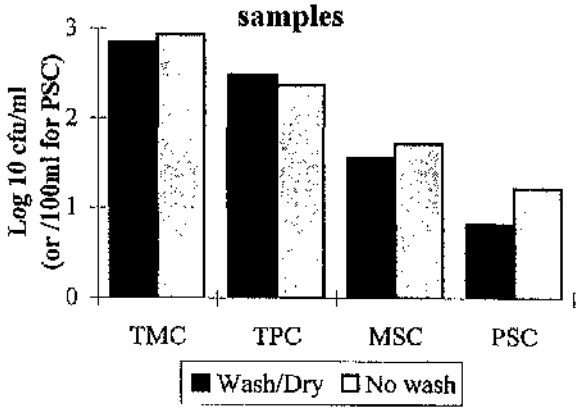
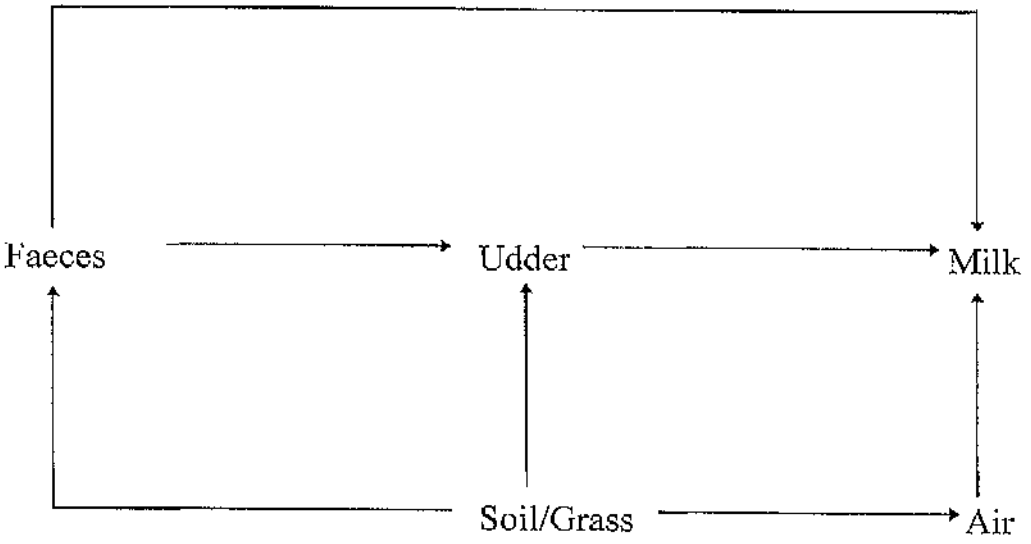


Figure 2:27 Proposed routes of entry of *Bacillus* spp. into raw milk



CHAPTER 3.

TOXICITY STUDIES

ON *BACILLUS CEREUS*

AND OTHER *BACILLUS* SPP.

3:1 INTRODUCTION TO TOXCICITY STUDIES ON *BACILLUS CEREUS* AND OTHER *BACILLUS* SPECIES

Bacillus cereus is a problem to the food industry for several reasons. It causes spoilage, especially in milk and dairy products. It is also associated with two distinct types of food poisoning.

The first type of food poisoning is a diarrhoeal syndrome. This is characterised by a delayed onset of 8 to 16 hours after consumption of the incriminated food (Kramer and Gilbert, 1989). It has been associated with a wide range of food stuffs including milk, sauces, pasta, desserts and cakes. The sufferer has symptoms of abdominal cramps and watery diarrhoea, which last for 12 to 24 hours (Kramer and Gilbert, 1989).

The second syndrome is caused by a heat stable emetic toxin. Onset following consumption is <1-5 hours (Kramer and Gilbert, 1989). The emetic syndrome is characterised by nausea and vomiting, which often is accompanied by diarrhoea, although this is not the major symptom (Kramer and Gilbert, 1989; Granum 1994). It is most commonly associated with rice dishes (Kramer and Gilbert, 1989).

Traditionally, the detection of *B.cereus* toxins has been through the use of *in vivo* biological tests. The ligated rabbit ileal loop test (Spira and Goepfert, 1972), and vascular permeability reactions in rabbit or guinea pig skin may be used to test for the diarrhoeal toxin (Glatz and Goepfert, 1973). Detection of the emetic toxin can be made by feeding rhesus monkeys with rice culture slurries, and observing

physiological effects (Granum, 1994). Toxin can be detected by cell cytotoxicity studies (Hughes *et al.*, 1988; Wong *et al.*, 1988; Shinagawa *et al.*, 1991; Hostiacka *et al.*, 1992).

The need in the food industry for *in vitro* assays has prompted the development of two commercial test kits, OXOID BCET-RPLA and TECRA BDE VIA, which use antibody-based reactions to detect components of the diarrhoeal toxin.

In this work a modified cytotoxicity assay for detection of diarrhoeal and emetic toxins is described. In addition, assessment was made of the ability of *B.cereus* isolates from raw milk, the farm and dairy environment to produce toxins. Measurements were made using OXOID BCET-RPLA, TECRA BDE VIA, and the modified cell cytotoxicity assays. Isolates of other species of the genus *Bacillus* were also assessed for their ability to form toxins using the same methods. These *Bacillus* spp. were isolated from either raw milk and the dairy environment in Scotland, or from raw milks and fermented sausage in Spain.

3:2 TOXICITY STUDIES METHODS

3:2:1 Culture of Bacillus spp.

Single colonies were selected from pure cultures and inoculated into 10ml brain heart infusion broth (BHI) for overnight culture. An aliquot (250µl) of the overnight culture was inoculated into 25ml BHI or 10% sterile skim milk (SMP) (OXOID), and incubated at 30°C. Flasks were shaken (200rpm) using an orbital incubator (L.H. Engineering Co. Ltd., Stoke Poges). Cultures were harvested after 18h, by centrifuging (5000 ***g***, 4°C for 30 min). The culture supernatants were removed and filtered through sterile 0.45µm porosity filters (Millipore) to remove residual bacterial cells.

Before testing for emetic toxin, the sterilised culture supernatants were heated for 10 min at 80°C.

3:2:2 Cell cytotoxicity

3:2:2:1 Tissue culture maintenance

The immortal monolayer cell lines Chinese hamster ovary (CHO) and INT 407 cells were used in the cytotoxicity assay for the detection of *Bacillus* spp. toxins. The tissue culture cells were cultured in 30ml RPMI 1640 media (GIBCO, Life Technologies Ltd., Paisley) supplemented with 5% foetal bovine serum (FBS), 1% 200mM glutamine and 1% penicillin (streptomycin), in 260ml tissue culture flasks (InterMed, Denmark). Cells were incubated at 37°C, in a humid atmosphere containing 5% CO_2.

The cells were subcultured by pouring off the culture media, and washing the cell

layer with approximately 15ml sterile phosphate buffered saline (PBS) (pH7.3) at room temperature, in order to remove residues of media from the surface of the cells. The cells were incubated with approximately 5ml trypsin (0.25%) for 3min at 37°C in a humid atmosphere containing 5% CO_2 to remove them from the surface of the flask. Trypsin enzymatically degraded the proteinaceous bonds by which the cells adhered to the flask surface. Having been removed from the flask surface, approximately 1ml of cell suspension was inoculated into a fresh tissue culture flask, containing 30ml of RPMI 1640 media supplemented with FBS (5%), glutamine (1%) and streptomycin (1%). The cells were subcultured by this method every 3-4d, to maintain an actively growing monolayer.

3:2:2:2 Setting up cell cytotoxicity plates

96 well, flat bottomed, gamma-irradiated, microtitre plates with lids (InterMed, Denmark) were used for cell cytotoxicity studies.

Column 1 of the plate was used for a positive control (Figure 3.1). For this 25µl of 0.1% triton-X 100 was added to each well in the column. Triton-X is a surfactant which kills the inoculated tissue culture cells, thus leaving a background value corresponding to 100% toxicity. The cells in the wells of column 12 were used as a negative control (Figure 3.1). These cells were cultured under normal culture conditions, and represented a 100% survival and proliferation level.

Sterile PBS (25µl) was added to all of the wells in columns 3-12 (PBS is added to the wells in column 12 to maintain a standard volume in the wells) (Figure 3.1). Into columns 2 and 3, 50µl filter sterilised, cell free bacterial culture supernatant was added. 25µl from column 3 was pipetted into column 4 and mixed. To prepare a dilution series 25µl was removed in turn from column 4, and added to the wells in

column 5; serial dilutions were performed sequentially in this manner to column 11. At column 11, 25µl volume was removed after mixing, and discarded to maintain standard volume. The dilution series obtained is thus an arithmetic progression from a 2 to a 256 fold dilution.

The wells were inoculated aseptically in a laminar flow cabinet (BassAire, Southampton) with 100µl cell suspension containing $5x10^4$ cells/ml. The plates were incubated under the incubation conditions described (section 3:2:2:1) for 72h.

3:2:2:3 Cytotoxicity assessment

3:2:2:3a Visual assessment of the cytotoxic effects of *Bacillus* spp. cell free culture supernatants

After 72h incubation, plates were scored by visually assessing cell growth and morphology under an inverted light microscope (magnification x100) .

Cells were awarded a score from 0-4 depending upon their condition and amount of growth. "4" corresponded to complete toxicity, and other scores ranged through to "0", which corresponded to complete survival and proliferation of the cells.

3:2:2:3b Metabolic assessment of cytotoxic effects of *Bacillus* spp. cell free culture supernatants using MTT

Following the 72h incubation of cells, 50µl of an aqueous solution of 3-(4,5,-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT) (2mg/ml) was added to each well of the microtitre plate. The plates were then incubated for a further 4h in the tissue culture incubator. After this time, the liquid media in the wells was pipetted off,

and 50µl dimethyl sulphoxide (DMSO) was added to each well. The DMSO lysed the cells, releasing metabolised MTT into the well. When MTT has been metabolised it changes colour from pale yellow to purple. Only actively metabolising cells can produce this colour change of MTT, and it was this colour change which was detected in this assay. The plates were then read at 540nm on an ELISA plate reader. The toxic effect of the cell free bacterial culture supernatants on the cells was calculated using

$$1-(OD_{Negative\ Control}-OD_{Test})\ x100\%$$

Samples were considered to be toxic if the optical density of the test well was ≥20% less than that detected in the negative control wells.

3:2:3 TECRA immunological assay

The TECRA *Bacillus* Diarrhoeal Enterotoxin (BDE) Visual Immunoassay is an immunological assay, using an ELISA method, which has been developed to provide a rapid specific screening system for the *in vitro* detection of *B.cereus* diarrhoeal enterotoxin in food and food-related samples. The manufacturers do stress that the results of the test should be confirmed using *in vivo* methods.

3:2:3:1 Sample Preparation

Bacillus spp. cultures were prepared as for the other toxin tests described (3:2:1). Cultures were grown in either sterile 10% w/v skim milk (SMP) (OXOID, Unipath, Basingstoke) or brain heart infusion (BHI) broth (OXOID).

Sample additive supplied in kit (50µl) was added to the supernatant, and thoroughly mixed. The sample additive contains a detergent, which is added prior to testing in the

visual immunoassay in order to minimise non-specific binding of food components in the test wells.

3:2:3:2 Reagent Preparation

Kit reagents (positive control, conjugate, and substrate) were made up in accordance with the instructions of the manufacturer, supplied with the kit. The positive control contained a suspension of diarrhoeal enterotoxin (BDE) antigen which the TECRA kit reacts against; the positive control comes in a dried format, which is then resuspended using supplied diluent. The negative control was the same diluent that had been used to dissolve the positive control, but without additions. The conjugate is an enzyme labeled antibody, specific for BDE. The conjugate binds to associated BDE, and its enzyme reacts with the substrate. The substrate is initially colourless, but the conjugate enzyme, can cleave the substrate producing a green coloration.

3:2:3:3 Performing the TECRA Assay

Before the assay may be conducted the antibody coated test wells, have to be presoaked. This was done by filling the wells with the wash solution (supplied with the kit), and allowing them to stand for 10 min at room temperature (20-25°C).

The wells were then emptied, and samples (200µl) and positive and negative controls (200µl) were pipetted into individual wells. The wells were covered to avoid evaporation, and incubated for 2h at 37°C.

Following incubation, the wells were emptied again, and washed four times using the provided wash solution.

Into the empty wells conjugate (200µl) was added. The conjugate is an enzyme labeled antibody, which is specific for the antigen which the manufacturers claim is the diarrhoeal enterotoxin. The wells were again covered, and incubated for 1h at room temperature (20-25°C) to allow the conjugate to bind.

The wells were emptied again, and washed 5 times. Then 200µl of substrate (supplied with the kit) was added into each well. The substrate is clear in colour when it is added into the test wells. The wells were incubated at room temperature (20-25°C) for a minimum of 30 min. If conjugate had bound in the wells, the enzyme which was linked to the conjugate, reacted with the substrate, changing its colour from clear to green.

If after the incubation period, the colour of the positive control well was equivalent to Panel 4 of the supplied colour card, or had an absorbance reading of at least 1.0 at 405nm on a plate reader, then the test proceeded to the next step. If the criteria for the positive control was not met at this point, the wells were incubated for an additional period of up to 15 min. If after this time the control had still failed to reach the designated reaction level the test is void.

When the positive control reaction had reached the correct level, 20 µl of stop solution (supplied with kit) was added to each well. The stop solution prevents further colour development from taking place. The contents of the wells were gently mixed.

The results were then read against the provided colour card and also on a plate reader at 405nm. For the test to be valid in addition to the positive control reaching its specified reaction level, the negative control had to be of a colour within the range specified as negative on the colour card chart, or have an absorbance of less than 0.2 at 405nm.

The sample was considered negative if its colour reaction fell within the negative range on the colour chart, or if its absorbance reading was less than 0.2 at 405nm.

A positive sample produced a colour reaction in the positive range on the colour chart, or had an absorbance equal to or greater than 0.2 at 405nm.

3:2:4 OXOID test kit for the detection of diarrhoeagenic toxin

Bacillus spp. tested for toxin production using the OXOID BCET-RPLA were cultured at 30°C for 18h in an orbital incubator (200rpm) in BHI broth. Cell free bacterial culture supernatants were prepared as described (3:2:1).

Two rows of 8 wells on a 96 V-well microtitre plate (InterMed, Denmark) were required to test a cell free culture supernatant for toxicity using the OXOID kit. Sample diluent (25 µl) supplied with the kit was added to 7 wells of the 8 in each row. Test sample (25 µl) was added into the first and second well of each column. Serial dilutions of test sample were made by removing 25µl with a pipette, from the second well of each column, and mixing this with the 25µl of diluent in the third well of each column. A further 25µl was removed from the third well in each column, and the same dilution procedure was carried out down until the seventh well. From the seventh well, 25µl test solution was removed after mixing, and this was discarded. The eighth well is left containing only diluent for both columns. This is used as a negative control. The reconstituted control enterotoxin, supplied with the kit was used as a positive control. This agglutinates with the sensitised latex, and verified the reactivity of the sensitised latex.

Polystyrene latex reagents were supplied ready to use in the test kit. These needed to

be shaken to allow thorough mixing before dispensing. The sensitised-latex particles were created using purified antiserum taken from rabbits, that had been immunised with purified *B.cereus* enterotoxin. The control-latex consisted of the polystyrene latex particles, sensitised with non-immune rabbit globulins. Into each well of the first test row, 25µl of sensitised latex were added. Into the second row 25µl of control latex was added. The plate was then agitated by hand to mix the test, and sealed to avoid evaporation. The plate was incubated at room temperature for 24h.

Following incubation, each well was examined for agglutination. The agglutination pattern was assessed by comparison to the illustration provided by OXOID (Technical Information leaflet), and was scored from "0" which represented no reaction, through to "+++" for a highly toxic response.

The results in the row of wells containing sensitised latex were compared with those in the latex control row. The latex control wells had to give a negative result, as must the diluent only wells, in order for a positive result in the sensitised row to be considered positive. If either of the controls were also positive, then the test would be considered void.

3:3 TOXICITY STUDIES RESULTS

3:3:1 Preliminary work

A preliminary series of experiments were carried out in order to develop a cell cytotoxicity assay for the detection of *Bacillus cereus* diarrhoeal and emetic toxins.

3:3:1:1 Cell line

Two different cell lines were compared to assess their sensitivity to *B.cereus* toxins. The cell lines used were INT407, which is a human intestinal cell line, and Chinese hamster ovary (CHO) cells. Both cell lines are immortal and form monolayers in tissue culture.

The cell free culture supernatants of *B.cereus* strains NCTC 11143 (emetic) and NCTC 11145 (diarrhoeagenic) were used to test the cell lines. The cell lines responded in different ways to the toxic effect of the cell free culture supernatants. The CHO cells became detached from the surface of the flat bottomed 96 well plate in which the assays were performed (Figure 3:2a). The cytoplasm of the INT407 cells appeared granular under an inverted microscope (x100), and the cells became distended (Figure 3:2b). However, although the form which the reaction took was different in the two cell lines, there was 99%

correlation between the results obtained from INT 407 and CHO cell lines (Figure 3:3a-d). The CHO cell line was therefore chosen for further toxicity experiments because the cells grew more rapidly. This was advantageous in cell line maintenance because the stock flask of cells could be replenished more quickly.

No significant differences ($P>0.05$) were detected between the results obtained from repeat cell cytotoxicity assays performed on the same culture supernatants. High correlations (>95%) were also found between results from repeat culturings of NCTC 11143 and NCTC 11145, when tested on a single preparation of tissue culture cells. Experimental repeats which had separate preparations of both bacterial supernatants and tissue culture cells demonstrated good correlation (>95%) (Figures 3:3a-b). The greatest differences between repeats were found in BHI cultured supernatants; they occurred amongst the highest dilutions (128 and 256) in unboiled samples, and in the most concentrated samples (up to 16 fold dilution) of the boiled supernatants (Figures 3:3a-b).

The lowest dilution which produced a positive response was used as an indication of the toxicity of the test isolate. However, because a cut off point for positive toxic effect had to be assigned, some variation in results arose due to border line responses. For instance, with the boiled SMP samples for NCTC 11143 (Figure 3:3a), although the average result was positive at 256 dilution, the deviation from the mean falls below the 20% toxicity cut off point. Therefore, if the data are examined as a single value at the lowest positive dilution, a

misleading indication of toxicity may sometimes be obtained. Nevertheless, it was considered that summary information on toxicity given in this manner was an informative way of considering results.

3:3:1:2 Comparison of visually scored toxicity results with metabolic assessment

Visual scoring and measurement of total metabolic activity of cells using 3-(4,5,-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT) were compared as methods of assessing cell cytotoxicity. Toxicity results were similar for both visual scoring and metabolic assessment in both INT 407 cells and CHO cells (Figures 3:3a-d and 3:4a-d).

3:3:1:3 Cell concentration

In the cytotoxicity assay, inoculated cells were incubated in the presence of cell free culture supernatants for 72h before the metabolic status of the cells was measured. Different concentrations of inoculated cells ranging from $1x10^4$ to $5x10^5$ cells/ml were assessed against cell free culture supernatants of NCTC 11145. It was found that the different concentrations influenced the toxicity results with CHO cells (Figure 3:5). Cells inoculated at $1x10^4$ cells/ml (log 4 cells/ml) gave the highest toxicity results, still showing 60% toxicity at 256 dilution with CHO cells. The cell concentration $5x10^4$ cells/ml (log 4.7 cells/ml) was chosen for use in the cell assay, because this number consistently produced a confluent lawn of growth in the positive control wells after 72h incubation. At a

cell concentration of $1x10^4$ cells/ml the cytotoxicity assay appeared to be more sensitive to toxic effect than at higher cell concentrations; this was possibly because there were fewer cells per unit amount of toxin. However, it was considered that at the higher concentration of cells ($5x10^4$ cells/ml) a better spectrum of toxicity was recorded, rather than just the high toxicity responses achieved using lower cell concentrations ($1x10^4$ cells/ml).

3:3:1:4 Effect of storage conditions on toxicity of cell free culture supernatants

The effects of storage on the toxicity of cell free culture supernatants of NCTC 11143 and NCTC 11145 were examined. Culture supernatants were measured for toxicity in their fresh state, after storage at 6°C for 72h and storage at -70°C for 7d (Figure 3:6a-d).

Samples of NCTC 11143 (Figure 3:6a) grown in SMP were unaffected by freezing, but lost approximately 30% of their toxic effect when stored at refrigeration temperatures. The toxicity of boiled supernatants of NCTC 11143 cultured in SMP were unaffected by storage at either 6°C or -70°C (Figure 3:6b).

The culture media used for the growth of the bacteria was important for the retention of toxic effect on storage. Culture supernatants of NCTC 11145, grown in SMP and stored at 6°C for 72h (Figure 3:6c), lost approximately 60% of toxic effect over the dilution series. However, when supernatants derived from the

BHI culture of NCTC 11145 were stored under the same conditions of 6°C for 72h, they retained full toxicity (Figure 3:6d). The toxicity of cell free culture supernatants of NCTC 11145 grown in BHI or SMP were unaffected by freezing for 7d at -70°C (Figures 3:6c-d).

3:3:1:5 Effect of dialysis of cell free culture supernatants on toxicity

When NCTC 11143 and NCTC 11145 were grown in SMP, dialysis of their culture supernatants reduced the toxicity over the dilution series by 65% and 68% respectively compared with comparative undialysed samples (Figure 3:7a-b). When cultured in BHI the dialysed supernatants of NCTC 11143 (Figure 3:7c) and NCTC 11145 (Figure 3:7d) were more toxic (5-10%) than their undialysed counterparts at the higher dilutions, but from 32 and 64 fold dilutions for NCTC 11143 and NCTC 11145 respectively, toxic effect was lower than in the undialysed samples.

Dialysis followed by boiling of the NCTC 11143 culture supernatants grown in SMP resulted in an increase of toxicity down to 16 fold dilution compared to boiled but undialysed samples (Figure 3:7a). Beyond 16 fold dilution toxicity was lost rapidly. The dialysed and boiled samples had lower toxic effect than dialysed only samples. With NCTC 11145 cultured in SMP, dialysis followed by boiling reduced the toxicity of the samples even at the highest concentrations of supernatants (Figure 3:7b); the initial toxicity (25µl supernatant) was 32%, compared with 60% in undialysed, boiled samples of NCTC 11145 cultured in

SMP. When cultured in BHI the dialysed and boiled supernatants of NCTC 11145 were non-toxic (Figure 3:7d).

3:3:2 Optimisation of conditions for toxin production for detection using MTT cytotoxicity assay

3:3:2:1 Effect of growth media on toxin production by *B.cereus*, and the effects of boiling cell free culture supernatants

Examination of skim milk (10% w/v) (SMP) and brain heart infusion (BHI) was made to assess the toxicity of the media themselves against the cells (Figure 3:8). Toxic effect against CHO cells was zero with 25µl of BHI (3.7% w/v and 7.4% w/v). 25µl was regarded as 1 unit in this assay, with 2, 4, 8 etc. being serial dilutions made from this start point. Skim milk (25µl) produced 13% toxicity, but this reduced to zero toxicity by 2 fold dilution. INT407 cells were slightly more sensitive to the media, but by 2 fold dilution SMP and BHI (3.7% w/v) were non-toxic to the cells. BHI (7.4% w/v) was only 8% toxic. Reduction in the metabolic activity of the cell line was therefore considered to be caused by the toxic effect of the cell free culture supernatants if it occurred at or beyond a 4 fold dilution.

The media in which the *B.cereus* isolates were grown appeared to influence the toxicity exhibited by the organism. Comparisons of BHI and SMP(10% w/v) suspension were made (Figures 3:3a-b). The emetic *B.cereus* strain NCTC 11143

produced greater amounts of toxin when grown in SMP than in BHI, and produced toxin with greater consistency in SMP (Figure 3:3a). The *B.cereus* diarrhoeal strain NCTC 11145 produced the greatest levels of toxin in BHI, retaining toxicity of 50% at 256 dilution. When cultured in SMP it was also still positive at 256 dilution, but at a lower level (29%).

Boiling cell free culture supernatants of *B.cereus* had different effects depending on the characteristics of the particular *B.cereus* isolate, and the culture media in which the isolate was grown (Figures 3:3a-b). The boiled cell free culture supernatants of NCTC 11143 grown in skim milk (10% w/v) still had toxic effect (20%) at x256 dilution against CHO cells. The sample dilutions retained on average 60% of their preboiled toxicity. The boiled supernatants of NCTC 11143 cultured in BHI retained on average 28% of their preboiled toxicity, and lost toxic effect on dilution by 16 fold (Figure 3:3a). NCTC 11145 retained on average 36% and 26% of its pre-boiled toxic effect when SMP and BHI culture supernatants respectively, were boiled; toxicity was lost by a 4 or 8 dilution when grown in BHI (Figure 3:3b).

The effect of the length of time for which the samples were boiled (from 10 to 60 min), was tested (Figure 3:9 a-b). Increasing the boiling time beyond the standard 10 min, caused no additional reduction in toxicity levels in either NCTC 11143 or NCTC 11145.

3:3:2:2 Growth time of *B.cereus* cultures

Growth and toxin production curves were made of NCTC 11143 (Figures 3:10a-b) and NCTC 11145 (Figures 3:11a-b) in skim milk (10%w/v) (SMP), static and shaken (200rpm) culture, at 30°C, and in static culture at 21°C, to assess when toxin production occurred in relation to time and growth.

In static culture at 30°C, NCTC 11143 (Table 3:1a) was growing slowly throughout. At 30°C in shaking culture NCTC 11143 (Figure 3:10a) grew faster and to greater concentrations than under static conditions, even though the inoculum concentration of *B.cereus* was lower; growth was occurring within the first hour of inoculation and had entered growth phase by 3h. The stationary phase was reached at 11h with a concentration of approximately $9x10^8$ cfu/ml. In the 30°C static culture a positive toxic effect occurred by 7h (Table 3:1a), with the highest toxicity (positive at dilution 256) in unboiled culture supernatants occurring at 14h. In boiled samples of the 30°C static culture, toxicity was first detected at 11h, with the peak (positive at dilution 128) at 18h. The shaken NCTC 11143 culture had toxic effect by 6h (Figure 3:10b)when the growth was at $5.9x10^6$ cfu/ml. The shaken culture reached its maximum toxicity levels (positive at dilution 512) by 7h in unboiled samples and by 14h in boiled samples. The high levels of toxin continued until the end of the experiment at

24h.

At 21°C, static culture, the maximum concentration reached by NCTC 11143 (Table 3:1a) was $8x10^7$ cfu/ml, as it had done in the 30°C static culture; but the maximum toxicity of unboiled toxin was at a dilution of x16 (Table 3:1a); there was no positive toxic effect with boiled samples. Positive toxic effect produced by NCTC 11143 occurred after 16h in unboiled samples from 21°C static culture.

When NCTC 11145 was grown in 10% skim milk powder at 30°C under static conditions (Table 3:1b), it entered the stationary phase at approximately 15h, with a concentration of $2.5x10^8$ cfu/ml. At 10h growth, a positive toxic effect was first detected against tissue culture cells (Table 3:1b). At 15h toxicity reached a maximum (32), toxicity remained at this level until 19h when it declined. A positive toxic effect by NCTC 11145 was detected after 8h in static culture at 21°C (Figure 3:11b). In static culture at 21°C, the concentration of cells was still increasing at 24h ($4x10^8$ cfu/ml); toxicity levels were low for NCTC 11145 (Figure 3:11b). However, with shaking (200rpm) at 30°C positive toxicity was apparent after 4h, with the maximum level (256) of toxicity being reached by 10h shortly before the culture entered the stationary phase (11h) (Table 3:1b). Toxicity remained at its maximum level until 20h when it declined slightly (128). The emetic strain of *B.cereus* differed from the diarrhoeagenic, in that no decline in toxin concentration was seen after 20h

(Table 3:1a).

3:3:2:3 Effect of molecular weight separation of cell free culture supernatants on toxicity

The cell free culture supernatants of NCTC 11143 (Figure 3:12a) and NCTC 11145 (Figure 3:12b) grown in BHI were fractionated on the basis of molecular mass by membrane ultrafiltration, and assessed for toxicity. The SMP samples blocked the molecular mass separation filters, and therefore were not tested.

When grown in BHI the NCTC 11143 >30 kDa fraction was more toxic than the whole sample. The <30 kDa fraction in BHI was non-toxic before and after boiling. Boiled whole supernatants of NCTC 11143 cultured in BHI did not exhibit toxicity.

The >30 kDa ultrafiltrates of the culture supernatants of NCTC 11145 (Figure 3:12b) were more toxic than the unfractionated supernatant of BHI cultured samples. The <30kDa fractions were not cytotoxic in BHI; the boiled samples also exhibited no toxicity.

The separated molecular mass fractions of BHI cultured NCTC 11143 and NCTC 11145 culture supernatants were tested for toxicity against TECRA and OXOID commercial kits (Table 3:2a-b). Using the TECRA BDE VIA ELISA assay, the whole supernatants and >30 kDa fractions of NCTC 11143 and NCTC

11145 all produced positive toxicity results. Boiling the supernatant fractions reduced the amount of toxin detected by the TECRA test kit, but the results were still positive in whole and >30 kDa fractions for both strains. The <30 kDa fractions of NCTC 11143 was positive, but the boiled <30kDa and both unboiled and boiled <30 kDa fractions of NCTC 11145 culture supernatants did not react with the TECRA assay.

When tested against the OXOID kit, all of the NCTC 11143 samples were negative. The whole and >30 kDa samples were positive for NCTC 11145; following boiling these samples were still positive, but had lost some of their reactivity.

3:3:3 Utilisation of cytotoxicity assay for detection of Bacillus spp. toxins

3:3:3:1 Screening *Bacillus cereus* isolates for toxin production

Bacillus cereus isolates (65) from raw milk, the farm and dairy environments were assessed for toxin production using cell cytotoxicity, OXOID BCET-RPLA and TECRA BDE VIA methods (Table 3:2). All of the isolates produced toxin detected by one of the three methods, although only 37% gave positive results to all tests. The amount of toxin detected varied from isolate to isolate.

The cell cytotoxicity assay was found to be the most sensitive method of

detecting toxic effect caused by *B.cereus*. Using this method approximately 91% of isolates produced toxic effect, and 40% of these isolates retained toxicity after samples were boiled. The TECRA and OXOID test kits detected toxin in 84.6% and 50.8% of samples, respectively. The cell cytotoxicity assay detected toxin in culture supernatants of 4 isolates for which TECRA responded negatively, and 16 supernatants where OXOID had been negative.

3:3:3:2 Screening isolates of *Bacillus* spp. other than *B.cereus* for toxin production

Members of various species of *Bacillus* were tested for toxin production using cell cytotoxicity, OXOID and TECRA assays (Table 3:4). The culture supernatants of all 15 isolates of *B.mycoides* examined, were toxic as determined by cell cytotoxicity and OXOID. None of the isolates were tested against TECRA. Boiled samples of 14 of the 15 culture supernatants (93%) retained marginal toxicity (x4 or x8 dilution), but none exhibited a high level of heat stable toxic effect. Different isolates were found to produce different amounts of toxin, as detected by both OXOID and cell cytotoxicity assays.

Of 13 *B.thuringiensis* isolates 12 (92%) caused cytotoxic effect to cells, of which 10 retained toxicity after boiling. Two of the *B.thuringiensis* isolates were tested against TECRA and both were positive. Differing levels of toxin were produced by different isolates.

None of the 10 *B.subtilis* isolates were positive when tested on the OXOID assay. When tested for cell cytotoxicity 3 were positive. After boiling 2 isolates retained positive toxic effect, but only to marginal levels. None of the isolates were tested against TECRA.

All 6 *B.lentus* isolates were positive against cell cytotoxicity assay, and 2 of the isolates were toxic after boiling. All of the 4 isolates which were tested on TECRA were also positive, but only 2 of these isolates reacted with the OXOID assay. One isolate of those tested from each of *B.circulans* (7 tested), *B.licheniformis* (4 tested), and *B.laterosporus/cereus* (2 tested) were positive on all 3 toxin detection assays. A further isolate of *B.circulans* was cytotoxic and retained toxicity after heat treatment. Two isolates of *B.brevis* (5 tested) exhibited cell cytotoxicity. None of the *B.sphaericus* (2 tested) or *B.polymyxa* (2 tested) were toxic by any detection method.

3:3:3:3 Screening *Bacillus* spp. isolated in Spain from fermented sausage and raw milk for toxin production

The toxicity of 54 isolates of various *Bacillus* spp. recovered from raw milk and fermented sausage in Spain were measured using cell cytotoxicity and OXOID assays (Table 3:5). The isolates were made available by Dr. M.R. Garcia Armesto, University of Leon, Spain. There were 8 isolates tested belonging to the *B.cereus* group, of which

100% had positive toxic effect as detected by the cell cytotoxicity assay, and 75% were also positive on the OXOID kit. Outwith the *B.cereus* group, the only other isolate to give a positive response to the OXOID assay was identified as *B.laterosporous/alvei*. Cytotoxic effect was detected in isolates of *B.polymyxa, B.polymyxa/circulans, B.subtilis, B.subtilis/amyloliquefaciens, B.subtilis/licheniformis, B.stearothermophilus, B.firmus/lentus, B.lentus/firmus, B.pumilus/licheniformis, B.laterosporus/alvei*. Also 4 of 5 isolates which gave unacceptable identification profiles on API 50 CHB were positive for cytotoxic effect.

3:4 TOXICITY STUDIES DISCUSSION

Bacillus cereus has been associated with food poisoning for many years. It causes two types of food borne disease, the diarrhoeal and emetic syndromes. These syndromes are caused by different toxins. The structure of the diarrhoeagenic toxin remains unclear, with some believing it to be a single protein (Turnbull *et al.*, 1979; Shinagawa *et al.*, 1991b and 1991c), and others a tripartite protein complex (Thompson *et al.*, 1984; Beecher and MacMillan, 1991; Granum and Nissen, 1993). Recent evidence suggests diarrhoeal toxic effect may be caused by several different toxins (Granum, 1996), with the possibility that strains of *B.cereus* may be capable of producing more than one type of enterotoxin (Ombui *et al.*, 1997).

Traditionally the toxins have been detected using *in vivo* assays. These have included for diarrhoeal enterotoxin detection by the rabbit ileal loop test (Spira and Goepfert, 1972; Singh *et al.*, 1984), the mouse lethality test, vascular permeability reactions, and Rhesus monkey feeding trials. The detection of the emetic toxin was restricted to Rhesus monkey feeding trials, because only primates are affected by this toxin (Granum, 1994). Such tests are expensive to perform, and require licensed staff to effect due to the involvement of animals. There was a requirement for *in vitro* alternatives which could replace *in vivo* assays in the detection of *B.cereus* toxin. Alternatives included cell cytotoxicity assays (Hughes *et al.*, 1988; Szabo *et al.*, 1991), commercially available antibody based test kits (Oxoid, Unipath; TECRA), and a fluorescent immunodot assay (Jackson, 1989).

Cell cytotoxicity assays have proved useful in the detection of both diarrhoeagenic (Hughes *et al.*, 1988) and emetic (Szabo *et al.*, 1991) toxins. They have been based on the microscopic assessment of the effect on cells caused by *B.cereus* cell free culture supernatant.

3:4:1 Development of cell cytotoxicity assay

3:4:1:1 Methods of cytotoxic assessment and selection of cell line for cytotoxicity assay

Hughes *et al.* (1988) were the first to describe the use of a cell cytotoxicity test for the evaluation of *B.cereus* toxicity. Assessment of *B.cereus* cytotoxic effect was made by microscopic examination of the toxic effect on the cells (Hughes *et al.*, 1988; Szabo *et al.*, 1991). The culture filtrates of emetic syndrome *B.cereus* produced vacuoles in HEp-2 cells (Hughes *et al.*, 1988), and it was the presence of these vacuoles which were detected by microscope.

The cell cytotoxicity assay described in this piece of work was based on cell proliferation. A cytostatic response is one of the effects of *B.cereus* toxin upon cultured cells (Mikami *et al.*, 1994). In the cytotoxicity assay described here, cells were inoculated at a relatively low level, and incubated over the course of 72h. The incubation period allowed viable cells to adhere and multiply, whilst affected cells were inhibited. After 72h there was a confluent lawn of cell growth in negative control wells which contained no toxin. After exposure to moderate levels of toxin some of the cells were affected, while some remained viable. The cells in wells containing high levels of toxic supernatant were unable to recover, and expressed no cell growth.

The growth media used in these experiments, brain heart infusion (BHI) and skim milk (10% w/v) (SMP) were assessed for toxic effect against the cells because the tissue culture cells may be inhibited by the *B.cereus* growth medium. It was found that the BHI and SMP had no toxic effects beyond a 2 fold dilution level, and even at the highest concentrations their toxic effect was low. Therefore to ensure that the effects

of media were not incorporated into the cytotoxicity results, positive responses in the cytotoxicity assay, had to occur at a minimum of a 4 fold dilution level of the cell free culture supernatant.

Scoring of toxic effect was made by visually assessing the difference between growth in the test wells and compared to that in the negative controls. The use of microscopic visual assessment of cytotoxic effect was restricted because it was subjective, giving rise to the potential for variation derived from non-standardised interpretation of results. Therefore to move away from subjective assessment, the measurement of total cellular metabolic activity was be performed using the tetrazolium salt 3-(4,5,-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT).

Assessment of the metabolic status of tissue culture cells using MTT has been made previously (Mosmann, 1983). The tetrazolium ring of MTT is cleaved in the mitochondria of metabolically active cells, resulting in a colour change from pale yellow to dark blue (Mosmann, 1983). Only viable cells can produce this formazan reaction product, which makes MTT a sensitive compound for assessment of living cells (Mosmann, 1983).

Tetrazolium salts have been used to assess *Pasterurella haemolytica* A1 leukotoxin (Vega *et al.*, 1987; Craig *et al.*, 1990), the cytotoxin of *Campylobacter jejuni* (Coote and Arain, 1996) and were recently used to investigate *B.cereus* toxicity (Seidel *et al.*, 1996).

After incubating tissue culture cells in the presence of MTT there was no requirement for excess MTT to be washed from the cell surface. This was because only the dark blue formazan reaction product of MTT was detected in the assay. The lack of washing step increases the reproducibility of the MTT assay compared to other

metabolic assessment compounds, such as neutral red (Borenfreund and Puerner, 1984) where washing is required. Neutral red assays are based on the uptake of chemical by viable cells without a colour change occurring. Therefore the cells have to be washed before measurement of absorbance can be made, in order to remove any free neutral red from the tissue culture test well. Washing can result in the disruption of cells adhering to the tissue culture plate surface, which leads to increased variability of results.

Other methods of detecting *B.cereus* toxin using cytotoxicity have included measuring the acid production by HEp-2 cells. This is done by spectrophotometrically measuring the colour changes in the media of cells incubated in the presence of cell free culture supernatants of *B.cereus* (Mikami *et al.*, 1994). Also the number and viability of cells following 24 and 48 h of incubation in the presence of *B.cereus* culture supernatants were assessed using trypan blue dye (Mikami *et al.*, 1994).

Different cell lines respond differently to the effects of *B.cereus* toxin (Szabo *et al.*, 1991). Therefore two cell lines, INT407, a human intestinal cell line, and Chinese hamster ovary (CHO) cells were tested in this study to assess their sensitivity to *B.cereus* toxins. These cell lines are immortal, and formed adherent monolayers of growth. They were selected because in previous cytotoxicity studies they had been found to be amongst the most sensitive cell lines for *B.cereus* emetic toxin detection (Szabo *et al.*, 1991). The Hep-2 cell line has been used (Hughes *et al.*, 1988; Szabo *et al.*, 1991; Buchanan and Schultz, 1992; Sakuria *et al.*, 1994), and was shown to be as sensitive as CHO and INT 407 cells to the toxic effects of *B.cereus* toxins (Szabo *et al.*, 1991). Interpretation of toxicity results using HEp-2 cells were harder than with CHO or INT 407 cells, (Szabo *et al.*, 1991), and therefore it was not selected for the work presented here. Cells of the CHO line have been used for the detection of *B.cereus* toxin (Buchanan and Schultz, 1992; Buchanan and Schultz, 1994).

Differences existed in the nature of the reaction of the CHO and INT 407 cell lines to the toxic effects of *B.cereus*; this had been found previously (Szabo *et al.*, 1991). The INT407 cells produced prominent vacuoles in the cytoplasm of the cells at high dilutions, and were granular and round at the lower dilutions (Szabo *et al.*, 1991). The CHO cells had no vacuoles, and became rounded and with granular cytoplasm (Szabo *et al.*, 1991). In this piece of work similar effects were observed: CHO cells became disassociated from the surface of the flat bottomed 96 well plate in which the assays were performed, while the cytoplasm of the INT407 cells appeared granular under an inverted microscope at x100 magnification. The cells also became distended. The extent of the reactions of the two cell lines were similar, both by microscopic and MTT metabolic assessment, therefore CHO cells were selected for use in all further experiments.

The reproducibility of the cell cytotoxicity was found to be good. Cultures of *B.cereus* were found to be consistent in their production of toxins under standardised conditions, as had been found previously (Jackson, 1989), when enterotoxin was measured using a fluorescent immunodot assay.

3:4:1:2 Cell concentration

The cytotoxicity assay described in this piece of work is based upon changes in the ability of tissue culture cells to proliferate brought about by the toxic effect of *B.cereus* cell free culture supernatants. *Bacillus cereus* toxins arrest cell proliferation (Mikami *et al.*, 1994). The cytotoxicity assays were run for 72h hours in order to allow unaffected cells and negative control cells to multiply, while affected cells could not.

The cell concentration selected for use in the cytotoxicity assay was $5x10^4$ cells/ml.

This concentration was selected because it gave consistent results with the cell line, and gave a good dilution response with emetic and enterotoxigenic strains NCTC 11143 and NCTC 11145, with toxicity at high concentrations of supernatant being approximately 80-90%. As supernatants were diluted the toxic effect was gradually lost. Response is not linear so the assay as described cannot be used to quantitatively assess the amount of toxin present in a sample. Although the cell concentration $1x10^4$ cells/ml appeared to be the most sensitive concentration, it was not used because there was less change in the response of the cells to toxic effect as the dilutions increased. With assays performed at $1x10^4$ cells/ml initial toxic effect was lower (approximately 60-70% toxicity) than was found with higher cell concentrations, and toxic effect tailed off very little as the dilutions increased, so that at dilutions of 128x and 256x toxic effect remained approximately 50-60%. Relationship of cell numbers to toxin level should be explored further to improve linearity of response for quantitative assay system.

When cell concentrations of $1x10^5$ cells/ml or above were used in the cytotoxicity assay, they gave good toxicity responses at high supernatant concentrations, but lost toxic effect quicker than when $5x10^4$ cells/ml were inoculated. This could be because with the 72h incubation time the maximum number of cells able to adhere to the bottom of the positive control wells was exceeded. Microscopic examination confirmed that several layers of cells growing on top of one another were present. Therefore, at dilutions where the toxin had begun to lose effect, the remaining healthy cells in those wells could have been able to multiply to such an extent that they also reached maximum concentrations for the well, resulting in similar metabolic results as in the negative control wells, and consequently masking the effects of low concentrations of toxin.

The principal use of this cytotoxicity assay was for the screening of *B.cereus* isolates

whose capacity for toxin production was unknown. Therefore the concentration of cells used in the assay was optimised for the detection of a wide spectrum of toxicity levels, whilst retaining sensitivity to toxic effect. A cell concentration of $5x10^4$ cells/ml best met both of these requirements, and was used in every following experiment. This aided standardisation of results obtained from experiments performed on different days.

3:4:1:3 *In vitro* detection methods for *B.cereus* toxins other than cytotoxicity

In addition to cell cytotoxicity assays other *in vitro* assays have been developed for the detection of *B.cereus* toxin. These include the TECRA and OXOID commercial immunoassays, a fluorescent immunodot assay (Jackson, 1989) and a discontinuous haemolysin pattern which indicates the presence of diarrhoeal enterotoxin (Beecher and Wong, 1994). The TECRA and OXOID immunoassays will be further discussed in Section 3:4:3:3. The fluorescent immunodot assay detected enterotoxin production in 94% of *B.cereus* isolates tested (Jackson, 1989); this was very comparable to the 91% detection rate determined by the MTT cytotoxicity assay in the screen of *B.cereus* isolates presented in this piece of work. Negative toxicity results were obtained from other species of *Bacillus* tested using the fluorescent immunodot method. The cell assay, which detects non-specific toxic effect was able to detect toxic effect from isolates belonging to *Bacillus* spp. other than *B.cereus*. The TECRA and OXOID immunoassays also detected toxin in *Bacillus* spp. other than *B.cereus*, which suggests that there is similarity between toxins derived from different *Bacillus* spp..

Haemolysin BL, which has been shown to be a *B.cereus* enterotoxin complex (Beecher and Macmillan, 1991), produces a discontinuous haemolytic pattern in blood agar (Beecher and Wong, 1994). This method will differentiate strains of *B.cereus* which would test positive on the OXOID test kit, because OXOID detects one of the

proteins which make up haemolysin BL (Beecher and Wong, 1994). However, although the discontinuous haemolysin pattern is specific, simple and inexpensive (Beecher and Wong, 1994), it is not highly reproducible as a method (Beecher and Wong, 1994), and therefore was not used in this study. A spectrometric haemolysis assay system which measures the activities of the individual haemolysin BL components has recently been developed (Beecher and Wong, 1997).

3:4:2 Optimisation of conditions for toxin production for cytotoxicity assay

3:4:2:1 Media for growth of *B.cereus* cultures

The ability of *B.cereus* isolates to produce toxin was influenced by the media in which they were cultured. This has been found previously (Melling *et al.*, 1976; Szabo *et al.*, 1991). Skim milk (10% w/v) and BHI were found to be successful media for the production of toxin.

The emetic toxin of *B.cereus* is highly heat stable (Melling and Capel, 1978), while the diarrhoeal enterotoxin is heat labile (Turnbull, 1976). Therefore to assess toxicity derived from the emetic-type toxin, culture supernatants were boiled before cytotoxicity testing was performed. When grown in SMP the emetic strain NCTC 11143 retained on average 60% of its cytotoxic effect after boiling. However, when grown in BHI the majority of the toxicity was lost following boiling, even though toxin production had been high in unboiled samples. BHI was not therefore considered to be a good medium for the production of emetic toxin; this had been found previously by Shinagawa *et al.* (1992). Emetic strains of *B.cereus* maybe producing two different toxins, one which is heat stable and one which is heat labile. If this were the case, this could provide an explanation for the 30% (Gilbert, 1979) of *B.cereus*

food poisoning cases where the symptoms are both of vomiting and diarrhoea. Such a theory is supported because all three of the known emetic toxin producing strains tested here, reacted positively to the TECRA VIA immunoassay, which is meant to be specific for the diarrhoeagenic enterotoxin. The antibody raised for the TECRA kit is specific for an antigenic faction, the so called Antigen 577, which is thought to be the diarrhoeagenic toxin (Bennett *et al.*, 1993; Baker and Griffiths, 1995). Therefore, unless the emetic strains tested here were expressing both toxins, they should not have reacted with the TECRA VIA immunoassay.

Emetic syndrome food poisoning is primarily associated with the consumption of rice dishes from Chinese restaurants (Kramer and Gilbert, 1989). In studies examining emetic toxin production in different types of media, rice slurry was found to be a good media for the production of emetic toxin, but milk was found to be even better (Szabo *et al.*, 1991). Nevertheless, there have been few cases of *B.cereus* food poisoning associated with dairy products, in spite of the problems associated with contamination of milk and dairy products by *B.cereus*. A reason for the low levels of food poisoning associated with milk may be due to the other spoilage problems which occur when *B.cereus* contaminates milk. Spoilage defects such as bitty cream and off flavours provide a warning of *B.cereus* contamination of the milk, and so consumption of infected milk is usually averted.

Samples of the diarrhoeal enterotoxin strain were more resistant to the effects of heating when grown in milk, than they had been in BHI; this was in agreement with earlier work (Baker and Griffiths, 1995). It is possible that the greater resistance which occurs in milk, may be due to a component of milk which might protect the diarrhoeal toxin from the denaturing effects of heating, perhaps by forming a complex with the toxin. Alternatively *B.cereus* may be capable of forming more than one type of diarrhoeal enterotoxin. If this is the case, as the latest research suggests (Lung and

Granum, 1996), then it is feasible that a single strain of *B.cereus* may hold the genes which code for several of the enterotoxins described, and may express them differently under various culture conditions. The different enterotoxins described may have variable heat stability, with the one produced in milk being more stable than that expressed in BHI. It is known that the production of diarrhoeal enterotoxin is influenced by nutrient availability (Sutherland, 1993; Sutherland and Limond, 1993). Starch was found to enhance enterotoxin production (Sutherland and Limond, 1993), whereas high levels of sugars inhibited production (Sutherland, 1993; Sutherland and Limond, 1993). In both of these pieces of work toxin was detected using the OXOID kit. The repression of toxin production by high concentrations of sugars in the bacterial growth media may be due to the lowering of media pH because of acid produced as the bacteria ferments sugars, because low pH prevented toxin production (Sutherland, 1993), and a pH outside the 5-10 pH range results in the rapid loss of toxic activity (Spira and Goepfert, 1975; Granum *et al.*, 1993b). Diarrhoeal enterotoxin is unstable over a wide range of conditions, with ionic strength being especially critical (Spira and Goepfert, 1975). Water activity also has a significant effect on toxin production and growth of *B.cereus* (Baker and Griffiths, 1993).

3:4:2:2 Growth time of *B.cereus* cultures

The best conditions for growth and toxin production in both emetic and diarrhoeagenic strains were when they were cultured at 30°C under shaking conditions. Although *Bacillus* spp. are facultative anaerobes, which are capable of growth (Williams and Withers, 1983), and *B.cereus* of toxin production (Granum *et al.*, 1993a), under anaerobic conditions, both are better when a good level of aeration is provided. It is unclear whether sub-optimal temperatures and, or restricted oxygen concentrations themselves impede toxin production, or whether toxin levels are lower under restricted conditions simply because bacterial growth is limited.

The diarrhoeal strain of *B.cereus* tested (NCTC 11145) produced toxin during the logarithmic stage of growth; these findings were consistent with earlier work (Glatz *et al.*, 1974; Spira and Goepfert, 1975; Fermanian *et al.*, 1996). With the emetic toxin producing strain tested, maximum levels of toxicity were reached before the stationary phase of growth was reached. However, maximum levels of toxicity in boiled samples did not occur until the stationary phase was entered.

3:4:9 Molecular weight separation of *B.cereus* cell free culture supernatants

Work has been carried out to determine the molecular masses of the purified *B.cereus* diarrhoeal enterotoxins. The molecular masses of the component parts of haemolysin BL have been determined as 37.8 kDa, 38.5 kDa and 43.2 kDa respectively (Beecher *et al.*, 1995), while the constituents of the enterotoxin complex described by Lund and Granum (1996) have masses of 39 kDa, 45 kDa and 105 kDa. The single protein enterotoxin-T has a molecular mass of 41 kDa (Agata *et al.*, 1995a).

Dialysis and molecular mass separations were performed on cell free culture supernatants by membrane ultrafiltration, in order to examine which molecular mass fraction of the supernatants contained the toxic components. Difficulties arose when supernatants of cultures grown in milk were put through the molecular mass filters, because the filters became blocked before samples could be collected. This was unfortunate because the heat stable toxin produced by emetic strain NCTC 11143 was produced when cultured in skim milk, but not when it was grown in BHI. The emetic toxin is a cyclic dodecadepsipeptide ionophore named cereulide (Isobe *et al.*, 1995; Agata *et al*, 1995b). It has a very low molecular mass, 5-7 kDa (Shinagawa *et al.*, 1992a); it was originally proposed that it was a metabolic by-product, possibly a lipid (Shinagawa *et al.*, 1992a). Therefore it would have been anticipated that supernatants

expressing emetic toxin, would have been toxic in the <30 kDa fraction. However this could not be tested because of the difficulties encountered in the ultrafiltration of the skim milk samples.

Samples cultured in BHI were more toxic in the >30 kDa samples than they had been in whole samples. This indicates a concentration effect caused by the removal of the <30 kDa fraction, which was non toxic.

Dialysis of samples removes the fraction which is approximately <10 kDa. When culture supernatants of both the emetic and diarrhoeal toxin producing strains which had been grown in skim milk were dialysed, they were less toxic than comparable undialysed samples. This indicates that some toxic effect was derived from the low molecular mass fraction. Maintenance of culture supernatants at 4°C overnight while dialysis was performed may also contribute to the loss of toxic effect. Storage of the SMP cultured supernatants of NCTC 11143 and NCTC 11145 at 6°C for 72h resulted in reduction of toxic effect. The dialysis of supernatants from culture in BHI resulted in increased toxicity, which was similar to the results of the molecular mass separations using >30 kDa molecular mass cut off filters.

3:4:3 Use of cytotoxicity assay to evaluate toxin production by strains of Bacillus spp. and comparison with commercial detection methods

3:4:3:1 Incidence of toxins in isolates of *B.cereus* from raw milk and the dairy environment

All of the psychrotrophic *B.cereus* isolates from the farm environment, many from raw milk, tested positive for producing food poisoning toxins on at least one of the three detection methods implemented. The individual isolates varied considerably in the

amount of toxin produced, which was in agreement with earlier work (Dufreinne *et al.*, 1995). Although a high number of isolates were capable of producing toxin, it is unknown how many would be capable of producing sufficient amounts of toxin to actually cause food poisoning. It has been suggested that there are only a small number which are able to produce large enough amounts of diarrhoeal enterotoxin to cause a danger of food poisoning (Ronner and Andersson, 1995). However, some of the isolates of *B.cereus*, such as FSS 063, FSS 099, were still cytotoxic at a 256 fold dilution, which was comparable with diarrhoeal strain NCTC 11145, which was isolated initially from meat loaf as the causative microorganism in a food poisoning outbreak in the USA (Midura *et al.*, 1970). These results suggest that there is a potential health risk arising from the contamination by *B.cereus* of milk and dairy products. However, despite a high incidence of *B.cereus* in milk, there are few reports of associated food poisoning (Christiansson, 1995). The *B.cereus* cultured in these experiments were incubated at 30°C, but it is considered that if milk and dairy products are maintained in the cold chain, the production of diarrhoeagenic enterotoxin is unlikely to occur (Sutherland, 1993). However, psychrotrophic strains of *B.cereus* have been shown to be capable of producing enterotoxin when grown at 8°C (Christiansson *et al.*, 1989), but a temperature of 4°C prevented growth and toxin production (Van Netten *et al.*, 1990). If temperature abuse during storage of milk or dairy products occurs the risk from *B.cereus* may be increased. In other food products, such as rice the presence of *B.cereus* is of concern. *Bacillus cereus* was isolated in rice in Taiwan (Chung and Sun, 1986), and in rice for sale in Hong Kong 69% of samples contained *B.cereus* (Lee *et al.*, 1995).

Low aeration of culture media restricted toxin production (Christiansson *et al.*, 1989; Notermans and Tatini, 1993), and this observation was confirmed in the *B.cereus* growth studies. However, toxin formation in whipped cream has been demonstrated at 8°C (Christiansson *et al.*, 1989), and so the risk arising from highly aerated dairy

products may be greater.

3:4:3:2 Toxin production by *Bacillus* spp. other than *B.cereus*

As well as *B.cereus*, isolates of the species *B.mycoides, B.thuringiensis, B.subtilis, B.lentus, B.circulans, B.licheniformis* and *B.laterosporus/cereus* also produced detectable toxin. Positive detection of toxic effect was most frequently made by cell cytotoxicity, but isolates belonging to *B.thuringiensis* and *B.lentus* reacted positively with the TECRA assay, which indicates that these isolates were producing proteins which were very similar to those of *B.cereus*. Of the isolates from Spanish fermented sausage and raw milk, members of the *B.cereus* group were toxic by both cell cytotoxicity and OXOID methods; these isolates were not tested against TECRA. Cytotoxic effect was also detected in *Bacillus* spp. isolates from either Scotland or Spain of *B.brevis, B.circulans, B.polymyxa, B.polymyxa/circulans, B.subtilis, B.subtilis/amyloliquefaciens, B.subtilis/licheniformis, B.stearothermophilus, B.firmus/lentus, B.lentus/firmus, B.pumilis/licheniformis* and *B.laterosporous/alvei*. None of the *B.subtilis* isolates tested were positive against TECRA. Species of *Bacillus* other than *B.cereus* have been implicated in outbreaks of food borne disease, including *B.subtilis, B.licheniformis, B.pumilis* and *B.thuringiensis* (Kramer and Gilbert, 1989). Toxin has previously been detected in isolates of *B.circulans, B.lentus, B.pumilus, B.polymyxa* and *B.carotarum,* as well as members of the *B.cereus* group, using the OXOID kit (Griffiths, 1990).

Although *B.thuringiensis* is widely used as an insecticidal pathogen, it is capable of producing food poisoning toxins. This was shown in work presented here, where isolates of *B.thuringiensis* were very similar in terms of toxin production to *B.cereus,* with many of the isolates retaining toxic effect after the sample was boiled. In other work *B.thuringiensis* has also been shown to produce toxin (Griffiths, 1990; Mikami

et al., 1995), and even strains isolated from commercial *B.thuringiensis*-based insecticides have been shown to produce diarrhoeal enterotoxin (Damgaard, 1995). The widespread use of this microorganism perhaps should be re-evaluated in light of these recent findings.

3:4:3:3 Evaluation of TECRA and OXOID immunological diarrhoeagenic detection kits

Evaluation of the TECRA and OXOID immunological tests compared with the cytotoxicity assay were made. Different isolates of *B.cereus* produced positive results with various combinations of the three tests, suggesting that different isolates are producing different proteins. The cell cytotoxicity assay was found to be the most sensitive of the three test methods. Another CHO cytotoxicity assay, which assessed the number of detached cells following exposure to culture supernatants, was also more sensitive to toxic effect than TECRA or OXOID test kits (Buchanan and Schultz, 1994). A higher proportion of *B.cereus* isolates (91%) produced a detectable toxic effect in the work presented here using CHO cells, than had been determined using human embryonic lung (HEL) cytotoxicity (73%) (Christiansson *et al.*, 1989). However, different cell lines have various levels of sensitivity to *B.cereus* toxins (Szabo *et al.*, 1991). Therefore it is unclear whether the higher proportion of cytotoxic *B.cereus* isolates detected in this study was owing to greater sensitivity of CHO cells to toxic effect, or whether there were actually greater numbers of toxic isolates in this sample.

The positive controls provided with TECRA and OXOID kits failed to react with each other, indicating that the kits detect different proteins; this had been found previously (Day *et al.*, 1994; Beecher and Wong, 1994), and has led to debate concerning which kit is actually detecting the diarrhoeal enterotoxin.

There was good agreement between CHO cytotoxicity results and those obtained from TECRA, with 91% of results being the same on both tests. A qualitatively good agreement was also found between cytotoxicity results using HEL and vero cells, with the TECRA assay (Christiansson, 1993; Baker and Griffiths, 1995). The TECRA assay reacted positively with a greater number of isolates than the OXOID kit (85% compared to 51% respectively). Previously there was no correlation found between the results of TECRA and OXOID kits (Christiansson, 1993; Notermans and Tatini, 1993; Day *et al.*, 1994); in the isolates screened here for toxin production there was agreement between TECRA and OXOID in 41% of results. Differences in toxicity results of *B.cereus* isolates obtained using cell cytotoxicity, TECRA and OXOID kits have been attributed to deficiencies in detection methods (Notermans and Tatini, 1993; Beecher and Wong, 1994; Buchanan and Schultz, 1994; Day *et al.*, 1994; Andersson *et al.*, 1995). Some strains of *B.cereus* which have either been positive in monkey feeding trials or which have been isolated from outbreaks of diarrhoeal food poisoning have produced negative results when tested on the OXOID kit (Day *et al.*, 1994). The variation between toxin detection may signify that different enterotoxins are being produced by isolates. Evidence has been provided of three different enterotoxins produced by *B.cereus*. These are haemolysin BL (Beecher and Macmillan, 1991), a non-haemolytic enterotoxin complex (Lund and Granum, 1996) and a single protein enterotoxin (Turnbull *et al.*, 1979; Shinagawa *et al.*, 1991b and 1991c; Agata *et al.*, 1995a). The single protein enterotoxin coded by the bceT gene has been named enterotoxin T (Agata *et al.*, 1995a).

Haemolysin BL is a tripartite protein complex purified by Beecher and Macmillan (1991), which appears to be the same complex described by Thompson *et al.* (1984). The three component enterotoxin complex described by Granum and Nissen (1993) had haemolytic activity, and could be the same complex as haemolysin BL. The

haemolytic protein of the complex is considered to be sphingomylinase (Granum and Nissen, 1993). The second enterotoxin complex (Lund and Granum, 1996) had no haemolytic activity. Haemolysin BL comprises of components B, L_1 and L_2, and causes fluid accumulation in the ligated rabbit ileal loop (Beecher and Wong, 1994). The OXOID kit is specific for the L_2 component of haemolysin BL (Beecher and Wong, 1994). Although haemolysin BL has been confirmed as an enterotoxin (Beecher and Wong, 1994), a high rate of false negatives had also been found using the OXOID kit (Buchanan and Schultz, 1992; Notermans and Tatini, 1993; Day *et al.*, 1994). This supports the suggestion that there are several discrete enterotoxins produced by *B.cereus*.

It has been suggested that the TECRA VIA detects two non-toxic proteins (Beecher and Wong, 1994), probably of molecular mass 40 and 41 kDa respectively (Beecher and Wong, 1994). The protein described as enterotoxin-T has a mass of 41 kDa (Agata *et al.*, 1995a), therefore the TECRA kit may be detecting the single enterotoxin protein. It has been suggested recently that TECRA detects the 45 kDa protein of the non-haemolytic enterotoxin complex (Lund and Granum, 1996).

3:4:4 Summary

The use of a cytotoxicity assay measuring metabolic activity to assess the toxic effect of *B.cereus* was successful. The method was more sensitive than commercial test kits OXOID and TECRA. Using the cell cytotoxicity assay it is only possible to determine toxic effect, but with development of this assay it might be possible to quantify the amounts of *B.cereus* toxin present in this sample. This study has shown that *B.cereus* isolates with the capacity to produce toxins are widespread in the environment. However, it is unclear how many of these isolates produce sufficient amounts of toxin

to cause clinical symptoms if consumed. The potential also exists for toxin formation by species other than *B.cereus*. Therefore future work might assess how the amount of toxic effect observed in cell cytotoxicity assays relates to the pathogenicity of the strain and its aetiology in food-poisoning outbreaks.

TABLES AND FIGURES

FOR TOXICITY STUDIES

Tables 3:1 a-b Toxin production, measured by cell cytotoxicity, and growth by *B.cereus* emetic (NCTC 11143) and diarrhoeagenic (NCTC 11145) strains cultured in skim milk (10% w/v) at 30°C under static and shaking (200rpm) conditions, and at 21°C under static conditions.

N/B = Not boiled; B = Boiled culture supernatants; Shak = *B.cereus* cultured under shaking conditions (200rpm); Stat = *B.cereus* cultured under static conditions

Table 3:1a Growth and toxicity of NCTC 11143 grown in skim milk

	Shaking culture (30°C; 200rpm)			Static culture (30°C)			Static culture (21°C)		
	Log cfu/ml	Toxicity		Log cfu/ml	Toxicity		Log cfu/ml	Toxicity	
Time (h)		N/B	B		N/B	B		N/B	B
0	4.89	1	4	7.00	0	0	5.30	1	1
1	5.26	4	4	7.02	0	0	NR	NR	
2	5.60	1	4	7.03	0	1	5.41	2	1
3	6.43	1	2	7.21	0	0	NR	NR	
4	7.19	2	2	7.07	0	0	5.59	2	2
5	7.00	2	2	7.12	1	1	NR	NR	
6	6.77	128	4	7.22	0	0	6.33	2	2
7	6.63	512	8	7.58	4	2	NR	NR	
8	6.67	512	64	7.59	4	1	7.22	2	1
9	7.64	512	128	7.68	8	2	NR	NR	
10	7.69	512	256	7.72	8	1	7.33	2	1
11	8.67	512	128	7.82	64	8	NR	NR	
12	8.90	512	256	7.99	64	16	7.16	1	1
13	8.93	512	256	8.21	64	32	NR	NR	
14	8.97	512	512	8.34	128	16	7.49	1	1
15	8.97	512	512	8.27	256	64	NR	NR	
16	8.97	512	512	8.32	128	64	7.49	4	1
17	8.98	512	512	8.15	256	64	NR	NR	
18	9.09	512	512	8.34	256	128	7.45	4	1
19	8.75	512	512	8.34	128	128	NR	NR	
20	8.54	512	512	8.30	128	128	7.88	4	1
21	8.54	512	512	8.38	128	128	NR	NR	
22	8.51	512	512	8.48	256	128	7.87	8	1
23	8.59	512	512	8.53	256	128	NR	NR	
24	8.45	512	512	8.45	256	128	7.92	8	1
32	NR	NR	NR	NR	NR	NR	8.21	8	1
42	NR	NR	NR	NR	NR	NR	8.33	16	1

NR = Not recorded

Table 3:1b Growth and toxicity curves for NCTC 11145 grown in skim milk

Time (h)	Shaking culture (30°C, 200rpm)		Static culture (30°C)		Static culture (21°C)	
	Log cfu/ml	Toxicity	Log cfu/ml	Toxicity	Log cfu/ml	Toxicity
0	4.64	0	7.01	0	5.56	2
1	5.00	4	7.11	4	NR	NR
2	5.15	4	6.74	1	5.60	1
3	5.56	2	6.69	0	NR	NR
4	5.60	8	6.89	0	6.25	1
5	7.00	8	6.42	1	NR	NR
6	7.62	8	6.31	0	6.95	2
7	8.01	32	6.85	1	NR	NR
8	8.06	32	7.33	1	7.33	4
9	7.88	128	7.43	2	NR	NR
10	8.29	256	7.67	4	7.26	4
11	8.44	256	7.75	8	NR	NR
12	8.38	256	7.8	8	6.83	4
13	8.54	256	7.76	8	NR	NR
14	8.49	256	7.77	8	7.29	4
15	8.09	256	8.08	32	NR	NR
16	8.48	256	8.16	32	7.46	4
17	8.48	256	8.47	32	NR	NR
18	8.42	256	8.24	32	7.60	4
19	8.71	256	8.48	32	NR	NR
20	8.69	128	8.35	2	7.78	4
21	8.59	128	8.38	2	NR	NR
22	8.75	128	8.44	2	7.87	16
23	8.75	128	8.45	2	NR	NR
24	NR	NR	8.35	2	7.91	64
26	NR	NR	NR	NR	8.16	64
28	NR	NR	NR	NR	8.29	64
40	NR	NR	NR	NR	8.48	128
42	NR	NR	NR	NR	8.63	256

NR = Not recorded

Table 3:2a Toxicity of molecular mass separated fractions of emetic strain of *B.cereus* (NCTC 11143) cell free culture supernatants grown in BHI

Supernatant Fraction	Toxicity Test		
	TECRA*	OXOID[#]	Cell Cytotoxicity[δ]
Whole N/B	+ (4.13)	-	128
Whole B	+ (1.67)	-	4
<30 kDa N/B	+ (1.49)	-	1
<30 kDa B	+/- (0.26)	-	2
>30 kDa N/B	+ (4.04)	-	256
>30 kDa B	+ (3.06)	-	2

Table 3:2b Toxicity of molecular mass separated fractions of the diarrhoeagenic strain of *B.cereus* (NCTC 11145) cell free culture supernatants grown in BHI

Supernatant Fraction	Toxicity Test		
	TECRA*	OXOID[#]	Cell Cytotoxicity[δ]
Whole N/B	+ (4.24)	64	128
Whole B	+ (1.18)	8	4
<30 kDa N/B	- (0.15)	-	4
<30 kDa B	- (0.15)	-	4
>30 kDa N/B	+ (4.31)	64	128
>30 kDa B	+ (2.02)	16	4

* Absorbance at 414 ± 10 nm

[#] Lowest dilution with agglutination

[δ] Lowest dilution causing ≥20% reduction in total metabolic acticity of CHO cells

Table 3:3 Toxicity of *B.cereus* isolates tested on TECRA, OXOID and cell cytotoxicity methods

Isolate	Source	TECRA	OXOID	Cytotoxicity	
				N/B*	B#
FSS 2	F	+	+	16	2
FSS 4	F	+	+	128	4
FSS 7	F	+	-	64	4
FSS 15	S	+/-	+	4	1
FSS 17	F	+	-	16	4
FSS 19	F	+	-	8	2
FSS 22	G	-	+	2	2
FSS 23	F	+	+	128	4
FSS 25	M	+	-	32	4
FSS 27	M	+	+	64	2
FSS 29	S	-	-	4	2
FSS 30	M	+	-	16	4
FSS 31	M	+	-	32	8
FSS 39	F	-	+	2	2
FSS 40	F	+	+	32	4
FSS 41	M	+	+	32	4
FSS 47	F	+/-	-	4	2
FSS 48	F	+	-	64	4
FSS 49	F	+	+	64	4
FSS 56	G	-	+/-	8	4
FSS 62		+	-	128	64
FSS 63	UW	+	-	128	4
FSS 64	UW	+	+	256	1
FSS 65	UW	+	+	256	2
FSS 66	UW	+	+/-	256	2
FSS 68	S	+/-	+	32	4
FSS 70	UW	+	-	256	32

Table 3:3 Toxicity of *B.cereus* isolates tested on TECRA, OXOID and cell cytotoxicity methods continued

Isolate	Source	TECRA	OXOID	Cytotoxicity	
				N/B*	B#
FSS 75	F	-	+/-	2	2
FSS 80	WF	+	-	8	2
FSS 83	UW	+	-	256	16
FSS 86	UW	+	-	64	16
FSS 88	WF	-	-	4	1
FSS 89	F	-	+/-	4	2
FSS 91	WF	+/-	+	8	1
FSS 96	UW	+	+	256	2
FSS 99	UW	+	+	256	8
FSS 101	S	+/-	-	32	4
FSS 106	F	+	+/-	8	2
FSS 110	F	+	+/-	16	1
FSS 112	UW	+	-	128	16
FSS 114	UW	+	-	32	1
FSS 115	UW	+	-	128	2
FSS 117	UW	+	-	64	1
FSS 121	UW	+	-	256	2
FSS 125	F	-	+/-	1	2
FSS 127	F	+	-	32	1
FSS 129	UW	+/-	-	8	2
FSS 132	F	+	-	32	2
FSS 134	UW	+	-	32	2
FSS 136	F	+/-	+/-	64	2
FSS 137	UW	+	-	8	1
FSS 142	F	+	-	8	4
FSS 144	F	-	+/-	1	1

Table 3:3 Toxicity of *B.cereus* isolates tested on TECRA, OXOID and cell cytotoxicity methods continued

				Cytotoxicity	
Isolate	Source	TECRA	OXOID	N/B*	B#
FSS 145	UW	+	-	64	4
FSS 155	F	-	+/-	4	2
FSS 157	F	+	+	2	2
BC 1	C	+/-	-	256	8
BC 2	C	+/-	+ (8)	32	2
BC 3	C	+	-	128	2
BC 4	C	+	-	128	2
BC 7	C	+	+ (16)	128	2
CM 1	C	+	+ (64)	128	4
CM 2	C	+	+ (32)	256	4
CM 3	C	+	+ (64)	128	2
CM 4	C	+	+ (32)	128	2
TOTAL		55 (9 +/-)	33 (10 +/-)	59 (6 +/-)	26 (18 +/-)

* Unboiled cell free culture supernatants
# Boiled cell free culture supernatants
C Vegetarian cheddar cheese
F Faeces of dairy cattle
G Grass
M Raw milk
S Soil
UW Udder washing
WF Winter feed given to dairy cattle

Table 3:4 Toxicity of *Bacillus* spp. isolates.

Species	Isolate	TECRA	OXOID	Cytotoxicity	
				N/B*	B#
B.mycoides	FSS 010	NT	+ (16)	256	4
	FSS 011	NT	+ (16)	256	4
	FSS 012	NT	+ (16)	64	4
	FSS 013	NT	+ (16)	256	2
	FSS 100	NT	+ (8)	16	4
	FSS 102	NT	+ (16)	16	4
	FSS 107	NT	+ (4)	32	4
	FSS 108	NT	+++ (4)	64	4
	FSS 109	NT	+ (16)	128	4
	FSS 118	NT	+++ (4)	64	4
	FSS 119	NT	+ (4)	256	4
	FSS 120	NT	+ (4)	256	4
	FSS 124	NT	+ (4)	16	4
	FSS 131	NT	+++ (4)	16	4
	FSS 133	NT	+++ (4)	64	8
B.thuringiensis	PM 003	NT	-	32	16
	PM 004	NT	-	128	4
	PM 028	NT	-	1	1
	PM 065	+	+/- (4)	64	4
	PM 068	NT	+/- (8)	128	4
	PM 073	NT	-	16	4
	HRM 053	NT	-	64	4
	HRM 067	NT	-	32	4
	HRM 074	NT	+ (4)	16	4
	HRM 083	NT	+/- (4)	128	4
	MRM 218	+	+/- (1)	128	1
	MRM 223	NT	+/- (1)	64	1
	MRM 227	NT	+/- (1)	256	4

Table 3:4 Toxicity of *Bacillus* spp. isolates continued.

Species	Isolate	TECRA	OXOID	Cytotoxicity	
				N/B	B
B.subtilis	FSSM 013	NT	-	16	8
	FSSM 019	NT	-	8	4
	FSSM 128	NT	-	4	2
	FSSM 048	NT	-	32	8
	MRM 290	NT	-	8	4
	119	NT	-	32	8
	121	NT	-	1	1
	133	NT	-	1	4
	153	NT	-	4	2
	FSS 094	NT	NT	1	0
B.lentus	PM 044	+	+	32	2
	PM 054	+	-	4	2
	PM 055	+	-	4	2
	PM 063	+	+	4	2
	FSS 081	NT	NT	4	4
	FSS 149	NT	NT	8	2
B.circulans	PM 013	+	+	8	4
	FSS 024	-	-	16	4
	FSS 092	-	-	1	2
	FSS 093	-	-	2	2
	FSS 095	-	-	1	1
	FSS 098	-	-	1	1
	FSS 104	-	-	1	1
B.licheniformis	FSSM 152	-	-	1	1
	FSSM 171	-	-	2	1
	MRM 071	NT	NT	16	8
	MRM 080	+	+	8	2

Table 3:4 Toxicity of *Bacillus* spp. isolates continued.

Species	Isolate	TECRA	OXOID	Cytotoxicity	
				N/B	B
B.brevis	FSS 071	-	-	0	0
	FSS 081	-	-	1	1
	FSS 149	-	-	0	0
	FSSM 152	NT	NT	2	2
	PM 044	NT	NT	8	2
	PM 055	NT	NT	16	4
B.lacterosporo-us/cereus	FSS 67	-	-	2	1
	FSS 69	+	+	16	2
B.sphaericus	FSS 130	-	-	1	1
	FSS 123	-	-	1	1
B.polymyxa	FSS 153	-	-	1	1
	MRM 304	-	-	1	1

* Unboiled cell free culture supernatants
# Boiled cell free culture supernatants

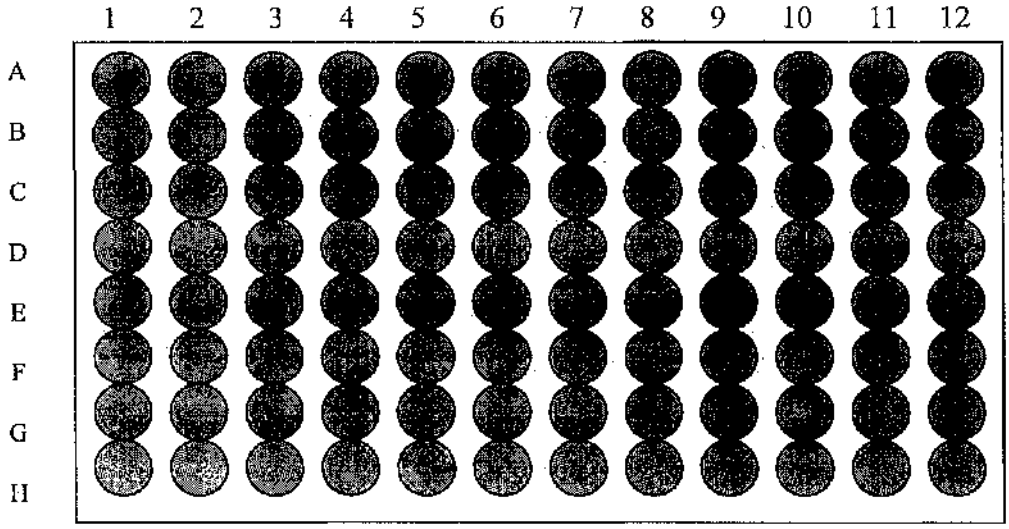
Table 3:5 Toxicity of *Bacillus* spp. isolated from fermented sausage and raw milk in Spain

Species	Number Tested	% Isolates Tested Postive	
		Cytotoxicity[#]	OXOID
B.cereus	2	100	50
B.mycoides	4	100	75
B.cereus/mycoides	2	100	100
B.circulans	7	0	0
B.circulans/macerans	1	0	0
B.circulans/polymyxa	2	0	0
B.lentus/pumilus	1	0	0
B.sphaericus	1	0	0
B.polymyxa	2	50	0
B.polymyxa/circulans	4	50	0
B.subtilis	2	100	0
B.subtilis/amyloliquefaciens	1	100	0
B.subtilis/licheniformis	1	100	0
B.licheniformis	3	0	0
B.stearothermophilus	2	50	0
B.amyloliquefaciens	1	0	0
B.licheniformis/ amyloliquefaciens	1	0	0
B.macerans	1	0	0
B.badius/firmus	1	0	0
B.firmus/lentus	5	40	0
B.lentus/firmus	1	100	0
B.pumilus/licheniformis	1	100	0
B.licheniformis/subtilis	1	0	0
B.stearothermophilus/lentus	1	0	0
B.laterosporus/alvei	1	100	100
UP*	5	80	0

* Unacceptable profile on API 50 CHB

[#] Positive cytotoxicity taken as 20% reduction at 4 fold dilution of cell free culture supernatant

Figure 3:1 Diagram illustrating a 96 well microtitre plate, set up for a cell cytotoxicity assay



Column 1 = Positive control (50µl triton-x)

Column 2 = 50µl sample

Column 3 = 50µl sample

Column 4-12 = 25µl phosphate buffered saline (PBS)

Serial dilutions made by removing 25µl from wells in Column 3 and mixing with the PBS in the wells in Column 4. A further 25µl were removed from the wells in Column 4 and mixed with the PBS in Column 5. This procedure was repeated down until Column 11. Introduction of the 25µl sample from Column 10 was made into Column 11, and 25µl was removed from the wells of Column 11 and discarded.

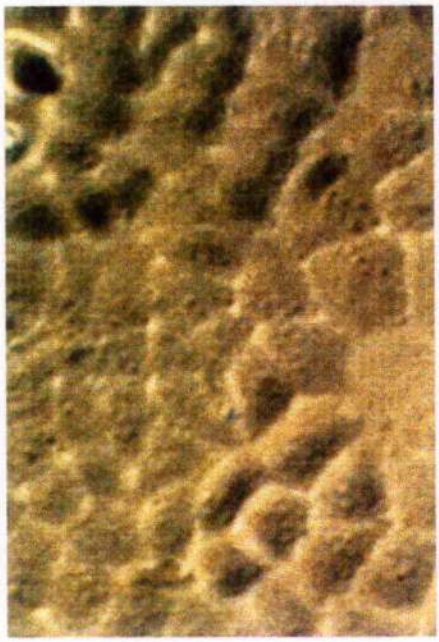
Column 12 = Negative control. Tissue culture cells with no addition of bacterial culture supernatants.

Figure 3:2a-b Photograph illustrating a) Chinese hamster ovary cells and b) INT 407 cells before (i) and after (ii) exposure to *B.cereus* cell free culture supernatant. (Magnification x2000)

a i

a ii

b i



b ii

Figures 3:3a-d Toxic effects of *Bacillus cereus* emetic (NCTC 11143) and diarrhoeagenic (NCTC 11145) strains, against Chinese hamster ovary (CHO) and INT 407 cell lines respectively, measured by metabolic assessment of cultured cells using the tetrazolium salt MTT. Unboiled (N/B) and boiled (B) culture supernatants were tested. The *B.cereus* strains were cultured in skim milk (10% w/v) (SMP) or brain heart infusion broth (BHI). Graphs show mean data of at least 6 repeats.

Figure 3:3a Toxicity of NCTC 11143 using CHO cell cytotoxicity

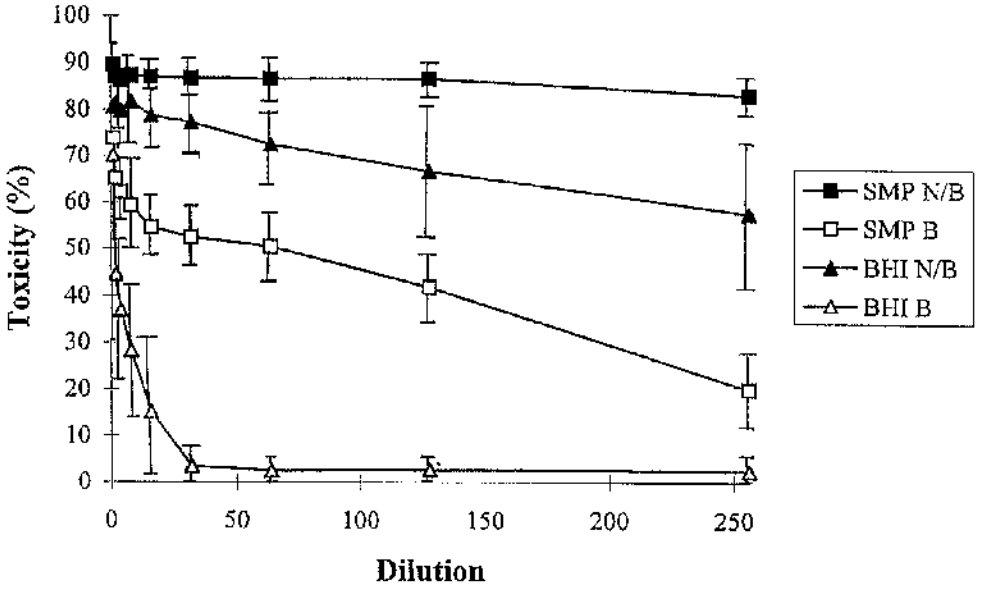


Figure 3:3b Toxicity of NCTC 11145 using CHO cell cytotoxicity

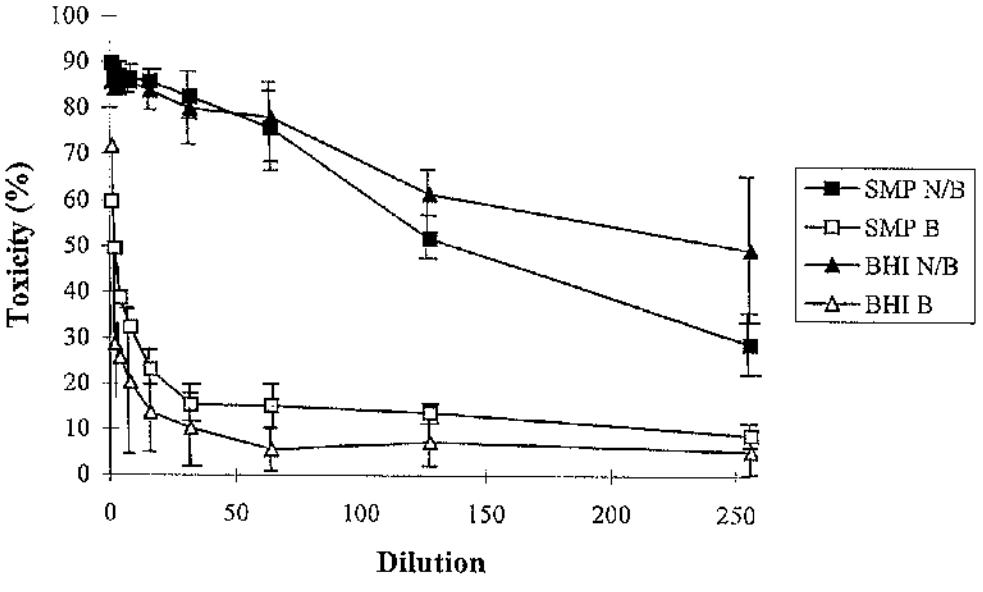


Figure 3:3c Toxicity of NCTC 11143 using INT 407 cell cytotoxicity

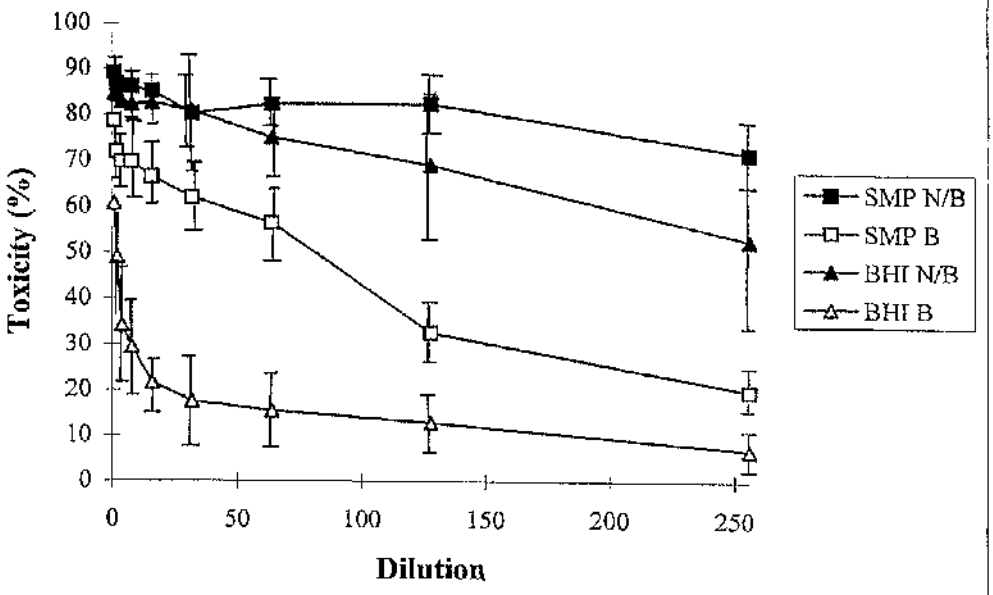
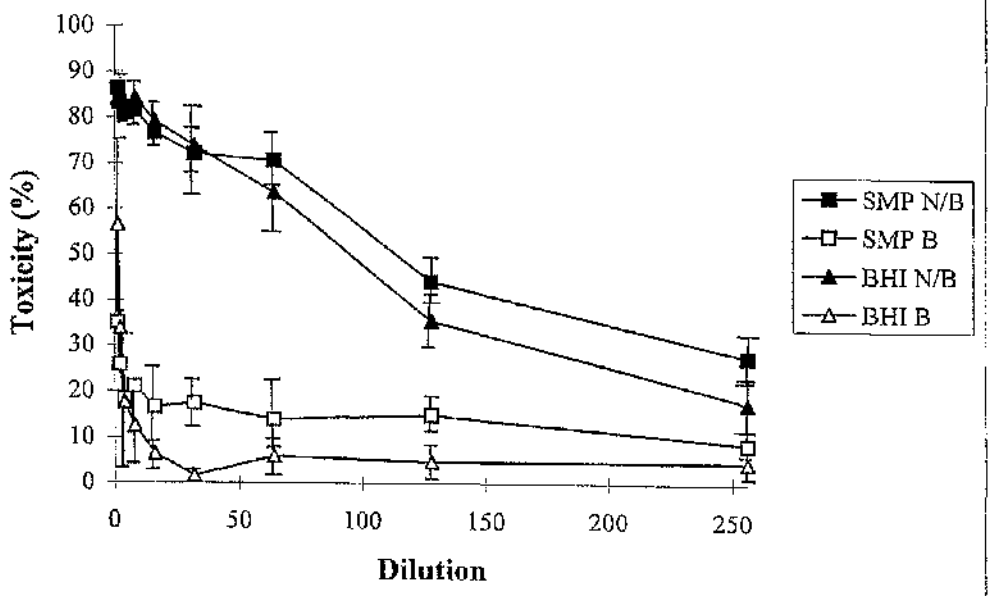


Figure 3:3d Toxicity of NCTC 11145 using INT 407 cell cytotoxicity



Figures 3:4a-d Toxic effects of *Bacillus cereus* emetic (NCTC 11143) and diarrhoeagenic (NCTC 11145) strains, against Chinese hamster ovary (CHO) and INT 407 cell lines respectively, scored using microscopic assessment.Unboiled (N/B) and boiled (B) culture supernatants were tested. The *B.cereus* strains were cultured in skim milk (10% w/v) (SMP) or brain heart infusion broth (BHI). Graphs show mean data of at least 6 repeats.

Scoring of microscopic assessment:-

4 = Total toxicity

3 = Only few surviving cells; majority of cells unable to adhere.

2 = Half cells surviving

1 = Greater than half cells surviving, but some still showing signs of toxic effects

0 = All cells healthy

Figure 3:4a Assessment of cytotoxicity by NCTC 11143 on CHO cells by visual scoring

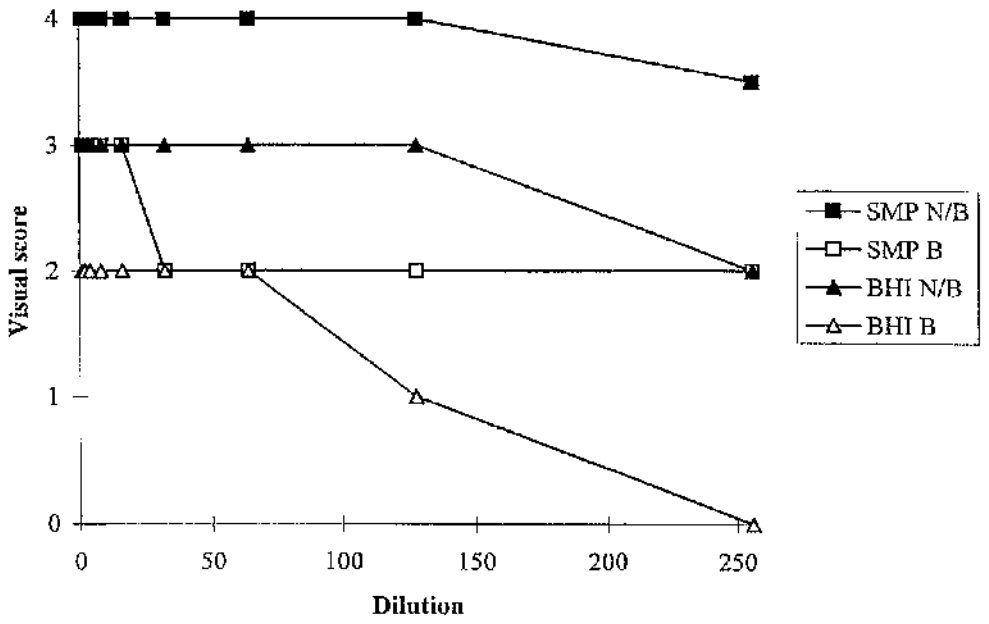


Figure 3:4b Assessment of NCTC 11145 cytotoxicity on CHO cells by visual scoring

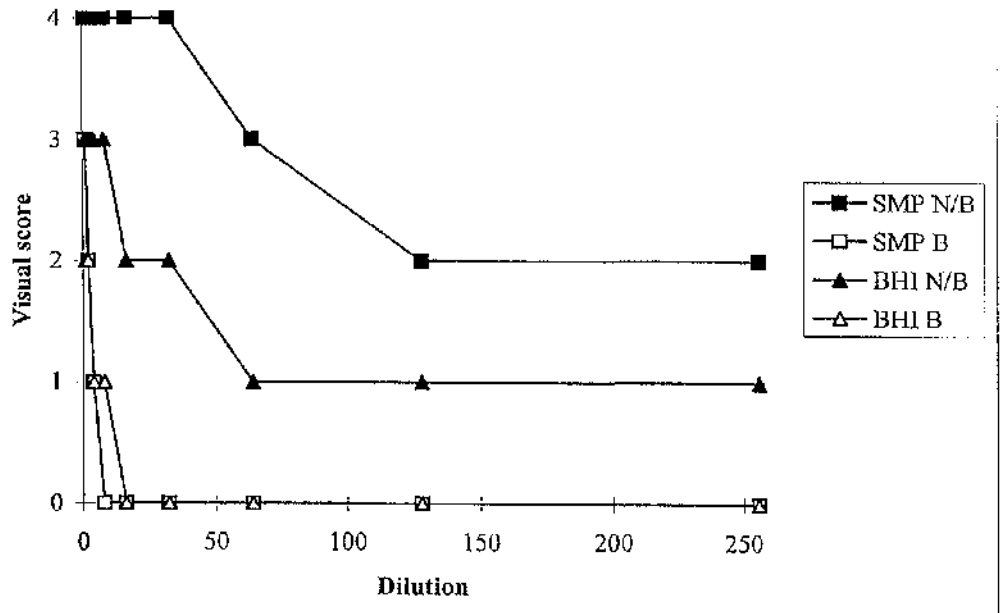


Figure 3:4c Assessment of cytotoxicity of NCTC 11143 on INT 407 cells by visual scoring

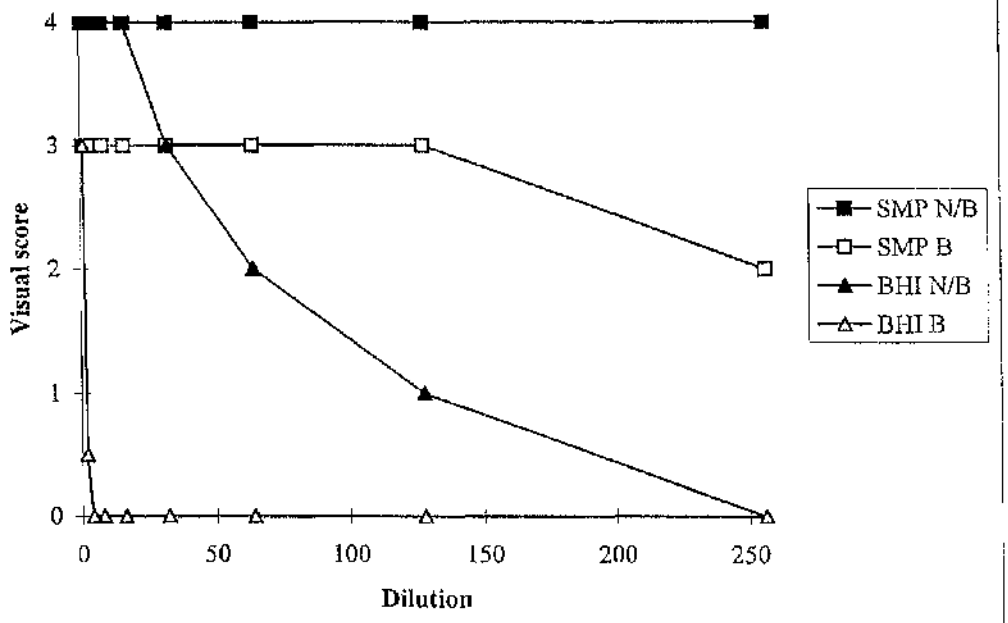


Figure 3:4d Assessment of cytotoxicity by NCTC 11145 on INT 407 cells by visual scoring

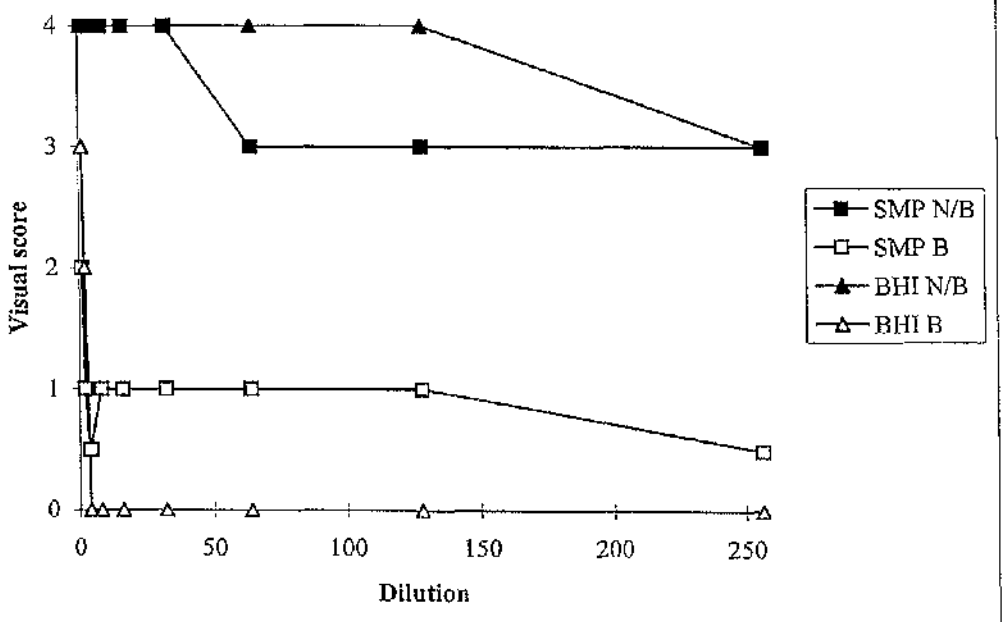


Figure 3:5 Effect of inoculated CHO cell concentration on MTT measured cell cytotoxicity results. Toxicity is represented as the highest dilution on the cell cytotoxicity assay which had a positive toxic response (>20% toxic effect).

Figure 3:5 Effect of inoculated CHO cell concentration on MTT toxicity results

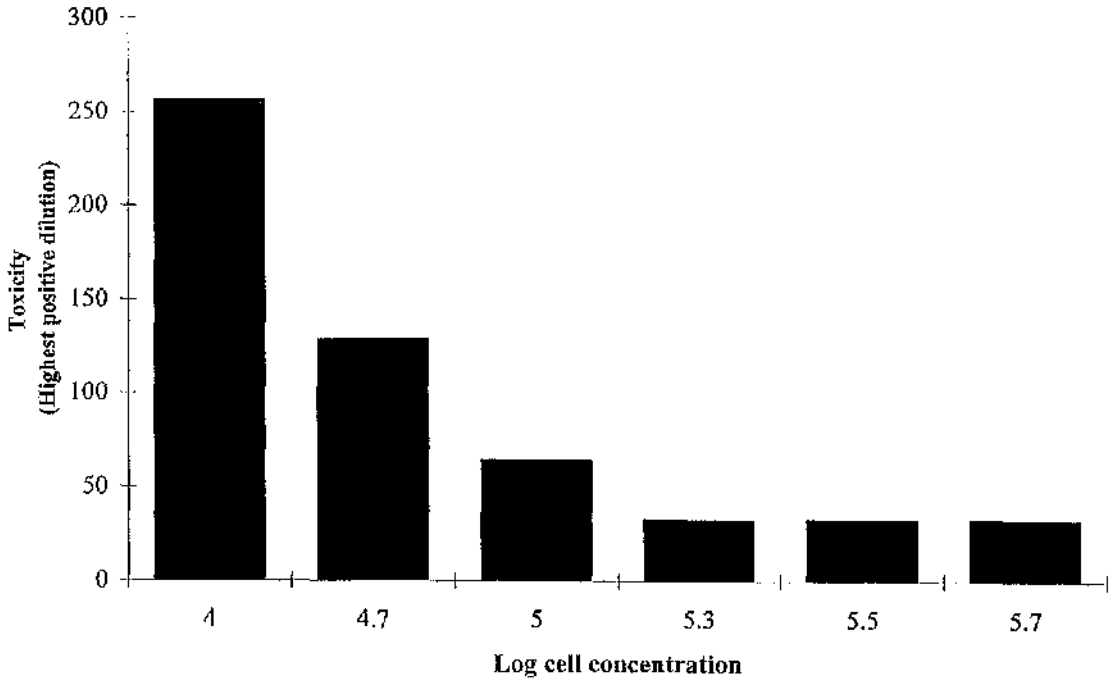


Figure 3:6a Effects of storage conditions on toxicity of NCTC 11143 culture supernatants grown in SMP

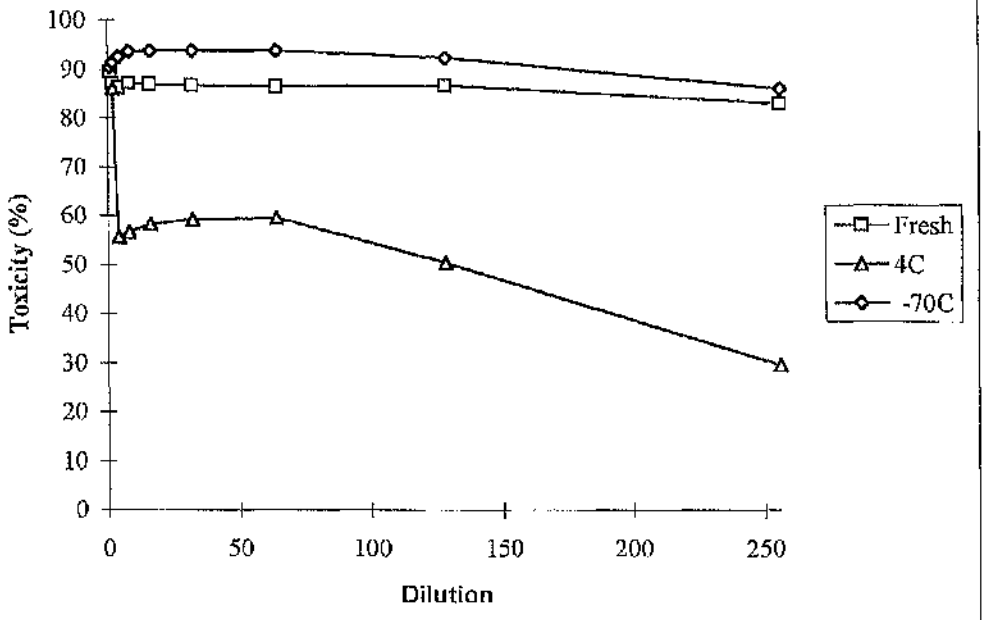


Figure 3:6b Effect of storage on boiled NCTC 11143 culture supernatants grown in SMP

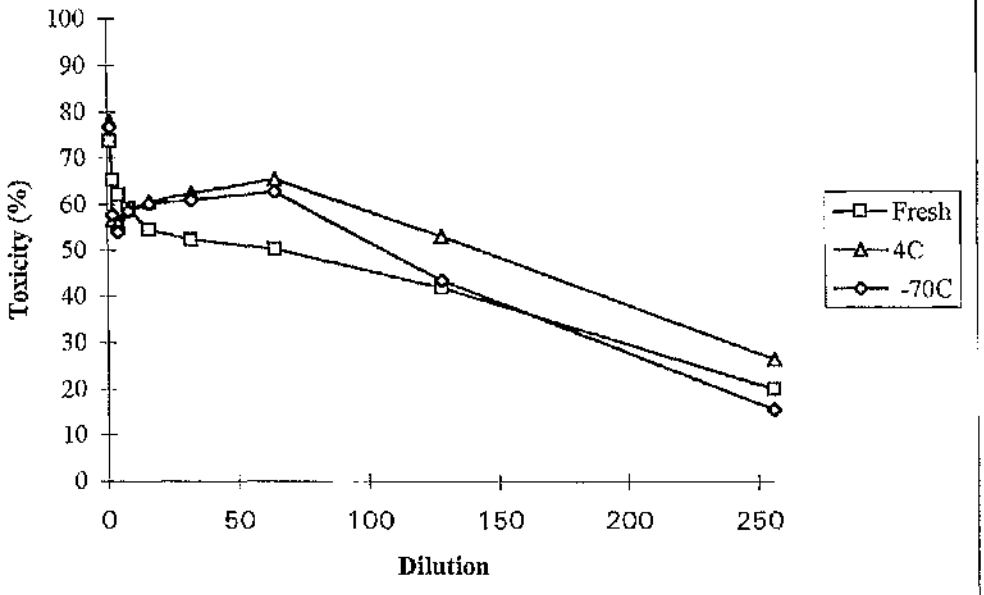


Figure 3:6c Effect of storage conditions on toxicity of NCTC 11145 culture supernatants grown in SMP

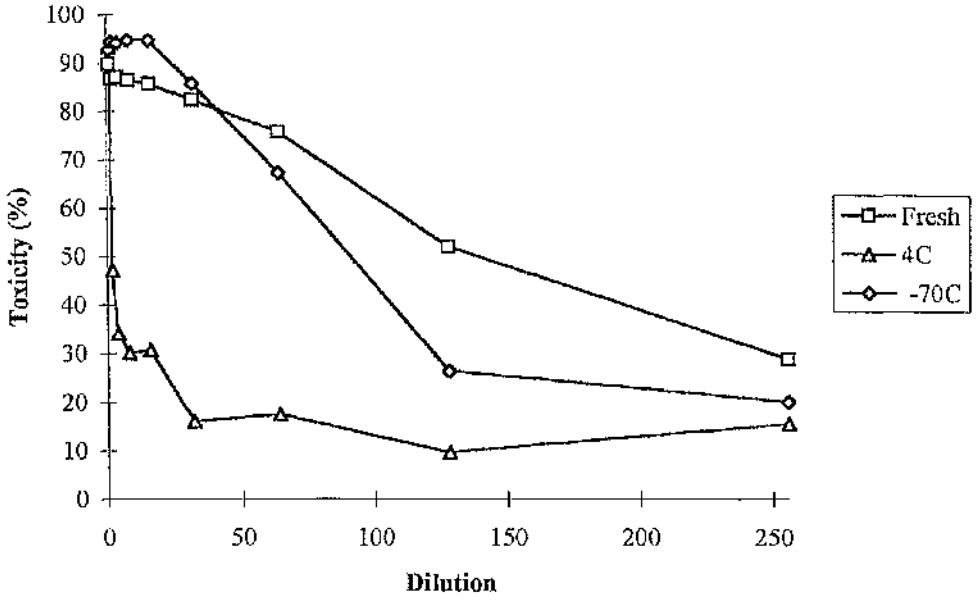
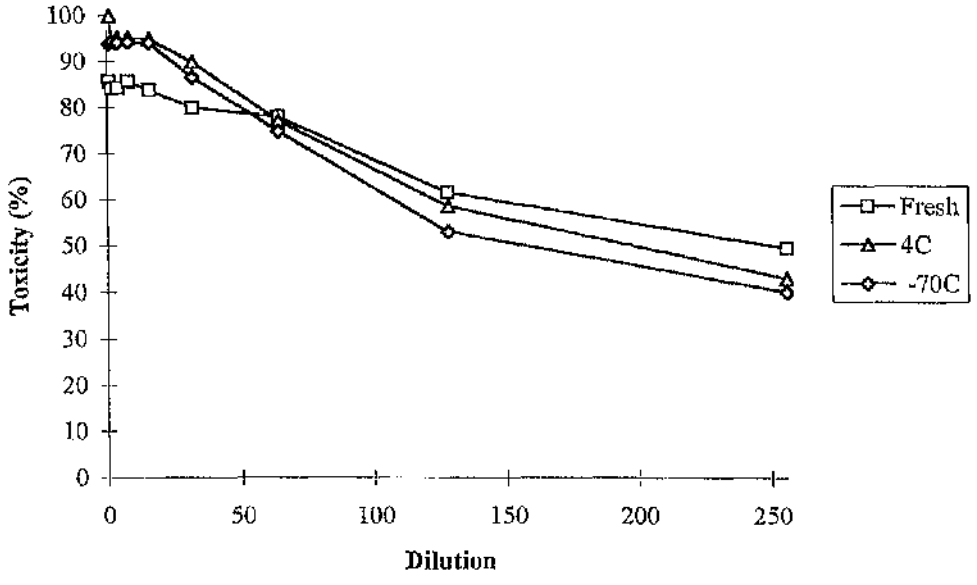


Figure 3:6d Effect of storage conditions on NCTC 11145 culture supernatants grown in BHI



Figures 3:7 a-d Effect of dialysis and boiling of cell free culture supernatants of *B.cereus* emetic (NCTC 11143) and diarrhoeagenic (NCTC 11145) cultured in skim milk (10% w/v) (SMP) and brain heart infuion broth (BHI).

N/B = Not boiled; B = Boiled; D = Dialysed; D/B = Dialysed and boiled culture supernatants

Figure 3:7a Effect of dialysis and boiling on NCTC 11143 culture supernatants cultured in SMP

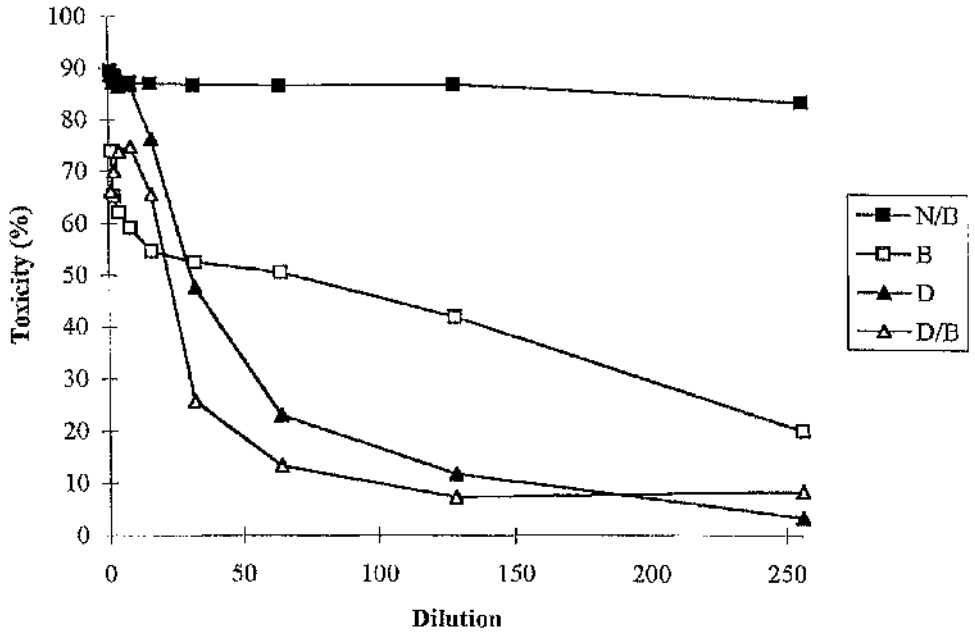


Figure 3:7b Effect of boiling and dialysis on NCTC 11145 supernatants cultured in SMP

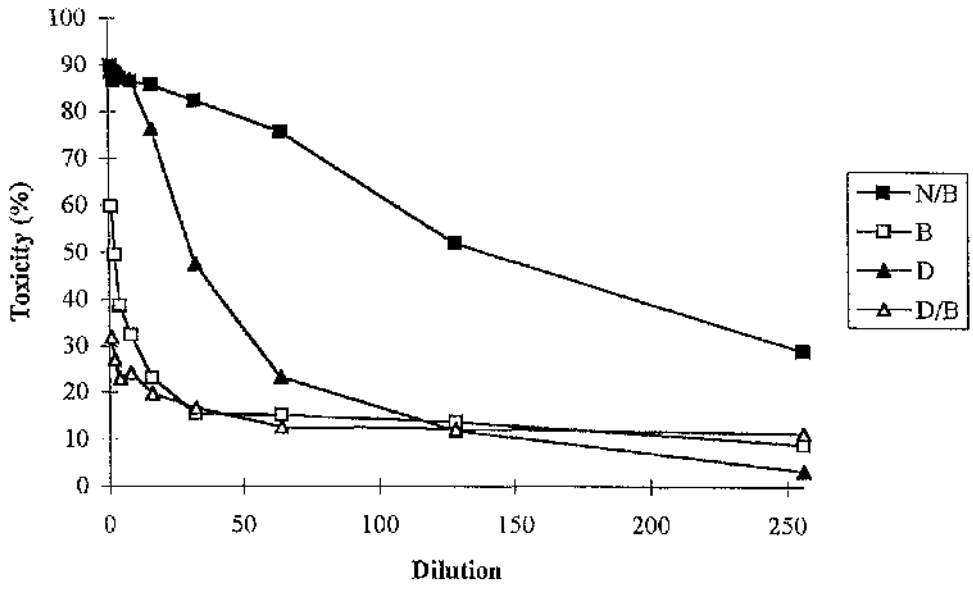


Figure 3:7c Effect of dialysis and boiling on BHI cultured NCTC 11143 supernatants

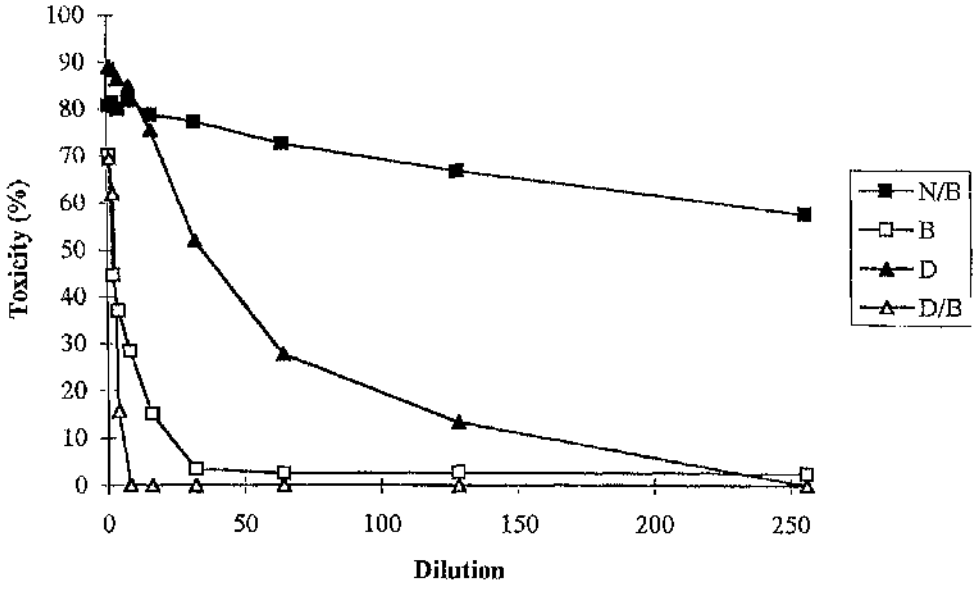
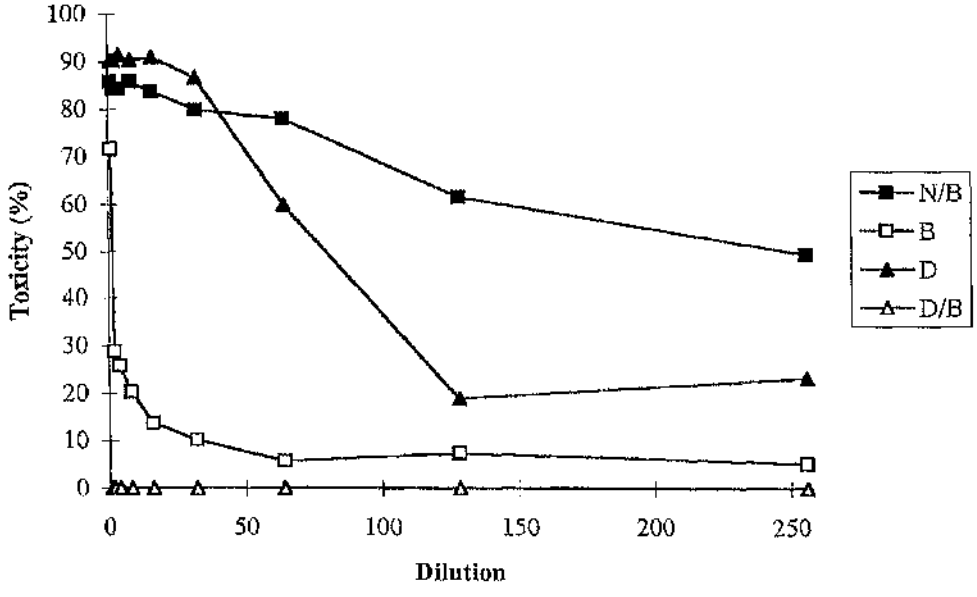


Figure 3:7d Effect of boiling and dialysis on NCTC 11145 supernatants cultured in BHI



Figures 3:8 a-b Effects of bactrial growth media on cell culture cell lines, Chinese hamster ovary (CHO) and INT 407. Growth media tested were skim milk (10% w/v) (SMP) and brain heart infusion broth (3.7% and 7.4% w/v) (BHI).

Fig 3.8a Effect of bacterial growth media on CHO cells

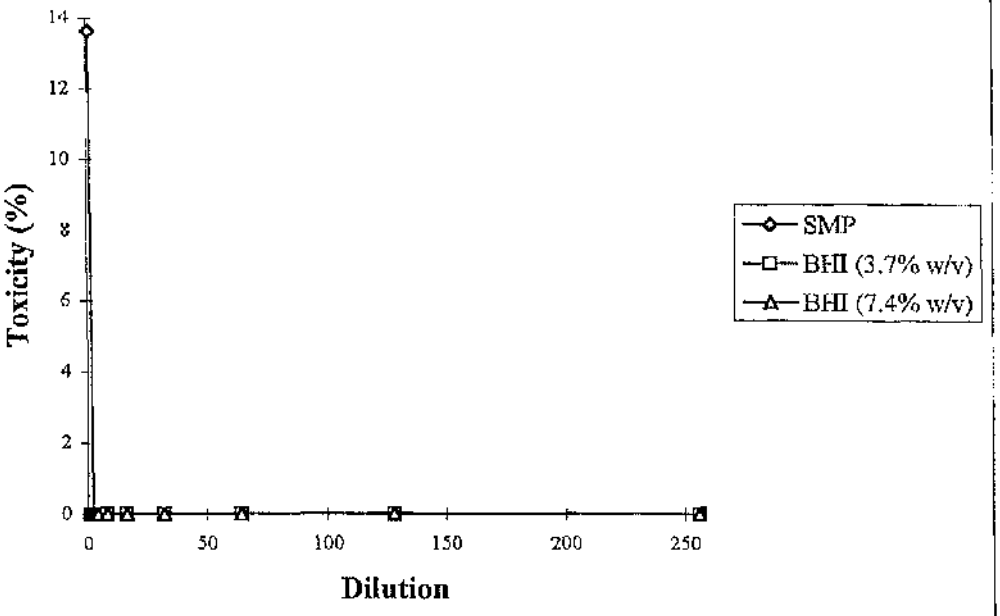


Fig 3.8b Effect of bacterial growth media on INT 407 cells

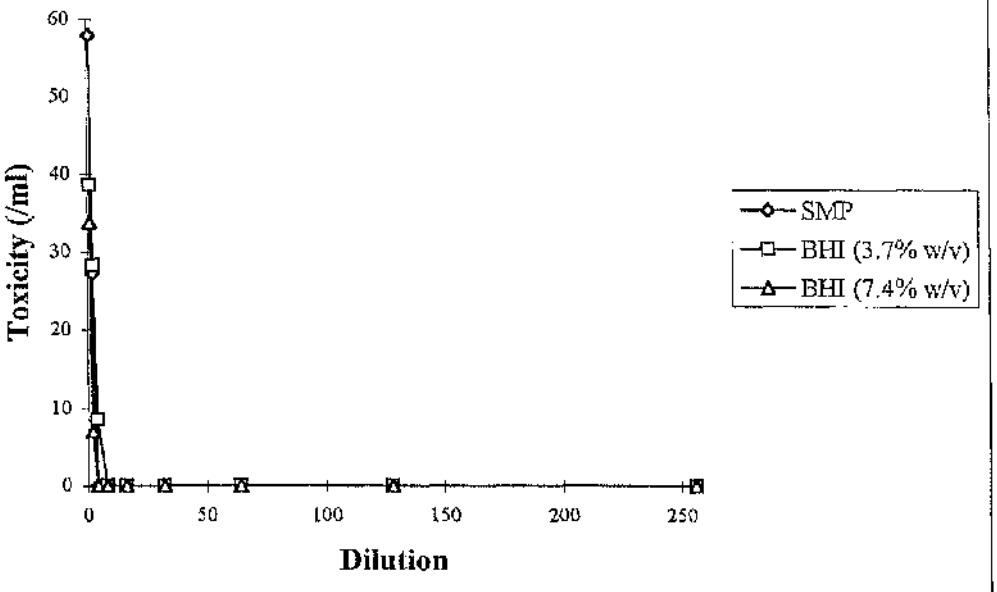


Figure 3:9a Effect of boil times on SMP cultured NCTC 11143 cell free culture supernatants with CHO cells

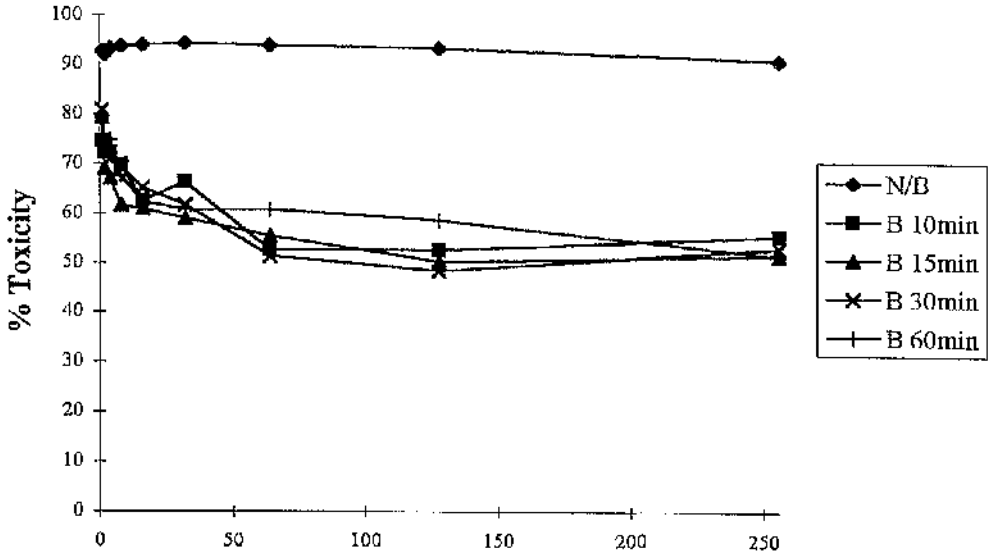
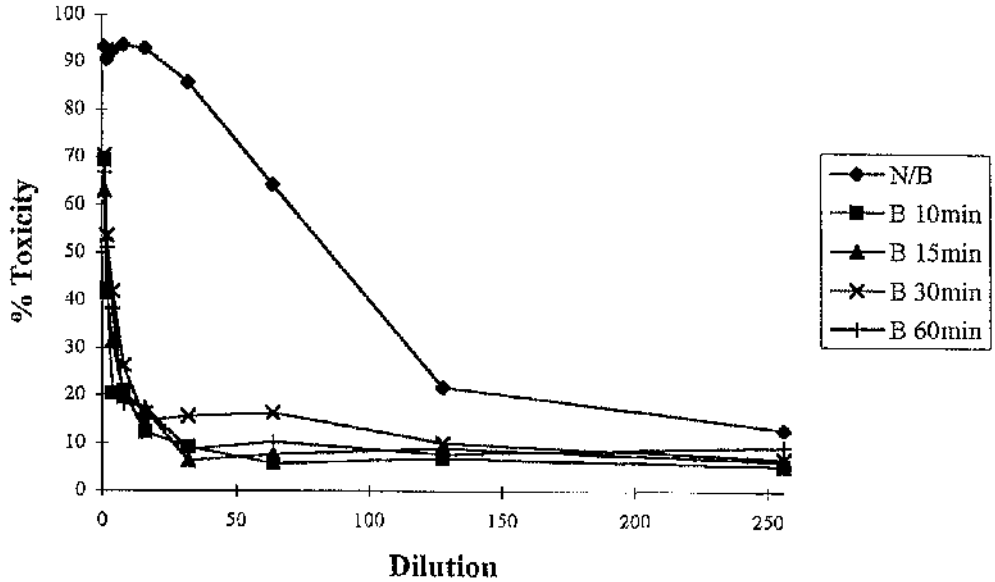


Figure 3:9b Effect of boil time on toxicity of SMP cultured NCTC 11145 cell free culture supernatants with CHO cells



Figures 3:10 a-b Molecular mass separated fractions of B.cereus emetic (NCTC 11143) and diarrhoeagenic (NCTC 11145) strains cell frec culture supernatants,following growth in brain heart nfusion broth (BHI).

N/B = Not boiled; B = Boiled

Figure 3:10a Molecular mass separation of NCTC 11143 supernatants cultured in BHI

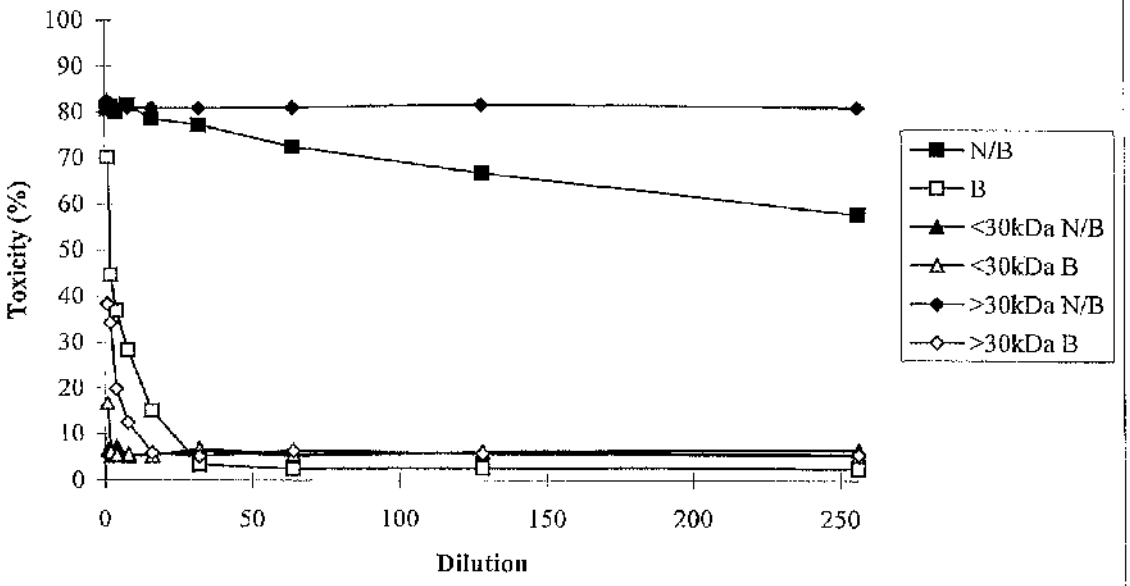
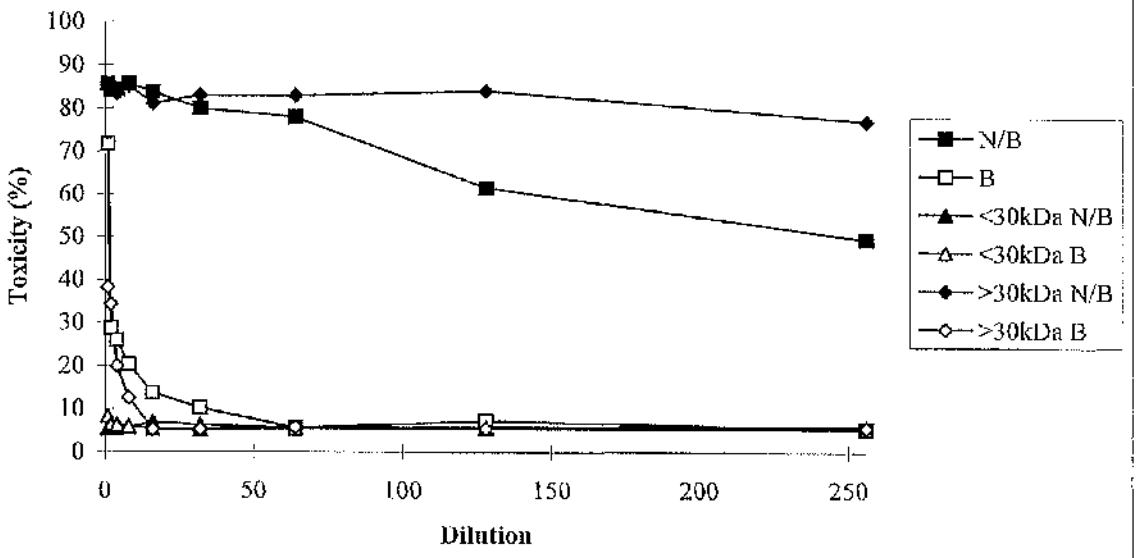


Figure 3:10b Molecular mass separation of NCTC 11145 supernatants cultured in BHI



CHAPTER 4.

FOURIER TRANSFORM INFRARED SPECTROSCOPY STUDIES ON *BACILLUS* SPP.

4:1 FTIR SPECTROSCOPY INTRODUCTION

In recent years molecular biological methods have been applied to the identification of bacteria. The requirement for molecular analysis or physico-chemical methods has come about because microbial classification using traditional methods may be too time consuming and labour intensive for use in some quality control (Tatzel *et al.*, 1994) or therapeutic treatments. Many of the classical identification tests require special growth media, for instance for *Bacillus* spp. media such as blood agar or polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) are used. These can be expensive and require preparation time. Improvements have been made in traditional techniques, including miniaturised carbohydrate utilisation kits made commercially by companies such as bioMérieux (Basingstoke), who produce the API identification systems. Nevertheless, these kits still rely on incubation of the bacteria, and therefore still take several days in order to generate results.

Molecular methods used include random amplification of polymorphic DNA (RAPD) (Williams *et al.*,1990; Stephan, 1996), and nucleic acid probes (Tatzel *et al.*, 1994). Physico-chemical methods include mass spectrometry (Wunschel *et al.*, 1996), pyrolysis mass spectrometry (Helyer *et al.*, 1997), pyrolysis gas-liquid chromatography (O'Donnell *et al.*, 1980), phage typing (Vaisanen *et al.*, 1991; Ackermann *et al.*, 1995), fatty acid analysis (Vaisanen *et al.*, 1991) and infrared (IR) spectroscopy (Naumann *et al.*, 1988).

Infrared radiation extends from the visible region of the electromagnetic spectrum, to the microwave region (10,000 to $10cm^{-1}$) (Naumann *et al.*, 1991). Information is generated by IR spectroscopy regarding the vibrational and rotational motions of atoms and molecules (Huston *et al.*, 1988). The IR spectra of a biological sample are vibrational spectra, with highly characteristic absorption bands, which produce a

"fingerprint" of the sample (Naumann *et al.*, 1991). FTIR spectroscopy measures all of the different wavenumbers of the IR spectrum at once using an interferometer, generating an interferogram. From the interferogram, the IR spectrum is obtained by means of fast Fourier transform algorithm (Naumann *et al.*, 1991), so that every data point of the signal contains information over the entire IR region (Naumann *et al.*, 1991). Fourier transform (FT) instruments have two principal advantages which contribute to a high signal to noise ratio compared to conventional continuous wave instruments. These advantages derive from (a) the multiplexing of the signal forming an interferogram so that effectively the whole spectrum is measured continuously, and (b) the absence of slits means that a higher through put of light is possible. An Fourier transform instrument can routinely achieve a signal to noise ratio of 10^4, which is 1 or 2 orders of magnitude higher than is commonly achieved in a continuous wave instrument. However, FT instruments have another advantage over continuous wave instruments in that the wavenumber is derived in the interferometer by a laser to a precision of 1 part in 10^4. Lastly, FT instruments are more stable to drift, so spectra can be averaged for long times and remain reproducible for many years without recalibration.

FTIR spectroscopy is a non-destructive, non-radioactive procedure, which provides a safe method of molecular analysis of bacteria. This should be compared with methods such as nucleic acid probes which can be labeled using radioactive markers (Tatzel *et al.*, 1994). FTIR spectroscopy allows the rapid characterisation of structural features of biological molecules when in a pure form (Zeroual *et al.*, 1994). It can also be utilised to generate infrared spectra of whole cells, such as bacteria. The entire chemical composition of the cell, including everything from cell membrane material to intracellular products contributes to the spectrum (Van der Mei *et al.*, 1993). Fourier transform infra-red (FTIR) spectroscopy was introduced as a method for the classification and possible identification of bacteria by Naumann *et al.* (1988) and

Naumann *et al.* (1991). Fourier transform instruments revolutionised the classification of microorganisms by their IR spectra, and made possible routinely what had previously, with continuous wave instruments, been achieved only with difficulty. FTIR spectroscopy has been employed in the identification of *Listeria* spp (Holt *et al.*, 1995; Leifer *et al.*, 1996), *Lactobacillus* spp. (Curk *et al.*, 1994), and *Staphylococcus, Streptococcus, Clostridium, Legionella* and *E.coli* (Helm *et al.*, 1991). FTIR is a highly discriminating method which, like all analytical methods, yields optimal results when a high degree of standardisation is used in preparing the samples. In order to produce standardisation, growth times and conditions for the bacteria, sample preparation procedures, and cell concentrations all must be carefully controlled.

In the case of *Bacillus* spp. while isolation and culturing is not difficult, identification to species level is considered to be complicated because it can generate ambiguous results (Logan and Berekley, 1984; Kramer and Gilbert, 1989). In many laboratories *Bacillus* spp. are simply classified as "aerobic endospore forming rods" (Logan and Berkeley, 1984). Many traditional methods, such as morphological description of colony size, shape and structure, rely on appraisal of phenotype which may be influenced by the physiological state of the cell and the particular growth conditions (Tatzel *et al.*, 1994).

Members of the *B.cereus* group, namely *B.cereus, B.mycoides*, *B.thuringiensis* and *B.anthracis* are very closely related species. There has been debate whether, in spite of distinct physiological properties, the *B.cereus* group members should be considered as variants of one species, as opposed to four separate species (Smith *et al.*, 1952; Wolf and Barker, 1968; Logan, 1980; Logan and Berkeley, 1981; Logan and Berkeley, 1984; Ash *et al.*, 1991; Ash and Collins, 1992; Henderson *et al.*, 1994; Nakamura and Jackson, 1995).

It is virtually impossible to discriminate among members of the *B.cereus* group using carbohydrate utilisation profiles (Logan and Berkeley, 1984). The bioMérieux API system for the identification of *Bacillus* species works by comparing the carbohydrate utilisation profile generated by an unknown Gram positive, spore forming rod, with the profiles contained in the bioMérieux data base. The probability that a particular species of bacteria has generated the carbohydrate utilisation profile is calculated, and the most likely species assigned, with an indication of the confidence of the identification. Different strains of a bacterial species can give rise to many different carbohydrate utilisation patterns. In a recent study of *B.cereus* isolated from dairy plants in the Netherlands, more than 20 different carbohydrate utilisation patterns for *B.cereus* were revealed (te Giffel *et al.*, 1996a). The carbohydrate utilisation patterns originating from *B.cereus*, *B.mycoides* or *B.thuringiensis*, are so similar that they could also be produced by any of the other group members.

Molecular methods, including randomly amplified polymorphic DNA (RAPD) techniques (Stephan, 1996), low molecular weight (LMW) RNA profiles (Demezas and Bell, 1995), restriction fragment length polymorphisms (RFLP) (Demezas and Bell, 1995), and nucleic acid probes (Tatzel *et al.*, 1994) have all been employed with varying success to differentiate between *Bacillus* spp..

Randomly amplified polymorphic DNA (RAPD) techniques (Williams *et al.*,1990), have been used to separate *B.cereus* and *B.lentus* species (Stephan, 1996). RAPD has also been used to distinguish strains in the species *B.thuringiensis* (Brousseau *et al.*, 1993). A high specificity was demonstrated between the members of the *B.cereus* group using three polymerase chain reaction (PCR) primers within the sequences cereolysin AB (Schraft and Griffiths, 1995). Although it was not possible to differentiate between members of the *B.cereus* group using these primers, they were able to distinguish between the *B.cereus* group from other *Bacillus* species (Schraft

and Griffiths, 1995).

Low molecular weight (LMW) RNA profiles have been used to differentiate between species of bacteria (Höfle, 1988). This technique is based on the high resolution electrophoretic separation of low molecular weight RNA (5S rRNA and tRNA). LMW RNA profiles were unable to separate the members of the *B.cereus* group into species (Demezas and Bell, 1995).

The use of restriction fragment length polymorphisms (RFLP) based on probing for ribosomal DNA sequences was also unable to distinguish between the members of the *B.cereus* group (Demezas and Bell, 1995). Even when the results of LMW RNA and RFLP were taken in combination, separation was not achieved (Demezas and Bell, 1995).

In a survey of milk and cream sampled in a dairy production plant *B.licheniformis* was most commonly identified (Tatzel *et al.*, 1994). Classical identification methods were found to be too time consuming, and therefore a colony hybridisation method was developed for the identification of *B.licheniformis* strains. The method utilised non-radioactive labeled 23S rRNA targeted oglionucleotide probes (Tatzel *et al.*, 1994).

Fatty acid analysis was able to separate members of the *B.cereus* group from other *Bacillus* spp, but was unable to differentiate between the members of the group (Vaisanen *et al.*, 1991). Analysis of sugar profiles of vegetative cells by gas chromatography mass spectroscopy also did not separate *B.cereus* from *B.thuringiensis* (Wunschel *et al.*, 1996). However, using pyrolysis gas-liquid chromatography with canonical variates analysis, it was possible to separate strains of *B.cereus*, *B.mycoides* and *B.thuringiensis* from one another (O'Donnell *et al.*, 1980). Pyrolysis mass spectrometry studies were successful at distinguishing *B.anthracis*

from *B.thuringiensis* and emetic strains of *B.cereus* (Helyer *et al.*, 1997).

Although the success rate of distinguishing between members of the *B.cereus* group using molecular methods was low, the ability to achieve species separation using pyrolysis gas-liquid chromatography (O'Donnell *et al.*, 1980) and pyrolysis mass spectrometry (Helyer *et al.*, 1997) have demonstrated that differentiation was possible.

Interpreting the information produced by FTIR spectroscopy is helped by multivariate statistical analysis. Linear discriminant analysis is one member of a group of multivariate statistical methods used in discriminating between similar objects (Jolliffe, 1986). The principle is to represent a spectrum as a point in a multi-dimensional space where the dimensions are the wavelengths of the absorption measurements. Similar objects would then occupy points nearby in space. For practical reasons, the dimensionality of the space is reduced to give canonical variates that are linear combinations of the dimensions. To maximise the discrimination among a selected group of objects, weighting factors (loadings) can be applied to the dimensions.

The object of the piece of work described here was to attempt to differentiate between *Bacillus* spp. using FTIR spectroscopy. Because the separation of the *B.cereus* group of species has been so problematical, it was decided to focus particularly on three members of this group. *B.anthracis* was not included for examination in this piece of work owing to its pathogenicity.

4:2 FTIR SPECTROSCOPY METHOD

4:2:1 Cell preparation

The *Bacillus* spp. type cultures used in this study were obtained from the National Collection of Food Bacteria, Reading, and the National Collection of Industrial and Marine Bacteria, Aberdeen. The type cultures used were *B.cereus* NCFB 1771, *B.mycoides* NCFB 681, *B.thuringiensis* NCIMB 9134, *B.circulans* NCFB 1775, *B.licheniformis* NCFB 1772, *B.megaterium* NCFB 1173, *B.pumilus* NCFB 1766, *B.subtilis* NCFB 1769, *B.firmus* NCFB 1762. Other isolates of *B.cereus*, *B.mycoides* and *B.thuringiensis* were isolated from raw milk or the farm environment.

Single, well isolated colonies, were selected from a pure culture milk agar plate, and subcultured onto another milk agar plate. This subculturing procedure was performed in triplicate for each type strain tested, except for type strains of the members of the *B.cereus* group where the procedure was replicated 9 times. Replicates were formed in this way to ensure the independence of the replicates as far back in the method as possible. The subcultured plates were incubated at 30°C for 24h. From the subcultured plates a single, well isolated colony was selected, inoculated into 10ml brain heart infusion broth (BHI) and incubated at 30°C overnight. An aliquot (250µl) of this suspension was then inoculated into 25ml BHI, and incubated on an orbital incubator (LH Engineering Co. Ltd., Stoke Poges) (200rpm) at 30°C for 18h.

The cells were harvested in a pre-weighed tube, by centrifugation of the broth (5,000*g* for 30min, at 4°C); the pellet was resuspended in 0.85% saline solution (2ml) and pelleted again (11,000 *g* for 10min at 4°C). The saline wash was repeated, and the wet weight of the cell pellet determined. The cells were resuspended to a concentration of 100mg wet cells per ml of sterile water. A 200µl aliquot of fresh bacterial suspension

was placed onto the centre of a zinc selenide (ZnSe) optical plate and dried at 50°C for 45-60 minutes.

For each *Bacillus* spp. type culture 3 replicate samples were taken, except for *B.cereus*, *B.mycoides* and *B.thuringiensis*, which were each repeated 9 times. Replicate samples were produced on different days to maximise the variance of the sample preparation, and thus ensure the greatest independence of repeat samples.

4:2:2 FTIR measurements

Spectra were recorded on a Mattson Galaxy 7000 spectrometer equipped with a deuterated triglycine sulphate (DTGS) detector by co-addition and averaging 100 scans. Spectral resolution was $4cm^{-1}$. FTIR spectra were measured from 500-$4000cm^{-1}$, at approximately $1cm^{-1}$ sampling interval. A background spectrum was taken before each test spectrum was measured to minimise the problem of the instrument detecting changes in atmospheric gases along the optical path which should not be incorporated into the test spectrum. An example of selected FTIR spectra are shown Figure 4:1 a-d.

4:2:3 Statistical analysis

The spectra recorded on the FTIR spectrophotometer were standardised by setting the baseline of each spectum to zero, scaled and normalised before statistical analysis was performed; these standardised data form the identity matrix (X). To reduce the dimensionality of the data, principal components analysis of the identity matrix was performed, which generated a score matrix (A).

Principal components (PC) analysis :-

(Uses the original spectra)

$A=XE$	A= PC score matrix	(n x r)
	X= Scaled normalised spectra	(n x l)
	E= PC loadings	(l x r)
	n= number of spectra	
	l= number of sampling points	
	r= number of PC's	

90% of the total variance was held in the first 20 principal components, and it is these 20 which were taken into the canonical variate analysis.

Canonical variates are linear combinations of the principal components in *A*, which maximise the ratio of between groups to within group variance. Each bacterial spectrum has its own position in canonical variate space. To separate the 9 *Bacillus* spp. type cultures, 4 canonical variates (CV) were required, and to separate the 3 species of the *B.cereus* group 2 CV's were used. Fewer CV's were used to separate the 3 *B.cereus* group species because more dimensions than groups cannot be used.

CV analysis:-

(Used *A*)

$D=AC$	D= CV score matrix	(n x l)
	A= PC score matrix	(n x r)
	C= CV loadings	(l x m)
	m= number of CV's	

The Mahalanobis distances between spectra are a measure of statistical significance of pattern differences (Goodfellow and O'Donnell, 1994). Mahalanobis distances are the square root of the sum of the squared differences between spectral coordinates; they were calculated from the canonical variates for the set of samples being assessed. Mahalanobis distances of more than 3, separate populations with little overlap and the populations can therefore be regarded as distinct.

The bootstrap estimate was used to validate the CV model. The bootstrap estimate worked by withdrawing some sample spectra, and randomly selecting the remaining absorbance spectra with repeats, so that the sample size was enlarged. The amplification procedure was repeated 100x, and CV analysis was performed on each set of generated data. The average of the 100 principal components results formed the classification matrix (*B*). The withdrawn spectra were then reintroduced into the classification matrix and classified, which generated a resubstitution matrix (*R*). To determine the efficiency of the classification matrix, values "*B*" and "*R*" were then introduced into the bootstrap estimation equation.

Validation of the model (bootstrap estimation):-

(Used a classification matrix)

$$B632 \text{ estimate } = 0.632B + 0.268R$$

B = Classification matrix based on amplified data set, with missing samples (g x g)

R = Classification matrix following reintroduction of missing values.

g = number of groups

If the classification matrix was reliable, then the resubstituted data would be correctly classified.

4:3 FTIR SPECTROSCOPY RESULTS

FTIR spectroscopy was applied to some members of the genus *Bacillus* to see if it is useful as a classification method for these species. Type cultures of eight Group I *Bacillus* spp. were examined together with *B.circulans*, which is a member of Group II *Bacillus* spp., but is commonly isolated from milk.

4:3:1 Identification of 9 Bacillus type species using biochemical tests

Miniturised biochemical test kits API 50 CHB (bioMérieux) were used to confirm the identity of the *Bacillus* spp. type cultures through the organisms' carbohydrate utilisation profiles. The API kits were able to correctly identify 7 out of the 9 *Bacillus* spp. type cultures. The identity of *B.firmus* and *B.thuringiensis* could not be confirmed biochemically. Also, although the *B.cereus* type culture strain was identified correctly, its carbohydrate utilisation profile was inconclusive because it could also have been generated by *B.mycoides* or *B.thuringiensis* strains. The *B.thuringiensis* type culture produced a carbohydrate utilisation profile which had a 99.9% probability of being *B.cereus*, with the possibility of being *B.thuringiensis*. These three species, *B.cereus*, *B.mycoides* and *B.thuringiensis* are members of the *B.cereus* group and are very closely related. Although there were small differences between the carbohydrate utilisation profiles of the three bacteria (Table 4:1), these differences were insufficient to discriminate between the species. The reason for this is that the API system classifies unknown bacteria on the basis of statistical probability.

The type culture of *B.firmus* (NCFB 1762) failed to produce any carbohydrate utilisation profile. Young actively growing, cultures of NCFB 1762 were tested 4 times on API 50 CHB kits, but no growth was apparent in any of the wells. The isolate was a Gram positive spore forming rod and for the purpose of the FTIR examination it

is referred to as *B.firmus*, on the basis of the classification provided by the type culture collection that supplied the strain.

4:3:2 FTIR spectral range

Assessment of the value of FTIR spectroscopy as a method of classifying *Bacillus* spp. was made using the 9 type culture spectra recorded in the range of 500-4000cm^{-1}. Using the "bootstrapping" estimation method, discriminant analysis was only able to identify 4 of the 9 *Bacillus* spp. tested (Table 4:2). The identified species were *B.firmus*, *B.circulans*, *B.megatarium* and *B.mycoides*. Although 89% of *B.mycoides* spectra were correctly classified by the classification matrix, the group was only 1.8 and 1.3 Mahalanobis distances from the *B.cereus* and *B.thuringiensis* spectra, respectively, indicating how closely related the three species are (Table 4:3).

In an attempt to increase the discrimination between *Bacillus* spp. by FTIR spectroscopy, the spectral range examined was shortened from 500-4000cm^{-1} to 500-2000cm^{-1}. When the same spectral results for the nine *Bacillus* spp. were examined, using the truncated spectra (500-2000 cm^{-1}) all of the nine type cultures could be separated from one another using four canonical variates (Figure 4:2). The matrix for their identification is shown in Table 4:4. When full spectra were used for the classification matrix the proportion assigned to the correct species was 58.6%. Using the shortened spectra 95.6% of results were correctly assigned. The Mahalanobis distances between the nine species were greater when truncated spectra were used, which indicated a greater degree of separation between the species than had been achieved using the full spectral range (Table 4:5). For example, the Mahalanobis distance between *B.mycoides* and *B.cereus* increased from 1.8 to 7.6 when the spectral range was shortened.

4:3:3 Bacillus cereus group type cultures

FTIR spectroscopy based on the discrimination model for the 9 *Bacillus* spp. tested had a success rate for classifying the 3 type cultures of the *B.cereus* group of 88.7%, when the wavenumbers 500-2000cm^{-1} were used. Canonical variate analysis was carried out, creating a classification matrix based on the loadings for the *B.cereus* group species alone. This was done to see whether the classification of the 3 *B.cereus* group species which had been derived from information from all 9 *Bacillus* spp., could be improved when the data from non-*B.cereus* group bacteria were removed from the matrix. The classification matrix for the *B.cereus* group alone correctly assigned 86% of the spectra (Table 4:6). The assignment was marginally worse than it had been with the 9 species model.

4:3:4 Discrimination among Bacillus cereus group isolates

From the library of bacterial isolates at the Hannah Research Institute, 38 *Bacillus* spp. isolates were selected which were catalogued as either *B.cereus*, *B.mycoides* or *B.thuringiensis*. The isolates came from milk samples, the farm or dairy environments. The selected isolates were examined for morphology, under the microscope, for haemolytic activity, growth on *B.cereus* selective media (PEMBA), motility tests and classification using the API 50 CHB test kit. The results are summarised in Tables 4:7 (a-c) and 4:8 (a-c).

It was confirmed using the API 50 CHB biochemical test kit that all 38 isolates belonged to the *B.cereus* group, but the technique did not discriminate the 3 species. When combined with other tests, the *B.mycoides* isolates could be distinguished from the two others: *B.mycoides* has rhizoidal morphology on an agar plate, and is non-

motile.

FTIR spectra were recorded on the isolates and the bootstrap method was used for validation of the statistical analysis. The same spectra of the isolates were grouped in two ways, first by treating the spectra of the isolates as random samples from the species which had been defined by the library storage classification; and then without assigning any species identification, but recognising isolate groups of replicate spectra. ie. The first analysis was designed with prior classification of the spectra into 3 groups, and in the second analysis into 38 groups.

A measure of the performance of the linear discrimination analysis is the agreement between the classification matrix derived from the bootstrap validation procedure and the prior classification based on the results of conventional tests or the source of the samples. Agreement of the classification matrices by the two methods of prior classification can also be considered to support the assignment of species. The results of the canonical variate analysis for classifying the isolates first as species and then as unknown species were encouraging. When the isolates were analysed having been assigned to species, the proportion correctly classified by FTIR spectroscopy was 96%; the classification matrix is shown in Table 4.9. When the isolates were assessed with prior classification as isolates, only 70% of the *B.cereus* isolates, 45% of *B.mycoides* and 59% of *B.thuringiensis* were correctly classified. However, although the classification to isolate level was low, 95% of the isolates had been grouped into their correct species (Table 4.10).

The proportion of isolates correctly classified by FTIR when the isolates had not been previously allocated to known species was only 1% lower than when the species of the isolate had been designated. When the spectra were pre-classified as species, different canonical variate results (Figure 4:3), and different loadings (Figure 4:4) were

obtained than if the isolates were not pre-classified as species. The most important wavenumber for discrimination of the isolates when not assigned into species was 1740 cm^{-1} (0.67 root sum of square loading), but when the isolates were first classified as species the most important wavenumber was approximately 1520 cm^{-1} (0.26 root sum of square loading). The wavenumber 1740 cm^{-1} was also the most significant in the *B.cereus* group type culture discriminant model (0.63 root sum of square loading). Although the significance of individual wavenumbers differed depending upon the pre-classification of the data, the same wavenumbers were significant in the discrimination of all sets of *B.cereus* group data. Table 4.11 shows the most important wavenumbers in the classification of *B.cereus* group species type cultures, *B.cereus* group isolates when preclassified and also when regarded as unknowns.

Using the *B.cereus* group type culture discrimination model it was not possible to correctly classify the isolates as species.

4:4 FTIR SPECTROSCOPY DISCUSSION

Fourier transform infrared spectroscopy was an effective technique for discrimination between the 8 members of Group I *Bacillus* spp. tested, correctly assigning 95% of samples.

4:4:1 Spectral range

The spectral range used in FTIR analysis was found to be important. When spectra from 500-4000cm^{-1} were used the success rate of classification among the 9 *Bacillus* spp. examined was 58.6%. When shortened spectra, taking wavenumbers from 500-2000 cm^{-1} were used, the success rate was 95.6%.

When the FTIR spectra were recorded, readings were made without purging the counting chamber of gases. Therefore, included in the readings of the bacterial spectrum, were also absorption lines from the atmosphere. To minimise the effect of absorbance derived from sources other than the bacterial sample, a background spectrum was recorded immediately before each bacterial spectrum. Nevertheless, carbon dioxide, which strongly absorbs at about 2350cm^{-1}, could clearly be detected on the loadings for canonical variates when the full spectra from 500-4000cm^{-1} was analysed. By shortening the spectral range to 500-2000cm^{-1}, the wavenumbers at which carbon dioxide is absorbed are removed from the analysis, and therefore do not bias the loading.

Shortening the spectral range was considered to be a more practical solution to the problem of atmospheric changes, than purging the counting chamber of gases. When the chamber is purged, the time taken to collect the sample spectra is increased two to

three fold. This is a significant increase, especially when a lot of samples are to be recorded.

Other information, such as water absorbtion, which occurs between 3100-3600cm^{-1} (Naumann *et al.*, 1991), was also removed from the loading data when the shortened spectra were used. The infrared spectrum can be separated into 5 broad windows, which correspond to different parts of the bacterial cell (Naumann *et al.*, 1991; Van der Mei *et al.*, 1993) (Table 4:12), but the spectral bands are so highly overlapped that only in rare instances is it possible to assign a band to a particular substance. Nearly all substances have more than one band. By shortening the FTIR spectrum from 500-4000cm^{-1}, down to 500-2000cm^{-1} the fatty acid region, corresponding to Window 1 (Table 4:12), was removed from the analysis, but this does not remove all information about fatty acid composition and content.

Discrimination among the *Bacillus* spp. was made from 500-2000 cm^{-1} wavenumbers, which loosely corresponds to windows 1-4. The loadings (Figures 4:3 a-d) of the data identify areas on the spectrum which are important for discrimination (Holt *et al.*, 1995). A peak at a particular wavenumber indicates the importance of this wavenumber in the discrimination. Loadings are based on all of the groups in a classification. Therefore the loadings for classification of the nine *Bacillus* spp. together, are different to the loadings for the *B.cereus* group alone.

When the nine *Bacillus* spp. were classified together, the wavenumbers of greatest importance for discrimination fell in spectral window 4 (1300-900 cm^{-1}). This area is the phosphate and polysaccharide region.

Window-4 was still of importance in the discrimination of the *B.cereus* group, but there were also high loadings in window 3 (1500-1300 cm^{-1}) and window 2 (1300-900

cm^{-1}). These are the "mixed" and amide regions respectively.

4:4:2 B.cereus group species

There has been considerable debate concerning the classification of members of the *B.cereus* group of bacteria, which consists of *B.cereus, B.mycoides, B.thuringiensis* and *B.anthracis*. It has been suggested that these Group I members should be regarded as variants of *B.cereus*, as opposed to separate species (Smith *et al.*, 1952; Logan, 1980; Logan and Berkeley, 1981). The DNA homology between the members of the *B.cereus* group is very high (Kaneko *et al.*, 1978; Stadhouders, 1992), suggesting they are in fact a single species. Conversely though, it has been argued that because of their highly different pathogenicities, they ought to remain as separate species (Heimpel, 1967). In this study the use of biochemical methods was insufficient to separate *B.cereus, B.mycoides* and *B.thuringiensis* from one another, both with the type cultures and the 38 isolates which were tested. This is similar to earlier findings (Logan, 1980; Logan and Berkeley, 1981; Vaisanen *et al.*, 1991). Using FTIR spectroscopy the three species could be distinguished from one another, and the generated identity matrix was able to classify 95% of the *B.cereus* group isolates tested.

Other molecular methods have had varying degrees of success in attempting to differentiate between members of the *B.cereus* group. Pyrolysis gas-liquid chromatography analysed by canonical variate analysis, was able to separate *B.cereus, B.mycoides* and *B.thuringiensis* into three distinct groups (O'Donnell *et al.*, 1980), and pyrolysis mass spectrometry studies were successful at distinguishing *B.anthracis* from *B.thuringiensis* and emetic strains of *B.cereus* (Helyer *et al.*, 1997). Fatty acid analysis was however, unable to distinguish between members of the *B.cereus* group (Vaisanen *et al.*, 1991).

The use of phage typing has had variable success rates, with some phages being species selective (Vaisanen, 1991), and others being unable to distinguish between *B.cereus* and *B.thuringiensis* (Ackermann *et al.*, 1995).

FTIR spectroscopy has been successful in differentiating between *Staphylococcus* spp. (Helm *et al.*, 1991), *Streptococcal* spp. (Helm *et al.*, 1991; Van der Mei *et al.*, 1993), *Clostridum* spp. (Helm *et al.*, 1991), *Legionella* spp. (Helm *et al.*, 1991), *E.coli* (Helm *et al.*, 1991), *Lactobacillus* spp. (Curk *et al.*, 1994),and *Listeria* spp. (Holt *et al.*, 1995).

FTIR spectroscopy also has potential as an identification tool. For FTIR spectroscopy to be used in this manner, the generation of a data base containing spectral information obtained from a wide range of bacterial isolates for each species, would be required. The spectra would have to be gathered under highly standardised conditions. The importance of sample standardisation was highlighted when pyrolysis gas-liquid chromatography was used to characterise *Bacillus* spp.. There it was found that changes in the physiological state, such as sporulation or pigmentation, resulted in marked changes in the pyrograms (O'Donnell and Norris, 1980). Identification in systems such as API (bioMérieux) rely on large databases, which contain information regarding the carbohydrate utilisation patterns of the general bacterial group to which an unknown isolate belongs. Statistics are employed to say with what likelihood a bacterium which generates a specific profile, will have of belonging to a specific species. An FTIR spectroscopic data base could work using these same basic statistical principals.

Using the classification matrix based on the *B.cereus* group type cultures, an attempt was made to classify the *B.cereus* group isolates. This met with limited success,

because although the type cultures are the microbiologically accepted standards for classification, they represent only one profile as an infrared spectra, which is not necessarily at the mean point of the variance found across the entire species. Therefore it is not feasible to classify unknown isolates against the profiles generated against type cultures alone. However, with databases which include strain variation, FTIR spectroscopy has the potential to be useful for identification purposes.

FTIR spectroscopy equipment is expensive, but following the initial investment, the technique is economical to run. This should be compared with the costs of conventional identifications. Biochemical identification using miniaturised test kits, such as API, are expensive, with each isolate requiring a new test. Thus, even though the initial equipment cost may be lower, if many bacteria are to be identified routinely, conventional methods may be more expensive to perform in the long run than FTIR spectroscopy.

It is possible to discriminate among isolates within a species of *Bacillus* using FTIR spectroscopy. In the results here, 70% of the *B.cereus* isolates which were correctly identified to species level, were also assigned to the correct isolate. In the future it may be possible to utilise isolate discrimination to identify specific features which isolates express. For instance, diarrhoeagenic or emetic toxin producing strains of *B.cereus* could be examined for potential separation. If the FTIR spectra for toxin producing strains were found to be different from non-toxic strains this information would have considerable potential in clinical and quality control situations. At present identification and toxicity testing occur separately. Using FTIR spectroscopy one identification test could potentially, not only identify an organism, but also provide an indication of its pathogenic potential. In clinical situations this could result in the hastening of treatment actions, and in quality control this information would make risk assessment more accurate. With further work FTIR spectroscopy has the potential to

become a more useful tool in the field of microbiology.

TABLES AND FIGURES

FOR FTIR SPECTROSCOPY STUDIES

Table 4:1 Carbohydrate utilisation profiles for type culture isolates of members of *Bacillus* Group I species. Carbohydrates which were not utilised by any of the *Bacillus* species tested have not been included in the table. A "+" score represents a score of 3-5 as defined according to the API 50CHB instructions. A "+/-" score represents a borderline score between 2 and 3.

Table 4:1 Carbohydrate utilisation profiles of *Bacillus* species

ISOLATE	Control	Glycerol	L-Arabinose	Ribose	D-Xylose	Beta- Methyl-xyloside	Galactose	D-Glucose	D-Fructose	D-Mannose	Inositol	Mannitol	Sorbitol	Alpha-Methyl-D-mannoside	Alpha-Methyl-D-glucosid	N Acetyl glucosamine
B.cereus				+				+								+
B.mycoides				+				+	+							+
B.thuringiensis				+				+	+	+						+
B.circulans			+		+	+	+	÷	+	+	+	+	+			÷
B.lichemiformis		+	+	÷	÷		+	+	+		+	+	+		+	+
B.megaterium		+/-	+	+			+/-	+	+			+				+
B.pumilis				+/-			+/-	+	÷	+	+					
B.subtilis		÷	÷	+				+	÷	+	+	+			+	
B.firmus																

ISOLATE	Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	Maltose	Lactose	Melibiose	Sucrose	Trehalose	Inulin	Melezitose	D-Raffinose	Starch	Glycogen
B.cereus		+	+	+	+	÷			+	÷				+	÷
B.mycoides	+/-	+	+	+		+			÷	+				+	÷
B.thuringiensis		+	+	+	+	+			+	+				+	+
B.circulans	+	+	÷	+	+	+	+		+	÷	+	÷	+	÷	+
B.lichemiformis	+	+	+	+	+	+		+	+	+				+	+
B.megaterium		+	+	+		+	+	+	+	+		+	+	+	+
B.pumilis		+	+	+	+				+	+					
B.subtilis	+	+	+	+	+	+		+	+	+	+		+	+	+
B.firmus															

ISOLATE	Xylitol	B-Gentiobiose	D-Turanose	D-Lyxose	D-Tagatose	D-Arabitol	L-Arabitol	Gluconate
B.cereus			+					
B.mycoides								
B.thuringiensis	+	+	+			+	+	+
B.circulans		+			+			
B.lichemiformis								
E.megaterium		+			÷			
B.pumilis			+					
B.subtilis								
B.firmus								

Table 4:2 Identification matrix for members of Group I *Bacillus* spp. using FTIR spectroscopy, with a spectral range 500-4000cm^{-1}

True	Assigned Species								
	cere	*myc*	*thur*	*circ*	*firm*	*lich*	*mega*	*pum*	*subt*
cere	0.50	0.25	0.13	0	0	0.13	0	0	0
myc	0	0.89	0	0	0	0.11	0	0	0
thur	0.22	0.56	0	0	0	0	0	0	0.22
circ	0	0	0	0.89	0	0.11	0	0	0
firm	0	0	0	0	1.0	0	0	0	0
lich	0	0	0	0	0	0.66	0	0.11	0.22
mega	0	0	0	0	0	0	1.0	0	0
pum	0	0	0	0	0	0.44	0	0.55	0
subt	0	0	0	0	0	0.44	0	0	0.55

Proportion correct 0.59

Key (Applies to Tables 4:2, 4:3, 4:4 and 4:5): *cere = B.cereus; myc = B.mycoides; thur = B.thuringiensis; circ = B.circulans; firm = B.firmus; lich = B.licheniformis; mega = B.megaterium; pum = B.pumilus; subt = B.subtilis*

Table 4:3 Mahalanobis distances for 9 *Bacillus* spp. using FTIR infra-red spectra from 500-4000cm^{-1}

	cere	*myc*	*thur*	*lich*	*pum*	*subt*	*firm*	*mega*	*circ*
cere	0.0								
myc	1.8	0.0							
thur	1.0	1.3	0.0						
lich	3.4	2.6	3.4	0.0					
pum	4.3	4.0	4.5	1.9	0.0				
subt	3.0	2.8	3.3	1.0	1.4	0.0			
firm	6.6	8.1	7.2	8.0	7.2	7.0	0.0		
mega	10.7	11.2	10.3	12.4	12.6	11.9	11.4	0.0	
circ	8.0	7.3	8.1	6.4	7.7	7.0	12.9	15.7	0.0

Table 4:4 Identification matrix for members of Group I *Bacillus* spp. using FTIR spectroscopy, with a spectral range 500- 2000cm^{-1}

True	Assigned Species								
	cere	*myc*	*thur*	*circ*	*firm*	*lich*	*mega*	*pum*	*subt*
cere	0.87	0.01	0.11	0	0	0	0	0	0
myc	0.01	0.85	0.15	0	0	0	0	0	0
thur	0.04	0.02	0.94	0	0	0	0	0	0
circ	0	0	0	0.94	0	0.04	0	0	0.02
firm	0	0	0	0	1.0	0	0	0	0
lich	0	0	0	0	0	1.0	0	0	0
mega	0	0	0	0	0	0	1.0	0	0
pum	0	0	0	0	0	0	0	1.0	0
subt	0	0	0	0	0	0	0	0	1.0

Proportion correct 0.96

Table 4:5 Mahalanobis distances for 9 *Bacillus* species using the FTIR infra-red spectra from 500-2000cm^{-1}

	cere	*myc*	*thur*	*circ*	*firm*	*lich*	*mega*	*pum*	*subt*
cere	0.0								
myc	7.6	0.0							
thur	4.3	4.5	0.0						
circ	26.3	25.9	27.3	0.0					
firm	16.3	17.6	14.9	34.9	0.0				
lich	28.5	27.4	28.9	8.9	37.4	0.0			
mega	50.5	45.9	49.7	44.6	57.5	48.9	0.0		
pum	46.3	43.8	46.0	23.2	53.4	18.8	51.1	0.0	
subt	26.3	24.5	25.9	10.8	32.6	7.0	49.0	21.2	0.0

Table 4:6 Classification matrix for *B.cereus* group species using FTIR spectroscopy with a spectral range of 500-2000cm^{-1}

True Species	Assigned Species		
	B.cereus	*B.mycoides*	*B.thur.*
B.cereus	0.82	0.03	0.15
B.mycoides	0.03	0.90	0.06
B.thur.	0.11	0.02	0.88

Proportion correct 0.86

B.thur = B.thuringiensis

Table 4:7a Microbiological characteristics of *B.cereus* isolates

Isolate	Morphological description	Microscopic description	Haemolysis	Motility	PEMBA
FSS 101	Effuse, crenated, waxy sheen	Single cells	+	+	+
FSS 103	Effuse, crenated, waxy sheen	Single cells	+ (Weak)	+	+
FSS 106	Effuse, crenated, waxy sheen	Single cells	+	+	+
FSS 110	Effuse, crenated, waxy sheen	Chains of 2 or 3 cells	+	+	+
FSS 112	Effuse, crenated, waxy sheen	Single cells	+	+	+
FSS 114	Effuse, crenated, waxy sheen	Single or paired cells	+	+	+
FSS 129	Effuse, crenated, waxy sheen	Single or paired cells	+ (Weak)	+	+
FSSM 155	Effuse, crenated, waxy sheen	Paired cells	-	+	+
NCTC 11143	Effuse, crenated, waxy sheen	Single or paired cells	+	+	+
HRM 44	Effuse, crenated, waxy sheen	Chains of 2-3 cells	+	+ (Weak)	+
NCTC 11145	Effuse, crenated, waxy sheen	Mostly pairs, but some single cells	+ (Strong)	+	+
F4108	Effuse, crenated, waxy sheen	Single or paired cells	+ (Weak)	+ (Weak)	+
F2105	Effuse, crenated, waxy sheen	Single cells	+ (Weak)	+	+
F1078/89	Effuse, crenated, waxy sheen	Single or paired cells	+ (Strong)	+	+

Table 4:7b Microbiological characteristics of *B.mycoides* isolates

Isolate	Morphological description	Microscopic description	Haemolysis	Motility	PEMBA
FSS 010	Rhizoid, opaque, waxy sheen	Chains of cells (~6)	-	-	+
FSS 011	Rhizoid, opaque, waxy sheen	Chains of cells (~6)	-	-	+
FSS 012	Rhizoid, opaque, waxy sheen	Chains of cells (~6)	-	-	+
FSS 013	Rhizoid, opaque, waxy sheen	Long cells, some chains	-	-	+
FSS 100	Rhizoid, opaque, waxy sheen	Long, thin cells, some chains (3 or 4 cells long)	-	-	+
FSS 102	Rhizoid, opaque, waxy sheen	Chains of cells (~6)	-	-	+
FSS 107	Rhizoid, opaque, waxy sheen	Small, thin cells. Chains (~6)	-	-	+
FSS 108	Rhizoid, opaque, waxy sheen	Chains of cells (~6)	-	-	+
FSS 109	Rhizoid, opaque, waxy sheen	Long thin cells. Chains (~4)	-	-	+
FSS 118	Rhizoid, opaque, waxy sheen	Long, thin cells. Chains (~3)	-	-	+
FSS 120	Rhizoid, opaque, waxy sheen	Long, thin cells. Chains (~3)	-	-	+
FSS 126	Rhizoid, opaque, waxy sheen	Chains (2-3 cells long)	-	-	+

Table 4:7c Microbiological characteristics of *B.thuringiensis* isolates

Isolate	Morphological description	Micro-scopic description	Haemolysis	Motility	PEMBA
PM 003	Effuse, crenated, waxy sheen	Mostly paired cells	+	+ (spiral)	+
PM 004	Effuse, crenated, waxy sheen, slight feathering at edge	Mostly paired cells	+	+ (spiral)	+
PM 065	Effuse, entire, waxy sheen	Mostly paired cells	+	+ (spiral)	+
PM 073	Effuse, irregular, waxy sheen	Long thin cells. Chains of 3 or 4 cells	+	-	+
HRM 053	Effuse, crenated, waxy sheen	Paired cells	+	+	+
HRM 074	Effuse, crenated, waxy sheen	Single	+	+	+
HRM 083	Effuse, crenated, waxy sheen	Mainly single or paired cells, some chains	+ (strong)	+	+
HRM 087	Effuse, crenated, waxy sheen	Single	+ (weak)	+	+
MRM 218	Effuse, crenated, waxy sheen	Single or paired cells	+	+	+
MRM 223	Effuse, crenated, waxy sheen	Single or paired cells	+	+ (weak)	+
MRM 227	Effuse, crenated, waxy sheen	Single or paired cells	-	+	+
MRM 370	Effuse, crenated, waxy sheen	Single or paired cells	+ (weak)	+ (weak)	+

Table 4:8 a-c Carbohydrate utilisation profiles of isolates of *B.cereus*, tested using API 50CHB. Carbohydrates which were not utilised by any isolates have not been included in the table. Scoring of carbohydrate utilisation is according to API 50CHB instructions. "1" represents low utilisation, through to "5" which is high utilisation. Scores of "3" and above are regarded by API 50CHB identification system to be positive utilisation.

Table 4:8a Carbohydrate utilisation profiles for *B.cereus* isolates

Isolate	Control	Glycerol	Ribose	D-Glucose	D-Fructose	D-Mannose	a-Methyl-D-mannoside	a-Methyl-D-glucoside	N Acetyl glucosamine	Amygdalin	Arbutin	Esculin	Salicin
FSS 101			2	5	5				2.5	3	2.5	5	2.5
FSS103				5	5					2.5	4	5	5
FSS 106		1	2	5	5				5		3	5	3
FSS 110				3	3				3			5	3
FSS 112		1	5	5	5				5	2	3	5	3
FSS 114		1		4	5	5			4	1	1	4	4
FSS129				5	5				5		5	5	5
FSSM 155			1	5	5		5	5	5	5	5	3	5
11143			2	5	5				5			3	
HRM 44													
11145									5		5	5	5
F4108			1	5	4				5		1	3	
F2105			2	5	4				5		2	5	
F1078/89			1	5	4				5			5	

Table 4:8a Carbohydrate utilisation profiles for *B.cereus* isolates continued

Isolate	Cellobiose	Maltose	Sucrose	Trehalose	Starch	Glycogen	B-Gentiobiose
FSS 101	2.5	3		2.5 2 or 3		3	
FSS103	2.5			3 2 or 3			
FSS 106	3	4	5	5	4	5	
FSS 110		3		5	3	3	
FSS 112	2	5		5 2 or 3		4	
FSS 114	5	3		5		2	2
FSS129	5	5		5	5	5	
FSSM 155				5	5	5	
11143		5	5	5			
HRM 44							
11145	5	5		5	5	5	
F4108		5	5	5			
F2105		5	5	5	2		
F1078/89		5	5	5			

Table 4:8b Carbohydrate utilisation profiles for *B.mycoides* isolates

Isolate	Control	Glycerol	Ribose	D-Glucose	D-Fructose	N Acetyl glucosamine	Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	Maltose	Sucrose	Trehalose
FSS 010			2	5	5	5	5	5	5	5	5	5		5
FSS 011			1	5	4	5	3	4	5	5	4	5		5
FSS 012				5	5	4	2	3	5	5	2	5		5
FSS 013		1	3	5	5	5	5	5	5	5	5	5		5
FSS 100			1	5	4	5	3	4	5	5	4	5		5
FSS 102		1	3	5	5	5	3	5	5	5	3	5		5
FSS 107		1	3	5	5	5		3	5	5	3	5	5	5
FSS 108			2	5	5	5	2	5	5	5	2	5		5
FSS 109				5	5	4	3	3	5	5	2	5		5
FSS 118			2	5	5	5	2	4	5	5	2	4		5
FSS 120		2	3	5	5	5	4	4	5	5	4	5		5
FSS 126		1	3	5	5	5	3	5	5	5	3	5		5

Table 4:8b Carbohydrate utilisation profiles for *B.mycoides* isolates continued

Isolate	Starch	Glycogen	D-Turanose
FSS 010	5	5	
FSS 011	4	5	
FSS 012	5	5	
FSS 013	5	5	
FSS 100	4	5	
FSS 102	5	5	
FSS 107	5	5	
FSS 108	5	5	
FSS 109	5	5	2
FSS 118	4	5	
FSS 120	5	5	
FSS 126	4	5	

Table 4:8c Carbohydrate utilisation profiles for *B.thuringiensis* isolates

Isolate	Control	Glycerol	Ribose	D-Glucose	D-Fructose	D-Mannose	N Acetyl glucosamine	Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	Maltose	Trehalose	Starch	Glycogen	B-Gentiobiose
PM 3		1	1	5	5		4	2	2	5	4	4	5	5	4	5	
PM 4			2or 3	4	5		4	2 or 3		5	5	2 or 3	4	5	3	5	
PM 5			2	5	5		5	2	5	5	5	2	5	5	5	5	
PM 65			4	5	5	5	5	2	5	5	5	2 or 3	4	5	5	5	
HRM 53		2	2	5	5	5	5	2	4	5	5	5	5	5	2	4	2 or 3
HRM 74			1	5	5		5		2	3	4		5	5	5	5	
HRM 83			2	5	5		5	1	2 or 3	5	5	1	5	5	4	5	
HRM 87		2	2	5	5		5	3	4	5	5	2	5	5	4	5	
MRM 218		1	2	5	5		5	3	3	5	5	1 or 2	5	5	3	5	
MRM 223			1	5	5		5		1	5	3		3	5		5	
MRM 227			2	5	5		5	5	5	5	5	3	5	5	5	5	
MRM 370			2	5	5		5	4	5	5	5	2	5	5	4	5	

Table 4:9 Matrix of classification of *B.cereus* group isolates with prior assignment into species using FTIR spectroscopy with a spectral range 500-2000cm^{-1}

	Assigned Species		
True Species	*B.cereus*	*B.mycoides*	*B.thur.*
B.cereus	0.97	0	0.03
B.mycoides	0	0.95	0.05
B.thur.	0.01	0.02	0.97

Proportion correct 96.3%

B.thur = B.thuringiensis

Table 4:10 Matrix of classification of *B.cereus* group isolates without prior designation into species, using FTIR spectroscopy with a spectral range from 500-2000cm^{-1}

	Assigned species		
True Species	*B.cereus*	*B.mycoides*	*B.thur.*
B.cereus	0.99	0	0.01
B.mycoides	0	0.96	0.04
B.thur.	0.08	0.02	0.90

Proportion correct 95%

B.thur = B.thuringiensis

Table 4:11 Important wavenumbers in the classification of the *B.cereus* group species using FTIR spectroscopy with a spectral range from 500-2000cm^{-1}

Important Wavenumbers	Type Cultures	Preclassified Isolates	Isolates As Unknowns
1025	0.249 (6)	0.187 (3)	0.649 (2)
1050	0.157 (8)	0.140 (7)	0.438 (3)
1100	0.368 (3)	0.181 (4)	0.349 (6)
1412	0.258 (5)	0.195 (2)	0.309 (8)
1510	0.281 (4)	0.265 (1)	0.400 (4)
1620	0.493 (2)	0.142 (6)	0.362 (5)
1689-1694	0.242 (7)	0.048 (8)	0.331 (7)
1740	0.631 (1)	0.162 (5)	0.670 (1)

Table 4:12 Windows of the infrared spectrum

Wavenumber (cm^{-1})	Region
3000-2800	Fatty acid region
1800-1500	Amide region
1500-1300	"Mixed region"
	Information from proteins and fatty acids
1300-900	Phosphate and polysaccharide
900-700	"The true fingerprint"
	Shows specific patterns which can not yet
	be assigned to functional groups

Naumann *et al*, 1991

Figure 4:1a FTIR absorbance spectra of selected Group I *Bacillus* spp. type cultures

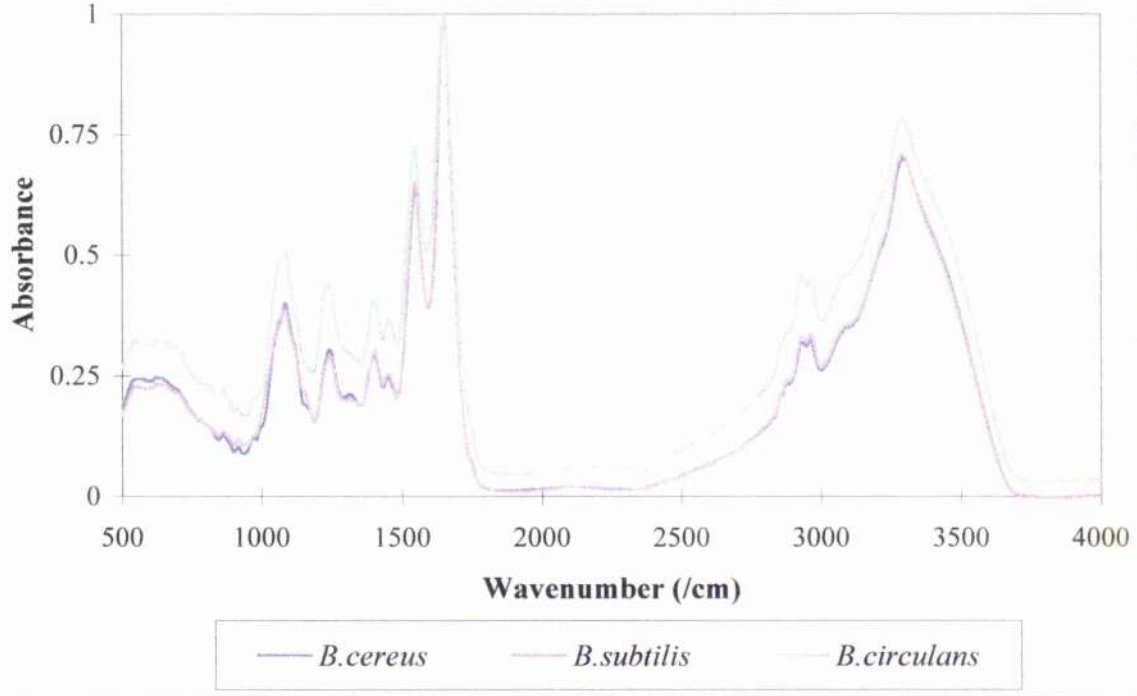


Figure 4:1b Truncated FTIR absorbance spectra of selected Group I *Bacillus* spp. type cultures

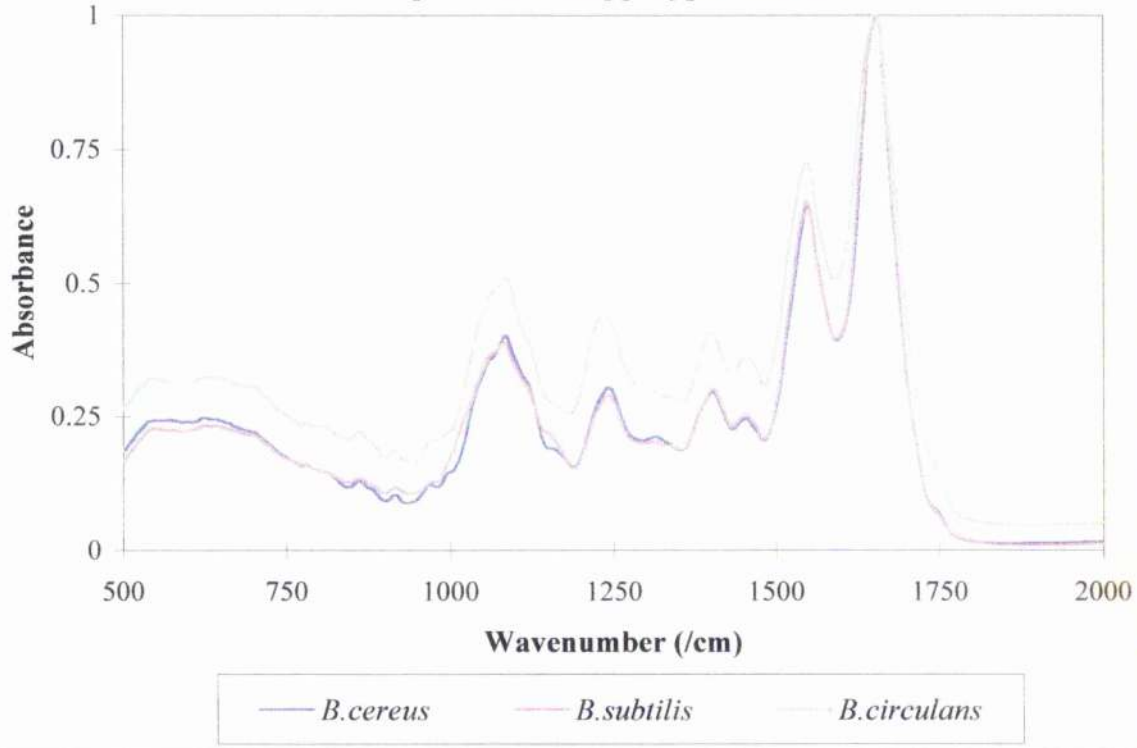


Figure 4:1c FTIR absorbance spectra for members of the *B.cereus* group

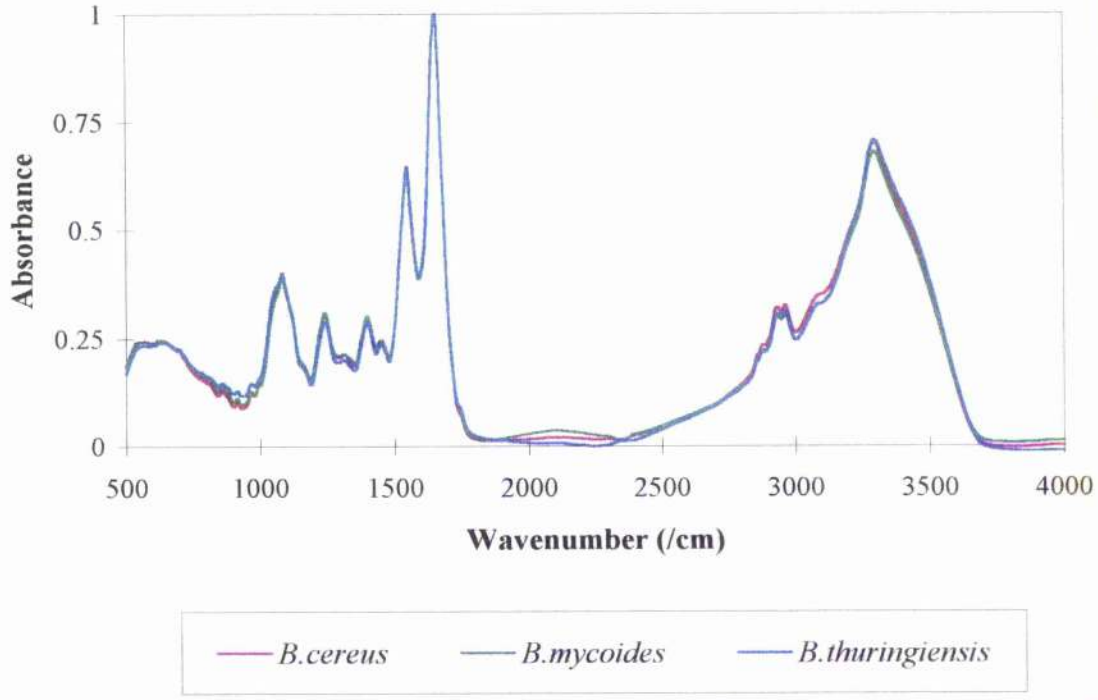


Figure 4:1d Truncated FTIR absorbance spectra for members of the *B.cereus* group

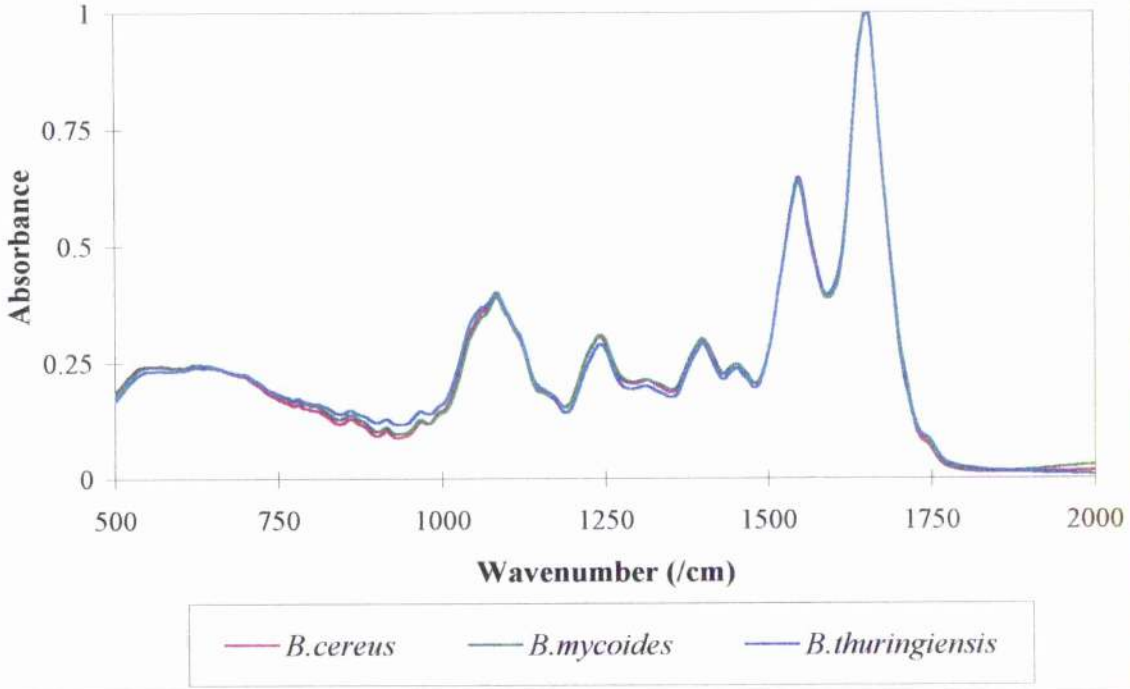


Figure 4:2 Graph showing discrimination between type species of 9 Group I *Bacillus* species using FTIR spectroscopy, illustrated using canonical variates 1, 2 and 3.

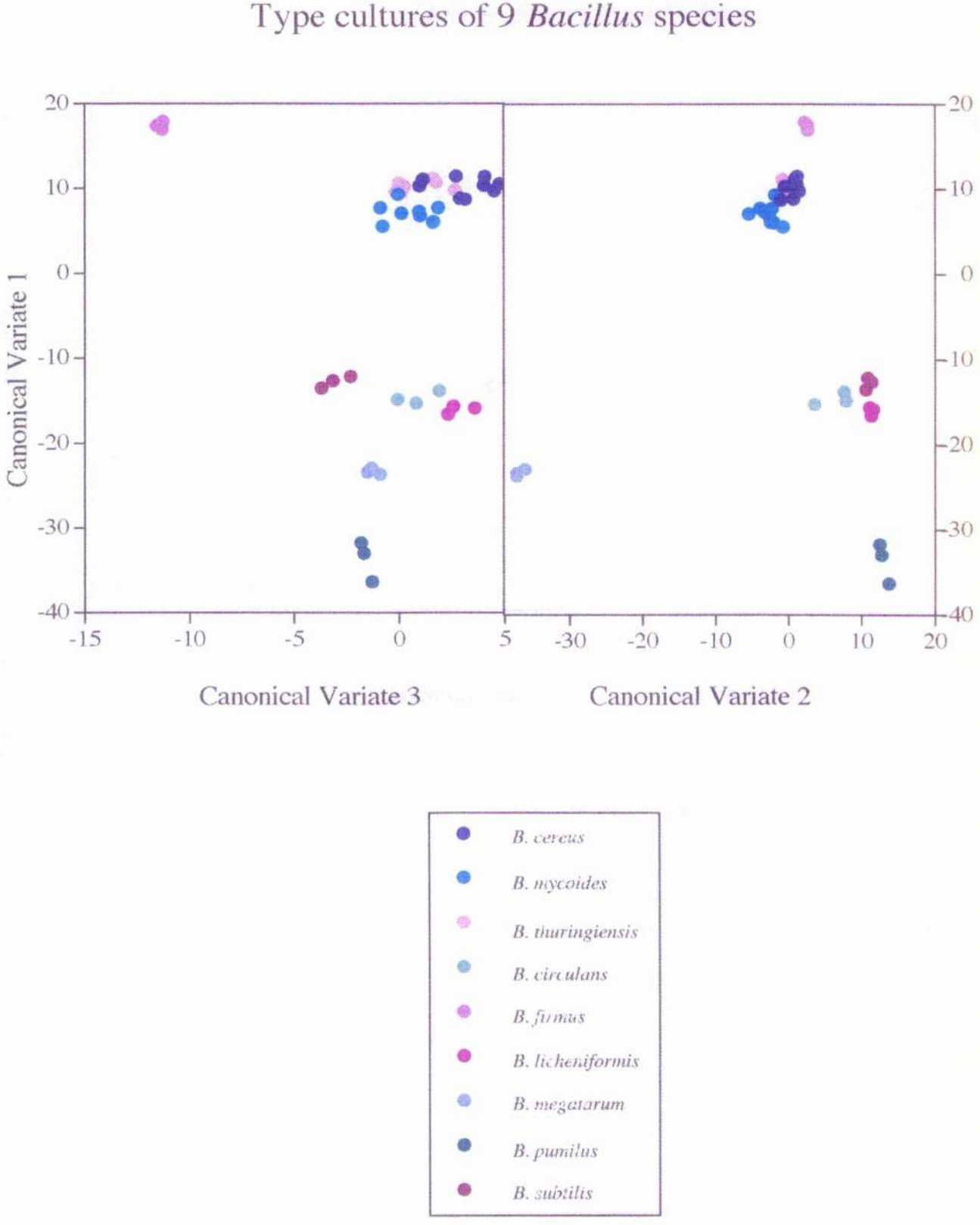

Type cultures of 9 *Bacillus* species
Canonical Variate 1
Canonical Variate 3
Canonical Variate 2
20
10
0
-10
-20
-30
-40
-15
-10
-5
0
5
-30
-20
-10
0
10
20
B. cereus
B. mycoides
B. thuringiensis
B. circulans
B. firmus
B. licheniformis
B. megaterium
B. pumilus
B. subtilis

Figure 4:3 a-b Graphs showing discrimination between 38 isolates of *B.cereus* group using FTIR spectroscopy when pre-classified by (a) isolate identification and (b) species identification, illustrated using canonical variates 1 and 2.

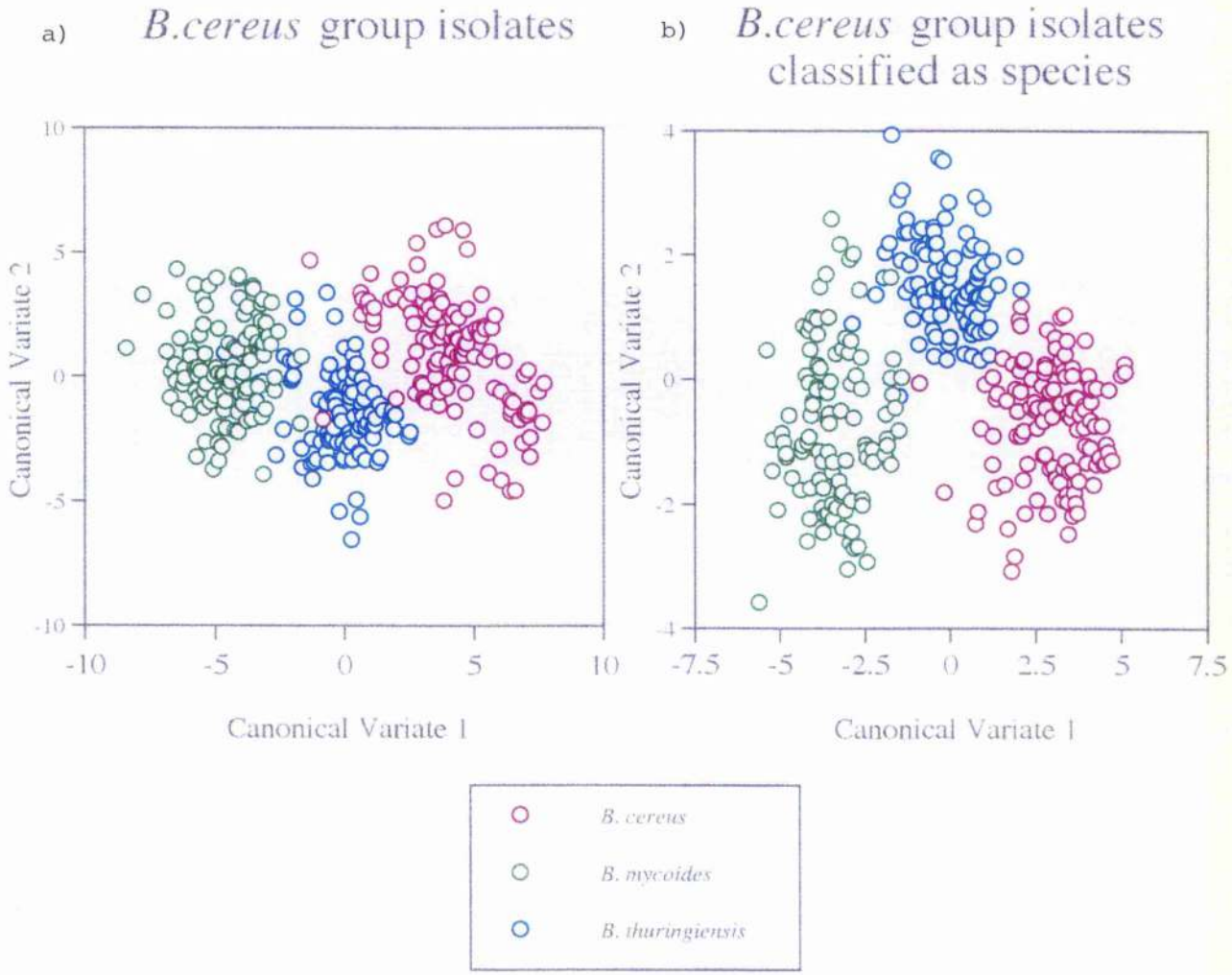

a)
B.cereus group isolates
b)
B.cereus group isolates classified as species
Canonical Variate 2
Canonical Variate 1
10
5
0
-5
-10
-10
-5
0
5
10
4
2
0
-2
-4
-7.5
-5
-2.5
0
2.5
5
7.5
B. cereus
B. mycoides
B. thuringiensis

Figure 4:4a-b Graphs illustrating the wavenumber loadings for discriminant models for a) the 38 *B.cereus* group isolates b) *B.cereus* group isolates pre-classified as species.

Figure 4:4a Loadings for 38 *B.cereus* group isolates discrimainant model

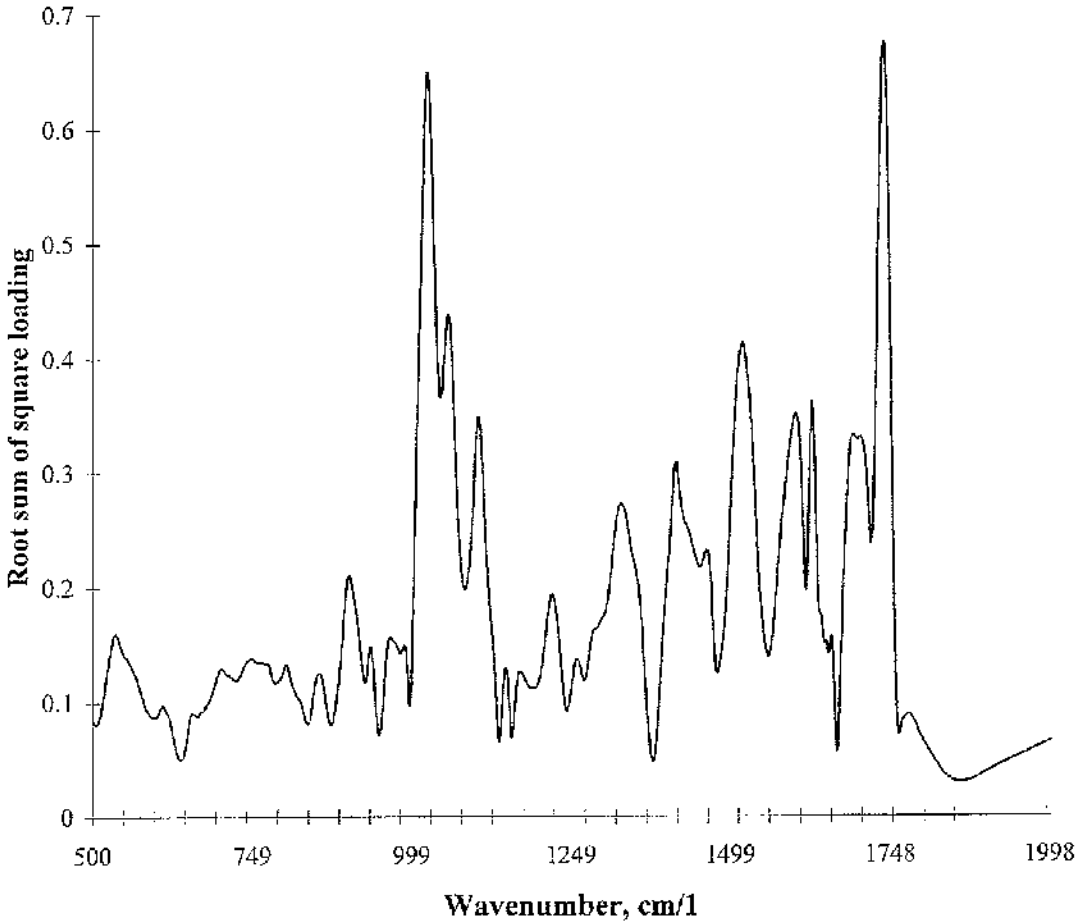
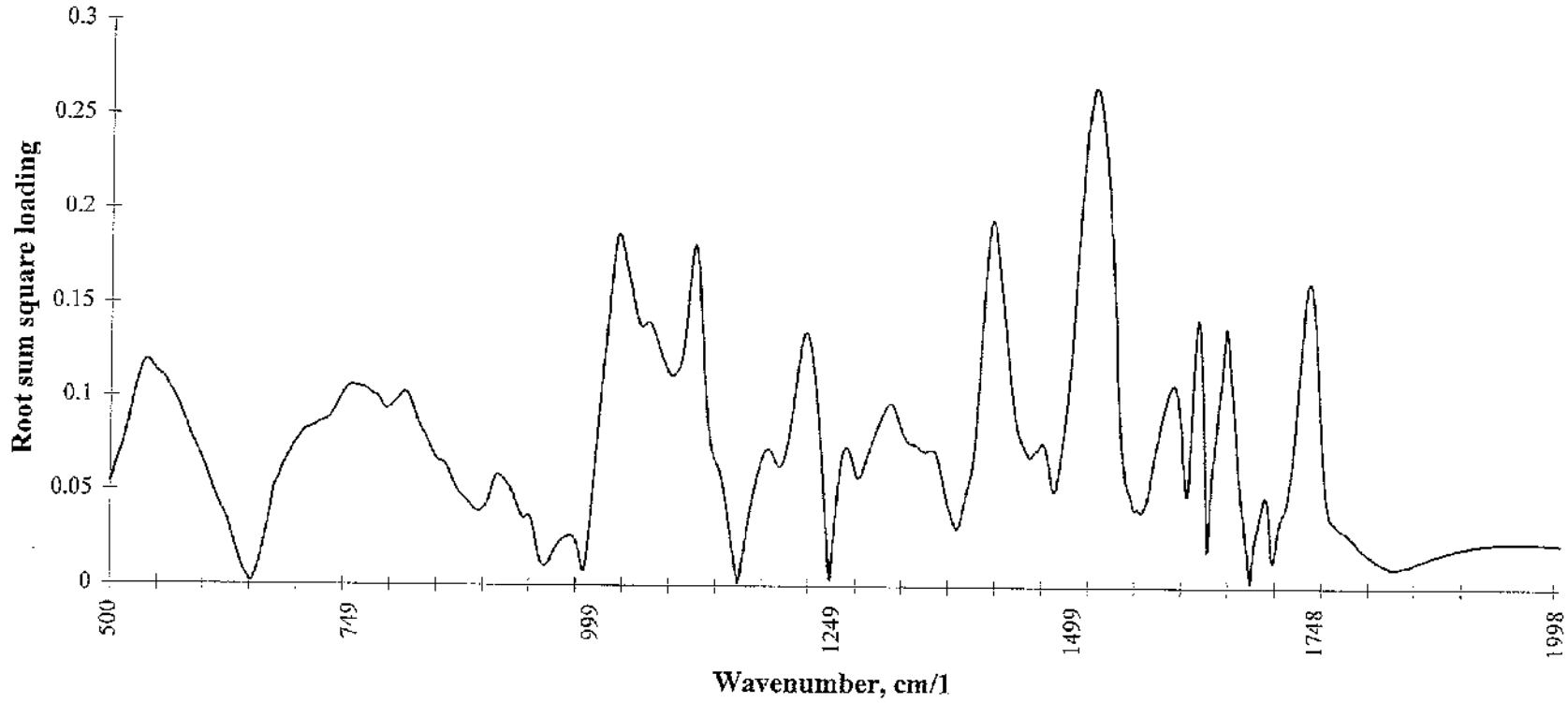


Figure 4:4b Loadings for discriminant model of *B.cereus* group isolates pre-classified as species



CHAPTER 5.

STUDIES OF INTERACTIONS EXISTING BETWEEN *BACILLUS* SPP.

5:1 INTRODUCTION TO

BACILLUS SPP. INTERACTIONS STUDIES

Interspecies microbial interactions are widespread in nature. They may occur between fungi and bacteria, for example *Penicillium* spp. produce antibiotics which are inhibitory to various bacteria. Interactions may also occur between different genera of bacteria, for example *Lactobacillus* and *Bacillus* spp. (Varadaraj, 1993), or between different species within the same genus, as with *B.subtilis* and *B.cereus* (Sutherland and Murdoch, 1994). Organisms generally produce inhibitory factors to procure themselves an advantage over other organisms which compete for the same nutrient sources.

Interactions between mesophilic and psychrotrophic species of *Bacillus* may be responsible for the seasonal changes which occur in their populations. As has been discussed in Chapter 2, psychrotrophic and mesophilic populations of *Bacillus* spp. have been shown to exist in milk with seasonality (McKinnon and Pettipher, 1983; Phillips and Griffiths, 1986; Sutherland and Murdoch, 1994). Psychrotrophic sporeformers have been shown to be more prevalent during the summer-autumn months, with mesophilic populations predominating in the winter (Phillips and Griffiths, 1986; Sutherland and Murdoch, 1994). Sutherland and Murdoch (1994) have demonstrated that the mesophilic and psychrotrophic species exist in milk only in the absence of the other, and proposed that interactions between mesophilic and psychrotrophic *Bacillus* spp. could be responsible for the observed seasonality (Sutherland and Murdoch, 1994). It was suggested that mesophilic species of *Bacillus* repress the growth of psychrotrophs for much of the year. However, in the summer-

autumn when they decline, psychrotrophs are then able to prevail (Sutherland and Murdoch, 1994).

The objective of this study was to investigate interspecies interactions in the genus *Bacillus* and specifically to establish the nature and potential application of the antagonism between *B.cereus* and *B.subtilis*. *Bacillus subtilis* produces bacillopeptins, which are antifungal antibiotics (Kajimura *et al.*, 1995), and has been shown to inhibit the growth of *B.cereus* (Sutherland and Murdoch, 1994). Studies were undertaken to establish whether inhibitors were cell associated or extracellular, and whether different strains of *B.subtilis* differed in inhibitory effect, or different strains of *B.cereus* varied in sensitivity to inhibitor effect.

Bacillus cereus itself can produce antimicrobial agents. These include cerein (Naderio *et al.*, 1993), zwittermicin A (Stabb *et al.*, 1994; Raffel *et al.*, 1996) and kanosamine (Milner *et al.*, 1996). Cerein is produced by a strain of *B.cereus*, but is active against other *B.cereus* strains (Naderio *et al.*, 1993). Zwittermicin-A suppresses the plant disease caused by *Phytophthora medicaginis*, which affects alfalfa growth (Naderio *et al.*, 1993; Raffel *et al.*, 1996). Kanosamine is inhibitory to the oomycetes of plant pathogens; in addition to these inhibitory effects on certain fungi it also inhibits a few bacterial species (Milner *et al.*, 1996). Some strains of *Bacillus cereus* can also inhibit rumen populations (Gedek *et al.*, 1992). Therefore the ability of *B.cereus* to inhibit *B.subtilis* was also investigated.

During recent years there has been both an increase in the incidence and detection of foodborne diseases, and a consumer driven demand for foods which are preserved naturally (Roller *et al.*, 1995). These factors have led to a rise in interest in bacteriocins, which are microbial inhibitors from bacterial sources. The most

commonly used bacteriocin is nisin (Abee *et al.*, 1992). Nisin is produced by *Lactococcus lactis;* it has a broad spectrum of activity, including effect against *B.cereus* (Abee *et al.*, 1992). It works by disrupting the plasma membranes of sensitive organisms (Abee *et al.*, 1992). *Bacillus cereus* decreases rapidly from young cheeses when lactic acid bacteria are present (Lodi and Malaspina, 1992). If non-toxic strains of *B.subtilis* could be identified, they may potentially be of benefit by inhibiting the growth of toxin forming *B.cereus* strains in milk and dairy products.

5:2 METHODS USED TO STUDY

BACILLUS SPP. INTERACTIONS

5:2:1 Assessment of inhibition of B.cereus by B.subtilis

The strains of *B.cereus* which were selected for use in this experiment were NCTC 11143 and NCTC 11145; these are emetic and diarrhoeagenic strains of *B.cereus* respectively. The strains of *B.subtilis* chosen were MRM 057 and MRM 079. These had been isolated from raw milk, and were strains which had been shown to inhibit the growth of *B.cereus* in previous work (Sutherland and Murdoch, 1994).

The interactions between species were assessed by the contemporaneous spot on the lawn technique (Sutherland and Murdoch, 1994). In this method lawns of *B.cereus* (18h BHI cultures) were sown onto milk agar plates using a spiral plater set to deliver a constant concentration of bacterial cell suspension across the whole surface of the agar plate. Cultures of *B.subtilis* were grown (18h, 30°C) in BHI broth. The cultures were centrifuged (5000 ***g***, 4°C); the cell free culture supernatant was collected and filter sterilised (0.22 μm porosity). The cell pellets were resuspended in sterile MRD to the same concentration as they had been in the 18h culture. *Bacillus subtilis* cell suspensions (10 μl) or cell free culture supernatants (10 μl), were spotted using pipettes on to the surface of the newly inoculated *B.cereus* lawn plate. The plates were allowed to dry, and were incubated at 30°C for 24h. After incubation, qualitative assessment of inhibition was determined, by measuring the size of the zone of inhibition surrounding the area of *B.subtilis* cell suspension, or the cell free culture supernatants. A subjective estimation of the reduction of *B.cereus* growth in the inhibition zone was made.

This method was also used to assess the effects of MRM 057 and MRM 079 against 63 different *B.cereus* strains, and was also used to assess 42 different strains of *B.subtilis* for inhibitory effect against NCTC 11143 and NCTC 11145.

5:2:2 Inhibitory effects of other Bacillus spp. against B.cereus

Isolates of *B.licheniformis* (MRM 080; MRM 071; FSSM 171), *B.lentus* (FSS 081; FSS 149), *B.circulans* (PM 013; FSS 024) and *B.brevis* (PM 44; PM 55; FSSM 152) were tested for antagonistic effects against *B.cereus* strains NCTC 11143 and NCTC 11145 using the method described in section 5:2:1. The *Bacillus* spp. had initially been isolated from raw or pasteurised milk.

The inhibitory effects of *B.cereus*, *B.licheniformis* and *B.subtilis* against one another was also assessed. Isolates of *B.cereus* (NCTC 11143, NCTC 11145, HRM 044), *B.licheniformis* (HRM 013, HRM 014) and *B.subtilis* (HRM 057, HRM 079) were grown as described in section 5:2:1. Lawns of each isolate were inoculated onto milk agar plates, and 10µl whole cell suspension from each of the other isolates were spot inoculated onto the plate. The plates were incubated and assessed for inhibition as in section 5:2:1.

5:2:3 Growth, inhibition and toxicity curves for selected strains of B.subtilis

Strains of *B.subtilis* (FSSM 013; FSSM 019; FSSM 048) which had been among the strongest inhibitors of *B.cereus* were selected. They were grown in skim milk powder (10% w/v) or BHI for 24h or 36h. At 6 hourly intervals during this time period growth,

the presence of inhibitory factors and toxin production were monitored using the detection method described in sections 5:2:1 and 3:2:3:3b respectively.

5:3 INTERACTION STUDIES RESULTS

5:3:1 Interspecies antagonism

Investigation of the interspecies antagonistic effects between strains of *B.cereus* (NCTC 11143, NCTC 11145, HRM 044), *B.subtilis* (HRM 057, HRM 079) and *B.licheniformis* (HRM 013, HRM 014) were made. The results (Table 5:1) showed both strains of *B.subtilis* produced an inhibitor which had effect against all three *B.cereus* strains, but did not inhibit *B.licheniformis*. The inhibitory effect of the two *B.subtilis* strains was greater with NCTC 11145 and HRM 044 than it was with NCTC 11143. *Bacillus licheniformis* (HRM 013 and HRM 014) spotted onto *B.cereus* (NCTC 11145) lawn plates, were able to grow but not produce a zone of inhibition.

Isolates of *B.licheniformis* (MRM 071, MRM 080, FSSM 171), *B.lentus* (FSS 081, FSS 149), *B.circulans* (PM 013, FSS 124), and *B.brevis* (PM 044, PM 055, FSSM 152) were tested for inhibitory effect against *B.cereus* (NCTC 11143, NCTC 11145) and cell cytotoxicity having been grown in skim milk (10% w/v) and in BHI broth (Table 5:2 a-b). Only *B.brevis* PM 055 and *B.licheniformis* FSSM 171 caused any inhibition when grown in skim milk, but even these responses were weak. The culture supernatants of the *Bacillus* spp. isolates did not cause an inhibitory effect when grown in SMP or BHI. All 3 isolates of *B.licheniformis* caused inhibition as whole cells when grown in BHI, but the amount of inhibition was slight. Isolates of *B.lentus* (FSS 081), *B.circulans* (PM 013), and *B.brevis* (PM 055) caused slight inhibition of *B.cereus* as whole cells when grown in BHI. Isolates of *B.brevis* (PM 055) and *B.licheniformis* (FSSM 171) caused slight inhibition when grown in SMP.

5:3:2 Screen of B.subtilis isolates for antagonistic effect against B.cereus

Strains of *B.subtilis* isolated from milk or the dairy environment, were selected to test for their antagonist effect against *B.cereus* (NCTC 11143 and NCTC 11145) (Table 5:3). Of the 33 *B.subtilis* tested against NCTC 11143, 22 inhibited *B.cereus* growth when inoculated as a whole cell broth. When the cell free culture supernatants were tested against NCTC 11143, 20 of the 33 had an inhibitory effect. Of the 22 *B.subtilis* isolates which were positive with whole cells, 14 strains were also positive when their cell free culture supernatant was tested. Conversely, 6 of the supernatant positive strains, were negative with their whole cells. The extent of inhibition both by the whole cells and by the cell free culture supernatants was variable from strain to strain.

Some the *B.subtilis* strains were tested for inhibition against NCTC 11145. Of these 14 strains, 9 whole cell samples were antagonistic, and 10 of the cell free culture supernatants had inhibitory effects. Some of the strains of *B.subtilis* were inhibitory against only one of the *B.cereus* strains tested. The *B.subtilis* isolates which demonstrated the highest antagonistic effects were positive against both *B.cereus* strains.

5:3:3 Screen of B.cereus isolates for sensitivity of response to B.subtilis antagonism

Isolates of *B.cereus*, including 51 psychrotrophic strains isolated from milk or the dairy environment, 2 emetic toxin producers (F2105 and F4108), and 9 isolates from vegetarian Cheddar cheese, were tested for their response to the antagonistic effects of *B.subtilis* (HRM 057 and HRM 079) (Table 5:4). Of the *B.cereus* isolates 35% and

19% were inhibited by HRM 057 whole cell suspension or cell free culture supernatant respectively. HRM 079 whole cells were inhibitory to 90% of *B.cereus* isolates, as were 48% of culture supernatants. Only 1 culture supernatant caused an inhibitory effect without a corresponding effect occurring in the whole cell test; this supernatant only caused approximately 10% reduction in *B.cereus* growth where the supernatant had been placed. This was a mild effect compared to the response derived from other strains.

5:3:4 Toxin production by B.subtilis isolates

Isolates of *B.subtilis* (NCFB 1769, HRM 057, HRM 079, FSSM 013, FSSM 019 and FSSM 048) which had been found to be strong inhibitors of *B.cereus* were tested for toxin production by cytotoxicity of CHO cells and using the OXOID BCET-RPLA test kit (Table 5:5 a-b). All of the isolates produced positive responses against the cell cytotoxicity assay, but were negative against the OXOID BCET-RPLA test.

High levels of growth, the production of inhibitory and toxic factors by *B.subtilis* isolates occurred in both skim milk (10% w/v) and BHI broth.

5:3:5 Growth, and production of inhibitory compounds by selected isolates of B.subtilis

Isolates of *B.subtilis* FSSM 013, FSSM 019 and FSSM 048 were selected for being good inhibitors as whole cells and cell free culture supernatants, against both of the strains of *B.cereus* which they were tested against. The *B.subtilis* isolates were cultured and monitored over the course of 24h to determine when inhibitory factors and toxins develop (Tables 5:6 a-f). The production of the inhibitory factor had begun

by 6h in all three isolates, and in both culture media. The inhibitory factor in whole cells and culture supernatants reached their highest level at 12h for FSSM 013 (Table 5:6 a) and FSSM 019 (Table 5:6 c) in SMP; by 24h some inhibitory effect had been lost. When grown in BHI the maximum inhibition occurred at 24h for these strains. Strain FSSM 048 (Tables 5:6 e-f) was slower to produce inhibitory factors, with its maximum level occurring at 24h in both SMP and BHI. The cell free culture supernatants of FSSM 048 reduced the growth of *B.cereus* by approximately 80% at 24h.

5:4 INTERACTION STUDIES DISCUSSION

The inhibitory effect of *B.subtilis* against *B.cereus* has been demonstrated (Sutherland and Murdoch, 1994), and was confirmed in the study presented here. Isolates of *B.subtilis* from milk or the farm environment, were tested for the ability to inhibit *B.cereus*, and were found to vary widely in the ability to antagonise. Therefore the extent of the effect of *B.subtilis* on *B.cereus* populations in the environment would depend on the specific strains of *B.subtilis* present. The exclusive seasonality patterns which have been observed for mesophilic and psychrotrophic populations of *Bacillus* spp. (Sutherland and Murdoch, 1994), may be influenced by interactions arising from non-*Bacillus* spp. bacteria.

Although *Bacillus subtilis* was the most frequently isolated mesophilic species in the farm survey described in Chapter 2, and previously in other milk surveys (Sutherland and Murdoch, 1994), other mesophilic species of *Bacillus* may contribute to psychrotrophic repression in the environment as well as *B.subtilis*. Therefore isolates of *B.licheniformis*, *B.brevis*, *B.lentus* and *B.circulans* were also tested for their ability to inhibit *B.cereus*. The isolates of these species which caused inhibition only did so as whole cell suspension, and in a minimal way. Therefore, these isolates may have been better adapted to growth conditions, and simply out compete the *B.cereus* for growth on the agar plate. A study of substrate affinities is required to determine whether the growth conditions were equally suited to both species inoculated onto an agar plate. In the absence of this information it is premature to make definitive conclusions on the production and effects of antagonistic factors. Since differences exist in the extent of the antagonistic effect caused by different *B.subtilis* isolates against *B.cereus*, similar variation may also occur in strains belonging to the other *Bacillus* spp. tested. It is unknown therefore whether the isolates tested in this study were representative of their

species as a whole. However, on the basis of the responses of the isolates tested here, the effects on the repression of *B.cereus* strains was minimal.

Although there is evidence of antagonism existing between different species of *Bacillus,* the seasonal effect observed in milk (Sutherland and Murdoch, 1994), may have been caused by interactions occurring between *Bacillus* spp. populations and other contaminants of milk, or by a factor such as a germinant which might favour the grow of particular species above others (Phillips and Griffiths, 1986). It is probable that more than one factor is responsible for the seasonality changes.

Inter-genus interactions have been demonstrated. *Bacillus* spp. including *B.cereus* and *B.subtilis,* can be inhibited by *Lactobacillus* spp. and *Lactococcus lactis* (Varadaraj, 1993). Lactic acid bacteria produce a variety of metabolic products called bacteriocins, that are able to antagonise the growth of other microbes (Vandenbergh, 1993; Dodd and Gasson, 1994). The antimicrobial bacteriocin proteins are ribosomally synthesised peptides, which are 30-60 amino acids long (Jack *et al.*, 1995). The best known and most widely used bacteriocin is nisin (Abee *et al.*, 1992). Nisin has a broad spectrum of activity and is effective against pathogenic bacteria including *B.cereus* and *Listeria monocytogenes.* Other bacteriocins, such as cerein, are highly specific. Cerein is a bacteriocin produced by a strain of *B.cereus* is only active against other *B.cereus* strains, but was inactive against other bacterial species (Naderio *et al.*, 1993).

The antimicrobial action of bacteriocins is caused by the destabilisation of plasma membrane function (Jack *et al.*, 1995). Bacteriocins may be released into the environment or be bound to the cell wall (Jack *et al.*, 1995). Therefore whole *B.subtilis* cells and their cell free culture supernatants were tested for inhibitory effects to determine whether the inhibitory factor was cell bound or whether it was released

extracellularly. Some of the supernatants of the *B.subtilis* isolates caused repression of *B.cereus* growth, which indicates that the compound is extracellular. The repression levels caused by *B.subtilis* culture supernatants were found to vary between *B.subtilis* isolates. The response of *B.cereus* strains to *B.subtilis* inhibition was also found to vary from isolate to isolate, with some being more susceptible than others to the effects of *B.subtilis* antagonism.

Several of the most inhibitory strains of *B.subtilis* were selected to be tested for toxin production, because if strains of *B.subtilis* were to be used to suppress *B.cereus* growth, it is important that they themselves are not toxigenic. *Bacillus subtilis* has been implicated in several outbreaks of food borne illness (Griffiths, 1995 , Gould *et al.*, 1995; Duretic *et al.*, 1996). The strains of *B.subtilis* tested for cell cytotoxicity were found to produce a toxic compound, which was still causing cell damage after boiling. The predominant symptom of *B.subtilis* food poisoning is vomiting; in about half of the cases diarrhoea followed (Griffiths, 1995). The cell cytotoxicity response was comparable to that observed by emetic toxin producing strains. When the cell free culture supernatants of the *B.subtilis* isolates were tested against the OXOID BCET-RPLA kit for toxin production, all strains were negative. Many emetic strains of *B.cereus* are also negative against the OXOID test, including NCTC 11143 which was used in this experiment.

The *B.subtilis* inhibitory factor was produced during the logarithmic growth phase of the bacterial life cycle. Toxicity also occurred at a similar time; it is possible that the inhibitory factor could be the same compound as the cytotoxin. *Bacillus subtilis* produces peptide antibiotics which may inhibit competing bacteria; recently a new cyclic lipopeptide antibiotic produced by *B.subtilis* has been described (Kajimura *et al.*, 1995). *Bacillus subtilis* also produces serine and metallo proteases, the antibiotic

mycobacillin and has surfactant activity (Priest, 1989). Under moderately acid or alkaline conditions the growth of *B.cereus* is inhibited (Garcia-Arrabas and Kramer, 1990). Therefore inhibition by *B.subtilis* could even be caused by the production of acid as it ferments sugars. Any of these factors could cause or contribute to the inhibition of *B.cereus*.

Bacillus cereus was shown here to have no inhibitory effect on *B.subtilis* and *B.licheniformis*. Rumen bacterial populations have been inhibited by the presence of *B.cereus* (Gedek *et al.*, 1992). Strains of *B.cereus* are known to produce the antibiotics zwittermicin A (Stabb *et al.*, 1994; Raffel *et al.*, 1996), and kanosamine (Milkner *et al.*, 1996). These antibiotics suppress certain plant diseases, such as the damping off of alfalfa by *Phytophthora medicaginis* (Stabb *et al.*, 1994; Raffel *et al.*, 1996; Milner *et al.*, 1996). Kanosamine can also inhibit certain fungi (Milner *et al.*, 1996).

Although inhibition of *B.cereus* by *B.subtilis* clearly exists, the extent to which such antagonisms affect environmental populations of the species is unclear. In a survey of the incidence of *Bacillus* species in foods in the Netherlands, the two species were found to exist together in 16% of foods sampled (te Giffel *et al.*, 1996b). In the work in the Netherlands and also in the farm survey reported in Chapter 2, *B.cereus* and *B.subtilis* were not mutually exclusive.

The commercial application of bacteriocins are as food additives to reduce the growth of pathogenic or spoilage organisms (Roller *et al.*, 1995). Nisin is produced by food grade bacteria, (Roller *et al.*, 1995), and is accepted as safe by the World Health Authority (Abee *et al.*, 1992). Therefore any application of inhibitory factors produced by strains of *B.subtilis* for suppressing *B.cereus* growth is limited by the cytotoxic

effects of this species. *Bacillus subtilis* has been implicated in outbreaks of foodborne disease (Griffiths, 1995; Gould *et al.*, 1995; Duretic *et al.*, 1996). As well as being a potentially pathogenic organism, *B.subtilis* can cause spoilage of food products. Consequently *B.subtilis* could not be introduced into food products as a whole organism, but only an extracted inhibitor. Therefore for the inhibitory compounds of *B.subtilis* to be of benefit, further work would be required to purify the inhibitory compound, and to confirm that it is distinct from toxins which the organism may also produce.

TABLES AND FIGURES

FOR INTERACTIONS STUDIES

Table 5:1 Inhibition of selected strains of *B.subtilis*, *B.licheniformis* and *B.cereus* on one another, measured by size (mm) of inhibition zone surrounding 10µm spotted inoculum

	Organism tested for inhibitory effects						
	B.subtilis		*B.licheniformis*		*B.cereus*		
Organism tested for susceptibility	HRM 057	HRM 079	HRM 013	HRM 014	HRM 044	NCTC 11143	NCTC 11145
HRM 057	NT	0	0	0	0	0	0
HRM 079	0	NT	0	0	0	0	0
HRM 013	0	0	NT	0	0	0	0
HRM 014	0	0	0	NT	0	0	0
HRM 044	1	1.5	0	0	NT	0	0
NCTC 11143	Trace	Trace	0	0	0	NT	0
NCTC 11145	1	1.5	0 *	0 *	0*	0	NT

0 = No visible growth of spotted colony, and no zone of inhibition.

0* = Spotted culture grew, repressing lawn culture's growth, but there was an absence of any zone of inhibition.

NT = Not tested

(Results average of 3 repeats)

Table 5:2a Growth, inhibition and toxicity of selected *Bacillus* spp. grown in SMP

Organism tested for inhibitory effects		cfu/ml	Organism tested for susceptibility					
			Culture*		Supernatant #		Toxicity+	
			11143	11145	11143	11145	N/B	B
B.licheniformis	MRM 071	$7.4x10^7$	0	0	0	0	4	4
	MRM 080	$1.4x10^6$	0	0	0	0	4	4
	FSSM 171	$4.2x10^7$	0	0.25	0	0	4	2
B.lentus	FSS 081	$7.3x10^6$	0	0	0	0	4	4
	FSS 149	$4.4x10^7$	0	0	0	0	2	2
B.circulans	PM 013	$4.1x10^7$	0	0	0	0	64	4
	FSS 024	$5.7x10^7$	0	0	0	0	8	8
B.brevis	PM 044	$5.9x10^7$	0	0	0	0	128	4
	PM 055	$1.4x10^8$	0.25	0.25	0	0	32	4
	FSSM 152	$1.2x10^8$	0	0	0	0	16	2

* Whole cell inhibition measured in mm of clearing zone

Cell free culture supernatants inhibition measured as % of *B.cereus* growth restriction

+ Toxicity as highest positive dilution tested by cell cytotoxicity

Not boiled culture supernatants

Boiled culture supernatants

Table 5:2b Growth, inhibition and toxicity of selected *Bacillus* spp. grown in BHI

Organism tested for susceptibility								
			Culture*		Supernatant #		Toxicity+	
Organism tested for inhibitory effects		cfu/ml	11143	11145	11143	11145	N/B	B
B.lichenifor -mis	MRM 071	$1.8x10^8$	0.25	0.25	0	0	16	8
	MRM 080	$3.7x10^7$	0.25	0.25	0	0	4	1
	FSSM 171	$4.7x10^6$	0	0.25	0	0	2	2
B.lentus	FSS 081	$3.9x10^9$	0.25	0	0	0	4	4
	FSS 149	$1.4x10^9$	0	0	0	0	8	2
B.circulans	PM 013	$7.7x10^8$	0	0	0	10%	8	4
	FSS 024	$4.3x10^8$	0	0.25	0	0	8	8
B.brevis	PM 044	$2.8x10^8$	0	0	0	0	8	2
	PM 055	$8.1x10^8$	0.25	0.25	0	0	16	4
	FSSM 152	$1.2x10^8$	0	0.25	0	0	2	2

* Whole cell inhibition measured in mm of clearing zone

Cell free culture supernatants inhibition measured as % of *B.cereus* growth restriction

+ Toxicity as highest positive dilution tested by cell cytotoxicity

N/B Not boiled culture supernatants

B Boiled culture supernatants

Table 5:3 Antagonistic effects of isolates of *B.subtilis* against *B.cereus* strains NCTC 11143 and NCTC 11145

Isolate tested for inhibitory effects (*B.subtilis*)	Organism tested for susceptibility (*B.cereus*)					
	NCTC 11143			NCTC 11145		
	Cell suspension	Supernatant		Cell suspension	Supernatant	
	Zone * (mm)	Zone# (mm)	%	Zone* (mm)	Zone# (mm)	%
FSSM 013	1	10	40	0.5	10	80
FSSM 015	1	0	0	NT	NT	
FSSM 019	0.75	10	40	0.5	16	100
FSSM 025	2	0	0	NT	NT	
FSSM 048	0.4	7.5	35	1	14	90
FSSM 057	0	0	0	NT	NT	
FSSM 062	0.4	0	0	0	0	0
FSSM 066	0.75	5	30	NT	NT	
FSSM 070	0	0	0	NT	NT	
FSSM 078	2	5	30	NT	NT	
FSSM 096	0.5	5	30	NT	NT	
FSSM 097	0	5	30	NT	NT	
FSSM 100	0.25	5	20	NT	NT	
FSSM 109	1	7.5	30	NT	NT	
FSSM 119	0	0	0	0	13	90
FSSM 121	0	5	20	0	14	50
FSSM 128	1	7.5	30	0.5	7.5	50
FSSM 148	1.75	0	0	NT	NT	
FSSM 151	1	7.5	5	NT	NT	
FSSM 153	0	0	10	0	0	0
FSSM 168	0	10	20	NT	NT	
FSSM 170	0	7.5		NT	NT	
FSSM 174	1.25	7.5	10	NT	NT	

Table 5:3 Antagonistic effects of isolates of *B.subtilis* against *B.cereus* strains NCTC 11143 and NCTC 11145 continued

Isolate tested for inhibitory effects (*B.subtilis*)	Organism tested for susceptability (*B.cereus*)					
	NCTC 11143			NCTC 11145		
	Cell cuspension	Supernatant		Cell suspension	Supernatant	
	Zone (mm)	Zone (mm)	%	Zone (mm)	Zone (mm)	%
FSSM 178	0	5	20	0.25	0	0
FSSM 350	0.25	0	0	NT	NT	
FSSM 373	0.5	5	25	NT	NT	
MRM 272	1.5	0	0	2	10	50
MRM 285	1.5	0	0	1.5	10	60
MRM 290	0	0	0	1	12.5	75
MRM 331	0	7.5	15	0	0	0
MRM 329	1	7.5	20	1.5	7.5	30
MRM 350	1	7.5	30	NT	NT	
MRM 373	0.75	0	0	NT	NT	
Total Positive	22/33 (66%)	20/33 (60%)		9/14 (64%)	10/14 (71%)	

* Zone of inhibition surrounding *B.subtilis* cell culture

Diameter of clearing caused by 10µl spot of *B.subtilis* culture supernatant

% The reduction in growth of *B.cereus* culture where cell free culture supernatant was spotted

NT Not tested

Table 5:4 Antagonistic effect of *B.subtilis* HRM 057 and HRM 079 on isolates of *B.cereus*

Organism tested for susceptibility (*B.cereus*)	Organism tested for inhibitory effects (*B.subtilis*)			
	HRM 057		HRM 079	
	Cell suspension*	Supernatant#	Cell suspension*	Supernatant#
NCTC 11143	0.25	NT	0.4	NT
NCTC 11145	1	NT	1.5	NT
F2105	1	0	0.75	0
F4108	1	0	0.5	0
FSS 002	0	0	0	0
FSS 004	0	0	0.25	0
FSS 007	1	25	0.5	40
FSS 15	0	0	1.25	10
FSS 017	0	0	0	0
FSS 019	0.2	10	0.2	5
FSS 022	0	0	2.5	0
FSS 023	0.1	5	1	5
FSS 025	1	0	0.5	90
FSS 027	1	0	0.75	90
FSS 029	0	0	0.75	20
FSS 030	1	0	0.5	75
FSS 031	0.5	0	0.25	0
FSS 032	0	0	2	0
FSS 039	0	0	0.25	20
FSS 040	0	0	0	0
FSS 041	1.5	0	1	40
FSS 047	0.25	10	0.25	0
FSS 048	0	5	0.1	5
FSS 049	0	0	0	0
FSS 056	0	0	1.25	10

Table 5:4 Antagonistic effect of *B.subtilis* HRM 057 and HRM 079 on isolates of *B.cereus* continued

Organism tested for susceptibility (*B.cereus*)	Organism tested for inhibitory effects (*B.subtilis*)			
	HRM 057		HRM 079	
	Cell suspension*	Supernatant#	Cell suspension*	Supernatant#
FSS 362	1.5	0	1	60
FSS 063	0	0	0.75	50
FSS 064	0	0	1.5	0
FSS 065	0	0	0.5	0
FSS 066	0	0	0.25	20
FSS 068	0	0	0.25	5
FSS 080	0	0	1.25	30
FSS 088	0	0	0	0
FSS 091	0	0	2.5	50
FSS 096	0	0	1.25	0
FSS 099	0	0	1.5	0
FSS 101	0	0	1	30
FSS 105	0	0	1.25	5
FSS 112	0	0	1	0
FSS 114	0	0	0.5	0
FSS 115	0	0	1	0
FSS 117	0	0	1.25	0
FSS 121	0	0	1.5	0
FSS 125	0.1	25	0.1	25
FSS 127	0.2	25	0.25	20
FSS 129	0.1	40	0.15	50
FSS 134	0.1	10	0.1	0
FSS 137	0.1	5	0.2	5
FSS 142	0.3	30	1	15
FSS 145	0	0	0	10

Table 5:4 Antagonistic effect of *B.subtilis* HRM 057 and HRM 079 on isolates of *B.cereus* continued

Organism tested for susceptibility (*B.cereus*)	Organism tested for inhibitory effects (*B.subtilis*)			
	HRM 057		HRM 079	
	Cell suspension*	Supernatant#	Cell suspension*	Supernatant#
FSS 152	0	0	1	0
FSS 156	0.75	0	0.75	25
FSS 157	0.5	0	1.25	40
FSS 160	0	0	1	5
BC 1	0	0	1	0
BC 2	0	0	0.2	0
BC 3	0	0	0.5	0
BC 4	0	0	0.75	0
BC 7	0	0	1	0
CM 1	0	0	1.25	0
CM 2	0	0	1.5	0
CM 3	0	0	1.25	0
CM 4	0.1	0	1.25	25
TOTAL POSITIVE	24/64 (37.5%)	12/62 (19%)	58/64 (90%)	30/62 (48%)

* Zone of inhibition around spot of cell suspension (mm)

Reduction in growth caused by cell free supernatant (%)

NT Not tested

Table 5:5a Growth, inhibition and toxicity of selected strains of *B.subtilis* grown in SMP

Organism tested for inhibitory effects	cfu/ml	Organism tested for susceptibility: Culture* 11143	Culture* 11145	Supernatant # 11143	Supernatant # 11145	Toxicity+ N/B	Toxicity+ B
NCFB 1769	$1.16x10^9$	0	0	0	0	32	16
MRM 290	$1.12x10^9$	0.25	0.25	40%	30%	16	32
FSSM 013	$3.42x10^9$	2	1	90%	100%	64	32
FSSM 019	$1.93x10^9$	1	0.5	95%	100%	256	32
HRM 057	$1.08x10^9$	1	1	95%	80%	16	128
HRM 079	$2.28x10^9$	1	1	95%	95%	8	16
FSSM 048	$7.93x10^8$	0.5	0.25	25%	30%	64	16

Table 5:5b Growth, inhibition and toxicity of selected strains of *B.subtilis* grown in BHI

Organism tested for inhibitory effects	cfu/ml	Organism tested for susceptibility: Culture* 11143	Culture* 11145	Supernatant # 11143	Supernatant # 11145	Toxicity+ N/B	Toxicity+ B
NCFB 1769	$1.63x10^9$	0	0	0	0	8	16
MRM 290	$3.31x10^9$	1	1.25	95%	90%	32	8
FSSM 013	$2.62x10^9$	3	0.5	95%	100%	64	32
FSSM 019	$2.24x10^9$	1	0.25	90%	100%	64	16
HRM 057	$3.11x10^9$	2	1.5	100%	100%	16	8
HRM 079	$2.24x10^9$	3.5	2.5	100%	100%	16	16
FSSM 048	$1.91x10^9$	0.75	2	100%	100%	64	32

* Whole cell inhibition measured in mm of clearing zone

\# Supernatants inhibition measured as % growth restriction of *B.cereus*

Table 5:6a Growth, inhibition and toxicity of *B.subtilis* FSSM 013 over 24h in SMP

		Organism tested for susceptability				
		Culture		Supernatant [#]		
Time (h)	cfu/ml	11143	11145	11143	11145	Toxicity [+]
0	$7.11x10^4$	0	0	0	0	2
6	$1.75x10^7$	Some growth of *B.subtilis*, & 10% reduction of *B.cereus* for 0.5mm	0.25 zone	0	0	4
12	$4.07x10^7$	2mm zone	2mm zone	75%	100%	32
24	$4.09x10^8$	1mm zone & 50% reduction *B.cereus* for 1mm	0.5mm zone & 40% reduction of growth for 2mm	50%	70%	64

[#] % reduction of *B.cereus* growth

[+] Highest positive dilution using cell cytotoxicity

Table 5:6b Growth, inhibition and toxicity of *B.subtilis* FSSM 013 over 24h in BHI

		Organism tested for susceptability				
		Culture		Supernatant [#]		
Time (h)	cfu/ml	11143	11145	11143	11145	Toxicity [+]
0	$2.6x10^5$	0	0	0	0	2
6	$2.03x10^5$	Some growth of *B.subtilis*, but no inhibition	Some growth of *B.subtilis*, but no inhibition	0	0	2
12	$3.86x10^6$	Strong growth *B.subtilis*, & 70% reduction of *B.cereus* for 2mm	0.75mm zone	50%	90%	32
24	$2.08x10^8$	1mm zone & 50% reduction *B.cereus* for 1mm	0.75mm zone & 70% reduction of growth for 2mm	75%	100%	64

[#] % reduction of *B.cereus* growth

[+] Highest positive dilution using cell cytotoxicity

Table 5:6c Growth, inhibition and toxicity of *B.subtilis* FSSM 019 over 24h in SMP

		Organism tested for susceptability				
		Culture		Supernatant [#]		
Time (h)	cfu/ml	11143	11145	11143	11145	Toxicity [+]
0	$4.06x10^4$	0	0	0	0	2
6	$1.5x10^6$	Some growth of *B.subtilis*, & 20% reduction of *B.cereus* for 5mm	Some growth of *B.subtilis*, but no inhibition	0	0	4
12	$1.42x10^6$	2mm zone	1mm zone & 50% reduction 1mm	75%	75%	128
24	$1.47x10^8$	1mm zone & 50% reduction *B.cereus* for 1mm	0.5mm zone & 40% reduction of growth for 2mm	50%	50%	256

[#] % reduction of *B.cereus* growth
[+] Highest positive dilution using cell cytotoxicity

Table 5:6d Growth, inhibition and toxicity of *B.subtilis* FSSM 019 over 24h in BHI

		Organism tested for susceptability				
		Culture		Supernatant [#]		
Time (h)	cfu/ml	11143	11145	11143	11145	Toxicity [+]
0	$2.03x10^5$	0	0	0	0	2
6	$5.69x10^6$	Growth of *B.subtilis*, but no inhibition	0.25mm zone	0	0	16
12	$4.42x10^6$	Strong growth *B.subtilis*, & 40% reductin of *B.cereus* for 2mm	0.25mm zone & 40% reduction of *B.cereus* growth for 3mm	20%	20%	32
24	$8.13x10^6$	70% reduction *B.cereus* for 2.5mm	0.25mm zone & 45% reduction of growth for 3mm	40%	30%	64

[#] % reduction of *B.cereus* growth

[+] Highest positive dilution using cell cytotoxicity

Table 5:6e Growth, inhibition and toxicity of *B.subtilis* FSSM 048 over 24h in SMP

		Organism tested for susceptability				
		Culture		Supernatant [#]		
Time (h)	cfu/ml	11143	11145	11143	11145	Toxicity [+]
0	$1.79x10^5$	0	0	0	0	2
6	$7.88x10^7$	Some growth of *B.subtilis*, no inhibition *B.cereus*	Some growth of *B.subtilis*, no inhibition *B.cereus*	0	0	4
12	$2.26x10^8$	10% reduction *B.cereus* for 0.5mm	10% reduction *B.cereus* for 0.5mm	10%	40%	32
24	$8x10^7$	10% reduction *B.cereus* for 0.5mm	0.5mm zone & 40% reduction of growth for 2mm	80%	80%	64

[#] % reduction of *B.cereus* growth

[+] Highest positive dilution using cell cytotoxicity

Table 5:6f Growth, inhibition and toxicity of *B.subtilis* FSSM 048 over 24h in BHI

		Organism tested for susceptability				
		Culture		Supernatant [#]		
Time (h)	cfu/ml	11143	11145	11143	11145	Toxicity [+]
0	$3.9x10^5$	0	0	0	0	2
6	$5.28x10^6$	Some growth of *B.subtilis*, but no inhibition	Some growth of *B.subtilis*, but no inhibition	0	0	4
12	$8.74x10^6$	Strong growth *B.subtilis* but no inhibition	Strong growth *B.subtilis* but no inhibition	0	70%	16
24	$5.73x10^7$	50% reduction *B.cereus* for 1.5mm	1mm zone	90%	80%	64

[#] % reduction of *B.cereus* growth

[+] Highest positive dilution using cell cytotoxicity

Figure 5:1 Lawn culture of *B.cereus*, with two 10µl spots of *B.subtilis* culture supernatant. Spots have prevented *B.cereus* growth in these positions, and represent a subjective score of 100% reduction of *B.cereus* growth.

CHAPTER 6.

GENERAL DISCUSSION

6:1 GENERAL DISCUSSION

The objective of this project has been to further existing knowledge of the incidence, seasonality and toxinogenicity of *Bacillus cereus* and other *Bacillus* species, particularly in milk and the dairy farm environment. *Bacillus* spp. pose a threat to the dairy industry because members of the genus produce spores which may withstand pasteurisation temperatures; some strains are also psychrotrophic and pose a threat in cold stored products. As post pasteurisation contamination problems in milk decrease, psychrotrophic strains of *Bacillus* spp., such as *B.cereus* become of increasing importance to the dairy industry, because in addition to causing product spoilage, *B.cereus* may also cause food borne disease.

The occurrence of *Bacillus cereus* in milk is predominantly seasonal. This seasonality has previously been demonstrated throughout the milk chain (Sutherland and Murdoch, 1994). The study conducted here demonstrated that the same seasonality trends extend to raw milk, udder washes and faecal samples taken from dairy cattle. Mesophilic and thermophilic sporeforming populations also demonstrated seasonality patterns during the survey of raw milk and the farm environment. Mesophilic and psychrotrophic *Bacillus* spp. populations were not found to be exclusive of the other, as had been observed previously (Sutherland and Murdoch, 1994). Psychrotrophic sporeformers were detected outwith the summer-autumn season when they were anticipated. This was in agreement with the findings of McKinnon and Pettipher, (1983) and Slaghuis and Wolters, (1992). This indicates that there is a potential

problem arising from the presence of *B.cereus* in milk and dairy products throughout the year .

Contamination of raw milk appears to be derived from the udder teat surface. The teat surface may be itself contaminated by faecal material or soil, contamination may also occur from soiled winter bedding. Therefore thorough cleaning of the udder teat surface prior to milking is recommended to ensure minimal contamination of raw milk by *Bacillus* spp. and also by other bacterial contaminants of faecal origin. It was shown that the most efficient method of cleaning the teat surface for removal of bacteria was washing the teat surface with a disinfectant wipe, followed by drying. A further study to evaluate the efficacy of this method for reducing bacterial contamination of milk is warranted.

Other potential sources of contamination were also identified. The extent of udder contamination was affected by husbandry practices. If a strict cleaning regime is not adhered to then the different housing conditions may directly affect the microbiological quality of the raw milk.

Spores of psychrotrophic strains of *Bacillus* spp. occurred in raw milk in the survey reported here at low levels (<1 cfu/ml). In farm controls are needed to produce low count raw milk.

Effective maintenance of the cold chain throughout milk storage is thought to be the most effective means of controlling *B.cereus* outgrowth (Sutherland, 1993). Greater public awareness of the problems associated with *B.cereus* may lead to the reduction of temperature abuse to dairy products by the general public, who for instance often purchase milk from the supermarket and leave it in unrefrigerated conditions for extended periods of time. In a risk assessment exercise performed on household refrigerators in the Netherlands, 5% of pasteurised milk samples were found to contain >5000 cfu/ml *B.cereus* (te Giffel *et al.*, 1997b). In this study the temperature of 57% of refrigerators were above 7°C (te Giffel *et al.*, 1997b). These results confirm the need for increased public awareness.

Bacillus cereus causes food borne disease. The work presented here describes an improved cell cytotoxicity assay which may be used to assess *Bacillus* spp. strains for their ability to cause cytotoxic effect. The cell cytotoxicity assay had good correlation with commercial detection systems. The assay has the advantage over commercially produced antibody based detection methods because it detects toxic effect, and may therefore be used for the detection of both emetic and diarrhoeal toxins.

Screening of *B.cereus* isolates for toxin production demonstrated their widespread ability to produce toxins. Although this was the case, there was also a wide spectrum of levels of toxin produced by the isolates. Therefore it is unclear how many of the isolates would produce toxin to sufficient levels to cause food poisoning, however it does demonstrate the need to minimise contamination of milk by *B.cereus*.

Other species of *Bacillus* including *B.subtilis, B.licheniformis* and *B.pumilus* have been implicated in outbreaks of foodborne disease (Kramer and Gilbert, 1989). Species of *Bacillus* other than *B.cereus* were shown to be cytotoxic. *Bacillus mycoides* and *B.thuringiensis* strains were particularly toxic. The pathogenic potential of *B.thuringiensis* warrents further investigation because of the extensive use of this species as an insecticide. Isolates from *Bacillus* species other than the *B.cereus* group were also toxic. These species included isolates of *B.subtilis, B.lentus, B.brevis, B.circulans, B.licheniformis* and *B.polymyxa.*

The classification of bacteria using classical methods of identification, including biochemical testing and morphological studies, is time consuming, and in the case of certain species of *Bacillus,* such as the *Bacillus cereus* group, may lead to inconclusive results. The effectiveness of Fourier transform infrared spectroscopy (FTIR) for the discrimination between *Bacillus* spp. was investigated, and was found to be a useful tool for this purpose. Species within the *Bacillus cereus* group are very closely related. FTIR spectroscopy confirmed their close relation, but was able to distinguish between the three species of the *B.cereus* group tested. This indicates that it is correct to continue to regard these species as distinct, rather than as variants of *B.cereus.* If a large data base of FTIR spectra were compiled, FTIR spectroscopy may prove to be an accurate and rapid method for the identification of *Bacillus* species. It is also possible that in addition to identification, FTIR may be able to detect specific features of tested

organisms, such as toxigenic strains. Information of this sort would be of importance in clinical situations.

Interactions between mesophilic and psychrotrophic *Bacillus* species were confirmed. The factor produced by *B.subtilis* which inhibits *B.cereus* is released into its growth environment. Strains of *B.subtilis* which caused inhibition also had a cytotoxic effect on CHO cells. It was unclear whether the inhibitor and the toxin were separate compounds, and therefore the potential for exploitation of the inhibitory factor to repress *B.cereus* in milk or other food products is removed. The use of antagonistic strains, possibly of another genus of bacterium such as *Lactobacillus*, still offers a potential method of controlling *B.cereus*, and warrants study.

6:2 FUTURE WORK

Although the work presented in this Ph.D. thesis has answered many questions regarding *Bacillus cereus* and other *Bacillus* spp., it has also raised further areas for future investigation.

The cytotoxicity assay described here is useful for the detection of toxin, but would be unsuitable for use in clinical situations because of the long duration (72h) of the cell incubation step, which allows for healthy cells to proliferate. Therefore further work could be undertaken with the aim of shortening the duration of the cytotoxicity assay. Other assays could be based on the ability of tissue culture cells to adhere in the

presence of *B.cereus* toxins. It may also be possible to enhance the metabolism of the tissue culture cells by providing them with substrate. This would amplify the differences between toxin affected and unaffected cells, and remove the requirement for the proliferation step. If such a method were effective, it may also be possible to make the assay quantitative, as the intensity of absorbance could be directly related to the numbers of actively metabolising cells.

As was mentioned previously, further work could be performed using Fourier transform infrared spectroscopy on *Bacillus* spp.. Investigations could be made on toxigenic strains of *B.cereus* to determine whether it was possible to differentiate between strains which produced high or low levels of toxins, and between diarrhoeagenic or emetic strains. Also strains of *B.cereus* which reacted with the TECRA assay could be compared to those which reacted either only with the OXOID, or those strains which reacted with both assays. If parallel studies were performed using polymerase chain reaction techniques (RAPD), the differences detected with FTIR could be compared with genetic variations.

There is a need to assess the extent of the problem caused by *B.cereus*, including medical information, and the incidence of the organism in food products and pasteurised milk stored in refrigerators.

Work to determine whether the inhibitory factor of *B.subtilis* is the same as that causing cytotoxic effect is required. Molecular weight fractions of *B.subtilis* cell free

culture supernatants may be tested initially to ascertain whether the inhibitory and toxic elements were in the same fractions. The nature of diarrhoeal toxins of *B.cereus* requires further elucidation, as do the toxins of the toxigenic *Bacillus* spp.

CHAPTER 7.

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7. BIBLIOGRAPHY

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APPENDIX

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: OCTOBER 1993

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	22	115	133	168	172	22	115	133	168	172	22	115	133	168	172	
TMC	$1.2x10^4$ SP	$2.4x10^4$ SP	$4.4x10^4$	$1.2x10^4$	$1.2x10^4$	$7.9x10^7$	$1.8x10^7$	$3.0x10^6$	$2.7x10^5$	$5.8x10^1$						$1.7x10^4$
TPC	$5.2x10^2$	$2.7x10^3$	$5.6x10^2$	$3.0x10^3$	$8.7x10^2$	$5.1x10^4$	$9.6x10^6$	$7.2x10^5$	$2.9x10^5$	$1.1x10^5$						$8.2x10^3$
TTC	$5.0x10^2$	$2.0x10^1$ SP	0	0	$2.0x10^1$	$5.6x10^3$	$1.7x10^4$	$1.9x10^4$	$5.6x10^3$	$8.5x10^3$						0
MSC	$1.0x10^2$	$6.0x10^1$	0	0	$8.0x10^1$	$2.8x10^3$	$3.1x10^4$	$5.6x10^3$	$3.1x10^4$	$5.6x10^3$	$2.9x10^4$ SP	$2.9x10^5$ SP	$8.1x10^4$ SP	$7.0x10^4$ SP	$9.1x10^4$ SP	0
PSC	0	38/100 ml	0	0	1/100 ml	3/100 ml	26/100 ml	2/100 ml	0	79/100 ml	0	0	10	15	25	54/100 ml
TSC	$2.0x10^1$ SP	$2.0x10^1$ SP	0	$8.1x10^2$	$1.2x10^2$	0	$1.6x10^2$	$6.0x10^1$	$8.1x10^1$	0						0
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph		*B. mycoides*		*B.brevis*		*B. mycoides*	*B. mycoides*	*B. mycoides*	*B. mycoides*				*B.cereus*	*B.cereus*	*B. mycoides*	
		B.cereus				*B.cereus*			*B.cereus* *B. mycoides*					*B. mycoides*	*B.latero sporos/ B.cereus*	
									B.cereus							N/API

SP = spreading colony.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: OCTOBER 1993

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	200	392	189			200	392	189			200	392	189			
MC	$3.8x10^4$	1.0×10^4	4.2×10^5			8.9×10^5	1.0×10^6	2.2×10^6								
TPC	$2.5x10^3$	9.7×10^2	4.1×10^3			1.2×10^5	2.6×10^5	6.7×10^5								
TTC	0	4.6×10^1	2.0×10^1			1.4×10^4	2.2×10^4	8.5×10^3								
MSC	0	0	2.0×10^1			8.5×10^3	1.1×10^4	2.8×10^3			SP 3.8×10^4	SP 2.4×10^5	SP $1.4x10^4$			
PSC	37/100 ml	0	24/100 ml			137/100 ml	0	5/100 ml			0	20/ml	0			
TSC	SP $2.0x10^1$	2.0×10^2	8.1×10^3			4.0×10^1	8.1×10^1	1.6×10^2								

Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph	No resc. in API	No resc. in API				*B. mycoides*		*B. mycoides*			*B. cereus*					
						B. cereus		*B. cereus*			*B. mycoides*					

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: OCTOBER 1993

Bacterial count type	Sample cfu/ml unless otherwise stated						
	Pasture grass		Pasture soil				Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed	
TMC	$4.13x10^8$	$4.03x10^8$	$9.33x10^8$	$1.25x10^5$ SP			
TPC	$1.45x10^6$	$4.38x10^4$	$2.64x10^7$	$1.28x10^7$			
TTC	$2.46x10^4$	$7.34x10^4$	$1.13x10^5$ SP	$1.16x10^5$ SP			
MSC	$3.25x10^4$	$5.24x10^4$	$2.95x10^4$ SP	$2.12x10^5$ SP			
PSC	$4.0x10^1$	$8.1x10^1$	$1.0x10^2$	$3.25x10^2$			0
TSC	0	$1.76x10^4$	$1.75x10^4$	$5.73x10^4$			

Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples						
Psychrotroph	*B. mycoides*	*B. mycoides*	*B. mycoides*	*B. mycoides*			
	B.laterosporos/ B.cereus	*B.cereus*		*B.cereus B. mycoides*			

SP = spreading colony.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: November 1993

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	22	115	133	168	392	22	115	133	168	392	22	115	133	168	392	
MC	$1.69x10^3$	$1.05x10^4$	$1.40x10^3$	$3.62x10^3$	$1.71x10^3$	$1.40x10^7$	$4.20x10^5$	$1.38x10^6$	$1.38x10^6$	$1.10x10^5$						$2.1x10^3$
TPC	$3.9x10^3$	$6.7x10^2$	$5.3x10^2$	$1.4x10^3$	$7.9xx10^2$	$6.2x10^3$	0	$1.2x10^2$	$1.3x10^3$	0						$5.3x10^3$
TTC	$1.50x10^3$	$4.06x10^2$	$1.22x10^2$	$3.90x10^3$	$4.27x10^2$	SP $2.80x10^4$	$2.80x10^3$	$1.14x10^4$	$7.11x10^4$	$2.60x10^4$						$8.53x10^2$
MSC	0	0	$2.03x10^1$	$8.13x10^1$	0	SP $6.0x10^3$	$5.70x10^3$	$1.14x10^4$	$5.70x10^4$	$3.13x10^4$	$2.03x10^6$	$2.03x10^5$	$2.03x10^6$	$2.56x10^6$	$1.63x10^6$	0
PSC	0	0	0	0	0	0	0	0	0	0	$1.2x10^2$	0	0	$1.8x10^2$	0	0
TSC	0	0	$4.06x10^1$	$2.03x10^1$	$2.03x10^1$	$2.60x10^4$	$5.70x10^3$	$1.14x10^4$	$1.40x10^4$	0						$2.0x10^1$
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph											*B. mycoides*			*B. mycoides*		
														B. cereus		

SP = spreading colony.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: NOVEMBER 1993

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	200	172	189			200	172	189			200	172	189			
MC	$1.07x10^4$	$2.15x10^4$	$1.38x10^5$			$1.23x10^6$	$2.80x10^6$	$1.30x10^6$								
TPC	$3.0x10^3$	$7.8x10^3$	$5.3x10^3$			$2.4x10^5$	$2.4x10^5$	$2.7x10^5$								
TTC	$6.1x10^1$	$9.35x10^2$	$2.64x10^2$			$4.27x10^4$	$1.40x10^4$	$5.69x10^3$								
MSC	$1.83x10^2$	$7.93x10^2$	$2.03x10^2$			$6.30x10^4$	$1.40x10^4$	$1.70x10^4$			$3.2x10^5$	$1.42x10^6$	$6.1x10^5$			
PSC	0	0	0			0	0	0			0	0	0			
TSC	$4.06x10^3$	0	$1.0x10^2$			$4.60x10^4$	SP $8.50x10^4$	$1.14x10^4$								
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph																

SP = spreading colony.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: November 1993

Bacterial count type	Sample cfu/ml unless otherwise stated						
	Pasture grass		Pasture soil				Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed	
TMC	3.70×10^7		6.30×10^6		2.40×10^8	5.07×10^7	
TPC	1.5×10^7		3.2×10^4		5.7×10^6	8.8×10^7	
TTC	4.0×10^5		4.80×10^5		2.80×10^4	8.50×10^4	
MSC	SP 2.60×10^3		SP 3.60×10^3		2.60×10^5	9.50×10^4	
PSC	0		1.60×10^2		0	0	0
TSC	1.40×10^4		1.90×10^4		2.40×10^5	8.90×10^3	
***Bacillus* type**	**Species of psychrotrophic and mesophilic *Bacillus* isolated from samples**						
Psychrotroph			*B. mycoides*				

SP = spreading colony.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: DECEMBER 1993

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	22	115	133	168	392	22	115	133	168	392	22	115	133	168	392	
TMC	$1.12x10^4$	$1.95x10^4$	$3.50x10^3$	$5.12x10^3$	$8.33x10^2$	$2.10x10^6$	$2.0x10^6$	$6.70x10^5$	$2.81x10^5$	$1.70x10^6$						$9.30x10^3$
TPC	$1.89x10^3$	$3.25x10^3$	0	$6.0x10^1$	0	$2.36x10^5$	0	$1.42x10^4$	$2.16x10^5$	$4.27x10^4$						$1.24x10^3$
TTC	$2.0x10^4$	$7.50x10^2$	$4.06x10^1$	$1.82x10^2$	$1.22x10^4$	$2.30x10^4$	$8.54x10^3$	$2.0x10^4$	$5.7x10^3$	$5.7x10^5$						$2.6x10^2$
MSC	0	0	0	0	0	$2.84x10^3$	$2.84x10^3$	$5.7x10^5$	$8.53x10^5$	0	$1.58x10^5$	$3.69x10^5$	$1.0x10^6$	1.96x10	$6.5x10^3$	0
PSC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TSC	0	$6.10x10^2$	$6.10x10^1$	$2.03x10^1$	0	0	0	$8.54x10^3$	0	$8.54x10^3$						$2.0x10^1$
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph																
Mesophile						*B. cereus*	N/API	N/API	*B. pumilus*		*B. subtilis / B. licheniformis*	*B. subtilis / B. megaterium*	*B. subtilus / B. megaterium*	*B. pumilus*	*B. subtilis*	
											B. subtilis		N/API	*B. subtilis*	N/API	
											B. circulans B. megaterium					

N/API = no result in API.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: DECEMBER 1993

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	200	172	189			200	172	189			200	172	189			
TMC	$1.5x10^4$	$1.2x10^4$	$1.0x10^4$			$1.77x10^7$	$4.84x10^7$	$5.55x10^5$								
TPC	$5.54x10^3$	$1.28x10^3$	$1.69x10^3$			0	$1.22x10^5$	0								
TTC	$8.7x10^4$	$4.10x10^4$	$2.0x10^3$			$1.14x10^4$	$2.84x10^3$	$8.54x10^2$								
MSC	0	0	$2.0x10^1$			0	0	0			$1.5x10^4$	$2.97x10^5$	$1.13x10^5$			
PSC	0	0	0			0	0	0			0	0	0			
TSC	$2.0x10^1$	$2.0x10^1$	0			$5.69x10^2$	0	$2.56x10^4$								
***Bacillus* type**	**Species of psychrotrophic and mesophilic *Bacillus* isolated from samples**															
Psychrotroph																
Mesophile			*B. subtilis*								N/API	*B. panto-thenicus* *B. lentus*	*B. subtilis* *B. mageratium*			
												B. subtilis				
												B. mycoides				

N/API = no result in API.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: DECEMBER 1993

Bacterial count type	Sample cfu/ml unless otherwise stated						
	Pasture grass		Pasture soil				Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed	
TMC	6.70×10^8		2.5×10^4 SP		1.2×10^9	5.5×10^5	
TPC	1.42×10^6		2.39×10^8		2.41×10^6	1.81×10^5	
TTC	6.20×10^8		1.4×10^4 SP		1.1×10^9	3.7×10^5	
MSC	2.0×10^2		2.0×10^2 SP		4.26×10^5	4.0×10^2	
PSC	0		0		0	0	0
TSC	4.3×10^2		1.1×10^4		1.2×10^4	1.8×10^3	

Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples						
Psychrotroph							
Mesophile	*B. mycoides*		*B. mycoides*		*B. mycoides*	*B. cereus* / *B. mycoides*	
					N/API		

SP = spreading colony; N/API = no result in API.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: JANUARY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	189	115	392	200	133	189	115	392	200	133	189	115	392	200	133	
TMC	$3.66x10^4$	$5.70x10^2$	$2.23x10^4$	$6.30x10^4$	$1.62x10^4$	$8.82x10^6$	$2.84x10^5$	$1.45x10^7$	$3.70x10^4$	$1.17x10^7$						$8.13x10^3$
TPC	$4.80x10^3$	$3.14x10^4$	$1.24x10^4$	$2.92x10^4$	$1.95x10^3$	$1.71x10^4$	$6.26x10^4$	$1.02x10^5$	$9.39x10^4$	$1.36x10^5$						$4.67x10^3$
TTC	$2.10x10^3$	$8.13x10^2$	$4.00x10^1$	$4.47x10^4$	$7.52x10^2$	$8.54x10^3$	$2.28x10^3$	$4.55x10^4$	$5.97x10^4$	$1.10x10^5$						$2.84x10^2$
MSC	$9.14x10^2$	$2.44x10^3$	$2.0x10^1$	$2.43x10^1$	$3.04x10^2$	$2.85x10^3$	$2.84x10^3$	$4.84x10^4$	$4.55x10^4$	$6.10x10^4$	$4.0x10^6$	$4.0x10^6$	$9.10x10^5$	$4.81x10^5$	$2.20x10^6$	$1.22x10^2$
PSC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TSC	$2.54x10^3$	$6.91x10^2$	$6.53x10^3$	$5.87x10^3$	$3.03x10^6$	$4.80x10^3$	$2.17x10^3$	$4.0x10^3$	$1.02x10^4$	$6.05x10^5$						$1.21x10^2$
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph																

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: JANUARY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	148	168	15			148	168	15			148	168	15			
TMC	$4.26x10^{2}$	$1.24x10^{5}$	$8.33x10^{4}$			$2.87x10^{5}$	$1.36x10^{5}$	$3.41x10^{5}$								
TPC	$4.53x10^{3}$	$3.60x10^{2}$	$9.87x10^{3}$			$2.98x10^{5}$	$2.84x10^{3}$	$5.69x10^{3}$								
TTC	$2.0x10^{2}$	$8.10x10^{1}$	$2.03x10^{1}$			$7.97x10^{4}$	$5.69x10^{3}$	$1.14x10^{5}$								
MSC	$2.60x10^{3}$	$2.23x10^{2}$	$9.35x10^{2}$			$7.11x10^{4}$	$1.99x10^{4}$	$7.97x10^{4}$			$1.51x10^{6}$	$2.11x10^{6}$	$4.1x10^{5}$			
PSC	0	0	0			0	0	0			0	0	0			
TSC	$7.70x10^{2}$	$1.93x10^{2}$	0			$6.1x10^{1}$	$1.21x10^{2}$	$4.26x10^{2}$								
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph																

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: JANUARY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated								
	Pasture grass		Pasture soil						Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed			
TMC	1.36×10^{8}		9.35×10^{3}		8.17×10^{8}	8.33×10^{5}	1.46×10^{8}	4.61×10^{4}	
TPC	2.26×10^{5}		2.55×10^{5}		2.04×10^{8}	4.89×10^{8}	6.91×10^{3}	3.65×10^{4}	
TTC	7.11×10^{3} SP		2.84×10^{3}		1.07×10^{6}	3.20×10^{4}	6.78×10^{7}	2.84×10^{3}	
MSC	6.30×10^{3}		2.09×10^{4}		6.66×10^{5}	9.15×10^{5}	4.09×10^{5}	4.06×10^{2}	
PSC	0		0		0	0	0	0	0
TSC	8.54×10^{3}		1.02×10^{4}		8.33×10^{4}	1.32×10^{5}	3.33×10^{8}	1.21×10^{3}	
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples								
Psychrotroph									

SP = spreading colony.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: FEBRUARY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	115	392	133	148	168	115	392	133	148	168	115	392	133	148	168	
TMC	$1.37x10^4$	$2.12x10^4$	$1.02x10^4$	$9.85x10^3$ SP	$1.02x10^3$ SP	$1.14x10^5$	$3.64x10^5$	$3.64x10^5$	$1.05x10^5$ SP	$2.97x10^6$ SP						$7.95x10^3$
TPC	$2.0x10^3$	$2.53x10^3$	$7.19x10^3$	$2.0x10^1$	$3.65x10^3$	$8.54x10^4$	0	0	$4.55x10^4$	$1.10x10^5$						$5.49x10^2$
TTC	$2.64x10^3$	$2.26x10^3$	$5.89x10^2$	$8.13x10^1$	$8.13x10^2$	$5.69x10^4$	$1.28x10^5$	$1.74x10^5$	$5.98x10^4$	$2.92x10^6$						$8.74x10^2$
MSC	$2.84x10^3$	$2.26x10^3$	$1.21x10^2$	$2.0x10^2$	$7.32x10^2$	$3.13x10^4$	$7.68x10^4$	$9.96x10^4$	$5.41x10^5$	$2.27x10^6$	$7.2x10^5$	$3.52x10^4$	$5.7x10^5$	$7.04x10^5$	$7.41x10^4$	$4.06x10^2$
PSC	0	0	0	0	$6.0x10^1$	0	0	0	0	0	0	0	0	0	0	0
TSC	$2.03x10^1$	$1.89x10^1$	$8.13x10^1$	0	$2.84x10^2$	$2.84x10^3$	$4.27x10^4$	$4.84x10^4$	$1.93x10^5$	$1.06x10^6$						$1.62x10^2$
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph					Not identified											

SP = spreading colony.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: FEBRUARY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	15					15	Udder wash water				15					
TMC	1.59×10^4					2.86×10^6	5.0×10^6 SP									
TPC	1.6×10^3					7.97×10^4										
TTC	2.84×10^2					1.54×10^6										
MSC	1.83×10^2 SP					7.42×10^5	2.84×10^5				3.84×10^4					
PSC	0					0					0					
TSC	0					9.67×10^4										
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph																

SP = spreading colony

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: FEBRUARY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated								
	Pasture grass		Pasture soil						Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed			
TMC	1.09×10^5		8.97×10^4		1.13×10^9	F1 1.02×10^7	F2 2.22×10^8	C 1.06×10^5	
TPC	3.65×10^8		1.97×10^5		5.47×10^8	F1 8.54×10^3	F2 2.92×10^4	C 0	
TTC	1.25×10^5		2.44×10^3 SP		7.96×10^8	F1 8.97×10^7	F2 4.38×10^4	C 1.36×10^5	
MSC	1.47×10^5		2.85×10^5 SP		1.05×10^6	F1 3.45×10^6	F2 3.45×10^6	C 4.59×10^4	
PSC	2.03×10^3 SP		2.03×10^4 SP		3.05×10^8	F1 3.05×10^6	F2 0	C 0	4/100 ml
TSC	4.27×10^5		2.44×10^4		2.03×10^5	F1 2.66×10^4	F2 8.13×10^2	C 4.59×10^4	
***Bacillus* type**	**Species of psychrotrophic and mesophilic *Bacillus* isolated from samples**								
Psychrotroph	*B. mycoides*		*B. mycoides*		*B. mycoides*	F1 *B. mycoides*			
						F1 *B. cereus* (4)			Not identified
						F1 *B. brevis*			
						F1 (1) Not identified			

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: MARCH 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	150	168	172	115	15	150	168	172	115	15	150	168	172	115	15	
TMC	$1.68x10^4$	$1.07x10^4$	$3.65x10^3$	$1.60x10^4$	$2.68x10^4$	$2.86x10^6$ SP	$1.53x10^6$ SP	$8.17x10^5$	$2.16x10^5$	$1.96x10^6$						$8.31x10^3$
TPC	$1.06x10^4$	$8.71x10^3$	$9.35x10^2$	$3.41x10^3$	$3.04x10^2$	$2.84x10^3$	0	$2.28x10^4$	$5.70x10^3$	$3.40x10^4$						$1.63x10^2$
TTC	$2.64x10^2$	$1.63x10^2$	0	$6.50x10^2$	$3.25x10^2$	$2.14x10^6$	$1.23x10^6$	$9.19x10^5$	$1.31x10^5$	$3.98x10^6$						$2.44x10^2$
MSC	$3.25x10^2$	$8.13x10^1$	$2.0x10^1$	$1.12x10^3$	$1.42x10^2$	$1.23x10^6$	$1.33x10^6$	$2.61x10^5$	$6.26x10^4$	$1.33x10^6$	$1.33x10^6$	$3.0x10^3$	$1.8x10^5$	$2.5x10^5$	$3.7x10^3$	$3.05x10^2$
PSC	0	0	0	0	0	0	19/100 ml	6/100 ml	7/100 ml	6/100 ml						0
TSC	$6.0x10^1$	$4.06x10^4$	0	$3.45x10^2$	0	$1.14x10^5$	$1.08x10^5$	$8.54x10^4$	$5.40x10^4$	$1.34x10^5$						$6.1x10^1$
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph							*B. mycoides*									
							B. cereus									
Mesophile	Bp	Bl/Bs Bsp/br	Ba	Bp	Bs Bp	N/API Bp Bs Bs/Ba Bl/Bs	N/API Bp Bl/Bs Bs	N/API Bs Bp Bl/Bs	N/API Bm Bs/Ba Bp Bl/Bs	N/API Bl/Bs Bp	N/API Bs Ba Bp	Bp Bs	N/API Ba Bp Bs Bm	Bp Bs Ba	N/API Bp Bs Ba	Bp Bl

SP = spreading colony; N/API = no result in API.

Bacillus spp: Bp = pumilus; Bs = subtilus; Bm = mycoides; Bl = licheniformis; Ba = amyloliquefaciens; sp = sphaericus; br = brevis.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: MARCH 1994

Bacterial count type	Sample cfu/ml unless otherwise stated											
	Individual cow raw milk (Tag number)			Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)			Bulk tank
	148	392	155	148	392	155	Wash water		148	392	155	
							A	B				
TMC	4.16×10^3	3.50×10^3	4.38×10^3	1.43×10^6	1.10×10^5	1.30×10^6	1.01×10^5	2.98×10^3				
TPC	4.19×10^5	1.90×10^5	4.92×10^5	2.56×10^4	0	1.70×10^4	8.1×10^1	4.0×10^1				
TTC	6.0×10^2	2.0×10^1	8.13×10^1	8.27×10^5	9.19×10^5	1.70×10^6	5.18×10^4	5.25×10^5				
MSC	4.0×10^1	1.62×10^2	2.0×10^1	7.96×10^5	4.59×10^5	9.80×10^5	5.10×10^4	6.78×10^3	2.10×10^6	5.3×10^5	9.9×10^5	
PSC	0	0	0		5/100 ml	28/100 ml	0	0	0	0	0	
TSC	4.0×10^1	1.62×10^2	2.0×10^1	1.7×10^5	1.93×10^5	2.10×10^5	3.06×10^3	4.0×10^1				
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples											
Psychrotroph					Bm	Bm Bc						
Mesophile	Bl Bp	Ba	Bp	N/API Bl/Bs Bp Bs/Ba	N/API Bl/Bs Bp	Bp Bs/Ba Bs	Bm Bl Bs Bp	Ba Bp Bs	Bs/Ba Bp	Bs/Ba Bp Ba	Bp Ba	

Bacillus spp: Bl = licheniformis; Bp = pumilus; Ba = amyloliquefaciens; Bm = mycoides; Bc = cereus; Bs = subtilis.
N/API = no result in API.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: MARCH 1994

Bacterial count type	Sample cfu/ml unless otherwise stated								
	Pasture grass		Pasture soil						Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed			
						Feed 1	Feed 2	Conc.	
TMC	6.60×10^{8} SP		2.20×10^{6} SP		1.18×10^{8}	7.22×10^{4}	5.11×10^{7}	5.84×10^{4}	
TPC	5.10×10^{8} SP		4.06×10^{3}		1.40×10^{5}	5.10×10^{3}	5.10×10^{5}	5.54×10^{4}	
TTC	2.44×10^{4} SP		1.83×10^{4} SP		6.30×10^{6}	1.80×10^{4}	3.94×10^{7}	5.84×10^{4}	
MSC	1.14×10^{4} SP		1.22×10^{3} SP		2.70×10^{5}	2.20×10^{3}	2.40×10^{5}	4.47×10^{3}	0
PSC	8.13×10^{2}				0	0	0	2/100 ml	
TSC	4.67×10^{3}		6.91×10^{3}		5.28×10^{3}	0	2.0×10^{4}	6.1×10^{2}	
***Bacillus* type**	**Species of psychrotrophic and mesophilic *Bacillus* isolated from samples**								
Psychrotroph	B. mycoides							B. cereus	
Mesophile	Bm Bs/Mg		Bm		Bs/Ba Bp	Bp	Bs Bl	Bc Ba/Bs Bg/Bst	

SP = spreading colony.

Bacillus spp: Bs = subtilus; mg = megaterum; Bm = mycoides; Bst = stearothermophilus; Bc = cereus; Bl = licheniformis; Bp = pumilus; Ba = amyloliquefaciens.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: APRIL 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	148	168	155	150	115	148	168	155	150	115	148	168	155	150	115	
TMC	$2.23x10^3$	$4.67x10^2$	$9.55x10^3$	$9.47x10^3$	$7.78x10^4$	$1.45x10^5$	$3.58x10^5$	$1.99x10^5$	$4.59x10^5$	$4.55x10^4$						$1.73x10^5$
TPC	$2.23x10^2$	$2.64x10^2$	$4.47x10^2$	$4.47x10^2$	$2.35x10^4$	$5.41x10^4$	$7.68x10^4$	$2.84x10^3$	$6.83x10^4$	0						$1.24x10^5$
TTC	0	0	$4.06x10^3$	$4.06x10^1$	$4.06x10^1$	$1.14x10^4$	$1.99x10^5$	$1.99x10^4$	$2.56x10^4$	$8.54x10^3$						0
MSC	$6.10x10^1$	0	$1.83x10^2$	$1.83x10^2$	$2.03x10^1$	$5.70x10^3$	$1.99x10^4$	$8.54x10^3$	0	$1.14x10^4$	$4.0x10^5$	$1.4x10^6$	$1.3x10^6$	$3.1x10^6$	$2.4x10^6$	0
PSC	0	0	0	0	0	0	0	0	0	0	$1.44x10^4$	$7.20x10^4$	$1.08x10^4$	$3.4x10^4$	$3.76x10^4$	0
TSC	0	0	$2.03x10^1$	$2.03x10^1$	$6.10x10^1$	$5.69x10^3$	$4.22x10^4$	$8.54x10^3$	$1.99x10^4$	$2.84x10^3$						0
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph											N/API	N/API	N/API	*Bs*		
											N/API	N/API	N/API		*Bc*	

Bs = *Bacillus subtilis*; *Bc* = *Bacillus cereus*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: APRIL 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	392	15	172			392	15	172			392	15	172			
TMC	$1.42x10^3$	$8.33x10^3$	$8.10x10^4$			$4.40x10^6$	$3.36x10^6$	$3.18x10^6$								
TPC	$5.89x10^2$	$7.11x10^2$	$2.27x10^3$			$7.11x10^4$	$4.84x10^4$	$2.10x10^5$								
TTC	$4.0x10^1$	$2.03x10^1$	0			$2.56x10^4$	$1.99x10^4$	$1.71x10^5$								
MSC	$4.0x10^1$	$8.13x10^1$	$2.03x10^1$			$5.69x10^3$	$5.69x10^3$	$2.56x10^4$			$1.2x10^6$	$7.0x10^5$	$1.5x10^6$			
PSC	0	0	0			0	0	0			$2.64x10^4$	$1.92x10^4$	0			
TSC	$8.13x10^1$	0	$2.03x10^1$			$8.54x10^3$	$8.54x10^3$	0								
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph											*Bcl*	*Bcl*				

Bcl = Bacillus circulans.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: APRIL 1994

Bacterial count type	Sample cfu/ml unless otherwise stated								
	Pasture grass		Pasture soil			Winter feed			Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	C	F1	F2	
TMC	1.77×10^8		1.14×10^7		1.03×10^6	7.0×10^4	3.5×10^5	4.38×10^5	
TPC	8.33×10^7		1.07×10^7		4.96×10^8		1.74×10^5	3.03×10^4	
TTC	8.74×10^3		7.52×10^6		3.65×10^5	4.37×10^4	1.75×10^5	3.65×10^5	
MSC	7.72×10^5		6.09×10^5		4.07×10^5	1.02×10^4	6.09×10^3	4.07×10^5	0
PSC	0		0		0	2.03×10^3	0	0	0
TSC	4.88×10^3		2.19×10^4		1.02×10^5	6.5×10^3	6.09×10^2	1.02×10^5	
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples								
Psychrotroph						*Bc*			
						Bm			

Bc = *Bacillus cereus*; *Bm* = *Bacillus mycoides*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: MAY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	172	148	115	15	168	172	148	115	15	168	172	148	115	15	168	
TMC	$1.06x10^3$	$5.87x10^3$	$1.14x10^6$	$7.44x10^3$	$1.85x10^3$	$1.34x10^5$	$9.95x10^4$	$1.28x10^5$	$2.25x10^5$	$2.84x10^4$						$1.12x10^3$
TPC	$3.25x10^2$	0	$7.52x10^4$	$3.04x10^2$	$3.05x10^2$	$1.14x10^4$	$5.69x10^3$	$5.69x10^3$	$2.85x10^3$	0						$1.02x10^2$
TTC	0	0	$6.0x10^1$	$4.06x10^1$	0	$2.85x10^3$	$2.28x10^4$	$2.85x10^3$	$8.54x10^3$	$1.14x10^4$						0
MSC	$2.0x10^1$	$8.13x10^1$	$3.45x10^2$	$4.06x10^1$	$2.0x10^1$	$2.85x10^5$	$1.43x10^4$	0	$5.69x10^3$	$2.84x10^3$	$4.8x10^3$	$9.0x10^2$	$3.2x10^3$	$1.48x10^3$	$6.40x10^3$	0
PSC	0	0	0	0	0	0	3/100 ml	0	0	29/100ml	0	0	0	0	0	0
TSC	0	0	$2.0x10^1$	0	0	0	$2.85x10^3$	$2.85x10^3$	0	0						0
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph							*Bm*			*Bc*						
										Bcl						
										Bm						

Bm = Bacillus mycoides; Bc = Bacillus cereus; Bcl = Bacillus circulans.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: MAY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	155	150	392			155	150	392			155	150	392			
TMC	$8.54x10^2$	$5.69x10^2$	$7.52x10^2$			$2.60x10^4$	$5.69x10^4$	$2.67x10^5$								
TPC	$7.11x10^2$	$2.03x10^2$	$5.49x10^2$			$5.69x10^3$	0	$5.12x10^4$								
TTC	0	0	0			$2.84x10^3$	$2.28x10^4$	0								
MSC	$8.13x10^1$	$4.06x10^1$	0			$5.69x10^3$	$1.43x10^4$	0			$4.24x10^3$	$2.80x10^3$	$6.12x10^3$			
PSC	0	0	0			0	0	1/100 ml			0	$1.0x10^1$	0			
TSC	0	0	0			0	0	0								
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph								*Bc*				*Bcl*				

Bc = Bacillus cereus; Bcl = Bacillus circulans.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: MAY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated						
	Pasture grass		Pasture soil				
	Constant site	Varied site	Constant site	Varied site	Winter bedding	CONCENTRATE Winter feed	Water
TMC	1.40×10^{8}	2.76×10^{8}	5.89×10^{6}	2.03×10^{4}		1.75×10^{5}	
TPC	1.02×10^{6}	1.39×10^{7}	4.27×10^{6}	2.80×10^{4}		6.09×10^{5}	
TTC	8.13×10^{5}	1.83×10^{4}	4.06×10^{2}	8.13×10^{7}		1.63×10^{4}	
MSC	1.83×10^{5}	4.27×10^{4}	1.65×10^{4}	4.06×10^{4}		7.44×10^{4}	
PSC	0	0	0	0		0	0
TSC	4.06×10^{2}	4.06×10^{3}	1.79×10^{4}	2.44×10^{4}		7.72×10^{3}	
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples						
Psychrotroph							

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: JUNE 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	15	148	150	155	168	15	148	150	155	168	15	148	150	155	168	
TMC	$1.18x10^3$	$8.13x10^1$	$7.11x10^2$	$3.05x10^2$	0	$3.06x10^6$	$7.68x10^4$	$3.41x10^4$	0	$2.84x10^4$						$8.13x10^1$
TPC	$2.03x10^2$	$1.22x10^2$	$1.83x10^2$	$3.66x10^2$	$1.22x10^2$	$3.71x10^6$	$3.41x10^4$	$2.56x10^4$	$1.42x10^4$	$1.71x10^4$						$8.13x10^1$
TTC	0	0	0	$2.03x10^1$	0	0	$2.84x10^3$	0	$2.84x10^3$	0						0
MSC	0	$6.10x10^1$	0	0	0	0	0	0	0	0	0	0	0	0	0	$2.0x10^1$
PSC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TSC	0	0	0	0	0	0	$2.84x10^3$	0	0	0						0
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph																
Mesophile		*Bc*														*Bc*

Bc = Bacillus cereus.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: JUNE 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	172	392	200			172	392	200			172	392	200			
TMC	$4.06x10^1$	$7.31x10^2$	$6.09x10^1$			$5.69x10^3$	$1.31x10^6$	$6.83x10^5$								
TPC	0	$5.89x10^2$	$1.02x10^2$			$8.54x10^5$	$2.84x10^5$	$2.76x10^6$								
TTC	0	0	0			$2.84x10^3$	0	$8.54x10^3$								
MSC	0	0	0			0	0	$2.84x10^3$			$1.0x10^2$	0	0			
PSC	0	0	0			0	0	0			0	0	0			
TSC	0	0	0			0	$2.84x10^3$	0								
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph																
Mesophile								*Bl/Bs*			*Bl*					

Bl = Bacillus licheniformis; Bs = Bacillus subtilis.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: JUNE 1994

Bacterial count type	Sample cfu/ml unless otherwise stated						
	Pasture grass		Pasture soil				Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	CONCENTRATE Winter feed	
TMC	9.19×10^7	3.70×10^6	4.06×10^4 SP	3.25×10^4 SP		8.75×10^4	
TPC	4.06×10^7	6.02×10^7	1.42×10^6	4.06×10^6		1.90×10^5	
TTC	6.09×10^2 SP	4.06×10^2 SP	2.03×10^4	1.22×10^4 SP		1.16×10^4	
MSC	6.56×10^4 SP	4.06×10^2	5.28×10^3 SP	4.47×10^3 SP		1.02×10^3	
PSC	0	0	1.63×10^5	8.13×10^2		0	0
TSC	5.69×10^3	0	1.87×10^4	2.03×10^4		4.47×10^3	
***Bacillus* type**	**Species of psychrotrophic and mesophilic *Bacillus* isolated from samples**						
Psychrotroph			*Bc Bp/Bcl*	*Bm*			
				Bc			
Mesophile	*Bm*	*Bpm*	*Bl*	*Bm*		*Bpm*	
	Bs		*Bpm*	*Bc/Bm*		*Bs/Ba*	
	Bpm		*Bm*	*Bpm*		*Bc*	
				N/API			

N/API = no result in API;
Bm = Bacillus mycoides; *Bc = Bacillus cereus*; *Bcl = Bacillus circulans*; *Bp = Bacillus polymyxa*; *Bpm = Bacillus pumilus*; *Ba = Bacillus amyloliquefaciens*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: JULY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	172	168	155	200	392	172	168	155	200	392	172	168	155	200	392	
TMC	$1.02x10^5$	$1.85x10^5$	$3.03x10^5$	$4.47x10^2$	$4.47x10^2$	$1.17x10^5$	$6.57x10^3$	$2.9x10^5$	$1.65x10^5$	$7.52x10^5$						$1.02x10^3$
TPC	$2.0x10^4$	$7.41x10^4$	$8.13x10^4$	$2.03x10^2$	$6.09x10^1$	$1.14x10^5$	$1.70x10^3$	$3.41x10^4$	$5.69x10^3$	$8.53x10^4$						$4.06x10^2$
TTC	0	0	0	0	0	$1.42x10^4$	$8.54x10^3$	$2.84x10^4$	$2.84x10^3$	0						0
MSC	0	$1.18x10^3$ SP	$1.22x10^3$ SP	$4.0x10^4$	0	0	0	0	0	$2.28x10^4$	$4.0x10^3$	$2.46x10^4$ SP	$2.0x10^2$ SP	0	$6.6x10^3$	0
PSC	1/100 ml	0	0	0	0	2/100 ml	3/100 ml	0	0	3/100 ml	0	$1 x 10^4$	$2 x 10^4$	0	$1 x 10^4$	0
TSC	0	0	0	0	0	0		0	0	0	0					0
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph	Bm					Bc	Bm			Bc		Bm	Bm		Bm	
										Bsp/Bf						

Bm = *Bacillus mycoides*; Bc = *Bacillus cereus*; Bsp = *Bacillus sphaericus*; Bf = *Bacillus firmus*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: JULY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	15	148	150			15	148	150			15	148	150			
TMC	$7.72x10^2$	$9.48x10^3$	$1.11x10^3$			$2.59x10^5$	$8.54x10^5$	$1.14x10^5$								
TPC	$9.15x10^2$	$3.45x10^2$	$2.0x10^1$			$6.83x10^4$	$1.12x10^6$	$8.54x10^3$								
TTC	0	0	0			0	$5.69x10^3$	$2.28x10^4$								
MSC	$4.13x10^1$	0	0			0	0	$5.69x10^3$			$4.20x10^5$ SP	$4.30x10^5$	$7.40x10^3$			
PSC	0	0	0			1/100 ml	2/100 ml	4/100 ml			2×10^4	1×10^1	7×10^1			
TSC	0	0	0			0	$2.84x10^3$	0								
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph						Bc	Bc	Bc			Bc	Bm	Bm			
							Bm	Bm					Bc			
								Bsp/Bf								

Bc = *Bacillus cereus*; Bm = *Bacillus mycoides*; Bsp = *Bacillus sphaericus*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: JULY 1994

Bacterial count type	Sample cfu/ml unless otherwise stated						
	Pasture grass		Pasture soil				Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed	
TMC	4.82×10^8	1.02×10^8	8.03×10^7	1.83×10^6 SP		1.63×10^6	
TPC	2.27×10^7	1.50×10^7	2.39×10^4	1.51×10^4		1.29×10^4	
TTC	1.14×10^4	1.17×10^6	6.50×10^6	1.32×10^7		1.46×10^4	
MSC	4.27×10^2	2.23×10^6	4.47×10^6	2.03×10^4		2.44×10^3	
PSC	0	0	0	2/100 ml		0	0
TSC	1.83×10^3	2.23×10^6	5.11×10^4	4.06×10^3		1.66×10^2	
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples						
Psychrotroph				*B. mycoides*			

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: AUGUST 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	118	15	150	200	392	118	15	150	200	392	118	15	150	200	392	
TMC	$9.07x10^5$	$1.27x10^4$	$1.67x10^5$	$4.35x10^3$	$4.96x10^3$	$9.68x10^7$	$4.09x10^8$	$9.10x10^8$	$2.12x10^5$	$7.97x10^5$						$1.02x10^6$
TPC	$1.32x10^5$	$1.18x10^3$	$2.03x10^1$	$1.40x10^5$	$3.86x10^2$	$3.41x10^7$	$1.45x10^4$	$5.69x10^7$	$2.49x10^6$	$1.96x10^5$						$1.85x10^5$
TTC	$1.89x10^5$	$2.03x10^2$	0	$2.03x10^1$	$2.03x10^4$	$1.71x10^6$	$1.14x10^6$	$1.14x10^4$	$9.67x10^4$	$8.54x10^5$						$1.73x10^5$
MSC	$5.28x10^2$	$2.03x10^1$	$6.09x10^1$	$2.03x10^1$	0	$2.0x10^4$	$2.28x10^4$	$4.84x10^4$	$5.69x10^4$	$8.54x10^3$	$7.4x10^3$ SP	$2.73x10^4$ SP	$2.30x10^4$ SP	$2.0x10^3$	$2.14x10^4$ SP	$8.13x10^1$
PSC	1/100 ml	0	0	0	0	2/100 ml	0	38/100 ml	51/100 ml	2/100 ml	0	$1.5x10^3$	$9.0x10^2$	$3.0x10^2$	$1.1x10^3$	4/100 ml
TSC	0	0	0	0	$2.03x10^1$	0	0	$1.14x10^4$	0	0						
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph	Bm					Bm		Bm	Bc	Bc		Bm	Bm	Bm	Bm	Bm
								Bb				Bc	Bcl/Bp	Bc	Bc	
								Bf/Blt								

SP = spreading colony;
Bm = *Bacillus mycoides*; Bc = *B. cereus*; Bb = *B. brevis*; Bcl = *B. circulans*; Bp = *B. polymyxa*; Bf = *B. firmus*; Blt = *B. lentus*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: AUGUST 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	155	168	172			155	168	172			155	168	172			
TMC	$1.02x10^4$	$3.70x10^3$	$6.09x10^4$			$2.56x10^7$	$8.58x10^6$	$3.41x10^7$								
TPC	$6.91x10^2$	$1.71x10^3$	$1.58x10^3$			$1.10x10^6$	$5.0x10^5$	$1.34x10^5$								
TTC	$3.05x10^5$	0	$2.0x10^3$			$5.69x10^3$	0	$2.84x10^3$								
MSC	$4.06x10^1$	$2.03x10^2$	$4.06x10^1$			$1.42x10^4$	$1.42x10^4$	$5.69x10^4$			$8.4x10^3$ SP	$7.3x10^3$ SP	$7.8x10^3$			
PSC	0	0	0			60/100 ml	1/100 ml	20/1100 ml			$5.2x10^3$	$1.2x10^3$	$2.4x10^4$			
TSC	0	0	0			$2.84x10^3$	0	0								
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph						Bm	Bm	Bm			Bm	Bm	Bc			
						Bc		Bc			Bc	Bc	Bm			
								Bsp								

SP = spreading colony; Bm = *Bacillus mycoides*; Bc = *B. cereus*; Bsp = *B. sphaericus*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: AUGUST 1994

Bacterial count type	Sample cfu/ml unless otherwise stated						
	Pasture grass		Pasture soil				Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed CONCENTRATE	
TMC	3.06×10^8	5.33×10^8	4.83×10^5	6.50×10^6		7.07×10^4	
TPC	2.80×10^8	1.74×10^8	6.70×10^6	6.70×10^6		3.58×10^4	
TTC	1.04×10^4	1.1×10^2	1.02×10^6	1.46×10^4		3.58×10^4	
MSC	6.3×10^3	2.64×10^3	6.18×10^4	2.11×10^4		1.63×10^3	
PSC	0	0		0		0	16/100 ml
TSC	0	2.03×10^2	4.88×10^3	6.10×10^3		0	
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples						
Psychrotroph							Bm Bc

Bm = *Bacillus mycoides*; Bc = *B. cereus*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: September 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	15	168	392	200	172	15	168	392	200	172	15	168	392	200	172	
TMC	$7.52x10^5$	$1.63x10^6$	$4.0x10^5$	$2.24x10^3$	$4.27x10^3$	$1.04xx10^6$	$2.0x10^5$	$1.48x10^6$	$5.70x10^4$	$1.22x10^5$						$1.22x10^3$
TPC	$8.13x10^3$	$1.02x10^6$	$2.0x10^2$	$2.0x10^1$	$2.0x10^1$	$1.71x10^5$	$2.84x10^6$	$8.77x10^5$	$2.28x10^4$	$9.33x10^5$						$6.14x10^4$
TTC	$1.22x10^2$	$8.13x10^1$	0	0	$8.13x10^1$	$1.71x10^4$	$3.4x10^4$ SP	$1.42x10^4$	$8.54x10^3$	$3.70x10^4$						$2.0x10^1$
MSC	0	0	$2.0x10^1$	0	0	0	0	0	0	0	$4.0x10^3$ SP	$4.1x10^2$ SP	$2.9x10^3$ SP	$4.9x10^3$	$5.2x10^2$ SP	0
PSC	0	0	0	0	0	0	0	0	0	0	0	0	$3.0x10^1$	0	0	0
TSC	0	0	0	0	0	0	0	0	0	0						0
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph													Bc/Bm			
													Bc			
													N/API			
Mesophile			Bc								Bc	Bc	Bc	Bp	Bp	
											Bp	Bp	Bp		N/API	

SP = spreading colony; Bc = *B. cereus*; Bp = *B. pumilus*; Bm = *B. mycoides*; N/API = No result in API.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: September 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	118	150	155			118	150	155			118	150	155			
TMC	$2.67x10^3$	$1.0x10^3$	$2.4x10^3$			$5.6x10^7$	$5.7x10^6$	$5.6x10^5$								
TPC	$2.64x10^2$	$4.06x10^1$	$1.42x10^2$			$3.9x10^5$	$2.16x10^5$	$2.33x10^5$								
TTC	$6.1x10^1$	$3.45x10^2$	0			$2.28x10^4$	$1.99x10^4$	$8.54x10^3$								
MSC	0	0	0			0	0	0			$2.9x10^2$	$1.5x10^2$ SP	$2.3x10^2$ SP			
PSC	0	0	0			0	0	0			0	0	0			
TSC	0	0	0			0	0	0			0	0	0			
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph																
Mesophile											Bc	Bc	Bc/Bm			
											Bl					
											Bp	Bp	Bls/Bp			

SP = spreading colony; Bc = *B. cereus*; Bp = *B. pumilus*; Bl = *B. licheniformis*; Bls = *B. laterosporos*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: September 1994

Bacterial count type	Sample cfu/ml unless otherwise stated						
	Pasture grass		Pasture soil				Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed	
TMC	5.10×10^8	8.17×10^9	1.52×10^8	1.32×10^8		1.14×10^5	
TPC	5.48×10^5	7.30×10^8	6.10×10^4	3.45×10^7		2.13×10^4	
TTC	5.69×10^5	2.0×10^8	3.79×10^3	3.05×10^4 SP		8.94×10^3	
MSC	5.47×10^4	2.44×10^3	1.44×10^4	6.56×10^4		4.27×10^3	
PSC	0	0	4.06×10^2	0		0	
TSC	6.7×10^3	2.0×10^2	2.03×10^3	7.32×10^3		1.42×10^3	
***Bacillus* type**	**Species of psychrotrophic and mesophilic *Bacillus* isolated from samples**						
Psychrotroph			N/API				
Mesophile	*B. subtilis*	*B. licheniformis*	*B. cereus/mycoides*	*B. subtilis*		*B. licheniformis*	
	B. pumilus	*B. pumilus*	*B. subtilis/ amyloliquefaciens*				

N/API = no result in API ; SP = spreading colony.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: October 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	22	172	202	152	200	22	172	202	152	200	22	172	202	152	200	
TMC	$7.6x10^{1}$	5.84	$3.65x10^{4}$	$1.54x10^{3}$	$1.75x10^{3}$	$3.40x10^{4}$	$2.70x10^{5}$	$2.04x10^{6}$	$1.05x10^{5}$	$3.30x10^{5}$						$7.90x10^{3}$
TPC	$8.94x10^{2}$	$4.47x10^{2}$	$4.37x10^{3}$	$1.09x10^{1}$	$1.04x10^{3}$	$2.0x10^{4}$	$1.00x10^{4}$	$3.60x10^{5}$	$2.30x10^{4}$	$1.37x10^{3}$						$6.10x10^{2}$
TTC	$1.00x10^{3}$	$1.00x10^{2}$	0	$2.00x10^{1}$	0	$2.84x10^{3}$	$4.48x10^{4}$	$8.54x10^{4}$	$2.00x10^{4}$	$4.00x10^{4}$						$1.63x10^{2}$
MSC	$1.63x10^{2}$	$2.00x10^{1}$	$1.22x10^{2}$	0	0	$8.50x10^{3}$	$1.70x10^{4}$	$8.00x10^{4}$	$2.00x10^{3}$	$2.60x10^{4}$	$2.16x10^{7}$	$3.76x10^{7}$	$2.64x10^{7}$	$1.60x10^{7}$	$4.04x10^{7}$	$6.10x10^{1}$
PSC	1/100 ml	0	0	0	0	0	0	0	0	0	0	0	$4.20x10^{3}$	0	$3.00x10^{4}$	0
TSC	0	0	0	0	0	0	$3.40x10^{4}$	$6.00x10^{4}$	$1.14x10^{4}$	$6.00x10^{3}$						$6.10x10^{1}$
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph	N/API												N/API		Bs/Ba	

N/API = no result in API; Bs = *Bacillus subtilis*; Ba = *B. amyloliquefaciens*.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: OCTOBER 1994

Bacterial count type	Sample cfu/ml unless otherwise stated															
	Individual cow raw milk (Tag number)					Total cfu in individual cow udder wash (Tag number)					Individual cow faeces (Tag number)					Bulk tank
	150	155	392			150	155	392			150	155	392			
TMC	$4.60x10^3$	$7.70x10^2$	$1.30x10^3$				$1.00x10^4$	$1.54x10^5$								
TPC	$1.18x10^3$	$1.60x10^2$	$1.02x10^2$				$2.28x10^4$	$1.70x10^5$								
TTC	$2.03x10^2$	0	$2.64x10^2$				$7.10x10^4$	$4.00x10^5$								
MSC	$2.00x10^1$	$7.31x10^2$	0				$2.56x10^4$	$2.25x10^6$			$4.80x10^7$	$2.50x10^7$	$8.20x10^6$			
PSC	0	0	0				0	0			$3.40x10^3$	0	0			
TSC	$2.00x10^1$	$2.00x10^1$	$4.00x10^1$				$4.00x10^4$	$6.30x10^5$								
Bacillus type	Species of psychrotrophic and mesophilic *Bacillus* isolated from samples															
Psychrotroph											N/API					

N/API = no result in API.

FARM SOURCES SURVEY FOR *BACILLUS* SP.

MONTH: OCTOBER 1994

Bacterial count type	Sample cfu/ml unless otherwise stated							
	Pasture grass		Pasture soil					Water
	Constant site	Varied site	Constant site	Varied site	Winter bedding	Winter feed		
						Concentrate	F1	
TMC	5.84×10^8	2.65×10^8 SP	1.32×10^7 SP	1.46×10^6 SP	1.28×10^9	1.75×10^5	5.87×10^7	
TPC	1.53×10^8 SP	1.17×10^6 SP	8.54×10^3 SP	1.14×10^7 SP	5.00×10^9	1.93×10^4 SP	1.70×10^5	
TTC	2.03×10^6	8.13×10^5	4.68×10^6 SP	8.80×10^5	1.38×10^7	2.27×10^4	6.10×10^6	
MSC	7.20×10^4	6.10×10^5	1.44×10^8 SP	4.70×10^6	1.26×10^7	5.08×10^3	1.22×10^6	
PSC	0	0	4.00×10^2	9.80×10^3	2.00×10^2	0	0	
TSC	2.00×10^5	6.10×10^2	2.43×10^6	6.10×10^6	8.54×10^6	6.10×10^3	1.42×10^3	
***Bacillus* type**	**Species of psychrotrophic and mesophilic *Bacillus* isolated from samples**							
Psychrotroph			*B. cereus*	*B. mycoides*	*B. mycoides*			