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RISK PROFILE: BACILLUS CEREUS IN DAIRY PRODUCTS

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Prepared for MPI by Peter Cressey and Nicola King (ESR)
and Dr Tanya Soboleva (MPI)

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Publications Logistics Officer
Ministry for Primary Industries
PO Box 2526
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RISK PROFILE:

BACILLUS CEREUS IN DAIRY PRODUCTS

MAY 2016

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PREPARED BY:	**Peter Cressey and Nicola King, Risk and Response Group**
REVIEWED BY:	**Maurice Wilson, Microbiology (Food, Water and Environmental)**

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Manager	Peer reviewer	Authors
		 
Dr Rob Lake Manager, Risk and Response Group	**Maurice Wilson** Senior Scientist, Microbiology (Food, Water and Environmental)	**Peter Cressey[1]** **Nicola King[2]** [1]Senior Scientist, Risk and Response Group [2]Scientist, Risk and Response Group

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SUMMARY

This Risk Profile considers *Bacillus cereus* in dairy products. Additional information on the other closely-related members of the *B. cereus* group is included where relevant, particularly the psychrotrophic species *Bacillus weihenstephanensis*. The foods considered in this Risk Profile are dairy foods intended to be consumed as whole foods, including milk (raw, processed, concentrated, powder), cream, butter, yoghurt, cheese, ice cream and foods where dairy is the main ingredient (e.g. custard). Dairy foods produced from milk from cows, goats, sheep and buffalo are considered, although relevant information on non-bovine dairy foods is scarce.

B. cereus is an aerobic spore-forming organism that is widespread in the environment and occurs naturally in most foods. Human foodborne illness is caused by consumption of foods where *B. cereus* has been allowed to multiply to a high concentration. Doses in the range 10^5 to 10^8 *B. cereus* cells are believed to be necessary to cause illness. The symptoms of *B. cereus*-associated foodborne illness are caused by toxins. *B. cereus* can produce the toxin cereulide, which causes an emetic (vomiting) illness, and/or one or more enterotoxins that cause diarrhoeal illness. Cereulide is pre-formed in food while the diarrhoeal illness is caused by enterotoxins produced by *B. cereus* colonising the intestine. Both are usually self-limiting conditions and resolve within 24 hours.

The purpose of this Risk Profile is to critically review available information to answer four Risk Management Questions (RMQs). The responses are summarised below (see Section 5.3 for full text).

RMQ1: How does milk become contaminated with *B. cereus*, including consideration of feed, bedding and the milking shed environment?

The most important sources of *B. cereus* in New Zealand dairy products are likely to be soil and faecal contamination of animal teats, and subsequent transfer of bacilli to raw milk during the milking process. *B. cereus* is likely to be ubiquitous in the farm environment, making identification of specific risk factors difficult. The consumption of silage by dairy animals can lead to them excreting *B. cereus* in their faeces, and the concentration of *B. cereus* increases over time in bedding used by housed dairy animals, but the relative importance of these sources are not clear for New Zealand. Contamination of raw milk from bacilli in residual biofilms in the milking equipment was suggested in a study in The Netherlands. New Zealand studies are needed to better understand important risk factors in this country, and such studies need to consider non-bovine dairy herds, for which data are scarce.

RMQ2: What levels of *B. cereus* are present in milk and other dairy products and what are the major determinants of these levels?

A prevalence of 0.07% has been reported for *B. cereus* in raw milk in New Zealand. However, the basis for sample submission in this survey was not specified and this prevalence may not represent the actual national prevalence of *B.* cereus contamination of raw milk. No other New Zealand data are available. Overseas surveys of raw milk have detected prevalences of 5 to 90%, and concentrations ranging up to 10^7 CFU/mL. Prevalence in other dairy products are also wide-ranging.

Available data indicate that raw milk is the major determinant of the occurrence of *B. cereus* in milk and dairy products, although contribution from biofilms or added ingredients must also be considered. The ability of *B. cereus* to survive and grow in dairy foods appears to be strain-specific and is affected by temperature, competing microflora and changes in pH.

RMQ3: What dairy products are most likely to be contaminated with *B. cereus* and what is the relative likelihood of the organism surviving in different product types?

B. cereus is most likely to be detected in pasteurised milk but it may be present in most, if not all dairy foods, due to the ability of the organism to form resistant spores. Marked growth will occur under conditions of non-optimal refrigeration (≥7ºC) or conditions of temperature abuse. Growth of *B. cereus* in raw milk appears limited due to the presence of competitive microflora, but *B. cereus* can survive in this food. Growth can occur in pasteurised milk and cream, reconstituted powdered milk and infant formula. Many dairy processes will inhibit *B. cereus*, due to decreases in water activity, increases in acidity or increases in the population of competing microflora. *B. cereus* strains are able to form stable biofilms and, although there is a lack of conclusive evidence for New Zealand, there is potential for recontamination of dairy products within dairy processing plants.

RMQ4: What microbiological methods provide the most reliable information about presence and concentration of *B. cereus* in dairy products?

Methods used for the detection and quantification of *B. cereus* in New Zealand are consistent with the most commonly propagated international standard methods. However, it appears that the degree of species confirmation of isolates is limited and a report of *B. cereus* detection should be viewed as presumptive. New developments, including the use of chromogenic agar or species confirmation by PCR are not currently in wide use. *B. cereus* spores can be separately quantified by inclusion of an initial rapid heating step. Testing for *Bacillus* spp. diarrhoeal toxins (immunoassay) is carried out in New Zealand but there is currently no New Zealand capability to test for the emetic toxin (cereulide).

There are insufficient data to support a qualitative assessment of the risk of *B. cereus* intoxication for people consuming dairy products in New Zealand. This is chiefly because:

- There are no robust data on the prevalence and/or concentration of *B. cereus* in dairy foods in New Zealand. Overseas surveys indicate that the prevalence and concentration can vary widely, so it is not sensible to apply such data to New Zealand.
- *B. cereus* intoxication is underreported in New Zealand because the disease is non-notifiable (unless an outbreak is detected) and the relatively mild and self-limiting symptoms mean sick people rarely seek medical attention. Dairy products have not been implicated as the cause of any outbreaks of *B. cereus* intoxication in New Zealand.

Available data suggest that *B. cereus* is more likely to be detected in pasteurised milk than raw milk, and that growth to a concentration high enough to cause illness is possible for pasteurised milk held in consumer refrigerators, operating at mildly abusive temperatures. These data are for cows' milk and data on the behaviour of *B. cereus* in milk from other animals are insufficient to ascertain risk. The available data also indicate that *B. cereus* can probably be isolated from most other dairy foods at retail in New Zealand. Growth in the range of foods considered in this Risk Profile depends largely on temperature, but also on the characteristics of the food. Growth in low-pH foods such as yoghurt, and in some cheeses, appears to be restricted.

Overseas data suggest that diarrhoeal strains of *B. cereus* are more of a concern in dairy products than emetic strains, but this is possibly an artefact, since many studies did not attempt to identify markers for emetic strains. Emetic toxin production in culture media (tryptic soy agar, skim milk medium or oatmeal agar) appears to be maximal in the range 12 to 28°C, but the toxin can be produced at low concentrations at temperatures as low as 8°C. Cereulide production by emetic strains of *B. cereus* has been demonstrated in milk and dairy foods with neutral or high-pH, but mainly at temperatures >20°C. A study showing production of toxicity at refrigeration temperatures determined cytotoxicity, rather than cereulide concentrations. Further investigations are necessary to understand whether the risk in New Zealand lies

largely with the presence and growth of diarrhoeal strains of *B. cereus* in dairy foods prior to consumption, or the presence and growth of emetic strains. These investigations need to consider normal and mildly abusive time/temperature regimes rather than the higher temperatures used in many studies.

1. INTRODUCTION

Risk Profiles provide scientific information for risk managers relevant to a food/hazard combination and describe potential risk management options.[1] This document provides a Risk Profile considering *Bacillus cereus* in dairy products.

This Risk Profile considers the *B. cereus* species, but additional information on the other closely-related members of *B. cereus* group is included where relevant. The foods considered in this Risk Profile are dairy foods intended to be consumed as whole foods, including milk (raw, processed, concentrated, powder), cream, butter, yoghurt, cheese, ice cream and foods where dairy is the main ingredient (e.g. custard).

The purpose of this Risk Profile is to critically review available information to answer the following Risk Management Questions (RMQs):

1. How does milk become contaminated with *B. cereus*, including consideration of feed, bedding and the milking shed environment?

2. What levels of *B. cereus* are present in milk and other dairy products and what are the major determinants of these levels?

3. What dairy products are most likely to be contaminated with *B. cereus* and what is the relative likelihood of the organism surviving in different product types?

4. What microbiological methods provide the most reliable information about presence and concentration of *B. cereus* in dairy products?

The current Risk Profile reviews information relevant to the scope, with greater focus on more recent information (2005 and later).

[1] http://foodsafety.govt.nz/elibrary/industry/RMF_full_document_-_11604_NZFSA_Risk_Management_Framework_3.1.pdf (accessed 27 August 2014)

2. HAZARD AND FOOD

2.1 THE PATHOGEN: *BACILLUS CEREUS*

KEY FINDINGS

B. cereus sensu stricto is one of several closely related species in the *B. cereus* group. The taxonomy of this group is still the subject of debate. The species *B. weihenstephanensis* was proposed in 1998 to reclassify psychrotrophic *B. cereus* isolates.

B. cereus is an aerobic spore-forming organism that occurs naturally in most foods. *B. cereus* can produce cereulide, which causes emetic (vomiting) illness, and/or one or more enterotoxins that cause diarrhoeal illness. Cereulide is pre-formed in food while the diarrhoeal illness is caused by enterotoxins produced by *B. cereus* colonising the intestine.

It is not possible to predict potential pathogenicity of a *B. cereus* isolate by detecting the presence of the genes controlling toxin production. There appear to be a number of intrinsic and extrinsic factors that influence toxin production and the presence of one or more toxin genes does not confirm that an isolate will cause illness in humans.

The name *Bacillus cereus* can be used to refer to a group of related species, more accurately referred to as *B. cereus sensu lato*[2] or the *B. cereus* group. The species *B. cereus* (*B. cereus sensu stricto*) belongs to this group, along with *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* (Zwick *et al.*, 2012). A further five species have been proposed as part of this group: *B. cytotoxicus*, *B. toyonensis*, *B. gaemokensis, B. manliponensis* and *B. bingmayongensis* (Guinebretière *et al.*, 2013; Jimenez *et al.*, 2013; Liu *et al.*, 2015). *B. cytotoxicus* appears to be an uncommon thermotolerant species, but has been associated with several food poisoning outbreaks, involving vegetable purees or cooked semolina (Fagerlund *et al.*, 2007; Guinebretière *et al.*, 2013; Lund *et al.*, 2000).

Species in the *B. cereus* group are very closely related and the taxonomy is still the subject of debate (see Appendix A.1). At the species level, *B. cereus* refers to *B. cereus sensu stricto*. The current Risk Profile will consider *B. cereus sensu stricto*, and henceforth the use of *B. cereus* only refers to this species, unless otherwise specified. However, this will inevitably include consideration of *B. weihenstephanensis*. *B. weihenstephanensis* has been used to describe strains of *B. cereus* that grow at less than 7ºC, but not at greater than 38ºC (previously 43°C) i.e. are psychrotrophic (Lechner *et al.*, 1998; von Stetten *et al.*, 1999). *B. weihenstephanensis* are capable of causing food poisoning through production of emetic toxin at temperatures as low as 8°C (Thorsen *et al*., 2006) and have the potential for producing enterotoxins (Stenfors *et al.*, 2002). The species *B. weihenstephanensis* was first proposed in 1998 and psychrotrophic *B. cereus* isolates discussed in literature before or soon after this date may actually be *B. weihenstephanensis*. In addition, standard laboratory methods do not usually distinguish between *B. weihenstephanensis* and *B. cereus*. Thus it is not clear how important *B. weihenstephanensis* is as a food contaminant or cause of illness relative to *B. cereus*.

B. cereus is an aerobic spore-forming organism that occurs naturally in most foods (Christiansson, 2011) and is widespread in the environment (see Appendix A.4). Sporulation is induced by starvation, but is not induced by refrigeration (see Appendix A.5 for more information on *B. cereus* spores). The organism can cause two different forms of food

[2] In this context *sensu lato* means ‘in the widest sense’.

poisoning: an emetic illness and a diarrhoeal illness. The emetic illness is mediated by a highly stable toxin pre-formed in food, that survives high temperatures and exposure to trypsin, pepsin and pH extremes (i.e. can withstand passage through the human stomach). The diarrhoeal illness is mediated by heat- and acid-labile enterotoxins produced by *B. cereus* colonising the intestine. Enterotoxins can be formed in food, but are destroyed during passage through the human stomach.

In addition to causing food poisoning, *B. cereus* is also a food spoilage organism, due to the action of lipolytic and proteolytic enzymes produced by the bacterium. This can result in problems such as 'sweet curdling' of pasteurised milk and formation of flakes in cream when added to hot drinks (bitty cream) (Christiansson, 2011). For example, of 86 strains of *B. cereus sensu lato* isolated from dairy products in Brazil, 14 demonstrated lipolytic activity at 10°C and one strain at 30°C, although no lipolytic activity was detected at 7°C (Montanhini *et al.*, 2013). The proportion of strains exhibiting proteolytic activity increased with temperature, from 4.6% at 7ºC, to 72.1% at 10ºC, to 100% at 30ºC. In a separate study, no enzyme activity was seen at 4ºC during four months of storage (Janstova *et al.*, 2006a; Janstova *et al.*, 2006b). A useful overview of *Bacillus* spp. spoilage of dairy foods has been recently published (Gopal *et al.*, 2015).

2.1.1 Disease and pathogenicity

B. cereus emetic illness is caused by a single peptide toxin, cereulide. The diarrhoeal illness is caused by a range of protein enterotoxins, with possible involvement of other virulence factors. Both toxin types can cause illness in humans. See Appendix A.6 for more information on these toxins. Carriage of the genes for cereulide synthesis appears to be confined to a highly clonal group of *B. cereus* and *B. weihenstephanensis* strains, but carriage of enterotoxin genes is widespread among *B. cereus* (Ehling-Schulz *et al.*, 2015). Thus, the majority of *B. cereus* strains appear to be capable of producing either diarrhoeal toxins or the emetic toxin, and a significant number (36% in one report) of isolates could produce both (Beattie and Williams, 1999; Rusul and Yaacob, 1995).

The best characterised of the *B. cereus* enterotoxins are the haemolytic HBL and the non-haemolytic Nhe toxins. Both occur as complexes of up to three protein subunits. Strains of *B. cereus* may produce only Nhe or a combination of Nhe and HBL, but none have been found that produce HBL alone (Jessberger *et al.*, 2014). These toxins account for over 90% of the observed toxicity of *B. cereus* secretions, with cytotoxin K (cytK) making a lesser contribution (Jessberger *et al.*, 2014).

The enterotoxins appear to have different toxicities towards different cell types. Vero and primary endothelial cells (HUVEC) were the most sensitive cell types to a *B. cereus* strain expressing only Nhe, while Hep-G2, Vero and A549 cells were most sensitive cell types to a combination of Nhe and HBL. A *B. cereus* strain expressing only CytK exhibited its greatest toxicity towards CaCo-2 cells, but had relatively low toxicity to other cell types (Jessberger *et al.*, 2014). There is still uncertainty as to whether cytK is a true enterotoxin and it may be involved in diarrhoeal food poisoning as a non-toxin virulence factor (Castiaux *et al.*, 2015).

While it is becoming increasingly common to categorise *B. cereus* strains in terms of the enterotoxin genes they carry, this has not been found to be indicative of the toxicity of the strain (Jessberger *et al.*, 2015). The best indication of enteropathogenic potential is through measurement of secreted enterotoxins. There is evidence that the amount of NheB produced may be indicative of the diarrhoeagenic potential of *B. cereus* strains (Moravek *et al.*, 2006). However, it was concluded that post-transcriptional and post-translational factors must contribute to final levels of secreted enterotoxins and to the toxicity of *B. cereus* strains. Consequently, it is currently not possible to predict the pathogenicity of *B. cereus* strains from their enterotoxin gene profile.

2.2 THE FOOD: DAIRY PRODUCTS

KEY FINDINGS

This Risk Profile considered dairy foods intended to be consumed as whole foods (e.g. milk, reconstituted powders, cream, cheese, yoghurt, dairy-based desserts).

Cows' milk production is increasing in New Zealand. Milk from goats, sheep and buffalo is also available in New Zealand, as are products made from these milks. The majority of dairy products sold in New Zealand are made using pasteurised cows' milk.

Approximately 95% of New Zealand's milk production (by value) is exported. Imports of dairy foods are modest compared to exports, and most imported product comes from Australia.

Previous Risk Profiles have considered specific dairy products (e.g. raw milk, ice cream, cheese). The current Risk Profile considers a range of dairy foods, as described below.

Dairy products are foods produced from the normal secretion of the mammary gland of mammals (ICMSF, 1998). The International Commission on Microbiological Specification for foods (ICMSF) identifies a number of dairy product types (ICMSF, 1998):

- Milk for direct consumption (raw or processed);
- Cream, the fat-rich part of milk, separated by skimming or other techniques;
- Concentrated milks, such as evaporated or condensed milks;
- Dried dairy products, normally containing less than 5% moisture (e.g. milk powder, infant formula);
- Cultured or fermented milk products, intended to be consumed after lactic fermentation (e.g. yoghurt, cottage cheese);
- Cheese, the product of casein coagulation followed by separation of the liquid whey from the solid curd; and
- Ice cream and ice milk, formulated products intended for consumption in a frozen or semi-frozen state.

These descriptors encompass most of the products included in MPI's dairy product descriptions[3] and are the foods considered in this Risk Profile along with butter (as a cream product), dairy-based spreads, and milk and cream-based foods such as dairy-based sauces, dairy custard/desserts and flavoured milks. These dairy foods may be produced from milk from cows, sheep, goats or buffalo.

This Risk Profile does not consider specialty dairy products that are generally not consumed as whole foods but are instead produced as ingredients for other foods. Examples include protein products (e.g. casein, caseinate, milk proteins), colostrum products (except milk), lactose, whey products (e.g. whey powder) and specialty powders (e.g. cheese powder).

This Risk Profile also does not include foods where dairy is not the main ingredient (e.g. baked foods containing dairy ingredients).

[3] http://www.mpi.govt.nz/document-vault/165 (accessed 29 February 2016)

2.2.1 Dairy production in New Zealand

Cows' milk production has increased steadily in New Zealand over the last 35 years, from 5,868 million litres of milk processed in 1980/81 to 21,253 million litres in 2014/15 (LIC/DairyNZ, 2015).[4] This equates to 1,890,000 tonnes of milk solids processed in 2014/15.[5] Production typically peaks in October and is minimal through the winter months of June and July.

There are no official data on the volume of dairy products produced in New Zealand from cows' milk. A large proportion of cows' milk is used to produce milk powder, fluid milk, butter and cheese.

Over the last 30 years cows' milk production has become 'concentrated', with the number of herds decreasing from about 16,000 to about 12,000, while the average herd size increased from about 140 animals to about 420 animals (LIC/DairyNZ, 2015). This has resulted in an increase in total dairy cow numbers from 2.3 million in 1985/86 to just over 5 million in 2014/15. While the total land area used for dairy production has increased from about 1 million hectares in 1985/86 to about 1.7 million hectares in 2014/15, the average number of cows per hectare has increased from 2.3 to 2.9. Productivity has also improved, with milk solid production per cow and per hectare increasing steadily over the period 1992/93 to 2014/15.

The major regions for cows' milk production are the Waikato (23.4% of total dairy cows), North Canterbury (13.4%), Southland (11.4%) and Taranaki (9.9%) (LIC/DairyNZ, 2015).

There are no consolidated data on milk production from non-bovine species. Statistics from the Dairy Goat Co-operative, who manufacture goat milk powder products, show 69 supplying shareholders, 44,000 milking goats and an annual milk supply of 26 million litres.[6] There are also dairy goat farms that produce their own fluid milk and/or cheeses for sale to the public, or who supply milk to dairy product manufacturers. In 2015, it was reported that there were five commercial sheep dairy operations in New Zealand producing milk, cheese, yoghurt and milk powder.[7] There are a few herds of milking buffalo in New Zealand. It appears that the milk from these animals is mainly used for producing yoghurt and cheese.

No systematic source of information was found on the domestic dairy market in New Zealand. Domestic dairy product supply is dominated by Fonterra through their Anchor, Mainland and Tip Top brands, and Goodman Fielder through their Meadowfresh, Ornelle, Puhoi Valley and Tararua brands (MAF, 2011c).

2.2.2 Manufacture of dairy products in New Zealand

The processes used to manufacture each of the dairy products considered in this Risk Profile will not be described. In general, *B. cereus* vegetative cells will not survive well in products with low pH (e.g. yoghurt, some cheeses), do not survive in dried product (e.g. milk powder) and die quickly with any heat-treatment (e.g. pasteurisation, drying). Spores are fairly resistant to these hurdles.

The majority of dairy products sold in New Zealand are made using pasteurised cows' milk. The time/temperature requirements for pasteurisation are set out in the Animal Products (Dairy Processing Specifications) Notice 2011 (MAF, 2011a). Specifically:

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[4] It should be noted that statistics collected prior to 1998/99 only covered milk for export, while subsequent statistics cover total production for processing.
[5] Milk solids are the sum of total milk protein and fat
[6] http://www.dgc.co.nz/home.cfm (accessed 26 January 2016).
[7] http://www.massey.ac.nz/massey/learning/colleges/college-business/school-of-management/research/sheep-dairy-new-zealand/nz-sheep-dairying-conference-2015/nz-sheep-dairying-conference-2015_home.cfm (accessed 26 January 2016).

- ≥72°C for ≥15 seconds; or
- ≥63°C for ≥30 minutes; or
- a temperature/time combination with the same process performance; or
- approved criteria for temperature/time combinations to provide the same process performance as pasteurisation for liquid dairy material.[8]

Thermisation of milk, which is permitted for some cheese-making, requires the milk to be held at ≥64.5°C for ≥16 seconds.

Ultra high temperature (UHT) treatment uses heat to produce a commercially sterile product. The UHT regime for milk may vary, but usually involves temperatures of 135ºC or more for 1-5 seconds.

2.2.3 International trade

Exports

Approximately 95% of New Zealand's milk production is exported, with the major export products (by value) being milk powders, butter, cheese and casein.[9]

Imports

Despite New Zealand's position as a major exporter of dairy products, a wide range of dairy products are imported into New Zealand. Based on harmonised trade data from Statistics New Zealand[10], in 2015, New Zealand imported:

- 2338 tonnes of milk and cream, with 74% coming from Australia
- 15,990 tonnes of concentrated milk and cream, with 64% coming from Australia. A further 24% was due to re-importation of New Zealand product.
- 35,798 tonnes of yoghurt, buttermilk, fermented dairy products and dairy components. Whey was the main product imported in this category (58%), mainly from France, the USA and Australia. A further 39% of this category was non-whey natural milk constituents, imported from the USA.
- 819 tonnes of butter and other dairy fats and oils, with 58% from Australia and 21% re-imported New Zealand product.
- 8119 tonnes of cheese and curd, with 31% from Australia, 24% from Denmark, and 18% from the USA.

[8] DPC 3: Animal Products (Dairy): Approved criteria for the manufacturing of dairy material and product. Available at http://www.foodsafety.govt.nz/industry/sectors/dairy/manufacturing/approved-criteria.htm (accessed 26 January 2016).
[9] http://www.dcanz.com/about-nz-dairy-industry/dairying-today (accessed 12 January 2016)
[10] http://www.stats.govt.nz/infoshare/TradeVariables.aspx?DataType=TIM (accessed 10 February 2016)

2.3 CONTAMINATION OF DAIRY PRODUCTS BY *B. CEREUS*

KEY FINDINGS

The routes by which *B. cereus* can contaminate dairy products have not been well studied. The most important routes in New Zealand are probably:

- Contamination of raw milk from the soiled teats of milking animals; and
- Biofilms in milking equipment and dairy processing equipment.

Limited evidence suggests that *B. cereus* contamination of consumer-ready dairy products is mostly influenced by the presence and concentration of *B. cereus* in the raw milk, with contamination from biofilms and other ingredients making a lesser contribution.

No New Zealand-specific studies were located that investigated possible routes by which dairy products may become contaminated with *B. cereus*.

Two distinct questions need to be answered with respect to *B. cereus* contamination of dairy products:

- What is the mechanism(s) by which raw milk becomes contaminated with *B. cereus*?
- Is *B. cereus* contamination of dairy products due to contamination in raw milk or the processing environment or both?

2.3.1 Contamination of raw milk with *B. cereus*

Published studies from other countries, summarised in Appendix A.8.1, show that *B. cereus* has been detected on dairy farms (soil, feed, faeces, bedding) and in farm milking equipment (including equipment wash water). The identified risk factors for *B. cereus* contamination of raw milk varied between studies, and included season, animal housing, feed type, teat soiling and bedding (housed stock). The variable results are likely to be due to different farming practices and the ubiquitous nature of *B. cereus.*

Some of these overseas studies compared housed and pastured herds, since housing for some or all of the year is common in other countries. The results from these studies are not consistent. An evaluation of on-farm management practices that could be associated with *B. cereus* concentration in bulk tank milk across 63 dairy farms in Ireland found significantly lower counts of *B. cereus* in milk when cows were kept on pasture compared to being housed, and if fresh grass was allocated every 12 hours compared to less often (O'Connell *et al.*, 2013).[11]

Other studies have examined housing effects by testing the prevalence and concentration of *B. cereus* in bedding, soil, grass, silage and/or faeces, and attempting to ascertain which of these contamination routes influenced *B. cereus* in bulk tank milk. A study in the Netherlands concluded that the concentration of *B. cereus* spores in milk was not related to grazing, despite *B. cereus* spore concentrations in soil being the highest of all sample types measured (Vissers *et al.*, 2007b). Instead, milk contamination with *B. cereus* spores was associated with the concentration of *B. cereus* spores in faeces and silage, suggesting silage as the source of *B. cereus* spore contamination (with contamination of faeces being the result of cows eating the silage and milk being contaminated due to faecal contamination). In contrast, an older study (also in the Netherlands) found that milk from cows housed during the summer was less likely

[11] The researchers noted that excessive rainfall during the study forced farmers to implement on-off grazing to protect pastures, and the movement of animals between wet pasture and housing may have increased teat contamination.

to be contaminated with *B. cereus* spores than milk from cows kept on pasture during this period (Slaghuis *et al.*, 1997). A third study, in Sweden, found the *B.* cereus spore content of bedding was linked to *B. cereus* spore contamination in milk, indicating that milk from housed herds is more likely to be contaminated with *B. cereus* spores (Magnusson *et al.*, 2007a). Temporal studies of milk from European milk processing facilities found lower counts of *B. cereus* spores in silo milk during winter compared with other seasons (Bartoszewicz *et al.*, 2008; Svensson *et al.*, 2004). However, it is uncertain whether these observations were due to differences in ambient temperature, feeding practices or animal housing practices.

Despite the variable results highlighted above, the general consensus is that contamination of raw milk with *B. cereus* occurs through contamination of the teats, followed by contamination transferring from the teats to harvested milk. Teat cleaning and drying prior to milking has been shown to result in a 96% reduction in *B. cereus* contamination of milk (Magnusson *et al.*, 2006).

Three separate mechanisms for teat contamination have been proposed:

- Contamination with farm soil (Christiansson *et al.*, 1999; Vissers *et al.*, 2007a);
- Contamination with animal bedding material (e.g. sawdust) (Magnusson *et al.*, 2007a); and
- Contamination with faecal matter following consumption of contaminated feed (te Giffel *et al.*, 2002; Vissers *et al.*, 2007b).

Of these three possible mechanisms, the predominance of pasture feeding in New Zealand suggests that contamination of animal teats with farm soil or faeces are the more important routes for introducing *B. cereus* into raw milk in New Zealand. However, it should be noted that animal feeding and animal housing practices in New Zealand are becoming increasingly diverse and other mechanisms may become more important. A study of spore-forming bacteria (clostridia and *Bacillus* spp.) in calf faeces and dairy farm effluent from four New Zealand farms detected 16S rDNA indicative of *B. cereus* in samples from all four farms (Tanushree Gupta, AgResearch, personal communication). There are no other data on the prevalence of *B. cereus* in New Zealand soils or animal faeces from New Zealand farms. *B. cereus* has been detected in soils and faeces from dairy farms in other countries.

There have been no reports of direct contamination of milk following ingestion of *B. cereus* by milk-producing animals. While feeding of *B. cereus* spores to lactating cows did result in contamination of milk, the authors of the study ascribed this to teat contamination with faecal material (Magnusson *et al.*, 2007a). No information was located describing *B. cereus* contamination of animal feed in New Zealand, but *B. cereus* has been detected in feed tested in other countries (Appendix A.9). Ingestion of soil during pasture grazing may be a more significant source of exposure to *B. cereus* for New Zealand ruminants. It has been reported that dairy cows may ingest between 90 and 450 kg of soil per year (Healy, 1968).

In addition, *B. cereus* has been identified as an occasional cause of bovine, and possibly ovine, mastitis in New Zealand (Graham, 1998; Parkinson *et al.*, 1999).

2.3.2 Contamination of pasteurised milk with *B. cereus*

Due to the ability of *B. cereus* to form thermotolerant spores, the pasteurisation process has the potential to select for *B. cereus* (and other bacilli) by eliminating competing microflora.

B. cereus has been detected on surfaces and equipment in facilities producing pasteurised milk and milk powder in other countries (see Appendix A.8.1). Overseas studies have also reported that the prevalence of *B. cereus* increases along the milk processing chain (Appendix A.8.2), but mixing of the milk during processing will largely account for this finding, at least in countries with well-established cool chains for milk. For example, the prevalence of *B. cereus*

in samples taken from two dairy processing plants in Canada increased from 7-10% of raw milk samples, to 85-94% of pasteurised milk samples and again to 90-96% of the final packaged milk products (Lin, 1997). There are no published studies on *B. cereus* from New Zealand dairy facilities.

Studies using molecular techniques to compare the relatedness of *B. cereus* isolates from raw and pasteurised milk from the same processing facility have concluded that contaminated raw milk is the major source of contamination of pasteurised milk (Banyko and Vyletelova, 2009; Bartoszewicz *et al.*, 2008; Lin, 1997; Vidal *et al.*, 2016).

However, these studies also show that pasteurised milk can be contaminated by persistent *B. cereus* colonies in the processing facility (Banyko and Vyletelova, 2009; Bartoszewicz *et al.*, 2008; Lin, 1997). Another study suggests that raw milk silos in dairy plants can be persistently contaminated with *B. cereus*, and that some strains of *B. cereus* are well-adapted to this environment (Svensson *et al.*, 2004).

B. cereus is able to adhere to stainless steel surfaces, such as those in the dairy processing environment, and form stable biofilms (Kumari and Sarkar, 2014). These biofilms can be quite resistant to conventional clean-in-place (CIP) hygiene measures (Hornstra *et al.*, 2007; Kumari and Sarkar, 2014). Adhesion to stainless steel increases with increasing temperature (Bernardes *et al.*, 2013). These biofilms can detach and contaminate milk through shear force from moving fluid, abrasion from any solids, or sloughing (instant loss of part or all of the biofilm from the surface) (Gopal *et al.*, 2015). See Appendix A.7 for further information on biofilm formation by *B. cereus.*

Using molecular analyses (RAPD-PCR), Svensson *et al.* (2000) demonstrated a transition in *B. cereus* types in pasteurised milk produced in a factory over a four-hour period. Initially, the RAPD types were diverse and reflected the diversity of strains in the incoming raw milk. However, after one hour of processing most isolates (>70%) taken post-pasteuriser were identical, suggesting contamination from an inoculum established within the pasteuriser.

The spores of 23 *B. cereus sensu lato* isolates initially recovered from silo tanks in dairy processing facilities were examined to determine their survival tactics (Shaheen *et al.*, 2010). This included five probable *B. weihenstephanensis* (able to grow at 8ºC, containing *cspA* gene) and three mesophiles able to produce cereulide. None were related to *B. thuringiensis,* using ribotyping. The results of four experiments have been summarised in **Error! Reference source not found.**, and this shows that these isolates varied in their survival strategies. No isolates showed resistance to both hot acid and hot alkali, although not all strains were tested under acid conditions. There were seven isolates that demonstrated both high hot alkali resistance and the ability to form tenacious biofilms under milk. Isolate UM98 demonstrated multiple survival strategies applicable to dairy production, but it is not known how this isolate behaves under hot acid treatment. The ability to produce cereulide or grow at 8ºC did not appear to be correlated with the other behaviours tested.

Table 1. Behaviour of the spores of 23 *B. cereus sensu lato* isolates from dairy processing facilities in Sweden

Isolate No.	Mesophilic/ psychro-trophic[2]	Cereulide production	Hot alkali survival (≤1 log kill after 15 min)[3]	Hot acid survival (≤1 log kill after 15 min)[4]	Adherence to stainless steel under water at 4ºC[5]	Biofilm formation under milk at 21ºC[6]
KA111	M	-	-	NT	+	-
UM169	M	-	+	-	-	+
JO59	M	-	-	NT	+	-
GO159	M	-	-	NT	-	-
UM218	P	-	-	+	+	-
GO95	P	-	-	-	+	-
SU119	P	-	-	NT	-	-
GR117	M	-	-	NT	-	+
JO273	M	-	+	NT	-	+
SU285	M	-	+	-	-	+
UM284	M	-	+	NT	-	+
GO282	M	-	+	NT	-	+
GR225	M	-	-	NT	-	-
SU160	M	-	+	NT	-	-
VI104	M	-	-	-	-	-
KA155	M	-	-	NT	-	-
SU226	M	-	-	NT	+	+
UM98	M	-	+	NT	+	+
VI172	P	-	-	NT	+	-
GR53	P	-	+	-	-	+
GR177	M	+	-	NT	-	-
GR651	M	+	-	-	-	-
mjA1[1]	M	+	-	NT	+	-

Data from Shaheen *et al.,* 2010

[1] mjA1 was isolated from milk from a dairy farm.

[2] Psychrotrophic: Able to grow at 8ºC, contains *cspA* gene.

[3] 1% w/v sodium hydroxide (pH 13.1) at 75ºC.

[4] 0.9% w/v nitric acid (pH 0.8) at 65ºC. NT, not tested.

[5] + = RFU>5 as measured by fluorescence (an indicator of good adherence).

[6] Biofilm formation on polystyrene microplates.

2.3.3 Contamination of other dairy products with *B. cereus*

Four studies were located that investigated the source of *B. cereus* contamination of other dairy products, and three used some form of isolate typing to support their findings.

A study examined *B. cereus* isolates from milk powder and associated environment samples from the milk powder plant (Wiebe, 1999). Isolates were characterised by randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). Cluster analysis did not show similarities between the two groups of isolates indicating that contamination of milk powder was due to contamination of raw milk, rather than the presence of an 'in house' contaminating strain.

BOX-PCR typing was used to confirm that *B. cereus* types in pasteurised milk were often the same as types in the associated raw milk, but types in yoghurt from the same facility were often different to those in the raw milk, suggesting an alternative contamination source in the yoghurt production line (Banyko and Vyletelova, 2009). It should be noted that the conclusions drawn in this study were based on a small number of typed isolates (4 from raw milk, 3 from pasteurised milk and 5 from yoghurt).

A study of a ricotta-producing facility noted that while detection of *B. cereus* in raw materials and finished product was variable, *B. cereus* was consistently recovered from the mould culture at high concentrations (da Silva Fernandes *et al.*, 2014). Typing of isolates based on the presence of enterotoxin genes suggested multiple routes of contamination. The two most common types (36% and 19% of isolates, respectively) were mainly detected in mould and production points after mould addition. However, another type (14% of isolates) was detected in raw and pasteurised milk, cheese whey, cheese press and ricotta before packaging, suggesting that the raw milk was the source of *B. cereus* contamination with this strain.

An Iranian study measured concentrations of *B. cereus* at various points through three feta production lines and concluded that contaminated raw milk was the main source of *B. cereus* contamination of the finished cheese (Moradi-Khatoonabadi *et al.*, 2014).

2.4 BEHAVIOUR OF *B. CEREUS* IN DAIRY PRODUCTS

KEY FINDINGS

B. cereus does not appear to be able to grow in raw milk, but data are limited. Spores of *B. cereus* can survive pasteurisation and germinate in milk if the temperature is suitable. Growth of naturally present *B. cereus* in pasteurised cows' milk has been measured at 7-8ºC and can reach 10^3 CFU/mL in a week, although growth at these temperatures appears to be strain specific. Available data suggests that *B. cereus* spores may survive UHT treatment, although robust evidence is lacking. Studies also show that *B. cereus* can grow in cream stored at 7ºC.

B. cereus spores can survive the heating and drying steps used to make powdered milk and powdered infant formula, and can germinate and multiply in the reconstituted product, depending on temperature. Under temperature abuse (≥25ºC), naturally present *B. cereus* multiplies quickly to levels that could cause illness, within 24 hours. Data at refrigeration temperatures are limited; growth is possible but probably slow and strain-specific.

While results vary by cheese type, in general there appears to be potential for *B. cereus* to survive the cheese-making process. In most cases concentrations decreased during storage, probably due to pH reductions and the growth of competitive microflora.

Very little information is available on the behaviour of *B. cereus* in other dairy product types. *B. cereus* will not grow in frozen dairy products, but can survive. Growth of *B. cereus* in dairy-based desserts has been demonstrated at 21ºC, but not in non-dessert dairy foods (sauce, spread, mayonnaise). There is mixed evidence on the ability of *B. cereus* to survive and grow under conditions of lactic fermentation. Most studies suggest an initial rapid increase in *B. cereus* concentrations, followed by a decrease. *B. cereus* appear to be inhibited to a greater extent when rapid changes in pH occur, but appear to be able to adapt to slow changes in pH.

B. cereus strains carrying the genes for enterotoxin production are often present in dairy products. Production of emetic toxin has been demonstrated in a wide range of dairy products stored at non-refrigeration temperatures (>20ºC), but data are limited for toxin production in dairy products at refrigeration temperatures. The role of oxygen supply in emetic toxin production is unclear, with studies (mainly in culture) suggesting oxygen supply

is pivotal, while some studies (mainly in dairy products) suggest increased toxin product under non-aerated conditions.

In the remainder of this document, reference to *B. cereus*, without further qualification, should be taken as referring to vegetative cells. Studies that specifically identified inoculation with spores or determined concentrations of spores will be identified accordingly.

2.4.1 Fluid milk

Vegetative cells of *B. cereus* will not survive pasteurisation, but the spores can. Germination of *B. cereus* spores is stimulated by high-temperature, short-time (HTST) pasteurisation. It is possible that vegetative cells can be introduced to milk after pasteurisation if *B. cereus* has been able to establish colonies in milk processing plants, e.g. as biofilms.

B. cereus spore populations appear to be heterogeneous in their thermotolerance, so survival of milk heat treatment will be strain-specific. For example, upon measuring the heat resistance of spores from 39 *B. cereus sensu lato* strains separated into seven phylogenetically-related groups, the groups containing psychrotrophic *B. cereus* and *B. weihenstephanensis* were the most temperature sensitive, while the most heat-resistant group contained mesophilic *B. cereus* (Luu-Thi *et al.*, 2014). The results from this study suggested that even spores of heat-sensitive *B. cereus* strains may survive pasteurisation temperatures. These experiments were conducted using spores suspended in buffer, not milk. It has been suggested that survival at elevated temperatures may be improved in oily or fatty matrices, but experiments measuring the heat resistance of *B. cereus* spores in milks found that the addition of cream (20%) decreased heat resistance (Mazas *et al.*, 1999).

Luu-Thi *et al.* (2014) also found a positive correlation between heat-resistance and the optimum growth temperature of the *B. cereus* strains evaluated. This suggests that the *B. cereus* strains most likely to survive high temperatures (mesophilic) are least likely to grow at refrigeration temperatures.

B. cereus spores can be inactivated by ultra-high temperature (UHT) treatment, with D-times at 100ºC of 0.3 to 10 minutes reported (Jenson and Moir, 2003), but survival of UHT treatment is also strain-specific. When spores from a strain of *B. cereus* with high heat resistance were heated to 72 or 78ºC in skim milk, the concentration only reduced by 1 $\log_{10}$ after 90 minutes (Novak *et al.*, 2005). When these spores were heated at 100ºC in skim milk, the number of viable spores reduced by about 90% in the first five minutes, followed by a slow, but not complete loss of viability of the remaining spores over the following 30 minutes (Novak *et al.*, 2005). Even at UHT temperatures (135 or 150ºC) it was estimated that treatment times of about two minutes would be required to achieve a 12-D reduction in viable spores (commercial sterility).

In contrast, when the spores from six *B. cereus* strains were inoculated into UHT milk and heated at temperatures in the range 95 to 135ºC, inactivation of spores at 135ºC was reported to be instantaneous (Janstova and Lukasova, 2001). Mean D times decreased from 2.02 minutes at 95ºC to 0.02 minutes at 120ºC. The strains of *B. cereus* studied were originally isolated from milk or from the farm environment. An earlier study measured D times of ≤36 seconds at temperatures of ≥110ºC, for spores of three *B. cereus* strains inoculated into UHT milk or powdered milk reconstituted to different concentrations (Mazas *et al.*, 1999). Surveys of *B. cereus* contamination of UHT milk have given equivocal results (see Table 3, Appendix A.8.2) and further experimental studies will help to resolve whether *B. cereus* survives the UHT process. Such studies would be more informative if they measured the behaviour of *B. cereus* naturally present in milk undergoing UHT processing.

Experiments using naturally contaminated raw cows' milk found that psychrotrophic *B. cereus* were not detected in samples of milk submitted to ultra-pasteurisation (138ºC for 2 seconds),

super-pasteurisation (96ºC for 13 seconds) or pasteurisation (74ºC for 15 seconds) then stored at 4 ± 2ºC for up to six weeks (Aires *et al.*, 2009). Mesophilic *B. cereus* were detected in some samples of super-pasteurised and pasteurised milk at concentrations up to 40 and $7x10^5$ CFU/mL, respectively. Other than during heat treatment and a brief (1 hour) transport step, the milk was not stored above 4ºC, so this result suggests that the *B. cereus* strains contaminating the raw milk survived the pasteurisation and super-pasteurisation regimes, but not the ultra-pasteurisation regime.

Under favourable conditions (largely governed by temperature), *B. cereus* spores can germinate in fluid milk, but the ability to germinate may vary between strains. Of four strains incubated at 30ºC in pasteurised milk as spores, two germinated efficiently, while one showed only marginal germination and one did not germinate at all. The non-germinating strain was an emetic toxin-producing *B. cereus*, while the marginally germinating strain was a psychrotolerant *B. weihenstephanensis* (van der Voort *et al.*, 2010). Another study demonstrated efficient germination of spores of an emetic strain of *B. weihenstephanensis* inoculated into UHT milk, at 7 or 30ºC (Bartoszewicz *et al.*, 2013). An emetic mesophilic *B. cereus* strain also germinated at 30ºC, but not at 7ºC. Both strains multiplied at 30ºC, with concentrations of approximately 6-7 log CFU/mL achieved after one day and then remaining constant up to 10 days storage. At 7ºC, the *B. weihenstephanensis* strain multiplied steadily over the 10 day storage period, while concentrations of the *B. cereus* strain remained constant after an initial decrease of about 1 log CFU/mL.

In a survey of pasteurised milk sampled from domestic refrigerators in the Netherlands, *B. cereus* was detected (≥5 CFU/mL) in 133/334 samples (40%) (te Giffel *et al.*, 1997a). Of the samples taken on or after the expiration date stamped on the packaging, those that had been kept at ≥7°C were more likely to contain *B. cereus* compared with those that had been stored at <7ºC. The concentration of *B. cereus* was close to or exceeded 10^3 CFU/mL in 31 samples. The maximum reported concentration was $>5x10^4$ CFU/ml (one sample). The authors did not present the concentration data alongside data on milk age, but commented that increased numbers were found when the refrigeration temperature was higher and storage time longer. Of 106 *B. cereus* isolates taken from the milk samples, 56 were able to grow at 7ºC and 42 were able to grow at 7ºC and 37ºC. This study showed the potential for *B. cereus* to grow in domestic refrigerators, particularly when the temperature was greater than optimal and milk was stored past its expiration date.

The results of the survey by te Giffel *et al*. (1997a) are supported by measurements of *B. cereus* growth over time in naturally contaminated, pasteurised milk. An initial study showed that after eight days storage at 7ºC, *B. cereus* was detected in 89/152 samples of pasteurised whole milk and 88/152 samples of skim milk at mean concentrations of approximately $5x10^2$ CFU/mL in both sample types (Larsen and Jørgensen, 1997). In a second study of 27 naturally contaminated pasteurised milk samples stored at 7ºC, *B. cereus* was initially detected in 2/27 samples (10 CFU/mL), and after nine days storage *B. cereus* was detected in 24/27 samples, with 11 samples containing $>10^3$ CFU/mL (Larsen and Jørgensen, 1999). Concentrations close to or exceeding 10^3 CFU/mL *B. cereus* were measured at day seven. The concentration of *B. cereus* in one carton exceeded 10^5 CFU/mL at day nine. The lag phase was 2-4 days.

Naturally-present *B. cereus* was also detected in commercially-prepared extended shelf-life (ESL) microfiltered and pasteurised milk, and was able to grow under storage at 8ºC (Schmidt *et al.*, 2012). The pasteurisation treatments following filtration were 77°C for 30 seconds (milk) and 125°C for 4 seconds (cream), after which the milk and cream were combined. At the end of shelf life (≤4 weeks), 6/125 samples (5%) stored at 8°C contained *B. cereus*, and some contained concentrations as high as 10^6 CFU/mL. After storage for three days at 30ºC, 15/125 samples (12%) contained detectable *B. cereus*. In some packages, the final microbial populations were almost pure cultures of *B. cereus*. This paper also presented unpublished information from another study, which found that only psychrotolerant *B. weihenstephanensis*, but not mesophilic *B. cereus*, were able to outcompete and dominate the concomitant

microbiota in pasteurised milk stored under refrigeration. The authors speculated that mesophilic *B. cereus* may have been able to grow in ESL milk because the numbers of competing microorganisms were very low.

An older study observed the effect of temperature abuse on small boxes of skim milk served in cafeterias (Harmon and Kautter, 1991). *B. cereus* was detected in all 24 boxes of milk at the start of incubation, and after 20 hours at 26ºC the mean concentration was $3x10^6$ CFU/g (range 10^5-10^6 CFU/g). After a further four hours (total 24 hours), the concentrations had reached 10^7 CFU/g. In another study, *B. cereus* was the dominant spore former present in samples of raw and pasteurised cows' milk heat treated, then incubated at 20 or 30ºC for 24 hours (Crielly *et al.*, 1994).

Only one study was located that investigated growth of *B. cereus* in raw and pasteurised milk from different species (Necidova *et al.*, 2014). This study only used one strain of *B. cereus*, which was able to grow when inoculated into pasteurised cows' milk, but not in raw cows' milk. Growth in goats' or sheep milk (raw and pasteurised) was restricted with extended storage. Experiments were conducted at 8, 15 and 22°C, but it does not appear that replicates were undertaken so the results are indicative only. From the available information, the changes of *B. cereus* concentration at 8°C were:

- Pasteurised cows' milk: After four days of storage the inoculum concentration had increased by approximately 1.5 $\log_{10}$ CFU/mL and remained elevated for the remaining three days of the experiment.
- Pasteurised goats' milk: Initial increase of approximately 0.5 $\log_{10}$ CFU/mL during first two days, then decrease to just below the initial concentration.
- Pasteurised sheep milk: Decrease by approximately 2 $\log_{10}$ CFU/mL over seven days.
- Raw cows' milk: *B. cereus* was undetectable after 24 hours and remained so.
- Raw goats' and sheep milk: Increase in concentration in first day, then decreased over remaining six days to below the initial concentration.

Data on the changes of *B. cereus* concentration in milks stored at 15 and 22°C showed similar patterns to those at 8°C, but faster growth rates were observed during periods of growth. The authors discussed pH and competitive microflora as the likely factors influencing the changes in *B. cereus* concentration, but did not provide all the pH data for evaluation nor measure background microflora. It should also be pointed out that Necidova *et al*. (2014) did not specify whether the inoculum was in vegetative or spore form, although it was probably in the form of vegetative cells. Under realistic conditions, pasteurised milk would not contain vegetative *B. cereus* cells (unless these were introduced post-pasteurisation), so any growth would be preceded by a period of spore germination. The authors did, however, provide data on the time to first detection of enterotoxin. At 8ºC, enterotoxin was not detected in any of the raw milks and was only detected in pasteurised cows' milk after seven days. At higher temperatures enterotoxin was detected in pasteurised samples of all milk types after 2-4 days at 15ºC and one day at 22ºC, but enterotoxin was only detected in raw goats' and sheep milks after storage at 22ºC for one or four days, respectively.

A *B. cereus* strain inoculated into UHT milk (10^3 CFU/mL) showed survival and growth at temperatures in the range 4 to 45ºC (Kong, 2015). At a storage temperature of 55ºC, the *B. cereus* strain was reported to survive, but not grow.

Kinetic models for the growth of *B. cereus* in milk have been developed by fitting observed growth data at temperatures in the range 10 to 30ºC to a modified Gompertz model (Kim *et al.*, 2013). Growth was not predicted at 10ºC, which is not in agreement with other studies (above). The researchers did not undertake growth experiments at temperatures less than 10ºC, plus they measured the behaviour of only one *B. cereus* strain over just two days of

incubation. As expected, the duration of the lag phase decreased with increasing storage temperature, from 14 hours at 15ºC to 1.8 hours at 30ºC. The maximum pathogen concentration achieved also increased with storage temperature from 7.6 log CFU/mL at 15ºC to 8.9 log CFU/mL at 30ºC.

Another report, using *B. cereus* growth data in milk and predictive microbiological modelling (Combase or PredictorXL), evaluated the growth of *B. cereus* in milk at 7, 8 and 9ºC (Soleimaninanadegani, 2013). The predicted lag phase decreased from about 115 hours (4.8 days) at 7ºC to about 80 hours (3.3 days) at 9ºC. The estimated time to achieve a *B. cereus* concentration of 10^5 CFU/mL, a concentration with potential to cause illness, increased from 189 hours (7.9 days) at 9ºC to 301 hours (12.5 days) at 7ºC.

2.4.2 Cream

After 154 samples of pasteurised (92ºC, 15 seconds) double cream collected from three processing plants in Denmark were stored at 7ºC for eight days, *B. cereus* was detected in 80 samples (52%) (Larsen and Jørgensen, 1997). The mean count was $3x10^3$ CFU/mL.

In another study, germination and growth of spores of *B. cereus* was limited when they were inoculated into UHT-cream (30% fat) and incubated at 7ºC (Buck *et al.*, 1992). However, a brief period at elevated temperatures (7ºC, 19 days, 15ºC 12-24 hours, return to 7ºC) increased germination and outgrowth of spores and caused spoilage, indicating that spore germination can be stimulated by a brief interruption to the cool chain. *B. cereus* growth was measured under constant storage at 7ºC when a mixture of vegetative cells and spores were added to the cream.

Spores of *B. cereus* were inoculated into full fat cream and heat-treated for 10 minutes (70, 80 or 90ºC), followed by storage at 7 or 10ºC (Samapundo *et al.*, 2014). Spores heated at 70 or 80ºC resulted in the faster outgrowth, while spores heated at 90ºC showed a lag phase of about three days before outgrowth when stored at 7ºC, but not at 10ºC. By 18 days storage, little difference was seen in growth characteristics between storage at 7 and 10ºC, with maximum concentrations of about 10^7 CFU/g reached.

Cream was heat-treated (90ºC for 15 minutes) and stored at 8ºC for 5 weeks (Nissen *et al.*, 2001). *Bacillus* isolates ($n = 52$) were recovered and found to be predominantly *B. licheniformis* and *B. pumilis*. Six of the isolates were *B. cereus* or *B. thuringiensis* (the two species could not be distinguished by the method used). None of the isolated strains grew at 4ºC, while four of six *B. cereus* strains grew at 6ºC and the remaining two grew at 8ºC. Only the *B. cereus* strains tested positive for enterotoxin production when cultured in cream at 10ºC. In a parallel experiment, addition of nisin (5 IU/mL) to cream completely inhibited growth of *Bacillus* spp.

Growth at non-refrigeration temperatures has been reported. A mesophilic strain of *B. cereus* was able to grow in a cream substitute, fresh single cream and fresh whipping cream (not whipped) when incubated at 21ºC for 24 hours (Beattie and Williams, 2002). The final concentrations of *B. cereus* were 10^7-10^9 CFU/mL, although the inoculum (10 CFU/mL) would have included vegetative cells (overnight culture). A psychrotrophic strain of *B. cereus* did not grow in these foods under these conditions.

2.4.3 Powdered milk and infant formula

B. cereus spores may survive the heating and drying steps used to make powdered milk. Reconstitution of the milk powders provides the conditions necessary for *B. cereus* spores to germinate and multiply, but this activity will be governed by storage temperature. Only two studies were located that specifically investigated *Bacillus* spp. growth in reconstituted milk powders. When 18 non-fat milk powders purchased in the USA were reconstituted, *B. cereus* was detected in all of them (Harmon and Kautter, 1991). After storage for 20 hours at 26ºC, the concentration of *B. cereus* had reached 10^7 CFU/g. Further measurements showed that the *B. cereus* population exceeded 10^3 CFU/g after six hours at this temperature. The second

study, based on milk powders from the USA and Venezuela, did not measure *B. cereus* specifically, but showed considerable *Bacillus* spp. outgrowth at 20 and 30ºC, but not at 5ºC (Rodriquez and Barrett, 1986).

A number of studies have measured the initial concentration of naturally present *B. cereus* in reconstituted powdered infant formulae (PIF) and subsequent growth over a period of temperature abuse:

- Of 100 PIF samples from Ireland, 24 were positive for *B. cereus* (mean 190 CFU/g, maximum 570 CFU/g (Haughton *et al.*, 2010). After 24 hours storage of reconstituted formula at temperatures greater than 25ºC, *B. cereus* was detectable in 59/100 samples at concentrations >10^3 CFU/g. There was no significant change in the bacterial quality of reconstituted PIF held at 10ºC or less for 24 hours.
- Of 12 PIF samples from the USA, nine were positive for *B. cereus* (Harmon and Kautter, 1991). After 20 hours storage of reconstituted formula at 26ºC, the concentration of *B cereus* reached a mean of 3x10^6 CFU/g (range 10^5-10^6 CFU/g). After a further four hours (total 24 hours), the mean concentration increased to 7x10^6 (range 10^6-10^7 CFU/g). Further measurements showed that the concentration of *B. cereus* exceeded 10^3 CFU/g after nine hours at this temperature.
- From a survey of PIF (*n* = 261), samples naturally contaminated with *B. cereus* at counts of approximately 100 CFU/g were reconstituted and stored at 27ºC (Becker *et al.*, 1994). Concentrations of *B. cereus* of 10^5 CFU/g were reached after 7-9 hours.
- Of 100 PIF samples from the UK, 17 were positive for *B. cereus* at concentrations of 10^3 CFU/g or less (Rowan *et al.*, 1997). A variety of cooling and storage time/temperature combinations were subsequently investigated. The concentration of *B. cereus* remained below 10^3 CFU/g in formulae incubated at ≤10ºC for 24 hours, but not when kept at higher temperatures. Enterotoxin was only detected in formulae incubated at ≥25ºC.

Two studies have measured production of enterotoxin in reconstituted PIF inoculated with *B. cereus*:

- When *B. cereus* isolates initially obtained from reconstituted PIF were seeded as spores into a milk-based PIF and stored for 15 days, 1/20 isolates grew at 4ºC, 4/20 isolates grew at 6ºC and 14/20 isolates grew at 8ºC (Rowan and Anderson, 1998). Six isolates produced enterotoxin under storage at 8ºC, two at 6ºC and none at 4ºC. Enterotoxin was first detected after 10 days at 8ºC and 15 days at 6ºC. The researchers noted that enterotoxin was not detected until *B. cereus* populations exceeded 10^5 CFU/mL.
- When 10 strains of *B. cereus* containing HBL genes were inoculated into four different reconstituted infant formulae and allowed to multiply at 37ºC, in almost all cases the cell-free supernatants taken from these cultures caused Hep-2 epithelial cells to die (Rowan *et al.*, 2001). However, the formulation affected the ability of these strains to produce enterotoxin. Infant formulae containing glucose or maltodextrin (a derivative of starch hydrolysis) permitted a larger number of *B. cereus* strains to produce enterotoxin. Only 1/10 strains produced enterotoxin in infant formulae without these sugars.

2.4.4 Cheese

Five studies were located where the behaviour of *B. cereus* during cheese-making or in/on cheeses was investigated:

- As part of an investigation into the growth of *B. cereus* in sandwich ingredients, *B. cereus* was inoculated onto cheese and stored at 10 or 25ºC for up to 24 hours (Oh *et al.*, 2011). No significant change in *B. cereus* counts was seen at any time point during the 24-hour period.

- *B. cereus* was inoculated into pasteurised milk to a concentration of 100 spores/mL, for use in the production of Gouda cheese (Rukure and Bester, 2001). The spores germinated and reached a maximum concentration of 10^4 CFU/g at hooping (about 4 hours after renneting). After pressing (16 hours after renneting) the concentration of *B. cereus* was reduced to less than 100 CFU/g and was not detected after brining (40 hours after renneting).

- *B. cereus* spore concentrations in ultra-filtered (UF) feta cheese were monitored from production, through ripening (4 days) and subsequent refrigerated storage for up to 90 days (Moradi-Khatoonabadi *et al.*, 2015). Separate samples were taken of the cheese core, the rind and the brine. After production, UF feta cheese contained about 2 log CFU/mL of *B. cereus* spores. During one day of warm ripening and three days of cold ripening, concentrations decreased slightly (0.3-0.5 log CFU/mL). Negligible further decreases (mostly less than 0.2 log CFU/mL) occurring during 90 days refrigerated storage.

- *B. cereus* inoculated into cottage cheese increased by about 2 log organisms/g after 6-11 days at 10ºC or 1 day at 20ºC (Sims *et al.*, 1989). *B. cereus* concentrations subsequently dropped by 1-2 log organisms/g, either due to decreasing pH or increases in competitive microflora. In cottage cheese containing sorbic acid (approximately 500 mg/kg), *B. cereus* concentrations decreased steadily with storage time at either storage temperature.

- *B. cereus* was inoculated onto the surface of Brie cheese and stored at 4, 8 or 20ºC for up to 35 days (Little and Knochel, 1994). *B. cereus* multiplied at 20ºC, with maximum concentrations achieved after about 2 days, followed by a steady decrease in bacterial numbers. At 4 or 8ºC, *B. cereus* concentrations decreased gradually with storage over the entire storage period.

2.4.5 Other dairy products

Data from overseas surveys indicate that *B. cereus* survives in ice cream (Appendix A.8.2), but no studies measuring survival of the vegetative or spore states in frozen dairy products were located. The presence and concentration of enterotoxigenic *B. cereus* are of more concern when these products are consumed frozen (e.g. ice cream) or just thawed (e.g. cheesecake), unless conditions were suitable for emetic toxin production prior to freezing. Spores are more resistant to prolonged freezing than vegetative cells (White and Hall, 1984). No studies were located that measured the ability of *B. cereus* spores to germinate or vegetative cells to grow in dairy products after thawing. Such growth will be largely temperature dependant.

A mesophilic strain of *B. cereus* was able to grow in UHT-sterilised custard, canned creamed rice pudding, instant mousse desserts and an instant chocolate whip dessert (Beattie and Williams, 2002). The experiments were monitored for 24 hours at 21ºC, and the final concentrations of *B. cereus* were 10^7-10^9 CFU/mL, although the inoculum (10 CFU/mL) would have included vegetative cells (overnight culture). Enterotoxin was detected in some of these foods (individual foods not reported). A psychrotrophic strain of *B. cereus* only grew in the UHT-sterilised custard, mousse and instant chocolate dessert, reaching populations of approximately 10^7-10^8 CFU/ml and producing detectable enterotoxin. The instant chocolate dessert showed no change in aroma nor texture, despite containing mesophilic *B. cereus* at $1x10^8$ CFU/mL. Mayonnaise, bottled white sauce for lasagne and sandwich spread did not support *B. cereus* growth, possibly due to low pH (3.4-4.2). In further experiments, two mesophilic strains of *B. cereus* were unable to grow in UHT-sterilised custard at 10ºC, but two psychrophilic strains were (although no enterotoxin was detected even after seven days).

Using a model food system, where a mesophilic or psychrophilic strain of *B. cereus* was inoculated into a custard-type dessert, growth and enterotoxin production was not affected when concentrations of sucrose or starch were changed, or when the type of milk protein or

initial pH were altered (Beattie and Williams, 2002). Growth and toxin production was measured at 6, 10, 15, 21 and 30ºC. Only the psychrotrophic strain grew in this food at 10ºC (and produced toxin), but no growth was detected at 6ºC, even after storage for 21 days.

2.4.6 Lactic fermentation

Behaviour of *B. cereus* in co-culture with either *Lactobacillus casei* (a fast acid producer) or *L. acidophilus* (a slow acid producer) was examined (Rossland *et al.*, 2005). The pH was allowed to decrease to a final pH of 5.0, 5.5 or 6.0. Maximum *B. cereus* concentrations were reached after about 10 hours (about $5x10^6$ CFU/mL with *L. casei* and about $1x10^8$ CFU/mL with *L. acidophilus*). In co-culture with *L. casei* and a final pH of 6.0, *B. cereus* counts remained fairly constant until the end of the experiment (72 hours). At final pH 5.5, *B. cereus* counts reduced to 10-70 CFU/mL after 72 hours, while at pH 5.0 *B. cereus* was not detectable after about 48 hours. No endospores were formed under any pH regime. In co-culture with *L. acidophilus*, *B. cereus* counts remained at 10^4 CFU/mL or higher at the end of 72 hours, with endospore counts increasing from 12 hours onwards. The authors concluded that a rapid pH decrease prevents sporulation, while a combination of pH and competitive organisms was required for complete inhibition of *B. cereus*.

Sadek *et al.* (2006) investigated the ability of seven lactic acid bacteria (LAB) to inhibit growth of *B. cereus*. The most effective species was *Lactobacillus reuteri*, followed by *L. rhamnosus*. The authors ascribed inhibition to the secretion of inhibitory compounds by these species, rather than due to effects on pH. Growth of *B. cereus* strains in Tallaga cheese was inhibited by the presence of a starter culture of LAB, but not by a decrease in pH to approximately 4.5. While these results initially appear to contradict those in the previous paragraph, they may be consistent if the pH changes were sufficiently slow.

Acid adaptation (holding at pH 6.3 for 40 minutes) was found to improve the survival of *B. cereus* under conditions of lactic fermentation (Shen *et al.*, 2008). Adapted and non-adapted *B. cereus* populations increased during the first 12-18 hours of lactic fermentation (*Streptococcus thermophilus* or *Lactobacillus bulgaricus*) of skim milk, then declined through to the end of fermentation. However, the decline was less marked with the acid-adapted population.

Kefir is a fermented milk drink, with fermentation mediated by the microbiota present on kefir grains (LAB, acetic acid bacteria and yeasts) (Kakisu *et al.*, 2007). Kefir fermentation with 5% added kefir grains was shown to inhibit germination of *B. cereus* spores and prevent growth of vegetative cells. This was ascribed to a rapid decrease in the pH (to 4.0) during the first eight hours of fermentation. At 1% added kefir grains no inhibition occurred.

2.4.7 Toxin production in dairy products

Overseas studies summarised in appendices A.8.2 and A.8.3, and above, show that *B. cereus* strains carrying the genes for enterotoxin production are often present in dairy products, but only a few studies have identified whether the full suite of toxin genes are present or demonstrated the ability of isolates to produce enterotoxin.

Emetic strains have been detected in dairy foods and dairy products and have been shown to support emetic toxin production. A study in Germany used a bioassay to specifically investigate cereulide production by an emetic *B. cereus* strain inoculated into 70 foods, including some dairy foods (Messelhäusser *et al.*, 2014). The experiments were carried out at 24°C and using an inoculum of 10^3 CFU/g. The foods were classified as “low risk”, “risk” and “high risk” according to the amount of cereulide produced after 24 hours at 24°C:

- Low risk: Crème fraiche, diet chocolate with cream filling, cottage cheese, fresh cheese, yoghurt, curd cheese, whey drink, cabonara sauce and chocolate bar with milk/caramel filling. These products had a low pH (4.3 to 4.8).

- Risk: Reconstituted milk powder (organic), cheese slices, latte macchiato drink, camembert cheese, chocolate mousse, pasteurised milk (1.5% fat), pasteurised cream (30% fat), chocolate biscuit with milk cream filling. These products had neutral pH and higher fat.
- High risk: Dessert crème, reconstituted skim milk powder, reconstituted whole milk powder, milk drink with nut flavour, vanilla mousse.

Emetic toxin production has also been demonstrated in dairy-based infant foods and UHT milk at non-refrigeration temperatures (Rajkovic *et al.*, 2006; Rowan *et al.*, 1997; Rowan and Anderson, 1998; Rowan *et al.*, 2001; Shaheen *et al.*, 2006). While there is some disagreement concerning the ability of *B. cereus* strains to produce emetic toxin at temperatures less than 12°C (Carlin *et al.*, 2006; Finlay *et al.*, 2000; Haggblom *et al.*, 2002; Thorsen *et al.*, 2006; Thorsen *et al.*, 2009) in culture, studies on toxin production at refrigeration temperatures are limited for dairy products, with the most relevant study determining general cytotoxicity, rather than emetic toxin concentrations (Christiansson *et al.*, 1989).

While some studies, in culture media (Haggblom *et al.*, 2002; Jaaskelainen *et al.*, 2004) or milk (Agata *et al.*, 2002; Christiansson *et al.*, 1989), suggesting adequate oxygen supply is essential for toxin production, others, in dairy products (milk or reconstituted infant formula), suggest that aeration has a negative impact on toxin production (Rajkovic *et al.*, 2006; Shaheen *et al.*, 2006). All but one of these studies (Christiansson *et al.*, 1989) involved incubation at temperatures in the range 21 to 30°C.

2.5 EXPOSURE ASSESSMENT

KEY FINDINGS

One New Zealand study has reported a prevalence of 0.07% for *B. cereus* in raw cows' milk. However, the basis for sample submission in this survey was not specified and this prevalence may not represent the actual national prevalence of *B.* cereus contamination of raw milk. No other data are available for the prevalence and concentration of *B. cereus* in New Zealand dairy products.

Dairy products are commonly consumed foods in New Zealand.

If *B. cereus* are present in a dairy product, their ability to grow depends mainly on the extrinsic factors of temperature, background microflora and pH, and also on the ability of the strain(s) to grow under the cooler conditions of dairy product storage.

Evidence for the ability of isolates of *B. cereus* from dairy products to produce enterotoxins is scarce. Available data suggest that emetic *B. cereus* can be detected in dairy products.

While there is good evidence that cereulide can be produced in dairy foods at non-refrigeration temperatures (>20ºC), limited data on toxin production at refrigeration temperatures are available. In laboratory media, cereulide production has been reported to be very low at ≤8°C, while other studies have not detected toxin production at temperatures <12°C.

2.5.1 New Zealand prevalence studies

Only one source of data on the prevalence of *B. cereus* in dairy products was located for New Zealand. *Bacillus* species were isolated from 1007/25,288 samples (4.0%) of raw milk submitted by veterinary practitioners to veterinary laboratories in New Zealand during the period 2003 to 2006 (Petrovski *et al.*, 2011). *B. cereus* was identified from 18 of these positive samples (prevalence of 18/25,288 or 0.07%). Most (14/18) of the samples positive for *B.*

cereus originated from South Island sources, despite the majority of the samples overall being submitted from North Island sources (22,744/25,288, 89.9%). No data were recorded on whether the samples came from cows with clinical or subclinical mastitis, or the date of sample collection.

While New Zealand surveys of thermophilic species in milk powders have detected *B. licheniformis* and *B. subtilis*, the incubation temperature used (55ºC) was unfavourable for growth of *B. cereus* and detection of *B. cereus* would have been unlikely (Ronimus *et al.*, 2003; Rückert *et al.*, 2004).

2.5.2 Product recalls

A single product recall of a dairy product due to *B. cereus* contamination was issued in New Zealand during the period 2008 to 2015. The product was a frozen dairy-based dessert and the recall was issued in August 2015.[12]

2.5.3 Requirements for imported food

In 2015, the Food Regulations 2015 were issued.[13] The Food Regulations 2015 define foods of high regulatory interest (HRI) and foods of increased regulatory interest (IRI). The Food Regulations 2015 also specify that clearance requirements for these foods will be set out in notices under section 405 of the Food Act 2014. The most recent Food Notice: Importing Food (MPI, 2016b) identified two classes of dairy products as HRI; raw milk products, and fresh cheese, curd cheese and soft cheese (pasteurised). Clearance limits are specified for *Listeria monocytogenes* (both classes) and *Salmonella* (raw milk products only), but not for *Bacillus cereus*.

2.5.4 Exception reporting

The occasional presence of *B. cereus* in dairy products is captured by the exception reporting system. New Zealand dairy processors operating under risk management programmes (RMPs) test products to demonstrate compliance with regulatory and company defined pathogen limits. Non-complying results are notified to MPI, through an exception report raised by the processor and submitted to the recognised agency responsible for verifying the RMP (Dr Tanya Soboleva and Chris Tomlinson, MPI, personal communication). Exception reports raised due to high levels of *B. cereus* have increased from 12 in 2013, to 21 in 2014 and 24 in 2015. However, it is uncertain whether this represents an increase in the rate of *B. cereus* contamination, an increase in testing for *B. cereus* or an increase in reporting.

Products for which exceptions have been reported include cheese, butter, frozen dairy products, powdered milk and nutritional powders and protein products. All dairy products identified through the exception reporting system are removed from the food supply chain.

2.5.5 Dairy product consumption

Dairy products are very commonly consumed foods in New Zealand. In 1995-96, it was reported that annual per capita dairy food consumption in New Zealand included 91 L of fluid milk, 9 kg of cheese and 8 kg of butter (NZIC, 2008). Similar levels of dairy food consumption have been reported for Australia, including 98 L/capita/year of milk, 12 kg of cheese, 3 kg of butter, 6.5 kg of yoghurt and 16.3 kg of ice cream (FSANZ, 2006). Food balance sheets for

[12] http://www.foodsmart.govt.nz/elibrary/consumer/recall-everyday-entertainer-brand-chocolate-bavarian.htm (accessed 8 December 2015)

[13] http://www.legislation.govt.nz/regulation/public/2015/0310/latest/DLM6684211.html?src=qs (accessed 6 May 2016)

New Zealand for 2011 give domestic supply of dairy products, expressed as milk equivalents, as 149 kg/capita/year.[14]

These data are based on the supply of dairy products for domestic consumption. While not comprehensive, reported New Zealand food consumption information for the dairy foods; milk, cheese (soft and low moisture) and ice cream have been reviewed (Cressey *et al.*, 2006; Cressey, 2013). Information in these publications is mainly synthesised from 24-hour dietary recall microdata from the 1997 National Nutrition Survey (Russell *et al.*, 1999), the 2002 National Children's Nutrition Survey (Ministry of Health, 2003) and the 2009 Adult Nutrition Survey (University of Otago and Ministry of Health, 2011). Relevant data from these publications are summarised in Table 2.

Table 2. Consumption of selected dairy products in New Zealand

STATISTIC	ADULTS (15+ YEARS)		CHILDREN (5-14 YEARS)
	2009ANS	1997NNS	2002CNS
Number of respondents	4721	4636	3275
MILK			
Number of servings	11342	15199	4114
Number of consumers (percentage of total respondents)	3755 (79.5%)	4067 (87.7%)	2375 (72.5%)
Servings/consumer/day	3.0	3.7	1.7
Consumer mean (g/person/day)	241	272	271
Population mean (g/person/day)	192	239	197
Mean serving size (g)	79.9	72.9	157
Median serving size (g)	53.0	41.6	129
95th percentile serving size (g)	265	258	335
CHEESE (SOFT)			
Number of servings	274	309	43
Number of consumers (percentage of total respondents)	246 (5.2%)	263 (5.7%)	40 (1.2%)
Servings/consumer/day	1.1	1.2	1.1
Consumer mean (g/person/day)	27.0	30.2	31.0
Population mean (g/person/day)	1.4	1.7	0.4
Mean serving size (g)	24.2	25.7	28.8
Median serving size (g)	16.3	18.8	15.0
95th percentile serving size (g)	76.6	74.7	99.8
CHEESE (LOW MOISTURE)			
Number of servings	2559	2976	1632
Number of consumers (percentage of total respondents)	1928 (40.8%)	2111 (45.5%)	1178 (36.0%)
Servings/consumer/day	1.3	1.4	1.4
Consumer mean (g/person/day)	36.7	32.3	32.5
Population mean (g/person/day)	15.0	14.7	11.7

[14] http://faostat.fao.org/site/368/DesktopDefault.aspx?PageID=368#ancor (accessed 12 May 2016)

STATISTIC	ADULTS (15+ YEARS)		CHILDREN (5-14 YEARS)
	2009ANS	1997NNS	2002CNS
Mean serving size (g)	27.6	22.9	23.4
Median serving size (g)	21.0	16.9	18.0
95th percentile serving size (g)	70.8	60.0	60.0
ICE CREAM			
Number of servings	603	711	913
Number of consumers (percentage of total respondents)	578 (12.2%)	666 (14.4%)	719 (24.4%)
Servings/consumer/day	1.0	1.1	1.1
Consumer mean (g/person/day)	101.2	99.1	128.9
Population mean (g/person/day)	12.4	14.2	31.4
Mean serving size (g)	97.0	92.8	113
Median serving size (g)	75.0	73.0	100
95th percentile serving size (g)	259	246	234

2009ANS: 2009 Adult Nutrition Survey (University of Otago and Ministry of Health, 2011)
1997NNS: 1997 National Nutrition Survey (Russell *et al.*, 1999)
2002CNS: 2002 National Children's Nutrition Survey (Ministry of Health, 2003)

It should be noted that this analysis does not include some commonly consumed dairy products (yoghurt, cream). Points to note from the material in Table 22 and from the source reports include:

- The frequency of consumption of fluid milk by adult New Zealanders decreased during the period 1997 to 2009, although serving sizes have increased.
- Fluid milk is less frequently consumed by New Zealand children than adults, but quantities consumed by children are substantially greater. This reflects the use of milk as a component of hot drinks by adults, while children are more likely to consume milk as a beverage on its own.
- Older people (65+ years) are more likely to consume fluid milk than the population less than 65 years, but consume significantly smaller servings.
- Pregnant women consume fluid milk in similar proportions to the general population, but consume significantly larger servings.
- Soft cheeses are more likely to be consumed by adults than children, but serving sizes are similar.
- There is no significant difference in patterns of soft cheese consumption between older people and other adults, but pregnant women rarely consume soft cheeses.
- Low moisture cheeses are more likely to be consumed by adults than children. Although the frequency of adult low moisture cheese consumption decreased during the period 1997 to 2009, serving sizes have increased.
- Older people are significantly less likely to consume low moisture cheeses than adults under 65 years and consume significantly smaller servings. Pregnant women are more likely to consume low moisture cheese than the general population.

- Children consume ice cream more frequently than adults and in greater amounts. The frequency of adult consumption of ice cream decreased significantly during the period 1997 to 2009.
- There is no difference in the frequency of ice cream consumption between older people and other adults, although serving sizes consumed by older people are significantly smaller. Consumption of ice cream by pregnant women is not significantly different to the general population.

2.5.6 Potential for growth of *B. cereus* along the dairy product food chain

Any dairy product can be contaminated with *B. cereus* spores and may also contain vegetative cells. Dairy foods can support *B. cereus* growth, but the available data indicate that the extent of growth mainly depends on background microflora, pH and temperature, and how these change over the life of the food product. *B. cereus* will not grow in frozen dairy products, but may survive and germinate/grow if temperatures are raised, as long as a sufficient supply of nutrients is present.

In dairy products with low background microflora and low acidity, such as pasteurised cows' milk, temperature will be the main growth moderator. The available data indicate that some strains of *B. cereus* (and the closely-related *B. weihenstephanensis*) can grow at the temperatures most commonly used for storing milk (4-7°C), albeit slowly. Growth rate is increased with increasing temperature. Increased storage temperature will also support growth of a wider range of *B. cereus* strains.

Storage at mildly abusive temperatures (7-10ºC) can occur along the dairy food chain, particularly during retail display and in consumer homes. There are no published New Zealand data on the temperatures in retail dairy display spaces. A survey of domestic refrigerators in New Zealand found one third (43/127; 34%) operating at a mean temperature above 6°C (Gilbert *et al.*, 2007). A study in the Netherlands found the mean temperature of milk sampled from domestic refrigerators was 7.4°C (minimum -1°C, maximum 17.9°C), and was ≥7°C in 57% (178/313) of the refrigerators (te Giffel *et al.*, 1997a). After the expiry date of the milk, the prevalence of *B. cereus* in milk sampled from the refrigerators operating at ≥7°C was higher than those operating at lower temperatures (there was no consistent pattern prior to the expiration date). A study of *B. cereus* growth in naturally contaminated pasteurised milk held at 7ºC showed that the concentration of this pathogen could reach 10^3 CFU/mL after seven days and 10^5 CFU/mL after nine days. (Larsen and Jørgensen, 1999).

Prolonged storage at abusive temperatures (e.g. >15ºC) will also encourage growth of spoilage microorganisms. This outgrowth of spoilage microorganisms will influence the *B. cereus* population, but it is difficult to predict the effect, and whether the population of *B. cereus* will achieve a concentration high enough to cause illness before the product appears spoiled.

Toxin production must also be considered. Studies have shown that *B. cereus* isolates from dairy products often contain one or more enterotoxin genes, but evidence for the ability of isolates to actually produce these toxins is scarce. Available data suggest that emetic *B. cereus* can be detected in dairy products, but further studies are necessary to establish more robust prevalence data. Further work is also required to evaluate the potential for emetic strains to multiply and produce cereulide in dairy products under typical storage conditions. While there is good evidence that cereulide can be produced in dairy foods at non-refrigeration temperatures (>20ºC) (Agata *et al.*, 1999; Rajkovic *et al.*, 2006; Shaheen *et al.*, 2006), limited data on toxin production at refrigeration temperatures are available (Christiansson *et al.*, 1989). In laboratory media, cereulide production has been reported to be very low at ≤8°C (Haggblom *et al.*, 2002; Thorsen *et al.*, 2006), while other studies have not detected toxin production at temperatures <12°C (Carlin *et al.*, 2006; Finlay *et al.*, 2000).

2.6 DATA ON *B. CEREUS* IN DAIRY PRODUCTS FROM OTHER COUNTRIES

KEY FINDINGS

While there appears to be some evidence for inhibition of *B. cereus* during production of some cheeses and yoghurt, the weight of evidence suggests that any dairy product should be considered to be potentially contaminated with *B. cereus.* Both emetic and diarrhoeal strains have been detected in dairy foods.

Data on the prevalence and concentration of *B. cereus* in dairy products in other countries are detailed in Appendix A.8.2.

While the prevalence of *B. cereus* contamination in dairy products examined in overseas studies varies considerably, most studies have detected *B. cereus* in the range of dairy products tested.

The prevalence in raw milk ranged from 5 to 90%. Concentrations of *B. cereus* in raw milk have been reported to vary widely from less than 1 CFU/mL to 10^7 CFU/mL. It should be noted that the higher concentrations were reported from developing countries in which milk production is usually carried out by farmers with small holdings (Bedi *et al.*, 2005; Kivaria *et al.*, 2006; Rather *et al.*, 2011; Yobouet *et al.*, 2014). In developed countries, with well-established dairy industries, raw milk is likely to contain <100 CFU/mL of *B. cereus* (Bartoszewicz *et al.*, 2008; Jackson *et al.*, 2012; Notermans *et al.*, 1997; Svensson *et al.*, 2004; te Giffel *et al.*, 1997a).

The prevalence of *B. cereus* in pasteurised milk is generally greater than in raw milk. While this might be due to the ability of the endospores to survive pasteurisation and grow (if conditions are favourable), or due to contamination from biofilm, sampling may also be a factor (e.g. where the pasteurised milk is produced from a mixture of incoming raw milk from various sources). Prevalence of *B. cereus* contamination of up to 100% of pasteurised milk samples has been reported in several studies. Studies in the Netherlands suggest that in developed countries, the concentration of *B. cereus* in pasteurised milk soon after pasteurisation is low, but can become elevated during storage in consumers' refrigerators (Notermans *et al.*, 1997; te Giffel *et al.*, 1997a). A study in Germany did not detect presumptive *B. cereus* in any of 384 milk and milk product samples taken as part of foodborne intoxication investigations (Messelhäusser *et al.*, 2014).

Five studies reported on *B. cereus* in UHT milk (four conducted in Brazil, one in Malaysia) and *B. cereus* was detected in all but one of these studies (11-30% prevalence; 14% in one study that measured presence of the *B. cereus* group).

The prevalence of *B. cereus* in milk powder and powdered infant formula is in the range 0-75%. *B. cereus* concentrations in milk powders and powdered infant formula will generally be <10 CFU/g, although hygiene problems in the processing environment may result in concentrations as high as 1000 CFU/g (Christiansson, 2011).

Prevalence of *B. cereus* contamination in cheese is in the range 0 to 70%. Little quantitative information is available, but there appears to be potential for *B. cereus* concentrations to reach 10^4 CFU/g. It should be noted that most studies on *B. cereus* in cheese appear to be of soft cheese varieties. Experimental studies with Gouda cheese production suggest that *B. cereus* does not survive the production process for this cheese type (Rukure and Bester, 2001). It has been reported that a hard cheese implicated in an outbreak in Germany contained an estimated <100 CFU/g *B. cereus,* but 2 µg/g cereulide (Messelhäusser *et al.*, 2014).

Prevalence of *B. cereus* contamination in ice cream has been reported in the range 20-63%, with concentrations up to 10^4 CFU/g. *B. cereus* will not grow at ice cream storage temperatures.

Little information is available on *B. cereus* contamination in yoghurt. The acid environment and high levels of competing bacteria present during yoghurt production will inhibit growth of *B. cereus*. The one survey of *B. cereus* contamination of yoghurt (Egypt) reported low prevalence (1/50) and concentration (6 ± 5.9 cells/g) (Hassan *et al.*, 2010).

3. EVALUATION OF ADVERSE HEALTH EFFECTS

3.1 DISEASE CHARACTERISTICS

KEY FINDINGS

B. cereus-associated foodborne illness occurs as two distinct syndromes: Emetic (from toxin pre-formed by the bacteria in food) and diarrhoeal (from toxin produced by the bacteria in the intestine). Both are usually self-limiting conditions and resolve within 24 hours. In rare cases people with the emetic syndrome have died from liver failure.

The diarrhoeal enterotoxin disrupts the membrane of epithelial cells, but the mechanism is not understood (Notermans and Batt, 1998). The emetic toxin is believed to bind to the 5-HT3 receptor associated with the vagus nerve, which runs from the brain to various parts of the chest, including the throat and stomach (Stenfors Arnesen *et al.*, 2008). Stimulation of the nerve leads to vomiting (Notermans and Batt, 1998).

Further details of the clinical characteristics of the diarrhoeal and emetic syndromes are included in the pathogen data sheet for *B. cereus* on the MPI website.[15]

Fatalities from foodborne illness involving *B. cereus* are rare, but patients with the emetic syndrome have died:

- Liver failure attributed to emetic toxin detected in spaghetti with homemade pesto (Mahler *et al.*, 1997). The dish had been prepared four days earlier, and although stored in the refrigerator, on several occasions it had been left at room temperature for one or more hours before being reheated in a pan.
- Liver failure in a 7-year-old child attributed to emetic toxin detected in a pasta salad (Dierick *et al.*, 2005). The child was one of five children in a family outbreak. The salad was prepared on Friday and taken to a picnic on Saturday. Illness followed consumption of the remaining salad on Monday evening. *B. cereus* concentrations of 10^7 to 10^8 CFU/g were found in the pasta salad. While the salad was stored in a refrigerator between consumption events, the temperature of the refrigerator was found to be 14ºC.
- A 20-year-old man died approximately 10 hours after consuming a spaghetti meal contaminated with *B. cereus* at a concentration of approximately 10^8 CFU/g, and with a concentration of emetic toxin of 14.8 mg/kg of spaghetti (Naranjo *et al.*, 2011). The exact cause of death could not be determined due to delays in carrying out the autopsy. The dish had been prepared five days before and stored in the kitchen at room temperature.

B. cereus can occasionally cause invasive disease (see Appendix B.1), usually due to contamination of medical devices introduced into the central nervous system (CNS). Serious eye infections may occur following introduction of foreign objects into the eye as a result of traumatic injuries (Schoeni and Wong, 2005).

[15] http://www.foodsafety.govt.nz/elibrary/industry/Bacillus_Cereus-Spore_Forming.pdf (accessed 18 February 2016)

3.2 DOSE RESPONSE

KEY FINDINGS

Doses in the range 10^5 to 10^8 cells are believed to be necessary to cause *B. cereus* emetic or diarrhoeal illness. However, diarrhoeal toxin-producers differ in their rate of toxin production, while the heat-stable emetic toxin may be present in food even when bacteria have been destroyed by heat treatment.

The threshold dose for illness due to emetic toxin, cereulide, has been estimated at about 10 μg/kg body weight.

While there are various estimates of the number of *B. cereus* cells required to cause illness, there is general agreement that foods containing $<10^3$ CFU/g are safe for human consumption (Vilas-Boas et al., 2007). However, the risk of developing illness depends on multiple factors including the amount of contaminated food eaten, the strain(s) of *B. cereus* present and the susceptibility of the individual consuming the food.

Evidence from outbreaks suggests that a concentration of $\geq 10^4$ CFU/g food is likely to cause *B. cereus* intoxication (Appendix B.2).[16] Doses of 10^5 to 10^8 cells are believed to be necessary to cause *B. cereus* emetic or diarrhoeal illness (EFSA, 2005). However this is complicated by:

- Strain differences in toxin production, meaning that a greater dose of some strains will be required to achieve the same toxin dose. Different strains of *B. cereus* have been shown to differ in their production of toxins, even when they possess the same range of toxin genes (Jessberger *et al.*, 2015).
- The thermal stability of the emetic toxin means that levels of toxin, sufficient to cause illness, may be present in heat-treated foods, when no or very few *B. cereus* remain.

As illness due to *B. cereus* involves intoxication, rather than infection, it has been proposed that the usual approach to microbial dose-response needs to be reconsidered in terms of the dose of toxin, rather than the dose of bacteria (Rajkovic, 2014). While this may be practical for the emetic toxin, the diarrhoeal toxins are usually formed *in vivo* and direct measurement of the toxin concentration is not easily achieved.

A threshold dose for illness due to emetic toxin has been estimated at approximately 10 μg/kg body weight (Rajkovic, 2014). No equivalent estimates of the threshold dose have been estimated for enterotoxins.

3.3 NEW ZEALAND HUMAN HEALTH SURVEILLANCE

KEY FINDINGS

Available disease and outbreak surveillance data suggest that cases of *B. cereus* intoxication in New Zealand are uncommon and there is no evidence to suggest that dairy foods are a cause. However, *B. cereus* intoxication is only notifiable in New Zealand if an outbreak is detected, and the largely mild and self-limiting nature of the illness means that it is likely to be largely unreported.

[16] While the emetic illness is clearly an intoxication, the diarrhoeal illness is mediated by toxin production following establishment of a bacterial population in the gastrointestinal tract. Therefore, the diarrhoeal disease includes elements of infection or colonisation and intoxication. For simplicity, in this report both syndromes have been referred to as intoxications

B. cereus intoxication is not a notifiable disease in New Zealand, unless there is a suspected common source (i.e. an outbreak) or a 'high risk' person has been identified as being ill, such as a food handler or an early childhood service worker (signalling potential for an outbreak) (Ministry of Health, 2013). In these cases, *B. cereus* intoxication will be notified under the category of 'acute gastroenteritis'. This, combined with the disease being of short-duration and usually without complications requiring medical attention, means that cases of *B. cereus* intoxication in New Zealand will be under-reported. Reporting may also be complicated by similarities between the symptoms of the emetic disease and *Staphylococcus aureus* intoxication, or similarities between the diarrhoeal disease and that caused by *Clostridium perfringens* type A food poisoning (Stenfors Arnesen *et al.*, 2008).

3.3.1 Dairy product consumption as a risk factor for *B. cereus* intoxication in New Zealand

There were 18 cases of *B. cereus* intoxication registered in the notifiable disease database (EpiSurv) in the period 2005-2014.[17] Dairy products were not implicated as the cause of any of these cases. Confirming the food causing infection is rarely accomplished in investigations of sporadic cases.

Dairy products were not implicated in any of the outbreaks of *B. cereus* intoxication reported in the period 2005-2014.

ESR tests food samples collected during sporadic or outbreak case investigations of suspected foodborne illness. The database was interrogated for the period 2006-2014 and there was little evidence to support dairy products as a source of foodborne illness due to *B. cereus* intoxication. During this period there were 11 dairy food types tested for *Bacillus* spp., *B. cereus* or *Bacillus* diarrhoeal enterotoxin as part of investigating a food complaint or suspected food poisoning. Of these 11 foods:

- *B. cereus* was not detected (<10 CFU/g) in eight (including cream spread, cheeses, infant formula, yoghurts, yoghurt sauce, crème brule);
- *B. cereus* was not detected (<10 CFU/mL) in a flavoured milk, but the concentration of *Bacillus* spp. was estimated to be 8.5×10^5 MPN/mL. The species was identified as *B. mycoides* and it was considered that the bacterial concentration was unlikely to have resulted in illness.
- *B. cereus* was detected at 10 CFU/g in a cheese-based mousse and at 20 CFU/g in a sour cream-based mousse, but no diarrhoeal enterotoxin was detected in either food (the presence of diarrhoeal toxin would be unlikely at these concentrations of *B. cereus*).

A twelfth food was also recorded as a dairy product; this product was a "rice and curry mixed" and contained 2.1×10^2 *B. cereus*/g, but growth may have occurred in the rice.

3.3.2 *B. cereus* intoxication in New Zealand

A total of 18 cases of *B. cereus* intoxication were registered in EpiSurv in the period 2005-2014. During approximately the same period (2006-2014), 12 people were admitted to public hospitals in New Zealand and diagnosed with 'foodborne *Bacillus cereus* intoxication' (ICD 10 code A05.4),[18] as either the primary diagnosis or as one of 99 other diagnosis codes.

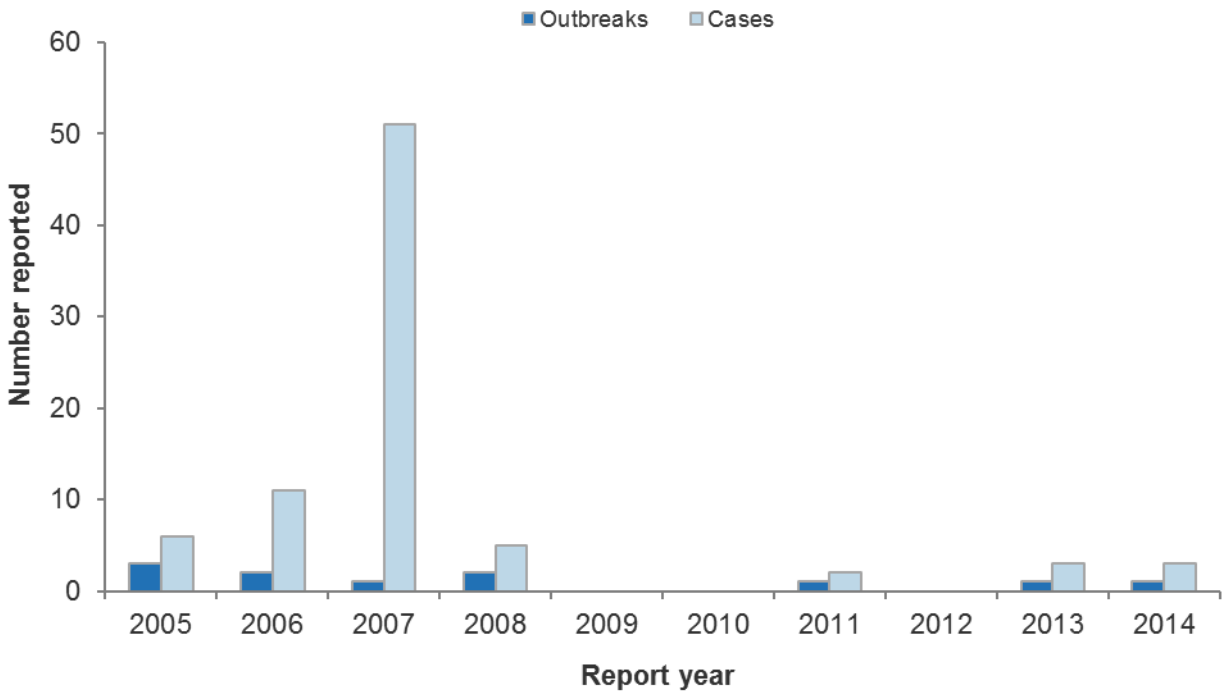
Outbreaks of *B. cereus* intoxication are rare, with three outbreaks reported in the last six years (Figure 1). Between 2005 and 2008 there were one to three outbreaks reported a year. The

[17] EpiSurv is New Zealand's notifiable communicable disease reporting database (https://surv.esr.cri.nz/episurv/index.php (accessed 23 February 2016)

[18] http://apps.who.int/classifications/icd10/browse/2015/en#/A05 (accessed 27 November 2015)

largest outbreak, with 51 associated cases, was reported in 2007, with lentil soup implicated as the cause. In 2014, outbreaks caused by *B. cereus* accounted for approximately 0.1% of enteric outbreaks and 0.02% of enteric outbreak cases.

Figure 1.Reported foodborne *B. cereus* outbreaks and associated cases reported by year, 2005−2014



Reproduced from Horn et al. (2015)

3.4 *B. CEREUS* INTOXICATION OVERSEAS

KEY FINDINGS

Unlike New Zealand, outbreaks of *B. cereus* intoxication have occasionally been linked to dairy products overseas (Appendix B.3). No detailed outbreak reports have been found since 1991, although recent outbreak summaries from Europe and the USA attribute small numbers of *B. cereus* outbreaks to dairy products.

As in New Zealand, *B. cereus* intoxication is not a notifiable disease in most countries so the available data are on outbreaks that have received the attention of public health authorities. While it appears that *B. cereus* outbreaks as a proportion of total outbreaks in Australia and the USA are similar to New Zealand (Appendix B.3.2), different surveillance and reporting methods mean directly comparing data between countries may be misleading.

4. EVALUATION OF RISK

4.1 EXISTING RISK ASSESSMENTS

KEY FINDINGS

A quantitative risk assessment for *B. cereus* intoxication in New Zealand due to consumption of dairy products is not available. Two international risk assessment related to *B. cereus* in pasteurised milk are available, but arrive at quite different conclusions, largely based on the approach taken to dose-response. Both risk assessments predict that approximately 7-10% of pasteurised milk servings may contain >10^5 *B. cereus*/mL.

4.1.1 New Zealand risk assessment

No quantitative risk assessments for *B. cereus* in dairy products have been conducted in New Zealand.

4.1.2 Risk assessments from other countries

Two human health risk assessments have been conducted related to *B. cereus* in pasteurised milk (Acai *et al.*, 2014; Notermans *et al.*, 1997). These studies arrived at contradictory conclusions. The risk assessment carried out for the Slovak Republic estimated that *B. cereus* contamination of pasteurised milk would result in less than one illness per annum per one million of population (Acai *et al.*, 2014). In contrast, a risk assessment for the Netherlands estimated that 7% of pasteurised milk servings would contain greater than 10^5 *B. cereus*/mL, sufficient to cause disease (Notermans *et al.*, 1997). Given the frequent consumption of milk by the Dutch population and the lack of epidemiological evidence linking illness from *B. cereus* intoxication to milk consumption, the authors of this study suggested that dose-response relationships for *B. cereus* in milk should be reconsidered.

It should be noted that both of these risk assessments derived very similar probabilities of a serving of pasteurised milk containing >10^5 *B. cereus*/mL – 7% for the Netherlands and 10% for the Slovak Republic. The major difference between the two risk assessments is that the Slovak Republic study calculated a dose-response equation “based on Slovak data”, which assumed that only one person in the Slovak Republic would become ill from *B. cereus* intoxication per year. This seems like an unrealistically low assumption.

4.2 EVALUATION OF RISK FOR NEW ZEALAND

KEY FINDINGS

There are insufficient data to support a qualitative assessment of the risk of *B. cereus* intoxication for people consuming dairy products in New Zealand.

4.2.1 Risk associated with dairy products

There are insufficient data to support a qualitative assessment of the risk of *B. cereus* intoxication for people consuming dairy products in New Zealand. This is chiefly because:

- Other than a small (and likely biased) survey of *B. cereus* in raw milk samples, which suggested prevalence in raw milk was 0.07%, there are no published surveys of the prevalence and/or concentration of *B. cereus* in dairy foods in New Zealand. Overseas surveys indicate that the prevalence and concentration can vary widely, so it is not sensible to apply such data to New Zealand.

- New Zealand public health surveillance data are limited because (i) *B. cereus* intoxication is non-notifiable (unless an outbreak is detected) and (ii) the relatively mild and self-limiting nature of the disease means sick people rarely seek medical attention (thus the disease is under-reported). Public health surveillance data from other countries are similar. Dairy products have not been implicated as the cause of any outbreaks of *B. cereus* intoxication in New Zealand, and are rarely implicated in outbreaks overseas.
- While the number of exception reports for *B. cereus* contamination of dairy products have increased over the period 2013 to 2015, it is not possible to say whether this is due to a greater prevalence of contamination or an increased rate of testing for *B. cereus*.

Available data suggest that *B. cereus* is more likely to be detected in pasteurised milk than raw milk, and that growth to a concentration high enough to cause illness is possible for pasteurised milk held in consumer refrigerators, operating at mildly abusive temperatures. These data are for cows' milk and data on the behaviour of *B. cereus* in milk from other animals are insufficient to ascertain risk. The available data also indicate that *B. cereus* can probably be isolated from most other dairy foods at retail in New Zealand. Growth in the range of foods considered in this Risk Profile depends largely on temperature, but also on the characteristics of the food. Growth in low-pH foods such as yoghurt, and in some cheeses, appears to be restricted.

Overseas data suggest that diarrhoeal strains of *B. cereus* are more of a concern in dairy products than emetic strains, but this is possibly an artefact, since many studies did not attempt to identify markers for emetic strains. Cereulide production by emetic strains of *B. cereus* has been demonstrated in milk and dairy foods with neutral or high-pH, but mainly at temperatures >20°C. Further investigations are necessary to understand whether the risk in New Zealand lies largely with the presence and growth of diarrhoeal strains of *B. cereus* in dairy foods prior to consumption, or the presence and growth of emetic strains. These investigations need to consider normal and mildly abusive time/temperature regimes rather than the higher temperatures used in many studies.

4.2.2 Risks associated with other foods

Starchy foods, particularly rice, are often associated with transmission of *B. cereus* (Lake *et al.*, 2004). An expert elicitation carried out in New Zealand derived an estimate for the proportion of foodborne *B. cereus* intoxication due to rice of 61.4% (Cressey and Lake, 2005). The potential for high concentrations of *B. cereus* to accumulate in reconstituted dried potato flakes in New Zealand has also been demonstrated (Turner *et al.*, 2006).

A survey of 4,396 food samples in Germany detected emetic *B. cereus* in 73 samples (32 from food poisoning investigations and 41 from monitoring programmes) and provides evidence of this pathogen being more widespread among food types than once thought (Messelhäusser *et al.*, 2014). The foods tested were obtained from suspected foodborne illness incidents, suggestive of emetic *B. cereus* intoxication (n = 3,654), and from foods tested as part of monitoring programmes (n = 742). Foods containing emetic *B. cereus* included vegetables, fruit products, sauces, soups, salads, cheeses, herbal teas, dried mushrooms and meat products, as well as cereal products.

4.3 RISK MANAGEMENT QUESTIONS

The section provides a response to each of the four specific RMQs.

4.3.1 RMQ1: How does milk become contaminated with *B. cereus*, including consideration of feed, bedding and milking?

The most important sources of *B. cereus* in New Zealand dairy products are likely to be soil and faecal contamination of animal teats, and subsequent transfer of bacilli to raw milk during the milking process. *B. cereus* is likely to be ubiquitous in the farm environment, making

identification of specific risk factors difficult. Silage is a source of *B. cereus* and its consumption by dairy animals can lead to them excreting *B. cereus* in their faeces. Overseas studies show that the concentration of *B. cereus* is higher in soils compared to silage (no New Zealand data are available) and the relative importance of these two sources is not clear. The concentration of *B. cereus* increases over time in bedding used by housed dairy animals, but year-round pasturing is more common in New Zealand, so this risk factor is of less importance. However, it should be noted that there is increasing diversity of animal feeding and housing practices in New Zealand.

A study in Ireland examined a number of risk/protective factors related to maintenance of the milking equipment and milking methods, and the effect of these on the mean *B. cereus* concentration in milk (O'Connell *et al.*, 2013). Several factors did not achieve statistical significance in the multivariate model, but the results suggested these were important, e.g. a hot detergent wash and dry wiping teats before milking were protective, feeding silage or reusing cleaning solution was risky. Contamination from bacilli in residual biofilms in the milking equipment was suggested in a study in the Netherlands (Slaghuis *et al.*, 1997). It is clear that New Zealand studies are needed to better understand important risk factors in this country, and such studies need to consider non-bovine dairy herds, for which data are scarce.

4.3.2 RMQ2: What levels of *B. cereus* are present in milk and other dairy products and what are the major determinants of these levels?

The prevalence and concentration of *B. cereus* in raw milk and dairy products varies. A prevalence of 0.07% has been reported for raw milk in New Zealand. However, the basis for sample submission in this survey was not specified and this prevalence may not represent the actual national prevalence of *B.* cereus contamination of raw milk. Overseas surveys of raw milk have detected prevalences of 5 to 90%, and concentrations ranging up to 10^7 CFU/mL. Prevalence in other dairy products are also wide-ranging.

Available data indicate that raw milk is the major determinant of the occurrence of *B. cereus* in milk and dairy products, although contribution from biofilms or added ingredients, such as mould cultures, must also be considered. The ability of *B. cereus* to survive and grow in dairy foods appears to be strain-specific and is affected by temperature, competing microflora and changes in pH.

Determination of the prevalence of *B. cereus* contamination of raw and/or pasteurised milk in New Zealand would be a useful first step in defining the scope of the food safety issue. Such a study should consider the prevalence of psychrotrophic and mesophilic strains to better predict risk of growth.

4.3.3 RMQ3: What dairy products are most likely to be contaminated with *B. cereus* and what is the relative likelihood of the organism surviving in different product types?

B. cereus is most likely to be detected in pasteurised milk. However, it may be present in most, if not all types of dairy product, due to the ability of the organism to form resistant spores. Although psychrotrophic strains have been shown to grow at refrigeration temperatures, it is more likely that marked growth will occur under conditions of non-optimal refrigeration (≥7ºC) or conditions of temperature abuse.

Growth of *B. cereus* in raw milk is likely to be limited due to the presence of competitive microflora, but *B. cereus* can survive in this food. Growth can occur in pasteurised milk and cream due to survival of *B. cereus* spores and removal of competing microflora. Available data indicate that *B. cereus* can grow in reconstituted powdered milk and infant formula. Many dairy processes will inhibit *B. cereus*, due to decreases in water activity, increases in acidity or increases in the population of competing microflora. However, evidence suggests that the presence of *B. cereus* in any dairy product cannot be excluded. *B. cereus* strains are able to

form stable biofilms and, although there is a lack of conclusive evidence for New Zealand, there is potential for recontamination of dairy products within dairy processing plants.

4.3.4 RMQ4: What microbiological methods provide the most reliable information about presence and concentration of *B. cereus* in dairy products?

Methods used for the detection and quantification of *B. cereus* in New Zealand (see Appendix A2) are consistent with the most commonly propagated international standard methods. However, it appears that the degree of species confirmation of isolates is limited and, in most cases, a report of *B. cereus* detection should be viewed as presumptive.

New developments, including the use of chromogenic agar or species confirmation by PCR, offer promise, but are not currently in wide use.

While not generally applied in New Zealand, standard methods for detection and quantification of *B. cereus* can be made specific for *B. cereus* spores by inclusion of an initial rapid heating step, to about 80ºC (Maurice Wilson, ESR, personal communication). This pre-heating step is employed in a mesophilic spore count test, used in the New Zealand dairy industry. However, this test is not specific for *B. cereus* spores.

Testing for *Bacillus* spp. diarrhoeal toxins (immunoassay) is carried out in New Zealand but there is currently no New Zealand capability to test for the emetic toxin (cereulide).

4.4 THE BURDEN OF *B. CEREUS* INTOXICATION IN NEW ZEALAND

KEY FINDINGS

There is no specific information on the burden of disease due to *B. cereus* intoxications in New Zealand, although an estimate of the annual number of cases has been derived (mean = 10,883, 90th percentile credible interval 0-40,652) using a US model.

4.4.1 Burden of disease from dairy products contaminated with *B. cereus*

No information is available on the burden of disease from dairy products contaminated with *B. cereus* in New Zealand.

4.4.2 Burden of disease from all *B. cereus* intoxications

A model developed in the USA (Scallan *et al.*, 2011) was used to estimate the number of domestically-acquired foodborne cases of *B. cereus* intoxication in New Zealand (Cressey and Lake, 2011). Based on New Zealand outbreak data for the period 2001 to 2009, it was estimated that 10,883 (90th percentile credible interval 0-40,652) domestically-acquired foodborne cases of *B. cereus* intoxication would occur in New Zealand per annum. It was further estimated that 3 of these cases (90th percentile credible interval 0-18) would be hospitalised, with no consequent fatalities. The mean estimated number of domestically-acquired foodborne cases of *B. cereus* intoxication was less than for norovirus (218,701), *Campylobacter* spp. (190,092), *Clostridium perfringens* (64,989), non-typhoidal *Salmonella* (22,570), or *Yersinia enterocolitica* (29,715), but greater than the cases estimated for 18 other pathogens.

No estimate of the burden of disease due to *B. cereus* intoxication, either in terms of disability adjusted life years (DALYs) or the cost of illness, has been derived for New Zealand.

4.5 DATA GAPS

KEY FINDINGS

There is a general paucity of New Zealand-specific information on the incidence of illness due to *B. cereus* in New Zealand and on the prevalence and toxigenic potential of *B. cereus* throughout the dairy production chain.

There is a paucity of data on *B. cereus* in the dairy food chain in New Zealand. Major data gaps limiting understanding of the situation include:

- Environmental studies on potential sources of *B. cereus* on New Zealand dairy farms, such as soil, faeces, feed, bedding and biofilms in milking equipment, supported by sub-typing;
- Prevalence and concentrations of *B. cereus* in raw milk in New Zealand, including sub-typing, markers of toxigenic potential and the proportion that are present as spores;
- The ability of *B. cereus* spores to survive heat treatments used to produce UHT milk (and prevalence in New Zealand UHT milk);
- Prevalence and concentrations of *B. cereus* in pasteurised milk in New Zealand, including sub-typing, markers of toxigenic potential and the proportion that are present as spores;
- Comparison of *B. cereus* growth in a range of dairy products available in New Zealand, including measurement of any cereulide production (particularly focussing on growth and toxin production at temperatures up to 15°C);
- Information on the incidence of *B. cereus* intoxication in humans in New Zealand; and
- Data on the temperature/time profiles for dairy products at retail and in consumer homes.

Data on the prevalence and concentration of *B. cereus* in New Zealand dairy products are possibly available from routine commercial testing data currently undertaken by New Zealand businesses.

Further studies of dairy products in New Zealand should also include *B. weihenstephanensis* since this species may be as important as *B. cereus* as a dairy food contaminant owing to its ability to grow and produce emetic toxin at refrigeration temperatures, and grow at 37ºC (i.e. able to multiply in the human intestine). Since current routine laboratory methods used for isolating *B. cereus* from human cases of suspected bacillus intoxication do not distinguish between *B. cereus* and *B. weihenstephanensis*, a future (or retrospective) study of these isolates is also recommended to better understand the importance of *B. weihenstephanensis* infection in New Zealand.

5. AVAILABILITY OF CONTROL MEASURES

5.1 CURRENT CONTROL MEASURES

KEY FINDINGS

No microbiological standards for *B. cereus* in any food are included in the Australia New Zealand Food Standards Code. However, limits have been specified for raw milk for sale to consumers. A general Product Safety Limit (PSL; 1000 CFU/g) has also been specified for dairy processing in New Zealand, while a specific PSL for infant formula has been specified (100 CFU/g). General requirements for controlling microbiological hazards may also control *B. cereus*, and dairy food producers may identify *B. cereus* as a specific hazard and put controls in place.

The regulatory controls over dairy products depend on the activities being undertaken, the intended market (domestic/export) and the end product. A Risk Management Programme (RMP; under the *Animal Products Act 1999*) or Food Control Plan (FCP; under the *Food Act 2014*) is required for primary and secondary processors of dairy products.[19,20] Under the *Animal Products Act 1999*, all primary processors of animal products must operate under a registered RMP. While secondary processors may operate under a RMP or FCP, if exporting and operating under an FCP to a country for which an official assurance is required, the product must meet all requirements specified under the *Animal Products Act 1999.* Retailers (supermarkets, dairies) selling dairy products operate under a FCP or one of three National Programmes, depending on their activities.

These risk management tools require general microbiological hazards to be identified and controls put in place (e.g. cleaning, storage temperature requirements) that may help to control *Bacillus* spp. As part of preparing their RMP or FCP a primary or secondary producer of dairy foods may identify *B. cereus* as a specific hazard and put controls in place.

5.1.1 Regulatory limits

New regulations governing the sale of raw milk for human consumption in New Zealand require microbiological testing of the raw milk.[21] Technical specifications have been issued in the form of an Animal Products Notice (MPI, 2016a). Under the requirements of the Notice, testing for *B. cereus* may be requested. Raw milk is considered to be acceptable if the concentration of *B. cereus* is <100 CFU/mL. For milk containing *B. cereus* concentrations in the range 101 to 1000 CFU/mL remedial action is required and one demerit point is incurred. Milk containing more than 1000 CFU/mL of *B. cereus* is considered to be a major non-conformance, with accrual of two demerit points.

According to section 6.14 of the Notice:

[19] http://www.legislation.govt.nz/act/public/1999/0093/latest/DLM33502.html (accessed 10 May 2016)
[20] Existing businesses registered under the *Food Act* 1981 or Food Hygiene Regulations 1974 (before 29 February 2016) will shift to the *Food Act* 2014 by 31 March 2018 (http://mpi.govt.nz/food-safety/food-act-2014/transition-timetable/ (accessed 29 February 2015)
[21] http://www.legislation.govt.nz/regulation/public/2015/0309/latest/DLM6671301.html?src=qs (accessed 6 May 2016)

(1) If a conformance test result indicates that remedial action is required, the farm dairy operator must:
   a) immediately take steps to identify the cause of the elevated result and to remedy the situation; and
   b) implement per-lot testing for the parameter concerned until at least 3 consecutive lots over at least 3 consecutive days result in acceptable outcomes not requiring remedial action.

(2) If a farm dairy operator incurs 10 or more demerit points over a 3-month period, the supply of RCS (Regulated Control Scheme) milk to consumers must be suspended until:
   a) the total demerit points over the past 3 months is reduced to 5 or less, with a minimum of 3 samples tested each month; or
   b) the verifier reviews all investigations into the cause of the accumulation of demerit points and the corrective actions implemented and is satisfied that the situation has been rectified.

Until 2016, Standard 1.6.1 of the *Australia New Zealand Joint Food Standards Code* specified limits for *B. cereus* in powdered infant formula, but not in any other dairy products.[22] However, in March 2016 a variation to the Standard was approved removing the limit for *B. cereus* (FSANZ, 2016). This change aligns the Australia New Zealand Standard with international (Codex) standards (Codex Alimentarius Commission, 2008).

The guidance document *DPC1: Animal Products (Dairy): Approved Criteria for General Dairy Processing* (MAF, 2011b) specifies Product Safety Limits (PSLs) for pathogens, including *B. cereus*. A general PSL of 1000 CFU/g and a specific PSL for infant formula of 100 CFU/g are specified. The guidance document further notes that "limits described must not be exceeded at any time during the product's shelf life (assuming the product is handled and stored according to the manufacturer's guidelines)".

5.1.2 On farm measures

While it is not clear which sources of *B. cereus* contamination are most important (feed, soil, bedding, etc.), it is clear that minimising teat/udder contamination will reduce the concentration of *B. cereus* in bulk milk. Experimental studies suggest that attention to teat cleanliness can decrease *B. cereus* contamination of bulk tank milk by more than 90% (Magnusson *et al.*, 2006). The Operational Code *NZCP1: Design and operation of farm dairies* specifies that "Animals' teats must be clean and should be dry before applying the clusters" (MPI, 2015).

Maintaining the milking equipment is also important for reducing the opportunity for bacilli to form biofilms, or to colonise rubber seals or scratched/damaged metal parts. NZCP1 also contains information on equipment maintenance and cleaning, and additional information is available from DairyNZ.[23] MPI maintains a list of approved dairy maintenance compounds that includes sanitisers/disinfectants for cleaning milking equipment.[24] Using a model system to replicate farm milking equipment with different levels of cleaning difficulty, chlorine-free alkaline detergents were not as effective at removing *B. cereus* spores as alkaline detergents with chlorine, but the mechanical action of the liquid was an important component of spore

[22] https://www.legislation.gov.au/Details/F2016C00200 (accessed 16 March 2016)

[23] http://www.dairynz.co.nz/milking/the-milking-plant/ (accessed 10 May 2016)

[24] Available from http://www.foodsafety.govt.nz/industry/sectors/dairy/farms-dairies/ (accessed 10 May 2016)

removal (Sundberg *et al.*, 2011). Cleaning at 55ºC was more effective than lower temperatures (see Appendix C.1 for more detail).

Practices that improve farm hygiene or maintain the farm are likely to have a positive impact on *B. cereus* contamination of milk. Examples include replacing bedding frequently for any housed animals, maintaining good pasture coverage to minimise soil exposure, maintaining animal access ways to minimise mud and dust, feeding good quality silage.

5.1.3 Dairy processing

The ability of *B. cereus* to form heat-resistant spores and CIP-resistant biofilms means this organism will be difficult to completely remove from the dairy process chain.

The guidance document *DPC 3: Animal Products (Dairy): Approved Criteria for the Manufacturing of Dairy Material and Product* (NZFSA, 2010) contains requirements for pathogen management and manufacturing premises cleaning that will contribute to *B. cereus* control.

5.2 ADDITIONAL CONTROLS

Appendix C details studies of additional control measures identified during preparation of this Risk Profile. A number of controls with potential for application to milk at processing were investigated. Those that demonstrated efficacy at reducing the concentration of *B. cereus* were:

- A combination of pulsed electric fields and nisin;
- Pressure combined with one or more other hurdles (e.g. temperature, nisin, carbon dioxide);
- Thermosonication;
- Ultra-violet; and
- Clean-in-place (CIP) after a treatment to pre-germinate spores.

A recent review has summarised and evaluated physical, chemical and biological controls for removing biofilms in the dairy industry (Gopal *et al.*, 2015). Enhancing traditional chemical CIP regimes with enzymes or bacteriocins showed promise.

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APPENDIX A: HAZARD AND FOOD

A.1. *BACILLUS CEREUS* CLASSIFICATION

Members of the *B. cereus* group were traditionally assigned a species based on phenotypic characteristics and pathogenicity. However, the taxonomy of *B. cereus sensu lato* is currently under debate as molecular analyses are used to investigate the relatedness between strains. Genetic similarities within the group means that standardised techniques such as 16S rDNA analysis are insufficient to provide species resolution (Maughan and Van der Auwera, 2011). A recent paper that explored genetic relatedness and taxonomic classification within the *B. cereus* group found that groupings based on genetic relatedness were more closely aligned with pathogenicity and isolate source than the species designation (Varghese *et al.*, 2015).

There is growing consensus that the members of the *B. cereus* group should be considered as forming one single species from which different ecotypes and pathotypes emerge in a dynamic fashion (Maughan and Van der Auwera, 2011). However, maintaining the current taxonomy allows separation of isolates that are medically and economically important (Lechner *et al.*, 1998; Vilas-Boas *et al.*, 2007). The similarities are exemplified by the four *Bacillus* species of *cereus*, *thuringiensis*, *anthracis* and *weihenstephanensis*. The virulence genes for *B. anthracis*, the toxin-producing genes for *B. thuringiensis* and the cereulide-producing genes of *B. cereus* and *B. weihenstephanensis* are carried on plasmids, which can be gained or lost by bacterial cells (Van der Auwera *et al.*, 2007). A number of *B. cereus* and *B. thuringiensis* strains have been identified that can produce anthrax toxin and carry plasmids closely related to the anthrax virulence plasmids (Liu *et al.*, 2015; Maughan and Van der Auwera, 2011). Loss of the toxin-producing plasmid by a *B. thuringiensis* isolate makes this species difficult to distinguish from *B. cereus*. Furthermore, while the enterotoxin genes of *B. cereus* are chromosomal, the same genes can be carried and actively expressed by some strains of *B. thuringiensis*. Plasmid exchange between *B. thuringiensis* and *B. cereus* has been shown to occur at much higher frequencies in milk than in culture media or rice pudding, at 30°C (Van der Auwera et al., 2007).

A useful review of the similarities and differences (phenotypical and molecular) between *B. cereus*, *B. thuringiensis* and *B. anthracis* has been published (Vilas-Boas *et al.*, 2007). A recent genomic analysis of 224 *Bacillus* strains suggested as many as 20 further novel species could be defined on the basis of genetic clustering (Liu *et al.*, 2015).

In the context of this Risk Profile, this information becomes important when considering surveys for *B. cereus sensu stricto* in foods. Standard methods do not distinguish between the *Bacillus* species *cereus*, *thuringiensis* or *weihenstephanensis* (EFSA, 2005).

A.2. *B. CEREUS* TESTING AND TYPING

RMQ4: What microbiological methods provide the most reliable information about presence and concentration of *B. cereus* in dairy products?

A.2.1 *B. cereus* test methods

The *Bacillus* genus is large and ubiquitous and comprises of 268 species and 7 sub-species. Traditionally, the species have been divided into three groups based on spore and sporangium morphology (*B. cereus*, *B. subtilis* and *B. sphaericus* groups). While the groups are readily distinguishable, members within each group are difficult to differentiate from each other by cultural methods.

The methods recommended or specified by most international reference method publications for the detection, enumeration and differentiation of the *Bacillus cereus* group in food products

are procedurally similar (Anonymous, 2004; Bennett *et al.*, 2015; Douey *et al.*, 2011; Tallent *et al.*, 2012b). The standard method used by many analytical laboratories is based on the simple and traditional technique of surface plating onto selective and differential agar. Presumptive isolates are further identified or confirmed by microscopy, biochemical and phenotypic characterisation. Most probable number (MPN) methods are also described in reference methods and are recommended for routine surveillance of products in which small numbers of *B. cereus* are expected (Anonymous, 2006; Bennett *et al.*, 2015; Tallent *et al.*, 2012b).

Alternative methods for detection, enumeration and differentiation have been described in recent years. The new techniques include molecular assays including polymerase chain reaction (PCR), chemical analyses such as liquid chromatography–mass spectrometry (LC-MS), cellular fatty acid analysis and Fourier transform infrared spectroscopy (FTIR), and use of chromogenic media specific for the *Bacillus cereus* group. However, with the exception of chromogenic media, these newer approaches have not yet been widely evaluated (Bennett *et al.*, 2015).

Surface plating

The *B. cereus* group can be differentiated from other *Bacillus* species by their haemolytic activity, egg yolk reaction (turbidity that develops in egg yolk due to activity of lecithinase and/or other extracellular substances) and their inability to ferment mannitol.

Many early techniques for the isolation of the *B. cereus* group used blood agar-based media, with haemolysis and colony morphology used to identify suspect colonies. These media were generally not selective and only useful in detecting high numbers of organisms. Many formulations of plating media with enhanced selectivity and differential features have since been described. Most of these media incorporate polymyxin B to inhibit competing bacterial flora, and mannitol and egg-yolk to enhance differentiation.

Most reference methods prescribe an incubation temperature of 30°C for primary isolation media in order to enhance selectivity. The exception is PEMBA agar which requires incubation at 37°C (Holbrook and Anderson, 1980).

Spores of most *B. cereus* strains germinate readily on plating media and, unless a count specifically for spores is required, heat shock is unnecessary before enumeration (Bennett *et al.*, 2015).

KG Agar

Kim-Goepfert (KG) agar is one of the agars recommended in the American Public Health Association (APHA) standard method. It has comparable sensitivity and selectivity to the other commonly used plating agars and contains egg-yolk emulsion and polymyxin B supplements to provide differential and selective properties.

Although less frequently used than other agars, KG medium has a feature that may give an advantage over other media. KG is nutritionally poor and was formulated to promote free spore formation within a 24-hour period. This property, in conjunction with the other selective and differential factors, allows direct confirmation by microscopic examination of the *B. cereus* group and differentiation of *B. cereus* and *B. thuringiensis* by visualisation of endotoxin crystals.

MYP Agar

Mannitol egg-Yolk Polymyxin B (MYP) agar is widely used in Europe and the United States and is the plating medium prescribed in several international standards (Anonymous, 2004; 2006; Bennett *et al.*, 2015; Tallent *et al.*, 2012b). The *B. cereus* group is differentiated from most other *Bacillus* species by its inability to ferment mannitol and its characteristic production

of egg yolk factor. MYP has little selectivity (polymyxin B) and one of its disadvantages is difficulty in distinguishing mannitol-fermenting from non-fermenting colonies when high numbers of non-target organism are present. Sporulation of *B. cereus* on this medium is poor and re-streaking of suspect colonies to media that promote sporulation may be necessary for confirmation tests.

PEMBA

Polymyxin Egg-yolk Mannitol Bromothymol blue Agar (PEMBA) was developed for the isolation and enumeration of *B. cereus* in foods (Holbrook and Anderson, 1980). As with MYP, the medium is selective by addition of polymyxin B (final concentration 100IU/mL). A low peptone concentration and addition of sodium pyruvate improves egg yolk precipitation and enhances spore formation. While there have been some reports critical of its performance (Bennett *et al.*, 2015), a number of other studies comparing methods have found quantitative recovery on PEMBA is not significantly different from counts on KG and MYP agars but selectivity is generally superior (Holbrook and Anderson, 1980; Jenson and Moir, 2003; Schulten *et al.*, 2000). The study by Schulten *et al.* (2000) commented that the 37°C incubation temperature specified for PEMBA might not recover all psychrotrophic isolates, which may be present in foods such as milk products.

PEMBA is used widely in the United Kingdom, Canada and a few other countries (Bennett *et al.*, 2015; Douey *et al.*, 2011). It is included as an optional plating medium for confirmation tests in the ISO 21871:2006 MPN method.

BACARA®

Bacillus cereus Rapid Agar (BACARA®) is a chromogenic selective and differential agar that promotes the growth and identification of *B. cereus,* but inhibits the growth of background flora. Methods using BACARA® are more rapid than those using MYP as characteristic colonies growing on the agar after 24 hours incubation are enumerated as *B. cereus* group without further confirmation.

BACARA® has been validated by the French national organisation for standardisation (ANFOR Certificate No. AES 10/10 – 07/10, 2014). Their comparison with the ISO 7932:2005 standard, showed the BACARA® method to be specific and selective and have similar reproducibility and repeatability to the reference method. A study by Tallent *et al.* (2012a) also found BACARA® to have better inhibition of competitive flora than MYP enabling lower concentrations of the target organism to be detected in contaminated samples. The authors suggested that BACARA® could replace MYP as a more rapid method to recover *B. cereus* from contaminated products.

BACARA® chromogenic medium is included in the FDA Bacteriological Analytical Manual method as an optional agar for enumeration of *B. cereus* in foods. However, the FDA method requires further confirmation of typical colonies (Tallent *et al.*, 2012b).

BACARA® can only be purchased as pre-poured plates or prepared bulk agar base. It has a proprietary formulation and cannot be purchased in a dehydrated form.

Most Probable Number Technique

The most probable number (MPN) technique is an acceptable alternative to spread plate methods for enumerating *B. cereus*. It is especially suitable for foods expected to contain low concentrations of *B. cereus* (less than 1000 per gram). The method can also be adapted for presence/absence testing.

MPN methods specified in ISO 21871:2006, APHA and FDA BAM references are similar with trypticase soy-polymyxin broth used in a three-tube MPN series. Tubes showing growth after 48 hours at 30°C are plated onto selective and differential agar for identification of *B. cereus*.

The MYP spread plating method and three-tube MPN method were compared in a study by Harper *et al.* (2011). In general the MPN method recovered higher counts of *B. cereus* in spiked and un-spiked raw and pasteurised milk samples that contained *B cereus* concentrations between 1.2 and 3.4 log CFU/mL. The MPN method recovered slightly more of the target population in spiked samples (one as low as 0.4 log CFU/mL difference) and significantly larger counts in the un-spiked samples (greater than 3.0 log CFU/mL in a raw milk sample).

The authors of the comparative study comment that MPN methods in general can give highly variable population estimates and the difference in results between the MPN and spread plate methods is not unexpected. MPN methods which incorporate initial culture in broth media offer an enrichment and resuscitative environment more favourable to bacterial growth than direct plating on solid media.

The main disadvantages of using the MPN method for routine laboratory testing are its complexity compared with the MYP test and the time required to obtain a result (the plating method requires 2 days for a presumptive result compared with 5 days for the MPN test).

Confirmation

The detection and enumeration methods routinely used for the examination of food are not exclusively selective for the *B. cereus* group, nor do they differentiate between *Bacillus cereus* and the other closely related members of the group (*B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides* and *B. weihenstephanensis*).

The growth of typically reacting strains of the *B. cereus* group on plating media is usually so characteristic that other *Bacillus* species are unlikely to be mistaken for them and for many purposes a simple rapid microscopic examination of a stained culture preparation is sufficient for confirmation. However, more comprehensive confirmation tests may be required for isolates which are atypical or which must be more definitively identified for regulatory purposes (Bennett *et al.*, 2015).

Three groups of tests may be used to identify presumptive isolates, depending on the level of confirmation required. They are: a rapid confirmation test for isolates exhibiting unambiguous reactions on plating agar; biochemical tests to distinguish the *B. cereus* group from other *Bacillus* species; and tests to differentiate between the members of the *B. cereus* group.

Rapid confirmation test for the Bacillus cereus group

The rapid confirmation test described by Holbrook and Anderson (1980) is a staining procedure to detect the presence of lipid globules in vegetative cells and to determine vegetative cell, sporangium and spore morphology. Isolates grown on KG or PEMBA agar can be examined directly from primary culture plates but isolates from MYP agar generally require sub-culture in order to produce spores.

Stained slides are examined microscopically under oil immersion for large rod shaped cells with red stained vegetative cells, the presence of lipid globules (blue) within the cytoplasm and pale to mid-green central-to-subterminal spores that do not obviously swell the sporangium.

These properties are not unique to *B. cereus* but are a good indication that typical isolates from MYP or KG agar are members of the *B. cereus* group. *B. thuringiensis*, picked directly for confirmation from PEMBA plates, do not have lipid globules in the cytoplasm (Jenson and Moir, 2003).

Biochemical confirmation of the Bacillus cereus group

The following tests can be used to differentiate members of the *B. cereus* group from other *Bacillus* species.

Reaction on MYP agar

This test can be omitted if the primary inoculation medium was BACARA® or PEMBA or if test results were unambiguous on initial MYP plates and there was no interference from other microorganisms. The test should be included for isolates from KG agar to test for mannitol fermentation.

On MYP agar members of the *B. cereus* group usually produce lecithinase but do not ferment mannitol.

Anaerobic glucose fermentation

B. cereus and other members of the *B. cereus* group anaerobically produce acid from glucose.

Nitrate reduction

Nitrates are usually reduced to nitrite by members of the *B. cereus* group

VP test

Members of the *B. cereus* group produce acetylmethylcarbinol from glucose in the modified Voges-Proskauer test.

Tyrosine Decomposition

B. cereus and other culturally similar members of the *B cereus* group (except *B. anthracis*) readily decompose tyrosine (3 days at 35°C).

Lysozyme Resistance

Members of the *B. cereus* group are resistant to lysozyme (0.001%).

Tests to differentiate members of the B. cereus group

B. mycoides and *B. weihenstephanensis* are relatively straightforward to differentiate but *B. cereus* is not easily distinguished from other closely related members of the group. *B. cereus* and *B. thuringiensis*, the only two members of the group likely to occur naturally in food products, are thought to be nearly identical except for the production of the protein toxin crystals encoded by the *cry* genes.

Commercial rapid *Bacillus* identification kits are available for confirmation, but some give unreliable identification of *Bacillus* species and poor discrimination between the closely related species (Public Health England, 2015). MALDI-TOF MS has been found useful for identification of *B. anthracis* (provided the samples are prepared under standardised conditions), but less useful for distinguishing other closely related *Bacillus* species including members of the *B. cereus* group (Public Health England, 2015).

Tests described below are usually adequate for distinguishing typical strains of *B cereus* from other members of the group. However, separation of members of the group into distinct species is not always possible. Other members of the group may differ from *B. cereus* by only a single characteristic which may be lost with repeated culturing. Atypical strains of *B. cereus* may also give variable results which prevent correct identification by these methods (Bennett *et al.*, 2015).

Psychrotolerance

B. weihenstephanensis is the only member of the group that will grow at 6°C within 28 days, but not at 43°C (4 days). *B. cereus* and *B. thuringiensis* both grow at 43°C, but generally not below 10°C. *B. mycoides* may grow at temperatures as low as 7°C, but is readily distinguishable by its phenotypic characteristics.

B. weihenstephanensis can also be identified using rRNA or cold shock protein A (*cspA*) targeted polymerase chain reaction (PCR) (Bennett *et al.*, 2015; Lechner *et al.*, 1998).

Rhizoid growth

Rhizoid growth, characterised by root or hair-like structures which may extend several centimetres from the point of inoculation, is typical for *B. mycoides* species. Rough irregular "galaxy-shaped" colonies are often formed by *B. cereus* species and should not be confused with typical rhizoid growth.

Haemolysis

B. cereus is typically strongly β-haemolytic. *B. thuringiensis* and *B. mycoides* are also β-haemolytic, but usually weaker than *B. cereus* and often showing complete haemolysis only directly beneath the colony. *B. anthracis* is usually non-haemolytic after 24 hours growth.

Emetic strains of *B. cereus* are weakly or non-haemolytic so may be misdiagnosed as not being *B. cereus* on laboratory media (non-haemolytic isolates are discarded in prevalence studies, which may be one reason why the prevalence of emetic strains is low) (Ehling-Schulz *et al.*, 2015)

Motility

Most strains of *B. cereus* and *B. thuringiensis* are motile by peritrichous flagella. Most *B. anthracis* and *B. mycoides* strains are non-motile.

Protein toxin crystal production

B. thuringiensis is the only member of the *B. cereus* group to produce a protein toxin crystal (an endotoxin which has insecticidal action). Demonstration of carriage or expression of the toxin gene is the only way of distinguishing *B. cereus* and *B. thuringiensis*. Unfortunately, the available methods are complicated.

While molecular approaches have been described for distinguishing the two species (Manzano *et al.*, 2003; te Giffel *et al.*, 1997b), they are not in common use in routine laboratories. The method described in most reference methods is direct observation of the toxin crystals by phase-contrast microscopy or examination of stained preparations of sporing cultures (Bennett *et al.*, 2015; Douey *et al.*, 2011; Tallent *et al.*, 2012b). This is a subjective test and relies significantly on suitable sample preparation and analyst expertise in examining the slides. *B. thuringiensis* isolates are often misidentified as *B. cereus* (Douey *et al.,* 2011). Isolates for which protein toxin crystal production can be demonstrated are confidently identified as *B. thuringiensis*. However, isolates that are negative for protein toxin crystals cannot be completely excluded as *B. thuringiensis*.

Interpretation

Isolates which are; large Gram-positive rods with spores that do not swell the sporangium; produce lecithinase; do not ferment mannitol; grow and produce acid from glucose anaerobically; reduce nitrate to nitrite; are VP positive; decompose L-tyrosine; and grow in the presence of 0.001% lysozyme can be tentatively identified as *B. cereus*.

Within the limitations described for the tests, isolates which also meet the criteria listed in the section *Tests to differentiate members of the B. cereus group* may be definitively identified as *B. cereus*. Non-motile and weakly haemolytic *B. cereus* strains can be differentiated from *B. anthracis* by their resistance to penicillin and gamma bacteriophage. Non-crystalliferous variants of *B. thuringiensis* and non-rhizoid *B. mycoides* strains cannot be differentiated from *B cereus* by the tests described above.

In order to achieve a practical test procedure for routine testing some reference methods such as ISO 7932:2004 restrict confirmation of isolates with typical appearance on MYP agar to just the haemolysis test. However, the standard acknowledges that the confirmatory phase does not enable differentiation between *B. cereus* and other members of the group and requires enumeration results to be reported as presumptive *B. cereus*.

Further testing

Not all strains of *B. cereus* isolated from food are associated with illness and, at times, it may be necessary to determine if an isolate poses a potential or actual hazard to health. This can only be accomplished by determining enterotoxigenicity.

B. cereus enterotoxins, collectively referred to as diarrhoeal toxin, cause a diarrhoeal form of illness which most often follows ingestion of contaminated food, local bacterial growth and subsequent toxin production in the gut. Immunoassays to detect the toxin are commercially available and include the *Bacillus* diarrhoeal enterotoxin visual assay (BDEVIA) ELISA kit (3M TECRA) and the *Bacillus cereus* enterotoxin-reversed passive latex agglutination (BCET-RPLA) diarrhoeal toxin detection kit (Oxoid, UK).

The *B. cereus* heat stable emetic toxin (cereulide) causes a vomiting form of illness which is the result of ingestion of food containing pre-formed toxin. Assays for the detection of cereulide are complex. Bioassays (including tissue culture and a boar sperm motility assays), a real-time PCR method, and a quantitative method using high performance liquid chromatography (HPLC) linked to ion trap mass spectrometry (MS) have been described (Bennett *et al.*, 2015; Fricker *et al.*, 2007; Jenson and Moir, 2003). However, there are no commercial kits or simple assays currently available for routine laboratory use.

***B. cereus* testing in New Zealand**

In New Zealand, 23 laboratories are currently accredited for analysis of *B. cereus* in foods or specifically in dairy products.[25] Laboratories can be broadly classified as dairy industry, meat animal industry or analytical laboratory industry. While a range of method references are used to describe the individual laboratory methods, the methods fall into two groups; spread plate methods using MYP agar and MPN methods using trypticase soy-polymyxin broth in a three-tube MPN series, with identification on MYP agar.

While method references do not include information on confirmatory testing, the experience of ESR staff suggests that confirmation other than examination of haemolytic activity and/or rapid confirmation staining would be unusual in New Zealand (Maurice Wilson, ESR, personal communication).

A.2.2 Genotyping

Genotyping of *Bacillus* isolates has been performed using polymerase chain reaction (PCR) analysis of short interspersed repetitive DNA elements. A range of different approaches to typing *Bacillus* isolates have been used, including the so-called BOX primers (Banyko and Vyletelova, 2009) and the GTG_5 sequence (Coorevits *et al.*, 2008; De Jonghe *et al.*, 2008).

Genetic screening of *Bacillus* isolates for toxin genes is also carried out (Bartoszewicz *et al.*, 2008). Specifically, genes encoding for the three-component HBL, Nhe and a cytotoxin (CytK) (*hblA, nheA* and *cytK*) can be detected by PCR.

Investigation of *Bacillus* isolates (*n* = 105, made up of *B. anthracis* (11), *B. cereus* (38), *B. thuringiensis* (53), *B. weihenstephanensis* (2) and *B. mycoides* (1)) by multi-locus sequence

[25] http://www.ianz.govt.nz/directory/ (accessed 10 March 2016)

typing (MLST; seven loci) was carried out to try to shed light on the evolution of the *B. cereus* group (Priest *et al.*, 2004). The isolates produced 59 distinct sequence types (STs). Phylogenetic analysis was used to classify the isolates into eight lineages, within two major lines of descent (clades). Clade 1 contained all *B. anthracis* isolates, a number of *B. cereus* isolates including the emetic lineage, and two rare *B. thuringiensis* strains. Clade 2 contained most of the *B. thuringiensis* strains and some *B. cereus* strains. The other *Bacillus* species and three *B. cereus* strains formed a third heterogeneous clade. Investigations using next generation sequencing found a similar structure of two main clades in the *B. cereus* group (Zwick *et al.*, 2012).

A.3. GROWTH, SURVIVAL AND INACTIVATION

General information on the growth, survival and inactivation of *B. cereus* is presented in a microbiological datasheet (ESR, 2015). Given the recent nature of the datasheet, information is only included here if it is more recent or more specific to the topic of the current Risk Profile.

A.3.1 Growth

Temperature: The optimum has previously been described as 30-37ºC and more recently as 28-35°C (Vilas-Boas *et al.*, 2007). Studies on growth temperatures of 168 *B. cereus* isolates showed that the growth range varied from 5-37°C from some, to 15-45°C for others (Guinebretiere *et al.*, 2008). Optimum growth temperatures were not measured in this study, but these data suggest that the optimum temperature for growth depends on the isolate. Some strains can grow up to 55ºC while others can grow at temperatures as low as 4ºC. A species, *B. weihenstephanensis*, has been defined to describe isolates from the *B. cereus* group that are psychrotrophic (capable of growing at less than 7ºC), but not mesophilic (typically grows optimally between 20 and 45ºC) (Lechner *et al.*, 1998). Classification of strains has conventionally been by culture at low temperature (7ºC) for 5 to 10 days. A PCR method has been developed to classify strains as psychrotrophic or mesophilic, based on the presence of genes for major cold shock proteins (Francis *et al.*, 1998). The presence of the *cspA* gene was predictive of growth at 7ºC. Many isolates from dairy products are able to grow at low temperatures. Of 80 isolates from milk and milk products in the Netherlands, 58 were classified as mesophilic, 5 as psychrotrophic and 17 as intermediate (Wijnands *et al.*, 2006).

A.3.2 Inactivation and inhibition of growth (CCPs and Hurdles)

The antibacterial lipopeptides, surfactin and fengycin, were able to inactivate *B. cereus* spores by approximately two log in 21 hours at 20ºC (Huang *et al.*, 2008).

A.4. SOURCES

Human: *B. cereus* has been isolated from pooled breast milk samples, but not from associated environmental swabs (Decousser *et al.*, 2013). However, the source of the bacteria was not established.

Animal: Animals can carry *B. cereus* on parts of their body and the organism may occasionally cause mastitis in cows. *B. cereus* was established as the cause of gangrenous mastitis in six lactating dairy goats (Mavangira *et al.*, 2013). *B. cereus* appear to be able to exist in the guts of certain arthropod species, in a symbiotic relationship (Stenfors Arnesen *et al.*, 2008).

Environment: *B. cereus* is widely distributed in the environment and can be found in soil, dust, air, water and decaying matter. While it is generally believed that *B. cereus* exists in soil in the form of spores, germination, growth and sporulation in soil has been demonstrated (Vilain *et al.*, 2006). *B. cereus* also exhibited the ability to translocate through a soil microcosm using a multicellular growth modes, forming chains.

A.5. SPORE FORMATION

Spores represent a metabolically dormant form of the organism derived from vegetative cells. Spore formation is generally induced by restriction in availability in one or more nutrients, or else a slowing of growth of cells. It also appears that spore production comprises part of the population of a growing culture (Setlow and Johnson, 1997). Sporulation of *B. cereus* can also be stimulated by the presence of magnesium or manganese ions (Christiansson, 2011).

Spores are more resistant to environmental challenges and control measures than vegetative cells. Such challenges include freezing, drying, pressure, radiation, ultraviolet light, chemicals, and heat. Germination of spores can be initiated by low pH, a number of chemicals (especially nutrients), and most commonly, sub-lethal heat.

As *B. cereus* is widespread in nature and survives extended storage in dried food products, it is not practical to eliminate low numbers of spores from foods. Instead, controls are directed at preventing spore germination and cellular multiplication, e.g. rapidly and efficiently cooling cooked foods that are not to be eaten immediately, and thoroughly reheating foods before serving (Setlow and Johnson, 1997).

A panel of strains of spore-forming organisms from the dairy processing environment, raw materials and processed foods (n = 467) was compiled (Lücking *et al.*, 2013). The predominant species were *B. cereus sensu lato* (n = 90) and *B. licheniformis* (n = 98). Spore formation was induced in all strains, followed by exposure to 100ºC in buffer for 20 minutes. Growth after heat treatment was measured for a total of 126 strains, including two *B. cereus* strains. The greatest number of heat-resistant spores were from *B. subtilis*. Both of the heat-resistant *B. cereus* strains also germinated after cold storage and were capable of vegetative growth at 10ºC. One of the *B. cereus* strains was the only heat-resistant strain to exhibit cytotoxic activity.

A.6. *B. CEREUS* TOXINS

B. cereus may produce two distinct types of toxin, responsible for the diarrhoeal and emetic syndromes. The majority of *B. cereus* strains appear to be capable of producing either diarrhoeal or emetic toxin, while a significant number (36% in one report) of isolates produce both (Beattie and Williams, 1999; Rusul and Yaacob, 1995)

Foods involved in diarrhoeal outbreaks are quite varied, ranging from vegetables and salads to meat dishes and casseroles. In contrast, emetic type outbreaks are usually associated with rice in some form, or else other starchy foods such as macaroni and cheese, or vanilla slices (Johnson, 1984).

A.6.1 Diarrhoeal toxin

The diarrhoeal type of food poisoning is caused by enterotoxins produced during vegetative growth of *B. cereus* in the small intestine (Granum, 1997). The toxin can be pre-formed in foods, such as bean curd (Wong, 1997), but it is unlikely this source of toxin would cause illness. One reason is that the enterotoxin is degraded in the gastrointestinal tract. The other reason is that the number of cells required to produce significant amounts of preformed toxin in food is much higher than the actual number of cells required to cause illness, and such high numbers of cells would make the food unacceptable for consumption. This suggests that there may be an 'optimum' level of food contamination – a sufficiently high concentration of *B. cereus* cells to result in infection of the small intestine and subsequent intoxication, but not so high that the food is unacceptable for consumption. Counts in foods associated with diarrhoeal illness have varied from 200 to 10^9 organisms/g (Granum and Lund, 1997).

B. cereus strains may produce a range of cytotoxic compounds, but the diarrhoeal syndrome is generally considered to be caused by one or more of three cytotoxins; two different three-component protein enterotoxins and a single protein cytotoxin (Stenfors Arnesen *et al.*, 2008):

- A three-component haemolysin (HBL) consisting of three proteins: B, L_1 and L_2 has been characterised. This has enterotoxin activity and has been suggested to be a primary virulence factor (Granum and Lund, 1997), although food poisoning has been caused by *B. cereus* strains that do not express HBL. The BCET-RPLA assay (Oxoid) is a reverse passive latex agglutination assay for L_2.

- A non-haemolytic three-component enterotoxin (Nhe) has also been characterised as comprising proteins of NheA and NheB (each approximately 40 kDa) and NheC (36 kDa), with a high degree of sequence homology to the subunits of the HBL enterotoxin (Heilkenbrinker *et al.*, 2013; Stenfors Arnesen *et al.*, 2008). Nhe is a pore-forming toxin and requires a specific binding order of the three subunits to exert maximum cytotoxicity (Lindbäck *et al.*, 2010). Binding to cell membranes appears to be initiated by NheC, but maximum cytotoxicity requires the presence of NheB, either applied at the same time as NheC or subsequent to NheC. NheA binding is the final step and triggers toxicity, by an unknown mechanism (Heilkenbrinker *et al.*, 2013). An immunoassay (TECRA-BDE) has been produced for the NheA component.

- Cytotoxin K (cytK) is a single protein toxin belonging to the family of β-barrel pore-forming toxins (Stenfors Arnesen *et al.*, 2008). CytK was originally isolated from a *B. cereus* strain that was responsible for a severe foodborne outbreak of diarrhoeal disease in a French nursing home in 1998 (Lund *et al.*, 2000). Several people involved in the outbreak developed bloody diarrhoea and three elderly people died. Based on the necrotic activity of CytK and the apparent lack of both Nhe and HBL in the outbreak-associated strain, CytK was initially implicated as the toxin responsible for the severe symptoms and uncharacteristic bloody diarrhoea presenting in this outbreak. However, a variant of *nhe* was later detected in this strain, along with production of NheB (Fagerlund *et al.*, 2007), and it has been proposed that this strain be reclassified as a novel species *B. cytotoxicus* (Guinebretière *et al.*, 2013). There is currently no commercially available kit for detection of the CytK toxin (Stenfors Arnesen *et al.*, 2008).

It should further be noted that the assays for HBL and Nhe do not indicate the presence of a biologically active toxin, as only one of the three toxin components is detected. PCR primers have been published for all relevant enterotoxin genes (Christiansson, 2011).

Enterotoxin activity is labile. It can be inactivated by heat at 56°C for 5 minutes, is unstable at pH beyond the range 4-11 (i.e. will be degraded by stomach acidity), and is sensitive to proteolytic enzymes (Jenson and Moir, 2003).

A large proportion of strains from the species *B. cereus, B. thuringiensis* and *B. weihenstephanensis* have the genes for at least one of these three diarrhoeal toxins (EFSA, 2005). Genes for the NheB subunit were detected in 24 of 50 (48%) *B. weihenstephanensis* strains (Stenfors *et al.*, 2002), while *nhe* genes were detected in all of 41 strains of *B. thuringiensis* (Gaviria Rivera *et al*., 2000). This suggests that the ability to cause diarrhoeal foodborne poisoning is distributed over *B. cereus* and some related species, in particular *B. thuringiensis*. A strain of *B. thuringiensis* was implicated in an outbreak of foodborne diarrhoea in a chronic care facility (Jackson *et al.*, 1995). Spice was the suspected transmission vehicle.

A.6.2 Emetic toxin (cereulide)

The emetic toxin from *B. cereus* was characterised in 1995 as a dodecadepsipeptide named cereulide (Agata *et al.*, 1995). This circular molecule consists of three repeats of a four amino acid sequence, and is closely related to the potassium ionophore valinomycin.

Cereulide is enzymatically synthesised rather than being a gene product, i.e. the genetic locus *ces* encodes the cereulide synthetases that, in turn, produce the toxin (Ehling-Schulz *et al.*, 2015). The *ces* gene cluster is located on a 270 kb megaplasmid named pCER270, which shows similarities to the toxin plasmid pXO1 of *B. anthracis*.

The mechanisms that regulate *ces* gene expression and cereulide production are poorly understood. A recent review by Ehling-Schulz *et al.* (2015) describes some of the intrinsic and extrinsic factors governing cereulide synthesis. Temperature and nutrient availability appear to be key factors.

The role of oxygen supply in toxin production is contentious, with some studies, in culture media (Haggblom *et al.*, 2002; Jaaskelainen *et al.*, 2004) or milk (Agata *et al.*, 2002), suggesting adequate oxygen supply is essential for toxin production, while others, in dairy products (milk or reconstituted infant formula), suggest that aeration has a negative impact on toxin production (Rajkovic *et al.*, 2006; Shaheen *et al.*, 2006). All of these studies involved incubation at temperatures in the range 21 to 30°C.

Rajkovic (2006) compared emetic toxin production in culture medium, under static and shaken conditions, at three temperatures (12, 22 and 30°C). No toxin was produced in shaken cultures, at any temperature, while toxin production was observed in static cultures at 22 and 30°C, but not 12°C. The same study also determined emetic toxin production in culture at 28°C under atmospheres with differing oxygen contents. Toxin production was greater at 10.6 and 4.5% oxygen than under fully aerobic conditions. However, no toxin was produced under atmospheres containing 1.6 or 0.7% oxygen. Christiansson *et al.* (1989) demonstrated cytotoxicity associated with a dairy-derived *B. cereus* strain inoculated into milk at 15°C under shaking, but not under static conditions. It should be noted that this study determined cytotoxicity against a panel of cell lines, but did not determine the identity of the toxic species present. Toxicity developed during the late stationary growth phase (68 to 92 hours). A dairy-derived strain acclimatised to low temperature growth was inoculated into milk and incubated at 8°C. Cytotoxicity was observed between 72 to 96 hours in aerated samples, but not in static samples.

While it seems plausible that the stress created by sub-atmospheric oxygen concentrations may stimulate toxin production and that very low oxygen concentrations may compromise the organism's ability to carry out the necessary metabolic processes for toxin production, the myriad of nutritional and physicochemical factors interacting at the food-bacteria interface suggests that no general conclusions can be drawn on optimum levels of oxygen for emetic toxin production in foods or the impact of different temperature-oxygen regimes on toxin production.

Emetic toxin production in culture media (tryptic soy agar, skim milk media, oatmeal agar) appears to be maximal in the range 12 to 28°C (Apetroaie-Constantin *et al.*, 2008; Finlay *et al.*, 2000; Haggblom *et al.*, 2002), but the toxin can be produced at temperatures as low as 8°C (Haggblom *et al.*, 2002; Thorsen *et al.*, 2006). The presence of some amino acids (valine, threonine, leucine) appears to be essential for emetic toxin production, while high concentrations of some amino acids (leucine, isoleucine and glutamic acid), as may be present in proteinaceous foods, may inhibit cereulide production (Agata *et al.*, 1999). Using an HPLC-MS detection method and three *B. cereus* isolates cultured in laboratory media (tryptic soy agar or trypticase soy broth), it was found that cereulide production commenced at the end of logarithmic growth, but was independent of sporulation (Haggblom *et al.*, 2002). Under these laboratory conditions, cereulide production was very low at ≤8˚C (0.016 to 0.071 μg/mL of broth after 7 days incubation) and at 40˚C (<0.2 μg/g bacteria, on a wet weight basis). By comparison, growth in broth at 21° for 7 days resulted in accumulation of 13.2 μg cereulide/mL of broth.

There is considerable variation in the literature concerning minimum growth temperatures and minimum toxin production temperatures for *B. cereus* strains. While some studies have reported that emetic toxin-producing strains of *B. cereus* are unable to grow below 10°C (Carlin *et al.*, 2006) or even 12°C (Finlay *et al.*, 2000), strains of *B. weihenstephanensis* have been reported to grow and produce emetic toxin at 8°C, although toxin production was limited (0.1 μg/g of biomass, on a wet weight basis, compared to 530-606 μg/g at 25°C, for the same

strains) (Thorsen *et al.*, 2006; Thorsen *et al.*, 2009). Low toxin production at this temperature (8°C) is consistent with the findings of Haggblom *et al*. (2002), although results from the studies are not directly comparable as toxin production is expressed on different bases. It should be noted that most normal testing for *B. cereus* would not distinguish *B. cereus sensu stricto* from *B. weihenstephanensis.*

In contrast to strains of *B. cereus* that carry the enterotoxin genes, carriage of the cereulide-producing genes appears to be restricted to highly clonal lineages (Ehling-Schulz *et al.*, 2015). Emetic strains are rarely isolated from environmental samples such as soil and animal faeces compared with foods. For example, based on the colourimetric method of Finlay *et al*. (1999), 3/177 *B. cereus* isolates recovered from samples of soil, faeces and raw and processed vegetables at 30°C were able to produce emetic toxin (Altayar and Sutherland, 2006). In addition, 1/148 *B. cereus* isolated at 7ºC (psychrotrophic) was also positive for emetic toxin production. In contrast, when *B. cereus* isolated from milk were inoculated into milk and incubated at 30°C for 7 hours (logarithmic phase) cytotoxic activity was not detected, but after 24 hours (stationary growth phase) cytotoxic activity was detected in 50-73% of isolates, depending on the cell line used (Christiansson *et al.*, 1989). As noted above, this study determined cytotoxicity against a panel of cell lines, but did not determine the identity of the toxic species present. However, in a study using assays specific for emetic toxin, only 0.05% (2 of 3401 positive by boar sperm motility microassay) of *B. cereus* isolates from the dairy processing environment were found to produce emetic toxin (Svensson *et al.*, 2006).

Cereulide is resistant to heat, proteolysis and pH, but is not antigenic (Granum and Lund, 1997).[26] The isolation and characterisation of this toxin was achieved after the discovery that the toxin causes vacuolation of Hep-2 cells, and this property formed the basis of an assay to detect the toxin present in foods or produced from isolates (Agata *et al.*, 2002). Real-time PCR assays specific to elements of the cereulide synthetase (*ces*) genes have also been developed (Fricker *et al.*, 2007). These assays have been shown to successfully distinguish potentially emetic *B. cereus* strains from non-emetic strains. Liquid chromatography-tandem mass spectrometry (LC-MS-MS) techniques have been developed for quantitative determination of cereulide (Haggblom *et al.*, 2002; Thorsen *et al.*, 2009).

A.6.3 Toxin production by other species of *Bacillus*

There is a popular misconception that *B. cereus* is the only species within the genus that is of public health concern, in terms of foodborne disease. However, a few other species are capable of causing foodborne disease, and prominent among these is *B. subtilis* (Jenson and Moir, 2003). In fact, Nichols *et al.* (1999) identified *B. subtilis* more frequently than *B. cereus* (41% vs 23%) in cooked rice samples containing *Bacillus* spp. at $\geq 10^3$ CFU/g. A similar finding (20 *B. subtilis,* 4 *B. cereus* isolates) was reported by Little *et al.* (2002) who also isolated *B. licheniformis* (3 isolates) and *B. pumilus* (1 isolate) from 28 samples of heavily contaminated cooked rice samples. *B. thuringiensis* has been reported as causing food poisoning when fed to volunteers (Granum and Lund, 1997). This species has also been implicated in one outbreak investigation (see Section A.1.5), although the isolation of norovirus from two of the outbreak cases makes the assignment of a causative pathogen questionable (Jackson *et al.*, 1995). Isolates of *B. circulans*, *B. laterosporus/cereus*, *B. lentus*, *B. licheniformis*, *B. mycoides*, *B. subtilis*, and *B. thuringiensis* have been shown to produce toxins (Beattie and Williams, 1999).

There is slightly equivocal evidence as to whether the common insecticidal forms of *B. thuringiensis*, *B. thuringiensis* var. kurstaki and *B. thuringiensis* var. israelensis, are able to produce enterotoxins. While recent studies have detected the full complement of enterotoxin

[26] Antigenic substances elicit an immune response and formation of antibodies when introduced into the body.

genes and reported secretion of enterotoxin (Kyei-Poku *et al.*, 2007), assessments of human populations in areas where the insecticides have been sprayed report no excess prevalence of gastrointestinal symptoms (Siegel, 2001).

A range of *Bacillus* species, including *B. subtilis, B, pumilus, B. circulans* and *B. licheniformis* have been detected in raw and pasteurised milk and other dairy products (Aouadhi *et al.*, 2014; Banyko and Vyletelova, 2009; Coorevits *et al.*, 2008; De Jonghe *et al.*, 2010; Huck *et al.*, 2007; Iurlina *et al.*, 2006).

A.7. BIOFILM FORMATION

Milk was shown to instigate 'bundle' formation in a range of *Bacillus* species, including *B. cereus* (Pasvolsky *et al.*, 2014). Bundles are biofilm-related aggregate structures. It was further determined that butyric acid, resulting from lipolysis of milk fat, was the component of milk that triggered biofilm formation. The authors suggested that butyric acid may act as a stress signal. A concentration of 0.1% butyric acid is toxic to *Bacillus* spp. cells; lower concentrations may act as a 'warning' of impending stress, initiating formation of biofilms, with greater resistance to the toxicity of butyric acid.

In vitro studies with a strain of *B. cereus,* isolated from a dairy processing chill tank, found that it could form biofilms at refrigeration temperatures (4ºC) and bacterial concentrations on stainless steel surfaces could reach 10^6 CFU/cm^2 (Kumari and Sarkar, 2014).

Raw milk samples (*n* = 50) were analysed for *Bacillus* spp. (Uraz and Gündüz, 2013). *B. subtilis* (22), *B. licheniformis* (11) and *B. cereus* (2) were isolated and tested for biofilm formation at 24 and 48 hours. All but three isolates had formed biofilms after 24 hours and all had formed biofilms after 48 hours. The *B. cereus* strains formed biofilms at both time points. It was not stated what substrate biofilm formation was tested on.

Analysis of biofilms in five dairy plants in Northwestern Algeria found that 21% of bacterial isolates were of the *B. cereus* group (Malek *et al.*, 2012). CIP had little impact on the total bacterial counts in plant pipelines. Treatment of experimental *B. cereus* group biofilms with quaternary ammonium compounds at concentrations up to 150 parts per million for 15 minutes showed that the biofilms were significantly resistant to inactivation.

A.8. *B. CEREUS* IN DAIRY PRODUCTS OVERSEAS

A.8.1 Environmental studies

Australia

A study of spore-forming bacteria on farms in Victoria found significant and consistent correlations between spore counts in raw bulk milk and spore concentrations on teat skin (Cook and Sandeman, 2000). Significant correlations were found between thermophilic and anaerobic, but not mesophilic, spore counts in faeces and spore counts in raw milk. The authors of the study interpreted this as indicative that faecal contamination of teat skin was more important than contamination with soil or bedding material. It should be noted that morphological and biochemical characterisation of isolates from raw milk suggested that *B. cereus* was a minor component of the bacterial population.

B. cereus sensu lato was determined in environmental samples from dairy farms (3 bovine, 1 ovine, 3 caprine) in Victoria, Australia (McAuley *et al.*, 2014). *B. cereus* was detected in most soil samples (93%, *n* = 14) and was present in soil from all farms. *B. cereus* was detected in 63% of faecal samples (*n* = 16), 14% of feed samples (*n* = 14) and 33% of milk filter samples (*n* = 9), but was not detected in any of 15 raw milk samples. The maximum concentration in any sample was 2.7 x 10^5 CFU/g, but it was not stated what type of sample this count was found in.

B. cereus sensu lato strains were isolated from environmental samples from the study described in the previous paragraph (Drean *et al.*, 2015). A total of 50 isolates were recovered from 27 samples of soil, faeces, feed (grain), raw milk and milk filters. All isolates had haemolytic activity and grew at 10ºC, indicating that none of the isolates were *B. anthracis* or *B. cytotoxicus*. The majority of the isolates (*n* = 40) were *B. cereus sensu stricto.* Pulsed-field gel electrophoresis (PFGE) typing revealed a high level of genetic diversity, with only two PFGE types occurring in more than one sample. No PFGE type was found on more than one farm.

Brazil

Sources of *B. cereus* contamination were evaluated in a ricotta processing plant in Brazil (da Silva Fernandes *et al.*, 2014). *B. cereus* was isolated from raw materials, environmental samples (mould, press, storage box, packaging table and whey sewage drain) and product. From a total of 42 isolates, 16 carried *hbl* toxin genes, while 39 carried *nhe* toxin genes. All isolates were resistant to ampicillin, penicillin, and trimethoprim; 9.5% were resistant to erythromycin. While the presence of *B. cereus* in raw milk and finished ricotta was variable, *B. cereus* was consistently present in the mould culture, at concentrations up to 7.23 log CFU/unit and it was suggested that this may the source of contamination of the processed ricotta. However, no typing was carried out to assess the relatedness of the *B. cereus* isolates. The highest concentration of *B. cereus* determined in packaged ricotta was 3.85 log CFU/g.

Post-pasteurisation surfaces and pasteurised milk were analysed in a dairy processing facility, for the presence and concentrations of *B. cereus* (Salustiano *et al.*, 2009). The mean concentration of *B. cereus* on post-pasteurisation surfaces was 0.646 log CFU/cm^2, with a maximum of 1.98 long CFU/cm^2. Ribotyping showed that the most common *B. cereus* type in pasteurised milk was also the type most commonly isolated from post-pasteurisation processing surfaces. It is uncertain whether surfaces were contaminated by milk or vice versa. However, contamination of post-pasteurisation surfaces with *B. cereus* indicates that there is potential for post-pasteurisation contamination of milk from this source.

Canada

Milk and environmental samples were taken from various points in dairy processing plants (Lin, 1997). Isolates were taken from positive *B. cereus* cultures and typed using fatty acid methyl ester (FAME) profiling. Based on FAME profile similarity, it was concluded that *B. cereus* in raw milk was the major source of *B. cereus* contamination of pasteurised milk and final milk products. There were low levels of *B. cereus* recovered from environmental swabs and it was concluded that dairy plant environmental contamination was a minor contributor to *B. cereus* contamination of final products.

Côte d'Ivoire

Samples of raw milk (from teats, bulk farm milk, sellers' bulk milk) and the farm environment (udder skin, household tap water, air, milkers' hands and farm milk storage containers) were analysed for the presence and concentrations of *B. cereus* (Yobouet *et al.*, 2014). The study noted generally poor hygiene standards on the farms included in the study. The prevalence of milk contamination increased from 27% (teat) to 41% (sellers) through the production chain. All environmental samples showed appreciable contamination, with the highest prevalence (65%) on udder skin and the lowest prevalence (33%) in farm storage containers. While no typing was carried out to track contamination, the authors assert that the major sources of contamination were udder skin, farm water and the farm environment (air). Given the high prevalence of contamination in teat milk, udder contamination is probably the major contributor to milk contamination.

Czech Republic

Isolates of *B. cereus* and *B. licheniformis* (*n* = 30), from raw and pasteurised milk and yoghurt collected from dairy farms and processing plants in the Czech Republic, were genotyped using BOX-PCR (Banyko and Vyletelova, 2009). Isolates from raw and pasteurised milk, for the same batch, showed a high level of concurrence with respect to strain type, suggesting that contamination of pasteurised milk originated in the raw source milk. Strain types in yoghurt were usually different to types in the raw or pasteurised milk from the same batch, suggesting contamination of yoghurt from the processing environment.

A three-year study on dairy farms in the Czech Republic examined samples (*n* = 70 of each sample type) of animal feed, animal faeces and bulk raw milk (Hanus *et al.*, 2004). *B. cereus* was detected in all sample types, but at generally low concentrations. Geometric mean concentrations were 7.1 (range 0-600) CFU/mL in feed, 14.1 (range 0-2000) CFU/mL in faeces and 3.4 (0-170) CFU/mL in bulk milk. Concentrations of *B. cereus* were significantly higher in faeces in winter and significantly higher in milk in summer. No significant seasonal differences were seen in the *B. cereus* content of animal feed.

In a study carried out in 2005 and 2006, product and environmental samples were taken from dairy farms and a dairy processing facility (Schlegelova *et al.*, 2010). *B. cereus* was not detected in raw milk at the farm or at dairy plant receipt, but was detected in 15% of pasteurised milk samples and 9% of surface swab samples from within the dairy processing plant. This may indicate that the milk was contaminated within the plant or may indicate that *B. cereus* numbers were able to increase post-pasteurisation. The majority of isolates were enterotoxigenic.

Estonia

Contact and non-contact surfaces in three Estonian dairy processing facilities were analysed for the presence of microbial contamination, including *B. cereus* contamination (Salo *et al.*, 2006). *B. cereus* was rarely detected, being isolated from one piece of equipment in one facility and from a sample of powdered raw ingredients in a different facility.

Iran

Samples were taken from various points in an ultra-filtered (UF) milk feta production facility (Moradi-Khatoonabadi *et al.*, 2014). It was concluded that raw milk was the main source of *B. cereus* contamination, with contamination of storage tanks and UF filters also contributing to contamination in the final product. Mean contamination levels in raw milk were in the range 1.1 to 1.9 log CFU/mL, while at the filling machine (last sampling point) mean concentrations were in the range 1.9 to 2.1 log CFU/mL.

Ireland

Farm management factors contributing to *B. cereus* contamination of bulk tank milk were examined across 63 dairy farms (O'Connell *et al.*, 2013). Factors were examined by regression analysis and analysis of variance. In the final multivariate model, *B. cereus* counts in bulk tank milk were four times higher if the cows were housed indoors (mean 209.9 CFU/mL) compared to left on pasture (mean 50.1 CFU/mL). *B. cereus* counts were lower in bulk tank milk if fresh grass allocation was carried out every 12 hours (mean 61.6 CFU/mL) than if there were lower periods between fresh grass allocation (mean 166 CFU/mL). Less explicable was that water testing for bacteriology in the last three years was associated with twice the level of *B. cereus* contamination than if no water testing was carried out. Other factors that were associated with lower *B. cereus* counts in bulk tank milk in the univariate analysis (p <0.1, but >0.05) were; higher wash solution starting temperature, use of plastic rather than cloth milk filters, not feeding silage in the last 14 days, not reusing wash solution, and teats dry-wiped prior to unit application.

Netherlands

Farms with cows pasture grazing and farms with cows housed in barns were compared for *B. cereus* contamination of milk and various environmental samples (Slaghuis *et al.*, 1997). The highest spore counts were seen in soil, feed material, used bedding and faeces. Raw milk from cows on pasture was more likely to be contaminated with *B. cereus* spores (23%) compared to bulk tank milk from cows kept in stables (3%).

A year-long survey on 24 Dutch dairy farms sought to determine critical factors for contamination of farm tank milk with *B. cereus* spores (Vissers *et al.*, 2007b). The mean spore concentrations were determined in farm tank milk (1.2 $\log_{10}$ spores/L), farm soil (4.9 $\log_{10}$ spores/g), animal faeces (2.2 $\log_{10}$ spores/g), bedding material (2.8 $\log_{10}$ spores/g) and silage feed (2.4 $\log_{10}$ spores/g). The spore concentration of farm tank milk increased during the period July to September, with similar increases in the concentrations in faeces, bedding and silage. The authors concluded that contamination of farm tank milk was predominantly transmitted from feed via faeces to milk.

Sweden

Farm-level factors contributing to *B. cereus* spore contamination of milk were investigated in a group of eight cows (Christiansson *et al.*, 1999). The spore content of farm tank milk varied in the range <10 to 880 spores/L. The spore content of milk was strongly associated with the degree of soil contamination of teats. High soil water content, low water evaporation rate and dirty access alleys were also correlated with high spore counts. Milking equipment did not contribute significantly to spore content, while spore contents in air and feed were too low to be major contributors. Genotyping of isolates by RAPD-PCR found the same types in soil and milk samples. Experiments to examine the impact of teat cleaning on spore contamination of milk were hampered by low spore counts, but when sufficient spore counts were present a significant reduction in the spore count of milk was seen following teat cleaning.

The presence of *B. cereus* in free stall bedding material for dairy cows was examined (Magnusson *et al.*, 2007b). The bedding material (sawdust) did not contain detectable *B. cereus*, in either the vegetative or spore form, before placement in stalls. *B. cereus* (spores and vegetative cells) were detected in all parts of the stall and at all depths measured (surface, 10 cm, 20 cm and 30 cm) from the first day after new bedding was applied. During two 14-day monitoring periods the average concentrations of *B. cereus* spores and total *B. cereus* in bedding material was 4.1 $\log_{10}$/g and 5.5 $\log_{10}$/g, respectively. Highest counts of spores and total organisms were found at the back of the stall and at 20 cm depth. *B. cereus* concentrations increased throughout the use period of the bedding. Some alternative bedding materials (peat) inhibited growth of *B. cereus*, while growth was also influenced by pH and the presence of faecal material, as a nutrient source.

The occurrence of *B. cereus* spores in fresh and used bedding material, air samples, feed, faeces, and the rinse water from milking equipment was compared with the spore level in bulk tank milk on two dairy farms, one of which had two different housing systems (Magnusson *et al.*, 2007a). A less extensive study was carried out on an additional five farms. Spores counts were high in rinse water from milking equipment (maximum 322 spores/L) and in bedding (maximum 87,000 spores/g). A significant positive correlation was found between the spore content of used bedding and the spore count in bulk tank milk. Genetic fingerprinting (random amplified polymorphic DNA PCR) indicated that used bedding material was the most likely source of contamination in milk. A further experiment, in which cows were fed *B. cereus* spores, demonstrated the potential for contaminated feed to contribute to milk contamination, probably through contaminated faeces resulting in teat contamination.

United Kingdom

A study of *Bacillus* spp. on-farm found that *B. cereus* (along with *B. licheniformis*) was the most frequently isolated spore-forming bacteria from raw milk sampled from farms over a period of 21 months (Crielly *et al.*, 1994). *B. cereus* was consistently detected in pelleted feed (up to 10^4 CFU/g), occasionally detected in silage (up to 10^5 CFU/g) and bedding (up to 10^7 CFU/g), and was occasionally present in large numbers in grass and soil sampled during summer months. *B. cereus* was detected on teat surfaces and milking clusters, but other *Bacillus* spp. dominated these samples.

A.8.2 *B. cereus* in dairy products

Table 3 summarises data from the scientific literature where dairy products were tested for *B. cereus*. These data show *B. cereus* to be a common contaminant in a range of dairy products sampled in both developing and developed countries, occasionally at high concentrations. It should be noted that standard methods do not distinguish between the *Bacillus* species *cereus*, *thuringiensis* or *weihenstephanensis*, so it needs to be assumed that samples reported as positive for *B. cereus* may be positive for any of these species.[27]

[27] *B. weihenstephanensis* can be distinguished by a specific test for a genetic marker or by growth range, *B. thuringiensis* can be distinguished by the presence of parasporal bodies (Vilas-Boas *et al*., 2007)

Table 3. Prevalence and concentration of *B. cereus* in dairy products from other countries

Dairy product	Food	Country	Number of samples	Number positive for *B. cereus* (%)	Concentration of *B. cereus*	Comments	Reference
Liquid milk	Raw milk	Brazil	30	15 (50)		Toxigenic potential demonstrated for some isolates	Rezende-Lago *et al.* (2007)
	Raw milk	Brazil	60	31 (52)		*B. cereus* group	Vidal *et al.* (2016)
	Raw milk	Egypt	50	15 (30)	Mean 911 cells/mL	All isolates haemolytic, most cytotoxic (Vero cells)	Hassan *et al.* (2010)
	Raw cows' milk (farm bulk tanks)	Finland	183	38 (21) *B. cereus sensu lato*	Mean 1 CFU/mL		Ruusunen *et al.* (2013)
	Raw milk	India	27	18 (67)	>10^5 CFU/g in 3 samples	Toxigenic potential demonstrated for some isolates	Bedi *et al.* (2005)
	Raw organic milk	Latvia	183[a]	10 (5)		*B cereus* prevalence increased with increasing somatic cell count (SCC), from 2.4% of samples with low SCC (<200,000/mL) to 9.3% in samples with high SCC (>500,000/mL)	Gulbe and Valdovska (2012)
	Raw milk	Pakistan	100	10 (10)			Shafee *et al.* (2013)
	Raw milk	Tanzania	128	8 (6)	Mean 1.1 x 10^7 CFU/mL	Factors identified that contributed to microbial contamination at retail included access to an operating refrigerator, milk container type and hygienic practices	Kivaria *et al.* (2006)
	Raw milk	Turkey	50	45 (90)	420-6600 CFU/mL		Gundogan and Avci (2014)
	Raw milk (commingled silo milk intended for pasteurisation)	USA	214	19 (9)	3-93 CFU/mL		Jackson *et al.* (2012)
	Pasteurised milk	Brazil	30	29 (97)		Toxigenic potential demonstrated for some isolates	Rezende-Lago *et al.* (2007)
	Pasteurised milk	Brazil	9	9 (100)	0.4-71 CFU/mL	One sample per week, over 9 weeks, from one dairy processing facility	Salustiano *et al.* (2009)
	Pasteurised milk	Brazil	60	49 (82)		*B. cereus* group	Vidal *et al.* (2016)

Dairy product	Food	Country	Number of samples	Number positive for *B. cereus* (%)	Concentration of *B. cereus*	Comments	Reference
	Pasteurised full fat milk	China	54	26 (48)	3-43 MPN/mL	One or more toxin genes (*nheA, nheB, nheC, hblA, hblC, hblD*) were detected in most of the 92 isolates obtained. All three *nhe* genes were detected in 47% of the isolates, all three *hbl* genes were detected in 34% of the isolates	Zhou *et al.* (2008)
	Pasteurised milk	Netherlands	38	38 (100)	Means for 3 sampling occasions: 0.46, 0.93 and 1.40 $\log_{10}$ CFU/100 mL (i.e. <0.3 CFU/mL)	Milk sampled one day after pasteurisation	Notermans *et al.* (1997)
	Reduced-fat pasteurised milk (domestic refrigerators)	Netherlands	334	133 (40)	Mainly low (<5 organisms/mL) concentrations >4.7 log organism/mL in some samples	In general, concentrations increased with increasing refrigerator temperature and storage time. 56/106 isolates could grow at 7ºC. 26/37 isolates produced enterotoxin (Vero cell method)	te Giffel *et al.* (1997a)
	Pasteurised milk	Thailand	18	18 (100)	50 to 1700 CFU/mL	Isolates (*n* = 125) were analysed for eight enterotoxin genes. Genes were detected in 59.2% (*nheA)* to 80.8% (*nheB*)	Chitov *et al.* (2008)
	UHT milk	Brazil	30	4 (13)		Toxigenic potential demonstrated for some isolates	Rezende-Lago *et al.* (2007)
	UHT milk	Brazil	180	25 (14)		*B. cereus* group	Vidal *et al.* (2016)
	UHT milk (2-4 seconds at 130-150ºC)	Brazil	6500	ND		Tested using incubation at 7 or 37ºC for 10 days	Pacheco-Sanchez and de Massaguer (2007)
	UHT milk	Brazil	135	15 (11)			Cattani *et al.* (2016)
	UHT milk	Malaysia	20	6 (30)	<3 - >1100 MPN/mL		Tong (2015)
	Infant formula	Malaysia	12	5 (42)	<3 - >1100 MPN/mL	Not stated whether infant formulae were dried or liquid. However, a companion report (Kong, 2015) suggests they were liquid	Tong (2015)
Milk powder	Skim milk powder	Australia	70	ND		High limit of detection (2 $\log_{10}$ CFU/g)	Eglezos *et al.* (2010)

Dairy product	Food	Country	Number of samples	Number positive for *B. cereus* (%)	Concentration of *B. cereus*	Comments	Reference
	Milk powder	Brazil	30	22 (73)		Toxigenic potential demonstrated for some isolates	Rezende-Lago *et al.* (2007)
	Milk powder	Costa Rica	50	25 (50)	3->100 MPN/g	5/19 isolates were positive for all three non-haemolytic toxin genes (*nheA, nheB, nheC*)	Rojas *et al.* (2014)
	Skim milk powder	Egypt	25	11 (44)		7/17 isolates able to grow at 7°C	Sadek *et al.* (2006)
	Milk powder	India	9	4 (44)	$<10^5$ CFU/g	Toxigenic potential demonstration for some isolates	Bedi *et al.* (2005)
	Milk powder	Germany	1365	146 (11)		Most isolates (97.9%) were positive for non-haemolytic toxin by immunoassay	Wiebe (1999)
Powdered infant formula	Powdered infant formula	Australia	20	ND		Method of analysis not stated. Limit of detection 100 CFU/g	Thompson (2010)
	Powdered infant formula	Italy	60	5 (8)		Of 12 isolates, all contained at least one *nhe* gene and all but one of the isolates contained at least one *hbl* gene. All three *nhe* genes were present in 50% of isolates. The cytotoxin gene, *cytK*, was detected in all isolates	Di Pinto *et al.* (2013)
	Powdered infant formula	Korea	99	29 (29)	0.69 ± 0.32 log MPN/g		Hwang *et al.* (2008)
	Dried infant formula	Various	261	141 (54)	0.3-600 CFU/g	Four samples contained more than 100 CFU/g, 27 contained more than 10 CFU/g	Becker *et al.* (1994)
	Powdered infant formula	Ireland	100	24 (24)	mean 190 CFU/g, maximum 570 CFU/g		Haughton *et al.* (2010)
	Powdered infant formula	USA	12	9 (75)			Harmon and Kautter (1991)
Other dried milk products	milk with rice, milk substitute, milk powder, milk cereal-rice, pudding milk, flan, mousse	Chile	381	175 (46) - spores	$3\text{-}10^4$ spores/g	Highest prevalence and mean spore counts in milk with rice products. 94 isolates tested for enterotoxin expression, 28 (29.8%) positive (no significant difference between different product types). None of the toxigenic strains were grew at 4 or 7ºC	Reyes *et al.* (2007)

Dairy product	Food	Country	Number of samples	Number positive for *B. cereus* (%)	Concentration of *B. cereus*	Comments	Reference
Cheese	Port Salut (soft cheese)	Argentina	30	15 (50)		75% *B. cereus* isolates were psychrotrophic. This cheese undergoes paste cooking which will reduce background microflora	Iurlina *et al.* (2006)
	Quartirolo Argentino (soft cheese)	Argentina	20	ND			Iurlina *et al.* (2006)
	Cheese, not further specified	Australia	31	ND			FSANZ (2006)
	Goats' milk cheese (made from pasteurised milk; 72ºC/20s)	Czech Republic	44	ND		Little information was provided on the detection method	Janstova *et al.* (2010)
	Soft white cheese	Egypt	25	8 (32)		11/15 isolates able to grow at 7°C	Sadek *et al.* (2006)
	Processed cheese	Egypt	20	5 (25)		2/8 isolates able to grow at 7°C	Sadek *et al.* (2006)
	Kareish cheese	Egypt	25	7 (28)		4/9 isolates able to grow at 7°C	Sadek *et al.* (2006)
	Artisanal cheeses (made from raw milk)	Scotland	25	8 (32)	100 to 10^4 CFU/g	All 20 isolates produced enterotoxin (latex agglutination test). *B. cereus* was not detected in three additional cheeses made with pasteurised milk	Williams and Withers (2010)
	Homemade white cheeses (open-air markets)	Turkey	200	8 (4)		All strains had lipolytic and proteolytic activity and were capable of causing spoilage. No assessment of their toxigenic potential was carried out	Ozdemir and Arslan (2011)
	White cheese	Turkey	50	10 (20)	200-6000 CFU/g		Citak *et al.* (2010)
	White cheese	Turkey	50	35 (70)	1000-2600 CFU/g		Gundogan and Avci (2014)
Ice cream	Ice cream	China	40	24 (60)	Mean 8.3 MPN/g, maximum 28 MPN/g		Zhou *et al.* (2010)

Dairy product	Food	Country	Number of samples	Number positive for *B. cereus* (%)	Concentration of *B. cereus*	Comments	Reference
	Ice cream	Egypt	50	24 (48)	Mean 6370 cells/mL	All isolates haemolytic, most cytotoxic (Vero cells)	Hassan *et al.* (2010)
	Ice cream	Germany	809	507 (63)	0.1-20 CFU/g (*ces*-positive samples only)	*ces* gene detected in 4.7% samples	Messelhäusser *et al.* (2010)
	Ice cream	India	15 packaged 15 opened	4 (27) 6 (40)	41-360 CFU/mL 210-4500 CFU/mL		Warke *et al.* (2000)
	Ice cream	Turkey	50	24 (48)	200-6000 CFU/g		Citak *et al.* (2010)
	Ice cream	Turkey	50	10 (20)	1000-2000 CFU/g		Gundogan and Avci (2014)
Other dairy products	Rice with milk	Egypt	30	18 (60)		15/20 isolates able to grow at 7°C	Sadek *et al.* (2006)
	Yoghurt	Egypt	50	1 (2)	6 ± 5.9 cells/g	All isolates haemolytic, most cytotoxic (Vero cells)	Hassan *et al.* (2010)
	Burfi (milk-based confectionary)	India	29	13 (45)	$>10^5$ CFU/g in 4 samples	Toxigenic potential demonstration for some isolates	Bedi *et al.* (2005)
	Dairy-based desserts	Turkey	100	7 (7)	20-500 CFU/g	PCR of 20 isolates: The HBL toxin genes (*hblA, hblB* and *hblC*) were detected in 6 isolates, the enterotoxin genes *nheA, nheB* and *nheC*, were detected in 19, 18 and 8 isolates, respectively, the cytotoxin K gene was not detected	Cadirci *et al.* (2013)

ND: not detected
CFU: colony forming units
MPN: most probable number
[a] The study report states that there were 155 samples taken, but Table 2 of the report includes results for 183 samples

In a seven-year intensive study carried out in Bavaria of foods collected during investigation of suspected food poisoning outbreaks, 16/131 cheese samples (12%) contained presumptive *B. cereus* with three positive for emetic-producing strains (Messelhäusser *et al.*, 2014). *B. cereus* was not detected in any of 384 samples of milk and milk products. One of the samples, a hard cheese implicated as the cause of illness in several students, contained emetic *B. cereus* at an estimated concentration <100 CFU/g, but the concentration of cereulide was 2 mg/kg.

In the same German study, 742 food samples of animal and plant origin were investigated for the presence of emetic *B. cereus* strains within different existing food monitoring programs (Messelhäusser *et al.*, 2014). This survey detected the following:

- Cream: 3/41 samples contained presumptive *B. cereus*; 0/41 samples contained emetic *B. cereus*;
- Ingredients for milk products (production level): 15/24 presumptive *B. cereus*; 3/24 emetic *B. cereus*;
- Other milk products (e.g. desserts): 3/12 presumptive *B. cereus*; 3/12 emetic *B. cereus*;
- Soft cheese: 6/12 presumptive *B. cereus*; 1/12 emetic *B. cereus*; and
- Mozzarella/pasta filata cheese: 92/123 presumptive *B. cereus*; 16/132 emetic *B. cereus*.

Two additional studies are worth noting:

- In Australia, *B. cereus* was isolated from a range of dairy products (raw milk, pasteurised milk, yoghurt, cheddar cheese, milk powder and ice cream), but was not isolated from UHT milk (Rangasamy *et al.*, 1993). The researchers used two different media; Kim and Goepfert (KG) and the more usual polymixin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA). The prevalence of *B. cereus* contamination ranged from 10 to 40%, depending on the food type and the media used. Concentrations did not exceed 500 CFU/mL or g, except in milk powder, where a concentration of 960 CFU/g was found.
- In France, *B. cereus* was detected by PCR in 6/30 samples (20%) of dairy products (raw and pasteurised milk, pasteurised and UHT cream, cheese and milk powder) (Postollec *et al.*, 2012).

A number of studies have also found that the prevalence of *B. cereus* tends to increase along the dairy food chain:

- Côte d'Ivoire: The prevalence of *B. cereus* in raw milk increased from 27% in teat milk samples (n = 119) to 41% in raw milk sold to the public (n = 17) (Yobouet et al., 2014). Mean *B. cereus* concentrations increased from 2.6 log CFU/mL in teat milk to 2.9 log CFU/mL in sellers' milk.
- Canada: In samples taken from two dairy processing plants, the prevalence of *B. cereus* increased from 7-10% of raw milk samples, to 85-94% of pasteurised milk samples and again to 90-96% of the final packaged milk products (Lin, 1997).
- India: *B. cereus* was detected in 4% of raw milk samples taken aseptically from the animal (*n* = 50), 33% of bulk farm raw milk samples (*n* = 36) and 38% of pasteurised milk samples (*n* = 74) (Rather *et al.*, 2011). Concentrations ranged from 50 to 2.6 x 10^5 CFU/mL. Prevalence of individual toxin genes varied between 66.7% (*cytK*) and 100% (*entFM*), with 54.8% of pasteurised milk isolates carrying all of the enterotoxin genes. The protein virulence factor, *entFM,* is referred to as a toxin gene in this study. Other studies have suggested that it is a cell wall peptidase (Tran *et al.*, 2010).

Temporal changes in the prevalence of *B. cereus* in milk have also been reported in studies in Sweden and Poland (Bartoszewicz *et al.*, 2008; Svensson *et al.*, 2004). Both studies suggest a pattern of higher prevalence and concentrations in spring/summer months, although winter indoor housing of cows is common in these countries:

- Sweden: Milk from silo tanks at eight dairy processing facilities in Sweden was sampled over the course of one year and analysed for *B. cereus* (Svensson *et al.*, 2004).[28] Spore counts in silo tanks were lower in winter (29 to 308 spores/L, median 86 spores/L) than summer (25 to 1355 spores/L, median 186 spores/L). The proportion of strains that were psychrotrophic, based on growth at 8ºC, varied between dairy plants, from 27 to 60%. The proportion of psychrotrophic strains was significantly higher in the grazing (summer) season, than the indoor (winter) season. Isolates were typed by RAPD-PCR, with a greater prevalence of unique types in the summer than the winter. This was interpreted by the authors as being indicative of high genetic diversity amongst soil-borne strains of *B. cereus*, which can contaminate teats during the summer grazing period. There was some evidence that silo tanks could develop their own 'resident' strains of (usually) mesophilic *B. cereus*.

- Poland: Raw milk samples from a dairy farm (n = 20) and raw and processed samples from two dairy plants (n = 24) were examined for *B.cereus sensu lato* over all four seasons (Bartoszewicz *et al.*, 2008). *B.cereus sensu lato* was detected in 19 of 20 dairy farm samples, with *B.cereus sensu stricto/B. weihenstephanensis* being the dominant species at each of four seasonal sampling times. Spore densities were highest in spring (mean 136 spores/L) and lowest in autumn (mean 28 spores/L). All samples from dairy plants were positive for *B.cereus sensu lato*, with little difference in spore counts in raw milk between spring, summer and autumn, but lower spore counts were seen in winter. Pasteurisation had a variable impact on spore density, but spores were present in all pasteurised milk samples. During summer, spore counts were higher in pasteurised milk from both dairy plants than from raw milk samples from the same plant. *B.cereus sensu stricto/B. weihenstephanensis* was the dominant species in dairy plant samples (85 of 111 randomly selected isolates). The toxin genes, *hblA, nheA* and *cytK*, were present in 80, 55 and 60% of isolates of *B.cereus sensu stricto/B. weihenstephanensis* and *B. thuringiensis*, respectively, while only *hblA* and *nheA* were detected in 30 and 70% of *B. mycoides/B. pseudomycoides* isolates, respectively.

Finally, in a small survey of donkey milk in Italy, *B. cereus* was the only pathogen detected, in milk from 1 of 5 farms sampled (Cavallarin *et al.*, 2015). The sample, taken in the spring of 2014, contained counts of 130 CFU/mL.

A.8.3 Toxin production by *B. cereus* isolated from dairy products

A number of studies have investigated the potential for enterotoxin production by *B. cereus* isolates from dairy products. These studies generally show that *B. cereus* strains carrying one or more of the enterotoxin genes are often detected in dairy products, but only a few studies demonstrated actual production of enterotoxin:

- Of 80 isolates from milk and milk products in the Netherlands, *nhe* was the sole virulence factor in 24% of isolates, while *hbl* and *nhe* were present together in 19% of isolates (Wijnands *et al.*, 2006). All isolates contained at least one virulence factor.

[28] In these dairy processing facilities, milk is stored in silo tanks after delivery from dairy farms and before processing, and may be stored in silo tanks for up to 24 hours, but usually only 10-12 hours

- *B. cereus* group isolates from raw milk (*n* = 7) in Belgium were all found to contain *hbl* or *nhe* genes or both (De Jonghe *et al.*, 2010). Five of the isolates also contained the gene for cytotoxin K. However, cytotoxin activity was only detected in one isolate.

- *B. cereus* isolates (*n* = 92, including 72 from raw milk) were analysed for the presence of enterotoxin genes (Karagoz *et al.*, 2015). Of the 72 raw milk strains, 44% carried *hblC*, 32% *nheA,* 8% *cytK* and 4% *EM1.* Over half of the raw milk isolates carried none of the genes analysed for. However, it should be noted that *hbl* genes do not occur in *B. cereus* strains in the absence of *nhe* genes. If the *nheB* subunit gene had been included a somewhat different picture is likely to have emerged.

- Of 69 *B. cereus* isolates recovered from 99 powdered infant formula samples, all but one carried *nhe* genes, while only 30% carried *hbl* genes (Hwang and Park, 2015a). The majority (65/69) also carried the *entFM* gene, while the *cytK* gene was only detected in 20% of isolates.

- *B. cereus* strains (*n* = 63) were isolated from 260 samples of pasteurised milk (36 isolates), powdered milk (15 isolates) and UHT milk (12 isolates) (Reis *et al.*, 2013). Of these isolates, 23 (36.5%) were found to carry all three genes encoding components of the HBL enterotoxin, 26 (41.2%) carried none of the genes, while the balance of isolates carried one or two enterotoxin genes. The 23 isolates with all three genes were either from pasteurised milk (14) or powdered milk (9), with 20 of the isolates (87.0%) testing positive for expression of the HBL enterotoxin, by immunoassay.

- *B. cereus* strains (*n* = 39) isolated from whipping cream in Norwegian dairies were assessed for diarrhoeal toxin potential by testing for cytotoxicity against Vero cells (Stenfors Arnesen *et al.*, 2007). Isolates were cultured at 25, 32 or 37ºC prior to testing. Following culturing at 37ºC, none of the strains were highly cytotoxic, suggesting low diarrhoegenic risk in humans. Some strains were moderately or highly cytotoxic when grown at the lower temperatures. Most of the strains (27 of 39, 62%) were able to grow at refrigeration temperatures.

- *B. cereus* strains, isolated from powdered infant formula, produced NheA when cultured in either synthetic media or reconstituted infant formula (Hwang and Park, 2015b).

- Representative *B. cereus* strains (*n* = 13) were isolated from extended shelf-life (ESL) milk after storage at 8ºC for their intended shelf lives (Schmidt *et al.*, 2012). The *nhe* gene complex was detected in all isolates, the *hbl* gene complex was present in 10 isolates, and the *cytK* gene was present in four. Three isolates carried the *cspA* cold-shock protein gene and were reclassified as *B. weihenstephanensis*.

- *B. cereus* isolates recovered from dairy farms (*n* = 100), dairy processing facility silo tanks (*n* = 100) and points through the dairy process (*n* = 196) were tested for psychrotrophic potential (*cspA* gene) and potential and actual production of enterotoxins (Svensson *et al.*, 2007). The proportion of isolates carrying the *cspA* genes increased from 25% in farm isolates to 49% in silo tank isolates to 80% in dairy process isolates. This suggests that the dairy process is 'selecting' for psychrotrophic strains of *B. cereus*. The *nheA* gene, was detected in 84% of isolates overall, with no obvious trend across sampling sites. The A subunit of the Nhe toxin was expressed by 74% of isolates. The gene *hblA* was detected in 51% of isolates, with a slight trend to increased prevalence from farm to silo tanks to dairy processing (46, 51 and 54% if isolates, respectively). The C subunit of the HBL toxin was expressed in 74% of isolates. The disparity between the detection of the gene, by PCR, and detection of the gene product, by immunoassay, was ascribed to the particular PCR primers not always being able to detect the toxin gene. The *cytK* cytotoxin gene was detected in 21% of isolates, with a clear decreasing trend through the production chain, from 43% of farm isolates to 27% of silo tank isolates to just 7% of diary process isolates.

All isolates carrying the *cytK* gene were mesophilic. Ten percent of the isolates examined were high producer of both of the toxin subunits, with the proportion of high producers decreasing through the dairy process.

Less well investigated is the prevalence of emetic *B. cereus* strains in dairy products and their potential for producing cereulide in these foods. Studies suggest that isolates containing the *ces* gene can sometimes be detected in dairy products, but there are only a few studies that have investigated their ability to grow and produce cereulide in dairy foods:

- Of 13 representative *B. cereus* strains isolated from extended shelf-life (ESL) milk after storage at 8ºC, none contained the *ces* gene (Schmidt *et al.*, 2012).
- *B. cereus* isolates (*n* = 100) were recovered from two infant foods (one dairy, one cereal and dairy), following reconstitution and incubation at 21-23ºC for 24 hours (Shaheen *et al.*, 2006). Cereulide was produced in culture by 11 isolates. Two of the infant foods were reconstituted and inoculated with a known emetic toxin-producing strain of *B. cereus* at concentrations in the range 10^1 to 10^7 CFU/mL and analysed for cereulide after storage at room temperature for 24 hours. High concentrations of cereulide (up to 3 mg/L) accumulated in the cereal and dairy infant food. The amount of cereulide produced was dependent on the *B. cereus* inoculum level.
- When UHT-skim milk was inoculated to 10^6 CFU/mL of a known emetic toxin-producing strain of *B. cereus*, cereulide (up to 1.14 mg/L) was produced in 48 hours under static conditions at 28ºC, but no toxin was detected after 48 hours of shaking (Rajkovic *et al.*, 2006). A second emetic toxin-producing strain produced 0.26 mg/L cereulide after 48 hours incubation at the same bacterial concentration and incubation temperature.
- *B. cereus* isolates from dairy farms (*n* = 1757) and dairy processing facilities (*n* = 3911) were examined for the presence of emetic toxin production by; phenotypic traits, RAPD-PCR typing, a boar sperm motility inhibition test and by LC-MS (Svensson *et al.*, 2006). No emetic strains were found among farm milk and environmental strains taken during the outdoor grazing period. During the indoor stall period, emetic strains were generally rare in milk (<1.0 to 3.8% of isolates), except for one farm on which 40% of milk isolates were emetic toxin producers. This appeared to be due to establishment of a particular strain, possibly due the use of sawdust bedding. With the exception of one silo tank, emetic strains were very rare in the dairy processing environment (<0.05% of isolates). However, 13% of isolates from one silo tank were emetic toxin producers, suggesting a persistent colonisation of this tank.
- Of 396 *B. cereus* isolates recovered from dairy farms, dairy processing facility silo tanks and points through the dairy process, only two isolates were positive for emetic toxin production (0.5%), one each from the farm environment and from silo tanks (Svensson *et al.*, 2007).
- Of 80 isolates from milk and milk products in the Netherlands, cereulide-like toxin production was detected in 12% of isolates, but only from isolates also carrying enterotoxin genes (Wijnands *et al.*, 2006).

A.9. *B. CEREUS* IN ANIMAL FEED OVERSEAS

Silage (fermented, high moisture green fodder) is a common feed source for dairy cows (Kalač, 2011). The high moisture content of silage makes it a suitable environment for bacterial growth. A study in the Netherlands found *B. cereus* in 67% of silage (*n* = 70) and 45% of maize silage (*n* = 20) samples, with spore counts up to 1000 spores/g (Slaghuis *et al.*, 1997). A Swedish study found a maximum *B. cereus* concentration in silage of 200 spores/g (Christiansson *et al.*, 1999). Hay was less frequently contaminated, although one very high *B.*

cereus concentration (4.5 x 10^4 spores/g) was detected in hay. Vissers *et al.* (2007b) found similar concentrations of *B. cereus* spores in silage (2.4 ± 0.07 log spores/g).

A survey of animal feed ingredients in Poland included analysis of meat meal and oilseeds (*n* = 49) for *B. cereus* (Kukier *et al.*, 2013). *B. cereus* was detected in two samples of soy and rape-derived ingredients at concentrations of 40 and 1200 CFU/g. An Australian study detected *B. cereus* in 2 of 14 feed samples, but further information on the type of feed material tested and the concentrations of *B. cereus* detected were not reported (McAuley *et al.*, 2014).

APPENDIX B: EVALUATION OF ADVERSE EFFECTS

B.1 NON-FOODBORNE (INVASIVE) DISEASE

B. cereus can occasionally cause invasive disease, usually due to contamination of medical devices introduced into the central nervous system (CNS), such as by spinal anaesthesia and ventricular tubes and shunts (Stevens *et al.*, 2012). A case of ventriculitis was reported following intrathecal (via the spinal canal) chemotherapy. A review of the literature identified 33 additional cases, including 12 fatal cases. Invasive disease can also follow sharing of needles by intravenous drug users (Schoeni and Wong, 2005). Invasive *B. cereus* infections generally have a high mortality rate (Uchino *et al.*, 2012).

Serious eye infections may occur following introduction of foreign objects into the eye as a result of traumatic injuries (Schoeni and Wong, 2005).

B.2 DOSE-RESPONSE

B.2.1 Diarrhoeal syndrome

Counts of *B. cereus* in foods associated with illness have been reported in the range 200 to 10^9 organisms/g (Granum and Lund, 1997). However, the lower number (200 CFU/g) was further investigated and found to be closer to 10^4 CFU/g (Stenfors Arnesen *et al.*, 2008). The reported concentration of *B. cereus* in foods causing illness generally exceeds 10^3 CFU/g. Gilbert and Humphrey (1998) reported the following numbers (per gram) of *B. cereus* in foods incriminated in outbreaks of diarrhoeal illness in the UK:

$<10^4$	1.8%
10^4-$<10^5$	4.1%
10^5-$<10^6$	22.0%
10^6-$<10^7$	28.0%
10^7-$<10^8$	21.7%
10^8-$<10^9$	15.8%
10^9-$<10^{10}$	4.8%
$>10^{10}$	1.8%

A commercially available immunoassay kit for *Bacillus* diarrhoeal enterotoxin was used to investigate a number of foods and faecal samples from food poisoning incidents in Australia (Tan *et al.*, 1997). The sensitivity of the kit was approximately 2 μg/kg (of faeces, the sensitivity in food samples was not reported). Foods that were positive for the enterotoxin were always contaminated with ≥10^5 CFU/g of *B. cereus*. However, the enterotoxin was not always detected in foods when *B. cereus* was present at 10^4-10^5 CFU/g. Overall the results supported the view that illness results from toxin production in the gut following the ingestion of between 10^5 and 10^8 enterotoxigenic cells or spores (EFSA, 2005).

The concentration of spores able to cause disease is probably lower, as spores are better equipped to survive passage through the gastric environment (Stenfors Arnesen *et al.*, 2008). A study in synthetic gastric medium, spore numbers decreased slightly at pH 1.0 or 1.4 in medium or medium containing pea soup, but remained unchanged in medium containing milk or chicken (Clavel *et al.*, 2004). A marked rapid decrease (>2 log CFU/mL in 2 hours) occurred pHs below 4.2, 4.0, 3.6, 3.5 for vegetative cells in chicken medium, medium, milk medium and pea soup medium, respectively. This suggests that milk has a protective effect for both spores

and vegetative cells under gastric conditions. A more recent study produced similar results, with survival of *B. cereus* vegetative cells in simulated gastric media (pH 4.5, 37°C for 6 hours) being greater in the presence of standard milk (3.2% fat, 85.4% survival) than non-fat milk (34.5% survival) or chicken (4.5% survival) (Berthold-Pluta *et al.*, 2014). It has been suggested that this phenomenon may be due to the bacteria being trapped in protein-lipid complexes and isolated from the direct effects of the low pH environment (Berthold-Pluta *et al.*, 2015)

B.2.2 Emetic syndrome

The numbers of organisms involved in emetic disease incidents vary from 10^3 to $5x10^{10}$ organisms/g with median values around 10^7 organisms/g (McElroy *et al.*, 1999). In most cases a bacterial concentration of at least 10^5 CFU/g will be required to result in emesis (Stenfors Arnesen *et al.*, 2008).

The minimum dose of cereulide capable of causing illness appears to be of the order of 8 µg/kg body weight (Jaaskelainen *et al.*, 2003; Rajkovic, 2014). This is similar to the minimum toxic dose determined in laboratory animals. The amount of emetic toxin present in food samples that had caused food poisoning in Japan ranged from 0.01 to 1.28 mg/kg (10-1280 µg/kg) (Agata *et al.*, 2002). Notermans and Batt (1998) reproduced a dose response curve for the activity of the emetic toxin in the husk shrew (a small rodent). The dose required to produce emesis in 50% of test subjects (ED_{50}) was 12.9 µg/kg body weight. The intraperitoneal ED_{50} was 9.8 µg/kg body weight.

It should be noted that the emetic toxin will survive heat treatment, such as cooking, while *B. cereus* cells may not. Consequently, measured concentrations of *B. cereus* may not necessarily reflect the risk of emetic poisoning (EFSA, 2005).

B.3 OUTBREAKS IN OTHER COUNTRIES

B.3.1 Outbreaks of suspected food poisoning due to *B. cereus* in dairy products

Reports from a number of countries suggest that *B. cereus* intoxication is responsible for approximately 1-5% of reported outbreaks in which a causative agent is identified (EFSA, 2005).

Outbreaks associated with dairy products appear to be relatively rare, although it is accepted that cases of *B. cereus* intoxication are probably heavily under-reported due to the mild nature and short duration of the illness caused. Table 4 summarises details of six outbreaks caused by dairy products, based on data presented by Christiansson (2011). Storage temperature was identified as a failure in some of these outbreaks. A search of the scientific literature did not find any further reports of outbreaks of *B. cereus* intoxication with links to consumption of dairy products. A wider search shows that outbreaks are still occasionally being detected (see Table 5), but detailed outbreak reports do not appear to have been published in the scientific literature.

Table 4. Outbreaks of illness caused by *Bacillus cereus* in dairy products*

Dairy food	Year	Country	People ill	Symptoms	Analytical data
Unpasteurised milk (heated and then kept at room temperature overnight)	1972	Romania	221 school children	Diarrhoea and abdominal cramps after 8–11 hours	2×10^7 *B. cereus*/mL in milk *B. cereus* found in children's faeces
Cream, pasteurised	1975	England	Two 15-year-old girls	Vomiting after 8–10 h One girl had diarrhoea	5×10^6 *B. cereus*/g in cream
Milk, pasteurised	1981	Denmark	1-year-old boy	Vomiting after 1.5 h, no diarrhoea	2.6×10^6 *B. cereus*/mL in milk Remaining milk was sweet curdled one hour after consumption
Milk powder, infant formula	1981	Chile	35 neonate children	Diarrhoea	*B. cereus* found in stool cultures
Milkshake[a]	1988	Canada	36 people		$>10^5$ CFU/g in milkshake mix
Milk, pasteurised	1988	Netherlands	42 elderly people	Nausea and vomiting after 2–14 hours	4×10^5 *B. cereus*/mL in milk
Milk[a]	1989	Canada	74 people		$1.8\text{-}8 \times 10^6$ CFU/g in milk, considered to be due to temperature abuse and poor stock rotation
Ultra-high temperature milk (process failure)	1991	Japan	201 people	Vomiting 95%, diarrhoea 55%	Milk distributed at room temperature

* Adapted from Christiansson (2011)
[a] British Columbia Centre for Disease Control (2002)

B.3.2 Outbreaks caused by *B. cereus* in other countries

Table 55 summarises international information on the prevalence of outbreaks caused by *B. cereus.* In the European Union, 7.1% of *B. cereus* outbreak cases were hospitalised (EFSA/ECDC, 2015). This is markedly higher than the hospitalisation rate of 0.8% for *B. cereus* outbreaks in the USA between 1998 and 2008 (Bennett *et al.*, 2013).

Fatalities due to *B. cereus* intoxication were not reported in these references.

Table 5. *B. cereus* outbreaks overseas

Country	Year(s)	Outbreaks (% total outbreaks)	Outbreak cases (% total outbreak cases)	Dairy implicated outbreaks	Reference
Australia[a]	1995-2000	2 (0.9)	28 (0.3)	NS	(Dalton *et al.*, 2004)
Australia[a]	2011	1 (0.7)	12 (0.6)	Unknown	(OzFoodNet Working Group, 2015)
Brazil	2000-2010	21 (10.8)	NS	NS	(Nunes *et al.*, 2013)
European Union[a]	2013	278 (5.4)[b]	2470 (5.9)	1 milk of 54 with identified food	(EFSA/ECDC, 2015)
Taiwan[a]	1991-2010	(5.4)	NS	NS	(Cheng *et al.*, 2013)
USA[a]	1998-2008	235 (1.8)	2050 (0.8)	2 outbreaks attributed to dairy[c]	(Gould *et al.*, 2013)
USA[a]	2013	5 (0.6)	25 (0.2)	NS	(CDC, 2015)

NS, not specified

[a] Foodborne outbreaks only.

[b] Of 278 foodborne *B. cereus* outbreaks in the European Union, 236 were reported from France. *B. cereus* outbreaks accounted for 19.3% of foodborne outbreaks reported from France.

[c] A more detailed analysis of the outbreaks during this period that were due to bacterial intoxications only identified one outbreak where dairy was the suspected transmission vehicle (Bennett *et al.*, 2013).

In an assessment of outbreaks attributed to cheese in the USA during the period 1998-2011, of 71 outbreaks with a known aetiology, one was due to *B. cereus* (Gould *et al.*, 2014). The outbreak involved cheese produced from pasteurised milk.

B.4 RISK ASSESSMENT AND OTHER ACTIVITIES OVERSEAS

B.4.1 Côte d'Ivoire

As an addendum to an environmental study, a cohort (*n* = 188) of raw milk purchasers were questioned regarding milk consumption habits and history and severity of illness following milk consumption (Yobouet *et al.*, 2014). Symptoms potentially associated with food poisoning linked to the consumption of milk were reported by 13% (23/183) of milk consumers and most of these symptoms (74%) occurred less than 24 hours after milk consumption. The occurrence of self-reported gastrointestinal illness was significantly related to the consumption of unheated local milk (Relative Risk = 2.9; 95% CI = 1.1–7.7).

B.4.2 Ireland

Gleeson *et al.* (2013) reviewed information on sources of thermoduric bacteria, including *B. cereus*, in bulk tank milk. Contamination of teat skin with bacteria-containing soil or bedding material, and subsequent translocation of the contamination into raw milk, is considered to be the cause of the majority of spore contamination of bulk tank milk. Contamination from this source can result in spore counts in bulk milk of up to 1000 spores/L. However, washing and

drying of teats prior to milking can reduce contamination by about 96% (Magnusson *et al.*, 2006). High water content of farm soil and dirty access roadways have been identified is important contributors to this contamination route. Contaminated animal feed can also contribute to spore contamination of bulk tank milk. However, this is considered to be due to teat contamination, rather than intestinal transmission. *B. cereus* biofilms can also form on stainless steel surfaces of milk-processing equipment, with subsequent release of spores into milk. Water quality also has the potential to contribute to the spore content of bulk tank milk.

B.4.3 Netherlands

Household surveys in the Netherlands found that pasteurised milk was consumed within 2-12 days after pasteurisation and was stored at temperatures in the range <5 to 13ºC (Notermans *et al.*, 1997). It was estimated that 7 and 4% of pasteurised milk portions consumed in the Netherlands would contain $>10^5$ and $>10^6$ *B. cereus*/mL, respectively. Given the large number of servings of pasteurised milk consumed in the Netherlands and the lack of epidemiological evidence linking milk consumption to human *B. cereus* intoxication, the authors of this study suggested that the dose-response relationship for the organism may need to be reconsidered.

Stochastic models were developed to predict the concentration of *B. cereus* in farm tank milk under various combinations of season and farm management practices (Vissers *et al.*, 2007a). The models used a combination of existing information and predictive microbiology. The models predicted that when teats are contaminated with soil (summer grazing period) 33% of farm tank milk would contain more than 3 $\log_{10}$ *B. cereus* spores/L. When animal feed was the main source of contamination, only 2% of farm tank milk would contain more than this level of contamination. During the grazing period the average spore concentration in raw milk stored at the dairy processor was predicted to be 3.5 $\log_{10}$ spores/L of milk and during the housing period to be 2.1 $\log_{10}$ spores/L. It was estimate that, during the grazing period, a 99% decrease in contamination could be achieved through optimum teat cleaning, while during the housing period, a 60% reduction could be achieved by limiting the spore content and controlling the pH of feed.

B.4.4 Slovak Republic

A risk assessment was conducted for *B. cereus* intoxication from consumption of pasteurised milk (Acai *et al.*, 2014). *B. cereus* was determined in 11 samples of freshly produced pasteurised milk, with results ranging from absent to 1.58 CFU/mL. Application of information on storage times and temperatures and microbial growth rates produced an estimate that 14% of cartons of pasteurised milk would contain $>10^4$ CFU/mL of *B. cereus* at the time of consumption. While no generally accepted dose-response model is available for *B. cereus* intoxication, application of an exponential dose-response model, based on Slovak data (details not provided), produced a mean annual estimate of *B. cereus* intoxication in the Slovak Republic of 0.054 cases per 100,000 from consumption of pasteurised milk. This is similar to the overall prevalence of *B. cereus* intoxication in the Slovak Republic of 0.02 cases per 100,000, estimated from outbreaks.

APPENDIX C: CONTROL MEASURES

C.1 ON FARM

A range of protocols for cleaning teats to remove spore-forming organisms was examined (Magnusson *et al.*, 2006). The most effective treatment involved cleaning of the teats with a damp washable towel (with or without soap), followed by drying with a dry paper towel for a total time of 20 seconds per cow (10 seconds wipe, 10 seconds dry). This resulted in a 96% decrease in *B. cereus* contamination in milk from the cows. The least effective measure was wiping the teats with a dry paper towel for 10 seconds. However, this still resulted in a 40 to 50% decrease in milk contamination. Cleaning effectiveness was independent of the contaminating matrix (soil, manure or sawdust), the spore type (*B. cereus* or *Clostridium tyrobutyricum*) or the degree of contamination.

Using a model system to replicate farm milking equipment with different levels of cleaning difficulty, researchers in Sweden evaluated the effectiveness of six commercial chlorine-free alkaline detergents on *B. cereus* spores introduced into the equipment (Sundberg *et al.*, 2011). They also studied the effects of a commercial alkaline detergent with chlorine, solutions containing sodium hydroxide or sodium hydroxide/hypochlorite and water. Using spore removal as the indicator of effectiveness, chlorine-free products performed similarly but were not as effective as those containing chlorine. The mechanical action of water was an important component of spore removal. Cleaning effectiveness was measured at 35, 45, 55 and 65ºC. Increasing the temperature of the cleaning solution improved spore removal, but there was no significant difference between 55 and 65ºC.

C.2 AT PROCESSING

C.2.1 Lactoperoxidase system

The lactoperoxidase/thiocyanate/hydrogen peroxide (LP) system is an indigenous antibacterial system in milk and human saliva (Codex Alimentarius Commission, 1991). The enzyme lactoperoxidase is present in bovine and buffalo milk in relatively high concentrations. It can oxidise thiocyanate ions in the presence of hydrogen peroxide. By this reaction, thiocyanate is converted into hypothiocyanous acid (HOSCN). At the pH of milk HOSCN is dissociated and exists mainly in the form of hypothiocyanate ions (OSCN-). This agent reacts specifically with free sulphydryl groups, inactivating several metabolic bacterial enzymes, consequently blocking their metabolism and ability to multiply. As milk proteins contain very few sulphydryl groups and those that are present are relatively inaccessible to OSCN-, the reaction of this compound is in milk quite specific and is directed against the bacteria present in the milk. The system can be activated in raw milk by addition of thiocyanate and hydrogen peroxide.

LP-activated milk inoculated with *B. cereus* (10^4 CFU/mL) exhibited no significant difference in microbial growth at 12 hours, when compared to a non-activated, inoculated control (Armenteros *et al.*, 2007). Total bacterial counts were significantly lower in the LP-activated milk.

C.2.2 Pulsed electric fields (PEF)

Pulsed electric fields (PEF) were examined, in combination with heat and a preservative (nisin), for their effectiveness in inactivating *B. cereus* spores in skim and whole milk (Bermúdez-Aguirre *et al.*, 2012). A combination of PEF and nisin at 65ºC was effective in reducing spore counts by 3.6 log, but no combination of treatments was able to completely inactivate spores.

C.2.3 High pressure (HP) treatment

Strains of *B. cereus* and *B. subtilis* in milk were treated with a combination of high pressure (HP; 100 or 500 MPa) and nisin (500 IU/mL) at 40ºC (Black *et al.*, 2008). While viability of *B. subtilis* could be decreased by almost 6 log by a combination of nisin and repeated cycles of 500 mPa pressure, *B. cereus* strains showed high variability in resistance. Inactivation of *B. cereus* ranged from complete inactivation (8 log reduction) of one strain to a 2 log reduction in viability in another strain.

Amador Espejo *et al.* (2014) used higher temperatures (55, 65, 75 or 85ºC) and 300 MPa pressure to inactivate strains of six *Bacillus* species in milk. Spore inactivation greater than 5 $\log_{10}$ CFU/mL was achieved at 75ºC for *B. cereus, B. licheniformis, B. sporothermodurans* and *B. coagulans*. *B. subtilis* and *Geobacillus stearothermophilus* demonstrated greater resistance to inactivation under these conditions, but a 5 $\log_{10}$ CFU/mL inactivation was achieved at 85ºC and 300 MPa pressure.

Inactivation of *B. cereus* (a mixture of spores and vegetative cells) was investigated using high pressure carbon dioxide treatment (HPCT) at elevated temperatures (Furukawa *et al.*, 2009). Compared to the studies summarised above, the pressure regime was moderate (30 MPa). Nine suspected food poisoning strains of *B. cereus* (initial concentrations 3-7 log CFU/mL) were treated for 120 minutes at 75ºC and 30 MPa of CO_2 pressure. For eight of the nine strains, complete inactivation was achieved in 120 minutes. However, for one strain approximately 2.5 log CFU/mL remained after 120 minutes of treatment.

Variability in inactivation of *Bacillus* species strains was also seen in a system involving 600 MPa for one minute at an initial temperature of 72ºC (Scurrah *et al.*, 2006). Inactivation ranged from nil to 6 $\log_{10}$ spores/mL. Strains of *B. cereus* showed reductions in spore concentrations of 3.6 to 5.6 $\log_{10}$ spores/mL.

Response surface analysis was used to assess to impact of hydrostatic pressure, temperature and process time on inactivation of *B. cereus* spores in milk buffer (Ju *et al.*, 2008). Optimum process conditions for a 6 log reduction in *B. cereus* spores was determined to be at 540 Mpa pressure and 71ºC for 16.8 minutes.

The impact of various combinations of pressure and temperature on inactivation of *B. cereus* spores in reconstituted milk was examined (Evelyn and Silva, 2015a). An increase in pressure from 200 MPa to 600 MPa resulted in an additional decrease in spore counts (40 minutes, 70ºC) of about 1 log, while an increase in temperature from 38 to 70ºC (40 minutes, 600 MPa) resulted in an additional 3.5 log reduction in spore counts. Application of 600 MPa pressure resulted in equivalent spore reduction at about 20ºC lower temperatures than for thermal treatment alone. However, the total energy inputs required to achieve a 5 log reduction by 70ºC-600 MPa-40 minutes was greater than to achieve the same reduction by thermal processing of 90ºC-10 minutes.

C.2.4 Thermosonication

Thermosonication is the simultaneous application of thermal and ultrasonic processes for the inactivation of microbial pathogens (Evelyn and Silva, 2015b). Thermosonication (1.5 minutes, 70ºC) resulted in four-fold greater reduction in spore counts (log scale) than thermal processing (same time and temperature) of a cheese slurry. Thermosonication was able to achieve the same pathogen reductions at 20-30ºC lower temperature than for thermal processing alone.

C.2.5 Ultra-violet (UV) radiation treatment

UV-C radiation was examined for its ability to inactivation *B. cereus* endospores in raw and skimmed cows' milk (Choudhary *et al.*, 2011). Maximum inactivation of *B. cereus* of 2.65 (raw cows' milk) and 2.72 (skimmed cows' milk) $\log_{10}$ CFU/mL were achieved with a narrower flow

tube and higher flow rate. By comparison, maximum inactivation of *E. coli* reached almost 8 $\log_{10}$ CFU/mL in skimmed cows' milk.

C.2.6 Electron bean irradiation

Powdered weaning foods, containing dairy ingredients, were inoculated with *B. cereus* to a concentration of 8-9 $\log_{10}$ CFU/g (Hong *et al.*, 2008). Samples were exposed to irradiation doses of 2, 8 or 16 kGy at 1 MeV, then stored for 12 days at 20ºC. Irradiation at 2 kGy reduced the *B. cereus* concentration by 1.64 $\log_{10}$ CFU/g, while the higher doses resulted in complete elimination of the organism. Little further change in bacterial populations occurred during 12 days of storage. It should be noted that these experiments are likely to have involved vegetative cells of *B. cereus*, while dry, pasteurised dairy ingredients are more likely to contain *B. cereus* endospores, which may be more resistant to the effects of irradiation.

C.2.7 Plant cleaning procedures

B. cereus endospores can adhere to stainless steel surfaces, such as those prevalent in dairy processing plants (Hornstra *et al.*, 2007). There are many published studies in the scientific literature investigating mechanical and chemical methods for removing these biofilms, with a focus on the clean-in-place (CIP) procedures used in dairy plants. Some relevant examples are included in this section.

When *B. cereus* spores attached to stainless steel were exposed to room temperature water under a flow pressure of 500 Pa, the mechanical action of the water was only able to remove 59-89% of the spores, depending on the *B. cereus* strain (Faille *et al.*, 2013). This percentage was increased to 79-96% using a CIP procedure of 5 min rinse with softened water (mean shear stress 1.4 Pa), 10 min clean with sodium hydroxide (0.5%, at 60ºC, mean shear stress 4 Pa), and 5 min rinse with softened water (mean shear stress 1.4 Pa). This experiment showed that chemical action was important in CIP procedures.

A clean-in-place (CIP) protocol of 15 minutes in 0.5 M sodium hydroxide at 60ºC did not result in any loss of spore viability. However, pre-germination of spores, initiated by L-alanine and inosine, followed by the CIP protocol resulted in a 3-4 log decrease in viable spores (Hornstra *et al.*, 2007).

Response surface methodology was used to optimise CIP procedures for decontamination of stainless steel tanks with *B. cereus* biofilms (Kumari and Sarkar, 2014). The standard CIP procedure (1% NaOH at 65ºC for 10 minutes - water rinse – 1% HNO_3 at 65ºC for 10 minutes - water rinse) achieved a 3.29 log/cm^2 reduction in a 24-hour biofilm, while an optimised procedure (1.5% NaOH at 65ºC for 30 minutes - water rinse - 1% HNO_3 at 65ºC for 10 minutes - water rinse) produced a 4.77 log/cm^2 reduction in biofilm cell count.

INSTITUTE OF ENVIRONMENTAL SCIENCE AND RESEARCH LIMITED

Kenepuru Science Centre
34 Kenepuru Drive, Kenepuru, Porirua 5022
PO Box 50348, Porirua 5240
New Zealand
T: +64 4 914 0700 **F:** +64 4 914 0770

Mt Albert Science Centre
120 Mt Albert Road, Sandringham, Auckland 1025
Private Bag 92021, Auckland 1142
New Zealand
T: +64 9 815 3670 **F:** +64 9 849 6046

NCBID – Wallaceville
66 Ward Street, Wallaceville, Upper Hutt 5018
PO Box 40158, Upper Hutt 5140
New Zealand
T: +64 4 529 0600 **F:** +64 4 529 0601

Christchurch Science Centre
27 Creyke Road, Ilam, Christchurch 8041
PO Box 29181, Christchurch 8540
New Zealand
T: +64 3 351 6019 **F:** +64 3 351 0010

www.esr.cri.nz