Assessment of Potential for Growth of B. cereus group organisms on Rocket

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Abstract

Bacilus cereus is a gram (+), facultative, spore-forming rod. It is ubiquitous, commonly isolated from soil and is a well known food borne pathogen, causing disease by toxin production. B. cereus and several other very closely related species, including B. thuringiensis which is used commercially as a bioinsecticide on vegetable crops, form the "B. cereus group". Commercially packaged rocket was found to have acceptable levels of contamination with B. cereus group bacteria. Rocket was inoculated with B. cereus and storage trials performed. Under conditions representative of commercial storage, distribution and retail display no growth was found. At 30°C B. cereus group bacteria were able to grow, but product spoiled before hazardous levels were reached. Thus, it is concluded, that unless there is a high initial contamination and high storage temperature, it is unlikely that B. cereus group bacteria would be able to grow to hazardous levels before product spoils. We were unable to differentiate between B. cereus and B. thuringiensis based on comparison of the 16s rRNA gene sequence, but all B. cereus group isolates tested produced the target protein for the Tecra® enterotoxin test. Further research into contamination levels and the toxigenic potential of B. thuringiensis used in bio-insecticides is required.

Introduction

Bacillus cereus is a ubiquitous bacterium, present in almost every environment including vegetable production areas (Murray *et al*., 2002). *Bacillus cereus* is classified as a Gram positive, catalase positive, facultatively anaerobic, spore-forming rod. Its spores readily survive heat treatment and routine chemical disinfection (EFSA, 2004). The importance of the bacterium arises because it can cause foodborne disease (Jenson and Moir, 2003). In the Netherlands, *B. cereus* was identified as the cause of 19% of all food poisoning outbreaks and in Taiwan it is the third most common cause of food poisoning (Schoeni and Lee, 2005).

There are two forms of *B. cereus* food poisoning. The rapid-onset emetic illness occurs one to five hours after the consumption of food contaminated with a heat stable enterotoxin produced by *B. cereus* during growth in the food (Helgason *et al.,* 2000). The second form is a diarrhoeal illness, which becomes evident eight to sixteen hours after ingestion of contaminated food. It is the diarrhoeal form that has been associated with the vegetable industry (Guinebretiere *et al*., 2002). Foods contaminated with >10^5 cfu.g^{-1} are most often associated with illness (EFSA, 2004). Up to 10^4 cfu.g^{-1} in a typical serving of food is considered the upper limit to "acceptable" levels of contamination (Montville and Mathews, 2004).

Bacillus cereus can reasonably be expected to be present in Tasmanian salad and vegetable production areas and is an important consideration for the industry. Recently there has been a

trend toward minimally processed, packaged and ready to eat foods such as fresh-cut leafy salad vegetables. Many of the packaged salad vegetable products have a shelf life of up to 16 days and are distributed over large distances. Producers in Tasmania supply national supermarket chains, including stores as distant as Darwin (A.Clark, *pers. comm*., 2005). Given the storage life and climatic variation during distribution, any contamination and growth of pathogenic bacterium on these products could pose a serious health risk.

The taxonomy of *Bacillus cereus* and closely related strains is complex. *Bacillus cereus* is one species within the '*Bacillus cereus* group', which includes *B. cereus*, *B. thuringiensis*, and four other species (EFSA, 2004). *B. thuringiensis* is used commercially as a bio-insectide. The *Bacillus cereus* group is a very homogeneous cluster, reported to have 99% similarity in their 16s rRNA sequences (Ash *et al*., 1991), a level of homology usually interpreted by bacterial taxonomists as being indicative of a single species. A practical consequence of this is that they are very difficult to differentiate, even using molecular biology techniques. For this reason within this paper the term *B. cereus* is used to refer only to that specific bacterium within the *B. cereus* group, recognised as a food-borne pathogen.

The genes for the toxins in *B. cereus,* which cause human illness, are plasmid-borne meaning they are transferable between cells. There are reports of some strains of *B. thuringiensis* having acquired these genes and causing human illness. Equally, genes encoding the insectidal parasporal crystal that is the main factor distinguishing *B. cereus* from *B. thuringiensis,* can be plasmid-borne. Thus, EFSA (2004) concluded that loss of this plasmid from a *B. thuringiensis* would render it a *B. cereus*.

Bacillus cereus group bacteria have been detected on commercial rocket, a type of lettuce, during routine microbiological testing for food safety assurance. Levels initially present were less than would be expected to cause human illness. A complication, however, is that Dipel®, a bioinsecticide that is essentially spores of *B. thuringienesis* is applied to rocket crops. With standard detection methods (i.e. culture on microbiological media) *B. cereus* and *B. thuringiensis* are virtually indistinguishable. Direct microscopic examination for parasporal crystals is diagnostic for *B. thuringiensis*.

Given these circumstances, the study described herein was undertaken to assess the public health significance of the *B. cereus* group bacteria on commercial rocket products. The specific aims of the studies were:

To assess the potential for growth of *B. cereus* on rocket under a range of conditions relevant to commercial storage, distribution and retail display.

To assess the toxigenic potential among *B. cereus* group strains present on rocket, including *B. thuringiensis* derived from Dipel®.

Materials and Methods

Materials

Strains used

Psychotropic strains of *B. cereus* were obtained from Food Science Australia, North Ryde, NSW. Four strains (2, 20, 16, 14) were used in all experiments. Presumptive *B. thuringiensis* colonies were isolated from Dipel®.

Microbiological Media

Nutrient Agar (NA; Oxoid CM3) was used for enumeration of total bacteria. Bacillus Cereus Selective Agar (BCSA; Oxoid CM617) was used to enumerate *B. cereus* group bacteria. Enumeration media were prepared according to manufacturer's instructions. BCSA included the selective agent polymyxin B (Oxoid SR99) and egg yolk suspension (Oxoid, SR47).
Sterile 0.85% NaCl, 0.1% peptone solution was used for all dilutions.

Other Experimental Materials

Toxin Test Kit
The Tecra® Bacillus Diarrhoeal Enterotoxin Visual Immunoassay (Tecra International Pty., Ltd.,) was used to detect *B. cereus* diarrhoeal toxin among selected strains.

Rocket and Packaging
A local commercial producer supplied rocket. Processed product and harvested, but unprocessed, product were used in different parts of the experiment. Commercial packaging film was also obtained.

Other Equipment

Salad Spinner
Centrifugation was required to remove excess moisture, containing *B. cereus* inoculum, from the rocket leaves and to simulate the drying step after chlorination in the commercial process. Centrifugation was performed using a plastic, household, salad spinner.

Inoculation Apparatus
Metal trays were used to contain the inoculum. A lower, wider, tray was used to capture any overflow from a deeper, but narrower, primary inoculum bath. A wire basket, constructed from galvanised wire mesh, was used to contain rocket leaves that were then submerged in the inoculum bath for a measured period of time.

Methods

Storage Trials

The overall process in the challenge trials was to:

- inoculate fresh, commercially grown rocket with *B. cereus* strains,
- spin the rocket dry,
- package the inoculated sample into bags made from a packaging film commercially used for fresh-cut salad products,
- heat seal the bags,
- incubate the bags under a number of temperature scenarios and
- enumerate *B. cereus* and total bacteria (aerobic total viable count; ATVC) at appropriate time intervals to characterise the potential for growth of *B. cereus* group bacteria and spoilage bacteria on the product.

Inoculation

B. cereus strains were grown on Nutrient Agar. After incubation at 37°C for 18 to 24 hours, cell material from colonies of four *Bacillus cereus* strains was suspended in 9ml of diluent to prepare a concentrated cell suspension. From this, a 3L suspension containing ~10^4 cfu.ml^{-1} was prepared by combining 3 L of sterile water and 3ml of a 10^7 suspension in an inoculation tray. Rocket

leaves were selected at random and ~ 25g placed into the inoculating cage. The entire cage was immersed into the inoculation bath for 5 minutes. The cage was inverted after 2.5 minutes and immersed for a further 2.5 minutes. The inoculum suspension was replaced periodically. Preliminary trials showed that this method enabled *B. cereus* cells to adhere to the rocket.

Removal of Excess Inoculation Media
Rocket leaves were transferred from the dipping cage with sterile forceps into a salad spinner. The leaves were spun for two rotations of the lid handle per second for thirty seconds, a total of 60 rotations. Preliminary trials showed that spinning for 30 seconds was the most effective method of removing excess suspending medium from the leaves.

Heat Sealing of Bags
Small versions of the bags used commercially were made from the same packaging film. All seams were double heat-sealed. Approximately 13g of inoculated plant material was placed into numbered pre-prepared bags, and sealed.

Incubation
To avoid bias from potential reduction of cell concentration in the bath with each successive dip, bags were randomly allocated to each temperature treatment and sampling time.

The shelf life required of the product is 16 days under refrigeration. Recognising that temperature abuse is likely, and that pre-packaged salad mixes are frequently displayed under ambient conditions several storage regimes were adopted.

- 4°C for 16 days; “ideal storage”.
- 4°C for 4 days followed by 10°C for 12 days; “realistic commercial conditions” (to simulate commercial distribution and retail display (Warton and Wills, 2002)).
- 30°C for 24 hours; “gross abuse” (to assess the consequences of gross temperature abuse).

Samples for the two lower temperature treatments were prepared simultaneously. Samples for the 30°C trial were prepared on a different day and using rocket purchased at retail.

Enumeration
B. cereus group bacteria and ATVC were enumerated at intervals appropriate to each challenge trial. Two bags were selected for enumeration at each sample time.
For the refrigeration treatments, 10g of rocket was weighed out, diluted with 90ml diluent and stomached for two minutes.

For the “gross abuse” trial unexpected weight loss occurred during the incubation, requiring that the dilution procedure be modified slightly. The entire sample (nominally 13 g initially) was stomached in a 1:10 dilution of diluent based on the weight before incubation.

Aliquots (0.1 ml) of appropriate dilutions of samples were spread-plated onto BCSA and NA. BCSA plates were incubated at 37°C for 12 to 15 hours. NA plates were incubated at 25°C for two days.

Un-inoculated rocket was processed as described above to determine background levels of *B. cereus* group bacteria. The inoculum suspension was also tested before dipping and after a series of inoculations to determine whether the concentration of the suspension decreased significantly over time.

Toxigenic Potential

The Tecra® Bacillus Diarrhoeal Enterotoxin detection kit was employed on Dipel® strains, challenge strains and representative trial isolates to test for the presence of a component of one of the diarrheal enterotoxins. Because pure cultures were available, the inoculum used was taken from resuspended colonies rather than through the enrichment procedure normally used.

Differentiation of *B. cereus* and *B. thuringiensis* by 16s rRNA gene sequencing

16s rRNA gene sequencing methods were employed to characterise known strains and to use this information to identify strains present at the completion of challenge trials. Sequencing of *B. cereus* challenge strains, presumptive *B. thuringiensis* strains isolated from Dipel®, and dominant colonies at the end of the trial was undertaken to determine whether growth, if it occurred, was due to growth of *B. cereus*, *B. thuringiensis* or both.

Results

Growth capability of *B. cereus* group on rocket under different storage conditions
Growth of *B. cereus* under the various storage conditions are shown in Figures 1-3.
On day 12 cool room testing revealed temperature of 2.9°C rather than the desired 4°C. Samples were subsequently transferred to an alternative incubator operating at 4°C.

Results from the "ideal storage" trial (Fig. 1) suggest that *B. cereus* group bacteria are incapable of growth on Rocket stored at ~4°C during the expected shelf life. ATVC increased by 1 – 2 log CFU.g^{-1} under the same conditions.

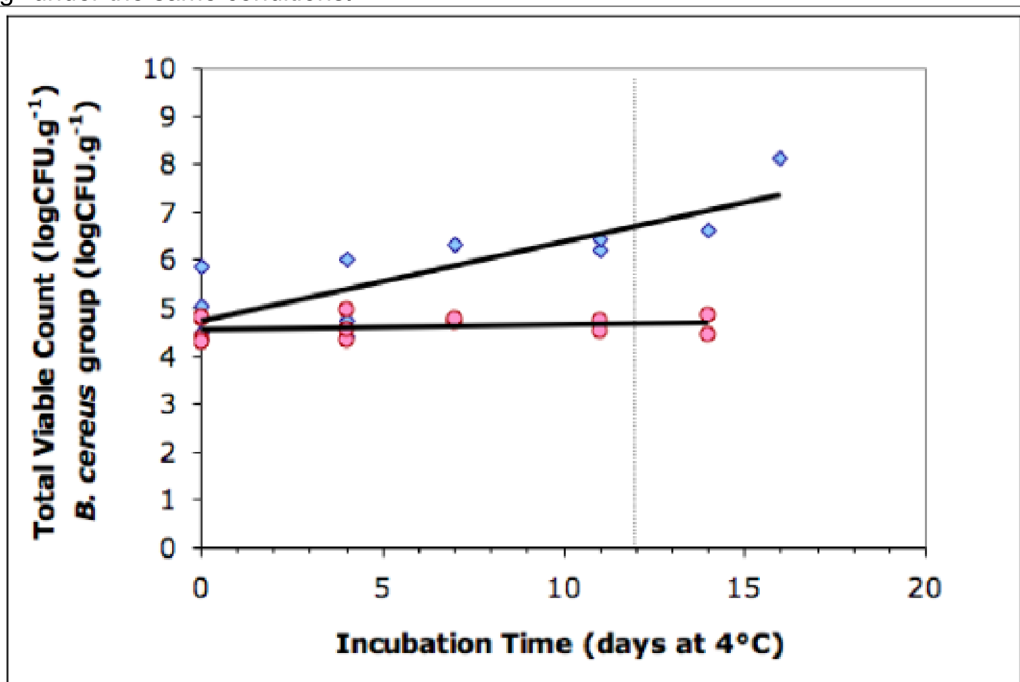

Figure 1: "Ideal storage". Growth of *B. cereus* group (●) and ATVC (■) on Rocket at 2.9°C for 12 days followed by 4°C for 4 days in normal commercial packaging conditions.

The results from the "realistic commercial conditions" trial (Fig. 2) extend the earlier observation that *B. cereus* group bacteria do not grow on rocket within the shelf life of the product, even at ~10°C. ATVC showed an increase of 4-5 log CFU.g^{-1} under the same conditions.

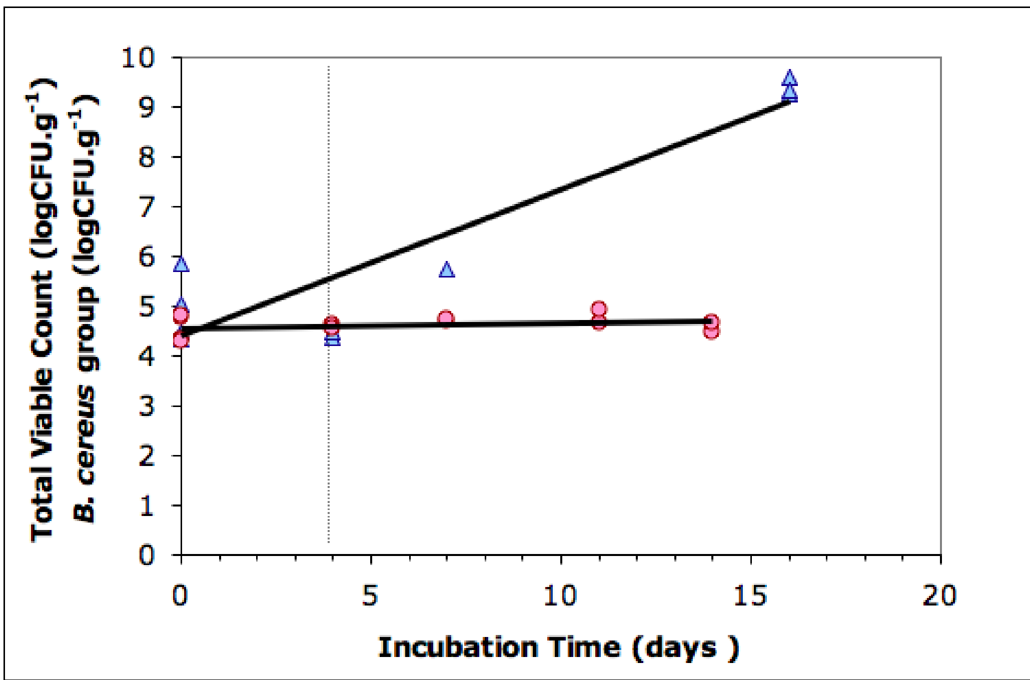

Figure 2: "Realistic commercial conditions". Growth of *B. cereus* group (●) and ATVC (▲) on Rocket at 2.9°C for 4 days followed by 10°C for 12 days in normal commercial packaging condition.

In the "gross abuse" trial (Fig.3), *B. cereus* group bacteria increased by about 0.7 log CFU.g^{-1} in 16 hours at 30°C. Under the same conditions, ATVC increased by ~3 log CFU.g^{-1} within 24 hours.

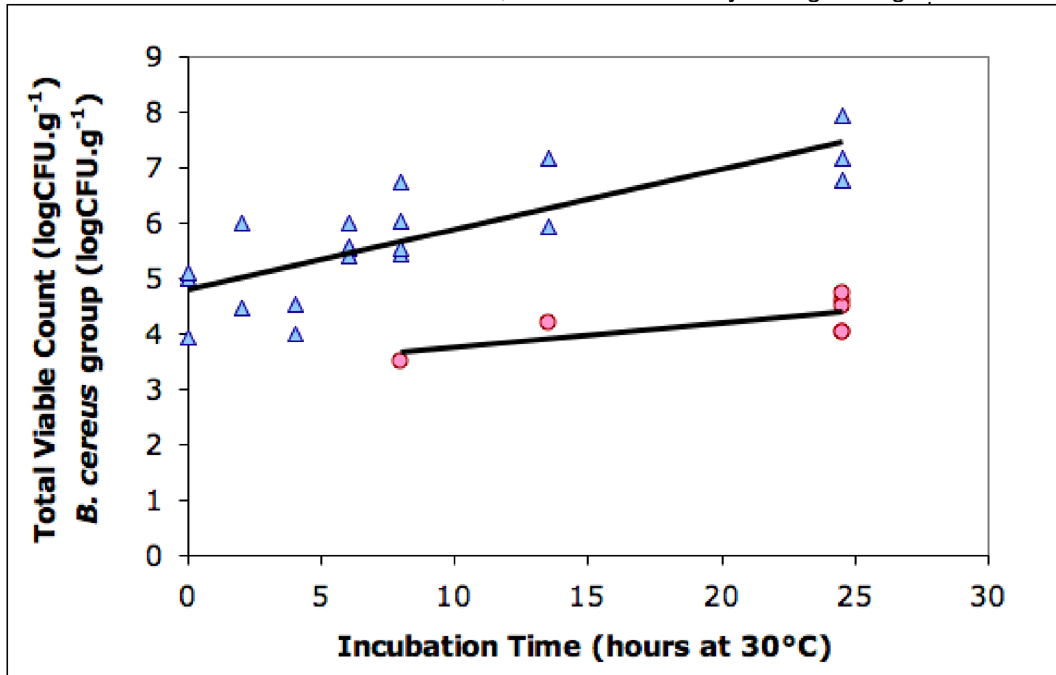

Figure 3: "Gross abuse". Growth of *B. cereus* group and ATVC (▲) on rocket at 30°C for 24 hours in normal commercial packaging.

Uninoculated Controls

On three samples of unprocessed rocket, levels of *Bacillus cereus* group bacteria ranged from 2.7×10^4 to 1.2×10^5 CFU.g^{-1}. Processed samples from a corresponding batch had ~2×10^3 CFU.g^{-1}, while other samples of commercially processed rocket had levels ranging from 4×10^2 to 4×10^3 CFU.g^{-1}.

Toxigenic Potential

The four *B. cereus* challenge strains, all twelve Dipel® isolates, and two representative samples from the end of the storage trials tested positive with the Tecra® immunoassay for Bacillus diarrhoeal enterotoxin.

Differentiation between B. çereus group organisms

Sequencing of 16s rRNA genes, using a number of primers, failed to differentiate the *B. cereus* group strains and species tested.

Discussion

B. cereus group bacteria were isolated from commercially packaged rocket. A comparison of *B. cereus* group numbers on untreated rocket with commercially washed and packaged revealed that a large proportion of *B. cereus* group bacteria are removed during processing, and secondly that a low, but acceptable, level of contamination does occur. These findings complement reports of the ubiquitous nature of *B. cereus* (EFSA, 2004; Murray *et al*. 2002). Using modern methods, we were unable to determine if the *B. cereus*-like bacteria were the pathogen *B. cereus* or *B. thuringiensis* from the Dipel®. This is perhaps unsurprising considering that taxonomists have proposed that they should be merged into a single species (Helgason *et al*., 2000).

We were unable to determine whether the *B. cereus* group bacteria that grew were those inoculated onto the rocket, or contamination already present. It was demonstrated however that *B. cereus* group bacteria can grow on rocket under favourable conditions. Results from the “gross abuse” trial showed a 0.7 log increase over a sixteen hour period. Given normal contamination levels of $<<10^3$cfu.g^{-1} (A.Clark, *pers. comm*., 2005) growth on commercial product would result in levels less than is considered harmful. *B. cereus* has “non fastidious growth requirements” (Murray *et al*., 2002) so it is curious that growth on rocket was so poor. Greater proliferation of *B. cereus group* bacteria was expected. It is possible that the growth of *B. cereus* group bacteria was restricted by a lack of nutrient availability, competition with high levels of other bacteria (Fig. 3), and/or an increase of CO_2 (which inhibits microbial growth) inside the package.

Whether *B. cereus* present on rocket could grow to hazardous levels under “ideal storage” conditions was addressed by incubation at constant 4°C for the shelf life of the product and during “realistic commercial conditions”. No consistent increase in *B. cereus* group numbers was found for either of the commercial temperature regimes. An increase in the ATVC however showed that other bacteria were able to grow (Fig.1, 2). Toward the end of the trial period rocket, under “realistic commercial conditions”, had spoiled with an off odour and viscous yellow exudate noted. At this time, the *B. cereus* group counts had still not increased. Thus the product had spoiled before growth of *B. cereus* could occur. Given the lack of growth it is unlikely that *B. cereus* could grow to hazardous levels during commercial handling of rocket.

16s RNA gene sequencing was unable to differentiate between the *B. cereus* inoculated on to the rocket and *B. thuringiensis* from initial contamination. Differentiation of the species, however,

has little practical relevance. The issue is the toxigenic potential of any *B. cereus* group bacteria encountered. The diarrhoeal enterotoxin is complex and multiple subunits must be present for biological activity. Using the Tecra® immunoassay, which detects one component of one of the Bacillus diarrhoeal enterotoxins, all twelve Dipel® isolates, the four *B. cereus* challenge strains, and representative samples from the end of the storage trial tested positive, indicating possible toxigenesis. Tests such as the ligated rabbit ileal loop assay, tissue culture assays, PCR for toxin genes and immunogel diffusion and aggregate-haemagglutination need to be employed, however, because they ascertain biological activity of the toxins (Jenson and Moir, 2003).

Conclusion

The main aim of this study was to determine if Bacillus cereus on the pre-packaged salad product, rocket, poses a threat to human health. We were unable to distinguish between the B. cereus inoculated onto the product and other B. cereus group bacteria on the product from Dipel® or soil contamination. All isolates tested, however, produced at least one component of one of the toxin complexes responsible for causing diarrhoea in humans. It was found that at 30°C B. cereus group bacteria were able to grow on packaged rocket. Under conditions relevant to commercial storage, distribution and retail display, however, no growth of B. cereus group bacteria was observed. Thus B. cereus on rocket is unlikely to pose a threat to human health unless there is a high initial load and the product is subject to high storage temperatures. Due to the homogeneity of the B. cereus group, the presence of plasmid-borne toxin genes, and widespread use of Dipel® further research should be directed toward determining initial contamination levels and the toxigenic potential of B. cereus group bacteria commonly encountered in the vegetable industry.

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