Review of current literature pertaining to the role of *Bacillus cytotoxicus* as a foodborne hazard

Author: Prof. Sophia Johler, Dipl. ECVPH

Institute for Food Safety and Hygiene

Vetsuisse Faculty

University of Zurich

Email: sophia.johler@uzh.ch

Executive Summary

Bacillus cytotoxicus is a member of the *Bacillus cereus* group. This group comprises closely related organisms of varying pathogenicity including the foodborne pathogen *Bacillus cereus sensu stricto*. Some members of the *B. cereus* group can cause two distinct forms of foodborne illness: Firstly, the diarrheal syndrome that is linked to three enterotoxins - Hbl, Nhe and CytK - and secondly, the emetic syndrome caused by cereulide toxin preformed in food. *B. cytotoxicus* was isolated for the first time in the context of a severe outbreak of diarrheal disease leading to fatalities and was described as a species in 2013. The organism harbors a specific variant of the gene encoding the enterotoxin cytotoxin K designated *cytK-1*. It has been reported that the cytotoxin K variant CytK-1 confers substantially higher toxicity than CytK-2. *B. cytotoxicus* is thermotolerant and might be missed using routine diagnostic screening for *B. cereus* group organisms at 30°C. Targeted screening at temperatures of at least 37°C has shown that dried food matrices containing potato starch very commonly harbor *B. cytotoxicus*. For instance, a recent study found a prevalence of 95% in mashed potato powder sold at retail level in Switzerland. Recent reports from the UK suggest that *B. cytotoxicus* also occurs in association with insect-based foods, which have recently gained popularity. In cytotoxicity assays, the few *B. cytotoxicus* strains that have been characterized to date range from non-toxic to extremely toxic, with toxicity surpassing the toxicity of a *B. cereus* outbreak strain (NVH 0075-95) used as a reference. Information on *B. cytotoxicus* is still limited and it is unclear if the organism's capacity to germinate, produce enterotoxins, and exhibit cytotoxic effects in the intestine is comparable to that of *B. cereus.* To allow for valid conclusions on the role of *B. cytotoxicus* in foodborne outbreaks, routine discrimination of *B. cereus s.s.* and *B. cytotoxicus* is needed. In addition, targeted screening at temperatures of 37-46°C as well as further studies on germination, growth and enterotoxin production under simulated intestinal conditions are essential for a better understanding of the role of *B. cyototxicus* as a foodborne pathogen.

1 The *Bacillus cereus* group

The *B. cereus* group, also designated *B. cereus sensu lato (s.l.),* is a group of Gram positive, facultative anaerobic, endospore forming bacteria that are phylogenetically closely related, but metabolically and ecologically diverse. *B. cereus sensu stricto* as well as *B. anthracis, B. cytotoxicus, B. mycoides, B. pseudomycoides, B. thuringiensis, B. weihenstephanensis,* and *B. toyonensis* are the most prominent species included in the *B. cereus* group (Ehling-Schulz M, Knutsson R, 2011; Okinaka and Keim, 2016). Members of this group range from thermophilic to phsychrophilic, allowing them to survive and grow under a wider variety of environmental conditions (Cairo et al., 2021). As the *B. cereus* group is ubiquitous in nature, it is a natural part of the microflora of various agricultural products (Stenfors Arnesen et al., 2008). While some of the bacteria belonging to this group are used as probiotics or biopesticides, others can lead to severe cases of foodborne diarrheal or emetic disease (Ehling-Schulz et al., 2019; Stenfors Arnesen et al., 2008). The emetic syndrome is caused by cereulide, a heat-stable, acid resistant peptide toxin, which is encoded on a 1.2 kDa plasmid and preformed in food. The diarrheal syndrome is linked to three chromosomally encoded, heat-labile enterotoxins – the Non-hemolytic enterotoxin (Nhe), Hemolysin BL (Hbl), and Cytotoxin K (CytK). Since the capacity for toxin production is broadly distributed among the *B. cereus* group, species other than *B. cereus s.s.* pose a risk for consumers (Johler et al., 2018; Prüß et al., 1999).

2 Characteristics of the species *B. cytotoxicus*

B. cytotoxicus is a Gram-positive, aerobic organism able to produce spores. The species description was based on five strains, four of which were linked to foodborne disease (Guinebretière et al., 2013). *B. cytotoxicus* is thermotolerant and able to grow at temperatures of up to 53°C. Further characteristic features are the absence of starch hydrolysis and the dependency from tryptophan for growth. *B. cytotoxicus* also characteristically harbors the *cytK-1* variant of the cytotoxin K gene (Guinebretière et al., 2013). Cytotoxin K is a pore-forming 34 kDa enterotoxin with necrotic and hemolytic activity (Fagerlund et al., 2007). The CytK-1 variant was reported to be more cytotoxic than the CytK-2 variant occurring in mesophilic *B. cereus* group species (Fagerlund et al., 2004).

3 *B. cytotoxicus* strains are missed by routine diagnostic screening of foods

Routine diagnostic screening of foods for members of the *B. cereus* group is executed at an incubation temperature of 30°C targeted at mesophilic *B. cereus* group species (EN ISO 7932, General guidance for the enumeration of *Bacillus cereus* in foods). If colonies showing a phenotype consistent with the *B. cereus* group are detected, these are commonly not further differentiated but subsumed under the term "presumptive *B. cereus*" (Ehling-Schulz and Messelhäusser, 2013).

Protocols that have successfully been used to screen for *B. cyototoxicus* employ enrichment in buffered peptone water or CGY medium at 37°C or 46°C, with subsequent plating on Mossel or alternatively sheep blood agar (Burtscher et al., 2021; Contzen et al., 2014). *B. cyototoxicus* will exhibit the same phenotype as *B. cereus sensu stricto*, with dry and rough colonies that on MYP (Mossel) agar are surrounded by pink zones due to egg yolk precipitation. Differentiation of *B. cytotoxicus* from other members of the *B. cereus* group can be achieved by PCR screening for the *cytK-1* gene (Guinebretière et al., 2013).

4 Foods linked to *B. cytotoxicus*

The first isolation of *B. cytotoxicus* was documented for vegetable puree in France in 1998 during an outbreak of diarrheal disease. A total of 44 persons became ill and three persons died (Lund et al., 2000). To date, all reported outbreaks related to *B. cytotoxicus* have been exclusively linked to plant-based food matrices (Cairo et al., 2021). Potato and products thereof (rehydrated mashed potatoes, dough for potato dumplings, potato chips, potato flour, instant soups), and in particular dried food matrices containing potato starch were frequently linked to *B. cyototoxicus* (Burtscher et al., 2021; Contzen et al., 2014; N. Heini et al., 2018). The organism has recently also been reported in association with insect-based foods (Cairo et al., 2021).

5 Hazardous potential of *B. cytotoxicus*

Several factors are crucial in determining the risk of foodborne diarrheal disease emanating from *B. cytotoxicus* strains:

a) Occurrence of B. cytotoxicus in foodstuff

There is only very limited data on the occurrence of *B. cytotoxicus* in foods. Food samples found positive for *B. cytotoxicus* that were collected after an outbreak include vegetable puree (Glasset et al., 2016; Lund et al., 2000), potato puree/ potato products (Fagerlund et al., 2007; Glasset et al., 2016; Rau et al., 2009), as well as semolina, mashed celery, and mashed fish (Glasset et al., 2016).

In Switzerland, *B. cytotoxicus* was identified in mashed potato powder samples with a prevalence of 45-95% (Burtscher et al., 2021; N. Heini et al., 2018). No *B. cytotoxicus* were found when screening flour products (Kindle et al., 2019), powdered infant formula and fruit powder (N. Heini et al., 2018). Contzen et al. (Contzen et al., 2014) detected *B. cytotoxicus* in 88% of mashed potato powders, flakes, and granules tested in Germany.

In the UK, food business operators notified the UK Food Standards Agency ten times within three years of potential *B. cereus* group contamination incidents with two out of these ten being linked to *B. cytotoxicus* (Cairo et al., 2021). The incidents took place in November 2019 (9.6 x 10^5 CFU/g) and December 2020 (1.9-5.4 x 10^6 CFU/g) and were associated with ambient food products that were made of insect flour (Cairo et al., 2021). These were the first reports of *B. cytotoxicus* in the UK, which led Cairo et al. to speculate that this might either indicate a lack of routine surveillance and/or a change in prevalence of food contamination (Cairo et al., 2021). Fasolato et al. (Fasolato et al., 2018) screened pre-packaged shelf-stable edible insects (n = 32) ordered online for foodborne pathogens and reported that out of 39 *B. cereus* group isolates detected in this study, 25 (64%) were assigned to *B. cytotoxicus* based on *pycA* sequence-based clustering.

b) Outbreaks associated with B. cytotoxicus

While most foodborne diseases caused by *B. cereus* group species are associated with bacterial counts above 10^5 CFU/g, rare cases of diarrheal disease associated with 10^3 CFU/g have been reported (EFSA BIOHAZ Panel, 2016). The *B. cytotoxicus* outbreak in a nursing home in France in 1998 had been caused by vegetable puree that exhibited 3.0 x 10^5 CFU of *B. cytotoxicus* per gram food. A total of 44 cases were reported, with six patients suffering from bloody diarrhea and with three fatalities due to necrotic enteritis, making this outbreak the most severe *B. cereus s.l.* related outbreak in France (Cairo et

al., 2021; Lund et al., 2000). Glasset and coworkers characterized isolates collected in association with foodborne outbreaks in France between 2007 and 2014 and reported that seven outbreaks were linked to *B. cytotoxicus* (Glasset et al., 2016). Linked to an outbreak in a school canteen with 61 cases of foodborne illness, strain AFSSA 08CEB44 BAC was detected at counts of 10^4 CFU/g in semolina. Other incidents took place in medico-social institutes and retirement homes, leisure centers, and centers for the disabled. Reported counts of *B. cyototoxicus* detected ranged from 4.0×10^2 to 9.2×10^5 CFU/g. Further outbreaks occurred in Germany and France in association with *B. cytotoxicus* strains CVUAS 2833 (Rau et al., 2009) and INRA AF2 (Fagerlund et al., 2007), respectively.

c) Prevalence of toxin genes and amounts of enterotoxins synthesized

Not only *cytK-1,* but also the *nhe* gene encoding enterotoxin Nhe is present in all *B. cytotoxicus. B. cytotoxicus* strains harbor a variant of the *nhe* gene that is missed by commonly used *nhe* primers and requires the use of nhe_{Bcyt} primers that were specifically designed to screen for *nhe* in *B. cytotoxicus* (Burtscher et al., 2021). In contrast, *B. cytotoxicus* strains seem to lack *hbl* encoding the Hbl enterotoxin and to date, no *B. cytotoxicus* strain harboring *ces* encoding cereulide has been reported (Fasolato et al., 2018; Stevens et al., 2019). The amount of Nhe and CytK-1 expressed by different *B. cytotoxicus* strains can vary substantially (Burtscher et al., 2021; N. Heini et al., 2018) and the number of strains for which cytotoxicity data is available is still very low. Presence of *cytK-1* alone is not indicative of highly toxic strains (Burtscher et al., 2021; Fagerlund et al., 2007). Still, occasionally extremely toxic isolates are being described such as NVH391-98 (Lund et al., 2000), INRA AF2 (Fagerlund et al., 2007), and CH_213 (Nicole Heini et al., 2018).

d) Growth/ inactivation under conditions encountered during food production and preparation

Only very limited data on growth or inactivation of *B. cytotoxicus* under conditions encountered during food production and preservation is available. The level of *B.*

cytotoxicus detected in foods is usually ≤ 10^2 CFU/g (Contzen et al., 2014; Koné et al., 2019). Levels of *B. cytotoxicus* in potato puree kept at room temperature were shown to increase from ≤ 10^2 CFU/g to 10^5 CFU/g within two days (Koné et al., 2019).

Due to their resistance against heat and desiccation, spores of *Bacillus* spp. can survive stresses during food production such as pasteurization or heating (EFSA BIOHAZ Panel, 2016). In order to avoid germination of spores and bacterial growth, the German Federal Institute for Risk Assessment recommends heating of foods to at least 70°C for 2 min, followed by either quick cooling to ≤ 7°C or by keeping them at ≥ 60°C (German Federal Institute for Risk Assessment, 2020).

e) Germination, motility, and enterotoxin production under intestinal conditions

The diarrheal syndrome is elicited when a member of the *B. cereus* group produces enterotoxins in the intestine. For this to happen, spores of the causative strain need to survive the stomach passage (e.g., in form of a spore), germinate, actively move and produce enterotoxins under conditions encountered in the intestine. Pronounced strain-dependent differences in germination patterns and motility were reported for other species of the *B. cereus* group, with some strains exhibiting the ability to germinate under simulated intestinal conditions and to actively move (Schwenk et al., 2020).

Filamentous motility has been shown for *B. cytotoxicus* strain DSM 22905 grown on 10% skim milk and whole blood absorbed onto 1.5% Mueller Hinton agar plates (Liu et al., 2020). However, this *B. cytotoxicus* strain did not show motility when grown on metabolically inactive lawns of *Campylobacter jejuni*, in contrast to other *B. cereus* group strains *B. cereus* ATCC 14579, *B. thuringiensis* DSM 2046, *B. wiedmannii* FSL W8-0169, and *B. weihenstephanensis* DSM 11821.

A recent study by Koné et al. demonstrated that CytK-1 is responsible for 90% of the cytotoxic effect of *B. cytotoxicus* supernatants on Caco-2 cells (Koné et al., 2021). To date, there is no data on the ability of *B. cytotoxicus* strains to germinate, actively move and produce enterotoxins under intestinal conditions.

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