

## REVIEW ARTICLE

# *Bacillus cytotoxicus*—A potentially virulent food-associated microbe

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Email: jessica.cairo@food.gov.uk**Abstract**

*Bacillus cytotoxicus* is a member of the *Bacillus cereus* group with the ability to grow at high temperatures (up to 52°C) and to synthesize cytotoxin K-1, a diarrhoeagenic cytotoxin, which appears to be unique to this species and more cytotoxic than the cytotoxin K-2 produced by other members of this group. Only a few isolates of this species have been characterized with regard to their cytotoxic effects, and the role of cytotoxin K-1 as a causative agent of food poisoning remains largely unclear. *Bacillus cytotoxicus* was initially isolated from a food-borne outbreak, which led to three deaths, and the organism has since been linked to other outbreaks all involving plant-based food matrices. Other studies, as well as food-borne incidents reported to the UK Food Standards Agency, detected *B. cytotoxicus* in insect-related products and in dried food products. With insect-related food becoming increasingly popular, the association with this pathogen is concerning, requiring further investigation and evidence to protect public health. This review summarizes the current knowledge around *B. cytotoxicus* and highlights gaps in the literature from a food safety perspective.

**KEYWORDS***Bacillus cytotoxicus*, cytotoxin K, food poisoning, food safety, food-borne pathogen**INTRODUCTION**

The *Bacillus cereus* group comprises eight formally recognized species: *Bacillus anthracis*, *Bacillus cereus sensu stricto*, *Bacillus cytotoxicus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, *Bacillus toyonensis* and *Bacillus weihenstephanensis* (Ehling-Schulz et al., 2019). Members of the *B. cereus* group are Gram-positive, aerobic, spore-forming bacteria commonly found in soil, dust, unprocessed dried plant material and foods such as rice, pasta and pastry (Auger et al., 2008). Species in the *B. cereus* group range from psychrophilic to thermophilic and are metabolically diverse, which enables them to survive in a wide range of environments.

Some species of the *B. cereus* group, including *B. cereus sensu stricto* and *B. thuringiensis*, are pathogenic and are responsible for gastrointestinal diseases manifested by diarrhoeic or emetic syndromes. The diarrhoeal type of food poisoning is caused by three chromosomally encoded toxins (non-haemolytic enterotoxin [Nhe], haemolysin BL and cytotoxin K [CytK]), which are produced during vegetative growth in the gastrointestinal tract, whereas the emetic disease is caused by a plasmid-encoded cyclic peptide (cereulide), produced while the bacteria are growing in food (Stenfors Arnesen et al., 2008). Symptoms arising from diarrhoeal and emetic toxins usually resolve within 24 h from onset (Tewari & Abdullah, 2015).

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*Bacillus cereus* is a concern in the food industry as it is a ubiquitous microbe in the environment and can easily contaminate food production or processing systems. Spores can survive pasteurization used to reduce levels of vegetative pathogens in food (Soni et al., 2016). Food business operators have official controls in place to guarantee the safety of food and are meant to notify the UK Food Standards Agency (FSA) of potential contamination incidents. In the last 3 years up to April 2021, the FSA has been notified of 10 food contamination incidents linked to *Bacillus* spp., of which two were linked to the detection of *B. cytotoxicus*. These two distinct incidents, from November 2019 to December 2020, both involved ambient food products made of insect flour. There had been no previous notifications to the FSA of incidents involving this pathogen, possibly reflecting a lack of routine surveillance and/or a change in the prevalence of food contamination. For this reason, a literature review was undertaken to collate the data available on *B. cytotoxicus* from a food safety perspective.

## METHODS

The literature search was performed on April 2021 with no earlier cut-off date. Three databases were searched to retrieve relevant literature. These were databases maintained by EBSCO: Food Science Source, PubMed and Scopus, which broadly returned similar results. The keyword used was '*Bacillus cytotoxicus*'. The known strains of the *B. cytotoxicus* cluster were also used as keywords, but none of these search terms reported new papers. Any results that did not contain at least one of the keywords '*Bacillus cytotoxicus*', 'cytotoxin K', 'thermotolerant', 'toxin gene', 'toxicity' and '*Bacillus cereus* group' in the abstract were excluded. Following title screening, a more specific screen of the abstracts and of the full text of the papers was performed manually to determine suitability for inclusion. This process was performed independently by two FSA microbiologists. Papers considered irrelevant, because they did not include information on *B. cytotoxicus*, were excluded based on reviewer interpretation. In case of disagreements, papers were discussed until a consensus was achieved.

## RESULTS

### Molecular and biochemical characteristics of *Bacillus cytotoxicus*

#### Genome size

The genome size of *B. cereus* group strains has been reported to be 5.2–5.4 Mb (Lapidus et al., 2008), which is notably larger than the genome size of *B. cytotoxicus* strain NVH

391-98 of 4.085 Mb with a circular plasmid of 7136 bp (Lapidus et al., 2008). A comparison of the chromosomal map of a *B. cereus* reference strain (ATCC 14579) and NVH 391-98 showed that the genome of the NVH 391-98 strain is collinear with that of ATCC 14579, with exception in some areas where only 39% of genes are conserved (Lapidus et al., 2008). However, the functions of these divergent areas of the genome are unclear.

#### Plasmid content

The *B. cytotoxicus* strain NVH 391-98 possesses a circular plasmid of 7136 bp (Lapidus et al., 2008). The 10 strains of *B. cytotoxicus* examined by Stevens et al. (2019) also contain one or more plasmids of size between 14 and 83 kb, but no plasmid-like sequences have been identified in the genomes of *B. cytotoxicus* strains AFSSA 08CEB44 BAC, NVH 883-00 and CVUAS 2833 (Stevens et al., 2019). It is possible that this could be due to poor assembly or loss during the bacterial isolation. The 18 plasmids from 11 *B. cytotoxicus* strains studied by Stevens et al. encode 1258 genes with over 50 different functions. The varied plasmid content of *B. cytotoxicus* strains show that it is flexible in exchanging genes and quick at adapting to its environment. Moreover, Stevens et al. suggested that the distribution of plasmids in strains from the different clades is related to horizontal gene transfer in *B. cytotoxicus*. Genes conferring resistance to antibiotic agents or encoding virulence factors have not been identified on any of the plasmids studied so far.

#### Cytotoxin K

Cytotoxin K is a single-component toxin of 34 kDa that belongs to the family of  $\beta$ -barrel pore-forming toxins. It has been shown that CytK exists in two different variants with 89% identity in the amino acid sequence (Ceuppens et al., 2013). The first CytK discovered, CytK-1, displayed stronger haemolytic and cytotoxic activity towards human intestinal cells (Caco-2 cells and Vero cells) than the second variant CytK-2 (Böhm et al., 2015a, 2015b; Ceuppens et al., 2013; Hardy et al., 2001; Lund et al., 2000). Indeed, Böhm et al. (2015a, 2015b) showed that, when expressed, the cytotoxic activity of the CytK-2 protein from a *B. cereus* strain (1230–88) was five times lower than that of the original CytK-1. All strains of the *B. cytotoxicus* cluster carry the *cytK-1* gene, as opposed to *B. cereus* strains, which harbour the *cytK-2* gene variant (Fagerlund et al., 2004, 2007; Guinebretière et al., 2013).

The enterotoxin CytK-1 was initially isolated from the strain NVH 391-98 (Lund et al., 2000) and has also been isolated from the INRA AF2 strain at high levels (Castiaux et al., 2015; Fagerlund et al., 2007). *Bacillus cytotoxicus* NVH

883-00 possesses the *cytK-1* toxin gene in its genome, but it is not cytotoxic due to the lack of CytK-1 expression (Ceuppens et al., 2013). The characterization of strain NVH 883-00 indicates that the presence of this gene is not on its own sufficient to assess the cytotoxicity of a strain. It remains unclear which factors are responsible for the lack of CytK-1 expression.

More recently, Burtscher et al. (2021) investigated the cytotoxicity of *B. cytotoxicus* strains isolated from 19 mashed potato powder samples. Strains, isolated from 8 out of 19 samples, showed no cytotoxic effect against Vero cells. The remaining isolates exhibited cytotoxic effects but were considered to be low level enterotoxin producers compared with the reference strain *B. cereus* NVH 0075-95 as described by Johler et al. (2018). Burtscher et al. hypothesized that the cytotoxicity of this species may have been overestimated; however, information about the cytotoxicity of *B. cytotoxicus* is still extremely limited.

The presence of the *cytK-1* gene has been exploited for a PCR diagnostic method that is able to detect *B. cytotoxicus* strains and has been validated by screening numerous *B. cereus* group strains (Guinebretière et al., 2013).

## Non-haemolytic toxin genes

The Nhe is one of the three chromosomally encoded toxins produced by the members of the *B. cereus* group. To capture the genetic and phenotypic diversity of strains within the *B. cereus* group, Miller et al. (2018) compiled a collection of *B. cereus* group isolates obtained from milk and dairy-associated environments throughout the north-eastern United States between 2005 and 2016. Overall, 51 out of 52 isolates tested contained at least one of three *nhe* genes. By contrast, the *B. cytotoxicus* type strain NVH 391-98 was negative for all three *nhe* genes.

Another study reported the presence of the *nhe* gene variant in *B. cytotoxicus* strains, which yields only about 80% identity in protein sequence compared with the Nhe toxin in other *B. cereus* group strains (Burtscher et al., 2021). The detection of this *nhe* gene variant was carried out using different primers (*nhe*<sub>Bcyt</sub>), and this explains why in previous studies (Auger et al., 2008; Böhm et al., 2015a, 2015b; Lapidus et al., 2008) that used standard primers targeting the *nhe* gene of *B. cereus* strains did not detect the Nhe toxin in *B. cytotoxicus* isolates. This *nhe* gene variant could account for potential cytotoxicity seen in some *B. cytotoxicus* strains. However, there are limited data available in the literature on this gene, and further investigation would be beneficial.

## Hydroxyphenylalanine operon

Stevens et al. (2019) found that the *B. cytotoxicus* species possesses a complete hydroxyphenylalanine (*hpa*) operon

that is absent in all other *B. cereus* group members. The presence of the *hpa* operon suggests that the bacterium can utilize aromatic compounds, including tyrosine, as a carbon source and potentially inhibit Gram-negatives in the intestine by producing the inhibiting agent para-cresol. Furthermore, *hpa*-encoded pathways are often associated with soil bacteria, thus indicating soil as the ecological niche of *B. cytotoxicus*, although the activity of the *hpa* operon and its role in the metabolism of *B. cytotoxicus* remain to be elucidated (Burtscher et al., 2021).

## Genes encoding the sigma transcription factors

Another important feature of *B. cytotoxicus*, compared with other *B. cereus* group strains, is the apparent absence of genes encoding the alternative transcription factor sigma B ( $\sigma^B$ ) and related regulatory proteins. Sigma B has been found in all other sporulating *Bacillus* spp. that have been examined and has been intensively studied in *Bacillus subtilis* (Lapidus et al., 2008). Some studies, using a *B. cereus* reference strain (14579), reported the importance of  $\sigma^B$  in stress response (Lapidus et al., 2008; Schaik & Abee, 2005). Two other sigma factors ( $\sigma^F$  and  $\sigma^G$ ), involved in regulation of the middle stage of sporulation, were detected in each member of the *B. cereus* group, including in *B. cytotoxicus* NVH 391-98 (Lapidus et al., 2008). The absence of genes encoding the  $\sigma^B$  in NVH 391-98 may indicate that this stress regulation system is a recent acquisition in the *B. cereus* group, or it has been recently lost in *B. cytotoxicus* NVH 391-98 (Lapidus et al., 2008). Whether the genes encoding the alternative transcription factor  $\sigma^B$  and related regulatory proteins are absent in other *B. cytotoxicus* strains remains unclear. Further investigation would be beneficial to understand the difference in stress response between *B. cytotoxicus* and other *B. cereus* group species, given the absence of genes encoding the  $\sigma^B$  in *B. cytotoxicus*.

## Tryptophan biosynthesis operon

The tryptophan biosynthesis operon (*trp*) genes are absent in all *B. cytotoxicus* strains, which have been studied. All other known representatives of the *B. cereus* group, as well as other *Bacillus* species, possess *trp* genes, which enable the bacterium to grow without tryptophan in the medium. This could be a phenotypic characteristic, which differentiates them from all other members of the *B. cereus* group with available genome sequences (Lapidus et al., 2008). The physiological significance of the absence of the tryptophan biosynthesis operon remains to be clarified.

## Proteolytic and keratinolytic capacities

Cavello et al. (2018, 2020) studied the ability of different thermophilic bacteria to degrade chicken feather waste and reported that compared with other aerobic keratinolytic bacteria, the *B. cytotoxicus* isolates from soil exhibited a higher hydrolysis rate of feathers. The expression of keratinolytic protease in *B. cytotoxicus* was inducible and occurred only in the presence of feathers as an exogenous inducer when incubated at 50°C (Cavello et al., 2018, 2020).

## Fatty acid profile

The fatty acid composition of the cell membrane varies according to environmental conditions, as it is involved in bacterial adaptation to environmental changes such as temperature, pressure and oxygen availability. The majority of the fatty acids identified in *B. cytotoxicus* strain NVH 391-98 are typically found in the *Bacillus* genus. However, lower amounts of anteiso-C<sub>15:0</sub> and the presence of particular fatty acids (iso-C<sub>12:0</sub>, iso-C<sub>13:0</sub> and anteiso-C<sub>13:0</sub>) are specific to the *B. cereus* group, supporting the affiliation of NVH 391-98 to the *B. cereus* group (Guinebretière et al., 2013). Diomandé et al. (2015) reported that the fatty acid profile of *B. cytotoxicus* NVH 391-98 is highly specific compared with that of the *B. cereus* phylogenetic groups I–VI: high proportion of iso-C<sub>15:0</sub> to that of iso-C<sub>13:0</sub> (i15/i13 ratio), the absence of the unsaturated fatty acids C<sub>16:1</sub> and the absence of the desaturase DesA. The fatty acids play a role in membrane fluidity and the consequent temperature tolerance (Los & Murata, 2004). Brillard et al. (2010) showed that a mutant *B. cereus* strain that could not grow at low temperatures had a much higher i15/i13 ratio than the wild type, and Diomandé et al. found that the percentage of unsaturated C<sub>16:1(5)</sub> in the membrane decreased with the thermotolerance of the *B. cereus* phylogenetic groups. Therefore, the high i15/i13 ratio and absence of the unsaturated fatty acids C<sub>16:1</sub> reported in *B. cytotoxicus* NVH 391-98 could be responsible for its thermotolerant strain characteristic.

## Cell surface carbohydrates

The cell surface of many pathogenic bacteria is composed of diverse and complex carbohydrate structures, some of which are known virulence factors. The nucleotide-sugar biosynthetic pathways in different bacterial species can provide an insight on the richness of diverse glycans and their importance in the cell adapting to diverse environments. Indeed, different *B. cereus* peptidoglycans and glycoproteins have been isolated, some of which are reported to play a role in spore formation and infection (Tocheva et al., 2016).

Gu et al. (2010) reported the first identification and characterization of two genes involved in the biosynthesis of glycan precursors (i.e., nucleotide sugars), such as the novel UDP-2-acetimido-2deoxyxylose, from *B. cytotoxicus* NVH 391–98. The identification of these enzymes provides insight into the formation of a new UDP-amino sugar, and similar genes have been detected in other *Bacillus* spp., such as *B. weihenstephanensis* and *B. subtilis* (Broach et al., 2012; Gu et al., 2010). However, the role of UDP-2-acetimido-2deoxyxylose, specifically in the *B. cytotoxicus* NVH 391–98 strain, remains to be determined, and further investigation would be beneficial.

## Growth temperature

*Bacillus cereus* group strains can be classified in seven phylogenetic groups (I–VII) based on their growth temperature (Guinebretière et al., 2013, Bundesinstitut Für Risikobewertung, 2020). *Bacillus cytotoxicus* strains have been shown to grow at temperatures between 18 and 52°C (Afchain et al., 2008; Auger et al., 2008; Carlin et al., 2013; Cavello et al., 2020), whereas strains of other *B. cereus* group species are reported to grow between 5 and 45°C (Guinebretière et al., 2013). Another study detected *B. cytotoxicus* strains from a mixture of water and biofilm samples collected from the Domuyo geothermal area in the Neuquen Province (Argentina) at a temperature around 50–60°C (Cavello et al., 2020). This characteristic has led to *B. cytotoxicus* strains being defined as the sole thermotolerant species within the *B. cereus* group (Guinebretière et al., 2013). The properties of a food and its environment include many intrinsic and extrinsic factors that can differ from those present under controlled laboratory conditions, and further work is required to study the behaviour of *B. cytotoxicus* strains in real food systems.

## Spores

Like other *B. cereus* group strains, *B. cytotoxicus* is capable of sporulation, a property that might allow the bacteria to persist on industrial surfaces and thus potentially contaminate food (Guinebretière et al., 2013). Luu-Thi et al. (2014) studied the thermal inactivation parameters of spores from *B. cereus* strains belonging to the seven phylogenetic groups. They found a large variation in heat resistance, with spores from psychrotrophic group VI strains being generally more heat sensitive particularly when compared with the moderately thermophilic group VII. The findings also indicated a potential for overestimation of the inactivation of *B. cytotoxicus* spores at temperatures below the reference temperature, which is generally 90°C for *B. cereus* spores.



## *Bacillus cytotoxicus* as a food-associated microbe

In total, 16 distinct isolates of *B. cytotoxicus* have been cited in the literature (NVH 391-98, INRA AF2, AFSSA 08CEB44 BAC, NVH 883-00, CVUAS 2833, CH\_1, CH\_2, CH\_3, CH\_13, CH\_4, CH\_15, CH\_23, CH\_25, CH\_38, CH\_39 and CH\_213), and four of these (NVH 391-98, INRA AF2, AFSSA 08CEB44 BAC and CVUAS 2833) were also linked to food poisoning outbreaks (Table 1). Strains of *B. cytotoxicus* from various food sources have been reported from France, Germany, Norway and Switzerland. *Bacillus cytotoxicus* strains have been compared by Stevens et al. (2019) and Carroll et al. (2020) and are considered members of a single clonal complex.

### Food poisoning outbreaks linked to *Bacillus cytotoxicus* strains

The first food poisoning outbreak linked to a *B. cytotoxicus* strain occurred in a nursing home in France in 1998. The strain NVH 391-98 was isolated from a vegetable puree sample at a level of  $3.0 \times 10^5 \text{ g}^{-1}$ . This outbreak reported 44 cases of illness, including six cases of bloody diarrhoea and three deaths from necrotic enteritis (Lund et al., 2000). This represented the most severe known case of diarrhoeal food poisoning in France caused by a presumed *B. cereus* group strain. The diarrhoeic enterotoxin, CytK-1, was identified, following work by Lund et al. (2000), and in 2013, multilocus sequence typing and 16S rRNA sequence comparisons classed NVH 391-98 within the novel species *B. cytotoxicus* (Guinebretière et al., 2013).

Epidemiological and microbiological characterization of food-borne outbreaks that occurred in France between 2007 and 2014 showed that seven food poisoning outbreaks were linked to *B. cytotoxicus* (Glasset et al., 2016). These occurred in school or staff canteens and medico-social institutes such as centres for disabled people, leisure centres and retirement homes. *Bacillus cytotoxicus* was isolated from mashed and boiled potatoes, mashed celery, mashed fish and semolina, and the levels of *B. cytotoxicus* detected were between  $4.0 \times 10^2$  and  $9.20 \times 10^5 \text{ CFU g}^{-1}$  (Glasset et al., 2016). One of these food poisoning outbreaks was linked to the strain AFSSA 08CEB44 BAC, which was isolated in cooked semolina at a level of  $10^4 \text{ CFU per gram}$  and linked to 61 cases of food-borne disease in a school canteen in 2008 (Glasset et al., 2016).

*Bacillus cytotoxicus* strains INRA AF2 and CVUAS 2833 were isolated from potato puree linked to food-borne disease in France in 2003 and in Germany in 2007, respectively (Fagerlund et al., 2007; Rau et al., 2009). The number of cases involved in these two outbreaks is unknown.

### Further detections of *Bacillus cytotoxicus* in food

Several papers have started to study and isolate, or attempt to isolate, *B. cytotoxicus* from different food matrices. A summary of these studies is shown in Table 2. To date, *B. cytotoxicus* has been found in potatoes and potato products (Burtscher et al., 2021; Contzen et al., 2014; Fagerlund et al., 2007; Glasset et al., 2016; Heini et al., 2018; Rau et al., 2009), semolina (Guinebretière et al., 2013), dried food products (Kone et al., 2019; Lund et al., 2000) and edible dried insects (Fasolato et al., 2018).

**TABLE 1** *Bacillus cytotoxicus* strains identified in food or food-borne outbreaks from the first detection in 1998 to March 2021

Strains	Origin and year of isolation	CYTK-1 toxin detection	References
NVH 391-98	Vegetable puree, France, 1998 (related to food poisoning)	+	Lund et al. (2000)
INRA AF2	Potato puree, France, 2003 (related to food poisoning)	+	Fagerlund et al. (2007)
NVH 883-00	Spices, Norway, 2000	–	Fagerlund et al. (2007)
AFSSA 08CEB44 BAC	Semolina, France, 2008 (related to food poisoning)	NK	Guinebretière et al. (2013)
CVUAS 2833	Potato puree, Germany, 2007 (related to food poisoning)	NK	Rau et al. (2009)
CH_1, CH_2, CH_3, CH_13, CH_4, CH_15, CH_23, CH_25, CH_38, CH_39	Mashed potatoes, Switzerland, 2014	NK	Stevens et al. (2019)
CH_213	Mashed potato powder, Switzerland, 2017	NK	Heini et al. (2018) and Stevens and Jöhler (2020)

Abbreviation: NK, not known.

The occurrence of *B. cytotoxicus* in commercial food products containing potatoes or other dehydrated food products was not exceeding concentrations of  $3.0 \times 10^2$  CFU per gram (Contzen et al., 2014, Kone et al., 2019), which is lower than the level of  $10^5$  CFU per gram isolated in France from food-related outbreaks, although the cooking and storage conditions are unknown. *Bacillus cytotoxicus* was not detected in some preheated foods made directly from raw potatoes, like fried potatoes or potato salad (Contzen et al., 2014; Heini et al., 2018).

Further studies have investigated the presence of *B. cytotoxicus* in spices and herbs, flour and dough products and soil and rice products (Bağcıoğlu et al., 2019; Frentzel et al., 2016; Kindle et al., 2019). All studies reported the use of a PCR diagnostic method to detect the presence of the *cytK-1* gene, but none of the tested isolates carried the *cytK-1* variant characteristic of *B. cytotoxicus*.

In the last 3 years up to March 2021, the UK FSA has been notified of 10 food contamination incidents linked to *Bacillus* spp., of which two were linked to the detection of *B. cytotoxicus*. The first was in November 2019 following the detection of *B. cytotoxicus* at a level of  $9.6 \times 10^5$  CFU per gram in a protein bar made with insect flour and the second related to an incident reported in January 2020 where *B. cytotoxicus* was detected at levels of  $1.9\text{--}5.4 \times 10^6$  CFU per gram in various food products made of insect flour. However, a linkage between these two incidents was not reported.

## DISCUSSION

### Molecular and biochemical characteristics of *Bacillus cytotoxicus*

Up until April 2021, 16 isolates have been formally classified in the literature as *B. cytotoxicus* strains. The main characteristic of *B. cytotoxicus* is the presence of the *cytK-1* gene, which is unique for this species. Since the first detection of *B. cytotoxicus* NVH 391-98 in 1998, many authors have focused on this strain, and therefore, there are limited data available on the molecular and biochemical characteristics of the other strains. Some strains of *B. cytotoxicus* (NVH 391-98 and INRA AF2) express high levels of the toxin CytK-1 and have been shown to be highly cytotoxic towards Vero cells (Fagerlund et al., 2007; Lund et al., 2000), whereas NVH 883-00 is not cytotoxic due to the lack of expression of CytK-1, despite carrying the *cytK-1* gene (Ceuppens et al., 2013). Indeed, these two more cytotoxic strains have been associated with two out of four outbreaks where the *B. cytotoxicus* strain was known (Table 1). A recent study showed low levels of cytotoxic effects in *B. cytotoxicus* strains against Vero cells, and the author hypothesized that the cytotoxicity of this species may have been overestimated (Burtscher et al.,

2021). Further research is crucial to identify which factors lead to the lack of CytK-1 expression and to evaluate the cytotoxicity of *B. cytotoxicus* strains.

It is important to consider that several other parameters other than cytotoxicity may contribute to food poisoning caused by *B. cytotoxicus*, including storage conditions of the consumed food product, its intrinsic properties and characteristics of the strain such as virulence factors, secreted enzymes, adhesion and spore germination. However, evidence on these virulence factors is limited, and the findings reported by Luu-Thi et al. (2014) suggest that the thermal inactivation of *B. cytotoxicus* spores is overestimated. This is important as current processing methods may be insufficient to eliminate spores. Studies looking at the thermal stability of CytK-1 and the effect of pH and water activity on growth, survival and toxin production would be beneficial. Further characterization of the *nhe* gene variant would shed light on the potential cytotoxicity seen in some *B. cytotoxicus* strains. Moreover, no studies have so far focused on antimicrobial resistance.

The extent of *B. cytotoxicus* contamination of food or in the wider environment is currently unknown, and further studies applying appropriate methodology to isolate and distinguish *B. cytotoxicus* isolates from other members of the *B. cereus* group are required. The standard method for differentiating *B. cytotoxicus* from other *B. cereus* group isolates is PCR detection of the *cytK-1* gene. However, due to the previously discussed issue, the presence of *cytK-1* gene is not synonymous with CytK-1 expression. Moreover, further studies on the presence of the *hpa* operon in *B. cytotoxicus* strains would be beneficial to understand whether this operon could be used for a discriminatory test. Guinebretière et al. (2013) also indicated that *B. cytotoxicus* can be differentiated from other species of the *B. cereus* group by the absence of starch hydrolysis. This is an interesting characteristic, given that *B. cytotoxicus* has been isolated mainly from potatoes and potato products.

### *Bacillus cytotoxicus* as a food-associated microbe

*Bacillus cytotoxicus* has been linked to nine food-borne outbreaks between 1998 and 2014 in France, and 11 out of the 16 known *B. cytotoxicus* strains have been isolated in Switzerland. The high number of isolations of *B. cytotoxicus* in Switzerland and France compared with other countries was prompted following detection of *B. cereus* group strains. In the case of microbiological food analysis, members of the *B. cereus* group are not usually further investigated, which could explain the limited amount of data available at the *B. cytotoxicus* species level. It is therefore entirely plausible that there are many more historical outbreaks in other countries linked to *B. cytotoxicus* that have been categorized as *B. cereus* due to a lack of further investigation. Moreover,

**TABLE 2** Summary of food and environmental studies that have looked for *Bacillus cytotoxicus*

Food products	Year	Detection of <i>Bacillus cytotoxicus</i>	Number of samples positive and tested	Reference
Vegetable puree	1998	+	Sample taken after an outbreak	Lund et al. (2000)
Potato puree	2003	+	Sample taken after an outbreak	Fagerlund et al. (2007)
Potato products	2007	+	Sample taken after an outbreak	Rau et al. (2009)
Semolina	2008	+	Sample taken after an outbreak	Glassset et al. (2016)
Potato products	2008	+	Sample taken after an outbreak	Glassset et al. (2016)
Vegetable puree	2008	+	Sample taken after an outbreak	Glassset et al. (2016)
Potato products	2009	+	Sample taken after an outbreak	Glassset et al. (2016)
Mashed celery	2011	+	Sample taken after an outbreak	Glassset et al. (2016)
Mashed fish	2012	+	Sample taken after an outbreak	Glassset et al. (2016)
Potato products	2014	+	53 out of 151 samples	Contzen et al. (2014)
Potato products	2018	+	NK	Heini et al. (2018)
Dairy products	2018	-	NK	Miller et al. (2018)
Spices and herbs	2018	-	NK	Frentzel et al. (2016)
Edible insects	2018	+	NK	Fasolato et al. (2018)
Flour and dough products	2019	-	0 out of 89 samples	Kindle et al. (2019)
Dried food products	2019	+	29 out of 210 samples	Kone et al. (2019)
Soil and rice products	2019	-	0 out of 6 samples	Bağcıoğlu et al. (2019)
Soil	2020	+	NK	Cavello et al. (2020)
Mashed potato powder	2021	+	19 out of 20 samples	Burtscher et al. (2021)

Abbreviation: NK, not known.

food-borne diseases are often under-reported due to generally mild and self-limiting symptoms, which do not require any medical treatments and/or further microbiological tests (Holland et al., 2020). *Bacillus cytotoxicus* has mostly been associated with commercial dehydrated vegetable products, such as mashed vegetable powders or potato puree (Burtscher et al., 2021). Bağcıoğlu et al. (2019) did not detect *B. cytotoxicus* from soil samples, whereas Cavello et al. (2020) managed to isolate *B. cytotoxicus* strains from soil in the Domuyo geothermal area in Argentina but did not provide any indication of potential food contamination pathways.

*Bacillus cytotoxicus* is likely widespread in the environment, given its characteristics, and, therefore, is likely to be a common contaminant on raw ingredients and plant materials during the production processes. Its sporulation ability could help the bacterium survive industrial processes or even establish residence in a food processing environment; hence, it appears mainly in plant and insect-based foods.

It is known that NVH 391-98 and INRA AF2 are highly cytotoxic, but there are no literature data on the infectious dose of these strains. Literature reports indicate that *B. cytotoxicus* is typically present in low concentrations in foods not exceeding  $10^2$  CFU per gram in potato products; however, the FSA has received notification of two instances of levels in food exceeding  $10^5$  CFU per gram. During food-borne outbreaks in France, *B. cytotoxicus* NVH 391-98 was detected at

a level of  $10^5$  CFU per gram from potato and vegetable puree samples, although we do not know the level of contamination at the point of consumption or the amount of product that was consumed. It also remains unclear how the products were stored before consumption or at what point the samples were tested. The cases of illness and death in one of these outbreaks occurred mostly in the vulnerable population, so it is possible that the infectious dose for non-vulnerable groups is higher. Infective doses ranging from  $10^5$  to  $10^8$  have been reported for *B. cereus* from implicated foods after food poisoning for the diarrhoeal and emetic syndromes (Granum & Lund, 1997). Thus, any ready-to-eat food containing a level of *B. cereus* higher than  $10^3$  g<sup>-1</sup> is considered unsatisfactory and not completely safe for consumption (HPA, 2009).

The FSA has been notified of the presence of *B. cytotoxicus* in two products containing insect flour in the last 3 years. In addition, another study, conducted in 2018, reported the detection of *B. cytotoxicus* from edible insects purchased in Italy (Fasolato et al., 2018). Contzen et al. (2014) hypothesized that soil is the natural habitat of *B. cytotoxicus*. However, the ecological niche and the contamination pathway of potato or insect-related products remain unclear. Given the increasing and wide utilization of insect flour by industry, further studies are crucial to investigate the association of these insect-related food products with *B. cytotoxicus* from a food safety perspective.

## CONCLUSION

*Bacillus cytotoxicus* is a novel pathogen, and relatively few studies have isolated it from food matrices. Its impact as a food-borne pathogen is difficult to assess as common detection methods do not usually differentiate it from *B. cereus*. The characterization of *Bacillus* isolates for the presence of *cytK-1* gene should become a standard practice in order to protect public health. The nature and extent of cytotoxicity in *B. cytotoxicus* require further investigation together with surveillance of a wider range of foodstuffs as well as different soils and other environments. Given the increasing utilization of insect-related products and the finding of *B. cytotoxicus* in certain edible insects and dried foods, there would be merit in examining the impact of heating, drying and other food processing steps on this bacterium to better understand the risks of *B. cytotoxicus* as a food-borne pathogen.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this work.

## AUTHOR CONTRIBUTIONS

All authors actively contributed to the writing and preparation of this manuscript.

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