

Detection of toxins involved in foodborne diseases caused by Gram-positive bacteria

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Funding information

Universiteit Gent, Grant/Award Number: GOA project no. 01G02213

Abstract

Bacterial toxins are food safety hazards causing about 10% of all reported foodborne outbreaks in Europe. Pertinent to Gram-positive pathogens, the most relevant toxins are emetic toxin and diarrheal enterotoxins of *Bacillus cereus*, neurotoxins of *Clostridium botulinum*, enterotoxin of *Clostridium perfringens*, and a family of enterotoxins produced by *Staphylococcus aureus* and some other staphylococci. These toxins are the most important virulence factors of respective foodborne pathogens and a primary cause of the related foodborne diseases. They are proteins or peptides that differ from each other in their size, structure, toxicity, toxicological end points, solubility, and stability, types of food matrix to which they are mostly related to. These differences influence the characteristics of required detection methods. Therefore, detection of these toxins in food samples, or detection of toxin production capacity in the bacterial isolate, remains one of the cornerstones of microbial food analysis and an essential tool in understanding the relevant properties of these toxins. Advanced research has led into new insights of the incidence of toxins, mechanisms of their production, their physicochemical properties, and their toxicological mode of action and dose-response profile. This review focuses on biological, immunological, mass spectrometry, and molecular assays as the most commonly used detection and quantification methods for toxins of *B. cereus*, *C. botulinum*, *C. perfringens*, and *S. aureus*. Gathered and analyzed information provides a comprehensive blueprint of the existing knowledge on the principles of these assays, their application in food safety, limits of detection and quantification, matrices in which they are applicable, and type of information they provide to the user.

KEYWORDS

Bacillus, bacterial toxins, Clostridium, detection, food safety, intoxication, toxico-infection, toxicity, Staphylococcus

1 | INTRODUCTION

Even though many efforts have been made by the food industry and competent authorities to ensure consumer protection, food safety remains a significant public health

challenge (Yiannas, 2009). The increasing attention for microbial toxins on the public health radar is a result of a number of factors including overall better surveillance and a general increase in the number of reported foodborne outbreaks, including those with toxins as etiological agents

(EFSA, 2009, 2011, 2012, 2013; 2015a, 2015b, 2016, 2017, 2018, 2019); Miller & Notermans, 2014; Sivapalasingam, Friedman, Cohen, & Tauxe, 2004; Van Doren et al., 2013). Moreover, a general impact of a globalized food supply chain and modern food-processing technologies on microbial food safety (Baines, 2010; Fung, Wang, & Menon, 2018; Holley, 2011; Van Boxtael et al., 2013) apply to microbial toxins, as well. Alternative methods and technologies, such as high hydrostatic pressure, ultrasound, intense light pulses, cold plasma, supercritical CO₂, and low-temperature long-time cooking, rose to replace established and proven heat treatments in an attempt to satisfy modern trends in food preparation and consumption (Atuonwu, Leadley, Bosman, & Tassou, 2020; Bahrami, Moaddabdoost Baboli, Schimmel, Jafari, & Williams, 2020; El Kadri, Alaizoki, Celen, Smith, & Onyeaka, 2020; Fein, Lando, Levy, Teisl, & Noblet, 2011; Qusted, Cook, Gorris, & Cole, 2010; Rajkovic, Smigic, & Devlieghere, 2010; Smith, Ng, & Popkin, 2013). These new technologies have a less destructive impact on food, but possibly also on resident microorganisms and their toxins.

While the focus of this review is on Gram-positive bacteria, it is worth noting that species belonging to both Gram-negative and Gram-positive bacteria can have representatives that produce these toxins. A specific bacterial pathogen may produce a single toxin or even multiple toxins. The multitoxin potential has been confirmed for *B. cereus* (Dietrich, Moravek, Bürk, Granum, & Märtlbauer, 2005; Moravek et al., 2006), *C. botulinum* (Hill et al., 2007), *C. perfringens* (McClane, 2010), and *S. aureus* (Dinges, Orwin, & Schlievert, 2000; Peles et al., 2007; Song et al., 2015). Moreover, bacterial toxins often act in concert. Each toxin possesses a unique mechanism of action, which is responsible for the elicitation of a specific pathology (Barbieri, 2009). The role of toxins in microbial pathogenesis is a long-known phenomenon (Alouf, 2006). Much less is known about reasons why a microorganism produces particular toxins, as for most of the toxins no final proof of ecological or evolutionary advantages has been established. Understanding the fundamental mechanisms and signals that regulate toxin production in the pathogenic bacteria may lead to the identification of the reasons why toxins are produced and how toxigenesis can be controlled in food safety. However, the toxin production resulting from (excessive) microbial growth may occur at almost any stage of the food production chain. Some of the toxins can remain resident and biologically active even when the toxin-producing microorganism has been inactivated by downstream food processing (Rajkovic, 2014; Rajkovic, Kljajic, Smigic, Devlieghere, & Uyttendaele, 2013). It is important to note that the mere presence of toxin-encoding genes, or genes-regulating toxin production, does not warrant actual toxin production (Duquenne et al., 2010; Frenzel, Letzel, Scherer, & Ehling-Schulz, 2011; Wang et al., 2012). The toxin production in food is influenced by extrinsic (e.g., tem-

perature, humidity, atmosphere) and intrinsic (e.g., pH, a_w , nutrients) properties in food, cell density, growth phase, cell stress, and injury (Wesche, Gurtler, Marks, & Ryser, 2009). The growth conditions for pathogens are not the same as the conditions that are permissible for toxin production. A well-known example of this is a minimum value of water activity at which *Staphylococcus aureus* can grow and at which it can produce enterotoxins, being 0.83 and 0.86, respectively (Ross & Nichols, 2014; Doyle & Buchanan, 2013; Labbe & Garcia, 2013; Pérez-Rodríguez, & Valero, 2013).

Prior research indicated the potential of sublethal injury to microbial pathogens as a result of mild processing and existence of different microbial subpopulations with distinct recovery, resistance, growth, and virulence characteristics (Jasson, Uyttendaele, Rajkovic, & Debevere, 2007; Pina-Perez, Rodrigo, & Lopez, 2009; Wesche et al., 2009). The surviving cells can be characterized by modified toxin production potential, both in food and in the gut (Berthold-Pluta, Pluta, & Leszcz, 2011; Even et al., 2009; Lee, Patriarca, & Magan, 2015; Martinovic, Andjelkovic, Gajdosik, Resetar, & Josic, 2016; McLeod, Mage, Heir, Axelsson, & Holck, 2016). In *S. aureus*, for example, ClpP proteases were previously shown to be essential for virulence expression and stress tolerance. Interestingly, the effect of ClpP on the expression of selected virulence genes was strain-dependent despite the fact that the expression of the global virulence regulators *RNAIII*, *mgrA*, *sarZ*, *sarR*, and *arlRS* was similarly changed under the conditions tested (Frees et al., 2012). Changes in gene expression constitute the main component of the bacterial response to stress and environmental changes and involve a myriad of different mechanisms. Especially in pathogenic bacteria a plethora of bacterial responses to distinctive stresses such as pH, reactive nitrogen and oxygen species, and antibiotic stress of direct importance to their pathogenicity and de novo toxin production in the human gut. Therefore, it is important to note that an overall potential of toxin production and the potential of the toxin to cause disease symptoms depends on multiple factors, including pathogenic species/strain, the amount of toxin produced, physico-chemical properties of toxins, interplay with food components and other microbial metabolites, stability in food and in the human gastrointestinal tract, possible metabolization of toxin molecules and characteristics of the resulting metabolites, inherent (sub)clinical dose of toxins, mode of action, effect of acute and (sub)chronic exposure, and targets and receptors in the human body.

The understanding of toxin production and toxic effect on humans is related to the ability to detect bacterial toxins in food and human samples. Consequently, the concept of this review is to outline, discuss, and position the analytical needs for the detection of bacterial toxins in relation to their toxicity and their role in foodborne diseases, that is whether the detection is more relevant in food or clinical samples and whether the detection needs to focus on toxin itself

or on the bacterial genetic potential to produce toxin. The review focuses on the most relevant properties of detection methods for their use in food-related diagnostics and provides concrete examples pertinent to toxins of *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, and *Staphylococcus aureus*. This choice has been made based on the respective public health and economic relevance of these pathogens, and the fact that their particular pathogenicity utterly resides on their toxigenesis. *S. aureus*, *B. cereus*, and *C. perfringens* create the group of three most important causative agents of bacterial exotoxin-related foodborne diseases. It is estimated that of 9.4 million foodborne illnesses caused by a known pathogen annually in the United States; 1.3 million are caused by *B. cereus*, *C. perfringens*, or *S. aureus*. Bacterial toxins were the second most important agent in terms of the number of hospitalizations in 2010 in the European Union. The number of outbreaks caused by bacterial toxins showed an overall increasing trend with 525 (9.8%), 558 (10.1%), 461 (8.8%), 730 (12.9%), 777 (14.5%), 834 (16.1%), 843 (16.05%), 849 (19.5%), 848 (17.7%), 818 (16.1%), and 950 (18.5%) outbreaks in 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, and 2018, respectively (EFSA, 2009, 2011, 2012, 2013, 2015a, 2015b, 2016, 2017, 2018, 2019). The reported toxins comprised those produced by *Bacillus* spp., *Clostridium* spp., and *Staphylococcus* spp. and involved almost all types of foods (EFSA, 2009, 2011, 2012, 2013, 2015a, 2015b, 2016, 2017, 2018, 2019). Table 1 shows updated information on some of the most important characteristics of these toxins, and sections below give a brief overview of key characteristics of selected Gram-positive foodborne pathogens and their main toxins related to foodborne diseases.

1.1 | *Bacillus cereus*

Bacillus cereus is among *Bacillus* spp. recognized as the most frequent cause of foodborne diseases. *B. cereus* is typically found in many raw and unprocessed foods, and the presence of low numbers of *B. cereus* in raw foods is found normal, but numbers above 5 log CFU/g (or per mL) are considered as a threat to food safety (Sánchez-Chica, Correa, Aceves-Diez, & Castañeda-Sandoval, 2020). *B. cereus* spores can survive thermal processes, and germinated vegetative cells can multiply and produce toxins under favorable conditions. It is necessary to formulate correct time/temperature profile for inactivation of *B. cereus*, which will be often specific for specific foods (Juneja, Osoria, Hwang, Mishra, & Taylor, 2020; Webb, Barker, Goodburn, & Peck, 2019) as well as to maintain cold chain due to psychrotrophic character of some strains of *B. cereus* (Webb et al., 2019).

B. cereus toxins cause two distinctly different forms of food poisoning—the emetic or vomiting type and the diarrheal type. The emetic type is an intoxication caused by the presence of emetic toxin, cereulide, in food. Cereulide is 1.2

kDa large, heat- and acid-stable, cyclic dodecadepsipeptide. Cereulide intoxication is characterized by rapid (0.5 to 6 hr) onset of symptoms, which include nausea, vomiting, and sometimes abdominal cramps and/or diarrhea that usually resolve within 24 hr. Fatalities have been reported (Dierick et al., 2005; Mahler et al., 1997). Foods typically involved comprise particularly rice, pasta, and potato-based meals.

The diarrheal type is caused after consumption of viable *B. cereus* vegetative cells and/or spores, by the formation and release of protein enterotoxins in the small intestine. The toxins known to be involved in this syndrome are hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe), and cytotoxin K (CytK). They are all heat labile, pH sensitive, and protease sensitive proteins, which is why preformed toxins in food typically do not result in foodborne intoxication. Hbl consists of a single B-component (37.8 kDa) and two L-components L1 (38.5 kDa) and L2 (43.5 kDa). All three subunits show certain degree of heterogeneity with variable molecular weights, and all three subunits are required for maximal activity. Nhe is comprised of NheA (41 kDa), NheB (39.8 kDa), and NheC (36.5 kDa) subunits, and all three components are necessary for maximal toxic activity (Bhunia, 2008). The genes encoding Nhe can be detected in nearly all enteropathogenic *B. cereus* strains, the *hbl* genes in about 45 to 65% (Jessberger et al., 2019). CytK is a single component of 34 kDa toxin and belongs to the family of β -barrel pore-forming toxins. Two CytK variants have since been described, CytK-1 and CytK-2. CytK-1 was described as more cytotoxic than the CytK-2, but also less prevalent in *B. cereus* group members (Koné, Douamba, De Halleux, Bougoudogo, & Mahillon, 2019). This form of food poisoning has an incubation time of 6 to 24 hr (typically 10 to 12 hr). Foods involved typically comprise dairy, meat, and versatile ready to eat foods products.

1.2 | *Clostridium botulinum*

A sporeforming anaerobic pathogen, *Clostridium botulinum*, as well as some strains of *C. argentinense*, *C. baratii*, *C. butyricum*, *C. sporogenes*, and *C. novyi* sensu lato, can produce different serotypes of protein neurotoxins (BoNTs) (Smith, Williamson, Hill, Sahl, & Keim, 2018). Typical serotypes are A, B, C, D, E, F, and G and made of a C-terminal 100-kDa heavy chain and an N-terminal 50-kDa light chain linked by a disulfide bond (synthesized as a single polypeptide chain), but novel BoNT types have been identified in the recent years (Dover, Barash, Hill, Xie, & Arnon, 2014; Zhang et al., 2017). BoNT type, originally named type H, was renamed into BoNT/FA when the investigation identified it as a chimeric toxin instead of a separate serotype, and Pellet et al. (2016) demonstrated that the existing type A antitoxins were effective to neutralize it. Following on from the work of Pellet et al. (2016), a novel BoNT/X type was identified (Maslanka et al., 2016). BoNTs are

TABLE 1 Some key food safety relevant properties of selected bacterial toxins

Bacteria/syndrome/toxin	<i>B. cereus</i> Diarrheal syndrome (Hbl, Nhe, bceT, CytK)	Emetic syndrome(cereulide)	<i>S. aureus</i> Enterotoxins	<i>C. perfringens</i> Enterotoxin CPE	<i>C. botulinum</i> (foodborne botulism <i>sensu stricto</i>)
Type	Toxico-infection	Intoxication	Intoxication	Toxico-infection	Intoxication
Symptoms	Abdominal pain, cramps, watery diarrhea (secretory type), and occasionally nausea	Nausea, vomiting, malaise, and ultimately a fatal liver failure	Nausea, vomiting, sometimes diarrhea	intense abdominal cramps, diarrhea, and flatulence	Fatigue, weakness, and vertigo, blurred vision, dry mouth, and difficulty in swallowing and speaking. Vomiting, diarrhea, constipation and abdominal swelling may occur. The disease can progress to weakness in the neck and arms and respiratory muscles, and muscles of the lower body are affected
Incubation time (hr)	8 to 24 (or longer)	0.5 to 5	1 to 5	6 to 24	12 to 36 (reported minimum 2, maximum 180)
Resolution time (hr)	12 to 24 (up to several days)	6 to 24	6 to 24	Within 24	Several weeks, gradually
Intoxication/Infection dose	Ingestion of more than 10 ⁵ CFU of diarrheal toxin producing <i>B. cereus</i> strains	ca. 10 µg/kg ⁻¹ bw, 0.01 µg/g ¹ of food (produced by <i>B. cereus</i> of more than 10 ⁵ CFU/g food, depending on the strain, food and conditions)	100 ng of ingested toxin, 0.05 ng/mL of food (produced when <i>S. aureus</i> counts reach ca. 10 ⁵ CFU/ mL (g ⁻¹))	10 ⁶ to 10 ⁷ CFU/g of food (ingested vegetative cells produce CPE during intestinal sporulation)	Extrapolated 1 µg/kg b.w. orally, for 70 kg man 0.09 to 0.15 µg intravenously or intramuscularly, 0.70 to 0.90 µg inhalationally
Toxin produced	In the small intestine of the host	Performed in the food	Performed in the food	In the small intestine of the host	Performed in the food
Toxin stability	Heat 5 min. at 56 °C	90 min at 121 °C	SEA 3 min at 80 °C, 1 min at 100 °C; SEB 87 min at 99 °C	5 min at 60 °C	80 °C for 10 min (function of pH and other factors); exact values are also toxin dependent. Substances in food such as divalent cations and organic acid anions protect the toxin from heat
pH	4 to 8	2 to 11	Wide range, resistant to gastric pH	5 to 10	2.7 to 10.2 (toxin and environment dependent)
Proteinases	Nonresistant	Resistant	Resistant	Increased activity	Sensitive, but proteolytic activity is needed for activation of toxins

divided into toxinotypes based on neutralization with specific corresponding antisera, and each toxinotype is subdivided into subtypes according to amino acid sequence variations. For instance, the BoNT/A, BoNT/B, and BoNT/F serotypes can be further classified into BoNT/A1 through to A8, BoNT/B1–B8 and BoNT/F1–F8, respectively. Additionally, BoNT/E can be separated into a larger subgroup covering BoNT/E1–12. Each serotype cleaves one of the SNARE proteins in a different site (Hobbs, Thomas, Halliwell, & Gwenin, 2019). There are now eight different serotypes of the toxin labeled A to FA and X. Most of the *C. botulinum* strains produce only one type of BoNT, whereas certain rare strains produce two BoNT types or three BoNT types. *C. botulinum* is a heterogeneous bacterial species that is subdivided into four groups according to the BoNT type produced (Rasetti-Escargueil, Lemichez, & Popoff, 2020). *C. botulinum* Group I (proteolytic *C. botulinum* producing BoNT types A, B, and F) and *C. botulinum* Group II (nonproteolytic *C. botulinum* producing BoNT types B, E, and F) are responsible for most cases of foodborne botulism. *C. botulinum* and its toxins create a threat to food safety not only for extreme toxicity of toxins but also both for the reasons of heat stability of its spores and ability to grow at low temperatures. *C. botulinum* group I forms very heat-resistant spores and is a concern in the safe production of canned foods. *C. botulinum* group II is able to grow and form neurotoxin at refrigeration temperatures, as low as 3.0 °C, and is a concern in minimally processed refrigerated foods. Foodborne botulism is a severe neuroparalytic disease resulting from the consumption of preformed botulinum neurotoxin (foodborne intoxication). Approximately 10% of cases of foodborne botulism are fatal, and full recovery is often longer than several months (Peck, 2005). Infant botulism is a special type of botulism, which is caused by infection and colonization of the gastrointestinal tracts of susceptible infants by live vegetative cells or spores of *C. botulinum*. Foods involved in botulism are different and include fruits and vegetables, meats, fish, and miscellaneous combined foods. Many outbreaks originate from household settings and are result mainly from improper preservation procedures (Johnson, 2013).

1.3 | *Clostridium perfringens*

Clostridium perfringens is an anaerobic pathogen able to produce several toxins that mediate a number of histotoxic and enterotoxic diseases in humans and animals (Timbermont et al., 2009). Using the latest 2018 *C. perfringens* toxin-based typing scheme *C. perfringens* is grouped into seven distinct types (A to G). It is *C. perfringens* type F that is relevant for foodborne toxico-infections. Strains belonging to *C. perfringens* type F are defined as isolates that carry the α -toxin gene and the *cpe* gene and produce CPE single polypeptide of approximately 35 kDa upon sporulation, but do not carry the structural genes for β -toxin, ϵ -toxin, or ι -toxin (Mi, Li,

& McClane, 2018; Rood et al., 2018; Yasugi et al., 2015). *C. perfringens* foodborne toxico-infection ranks as the second most common foodborne illness in most developed countries (Rood et al., 2018). There are approximately one million cases of this food poisoning each year in the United States (Freedman, Shrestha, & McClane, 2016; Hoffmann, Batz, & Morris, 2012; Scallan et al., 2011;). The leading food vehicles for *C. perfringens* food poisoning are meats and poultry products. *C. perfringens* food poisoning usually results from temperature abuse during the cooking, cooling, or holding of foods (McClane, Robertson, & Li, 2013).

1.4 | *Staphylococcus aureus*

Staphylococcus aureus is among the most established foodborne pathogens and staphylococcal enterotoxins (SEs) are the most notable virulence factors associated with *S. aureus*. SEs belong to a great family of staphylococcal and streptococcal pyrogenic exotoxins, characterized by common phylogenetic relationships, structure, function, and sequence homology. SEs function not only as potent gastrointestinal toxins causing emesis but also as superantigens that stimulate nonspecific T-cell proliferation. To date, 26 SEs and enterotoxin-like types have been described: enterotoxins A (SEA), B (SEB), C1 (SEC1), C2 (SEC2), C3 (SEC3), D (SED), E (SEE), G (SEG), H (SEH), I (SEI), J (SEJ), K (SEK), L (SEL), M (SEM), N (SE/N), O (SEO), P (SEP), Q (SE/Q), R (SER), S (SES), T (SET), U (SE/U), W (SE/W)), V (SE/V), X (SE/X), and Y (SE/Y) (Fisher, Otto, & Cheung, 2018). Enterotoxin and enterotoxin-like proteins are globular, single polypeptides with molecular weights ranging from 22 to 29 kDa. They can be encoded in prophages, plasmids, or chromosomal pathogenicity islands. The location of the SE genes on mobile genetic elements presents an additional risk factor in *S. aureus* food intoxication, due to possible horizontal gene transfer (Cafini et al., 2017; Lindsay, 2014). The transfer of genetic elements in *S. aureus* has contributed to strain variability and enhanced virulence. It is well known that *S. aureus* strains usually carry more than one SE encoding gene. *S. aureus* is predominantly of animal origin, although can be often isolated from the environmental sources, as well. They may be present as part of the normal microflora of humans and animals. *S. aureus* is carried on the skin and nasal cavities of about 30% of the healthy human population (Zeaki, Johler, Skandamis, & Schelin, 2019). The incidence of enterotoxin-producers among human isolates is reported to be much higher than among nonhuman isolates.

Foods that have been frequently incriminated in staphylococcal intoxication include meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, particularly cream-filled pastries and cakes, and sandwich fillings.

2 | FOOD SAFETY ASPECTS AND TOXIN-DEPENDENT DETECTION REQUIREMENTS

The involvement of bacterial toxins in foodborne diseases follows two scenarios, namely foodborne intoxications and foodborne toxico-infections (also known as toxin-mediated infections). Foodborne intoxications involve the ingestion of the toxin that was formed in the food prior to the consumption as a consequence of preceding bacterial outgrowth. The pre-condition for toxin production is that toxigenic microorganisms are present and that food (e.g., pH, a_w , food composition, and the background microbiome) and environmental conditions (e.g., temperature, humidity, and the atmosphere) allow the growth of a given toxigenic pathogen to the counts that are sufficiently high to result in an apparent toxin production. The number of bacteria required to produce toxins is dependent on the species, strain, and type of the toxin, as well as on the surrounding factors. For a food intoxicant emetic *B. cereus*, the number of bacteria assumed safe is below 5 log CFU/g and measurable amounts of cereulide are found only in later exponential or as of stationary growth phase (Dommel, Lucking, Scherer, & Ehling-Schulz, 2011; Rajkovic et al., 2013; Wang, Ding, & Oh, 2014). Also for *S. aureus* 5 log CFU/g is taken as an assumed safety limit (Bang, Hanson, & Drake, 2008; Delbes, Alomar, Chouqui, Martin, & Montel, 2006; Mohammadi & Hanifian, 2015; Rajkovic, 2012). This does not preclude limited toxin production at lower counts or that final counts detected are lower than previously attained higher outgrowth that resulted in toxin production. For example production of staphylococcal enterotoxin B was found already at 3 log CFU/g of *S. aureus* (Rajkovic et al., 2012) at the dose that corresponds to the lowest dose implicated in a foodborne outbreak. As for *C. botulinum* neurotoxins (BoNT), there the relationship between bacterial counts and toxin production is less known. Reported data by several authors show that detectable toxin amounts of BoNT were found already at *C. botulinum* counts of 10^3 CFU/g, but published experimental setups do not allow at this moment to critically assess this information. It is interesting to note that toxins were detected even though no active growth of *C. botulinum* was observed as the inoculum and the enumerated counts after the incubation remained similar (Odlaug & Pflug, 1979). Similar findings were observed when analyzing data of Carlin and Peck (1996) who found detectable toxin of nonproteolytic *C. botulinum* in sterilized cooked mushrooms at 10 °C after 5 to 7 days that corresponded still to the lag phase of all inoculated *C. botulinum* strains and counts of 1.8 to 2.8 log CFU/g. Furthermore, analysis of results reported by Austin, Dodds, Blanchfield, and Farber (1998) revealed that samples inoculated with proteolytic or nonproteolytic strains of *C. botulinum* became toxic usually when counts greater than 4 log CFU/g were observed, but in several conditions tox-

ins were found at counts between 2 and 3 log CFU/g. Other researches showed that measurable toxin production starts at 4.5 to 5 log CFU/g under conditions tested (Daifas, Smith, Blanchfield, & Austin, 1999b; Elliott & Schaffner, 2001). Based on these and several other publications, one should look for botulinum toxins even when *C. botulinum* counts of 100 CFU/g are found in food until a clear and comprehensive study with strict anaerobic control during plating (of spores and of total *C. botulinum* count) and the use of controlled BoNT-free inoculum is performed to provide a better relationship between growth and toxin production. However, a universally applicable rule is that the storage temperature and type of food markedly influences the toxin titers and time to toxin production. Another rule that applies for all toxins causing foodborne intoxication is that toxin needs to be resistant to acid pH, proteolytic enzymes and bile salts in order not to lose its toxic activity during the gastrointestinal passage.

The scenario of foodborne toxico-infections involves the ingestion of pathogens, which produce biologically active toxins in the small or large intestine. These toxins can be produced also in the food, but in general, are degraded during the gastrointestinal passage and therefore need to be produced de novo in the gut to cause foodborne disease. The survival of a specific pathogen during the gastric passage is dependent on its prior food history. The ability to adapt to gastric stress conditions, such as low pH, can influence their survival and in situ toxin production. Bacteria can initially adapt to low pH in the course of food production leading to higher acid resistance of the cells and, consequently, may facilitate the transit of the pathogen and toxin production in small intestines (Berthold-Pluta et al., 2011). Moreover, close contact with intestinal cells may induce higher toxin production, as it has been noticed for *B. cereus* diarrheal enterotoxins (Jessberger et al., 2017), as well as the spore germination (Wijnands, Dufrenne, van Leusden, & Abee, 2007) and overall pathogenicity (Castiaux, Laloux, Schneider, & Mahillon, 2016; Minnaard, Rolny, & Perez, 2013).

Figure 1 summarizes the global differences between intoxications and toxico-infections in terms of detection targets and rationale for toxin detection in different scenarios. These differences will in many ways influence the need for a specific strategy in toxin detection, that is whether an actual toxin is to be looked for in the food, a specific toxin-producing viable organism or presence of genetic potential for toxin production in an isolated viable microbial community.

3 | BIOLOGICAL ASSAYS

Bacterial (exo)toxins are a group of soluble peptides and proteins that are secreted by the bacterium and interact with

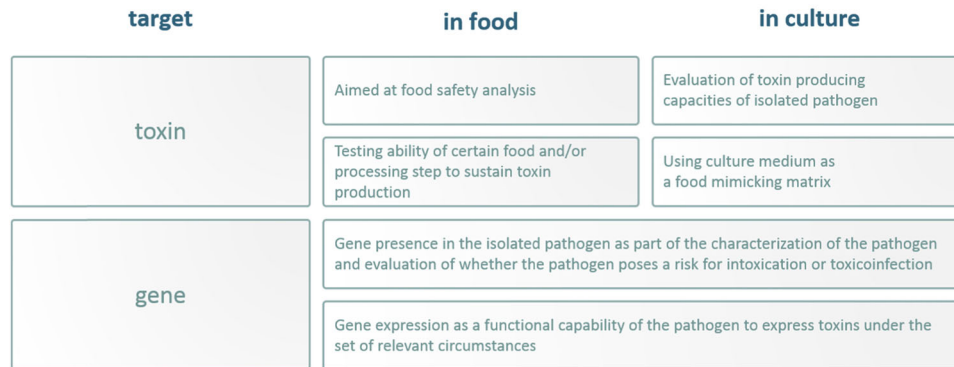


FIGURE 1 Detection targets and rationale for toxin detection in different scenarios

the host cell or its component(s) to alter overall host cell physiology. Each toxin possesses a unique mechanism of action, which is responsible for the elicitation of a specific pathology (Barbieri, 2009). One of the most effective microbial strategies in disease is the generation of toxins that modulate important functions of host cells. The central importance of toxins to the virulence strategies of pathogens is perhaps best illustrated in diseases such as foodborne botulism, emetic intoxication caused by cereulide and staphylococcal foodborne intoxication, where essentially all of the symptoms can be attributed to toxins acting upon their host cells (Blanke, 2006).

Toxins have a target in many different functions of eukaryotic cells and are divided into three main categories: (1) those that exert their toxicity by acting on the surface of eukaryotic cells simply by binding to the cellular specific or general receptors, by cleaving surface-exposed molecules, or by creating pores in the cell membrane breaking the cell permeability barrier; (2) those that have an intracellular target and hence need to cross the cell membrane (these toxins need at least two active domains, one to cross the eukaryotic cell membrane and the other to modify the toxin target); and (3) those that have an intracellular target and are directly delivered by the bacteria into eukaryotic cells (Blanke, 2006).

It seems therefore logical that toxins can be detected and identified by their biological effects. Even their (semi)quantification is in certain cases possible by observing their biological effects (Mohammed, Syed, & Aslan, 2016; Rasooly, Do, & Hernlem, 2017; Sesardic, 2012). In fact, bioassays are commonly used for detection of many microbial toxins (Andersson et al., 2007; Rajkovic, 2017; Rajkovic et al., 2014; Rasooly et al., 2017; Yamamoto et al., 1972) but also for all sorts of food and environmental and contaminants, such as organic pollutants, heavy metals, and pharmacological products (Hemachandra & Pathiratne, 2017; Kunz, Kienle, Carere, Homazava, & Kase, 2015; Lauschke, Hendriks, Bell, Andersson, & Ingelman-Sundberg, 2016; Ma, Wang, Liu, Gao, & Wang, 2017; Mondaca, Catrin,

Verdejo, Sauve, & Neaman, 2017; Neale, Leusch, & Escher, 2017).

A bioassay, a shortened form for biological assay, is a type of test in which the effect of a toxin (or any substance in general) on living material or organisms is investigated. In almost all of the cases, bioassays are used to mimic and predict what a toxin will do to humans by observing biological reaction of other animals, or by observation on in vitro biological systems, such as cell cultures. In other cases, the reaction of a certain organism or type of cell and tissue is used to identify an unknown toxin. The applicability of different bioassays for a variety of toxicological endpoints offers a possibility for measurements of acute and chronic toxicity (Kokkali & Van Delft, 2014).

There are two main reasons for using a bioassay approach besides or instead of an (instrumental) analytical test. First, when addressing the effects of toxins that have not been studied before, it is difficult to approximate the effects that the toxin will have on humans without testing it in biological systems. Analytical tests can tell what toxin molecules are, and quantify their presence, but life forms provide information on toxicity, determine the biological intactness of the toxin, and provide complementary information on the relevant dose/response. Such information is not only crucial for toxin characterization, but it also provides an important platform for detection and semiquantification of toxins. Second, different (components of) toxins may interact with each other, or with food components, additives and preservatives, or may undergo a change during food processing. Therefore, the finding that is most relevant when predicting the effect of the in-food present toxin on a consumer is the effect that it has had on a model biological system. Moreover, monitoring approach based on target lists of a priori defined single microbial toxins and microorganisms might not be sufficient to ensure the required food safety and/or to take the most appropriate measures to improve it. Advances in analytical sciences have resulted in the discovery of more and more not regulated pathogens and toxins

These assays can help to elucidate pathogen–toxin–host interactions under different conditions. Utilization of this principle results in two kinds of bioassays, namely one can distinguish whole animal assays and cell (tissue) culture assays. Also, microbial bioassays in which living bacteria are used to measure toxicity are well known (Kratasyuk & Esimbekova, 2015). These assays mainly rely on the bioluminescence and are often used in ecotoxicology but are inherently less relevant for the studies of host–pathogen interactions. Regardless of the type of the assay some of the issues remain troublesome, and the suitability of a bioassay must be determined for each application and cell model. Inherent variability of biological responses in cell culture and whole animal assays requires unprecedented standardization of the assays and complex statistical techniques in bioassay data interpretation. Also, *in vitro* bioassays may not be highly predictive of *in vivo* effects especially across species. Therefore, there is on side a need to collect reliable and reproducible *in vitro* toxicity data, and on the other side to translate it into meaningful *in vivo* effects (McKim, 2010).

3.1 | Whole animal assays

Table 2 shows selected examples of the whole animal assays applied to detect toxins produced by *B. cereus*, *C. botulinum*, *C. perfringens*, and *S. aureus*. Although whole animals have been used for a long time in science and their value has been proven in many ways, the ethical issues require this approach to be abandoned any time a reasonable alternative can be found. While their use may still have the foundation in evaluation of toxicity for the sake of human and animal health, one should try to utilize alternative strategies, such as *in silico* and *in vitro* methods (Caloni, Benfenati, & Sambuy, 2016). *In silico* and *in vitro* tools are being developed and verified against *in vivo* experiments, with focus on relevant selected endpoints, for example, liver toxicity, hormonal toxicity, developmental, immune toxicity, and intestinal toxicity. There is a long history of using both *in silico* and *in vitro* methods to predict adverse effects in humans where toxicity data are lacking (Madden, Rogiers, & Vinken, 2014). Intestinal models are very frequently used in both research and regulatory aspects as an alternative to animal testing. A variety of approaches are currently employed, ranging from the utilization of *ex vivo* tissue to reconstructed *in vitro* models, organoids, chip-based technologies, synthetic membrane systems, and, of increasing current interest, *in silico* modeling approaches (Gordon et al., 2015).

3.2 | Cell culture assays

By using diverse cell types in different biological assays relevant responses can be assessed. Determined toxic effects can be then related to the presence of particular (group of)

toxin(s) and their concentrations. There is ample information in the literature available on the use of different cells, whether from primary cell cultures or more often from the established cell lines, and cell culture conditions to evaluate pathogenicity and toxicity of many different pathogens and their toxins, respectively. Table 3 gives an overview of selected examples of *in vitro* toxicity studies on toxins of *B. cereus*, *C. botulinum*, *C. perfringens*, and *S. aureus*.

Cell culture–based assays can be used to screen for toxicity both by estimation of the basal functions of the cell (i.e., those processes common to all types of cells) or by testing specialized cell functions. General toxicity tests, aimed mainly at detection of the biological activity of toxins, can be carried out on many cell types. A number of parameters including vitality staining, cytosolic enzyme release, cell growth, and cloning efficiency are used as end points to determine toxicity (Adan, Kiraz, & Baran, 2016; Makris et al., 2011; Zhang, Ball, Panzica-Kelly, & Augustine-Rauch, 2016). Organ-specific toxic effects are tested using specialized cells by measuring alterations in membrane and metabolism integrity and/or in specific cell functions. Major problems in the interpretation of cell-specific results obtained in *in vitro* are related to the difficult separation of the effects on the basal and special cell functions, which are both under the strong influence of the experimental conditions. Moreover, different cell lines are characterized by different features that make them more or less responsive and representative for different toxicological assessments and toxin detection.

Furthermore, even when the appropriate cell type is used, intrinsic cell sensitivity depends on several of cell characteristics, which are probably only partially present *in vitro* models; these include chemical biotransformation and binding, membrane permeability characteristics and surface determinants, intracellular synthetic pathways, and adaptive and recovery mechanisms. It is also worth noting that many of the cell lines, if not most of the established ones, have cancer origins and may not fully reflect what occurs in healthy cells. For some toxic chemicals, it is the functional status of the cell rather than the cell type that determines the extent to which the inhibition of a given biochemical mechanism is critical to the function and survival of the cell, influencing the interpretation of the toxicity data and as such the value for detection of toxicity or toxins (Ekwall, Silano, Paganuzzi-Stammati, & Zucco, 1990). Intrinsic cell sensitivity is an important factor in determining the specificity of toxic action, but can also induce a bias as the assay sensitivity needs to be relevant for genuine dose-response. Other factors such as rates of absorption, biotransformation, distribution, and excretion, which influence the exposure at the level of target cells *in vivo* are important to understand the value of data coming from *in vivo* assays.

When cells are embedded in three-dimensional tissue constructs, they do not only signal the presence of biological

TABLE 2 Examples of the whole animal assays applied to toxins produced by *B. cereus*, *C. botulinum*, *C. perfringens*, and *S. aureus*

Method	Toxin	Lower sensitivity reported on the basis of doses used	Assay observations	Note	References
Rabbit ileal loop	<i>B. cereus</i> diarrheal enterotoxin Hb1 [†]	5 µg per component	Fluid accumulation	Injection of antibodies prevented symptom development in the ileal loop test, indicating that binding of the toxic components to cells might be weak	Beecher, Schoeni, and Wong (1995); Beecher and Wong (1997); Kramer and Gilbert (1989); Shinagawa, Sato, Konuma, Matsusaka, and Sugii (1991)
	<i>C. perfringens</i> enterotoxin (CPE)	6.25 µg	Fluid accumulation	The sensitivity of the rabbit's small intestine to the toxin increases from the upper duodenum downward, with the terminal ileum being the most responsive	Caballero, Trugo, and Finglas (2003); Garcia et al. (2014); Jay (2000); Navarro, McClane, and Uzal (2018); Sarker, Carman, and McClane (2000)
Mouse ileal loop	<i>C. perfringens</i> enterotoxin (CPE)	1 µg	Fluid accumulation	There is a certain controversy in observation of intestinal loop fluid accumulation. While CPE-treated mice do show some intestinal histologic damage, the reproducible intestinal fluid accumulation has not been entirely confirmed. In the first work of Yamamoto, Ohishi and Sakaguchi (1979) the increase in weight of the intestinal loop was proportional to the log dose of enterotoxin within a range from 1 to 16 µg, and a measurable quantity of fluid was accumulated within 10 min when treated with 20 µg of CPE. Intestinal loops inoculated with 10 µg of enterotoxin showed maximum fluid accumulation after 1 hr. Later research did not confirm a reliable intestinal loop fluid accumulation even when using much higher CPE doses and longer exposure time. Even 50 µg CPE treatment induced only minor intestinal damage during 4 hr exposure. Differences could be perhaps attributed to different experimental design and that the rapid onset of fluid accumulation observed in the previous mouse intestinal loop study involved the presence of another factor in addition to CPE	Caserta et al. (2011); Freedman et al. (2018); Navarro et al. (2018); Yamamoto, Ohishi, and Sakaguchi (1979)

(Continues)

TABLE 2 (Continued)

Method	Toxin	Lower sensitivity reported on the basis of doses used	Assay observations	Note	References
Rabbit skin	<i>B. cereus</i> diarrheal enterotoxin Hbl ^a	>0.5 µg per component	Vascular permeability and necrosis	Samples containing 0.5 to 1.5 µg of single components and binary combinations produced no reaction	Beecher and Wong (2000); Glatz, Spira, and Goepfert (1974)
Guinea pig skin	<i>B. cereus</i> diarrheal enterotoxin Hbl ^a	0.05 mL of <i>B. cereus</i> culture filtrate of 6 log CFU/mL (no information on toxin concentration).	A green or bloody necrotic reaction was produced in the guinea pig skin	Injection of culture filtrates from 21 of 24 <i>B. cereus</i> strains tested. Heat-treated filtrates (5 min, 56 °C) gave no pathological reaction	Glatz and Goepfert (1973)
Rhesus monkey emesis	<i>B. cereus</i> cereulide (emetic toxin)	10 µg/kg body weight of animal	Emesis		Melling and Capel (1978); Melling, Capel, Turnbull, and Gilbert (1976); Shinagawa, Konuma, Sekita, and Sugii (1995)
Suncus murinus emesis	<i>S. aureus</i> enterotoxins	5 µg/(2 to 3 kg body weight) of animal. Probably SE dependent/kg body weight of animal.	Emesis		Capel and Melling (1978); Munson, Tremaine, Betley, and Welch (1998)
Suncus murinus emesis	<i>B. cereus</i> cereulide (emetic toxin)	8 µg/kg body weight of animal	Emesis		Agata et al. (1994); Agata, Ohta, Mori, and Isobe (1995)
Mouse lethality	<i>S. aureus</i> enterotoxins SEA, SEB, SEC2, SED, SEE, SEG, SEH, and SEI	SE dependent. SEA was the most toxic, followed by SEI and SEE. SEB, SEC2, SED, SEG, and SEH showed emetic activity but required much higher concentrations	Emesis		Hu, Omoe, Shimoda, Nakane, and Shinagawa (2003)
Mouse lethality	<i>C. botulinum</i> BoNT (A-G)	20 to 30 pg/mL, 1 MLD/mL	Death		Lindström and Korkeala (2006)
	<i>C. perfringens</i> enterotoxin (CPE)	LD50 1.8 µg	Death		Jay (2000)

^aFor Hbl diarrheal enterotoxin, a combination of 5 µg of each component was sufficient to cause significant symptoms on rabbit ileal loop, and 25 µg was necessary to achieve the maximum enterotoxigenic activity.

TABLE 3 Overview of the cell-culture based assays applied to some toxins produced by *B. cereus*, *C. botulinum*, *C. perfringens*, and *S. aureus*

Cell line	Toxin	Approx. sensitivity ^a	Note	Reference
Vero	<i>C. perfringens</i> enterotoxin	40 ng/g in feces	In comparison to immunological methods, Vero cell assay was the least sensitive	Berry et al. (1988); Husain and Drasar (1986); Markovic, Asanin and Dimitrijevic (1993); Singh, Mitic, Wieckowski, Anderson, and McClane (2001)
	<i>B. cereus</i> Nhe and Hbl	na	Higher relative cytotoxicity of Hbl and Nhe for Vero than for Caco-2 cells	Didier et al. (2012); Fagerlund, Lindback, Storsset, Granum, and Hardy (2008); Jessberger, Dietrich, Bock, Didier, and Martlbauer (2014); Lindbäck et al., 2010; Lindbäck, Okstad, Rishovd, and Kolsto (1999)
Caco-2	<i>C. perfringens</i> enterotoxin	<1 µg/mL in solution	Morphological damage and DNA cleavage in CaCo-2 cells. CPE interacts with several eukaryotic proteins to form a large complex in the plasma membrane, which corresponds to a pore.	Chakrabarti, Zhou, and McClane (2003)
	<i>B. cereus</i> Nhe and Hbl	na	Cells develop blebs visually seen under the microscope	Clair, Roussi, Armengaud, and Dupont (2010); Fagerlund et al. (2008)
	<i>B. cereus</i> CytK	na	Off all tested cell lines Caco-2 was the most sensitive to Cyt K	Jessberger et al. (2014)
	<i>B. cereus</i> emetic toxin	0.125 ng/mL	MTT and SRB assays showed that differentiated Caco-2 cells were sensitive to low concentrations of CER (in a MTT reaction of 1 ng/mL after 3 days of treatment; in an SRB reaction of 0.125 ng/mL after 3 days of treatment). Cell counts revealed that cells were released from the differentiated monolayer at 0.5 ng/mL of CER. Additionally, 0.5 and 2 ng/mL of CER increased the lactate presence in the cell culture medium. Proteomic data showed that CER at a concentration of 1 ng/mL led to a significant decrease in energy managing and H ₂ O ₂ detoxification proteins and to an increase in cell death markers	Rajkovic et al. (2014)

(Continues)

TABLE 3 (Continued)

Cell line	Toxin	Approx. sensitivity ^a	Note	Reference
Rabbit intestinal brush border membranes	<i>C. perfringens</i> enterotoxin	na	CPE induces massive desquamation and villi shortening in the small intestine. This histopathological damage appears essential for CPE-associated fluid and electrolyte transport alterations. For example, only CPE doses that induce histopathological damage can cause fluid/electrolyte transport alterations in the rabbit ileum	McClane (2000); McClane and Wnek (1990)
Murine spleen cells	Staph. enterotoxins A, B, and E	na	Measured by ³ H-thymidine uptake and blast formation	Peavy, Adler, and Smith (1970); Warren (1977)
Human peripheral lymphocytes	Staph. enterotoxin A	0.1 to 0.001 µg/mL	Measured by ³ H-thymidine uptake and blast formation. SEA-treated cultures produce large amounts of interferon and stimulate maximal cell proliferation	Langford, Stanton, and Johnson (1978); Peavy et al. (1970)
Human fetal intestinal cells	<i>S. aureus</i>	40 to 60 µg/mL	The cytotoxicity was profoundly influenced by the age of the culture. No cytotoxicity was evident until after 2 days of growth had taken place, when the cell number was approximately 4.0×10^5 cells per culture	Schaeffer, Gabliks, and Calitis (1966, 1967)
Hep-2 and Hep G2	<i>B. cereus</i> cereulide	Hep G2 - 0.04 ng/mL, HEp-2 - 1 ng/mL.	Cell-vacuolation serves as an end-point toxicity. The number of vacuoles in the Hep G2 cells was greater, and the size of the vacuoles was larger than those observed in HEp-2 cells	Kamata et al. (2012)
Hep-G2	<i>B. cereus</i> Hbl, Nhe, CytK	na	CytK exhibited much smaller cytotoxicity than Hbl and Nhe. Nhe seems more toxic than Hbl, but Hbl in general cause pores on membranes sooner than Nhe	Jessberger et al. (2014)
Boar spermatozoa	<i>B. cereus</i> emetic toxin	LOD 2 ng/mL LOQ 20 ng/mL	Boar semen motility ceases in contact with cereulide	Rajkovic et al. (2006, 2007)
Primary rat spinal cord cell (RSC)	<i>C. botulinum</i> BoNT/A	50% cleavage at 5 pg BoNT/A	Rat spinal cord cells assay sensitively detects purified botulinum neurotoxin type A, and requires all biological properties of the toxin for detection	Pellet, Tepp, Toth, and Johnson (2010)
Cultured human motor neurons	<i>C. botulinum</i> BoNT/A, B, C, E, and F	EC50 varied from 0.006 to 17.3 mouse LD50 for respective BoNT ^b	The sensitivity of the isolated neurons and potency of BoNTs varied markedly for the BoNTs tested. Motor neurons were the most sensitive cell model for all BoNT serotypes tested except BoNT/D, greatly exceeding the sensitivity of the MBA by up to 160-fold, depending on the serotype	Pellet, Tepp, and Johnson (2019)

^ana, not available.^bEstimated EC50 values of 0.006 mouse LD50 U per well for BoNT/A1 and BoNT/F1, 0.008 U per well for BoNT/C1, 0.02 U per well for BoNT/E3, 0.1 U per well for BoNT/B1 and 17.3 U per well for BoNT/D1. The specific activity of each BoNT subtype preparation was determined by the intraperitoneal mouse bioassay and was 5.6 pg per LD50 (A1), 6 pg per LD50 (B1), 160 pg per LD50 (C1), 7.1 pg per LD50 (D1) 61 pg per LD50 (E3), and 72 pg per LD50 (F1).

or chemical agents but can also further indicate physiological consequences of exposure to these analytes.

Viable and in-suspension cells, such as boar semen cells proved to be very valuable for the detection of ionophoric toxins such as *B. cereus* emetic toxin. The use of mammalian spermatozoa for studying the cytotoxic effect of microbial toxins provides several advantages compared to other in vitro systems with different cell types. The swimming activity of spermatozoa is dependent on intact cellular structures and functions, which consequently offers an endpoint for semi-quantitative evaluation of the cytotoxic potential of various substances that may intervene with functions that control or influence their motility. By using computer-assisted semen analysis in the evaluation of cereulide impact on the boar semen motility, a software-based characterization of the observed motility and interpretation of the motility changes can be obtained. Software-generated data describe the dynamics with which the progressive motility (motility characterized with specific properties of speed and straightness in the movement) drops from the starting value to the level of 10% (Rajkovic et al., 2006). By plotting the change of progressive motility in the function of time, a semiquantitative information can be obtained about the sample toxicity and the amount of cereulide present in the sample. This assay has been successfully tested for the detection of cereulide in relevant foods, too, such as rice, pasta, and potato puree (Rajkovic, Uyttendaele, & Debevere, 2007). The assay is not cereulide specific, which is a general characteristic for bioassays.

Cell cultures approaches are sometimes modified or combined with immunological, electrical, or physico-chemical assays to create different biosensors. There are numerous examples of biosensors based on different cells for all toxins pertinent to this review. Three prototypes of the biosensor capable of handling different sample types were developed and tested with food for detection of bacterial foodborne toxins (Banerjee & Bhunia, 2009; Banerjee & Bhunia, 2010; Banerjee, Franz, & Bhunia, 2010; Banerjee, Lenz, Robinson, Rickus, & Bhunia, 2008). The sensing element in this sensor, a B lymphocyte Ped-2E9 cell-line, is encapsulated in collagen matrix in three-dimensional scaffold. The uniqueness of this biosensor is that it detects analytic interaction with mammalian cells and can distinguish active from inactive toxins, rendering accurate estimation of the risk associated with the toxin. This sensor measures the activity of enzymes such as alkaline phosphatase and already gave positive signals for a broad range toxins; α -hemolysin from *S. aureus*, phospholipase C from *C. perfringens*, and listeriolysin O from *Listeria monocytogenes*. Also for *C. botulinum*, Rust et al. (2017) reported a SiMa cells (human neuroblastoma cell line) based assay combined with reengineered VAMP molecules applied for BoNT/B detection with sensitivity of approximately 3 pg/mL. This approach combines the cell response with enzyme-linked immunosorbent assays (ELISA) in a one-step

assay that facilitates detection of BoNT/B via a luminescent enzymatic reaction. Also a B-cell based biosensor assay CANARY[®] (Cellular Analysis and Notification of Antigen Risks and Yields) Zephyr was developed to detect BoNT/A holotoxin at with limits of detection (LOD) ranging from 10.0 ± 2.5 ng/mL in assay buffer to 7.4 to 7.9 ng/mL in milk matrices, 32.5 to 75.0 ng/mL in carrot, orange, and apple juice, 14.8 ng/g to 62.5 ng/g in ground beef, smoked salmon, green bean baby puree) to LOD of 171.9 ± 64.7 ng/mL in viscous liquid egg matrix (Tam, Flannery, & Cheng, 2018).

However, biosensors do not always incorporate cell lines, and many of the reported bioassays are not based on living cells, but use other immobilized sensing elements, bioreceptors, such as antibodies, RNA, DNA, glycan, lectin, enzymes, tissues, and aptamers (Sharma & Mutharasan, 2013; Vidic, Manzano, Chang, & Jaffrezic-Renault, 2017; Xiong, Shi, Liu, Lu, & You, 2018).

4 | IMMUNOLOGICAL ASSAYS

4.1 | Enzyme-linked immunosorbent assays and reversed passive latex agglutination assay

The currently (commercially) available kits for the detection of *B. cereus*, *C. perfringens*, and SEs are portable immunoassays and have been the major choice for detection of all of these toxins in food and clinical samples. Due to the unmatched sensitivity of the gold standard mouse-bio assay, no commercially available ELISA assays exist for *C. botulinum* BoNTs. Nevertheless, several ELISA formats have been tested and published by different authors presenting possible alternative methods for screening of *C. botulinum* BoNTs (rather than for final confirmation of the positive samples) (Guglielmo-Viret, Attree, Blanco-Gros, & Thullier, 2005; Phillips & Abbott, 2008; Rajkovic et al., 2012; Weingart et al., 2010). Also for *B. cereus* emetic toxin, no immunological assays are marketed, nor have been reported in the scientific literature (Kramer & Gilbert, 1989; Rajkovic et al., 2006). The main reason seems to be related to the low immunogenicity of the toxin and the high toxicity for test animals (Kramer & Gilbert, 1989).

The sandwich ELISA with a solid phase of a microtiter plate is a common format while coated tubes or polystyrene balls are used in some kits. However, there are several ELISA formats, which are used, but almost all have the same principles and key operations. In a typical ELISA, the antigens are immobilized either by direct adsorption or via an antibody adsorbed to the wells of a microplate. The plate is blocked, and the antigen is detected with a specific detection antibody. The detection antibody can be directly labeled with a signal-generating enzyme or fluorophore, or it can be secondarily probed with an enzyme- or flour-labeled

TABLE 4 Some commercially available ELISA and RPLA assays for detection of bacterial toxins

Organism and toxin	Trade name	Manufacturer	Type of assay and description	Note	Reference
<i>Bacillus cereus</i> diarrheal nonhemolytic enterotoxin (Nhe)	3M™ Tecra™ Bacillus Diarrheal Enterotoxin Visual Immunoassay	3M™ Tecra™	ELISA: Rapid ELISA for the screening of <i>Bacillus</i> diarrheal nonhemolytic enterotoxin, (48 well kit)	Detects NheA component	Buchanan and Schultz (1992); Buchanan and Schultz (1994); Ceuppens et al. (2012); Day et al. (1994); Fermanian, Lapeyre, Fremy, and Claisse (1996); Odumeru, Toner, Muckle, Griffiths, and Lynch (1997); Rusul and Yaacob (1995)
<i>Bacillus cereus</i> diarrheal hemolytic enterotoxin (Hbl)	Oxoid BCET-RPLA kit	Oxoid	RPLA: A kit for the detection of <i>Bacillus cereus</i> enterotoxin (diarrheal type) in foods and culture filtrates by reversed passive latex agglutination	Detects L2 component	
<i>Staphylococcus aureus</i> enterotoxins	3M™ Tecra™ Staph Enterotoxins Visual Immunoassay	3M™ Tecra™	ELISA: Rapid ELISA for the screening of Staphylococcal enterotoxins A–E (48 or 96 well kit)	Detects SEA–SEE without identification/discrimination	English, Scalici, Hamilton, Destro, and Jimenez (1999); Häit, Tallent, Melka, Keys, and Bennett (2012); Park, Akhtar, and Rayman (1992); Park et al. (1996b); Vernozy-Rozand et al. (2004a)
	3M™ Tecra™ SET ID VIA	3M™ Tecra™	ELISA: Rapid ELISA for the identification of Staphylococcal enterotoxins A–E (48 or 96 well kit)	Identifies SEA–SEE	
	RIDASCREEN® SET A, B, C, D, E.	R-Biopharm GmbH, Darmstadt, Germany	ELISA: Kit detects and identifies enterotoxin types SEA–SEE		Clarisse et al. (2013); Park et al. (1994, 1996a)
	RIDASCREEN® SET Total		ELISA: Kit detects enterotoxin types		Ostyn et al. (2011)
<i>C. perfringens</i> enterotoxin	Oxoid™ PET-RPLA Toxin Detection Kit	Oxoid	RPLA: A kit for the detection of enterotoxin type A of <i>C. perfringens</i> in fecal samples or culture filtrates by reversed passive latex agglutination		Bajaj and Langfeldt (2003); Harmon and Kautter (1986); Lukinmaa, Takkunen, and Siitonen (2002)
	<i>Clostridium Perfringens</i> Enterotoxin Test, ELISA	TechLab	ELISA: In vitro diagnostics for detecting <i>C. perfringens</i> and its toxins in fecal specimens		Forward, Tompkins, and Brett (2003); Goldstein, Kruth, Bersenas, Holowaychuk, and Weese (2012)

secondary antibody (or avidin-biotin chemistry). For enzymatic detection, the appropriate enzyme substrate is added. The signal that visually observed or measured by the instrument is proportional to the amount of antigen in the analyzed sample. Washing between each of the steps ensures that only specific (high-affinity) binding is maintained to give the signal at the final step. ELISA principles, methods, and applications have been reviewed in numerous publications (Crowther, 1995, 2009; Gan & Patel, 2013; Hnasko, 2015). Table 4 provides an overview of some of the existing and commercially available immunological kits for detection of toxins of *B. cereus*, *C. botulinum*, *C. perfringens*, and *S. aureus*. The result may be read by eye (positive or negative) or by computerized measurements of absorbance.

The other common system is the reversed passive latex agglutination assay (RPLA), where latex beads coated with specific antibody or with normal serum are added to doubling dilutions of the test sample in a microtiter plate. If the antigen is present, the latex beads form a diffuse layer due to antigen-antibody reactions creating a lattice, while if the antigen is absent, the latex beads form a tight button (Mahon, Lehman, & Manuselis, 2015). The difference in the reaction with control and sensitized latex is read by eye. Two rapid tests, based on sandwich ELISA and RPLA, for detection of *B. cereus* enterotoxins are commercially available (see Table 4 for example). Both kits detect diarrheal enterotoxins, but the fact that they detect different antigens was not initially evident. Work of Day, Tatani, Notermans, and Bennett (1994) with fourteen strains of *B. cereus* examining their ability to produce diarrheal enterotoxin by two commercial immunoassay kits and the microslide immunodiffusion assay confirmed that the two commercial assays (BCET-RPLA kit marketed by Oxoid, Basingstoke, UK and produced by Denka, Japan; and TECRA-VIA kit, Bioenterprises Pty Ltd, Roseville, Australia) did not detect the same antigen. These results were in accordance with the reported results of Buchanan and Schultz (1992, 1994) who found that the positive controls of the BCET-RPLA and the TECRA-VIA kits are not detected by the other kit. Notermans and Tatini (1993) also showed no correlation between the results of two kits showing that isolates from a particular outbreak were giving only positive reaction with the TECRA-VIA kit. It is now well established that Hbl is detected with RPLA and Nhe is detected with ELISA and that both enterotoxins can be involved in foodborne outbreaks (Beecher & Wong, 1994; EFSA, 2005; Schoeni & Wong, 2005). According to the results of Beecher and Wong (1994), the antiserum of TECRA-VIA does not react with the enterotoxin Nhe, but reacts with two other proteins of 40 and 41 kDa that showed no toxicity in a vascular permeability assay. The same authors reported that the antiserum used in the BCET-RPLA reacts against L2 component of the enterotoxin. From both these results, it appears that the BCET-RPLA detects one component of the enterotoxin complex and the TECRA-

VIA detects two nontoxic components. The TECRA-reactive components may participate in human diarrhea, despite being nonreactive in the vascular permeability assay, but it also leaves a possibility for false-negative and false-positive results. In general, the usefulness of two commercial immunoassays for the detection of diarrheal enterotoxin of *B. cereus* remains somewhat unclear and the information provided by these tests is more indicative than final. As an additional drawback of serological assays for detection of *B. cereus* enterotoxins is that heat-inactivated and active Hbl was not differentiated by BCET-RPLA kit (Brett, 2006). However, there are currently no other ELISA and RPLA formats for *B. cereus* Hbl and Nhe enterotoxins. It is also worth noting that their use in food is less relevant as *B. cereus* Hbl and Nhe belong to the group of toxico-infectants, and therefore the main interest for their detection is related to characterization of enterotoxigenic *B. cereus* isolates, detection in clinical samples, and investigation of the effect of the host in situ factors on enterotoxin production. Unlike for Hbl and Nhe, currently no ELISA or RPLA, or related immune assay formats, are available for detection of *B. cereus* CytK-1 and CytK-2 enterotoxins, which is an existing gap on the market that surprisingly has not been filled in yet.

Opposite to *B. cereus* enterotoxins, immunoassays have been truly, so far, the major choice for SE detection in food. ELISA-based kits for enterotoxin detection are commercially available from various manufacturers like (1) 3 M™ Tecra™ (Staph Enterotoxins Visual Immunoassay; screening of the presence of SEA-SEE using AOAC International official method 3 M™ Tecra™ SET VIA, and/or specific identification of present SEA-SEE using 3 M™ Tecra™ SET ID VIA; both methods have limit of detection at about 1 ng/mL of sample extract according to the manufacturer) (Bennett, 2005; Bennett & McClure, 1994); (2) bioMérieux (VIDAS SET2, which is a reference method for the European screening method of the Committee Reference Laboratory for milk and milk products; it is also a recommended as the primary screening method for suspect food samples and culture isolates in FDA BAM chapter 13A and an Official method on a variety of foods by AOAC International) (Hennekinne et al., 2007; Vernozy-Rozand, Mazuy-Cruchaudet, Bavai, & Richard, 2004a); (3) Diffchamb AB (Transia Plate SE- Official Methods recommended by Ministère de l'Agriculture, France, Transia Tube SE) (Vernozy-Rozand, Mazuy-Cruchaudet, Bavai, & Richard, 2004b; Zourob, Elwary, & Turner, 2008); (4) R-Biopharm (RIDASCREEN-A, B, C, D, E with sensitivity of below 0.031 ng/mL in buffer for SEA, SEB, SEC, and SEE, and 0.062 for SED and performance equal or better than VIDAS SET2 in cheese) (Ostyn et al., 2011; Park, Akhtar, & Rayman, 1994, 1995; Park, Warburton, & Laffey, 1996a); (5) and Toxin Technology (SET-EIA) (McMeekin, 2003). Since as little as 100 to 200 ng of the toxin can cause symptoms of staphylococcal intoxication, the methods are

required to have very high sensitivity (Boyle, Njoroge, Jones, & Principato, 2010; Evenson, Hinds, Bernstein, & Bergdoll, 1988;). Studies have indicated that VIDAS SET2 can have a sensitivity of less than 0.5 ng/g for SEA and SEB; and less than 1 ng/g of SEC, SED, and SEE, with 100% specificity (Vernozy-Rozand et al., 2004a). The reported sensitivity of VIDAS SET2 is comparable with some other immunoassays (not commercially available): Western immunoblot with detection of SEA at a level of 0.1 ng/mL (Rasooly & Rasooly, 1998) and biosensor technologies with SEB detection limit of 1 ng/mL for a pure sample (O'Brien et al., 2000). The surface plasmon resonance biosensor reported by Homola et al. (2002) was capable of detecting SEB at concentration as low as 0.5 ng/mL in dry milk samples. Commercially available VIDAS SET and TRANSIA PLATE SE were characterized with somewhat lower sensitivity of 0.5 ng/g for SEA and SEC, and above 1 ng/g for SED and SEE (Vernozy-Rozand et al., 2004a). It is however very important to understand that the sensitivity of the detection methods is always to a greater or smaller extent influenced by the food matrix, most probably already in the toxin extraction phase, as well as in antigen-antibody recognition phase of the assay. Such findings were also reported by Park et al. (1994) who found food dependence of the obtained sensitivities with RIDASCREEN kit ranging from 0.20 to 0.75 ng/g depending on tested food matrix. Among the reported drawback of serological detection of SEs is that serological activity seems to be more heat-labile than their biological activity (toxicity), which could yield false-negative results (Brett, 2006) and biased safety assessment.

Several immunological assays have been reported for detection of *C. perfringens* enterotoxin (CPE), such as electroimmunodiffusion, fluorescent antibody, and single- and double-gel diffusion, but the most of the attention has been given to RPLA and ELISA (Berry, Rodhouse, Hughes, Bartholomew, & Gilbert, 1988; Labbe & Juneja, 2006; McClane & Strouse, 1984), of which some kits are commercially available (e.g., TechLab, Blacksburg, USA, and R-Biopharm AG, Darmstadt, Germany). Not much of new development has been witnessed regarding CPE detection in food in recent years. Probably, the among reasons is that in most of the cases CPE is being sought in human samples and less in the food, with the direct detection of CPE in outbreak stools being a good way for confirmation of a foodborne outbreak due to *C. perfringens*. Clinical symptoms associated with foodborne illness due to *C. perfringens* are due to enterotoxin production in the small intestine during sporulation of ingested vegetative cells. CPE and the mature spore are released from the mother cell together. Another reason is that CPE is not secreted outside of the cell. Although significant accumulation of CPE in *C. perfringens* cells occurs only during the sporulation process, very low levels of CPE have been detected in vegetative cells.

4.2 | Lateral flow immunoassay

Although ELISA has been widely used in many laboratories, this method still requires various equipment and trained personnel. Therefore, rapid and cheap, yet still reliable methods that can be conducted and interpreted at the site are needed (Zhao, Lin, Wang, & Oh, 2014). Lateral flow immunoassays (LFIAs) meet these requirements and are important for diagnostic purposes in food safety to detect contamination with specific pathogens and toxins including biowarfare agents in food (Ayong et al., 2005; Delmulle, De Saeger, Sibanda, Barna-Vetro, & Van Peteghem, 2005; Mettler, Grimm, Capelli, Camp, & Deplazes, 2005), feed or the environment (Posthuma-Trumpie, Korf, & van Amerongen, 2009).

LFIA is a form of immunoassay in which the test sample flows along an analytical nitrocellulose membrane due to capillary forces. After the sample is applied to the test, it encounters a colored reagent (antibody or antigen bound to a label), which mixes with the sample and runs over the membrane. At the membrane it encounters lines or zones that have been pre-treated with an antibody or antigen. Depending on the analytes present in the sample, the colored reagent can become bound at the test line or zone. LFIAs can be both qualitative with a defined cutoff level or quantitative when used with a photometric strip reader (Anfossi et al., 2011; Gomez, Pagnon, Egea-Cortines, Artes, & Weiss, 2010). Nowadays, colloidal gold is used most often as label followed by colored latex particles, chemiluminescent and fluorescent nanoparticles (e.g., Quantum dots) (Berlina et al., 2013; Zhao et al., 2014).

Until today commercially kits are available for the detection of toxins from *B. cereus*, *C. botulinum*, and *S. aureus*. However, no LFIAs are registered for the detection of the emetic toxin of *B. cereus* and the detection of enterotoxin of *C. perfringens*. Most of the available LFIAs for the detection of *B. cereus*, *C. botulinum*, and *S. aureus* use colloidal gold as a label for detection of the toxins. Many studies proved that LFIAs based on fluorescence have substantially greater sensitivities and dynamic ranges than the ones based on colloidal gold (Berlina et al., 2013; Posthuma-Trumpie et al., 2009; Sharma, Eblen, Bull, Burr, & Whiting, 2005; Yang et al., 2010). Nevertheless, only one commercial test is available that uses fluorescent-dyed latex particles for the detection of BoNT/A (RAMP[®] Bot Tox test). The development of such assays has been limited by the need for stable dyes that do not cause sample interference and because of the need of more expensive, sophisticated hardware and software to read the signal (Gessler, Pagel-Wieder, Avondet, & Bohnel, 2007).

The Duopath[®] Cereus Enterotoxins test (Merck KGaA) was developed as a fast LFIA for the detection of Hbl and Nhe enterotoxins of *B. cereus* in food and environmental samples. This test uses gold-labeled monoclonal antibodies to track the NheB components of Nhe and the L₂ component

of Hbl independently within the same test device, providing a substantial advantage given time and cost-effectiveness. Another advantage is the use of monoclonal antibodies of defined specificity thus avoiding false-positive results due to cross-reactivity with nontoxic exoproteins produced by *B. cereus* as reported for rabbit polyclonal antisera based test (Beecher & Wong 1994). Krause et al. (2010) evaluated the performance characteristics of the Duopath[®] *Cereus* Enterotoxins test and reported LOD higher than the ones given by the manufacturer for the BDE VIATM and the BCET-RPLA test kits (> 1 ng/mL NheB and 2 ng/mL Hbl L₂). Still, the Duopath[®] proved to be sufficiently sensitive to enable a reliable detection of *B. cereus* Hbl and Nhe enterotoxins and could also be a helpful tool for quality control purposes of processed food (Krause et al., 2010). For the moment no LFIA is commercially available for the detection of CytK (CytK-1 and CytK-2) enterotoxin of *B. cereus*.

Several LFIA are commercialized for the detection of *C. botulinum* because of their need for the detection of BoNT in biodefense and biosecurity. However, these LFIA are developed for environmental and (sometimes) serum samples and are not validated for food samples (Ching, Lin, McGarvey, Stanker, & Hnasko, 2012; Gessler et al., 2007; Sharma et al., 2005). Among commercially available LFIA test kits are BioThreat (Tetracore, Rockville, MD), SMART (New Horizons Diagnostics, Columbia, MD), BADD (ADVNT Biotechnologies, Phoenix, AZ), and RAMP (Response Biomedical, Burnaby, BC, Canada) assays (Table 5). Gessler et al. (2007) evaluated these commercially LFIA tests and found that the BioThreat and SMART assays were not able to recognize native holotoxin (Gu & Jin, 2013). This makes the assays susceptible to cross-reactivity with nonbotulinum metabolites. In general, cross-reactions were seen in all assays with all toxin types except for RAMP, which was specific for BoNT/A. This could be explained by structural similarities of the toxins (Singh & DasGupta, 1989; Smith et al., 2005) and have been reported by several authors (Chiao, Shyu, Hu, Chiang, & Tang, 2004; Dezfulian & Bartlett, 1984; Sharma et al., 2005). The BADD assay was found to be unsuitable for the testing of culture material. The BioThreat and RAMP assay were also evaluated with clinical sample matrices (serum, gastric, and rectum contents from pigs) and results indicated that the direct detection of the toxins was hampered by matrix effects and a low positive response (Gessler et al., 2007).

Chiao et al. (2004) used polyclonal anti-BoNT/B antibodies to develop a rapid and sensitive LFIA for BoNT/B detection. In order to detect very low levels of the toxin, silver enhancement reagent was used to amplify the signal of colloidal gold and the LFIA was tested with serum and urine samples. The analysis was complete in less than 10 min and had a LOD of 50 ng/mL BoNT/B in case of just using colloidal gold. Using silver enhancement, 50 pg/mL of

BoNT/B was detected, which brings the LOD close to the sensitivity of the mouse bioassay (Chiao et al., 2004).

Sharma et al. (2005) evaluated the Bot-Tox-BTA kit for the detection of BoNT/A (Alexeter Technologies), the BioThreat (Tetracore, Rockville, MD) assay, and a kit from the NMRC (Naval Medical Research Center, Silver Spring, MD) for the detection of BoNT/A, BoNT/B, and BoNT/E in foods (liquid, semisolid, and solid) and developed procedures to extract the toxin from food samples. When the tests were performed with undiluted milk-based samples (whipping cream, half-and-half, and raw milk), they exhibited low or no filtration, probably because of the high-fat content. Such problems were particularly encountered with the Alexeter Technologies kits. Extending the migration time with an extra 15 min, on top of the manufacturers' recommended time of 15 min, was in almost all the cases sufficient for the toxins to reach the control zone. Alternatively, dilution or centrifugation, in the case of orange juice, ice-cream, and honey, may also speed up the migration process. Slow filtration was also observed for some semisolid and solid food with a low-fat content, which suggests that fat is not the only factor that is responsible for slow filtration. Data showed that these LFIA can also be used to test undiluted cultured toxins of foods for qualitative identification of BoNT (Sharma et al., 2005).

Ching et al. (2012) developed an LFIA that is capable of detecting and differentiating BoNT/A and /B as two independent colorimetric lines on a single strip in spiked beverages. In contrast to currently available commercial LFIA, which utilize polyclonal antibodies that are cross-reactive for BoNT/A and /B, this LFIA can distinguish between BoNT/A and /B serotypes as it uses two sets of highly specific monoclonal antibody pairs. In these studies, the Alexeter strip demonstrated a lower LOD of 100 ng/mL when spiked milk products were diluted and 10 ng/mL when they were defatted. The LFIA achieved similar sensitivities in milk but outperformed the Alexeter Technologies strip in spiked orange juice samples by fourfold detecting of both BoNT/A and /B in orange juice spiked at 25 ng/mL (Ching et al., 2012). Although the LFIA kits exhibit sensitivities less than the LOD of the mouse bioassay (10 pg/mL for pure BoNT/A), they are capable of detecting the toxin concentration that can cause botulism disease symptoms in humans. Therefore, they can be considered for large-scale or presumptive test screening to be followed by a confirmatory mouse bioassay (Sharma et al., 2005).

Many applications of LFIA for the detection of SEs have recently been developed. For instance, Boyle et al. (2010) reported the detection of SEB in milk and milk products, by which a SEB concentration of 0.25 ng/mL was reliably detected within 20 min. However, to permit the capillary flow of the sample onto the test strip of the LFIA in some cases a dilution (1:4 to 1:20) of the sample was necessary. In the case of dilution, a longer reading time is recommended to compensate for the lower concentration of the sample (Boyle

TABLE 5 An overview of the (non) commercially available LFIA for the detection of toxins produced by *B. cereus*, *C. botulinum*, *S. aureus*

Organism and toxin	Trade name	Manufacturer	Description	LOD/cross-reactivity	Reference
<i>B. cereus</i> diarrheal nonhemolytic enterotoxin and diarrheal hemolytic enterotoxin (Nhe and Hbl)	Duopath® Cereus Enterotoxins	Merck KGaA	LFIA for the detection of NheB Hbl L ₂ in food and environmental samples in 30 min	NheB: 6 ng/mL Hbl L ₂ : 20 ng/mL	Krause et al. (2010)
<i>C. botulinum</i> Botulinum neurotoxin A (BoNT/A)	RAMP® Bot Tox test	Response Biomedical	LFIA with antibodies coupled to fluorescent-dyed latex particles for the detection of BoNT/A in 15 min	Purified BoNT/A: 50 ng/mL BoNT/A complex: 250 ng/mL In culture supernatants: 2500 MLD/mL No cross-reactivity reported	Gessler et al. (2007); Sharma et al. (2005)
	BADD™ Test Strip	ADVNT Biotechnologies	LFIA for the detection of BoNT/A in 15 min	Purified BoNT/A: 100 ng/mL BoNT/A complex: 100 ng/mL In culture supernatants: 100 MLD/mL Cross-reactivity	
	SMART™-II	New Horizons	LFIA for the detection of BoNT/A from environmental samples in 15 min	Purified BoNT/A: ND BoNT/A complex: 10 ng/mL In culture supernatants: 100 MLD/mL Cross-reactivity	
<i>C. botulinum</i> Botulinum neurotoxin B (BoNT/B)	Bot-Tox-BTA	Alexeter Technologies	LFIA for the detection of BoNT/A from environmental samples in 15 min	BoNT/A complex: 10 ng/mL Cross-reactivity with BoNT/B	Chiao et al. (2004)
<i>C. botulinum</i> Botulinum neurotoxin A (BoNT/A) and botulinum neurotoxin B (BoNT/B)	/	Agriculture Research Service (United States Department of Agriculture)	LFIA for the detection of BoNT/A and BoNT/B	Sensitivities >10 ng/mL for purified toxins and 10 to 500 ng/mL in toxin-fortified beverages	Ching et al. (2012)
<i>C. botulinum</i> Botulinum neurotoxin A (BoNT/A) and botulinum neurotoxin B (BoNT/B)	Bot Tox BioThreat Alert™ Test Strip	Tetracore	LFIA for the detection of BoNT/A and BoNT/B in 15 min	Purified BoNT/A: ND BoNT/A complex: 10 ng/mL In culture supernatants: 100 MLD/mL BoNT/B complex: 10 ng/mL BoNT/E: 20 ng/mL	Sharma et al. (2005); Gessler et al. (2007)
<i>S. aureus</i> enterotoxin SEA	/	Naval Medical Research Center (NMRC)	LFIA for the detection of BoNT/A, BoNT/B, and BoNT/C in 15 min	Purified BoNT/A: 10 ng/mL BoNT/B complex: 10 ng/mL BoNT/E: 20 ng/mL SEA in milk: 0.2 ng/mL	Jin et al. (2013)
<i>S. aureus</i> enterotoxin SEB	SEB BioThreat Alert™ Test Strip	Tetracore	LFIA for the detection of SEA in milk in 15 min by using specific anti-SEs IgYs LFIA for the detection of SEB in 15 min	Cross-reactivity with SED	Tallent et al. (2014)

et al., 2010). It is well known that the sensitivity of the assay can be affected when antigen capture is applied to certain foods or beverages that are extremely acidic. The performance of LFIA for the environmental detection of SEB was evaluated in orange juice and popular dark or clear-colored carbonated soda drinks. Dilution of the beverage sample with Phosphate-Buffered Saline (PBS) prior to application onto the LFIA could eliminate the problem of the pulp fibers and the pH (Principato et al., 2010).

Jin et al. (2013) used chicken immunoglobulin (IgY) to develop an LFIA to detect SEA. Unlike mammalian IgG, it does not react with protein A because it possesses a different structure of the Fc region, thereby avoiding a nonspecific reactivity (Jin et al., 2013). It resulted in an LFIA that could rapidly detect SEA in milk (15 min) with high sensitivity (0.2 ng/mL); similar results were obtained for dairy foods. These data suggest that LFIA could be successfully used for the direct detection of SEA (0.2 ng/mL to 50 µg/mL) without interference by milk components, with greater simplicity and rapidness than ELISA. Just like for most of the other detection technologies also for LFIA, further studies should be carried out to apply these methods to detect enterotoxins in solid foods (Jin et al., 2013).

In 2014, an incident involving a foodborne outbreak with the potential of a SEB bioterrorism event was reported. However, the event was mischaracterized due to the use of methods that had not been validated in food matrices. This example highlights the need for more research toward cross-reactivity and the use of different food matrices in LFIA. PCR screening of the egg salad and staphylococcal strains isolated from the egg salad indicated SED contamination, which was confirmed by ELISA. However, the SEB BioThreat Test Strip yielded positive results for SEB. Tallent, Hait, and Bennett (2014) investigated the cross-reactivity of the SEB BioTreat Test Strip. This testing clearly demonstrates that, unless processed correctly, the food matrix can interfere with antigen and antibody binding, yielding either false-positive results as seen with the egg salad and milk or false-negative results as observed with the acidic peaches. Additionally, SED can cause false-positive reactions with the LFIA assays. The BioThreat Test Strip did not show cross-reactivity with the SEA, SEC, or SEE (Tallent et al., 2014). The SEB BioThreat Test Strip had been evaluated with several food matrices including dairy products, orange juice, and carbonated beverages but the studies seemed to be incomplete since the other SEs were not tested for cross-reactivity (Boyle et al., 2010; Principato et al., 2010; Tallent et al., 2014). In general, it is important to process suspect food samples using concentration and extraction techniques and to use validated assays fit for purpose that accurately detect toxins in the samples (Tallent et al., 2014). Overall, more attention should be paid to cross-reactivity and interference coming from the food matrix with LFIA to ensure accurate reporting.

5 | MASS SPECTROMETRY

Mass spectrometry (MS) is becoming a very popular technique for the study of toxins due to possibilities offered for identification, structural analysis, and quantification. The turn to MS-based approaches for the study of toxins (usually proteins or peptides) was enabled by the development of two soft ionization techniques, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), which allow the analysis of macromolecules without causing extensive (insource) fragmentation (Fenn, Mann, Meng, Wong, & Whitehouse, 1989; Karas & Hillenkamp, 1988). A typical setup for ESI-based MS is usually coupled (online) with a liquid chromatography (LC) system, which allows the separation of the molecules in the sample based on their physicochemical properties. In contrast, prior separation of the analytes (electrophoresis, LC, etc.), especially for complex mixtures is required for MALDI-MS. Peptides and small proteins (10 to 20 kDa) can be easily analyzed using MS with high sensitivity, but larger proteins (larger than 20 to 30 kDa) require digestion prior to analysis for efficient detection and accurate quantification. The implementation of tandem MS (MS/MS) offers additional specificity (sequence oriented approach due to fragmentation potential) and increases the confidence in quantification due to increasing sensitivity. Tandem MS/MS hereby enables specific toxins to be (quickly) detected and quantified in relatively complex mixtures based on their characteristic fragmentation patterns. In addition, MALDI-TOF-MS and MALDI-TOF/TOF are evolving into an indispensable tool for proteomics research (Han, Aslanian, & Yates, 2008).

The study of *B. cereus* pathogenicity is complicated by the abundance of toxins. On the one hand, there is cereulide, a peptide that is produced in foods and causes emesis and, on the other hand, there are the various diarrheal enterotoxins, such as Nhe, Hbl, and CytK that are de novo produced in the small intestine. Various LC-MS methods have been presented for the detection of cereulide, mainly in rice and pasta (Biesta-Peters et al., 2010; Haggblom, Apetroaie, Andersson, & Salkinoja-Salonen, 2002). The shift from to tandem MS improved sensitivity (with detection limits up to 0.1 ng/g) and robustness of the analysis due to fragmentation of the molecular ions (Decler, Rajkovic, Sas, Madder, & De Saeger, 2016; Delbrassinne et al., 2011b; Ronning, Asp, & Granum, 2015; Yamaguchi, Kawai, Kitagawa, & Kumeda, 2013). Just recently an ISO method for detection of cereulide using LC-MS/MS has been published (in't Veld, van der Laak, van Zon, & Biesta-Peters, 2019). Several outbreak-related reports in penne pasta and several types of rice dishes were published based on the previously developed MS methods (Delbrassinne et al., 2011a, 2012; Shiota et al., 2010). It is relevant to note that for CER quantification in complex matrices, the dodecadepsipeptide valinomycin or in recent years,

a stable isotope of cereulide ($^{13}\text{C}_6$ -cereulide), is regularly used as an internal standard (Zuberovic Muratovic, Troger, Granelli, & Hellenas, 2014). MS/MS analysis has also proved its usefulness as a supplementary technique for toxicity and toxicokinetic studies (Cui et al., 2016; Rajkovic et al., 2014). Regarding proteomics in cereulide investigations, Rajkovic et al. (2014) established a study describing a proteomic profile of Caco-2 cell after exposure to different cereulide concentrations by tryptic in-gel digestion prior to MALDI TOF/TOF MS/MS analysis. Regardless of the drawbacks of selectivity, sensitivity, and robustness of the previously described serological kits for *B. cereus* enterotoxin detection only limited MS-based alternatives have been published. MALDI-TOF/MS was applied for the detection of CytK-1 and Nhe by Tsilia et al. (2012). With the use of 1D gel electrophoresis, tryptic peptides from CytK-1 and NheA produced by different pathogenic *B. cereus* strains were verified through MALDI-TOF/MS using peptide mass fingerprinting and/or MS/MS ion search. The identified enterotoxin tryptic peptides can be used as biomarkers for the screening of food or of potentially enterotoxigenic *B. cereus* isolates (Tsilia et al., 2012). Other publications used the MALDI strategy to study the *B. cereus* secretome and detected enterotoxins components (Gilois et al., 2007; Gohar et al., 2002). Our research group has recently used multiple reaction monitoring to detect and quantify enterotoxins produced from food poisoning strains of *B. cereus*, including the very heterogeneous strain *B. cytotoxicus* NVH 0391/98 described by Guinebretiere et al. (2013). Quantification was performed using in total seven stable isotope-labeled tryptic peptides selected after the screening of in silico enterotoxin digests. To circumvent the lack of detection methods for CytK, we have designed heavy tryptic peptides to discriminate among the two orthologues/variants of CytK. At the same time, five peptides were used to target all the components of Nhe.

There are seven known serotypes of *C. botulinum* neurotoxin proteins (BoNTs A–G) that can further be categorized into different subtypes or toxin variants of which some have as few as a single amino acid difference. In terms of diagnostics, BoNT toxins need to be identified by their enzymatic action on peptide substrates in combination with the detection of by their unique amino acid sequence. Several MS-based methods have been developed for the detection of BoNT. Van Baar and colleagues combined a MALDI-TOF and an ESI-QTOF instrument to gather sequence information needed to identify BoNT/A and /B (van Baar, Hulst, de Jong, & Wils, 2002). The development of the so-called Endopep-MS method enabled the detection of all seven BoNT types and the quantification of their enzymatic activities (Boyer et al., 2005). To assess the enzymatic activity, the unique peptide products produced by the toxin-specific cleavage of target peptide substrates are detected by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) or liq-

uid chromatography electrospray ionization tandem MS (LC-ESI-MS/MS). The different BoNT serotypes can be differentiated through the determination of their amino acid sequence. To identify the toxin by their amino acid sequence, toxins are digested with enzymes such as trypsin and the resulting peptides are then detected by MS/MS. Barr et al. (2005) developed simultaneously an Endopep-MS method where the product peptides derived from endopeptidase activities of BoNT serotypes A, B, E, and F were detected. This promising Endopep-MS method has been extended to various types of matrices (Kalb, Goodnough, Malizio, Pirkle, & Barr, 2005, 2006). A description of a validated Endopep-MS method was described by Bjornstad et al. (2014) for BoNTs A, B, C, C/D, D, D/C, E, and F in serum. Several studies followed where a proteomics approach was implemented after an antibody extraction method with Endopep-MS (Kalb et al., 2012; Moura et al., 2011). Endopep-MS is a reliable alternative to the traditional mouse bioassay (MBA) that provides detailed information to differentiate between the specific BoNT subtypes based on amino acid sequencing and the highly specific endopeptidase activity. Today, official detection and quantification methods for the small SEs are mainly based on the immunoassay principle. In contrast, high-throughput techniques based on MS theoretically allow detection of any kind of SE type in complex samples. Different LC-MS/MS-based methods have been published for the detection of whole toxins or their tryptic fragments. Callahan, Shefcheck, William, and Musser (2006) started to develop a whole protein LC/MS analysis to measure SEB in apple juice but switched to proteomics-based methods as they were not able to detect intact SEB. However, Sospedra, Soler, Manes, and Soriano (2011) published a whole protein method that successfully detects both SEA and SEB in apple, orange juice, and milk at levels around $0.05\ \mu\text{g}/\text{mL}$. Recently, an online SPE-LC-ESI-MS/MS method was developed for the detection of SEA and SEB in milk (Andjelkovic, Tsilia, Rajkovic, De Cremer, & Van Loco, 2016). In the pursuit of finding quantitative determination of SEs in complex biological samples, Brun et al. (2007) proposed an innovative strategy called protein standard absolute quantification (PSAQ), which is based on full-length isotope-labeled recombinant toxins (PSAQ standards). This approach was first developed for drinking water and human urine samples and further expanded to semihard cow-milk cheese (Dupuis, Hennekinne, Garin, & Brun, 2008). From a clinical point of view, the analysis of serum or plasma samples in case of staphylococcal sepsis might be crucial. It has been demonstrated that the coupling of immunoaffinity techniques with MS enhances specificity and sensitivity for the quantification proteins at low concentration levels, especially in highly complex samples such as serum or plasma (Ackermann & Berna, 2007). Therefore, Adrait et al. (2012) optimized an immunoaffinity-based sample preparation with MS analysis for the quantification of SEA in serum. The incorporation of

the PSAQ method and MS analysis in selected reaction monitoring improved, respectively, specificity and selectivity.

So far no reports are found on the use of the LC-MS method for detection of CPE, but the method has been used for *C. perfringens* epsilon toxin (Alam, Kumar, & Kamboj, 2012; Alam, Uppal, Gupta, & Kamboj, 2017) and a new type of enterotoxin, named *C. perfringens* iota-like enterotoxin (Irikura et al., 2015).

In conclusion, the identification of toxins carried out by MS-based provides new possibilities for the detection of protein/peptide sequencing. The two main methods based on MS regarding bacterial protein/peptide toxins are MALDI-TOF based protein fingerprinting and LC-MS/MS. Furthermore, very powerful analytical methods have been described when combining antibody affinity preconcentration and biochemical tests with MS. It is clear that the use of MS instrumentation with high specificity, good sensitivity, and quantitative characteristics is recommended for bacterial identification and characterization.

6 | GENE DETECTION

6.1 | Polymerase chain reaction

The polymerase chain reaction (PCR), either conventional or real time, is being increasingly applied for the detection, identification, and characterization of microorganisms in food and environmental samples. These techniques are particularly relevant where there is a lack of cultivation methods, when one deals with low levels of pathogens that are difficult to isolate, when within species toxigenic and nontoxigenic strains exist and when the potential of the isolated pathogen to produce toxin in host's gastrointestinal tract needs to be assessed. Therefore, PCR-based methods are routinely used to classify isolated pathogens based on the presence or absence of toxin-encoding genes (or in general other specific sets of virulence-encoding genes). An interesting aspect that endorses the use of gene(s) detection is that the distribution of various enterotoxin genes and other accompanying virulence factor genes in the same pathogen may result in higher virulence (and potentially higher risk for the consumer) than one could assume from the detection of one single (produced) toxin only. PCR, particularly real-time PCR, is an extremely expedite method that allows testing for a large number of samples, with multiple targets, in a single day. Furthermore, the enrichment procedure, although decreasing the limit of detection, is not always necessary for PCR. Therefore, it is a very useful tool to provide information on multiple toxins and virulence factors, as well as to identify isolated pathogens. However, the interpretation of the results of multiplex amplification from a mixed population of bacteria may be problematic. A general drawback inherent to gene detection is that sequence diversity among

the toxin genes could lead to false-negative results in PCR. Comparative analysis of the nucleotide sequences for different enterotoxins, for example, *seb*, *sec*, and *sed* genes in different *S. aureus*, showed that some gene sequences are more conserved than the others (Johler, Sihto, Macori, & Stephan, 2016). Another, but different kind of drawback of toxin, gene detection is caused by so-called silent genes. Despite carrying the toxin-encoding gene a pathogen is not able to express the toxin. The silent *cpe* sequences have been reported in *C. perfringens* that carried *cpe* gene-encoding *C. perfringens* CPE, but was not able to express CPE (Billington et al., 1998). Similar examples can be given for *sed* gene encoding for enterotoxin SED in *S. aureus* (Lawrynowicz-Paciorek, Kochman, Piekarska, Grochowska, & Windyga, 2007) or for *hbl* genes in certain strains of *B. cereus* group members, *B. thuringiensis* and *B. mycoides* (Oh, Ham, & Cox, 2012).

Since the beginning of the 1990s, several PCR assays were reported for the specific detection of multiple *B. cereus*, *C. botulinum* *C. perfringens*, and *S. aureus* toxins (Table 6). More interestingly, the PCR reactions accompanied the discovery of new toxins throughout the years. In general, several key steps are relevant for the development of a sensitive and specific PCR reaction. The first and the most significant step is a design of the primers to eliminate the chance of false-positive or negative results and increase PCR yield. Following the design of the primers, the PCR reaction itself must be optimized, particularly the annealing temperature (T_a). Relatively low T_a may lead to the amplification of unspecific PCR amplicons. In contrast, high T_a can lead to a decrease in the PCR yield. Generally, and empirically, T_a should be set and tested anywhere between -2 and 2 °C of the determined temperature of melting (T_m), with the possibility of using -5 and 5 °C of the T_m . The primers themselves should have a similar T_m for a more accurate PCR result. Another important issue to attend to while developing a PCR reaction is the extraction protocol. The extraction efficiency not only in terms of DNA yield but also in terms of removing PCR inhibitors is crucial. In a study by Ehling-Schulz et al. (2006), the authors tested the efficiency of three distinct protocols for the extraction of *B. cereus* DNA from culture collection strains, food, and clinical isolates. Two of reported methods were based on the commercially available kits (AquaPure Genomic DNA Isolation kit (Biorad, Germany) and the DNeasy Tissue kit (Qiagen, VWR International AB, Sweden)) and the third one was a simple boiling method. The authors determined that DNA extracted by the latter method, although working at a high level for isolates from a culture collection, was not suitable for extracting DNA from food and clinical samples. In another work focused on the detection of enterotoxigenic *C. perfringens* from meat samples, three types of DNA extraction kits were evaluated with respect to their sensitivity, convenience and their applicability for testing a large number of samples (Kaneko et al., 2011). The three kits applied were the QIAamp

TABLE 6 PCR assays for detection of targeted bacterial toxins

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
<i>B. cereus</i>	<i>ces</i>	EM1F	GACAAGAGAAATTTCTACGAGCAAGTACAAT	635	60	Ehling-Schulz, Fricker, and Scherer (2004)
		EM1R	GCAGCCTTCCAATTAATCTCTTCTGCCACAGT			
		<i>ces</i> _SYBR_F	CGTGTGAACGTGGTCAAATCA	176	60	Fricker et al. (2007)
		<i>ces</i> _SYBR_R	CACCTTCGTCCTTTTGATAATACG			
		<i>ces</i> _TaqMan_F	CGCCGAAAAGTGATTATACCAA	103	55	Fricker et al. (2007)
		<i>ces</i> _TaqMan_R	TATGCCCCGTTCTCAAACCTG			
		CES F1	GGTGACACATTATCATATAAGGTG	1271		Ehling-Schulz et al. (2005)
		CES R2	GTAAGCGAACCTGTCTGTAAACAACA			
		Forward	ATGAATCAACGATTATGGG	70	56	Forghani et al. (2015)
		Reverse	GTCGGATTCGATACAAATTC			
		Forward	TTCCGCTCTCAATAAATGGG	634	54	Kim et al. (2012)
		Reverse	TCACAGCACATTCCAAATGC			
	<i>ces b</i>	<i>ces</i> B-F	ACCCATCTTGCCTCATT	154	50	Zhang et al. (2014)
		<i>ces</i> B-R	CAGCCAAGTGAAGAATACC			
	<i>cer</i>	Forward	GCGTACCAAATCACCCGTTTC	546	54	Kim et al. (2012)
		Reverse	TGCAGGTGGCACACTTGTITA			
	<i>cytK</i>	CK F	ACAGATATCGG(G,T)AAAATGC	809	54	Guinebreiere, Brousolle, and Nguyen-The (2002)
		CK R	GAAGT(G,C)(A,T)AACTGGGTGGGA			
		CK F2	ACAGATATCGGICAAAATGC	421	49	Ehling-Schulz et al. (2006)
		CK R5	CAAAGTIACTGACCIGTTGC			
		Forward	AAATGTTTAGCATTATCCGC	106	56	Forghani et al. (2015)
		Reverse	TTTGCCCGATAATCTGTTACAAC			
		<i>cytK</i> -F	GCGCTGATAAACAGATTGCCGT	105	56	Ceuppens, Timmerly, Mahillon, Uyttendaele, and Boon (2013)
		<i>cytK</i> -R	TAGCGCCAGGATTGGGTAGTT			
		FCytK	CGACGTCACAAAGTTGTAAACA	565	54	Ngamwongsatit et al. (2008)
		R2CytK	CGTGTGTAATAATACCCCAAGTT			
		Forward	TGCTAGTAGTGTGTTAACTC	881	54	Kim et al. (2012)
		Reverse	CGTTGTTTCCCAACCCAGT			
		Forward	AAATGTTTAGCATTATCCGC	106	56	Forghani et al. (2015)
		Reverse	TTTGCCCGATATCTGTTACAAC			

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		cytK-F	ATCGGTCAAAAATGCAAAAACAC	800	52.2	Zhang, Feng et al. (2016)
		cytK-R	ACCCAGTTACCAGTTCGGAATG			
<i>cytK1</i>		CK1F	CAAATCCAGGGGCAAGTGTC	426	57	Guinebretiere et al. (2006)
		CK1R	CCTCGTGCATCTGTTTCATGAG			
		mp4CytKfor	GCTTTGTATAAGCAACTTGGATAG	389	60	Wehrle et al. (2010)
		mp4CytKrev	AGCCTCTGTAACACCAAGC			
<i>cytK2</i>		CK2F	CAATCCCTGGCCGTAGTGCA	585	57	Guinebretiere et al. (2006)
		CK2R	GTGIAGCCCTGGACGAAAGTTGG			
<i>hbl</i>		HD2 F	GTAATAATTAIGATGAICAAATTC	1091	49	Ehling-Schulz et al. (2006)
		HA4 R	AGAATAGGCATTTCATAGATT			
<i>hblC</i>		FHbIC	CCTATCAATACTCTCGCAA	695	54	Ngamwongsatit et al. (2008)
		RHbIC	TTTCCITTTGTATACGCTGC			
		HC F	GATAC(T,C)AATGTGGCAACTGC	740	54	Guinebretiere et al. (2002)
		HC R	TTGAGACTGCTCG(T,C)TAGTTG			
		L2aF	CGAAAATTAGGTGCGCAATC	411	51	Moravek et al. (2004)
		L2aR	TAATATGCCCTTGGCAGTTG			
		Forward	CGCAACGACAAATCAATGAA	421	54	Kim et al. (2012)
		Reverse	ATTGCTTCACGAGCTGCTTT			
<i>hblD</i>		HD F	ACCGGTAAACACTATTTCATGC	829	54	Guinebretiere et al. (2002)
		HD R	GAGTCCATATGCTTAGATGC			
		mp3LIR lfor	AGTTATTGCAGCTAATTGGAGG	148	60	Wehrle et al. (2010)
		mp3LIR lrev	GTCCATATGCTTAGATGCTGTGA			
		L1aF	AGGTCAAACAGGCAACGATTC	205	53	Moravek et al. (2004)
		L1aR	CGAGAGTCCACCAACAACAG			
		Forward	GCATGGTCAATTGGTGGT	163	56	Forghani et al. (2015)
		Reverse	CACCAGCTGCTGTTCCCTA			
		hblD-F	GTTAGATACAGCGAAGCCAC	465	52.2	Zhang, Feng et al. (2016)
		hblD-R	CCGCCAGTTACAACAATA			
<i>hblA</i>		HA F	AAGCAATGGAATACAATGGG	1154	54	Guinebretiere et al. (2002)
		HA R	AGAAATCTAAATCATGCCACTGC			
		FHbIA	GCAAAATCTATGAATGCCTA	884	54	Ngamwongsatit et al. (2008)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
<i>nhe</i>	RHbIA		GCATCTGTTCTCGTAAATGTTTT			
	NA2 F		AAGCIGCTCTTCGIATTC	766	49	Ehling-Schulz et al. (2006)
	NB1 R		ITIGTTGAAATAAAGCTGTGG			
	F2NheA		TAAGGAGGGGCAAAACAGAAG	759	52.2	Ngamwongsatit et al. (2008)
	RNheA		TGAATGCCGAGAGCTGCTTC			
	NA F		GTTAGGATCACAAATCACCGC	755	54	Guinebretiere et al. (2002)
<i>nheA</i>	NA R		ACGAATGTAATTTGAGTCGC			
	NA F2		GAATGTRCGAGARTGGATTG	551	54	Ehling-Schulz et al. (2006)
	NA R2		GCYGCTTCYCTCGTTTGRCT			
	mp3AR2for		TTCAAATTCAAAAGAATGTTGAAGAAGG	111	49	Wehrle et al. (2010)
	mp3AR2rev		GATTTGTTTGGCTTATTCATTTTCATCAC			
	45c1		GAGGGCAAAACAGAAAGTAA	186	60	Moravek et al. (2004)
<i>nheB</i>	45c2		TGCGAACTTTTGTGATGATTCG			
	Forward		GGAGGGCAAAACAGAAAGTAA	750	52	Kim et al. (2012)
	Reverse		CGAAGAGCTGCTTCTCTCGT			
	Forward		TGAAAATTGAAAATGCTGCAG	376	54	Forghani et al. (2015)
	Reverse		ATGTACTTCAACGTTTGTAAACG			
	39b1		CCGCTTCTGCAAAATCAAAT	281	52	Moravek et al. (2004)
<i>nheC</i>	39b2		TGCGCAGTTGTAACTTGTCC			
	NB F		TTTAGTAGTGGATCTGTACGC	743	49	Ehling-Schulz et al. (2006)
	NB R		TTAATGTTTCGTTAATCCTGC			
	F2NheB		CAAGCTCCAGTTCATGCGG	935	54	Ngamwongsatit et al. (2008)
	RNheB		GATCCCATTTGTACCATTG			
	NC F		TGGATCCAAGATGTAACG	683	54	Guinebretiere et al. (2002)
<i>beeT</i>	NC R		ATTACGACTTCTGCTTGTGC			
	FNheC		ACATCCTTTTGCAGCAGAAC	618	54	Ngamwongsatit et al. (2008)
	R2NheC		CCACCAGCAATGACCCATATC			
	BCET1 F		CGTATCGGTCGTTCACTCGG	661	54	Guinebretiere et al. (2002)
	BCET3 R		GTTGATTTTCCGTAGCCTGGG			
	BCET1 F		CGTATCGGTCGTTCACTCGG	924	54	Guinebretiere et al. (2002)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
<i>entFM</i>	BCET4 R	Forward	TTTCTTTCCCGCTTGCCTTT	488	56	Forghani et al. (2015)
		Reverse	GGAACTGGATACGTAAGC			
	FEntFM	Forward	TAGTGAATGAATCCACTGC	486	54	Ngamwongsatit et al. (2008)
		Reverse	GTTTCGTTACGGTGTGGTAC			
<i>C. botulinum</i>	BoNT_A_F	Forward	ATTAGAGGTTATATGTATCTTAAAGGGC	136	60	Kirchner et al. (2010)
		Reverse	CTAACAAATATATCTTYYATTTCCAGAAGC			
	CBMLA1	Forward	AGCTACGGAGGCAGCTATGTT	782	60	Lindström et al. (2001)
		Reverse	CGTATTTGGAAAAGCTGAAAAGG			
	NKB-1	Forward	GATACATTTACAAAATCCTGAAGGAGA	2278	50	Franciosa et al. (1994)
		Reverse	AACCGTTTAAACACCATAAGGGATCATAGAA			
IOA1a F	Forward	GGGCCTAGAGGTAGCGTARTG	101	55	Feniccia, Anniballi, De Medici, Delibato, and Aureli (2007)	
IOA2a R	Reverse	TCTTYATTTCCAGAAGCATATTTT				
AS-11	Forward	Forward	TGCAGGACAAAATGCAACCAGT	286	55	Binz, Wernars, and Kurazonosli (1990); Takeshi (1996)
		Reverse	TCCACCCCAAAAATGGTATTCC			
	Reverse	Forward	ACGGAAAATGGTTATGGYTCTACTC	142	62	Satterfield et al. (2010)
		Reverse	GTGCTAATGYTACYGCTGGATCTG			
<i>bont/b</i>	BoNT_B_F	Forward	TTGCATCAAGGGAAGGCT	116	50	Franciosa et al. (1994)
		Reverse	ATCCACGTCTATTAATAATACCTTGGC			
	B1-a	Forward	GATGGAACCAATTTGCTAG	1284	50	Franciosa et al. (1994)
		Reverse	AACATCAATACATATTCCTGG			
B3	Forward	CCAGGAATATGTATTGATGTT	1450	50	Franciosa et al. (1994)	
	Reverse	AAATCAAGGAACACACACTA				
B5	Forward	TGGATAAGAATACCTAAATATAAG	881	50	Franciosa et al. (1994)	
	Reverse	AAGCAACTGACAACACTATATGT				
JF-B1	Forward	ATGCCAGTTACAATAAAATTTAATTAT	3873	50	Franciosa et al. (1994)	
	Reverse	TTCAGTCTCCCTTCATCTTTAGG				
CBMLB1	Forward	CAGGAGAAAGTGGAGCGGAAA	205	60	Lindström et al. (2001)	
	Reverse	CTTGGCCCTTTGTTTCTTGG				

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		BS-11	CCTCCATTTGCGAGAGGTACG	315	55	Takeshi (1996)
		BS-22	CTCTTCGAGTGGAAACACGCTCT			
		Forward	AGTAATCCAGGAGAAGTGGAGCGA	136	62	Satterfield et al. (2010)
		Reverse	CRAAGCCTTCCCTTGATGCAAA			
	<i>bont/c</i>	BoNT_C_F	AGAGAAAACATATATAGAYCCAGAAAACIT	166	60	Kirchner et al. (2010)
		BoNT_C_R	GACTTAGAAAATCTACCCCTCTCCTACAT			
		CS-11	ATACACTAGCTAATGAGCCTG	290	55	Takeshi (1996)
		CS-22	TGGAGTATTTGTTATTTCCACGG			
	<i>bont/d</i>	BoNT_D_F	GGGTAATACCAGAAAAGATTTTCATC	91	60	Kirchner et al. (2010)
		BoNT_D_R	AGGATCATAATAACTTTGATACCTTTGAAGT			
		DS-11	GTGATCCTGTTAATGACAAATG	497	55	Takeshi (1996)
		DS-22	TCCTTGCAATGTAAGGGATGC			
	<i>bont/e</i>	BoNT_E_F1	TGAAAATAATGTCAATCTCACCTCTTCA	123	60	Kirchner et al. (2010)
		BoNT_E_R1	AAATAATGCTGCTTGCACAGGTT			
		CBMLE1	CCAAGATTTTCATCCGCCTA	389	60	Lindström et al. (2001)
		CBMLE2	GCTATTGATCCAAAACGGTGA			
		GF-1	AAAAGTCATATCTATGGATA	762	50	Franciosa et al. (1994)
		GF-3	GTGTTATAGTATACATTGTAGTAATCC			
		Forward	CACAGAAAGTGCCCGAAGGTGAAA	136	62	Satterfield et al. (2010)
		Reverse	GCTGCTTGCACAGGTTTATTGACA			
	<i>bont/f</i>	BoNT_F_F1	CCGGMTTTCATTARAGAACCGGAAG	152	60	Kirchner et al. (2010)
		BoNT_F_R1	TGATAITTTCTTSTAACAAAACCTTYCCCTG			
		CBMLF1	CGG CTT CAT TAG AGA ACG GA	543	60	Lindström et al. (2001)
		CBMLF2	TAA CTC CCC TAG CCC CGT AT			
		FS-11	CAATAGGAACGAATCCTAGTG	332	55	Takeshi (1996)
		FS-22	ATCAGGTCCTGCTCCCAATAC			
		Forward	GTGGAGGGMATMATA TAGTAGACAGA	155	62	Satterfield et al. (2010)
		Reverse	GGCTATCATAAGAGGTSCTYGCCTTT			
	<i>C. perfringens</i>	CPA F	TGCATGAGCTTCAATTAGGT	400	53	Heikinheimo and Korkeala (2005)
		CPA R	TTAGTTTTGCAACCTGCTGT			
		Forward	GCTAATGTTACTGCGGTTGA	324	55	Lin and Labbe (2003)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		Reverse	CCTCTGATACATCGTGTAAAG			
		Forward	GTTGATAGCCGAGGACATGTTAAG	402	72	Yoo et al. (1997)
		Reverse	CATGTAGTCACTCTGTTCCAGCATC			
		cpa F	TGCACATAATTTGGAGATATAGATAC	128	55	Gurjar, Hegde, Love, and Jayarao (2008)
		cpa R	CTGCTGTGTTAATTTTATACITGTTTC			
		CPA5L	AGTCTACGCTTGGGATGGAA	900	55	Baums et al. (2004)
		CPA5R	TTTTCTGGGTTGTCCATTTC			
		Forward	GCTAATGTTACTGCCCCTTGA	325	60	Adi, Pappithi, Veeraraghavulu, and Matcha (2015)
		Reverse	CCTCTGATACATCGTGTAAAGAATC			
	<i>cpb</i>	CPB F	GCGAATATGCTGAATCATCTA	196	53	Meer and Songer (1997)
		CPB R	GCAGGAACATTAGTATATCTTC			
		cpb F	ATTTCAITTAGTTATAGTTAGTTTCAC	93	55	Gurjar et al. (2008)
		cpb R	TTATAGTAGTAGTTTTGCCTATATAC			
		CPBL	TCCTTTCTTGAGGGAGGATAAA	611	55	Baums et al. (2004)
		CPBR	TGAACCTCCTAITTTTGTATCCCA			
	<i>cpb2</i>	Forward	AGATTTTAAATATGATCCTAACC	548	55	Garmory et al. (2000)
		Reverse	CAATACCCCTTCAACCAATACTC			
		Forward	ACTATACAGACAGATCAITTCACACC	236	72	Yoo et al. (1997)
		Reverse	TTAGGAGCAGTTAGAACTACAGAC			
		cpb2 F	TAAACCCATCAITTAGAACTCAAG	90	55	Gurjar et al. (2008)
		cpb2 R	CTATCAGAATAATGTTTGTGGATAAAC			
		P319BETA2	GAAAGGTAATGGAGAATTATCTTAATGC	574	48	Herholz et al. (1999)
		P320BETA2	GCAGAATCAGGATTTTGACCATATACC			
		CPB2L	CAAGCAATTTGGGGAGTTTA	200	55	Baums et al. (2004)
		CPB2R	GCAGAATCAGGATTTTGACCA			
	<i>etx</i>	ETX F	GCGGTGATATCCATCTATTTC	655	53	Meer and Songer (1997)
		ETX R	CCACTTACTTGTCTACTAACC			
		etx F	TAACTAATGATACTCAACAAGAAC	145	55	Gurjar et al. (2008)
		etx R	GTTTCAITTAAGGAACAGTAAAC			
		CPETXL	TGGGAACCTCGATACAAGCA	396	55	Baums et al. (2004)
		CPETXR	TAACTCATCTCCCATACTGCAC			
	<i>iA/cpi</i>	IA F	ACTACTCTCAGACAAGACAG	446	53	Meer and Songer (1997)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		IA R	CTTTCCTTCTATTACTATACG			
		Forward	GCGATGAAAAGCCTACACCACTAC	317	55	Yoo et al. (1997)
		Reverse	GGTATATCCTCCACGCATATAGTC			
		ia F	CAAGATGGATTTAAGGATGTTTC	89	55	Gurjar et al. (2008)
		ia R	TTTTGGTAATTTCAAATGTATAAGTAG			
		CPIL	AAACGCATTAAGCTCACACC	293	55	Baums et al. (2004)
		CPIR	CTGCATAACCTGGAATGGCT			
		JRP5507	GGAAAAGAAAATTATAGTGATTTGG	461	55	
		JRP5508	CCTGCATAACCTGGAATGGC			Rood et al. (2018)
	<i>cpe</i>	CPE F	GGAGATGGTTGGATATTAGG	233	53	Meer and Songer (1997)
		CPE R	GGACCAGCAGTTGTAGATA			
		Forward	ACTGCAACTACTACTCATACTGTG	541	72	Yoo et al. (1997)
		Reverse	CTGGTGCCCTTAATAGAAAAGACTCC			
		cpe F	AACTATAGGAGAACAAAATACAATAG	84	55	Gurjar et al. (2008)
		cpe R	TGCATAAACCTTATAATATACATAATC			
		CPEL	GGGGAACCTCAGTAGTTTCA	506	55	Baums et al. (2004)
		CPER	ACCAGCTGGATTTGAGTTTAATG			
	<i>netB</i>	JRP6656	CTTCTAGTGATACCGCTTCAC	738	55	Rood et al. (2018)
		JRP6655	CGTTATATTCACTTGTGGACGAAAG			
	<i>sea</i>	<i>sea</i> -3	CCTTTGGAAACGGTTAAAACG	127	55	Becker, Roth, and Peters (1998)
		<i>sea</i> -4	TCTGAACCTTCCCATCAAAAAC			
		<i>setA</i> -F	GGATATTGTTGATAAATATAAAGGAAAAAAG	439	45	Sergeev et al. (2004)
		<i>setA</i> -r	GTTAATCGTTTTTATTATCTCTATATATTTCTTAATAGT			
		GSEAR-1	GTTATCAATGTGCGGGTGG	102	57	Mehrotra et al. (2000)
		GSEAR-2	CGGCACTTTTTCTCTTCGG			
		SEA-F1170	TAAGGAGGTGGTGCCTAIGG	180	56	Cremonesi et al. (2005)
		SEA-R1349	CATCGAAAACCCAGCCAAAAGTT			
		<i>sea</i> F	GCAGGGAACAGCTTTTAGGC	521	68	Manfredi, Leotta, and Rivas (2010)
		<i>sea</i> R	GTTCTGTAGAAAGTATGAAAACAGC			
			GCAGGGAACAGCTTTAGGC	520	68	Monday and Bohach et al. (1999)
			GTTCTGTAGAAAGTATGAAAACAGC			
		SEA-1	TTGAAAACGGTTAAAACGAA	120	55	Johnson et al. (1991)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		SEA-2	GAACCTTCCCATCAAAAAACA			
		SEA-1	AAAGTCCCGATCAATTTATGGCTA	218	55	Akineden et al. (2001)
		SEA-2	GTAATTAACCGAAGGTTCTGTAGA			
<i>seb</i>	<i>seb-1</i>		TCGCATCAAAACTGACAAAACG	477	55	Becker et al. (1998)
	<i>seb-4</i>		GCAGGTACTCTATAAAGTGCCTGC			
	setB-F		AGATTTAGCTGATAAATACAAAGATAAATACG	494	45	Sergeev et al. (2004)
	setB-R		TCGTAAGATAAAACTTCAATCTTCACAICT			
	GSEBR-1		GTATGGTGGTGAACGTGAGC	164	57	Mehrotra et al. (2000)
	GSEBR-2		CCAAATAGTGACGAGTTAGG			
	seb-sec F		ACATGTAATTTTGATATTCGCACCTG	667	68 60	Manfredi et al. (2010)
	seb R		TGCAGGCATCATATCATACCA			
	SEB-1		TCGCATCAAAACTGACAAAACG	478	55	Johnson et al. (1991)
	SEB-2		GCAGGTACTCTATAAAGTGCC			
	seb-sec		ATGTAATTTTGATATTCGCAGTG	643	68 64	Monday and Bohach et al. (1999)
			TGCAGGCATCATATCATACCA			
<i>sec</i>	<i>setC-F</i>		AGATTTAGCAAAAGAAGTACAAAAGATG	490	45	Sergeev et al. (2004)
	<i>setC-R</i>		AAGGTGGACTTCTATCTTCACACTT			
	SEC-3b		CTCAAGAAGTACGACATAAAAGCTAGG	271	55	Becker et al. (1998)
	SEC-4b		TCAAAAATCGGATTAACACATATATCC			
	GSECR-1		AGATGAAGTAGTTGATGTATGG	451	57	Mehrotra et al. (2000)
	GSECR-2		CACACTTTTAGAATCAACCG			
	SEC-F97		ACCAGACCCTATGCCAGATG	371	56	Cremonesi et al. (2005)
	SEC-R467		TCCCATTATCAAAAGTGGTTTCC			
	sec F		CTTGTATGTATGGAGGAATAACAA	284	68 60	Manfredi et al. (2010)
	sec R		TGCAGGCATCATATCATACCA			
			CTTGTATGTATGGAGGAATAACAA	283	68 64	Monday and Bohach et al. (1999)
			TGCAGGCATCATATCATACCA			
	SEC-1		GACATAAAAGCTAGGAAATTT	257	55	Johnson et al. (1991)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		SEC-2	AAATCGGATTAACATTATCC			
	<i>sed</i>	<i>sed_newF</i>	GAGGTGTCACCCACACGAA	349		Varshney et al. (2009)
		<i>sed_newR</i>	TGAAGGTGCTCTGTGGATAATG			
		setD-F	AGATTTAGCAAAGAAGTACAAAAGATG	481	45	Sergeev et al. (2004)
		setD-R	CTACTTTTCATATAAATAGATGTCAAATATG			
		SED-3b	CTAGTTTGGTAAATATCTCCTTTAAAGG	319	55	Becker et al. (1998)
		SED-4b	TAAATGCTATATCTTATAGGGTAAACATC			
		GSEDR-1	CCAATAATAGGAGAAAAATAAAG	278	57	Mehrotra et al. (2000)
		GSEDR-2	ATTGGTAITTTTTTTCGTTC			
		SED-F578	TCAATTCAAAAGAAATGGCTCA	339	56	Cremonesi et al. (2005)
		SED-R916	TTTTCCGGCTGTATTTTT			
		sed F	GTGTTGAAAATAGATAGGACTGC	385	68 60	Manfredi et al. (2010)
		sed R	ATATGAAGGTGCTCTGTGG			
			GTGTTGAAAATAGATAGGACTGC	384	68 64	Monday and Bohach et al. (1999)
			ATATGAAGGTGCTCTGTGG			
		SED-1	CTAGTTTGGTAAATATCTCCT	317	55	Johnson et al. (1991)
		SED-2	TAATGCTATATCTTATAGGG			
	<i>see</i>	<i>seeI-f</i>	ACCGATTGACCCGAAGAAAAA	264		Varshney et al. (2009)
		<i>seeI-r</i>	ATTGCCCTTGAGCATCAAAC			
		setE-F	AGATTTAGCAAAGAAGTACAAAAGATG	473	45	(Sergeev et al., 2004)
		setE-R	TGTATAAATACAAAATCAATATGGAGGTTCTCT			
		SEE-3b	CAGTACCTATAGATAAAGTTAAACAAGC	178	55	Becker et al. (1998)
		SEE-2c	TAACTTACCGTGGACCCCTTC			
		GSEER-1	AGGTTTTTTCACAGGTCATCC	209	57	Mehrotra et al. (2000)
		GSEER-2	CTTTTTTCTTCGGTCAATC			
		see F	TACCAATTAACCTTGTGGATAGAC	121	68 60	Manfredi et al. (2010)
		see R	CTCTTTGCACCTTACCCG			
			TACCAATTAACCTTGTGGATAGAC	170	68 64	Monday and Bohach et al. (1999)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
			CTCTTTGCACCTTACCGC			
		SEE-1	TAGATAAAGTTAAAAACAAGC	170	55	Johnson et al. (1991)
		SEE-2	TAACTTACCGTGGACCCCTTC			
<i>seg</i>		<i>setG-F</i>	AGAAATTAGCTAACAAATTATAAAGATAAAAAAAG	496	45	Sergeev et al. (2004)
		<i>setG-R</i>	TCAGTGAGTATTAAGAAATACTTCCAT			
		SEG-1	AAGTAGACATTTTTGGCGTTCC	287	55	Omoe et al. (2002)
		SEG-2	AGAAACCATCAAACTCGTATAGC			
		SEG-F322	CCACTGTTGAAGGAAGAGG	432	56	Cremonesi et al. (2005)
		SEG-R753	TGCAGAACCATCAAAACTCGT			
		SEG-for	GTTAGAGGAGGTTTTATG	198	52	Bania et al. (2006)
		SEG-rev	TTCTTCAACAGGTGGAGA			
			CGTCTCCACCTGTTGAAGG	327	68 64	Monday and Bohach et al. (1999)
			CCAAAGTGATTGTCTATTGTGC			
		SEG-1	AATTATGTGAATGCTCAACCCGATC	641	55	Akineden et al. (2001)
		SEG-2	AAACTTATATGGAAACAAAAGGTACTAGTTC			
		SEG1	TGCTATCGACACACTACAACC	704	55	Boerema, Clemens, and Brightwell (2006)
		SEG2	CCAGATTCAAATGCAGAACCC			
		SEG1	GCTATCGACACACTACAACC	583	60	Chen, Chiou, and Tsen (2004)
		SEG2	CCAAAGTGATTGTCTATTGTGC			
		<i>setH-F</i>	TGATTTAGCTCAGAAAGTTTAAAAATAAAAAATG	466	45	Sergeev et al. (2004)
		<i>setH-R</i>	TTTCTTAGTATATAGAITTACATCAATATATG			
		SEH-1	GTCTATATGGAGGTACAACACT	213	55	Omoe et al. (2002)
		SEH-2	GACCTTTACTTATTTTCGCTGTC			
		SEH-F260	TCACATCATAATGCGAAAAGCAG	463	56	Cremonesi et al. (2005)
		SEH-R722	TCGGACAATATTTTCTGATCTTT			
		SEH-for	CAACTGCTGATTTAGGTCAGA	173	52	Bania et al. (2006)
		SEH-rev	CCCAAAACATTAGCACCA			
			CAACTGCTGATTTAGGCTCAG	360	68 64	Monday and Bohach et al. (1999)
			GTCGAATGAGTAATCTCTAGG			
		SEH-1	CAATCACATCATATGCGAAAAGCAG	371	55	Akineden et al. (2001)
		SEH-2	CATCTACCCCAAACATTAGCACC			
		SEHI	CGAAAGCAGAGATTACACG	495	55	Boerema et al. (2006)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		SEH2	GACCTTTACTTATTTTCGCTGTC			
		SEH1	CACATCATATGCGAAAAGC	548	60	Chen et al. (2004)
		SEH2	CGAATGAGTAATCTCTAGG		59	
	<i>sei</i>	<i>sei_newF</i>	TGGAACAGGACAAGCTGAAA	529		Varshney et al. (2009)
		<i>sei-newR</i>	TGTTTGCCATTAAACCCAAAAG			
		setI-F	TGATTTAGCTCAGAAAGTTTAAAAATAAAAAATG	505	45	Sergeev et al. (2004)
		setI-R	TTAGTTACTATCTACATATGATATTTCCGA			
		SEI-1	GGTGATATTGGGTAGGTAAC	454	55	Omoe et al. (2002)
		SEI-2	ATCCATATTTCTTTGCCTTTACCAG			
		SEI-F71	CTCAAGGTGATAITGGGTGAGG	529	56	Cremonesi et al. (2005)
		SEI-R637	CAGGCAGTCCCATCTCCTGTA			
		SEI-for	GGCCACTTTATCAGGACA	328	52	Bania et al. (2006)
		SEI-rev	AACTTACAGGCAGTCCA			
			CAACTCGAATTTTCAACAGGTAC	465	68	Monday and Bohach et al. (1999)
			CAGGCAGTCCATCTCCTIG			
		SEI-1	CTCAAGGTGATAITGGGTGAGG	516	55	Akineden et al. (2001)
		SEI-2	AAAAAACTTACAGGCAGTCCATCTC			
		SEI1	GATACTGGAACAGGACAAGC	789	60	Chen et al. (2004)
		SEI2	CTTACAG GCAGTCCATCTCC			
	<i>setJ</i>	<i>setJ-F</i>	ATGAAAAAACAATATTTTACTGATTTTCTCCC	807	59	Sergeev et al. (2004)
		<i>setJ-R</i>	TCTACAGAACC AAAAGGTAGACTTATTAATAC			
		SEJ-F349	GGTTTCAATGTTCTGGTGGT	306	45	Cremonesi et al. (2005)
		SEJ-R654	AACCAACGGTCTTTTGAGG			
		SEJ-for	GTTCTGGTGGTAAACCA	131	56	Bania et al. (2006)
		SEJ-rev	GCGGAACAACAGTTCTGA			
			CATCAGAACTGTTGTTCCGCTAG	142	52	Monday and Bohach et al. (1999)
			CTGAATTTTACCATCAAAGGTAC			
		SEJS1	CCCCGGATCCGATAGCAAAAAATGAAAC	732	68	Omoe, Hu, Takahashi-Omoe, Nakane, and Shinagawa (2003)
		SEJAS2	CCCCGAATTCCTAAACCAAAAGGTAGACTTATTA	64	64	
		ESJ1	CAGCGATAGCAAAAAATGAAACA	426		Boerema et al. (2006)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		ESJ2	TCTAGCGGAACAACAGTCTCTGA			
		SEJ-1	ATAGCATCAGAACTGTGTCCG	152	55	Omoe et al. (2005)
		SEJ-2	CTTCTGAAATTTACCACCAAAGG			
	<i>selk</i>	<i>setK-F</i>	ATGAATCTTATGATTTAATTCAGAAATCAA	545	57	Sergeev et al. (2004)
		<i>setK-R</i>	ATTTATATCGTTCTTTTATAAGAAATATCG			
		SEK-for	GGAGAAAAGGCAATGAA	516	45	Bania et al. (2006)
		SEK-rev	TAGTGCCGTTATGTCCA			
		SEKf1	GGGGATCCCAAGCGATATATAGGAATTGATAAT	678	52	Omoe et al. (2013)
		SEKRI	GGGGAATCTTATAATCGTTTCTTTATAAG			
		SEK-1	TAGGTGTCCTAAATAATGCCA	293		Omoe et al. (2005)
		SEK-2	TAGATAATTCGTTAGTAGCTG			
	<i>sell</i>	<i>setL-F</i>	ATGAAAAAAGATAATTAATTTGTAATGTTATTAC	723	57	Sergeev et al., 2004)
		<i>setL-R</i>	ATCATCTTTTGGAAATTCGACATCTAG			
		SEL-F158	CACCAGAATCACACCCGCTTA	240	45	Cremonesi et al. (2005)
		SEL-R397	CTGTTTGAATGCTTGCCATTG			
		SEL-for	CGATGTAGTCCAGGA	369	56	Bania et al. (2006)
		SEL-rev	TTCTTGTGCGGTAACCA			
		SEL-1	TAACGGCGATGAGGTCCAGG	383	52	Omoe et al. (2005)
		SEL-2	CATCTATTTCTTGTGCGGTAAC			
	<i>setm</i>	<i>setM-F</i>	ATGAAAAGAATACTTATCATTTGTTGTTTATTG	720	57	Sergeev et al. (2004)
		<i>setM-R</i>	CTTCAACITTCGTCCTTATAAGATAITTC			
		SEM-for	CATATCGCAACCCGCTGA	148	45	Bania et al. (2006)
		SEM-rev	TCAGCTGTTACTGTCCA			
		mpSEM-1	CTATTAAATCTTTGGGTTAAATGGAGAAC	300	52	Boerema et al. (2006)
		mpSEM-2	TTCAGTTTCGACAGTTTGTGTGTCAT			
		SEM-1	GGATAATTCGACAGTAACAG	379	55	Omoe et al. (2005)
		SEM-2	TCCTGCATTAATAATCCAGAAC			
		SEM1	CCAATTGAAGACCACCAAAG	517	57	Blaiotta et al. (2004)
		SEM2	CTTGCTCTGTCCAGTATCA			
	<i>setn</i>	<i>setN-F</i>	ATAAAAAAATATTAATAAGCTTATGAGATTGTTCC	777	45	Sergeev et al. (2004)
		<i>setN-R</i>	ACTTAATCTTTATATAAAAAATACATCAATATG			
		SEN-for	GGCAATTAGACGAGTCA	237	52	Bania et al. (2006)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		SEN-rev	ATCGTAACTCCTCCGTA			
		SEN1	CTTCTTGTGGACACCATCTT	135	55	Chiang et al. (2008)
		SEN2	GAAATAAAATGTGTAGGCTT			
		mpSEN-1	ATGAGATTGTTCTACATAGCTGCAAT	680	55	Boerema et al. (2006)
		mpSEN-2	AACTCTGCTCCCACCTGAAAC			
		SEN-1	TATGTTAAATGCTGAAAGTAGAC	282	57	Omoe et al. (2005)
		SEN-2	ATTTCCAAAATACAGTCCATA			
		SEN1	ATTGTTCTACATAGCTGCAA	682	55	Blaiotta et al. (2004)
		SEN2	TTGAAAAAACTCTGCTCCCA			
	<i>setO-F</i>		TATGTAGTGTAACAATGCATATGCA	685	45	Sergeev et al. (2004)
	<i>setO-R</i>		TCTATTGTTTTATTATCAATTATAAATTTGGCAAAT			
	SEO-for		GTCAAAGTGTAGACCCTA	288	52	Bania et al. (2006)
	SEO-rev		TGTACAGGCAGTATCCA			
	SEO1		AAATTCAGCAGATAITTCAT	172	56	Chiang et al. (2008)
	SEO2		TTTGTGTAAGAAGTCAAGTGTAG			
	mpSEO-1		AGTTTGTGTAAGAAGTCAAGTGTAGA	180	55	Boerema et al. (2006)
	mpSEO-2		ATCTTTAAATTCAGCAGATATCCCATCTAAC			
	SEO-1		TGTGTAAGAAGTCAAGTGTAG	214	57	Omoe et al. (2005)
	SEO-2		TCTTTAGAAAATCGCTGATGA			
	SEO1		AGTCAAAGTGTAGACCCTAAT	534	55	Blaiotta et al. (2004)
	SEO2		TATGCTCCGAATGAGAAATGA			
	<i>setP-F</i>		TTAGACAAAACCTATATCATAATGGAAGT	618	57	Sergeev et al. (2004)
	<i>setP-F</i>		TATATAAATATATATCAATATGCATATTTTTAGACT			
	SEP-for		TCAAAAAGACACCCGCAA	396	55	Bania et al. (2006)
	SEP-rev		ATTGCTCTTGAGCACCA			
			ATCATAACCAACCGAATCAC	148		Chiang, Fan, Liao, Lin, and Tsen (2007)
			AGAAAGTAACTGTTCCAGGAGCTA			
			TGATTTATAGTAGACCCTTGG	396	52	Omoe et al. (2005)
			ATAACCAACCGAATCACCCAG			
	<i>setQ-F</i>		GGAAAATACACTTTATATTCACAGTTTCA	539	45	Sergeev et al. (2004)
	<i>setQ-R</i>		ATTTATTCAGTTTTCTCATATGAAATCTC			
	SEQ-for		GGAATACGTTGGCGAA	330	52	Bania et al. (2006)

(Continues)

TABLE 6 (Continued)

Organism	Gene	Primer name	Primer sequence	Size	Ta	Reference
		SEQ-rev	AACTCTCTGCTTGACCA			
		SEQ1	TCAGGTCTTTGTAAATACAAAA	359	55	Chiang et al. (2007)
		SEQ2	TCTGCTTGACCAAGTTCCGGT			
		SEQ-1	AACTCTGGGTCAATGGTAAAGC	122	57	Omoe et al. (2005)
		SEQ-2	TTGTATTCGTTTTGTAGGTATTTTCG			
<i>ser</i>		<i>ser</i> -f	AGCGGTAATAGCAGAAAAATG	363		Varshney et al. (2009)
		<i>ser</i> -r	TCTTGACCCGTAAACCGTTTT			
		SER1	AGATGTGTTTGGAAATACCCCTAT	123	55	Chiang et al. (2007)
		SER2	CTATCAGCTGTGGAGTGCAAT			
		SER-1	GGATAAAGCGGTAATAGCAG	166	57	Omoe et al. (2005)
		SER-4	GTATTCCAAACACATCTAAC			
<i>ses</i>		<i>ORF6F</i>	TTCAGAAATAGCCAAATCAITTCAA	195		Ono et al. (2008)
		<i>ORF6R</i>	CCTTTTTGTGAGAGCCGTC			
<i>set</i>		<i>ORF5F</i>	GGTGATTAIGTAGATGCTTGGG	170		Ono et al. (2008)
		<i>ORF5R</i>	TCGGGTGTTACTTCTGTTTGC			
<i>setu</i>		<i>setu</i> -f	AATGGCTCAAAAATTGATGG	215		Varshney et al. (2009)
		<i>setu</i> -r	ATTTGATTTCCATCATGCTC			
		SEU1	ATTTGCTTTTATCTTCAT	167	51	Chiang et al. (2008)
		SEU2	GGACTTAAATGTTTGTCTTGAT			
<i>selv</i>		<i>SEVI</i>	GCAGGATCCGATGTCGGAGTTTTGAAATCTTAGG	720	55	Thomas et al. (2006)
		<i>SEV2</i>	TAACTGCAGTTAGTTACTATCTACATAT GATAITTCGACATC			
<i>lssf</i>		<i>lssf</i> -f	AAGCCCTTTGTTGCTTGGG	215		Varshney et al. (2009)
		<i>lssf</i> -r	ATCGAACTTTGGCCCAATACATTT			
		GTSSSTR-1	ACCCCTGTTCCCTTATCATC	326		Mehrotra et al. (2000)
		GTSSSTR-2	TTTTTCAGTATTTGTAACGCC			
		TST-3	AAGCCCTTTGTTGCTTGGG	447		Omoe et al. (2005)
		TST-6	ATCGAACTTTGGCCCAATACATTT			

DNA stool Minikit (Qiagen), Isogen (Wako), and InstaGene matrix (BioRad). Comparison of the kits demonstrated that the Qiagen kit required the highest initial number of *C. perfringens* cells spiked in meat ($\sim 10^4$ to $\sim 10^8$ cells/g), with the lowest amount of contaminated cells for a positive result being obtained with the Isogen kit ($\sim 10^3$ to 10^5 cells/g). The results using the InstaGene kit were similar to those obtained with Isogen at levels ranging from 10^2 to 10^6 cells/g. However, the InstaGene kit involved fewer steps for extraction, decreasing not only the possibility of cross-contamination but also the time for analysis, which is of paramount importance when analyzing a large number of samples, for instance, from an outbreak.

Numerous studies were carried out in environmental and food samples to determine if the lowest numbers of pathogens required for toxin production can be directly detected by PCR (Fricker, Messelh u ber, Busch, Scherer, & Ehling-Schulz, 2007; Kaneko et al., 2011; Kirchner et al., 2010; Lindstr om et al., 2001). For most of the toxigenic pathogens, this implies the detection of 5 log CFU/g. The limit of detection can be improved if needed if an enrichment step is added to the workflow, with a drawback being an extension of time-to-results. Lindstr om et al. (2001) performed tests in raw minced beef and smoked whitefish and reported PCR sensitivity of 10^{-2} to 10^{-1} spore/g for *C. botulinum* types A, B, E, and F in raw minced beef depending on the food type, inoculated *C. botulinum* strain, enrichment time, and temperature. Optimal enrichment lasted between 1 to 5 days, with all strains being detectable after 3 days. Fricker et al. (2007) performed a study on *B. cereus* in inoculated cooked rice and pasta investigating the effect of enrichment in the detection of cereulide producing *B. cereus* by PCR. Samples were taken at different times (0, 2, 4, and 6 hr) and subjected to real-time PCR using either SYBR green detection or TaqMan-based detection. The limit of detection using SYBR green technology without enrichment was 10^3 CFU/g rice (using just the boiling extraction method) decreasing to 10^0 CFU/g rice after just 4 hr of enrichment. Johnson et al. (1991) described one of the first tests on the usage of PCR for the detection of bacterial toxin genes. A set of eight pairs of primers were designed to detect internal regions of the genes for SEs A to E (SEA to SEE), exfoliative toxins A and B (ETA and ETB), and toxic shock syndrome toxin 1 (TSST-1) strains isolated from clinical specimens and contaminated foods. PCR results showed in general great concordance with biological and immunological assays performed previously for the chosen *S. aureus* strains. Especially for all tested enterotoxins, there was a 100% match between PCR results and immune assays. Only in two tested stains carrying SEB and TSST-1, or SEC and TSST-1 combination of genes was partial disagreement found between two methods. From the current perspective, some of the observed disagreements could be a result of the suboptimal sensitivity and specificity of the PCR assays, limited or impaired gene

expression for one of the toxins, or lack of stability of the genetic element carrying the toxin genes in tested strains.

Several multiplex PCR methods were designed for the simultaneous detection of multiple toxin genes. Multiplex PCR allows the detection of multiple genes in a single PCR tube, decreasing the time and costs associated with the final analysis of the results. Numerous multiplex PCR setups have been designed during the last decades for the detection of multiple toxin genes of *B. cereus*, *C. perfringens*, *C. botulinum*, and *S. aureus* in food, clinical, and environmental matrices (Baums, Schotte, Amtsberg, & Goethe, 2004; Cremonesi et al., 2005; Ehling-Schulz et al., 2006; Garmory et al., 2000; Guinebretiere, Fagerlund, Granum, & Nguyen-The, 2006; Guinebretiere, Fagerlund, Hwang et al., 2007; Kim et al., 2012; Lindstr om et al., 2001; Mehrotra, Wang, & Johnson, 2000; Monday & Bohach, 1999; Ngamwongsatit et al., 2008; Omoe, Hu, Takahashi-Omoe, Nakane, & Shinagawa, 2005; S anchez-Chica et al., 2020; Wehrle, Didier, Moravek, Dietrich, & M artlbauer, 2010; Yoo, Lee, Park, & Park, 1997). Use of multiplex PCR led to a number of very relevant tools that are used to distinguish between different subtypes of toxins and investigate genetic polymorphisms in toxin-encoding genes. For example, a duplex PCR was created for the rapid discrimination and detection of *cytK-1* and *cytK-2*, at the time, newly discovered forms for cytotoxin K, in *B. cereus* (Guinebretiere, Fagerlund, Granum, & Nguyen-The, 2006). In addition to the duplex PCR, strains that were negative following PCR were also subjected to Southern blot analysis to confirm unquestionably the absence of the *cytK* gene. This PCR was capable of amplifying both genes and showed 0% of false-positive and false-negative results, as confirmed by Southern blotting. Ehling-Schulz et al. (2006) described a multiplex PCR assay for the detection of enterotoxic and emetic *B. cereus* isolates based on the analysis of sequences that had been previously sequenced for cereulide synthetase genes and based on the analysis of partially sequenced material for enterotoxin genes. The analysis of data from enterotoxin gene sequencing revealed high levels of sequence polymorphisms. To overcome the potential issue of false-negative results due to sequence polymorphisms, the primers were designed to insert inosine nucleotide at variable positions. This approach allowed for the amplification of enterotoxin genes that had only been detected by Southern blot assay. In addition to good concentration and extraction procedures, amplification of nucleic acids from foodstuff may be an intricate process due to the presence of inhibitory substances, which may cause false-negative understanding of the results (D'Agostino, Cook, Rodr iguez-L azaro, & Rutjes, 2011; Rodr iguez-L azaro, D'Agostino, Pla, & Cook, 2004;). It is paramount to use appropriate controls to determine the nonexistence of interference due to the presence of inhibitory substances. Incorporating an internal control of amplification (IAC) helps to identify unsuccessful reactions. An IAC is a

nontarget sequence present in every reaction co-amplified with the target sequence (Cone, Hobson, & Huang, 1992). In the absence of an IAC, a negative result can either represent the absence of the target sequence or that the amplification is inhibited. A PCR reaction that includes an IAC and provides no result for the IAC and the target sequence is indicative of an unsuccessful amplification. Therefore, the sample must be retested to confirm the negative result. Several studies have resorted to an IAC to overcome the issues of false-negative results in PCR reactions from food samples (Fricker et al., 2007; Kirchner et al., 2010; Mehrotra et al., 2000; Ono et al., 2008; Thomas et al., 2006; Wehrle et al., 2010; Zhang et al., 2014). Different internal amplification controls were chosen depending on the tested bacteria. *B. cereus* IAC was usually based on a commercially available plasmid to which the sequences of interest were added. For staphylococcal toxins, the IAC was generally based on a gene encoded by the bacteria, whether the *femA* or the *gyrA* genes.

Although being a very powerful and fundamental tool in food and environmental diagnosis, PCR is incapable of distinguishing between live and dead cells, which is a trait paramount in risk analysis. Nonetheless, several methods may be used to circumvent this particular PCR drawback and which include viability PCR (v-PCR) or reverse transcriptase PCR (RT-PCR). Viability PCR has been extensively studied in the last years, particularly for the detection of foodborne pathogens (Cattani, Barth, Nasário, Ferreira, & Oliveira, 2016; Elízaquivel, Aznar, & Sánchez, 2013; Forghani et al., 2015; Monteiro & Santos, 2018; Randazzo, López-Galvéz, Allende, Aznar, & Sánchez, 2016; Zhang et al., 2014). This procedure is based on the integrity of membrane cells as the viability dyes are only able to penetrate compromised membrane cells. Once inside the cells, and after exposure to strong visible light, the dye covalently intercalates into the nucleic acid, interfering with PCR amplification. Two viability dyes have been reported, ethidium monoazide (EMA) and propidium monoazide (PMA). EMA was the first viability dye to be tested, but it is able to penetrate not only cells with the compromised membrane but also cells with their membrane intact, additionally becoming toxic to viable cells (Nocker, Cheung, & Camper, 2006; Pan & Breidt, 2007; Rudi, Naterstad, Dromtorp, & Holo, 2005). Due to the bigger size of PMA, this molecule is incapable of penetrating viable cells being thus used preferentially (Nocker et al., 2006; Pan & Breidt Jr., 2007). Zhang et al. (2014) tested the use of a combined multiplex PCR and PMA pretreatment for the detection of emetic and nonemetic *B. cereus* (dead or viable cells) in inoculated foodstuffs (cooked noodle, rice and sausage). The combined PMA-mPCR assay was able to detect both targeted bacteria in spiked food with complete removal of the PCR signal from dead cells without significantly affecting the LOD, a result similar to that of Forghani et al. (2015). Viability dyes have a

few disadvantages including not being able to fully remove the signal from dead bacteria if certain conditions are met, that is if the amplicon size of the PCR is very short, if the initial concentration of bacteria is high, and if the fat content of the food sample is high, among others (Elízaquivel, Sánchez, & Aznar, 2012; Liang & Keeley, 2012; Li & Chen, 2013; Martin, Raurich, Garriga, & Aymerich, 2013; Nocker et al., 2006; Nocker, Sossa, & Camper, 2007; Yang, Badoni, & Gill, 2011). RT-PCR is an interesting alternative for the discrimination of live and dead cells as it detects mRNA (Gonzalez-Escalona et al., 2009; McIngvale, Elhanafi, & Drake, 2002; Yaron & Matthews, 2002). A study conducted to determine the potential application of RT-PCR to detect viable *L. monocytogenes* cells demonstrated that the levels of PCR signal removal were highly influenced by the targeted gene (Xiao, Zhang, & Wang, 2012). Exposure of *L. monocytogenes* (concentrations of 10^6 and 10^9 CFU/mL) to heat treatment at 98 °C for 30 min revealed that 16S rRNA molecules were still easily detected but only trace amounts of mRNA transcripts of *inlA* and *rplD* genes were found, with a steeper tendency on the lowest concentration tested. RT-PCR is dependent on the targeted nucleic acid sequence but also relies heavily on the expression of the targeted gene, which may vary according to conditions of stress, an issue associated with the detection of RNA. Nonetheless, both approaches have proved to be promising tools for the sole detection of viable cells.

In addition to providing positive signals from live and dead cells, a positive signal in a regular PCR represents only the enterotoxic potential of an isolate and does not provide any insight on its enteropathogenicity. Tests to determine the expression levels of a toxin have been extensively described in previous chapters. In this context, several studies have compared data obtained by PCR and by classical methods for the determination of gene expression (Boerema, Clemens, & Brightwell, 2006; Chiang et al., 2008; Cremonesi et al., 2005; Ehling-Schulz et al., 2005; Franciosa, Ferreira, & Hatheway, 1994; Fricker et al., 2007; Guinebretiere, Broussolle, & Nguyen-The, 2002; Johnson et al., 1991; Kim et al., 2012; Kirchner et al., 2010; Lin & Labbe, 2003; Moravek et al., 2004; Ono et al., 2008; Satterfield et al., 2010; Sergeev, Volokhov, Chizhikov, & Rasooly, 2004; Takeshi, 1996; Varshney et al., 2009; Wehrle et al., 2009). Overall, the results obtained by the PCR reaction were substantiated by the classical methods chosen for the determination of the expression of enterotoxins. Fricker et al. (2007) tested isolates from two foodborne outbreaks by PCR to determine the presence of the emetic *B. cereus* toxin cereulide gene and confirmed the positive results using HEp-2 cytotoxicity assay. In another study, a panel of 176 strains, including only *B. cereus* strains, *B. cereus* group strains, and other *Bacillus* spp. were tested by PCR, cytotoxicity analyzed with Vero cells and HEp-2 cells and enzyme immunoassays (Wehrle et al., 2009). The

results showed an altogether good agreement between PCR and the corresponding component enzyme immunoassay. The Vero cell assay exhibited an excellent agreement with the immunoassay and PCR, with only three strains that expressed all Nhe components in very low amounts producing negative results in the cell assay. Additionally, two strains (one strain lacking NheB and the second one expressing only NheA) did not induce cytotoxic effects on Vero cells, since toxin expression of all three components is mandatory for the biological activity. Results from PCR for the emetic strains were confirmed with HEp-2 cells. Altogether, of the strains harboring a complete set of *hbl* genes and/or *nhe* genes, 98% and 100%, respectively, were positive when using the respective immunoassay, and 98% exhibited cytotoxicity. A study conducted in two food products determined the presence of *C. botulinum* toxin type E gene with no other toxin gene fragment being amplified. These results were confirmed by mouse bioassays with neutralization by antiserum against type E toxin. Much of the information on the type of toxins present in an isolate is provided by bacterial culture collections usually using mouse bioassays. Nonetheless, many of these toxins were determined decades ago, with perhaps less accurate and sensitive mouse bioassays. A study conducted in *C. botulinum* toxins using isolates obtained from different culture collections showed that in most cases, with few exceptions, the result from the PCR was equal to that of the mouse bioassay (Satterfield et al., 2010). However, there have been exceptions. Satterfield et al. (2010) reported two isolates that were previously purported to produce type BoNT/B and BoNT/E by bioassays, were by PCR found to be positive only for a type A gene. The PCR results were corroborated by the newly developed mouse bioassay. The third isolate, determined previously to produce type B toxin provided negative results in PCR, a result confirmed by new mouse bioassay. These contradictory findings may indicate that the gene was on a plasmid that got lost during repeated culturing.

PCR can produce signals for more than one toxin gene in a single run and is extremely sensitive and fast, providing results in just a few hours making PCR exceptionally useful in outbreak situations. Further automatization, miniaturization, and constant improvement of instruments and reagents, especially enzymes, and lab-on-chip type of design (Cocolin, Rajkovic, Rantsiou, & Uyttendaele, 2011) may offer additional advantages that will endorse the use of PCR and PCR-based methods in toxin and toxin gene detection. Moreover, reverse transcriptase PCR can be very valuable in detecting toxin production at RNA expression level when no other detection methods for toxin itself are available (e.g., for *B. cereus* CytK enterotoxin). This can help to understand often noticed strain-specific variation in toxin production at mRNA and protein levels, in particular under conditions of food matrix.

6.2 | Whole genome sequencing

The rampant and continuous development of high-throughput DNA sequencing and bioinformatics is reshaping various biology fields, including microbiology and toxicology. DNA sequences are a universal dataset from which, theoretically, any biological feature can be inferred, including toxin production potential in bacterial pathogens. Whole genome sequencing (WGS) has become a powerful tool in food safety and may play an extremely important role in food safety surveillance and risk assessment. WGS was initially extremely expensive impairing its use in routine food analysis. With the establishment of next-generation sequencing, third-generation sequencing, and less expensive technologies (including small, bench-top, sequencers), a decrease in the overall costs of sequencing was achieved, bringing it to the point where costs are comparable to the price range of traditional methods such as PCR and pulsed-field gel electrophoresis (PFGE). Moreover, WGS does the work of several other combined methods. Since the costs of WGS of prokaryotes have been dramatically dropping the WGS is on its way to replace many of other molecular and even phenotypic methods. As a result, the number of complete bacterial genomes available in public databases grows fast.

WGS can be divided into two categories based solely on the length of the targeted sequence reads: (i) short sequencing technologies, generating reads up to 600 base-pairs (bp) long; and (ii) long sequencing technologies, able to produce reads longer than 1000 bp with the possibility of achieving reads sometimes longer than 70,000 bp. Additionally, the sequencing analysis to determine genomic differences between isolates can be divided into two categories also (i) reference-based methods and (ii) de novo methods. The first bioinformatics tool depends on the alignment of the obtained reads to existing genome databases, whereas the second tool does not require an existing reference genome but instead attempts to find mismatches in smaller parts of the genome (i.e., k-mers) or try to recreate parts of the genome. De novo sequencing yields a novel genome for which no reference sequence is available for alignment. Sequence reads are assembled as contigs, and the coverage quality of de novo sequence data depends on the size and continuity of the contigs.

A growing body of sequencing data and machine or deep learning show that it is possible to predict biological features, including toxin production potential. Therefore, not only detection of previously known or putative toxin genes by WGS can be used as a screening technique, but also genes that encode for new bacterial toxins can be identified by bioinformatics and computational models deploying deep and machine learning based on previous knowledge obtained from decades of laboratory testing of known bacterial toxins.

Several bioinformatics tools are currently available for the prediction of bacterial toxins. Homology comparison between the unknown sequence and a known protein is the most straightforward and possibly the most reliable method to predict a bacterial toxin. Following the determination of a sequence, alignment can be conducted using different available databases, including BLAST or PSI-BLAST, and probabilistic models. In performing these analyses, it is important to consider the different information provided: (i) alignment coverage, which indicates if the homologs share only a partial homology of the full-length query toxin; (ii) the percentage of sequence similarity, evaluated by the E-value. Functional annotation of newly sequenced genomes can also indicate the presence of novel toxins, in addition to recurring to available searching databases. The identification of a possible new toxin by homology is just the first step, as the homologs may be nontoxic or may have a distinct function (Negi et al., 2017). Furthermore, the newly identified toxin may present a very distinct sequence, but contain high similarities in the “twilight zone” of homology (Chung & Subbiah, 1996; Otto et al., 2000; Rost, 1999;). As a result, other tools should be considered in addition to simple homology detection, including motifs, genome context, the conservation of the protein domain architecture, conservation of important functional residues, and the position of the newly identified toxins in comparison with known toxins (Fieldhouse & Merrill, 2008; Lobb & Doxey, 2016; Zhang, de Souza, Anantharaman, Iyer, & Aravind, 2012). The potential functional correlation of the new toxins with known ones can be determined more precisely by analyzing the position of the identified toxin within the toxin’s phylogenetic family tree. Inclusion of the toxin in the tree is symptomatic that the homolog is a possible member of the intended toxin family. On the other hand, the creation of a distinct branch outside the intended family may be indicative of a different toxin lineage and function (Mansfield et al., 2017). Studying the protein domain architecture may provide insights on the evolutionary and functional relation when the identity percentage is reduced and/or the new toxins form a distinct lineage branch. Neighboring genes may also yield further knowledge on the function of the newly discovered protein. In addition, other approaches may be used to predict new toxins, including the identification of unique sequences or structure similarities to host proteins, among others (Doxey & McConkey, 2013; Ho Sui, Fedynak, Hsiao, Langille, & Brinkman, 2009, Petrenko & Doxey, 2015).

WGS including whole-genome single nucleotide-polymorphism (SNP) analysis is an extremely important tool to rapidly differentiate bacterial strains harboring identical toxin gene subtypes and may be potentially helpful in discriminating strains that were described as indiscernible by PFGE and multilocus sequence typing (Jacobson, Lin, Whittam, & Johnson, 2008; Raphael et al., 2008). For

instance, many national centers for botulism have started to sequence clinical and environmental strains of *C. botulinum* and BoNT-producing *Clostridium*, which led to the discovery of new BoNT sequences (Doxey, Mansfield, & Montecucco, 2018). Such approach has authenticated the individuality of the seven known botulinum neurotoxin serotypes (A-G), represented in a large difference in the amino acids (37.2 to 69.6%) (Hill & Smith, 2013). Since March 2017, WGS is used for routine monitoring of *L. monocytogenes* and is being applied to support foodborne outbreaks investigations caused by a large number of other pathogens in the United States (Jackson et al., 2016). WGS contributed also with new and important knowledge on *C. perfringens* foodborne outbreaks that can be caused by strains carrying *cpe* either on the chromosome or on plasmid. It is important to note that chromosomal *cpe+* strains are more common cause of *C. perfringens* food toxico-infection than *cpe+* plasmid ones. The prevalence of *cpe+* *C. perfringens* in 24 different food poisoning outbreaks was reported to be 75% chromosomal, 21% (plasmid-borne IS1470-like-*cpe*), and 4% (plasmid-borne IS1151-*cpe*) (Lahti, Heikinheimo, Johansson, & Korkeala, 2008). This can be partially explained by higher resistance of both the spores and vegetative cells of chromosomal *cpe+* isolates to high and low temperatures, as well as to NaCl and nitrites (no difference in pH sensitivity was observed between chromosomal and plasmid *cpe+* strains) circumventing food preservation hurdles (Li & McClane, 2006). Both plasmid and to a lesser extent chromosomal *cpe+* *C. perfringens* type F strains have been found in the feces from healthy individuals, but in the environment, the most of the *cpe+* isolates contained a plasmidborne *cpe* (Brynstad, Synstad, & Granum, 1997; Carman et al., 2008; Lindström, Heikinheimo, Lahti, & Korkeala, 2011; Miki, Miyamoto, Kaneko-Hirano, Fujiuchi, & Akimoto, 2008; Miyamoto, Chakrabarti, Morino, & McClane, 2002, 2006; Mueller-Spitz, Stewart, Val Klump, & McLellan, 2010; Wen & McClane, 2004).

Gonzalez-Escalona, Timme, Raphael, Zink, and Sharma (2014) sequenced the genome of three *C. botulinum* strains producers of BoNT/A1 toxin and performed a whole-genome phylogenetic single-SNP analysis along with other available sequences of *C. botulinum* group I strains. The analysis determined the existence of five different lineages, with two of the sequenced strains clustering with *C. botulinum* type A1 Hall group and the last strain clustering with the *C. botulinum* type Ba4 strain 657.

Aung et al. (2017) applied WGS to isolates of *S. aureus* collected from the hands and nasal cavities of food handlers to determine the genetic variability within the *selw* gene. Following analysis by PCR, the authors determined that the most prevalent enterotoxin(-like) gene was *selw* with 98% of the isolates positive for this gene, followed by *selx* at a percentage of 97% of positive isolates. Additionally, the authors further

categorized *selw* gene using WGS into six different groups, with a sequence identity of > 99% within isolates from groups 2 to 5 and slightly lower sequence identity for group 1 and a difference in identity between groups ranging from 84 to 97%. On a different study, two distinct techniques were applied, DNA microarray and WGS, to determine *S. aureus* genes encoding for virulence and resistance genes (Strauß et al., 2016). The data showed that 96.8% of all typing results provided similar results with both techniques, whereas the inconsistencies amounted for only 3.2% in total. The abnormalities observed resulted mainly from WGS errors, microarray hybridization failures, and incorrect prediction of ambivalent microarray data. The higher levels of discrepancies were obtained for the virulence factors, which were caused by under detection of SEs or antigens by WGS or by polymorphisms in probe/primer binding sequences on microarrays. Contrary to microarrays, WGS allowed for the discrimination of allelic variants, essential for the prediction not only of the bacterial virulence but also of the resistance phenotypes. The incorrect assembly can be surpassed by mapping directly the generated sequences to known reference sequences (Zhang et al., 2015)

Although being an extremely powerful tool, the use of bioinformatics for the identification of bacterial toxins carries some shortcomings. The most important is the relatively low sensitivity compared to traditional enrichment-based methods. The lack of standardization is also a drawback since it is a requirement for a broader application of WGS. Several efforts have been made towards better and more standardized protocols for pathogen identification, food microbiology, and outbreak investigations in an attempt to obtain the best practices for the integrity, traceability, and reproducibility of the results obtained (Lambert et al., 2017). Having a diversified WGS allele library can introduce the risk of distinct, but analogous, genes. Moreover, due to the evolutionary ladder, it may be difficult to differentiate if a sequence is still an allelic variant of a given gene or if it belongs already to a different gene. Incomplete genome coverage or the unsuccessful de novo assemblies present also another challenge for the current use of WGS.

Integrating phenotypic- and omics-based data will potentiate for a more informed characterization of risks related to several groups of bacteria, with the possibility to develop more targeted food safety procedures and policies. Such predicament will ensure a lower reliance on simple premises, including moderately homogenous distribution of virulence-related characteristics in a certain species, enabling a strict characterization and identification of subgroups with specific virulence traits. Changes in the structure of the microbiome may anticipate both known and uncharacterized bacterial toxins and, as a result, such techniques would improve the detection of food safety hazards.

7 | CONCLUSIONS

A variety of methods exist to detect, identify, quantify, and characterize bacterial toxins or bacterial genetic ability to produce toxins. However, not every type of method is available for each toxin. This may be for various reasons, including lack of fundamental information on particular toxins, difficulties in their purification, high toxicity and limitations of detection technology. Especially quantitative methods for larger toxins (larger proteins) are not available.

The gold standard for the detection and biological characterization for some toxins are still animal bioassays. However, these assays are time consuming, which is not compatible with the needs of outbreak investigations and epidemiological surveys, and the toxins may have effects at lower doses than the acute intoxication dose. Moreover, the use of animals in diagnostic trials faces an increased contestation. Many immunological assays have been described for the detection of microbial toxins in food, water, and feces, but commercial and validated kits are not always available. Procedures based on mass spectroscopy have been developed, but the costs associated with the instrumentation and human expertise are high. Often, the detection of genetic signatures that code for the toxin production is equally important as the detection of the toxin itself. In such circumstances, use of molecular methods, such as PCR and NGS, is highly valuable. Especially sequencing approaches may be highly relevant for detection of unknown variants of toxins. Finally, a choice of detection of the toxins in food samples, or detection of toxin production capacity in the isolated foodborne pathogens, is dependent on the role of the toxin in microbial pathogenesis and on the type of foodborne illness they cause.

ACKNOWLEDGMENT

This work was financially supported by the Special Research Fund of Ghent University, GOA project no. 01G02213.

AUTHOR CONTRIBUTIONS

Andreja Rajkovic led the study, wrote the draft of the manuscript, and provided final scientific and technical editing. He wrote all of the biological assay and ELISA and RPLA methods and provided inputs regarding toxins to all other parts of the manuscript. Jelena Jovanovic and Silvia Monteiro wrote with Andreja Rajkovic mainly molecular methods (PCR and NGS) and made great efforts to control the references and the design and the content of all tables in the manuscript. Marlies Decler worked specially on the part related to mass spectrometry and worked on editing of the manuscript. Mirjana Andjelkovic worked on the part related to mass spectrometry and toxins of *B. cereus* and *S. aureus*. Astrid Foubert worked on the part related to lateral flow

assays. Natalia Beloglazova worked mainly on part related to mass spectrometry and immunological methods. Sarah De Saeger worked on the immunological and instrumental aspects. Varvara Tsilia worked on the part related to mass spectrometry, editing of the manuscript, and scrutinized information on toxins of *B. cereus*. Annemieke Madder and Benedikt Sas worked on overall chemical aspects of detection methods. Mieke Uyttendaele elaborated context, structure, and pros and cons of each detection methods in terms of food analysis in research and routine.

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How to cite this article: Rajkovic A, Jovanovic J, Monteiro S, et al. Detection of toxins involved in foodborne diseases caused by Gram-positive bacteria. *Compr Rev Food Sci Food Saf.* 2020;19:1605–1657. <https://doi.org/10.1111/1541-4337.12571>