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Review

Molecular detection of *Clostridium* and *Bacillus* species in foods: recent advances and applications

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ABSTRACT

Spore-forming bacteria, especially *Clostridium* spp. and *Bacillus* spp., are ubiquitous in food systems, and their ingestion can cause serious diseases in humans and animals. Their persistence in diverse food matrices and resistance to conventional treatments make rapid and accurate detection essential for effective monitoring and control. Traditional culture-based and biochemical assays remain the standard for identifying these bacteria but are often time-consuming, labor-intensive and limited in sensitivity. In contrast, nucleic acid-based methods provide rapid, specific and sensitive alternatives by directly targeting genetic markers of pathogenic or spoilage strains. This review summarizes how nucleic acid methods, including PCR, FISH, LAMP, RPA, WGS, and the emerging CRISPR/Cas systems, have been applied specifically to detect *Clostridium* spp. and *Bacillus* spp. in food systems. Each method offers unique advantages and limitations. PCR-based methods enable accurate quantification but require thermal cycling. FISH-based methods are simple but require microscopy and have limited validation in food. WGS-based methods provide strain-level characterization but depend on informatics and specialized equipment. Isothermal techniques such as LAMP- and RPA-based methods allow rapid field detection but involve complex primer design or poor discrimination of closely related genes. CRISPR/Cas-based platforms further enhance simplicity, specificity, sensitivity for on-site detection, though the validation for spore-forming bacteria remains limited. Overall, this review provides an overview of gene targets, methodological adaptations, and analytical performance of nucleic acid-based assays for detecting *Clostridium* spp. and *Bacillus* spp., highlighting current progress and future opportunities for improving food safety monitoring.

1. Introduction

Spore-forming bacteria, such as *Clostridium* (anaerobic) and *Bacillus* (aerobic) species, play significant roles in foodborne illnesses and food spoilage. *Clostridium* spp. and *Bacillus* spp. exist in two different forms: vegetative cells and spores. While vegetative cells are actively growing cells, spores are a dormant part of their life cycle under unfavorable conditions. Each vegetative cell produces only one spore that carries all the genetic material present in the vegetative form (Moeller et al., 2009). Both forms are found in diverse food materials, such as dairy products, rice, meat and vegetables (de Boer et al., 2011; Eckert et al., 2013; From et al., 2007; Guven et al., 2006; Kong et al., 2021; Morandi et al., 2015; Vidic et al., 2020).

Consumption of food contaminated with *Clostridium* spp. or *Bacillus* spp. can lead to a range of illnesses, including diarrheal and emetic syndromes, gas gangrene, and botulism, depending on the species and toxins involved (Bennett et al., 2013). *Clostridium perfringens* (*C. perfringens*) is one of the most commonly reported pathogens causing foodborne illness, with an estimated one million cases of food poisoning occurring annually in the USA (Sridapan et al., 2021). In England, it is estimated that 8-13% of gastrointestinal foodborne outbreaks are associated with *C. perfringens* (Bhattacharya et al., 2020). The Centers for Disease Control and Prevention (CDC) estimates 63,400 cases of foodborne illness and 20 hospitalizations from *Bacillus cereus* (*B. cereus*) infection (Scallan et al., 2011). Given the harmful effects of foodborne pathogenic *Clostridium* spp. and *Bacillus* spp. on human health, their

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timely and accurate detection is of great importance.

Conventional culture-based and biochemical methods are still widely used for enumerating and identifying vegetative cells or spores of these pathogens in food (Rajapaksha et al., 2019; Rhodehamel & Harmon, 2021). However, these methods are often complex, laborious and may lack sensitivity and specificity. In addition, culture-based detection of spore-forming bacteria typically requires specific growth conditions to promote spore germination and vegetative growth, which can delay detection (Gupta & Brightwell, 2023; Kawai & Nakano, 2025; Shams et al., 2020). In contrast, nucleic acid-based detection methods can directly detect target DNA or RNA, enabling faster and more reliable identification and confirmation.

To circumvent the limitations of conventional assays, various nucleic acid-based techniques have been developed to detect *Clostridium* spp. and *Bacillus* spp., including polymerase chain reaction (PCR) (Banger et al., 2021), fluorescence *in situ* hybridization (FISH) (Weerasekara et al., 2013), isothermal nucleic acid amplification technologies such as loop mediated isothermal amplification (LAMP) (Cecere et al., 2021) and recombinase polymerase reaction (RPA) (Guo et al., 2022). Whole genome sequencing (WGS) has also provided comprehensive insights into species-level differentiation and virulence gene profiling of these bacteria (Frentzel et al., 2022). Recently, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas)-based systems have been utilized to develop rapid, sensitive, and specific diagnostic assays for detecting various foodborne pathogens, including spore-forming bacteria (Gootenberg et al., 2017; Jiang et al., 2023; Xu et al., 2023; Zhang et al., 2021).

In this review, we summarized nucleic acid-based detection methodologies specifically applied to *Clostridium* spp. and *Bacillus* spp. in food systems, highlighting gene targets, methodological adaptations and analytical performance. In particular, the potential of CRISPR/Cas-based systems to improve the sensitivity and specificity of detecting spore-forming bacteria is described.

2. Nucleic acid-based detection methods

2.1. DNA extraction

Nucleic acid-based detection assays have emerged as reliable, rapid and sensitive tools for identifying both vegetative cells and spores of spore-forming bacteria. These technologies enable the detection of low levels of microorganisms within a short time, making them valuable for food safety monitoring.

Two key considerations are critical when applying nucleic acid-based methods for the detection of spore-forming bacteria. First, sample preparation plays an important role in nucleic acid analysis (Emaus et al., 2020). It involves the efficient release of DNA from target organisms in sufficient quantity for amplification and the removal of macromolecules, lipids, RNA or proteins (Gupta, 2019). This process can be influenced by several factors, including cell type (vegetative cell or spore) and the sample matrix.

Second, the inherent advantages and limitations of nucleic acid-based detection methods should be considered, as each method varies in sensitivity, specificity, speed, and suitability for detecting different spore-forming bacteria across various food matrices.

DNA from vegetative cells is relatively easy to extract using most commercial DNA extraction kits, as commonly reported in studies using nucleic acid-based detection methods (Table 1, 3-6). However, *Bacillus* spp. and *Clostridium* spp. are gram-positive bacteria, and DNA extraction from these organisms presents additional challenges due to their thick cell walls (Galperin, 2013).

For spores, their structural complexity makes DNA extraction challenging using commercial kits. Therefore, pre-treatment methods are often required to improve DNA yield. One common approach is to germinate spores into vegetative cells prior to extraction, as reported in several studies for the detection of spores (Guo et al., 2022; Knüpfer et al., 2020; Shams et al., 2020). Alternatively, direct DNA extraction methods have been utilized, including the use of strong lysis buffers (Filion et al., 2009), beads beating (Bassi et al., 2013), enzymatic lysis (Sánchez-Chica et al., 2020), mechanical disruption (Şahiner et al., 2022), or heat treatment up to 130 °C (Cecere et al., 2021) to enhance DNA release, purity and concentration.

2.2. Polymerase chain reaction

Polymerase chain reaction (PCR) is one of the most widely used molecular-based detection methods for detecting and confirming the type of microbes, including spore-forming bacteria, by amplifying specific gene sequences *in vitro*. It involves repeated cycles of denaturation (separating DNA strands), annealing (binding of primers to target sequences), and extension (synthesis of new DNA by DNA polymerase) (Law et al., 2015; Shahzad et al., 2020). In a PCR reaction, the target DNA template is amplified in a linear fashion to produce a large number of copies of the target DNA.

2.2.1. Clostridium spp.

In the 1990s, PCR-based approaches were used to detect *Clostridium* spp. in food, with studies targeting both vegetative cells and spores across diverse food matrices. For example, the detection of *C. perfringens* vegetative cells in chicken (Wang et al., 1994), *Clostridium botulinum* (*C. botulinum*) vegetative cells in pork and beef (Fach et al., 1993), and *C. botulinum* spores in dairy products, fish and vegetables (Hiel et al., 1996; Szabo et al., 1994).

Because food contamination often involves multiple *Clostridium* types, assays evolved from single gene PCR to multiplex PCR (mPCR) to simultaneously detect several toxin genes. De Medici et al. (2009) successfully developed a mPCR assay targeting the neurotoxin genes of *C. botulinum* type A, B, E, and F. This assay was validated in vegetables, honey, fish, and meat. The assay achieved 99.2% relative accuracy compared with the standard mouse bioassay. However, its sensitivity and limit of detection (LOD) were not reported. While these endpoint PCR methods enabled specific detection of *Clostridium* toxins, their reliance on gel electrophoresis and UV visualization limited rapid application and increased the risk of cross-contamination.

Real-time quantitative PCR (qPCR) is a modification of the PCR strategy that utilizes fluorescence-based chemistry, which enables monitoring of the target amplification in real-time during PCR (Artika et al., 2022; Mortari & Lorenzelli, 2014). The qPCR assays have been successfully used to detect *C. perfringens* vegetative cells in meat and vegetables (Chon et al., 2012), *Clostridium tyrobutyricum* (*C. tyrobutyricum*) spores in milk and dairy products (Arnaboldi et al., 2021; Bassi et al., 2013; Lopez-Enriquez et al., 2007), as well as *C. botulinum* spores in meat and vegetables (Yoon et al., 2005).

Multiplex qPCR further enables simultaneous detection of multiple toxin genes or various pathogens in a single assay by using different fluorophore quencher probe sets, enhancing efficiency and diagnostic capacity. For example, two independent studies developed multiplex qPCR assays to detect *C. botulinum* toxin types A, B, E, and F using vegetable and meat matrices for validation (Kirchner et al., 2010; Satterfield et al., 2010). Morandi et al. (2015) developed a triplex qPCR assay targeting *nifH*, *gerAA* and *enr* genes for simultaneous detection of *Clostridium beijerinckii* (*C. beijerinckii*), *Clostridium sporogenes* (*C. sporogenes*) and *C. tyrobutyricum* spores in raw milk. In this study, the LOD for different targets was defined as the quantification cycle (Cq) at which the fluorescence signal of the lowest concentration exceeds the background level. The LODs of the developed method were 300 CFU/50 mL (Cq=37.11) for *C. beijerinckii*, 2 CFU/50 mL (Cq=37.15) for *C. sporogenes*, and 5 CFU/50 mL (Cq=38.01) for *C. tyrobutyricum*. Similarly, (Şahiner et al., 2022) developed a multiplex qPCR targeting *nifH*, *gerAA* and *enr* genes to detect vegetative cells of *Clostridium butyricum* (*C. butyricum*), *C. sporogenes* and *C. tyrobutyricum* in cheese, with LODs ranging from 10 to 100 CFU/mL.

Table 1
The applications of PCR-based methods for detecting *Bacillus* spp. and *Clostridium* spp. in food.

Strains	Methods	Genes	Analytical sensitivity		Isolation methods		In food		References
			Spores	Vegetative	Spores	Vegetative	Food types	LODs	
C. perfringens	PCR	*16S rDNA*	-	2 cells	-	1% triton X100 + Boiling	Chicken	20 cells	(Wang et al., 1994)
C. botulinum	PCR	*BoNT/A*	-	12.5 fg/reaction	-	Boiling	Beef and pork	10-10^3 spores/g	(Fach et al., 1993)
C. botulinum	PCR	*BoNT/A* and *BoNT/B*	-	-	-	-	Pasteurized milk, UHT milk, infant formula, meat juice, canned tuna, mushrooms and blood sausage		(Szabo et al., 1994)
C. botulinum	PCR	*BoNT/E*	-	-	-	-	Fish, eggs, canned tuna and pickled herring		(Szabo et al., 1994)
C. botulinum	PCR	*BoNT/E* and *16S rRNA*	-	-	Boiling	-	Rainbow trout	10^2 CFU/kg	(Hielm et al., 1996)
C. botulinum	mPCR	*BoNT/A*, *BoNT/B*, *BoNT/E* and *BoNT/F*	-	-	-	Chelex 100 matrix	Vegetable, shellfish, canned meat, sausage, canned tuna fish and honey	-	(De Medici et al., 2009)
C. perfringens	qPCR	*plc*	-	10^2 CFU/mL	-	Boiling	Pork, beef, canned bean and vegetable	10^3 CFU/g	(Chon et al., 2012)
C. tyrobutyricum	qPCR	*pta*	10 CFU/mL	10 CFU/mL	Beads-beater + Enzymatic lysis	Beads-beater + Enzymatic lysis	Milk, curd and cheese	10 CFU/mL	(Bassi et al., 2013)
C. botulinum	qPCR	*BoNT/A*	10^2 spores/mL	-	Guanidine isothiocyanate treatment	-	Sausage slurry and canned corn slurry	10^2 spores/mL	(Yoon et al., 2005)
C. tyrobutyricum	qPCR	*pta*	-	-	DNA extraction kit	-	Milk	1 spores/mL	(Arnaboldi et al., 2021)
C. tyrobutyricum	qPCR	*fla*	300 spores	-	Pre-treatment + DNA extraction kit	-	Raw milk and UHT whole milk	25 spores	(Lopez-Enriquez et al., 2007)
C. perfringens	CRENME and qPCR	*plc*	-	-	-	-	Drinking water	4 spores/100 mL	(Maheux et al., 2013)
C. botulinum	multiplex qPCR	*BoNT/A*, *BoNT/B*, *BoNT/E* and *BoNT/F*	-	10-100 copies/assay	-	DNA extraction kit	Mackerel, frozen green beans, vacuum-packed and smoked black pudding with meat	10^3 to 10^5 CFU/mL	(Kirchner et al., 2010)
C. botulinum	multiplex qPCR	*BoNT/A*, *BoNT/B*, *BoNT/E* and *BoNT/F*	-	100 fg	-	DNA extraction kit	Sausage and vegetable	-	(Satterfield et al., 2010)
C. beijerinckii, C. sporogenes. and *C. tyrobutyricum*	multiplex qPCR	*nifH*, *gerAA* and *enr*	10 pg for *C. tyrobutyricum* and *C. sporogenes* and 60 pg for *C. beijerinckii*	-	Microwave treatment	-	Raw milk	300 CFU/50 mL for *C. beijerinckii*, 2 CFU/50 mL for *C. sporogenes*, 5 CFU/50 mL for *C. tyrobutyricum*	(Morandi et al., 2015)
C. butyricum, C. sporogenes, and *C. tyrobutryricum*	multiplex qPCR	*enr*, *gerAA* and *nifH*	-	10 copies/reaction	Mechanical digestion and DNA extraction kit	DNA extraction kit	Cheese	-	(Şahiner et al., 2022)
B. cereus group spp.	PCR	*motB*	-	10^3 CFU/mL	-	Boiling	Milk powder	10^3 CFU/g	(Oliwa-Stasiak et al., 2010)

(*continued on next page*)

Table 1 (*continued*)

Strains	Methods	Genes	Analytical sensitivity		Isolation methods		In food		References
			Spores	Vegetative	Spores	Vegetative	Food types	LODs	
B. cereus	PCR	*entFM* and *hblA*	-	0.1 ng	-	JKM M 3050 standard method	Ready-to-eat food and drink	0.1 ng	(Nooratiny & Sahilah, 2013)
B. cereus	mPCR	*entFM*, *hblC*, *nheA*, *cytK*, *ces* and *CER*	-	10^2 CFU/mL	-	DNA extraction kit	Milk	10^3 CFU/mL	(Kim et al., 2012)
B. cereus	mPCR	*nheA*, *hblD*, *cytK2* and *cesB*	-	-	Lysis, beads-beater and heat treatment before DNA extraction	Lysozyme and proteinase K processing	Milk powder and infant formula	10^3 CFU/g	(Sánchez-Chica et al., 2020)
B. cereus	mPCR	*nheA*, *groEL* and *ces*	-	10 CFU/tube for enterotoxin strain, 100 CFU/tube for emetic producing strain	-	DNA extraction kit	Milk	100 CFU/tube for enterotoxin strain, 1000 CFU/tube for emetic producing strain	(Lee et al., 2008)
B. cereus	PMA-mPCR	*nheA*, *entFM*, *hblD*, *cytK* and *ces*	-	10^2 CFU/mL	-	DNA extraction kit	Baby cereal, pasteurized milk and rice	10^3 CFU/g	(Forghani et al., 2015)
B. cereus group	PMA-mPCR	*cesB* and *16S rRNA*	-	10 CFU/mL	-	Boiling	Noodle, rice and sausage	10^3 CFU/g	(Zhang et al., 2014)
B. cereus	Van-PLL-MB-mPCR	*entFM*, *cesB*, *cer*, and *16S rRNA*	-	10^3 CFU/mL	-	Boiling	Milk	10 CFU/mL	(Li et al., 2021)
B. cereus, B. subtilis, and *B. licheniformis*	qPCR	*16S rRNA*	-	16.5 CFU/mL	-	DNA extraction kit	Fish	165 CFU/g	(Fernandez-No et al., 2011)
B. cereus	qPCR	*plc*	-	44 CFU/mL	-	DNA extraction kit	Liquid egg and infant formula	40 CFU	(Martinez-Blanch et al., 2009)
B. cereus	qPCR	*ces*	-	0.6 pg	-	DNA extraction kit	Rice and pasta	10^3 CFU/g	(Fricker et al., 2007)
B. subtilis, B. cereus	multiplex qPCR	*ccpA* and *cotQ*	-	10 CFU/mL	-	DNA extraction kit	Milk	-	(Kwon et al., 2021)
B. cereus	multiplex qPCR	*nheA*, *hblD*, *cytK1*, and *ces*	-	1 ng	-	DNA extraction kit	Rice pudding, carrot puree and baby food cereal	10 CFU/g	(Wehrle et al., 2010)
B. cereus	PMA qPCR	hemolysin gene	-	10^2 copies/mL	-	Phenol-chloroform-isoamyl	Milk	10^2 CFU/mL	(Cattani et al., 2016)
B. cereus	PMA-qPCR	*cesB*	-	10^2 CFU/mL	-	Boiling	Milk	10^2 CFU/mL	(Zhou et al., 2019)

Target strains, detection methods, target genes, analytical sensitivity, and DNA extraction approaches (from spores and vegetative cells using commercial kits or other methods) were summarized. Validation for detection in food samples was also included. '-' indicates not applied in the study.

Although multiplex qPCR enables rapid, quantitative detection of multiple *Clostridium* spp., it requires careful assay optimization to address challenges such as probe design, primer concentrations, and consistent annealing temperatures across targets (Dale et al., 2016; Elnifro et al., 2000; Emaus & Anderson, 2020; Zhou et al., 2013). These considerations highlight that the biological diversity of *Clostridium* spp., combined with food matrix complexity, should guide assay design to ensure reliable and accurate quantification.

2.2.2. *Bacillus spp.*

Basic PCR methods have been used to detect *Bacillus* spp. in food (Nooratiny & Sahilah, 2013; Oliwa-Stasiak et al., 2010). The mPCR methods targeting multiple toxin or housekeeping genes have also been used to identify *B. cereus* vegetative cells and/or spores in milk and dairy products (Kim et al., 2012; Lee et al., 2008; Sánchez-Chica et al., 2020). These results were visualized using gel electrophoresis. To enhance the selectivity and capture efficiency, Li et al. (2021) developed a novel mPCR method coupled with vancomycin (Van)-modified poly-L-lysine (PLL) magnetic beads (MBs) for the detection and genotyping of *B. cereus* in milk. Four genes (*entFM*, *cesB*, *cer* and *16S rRNA*) were targeted. In this approach, vancomycin functioned as a molecular ligand between the MBs and the d-alanyl-d-alanine residues on the *B. cereus* cell wall, while PLL served as a flexible molecular spacer to reduce steric hindrance and preserve vancomycin's biological activity. This MB-PLL-Van system enhanced the specificity of *B. cereus* isolation before mPCR analysis, though the MB-PLL-Van mPCR assay remained complex and required at least 6 h of enrichment to detect 10 CFU/mL of *B. cereus* in milk samples, limiting its suitability for rapid diagnostics. To improve viability discrimination, mPCR has also been combined with propidium monoazide (PMA), a DNA binding dying, that selectively penetrates non-viable cells and prevents amplification of their DNA (Lee et al., 2022). Zhang et al. (2014) developed a PMA-mPCR assay targeting *cesB* and *16S RNA* genes of *B. cereus* to differentiate between viable cells of emetic *B. cereus* and non-emetic *B. cereus* in cooked rice, noodle and sausage. The PMA treatment eliminated frequency of mPCR false positives by preventing amplification from non-viable vegetative cells. Forghani et al. (2015) developed a PMA-mPCR assay for simultaneous detection of four enterotoxin genes (*nheA*, *hblD*, *entFM*, and *cytK*) and an emetic toxin gene (*ces*) in *B. cereus*. When combined with PMA, the method successfully detected and differentiated viable enterotoxic and emetic *B. cereus* strains in dairy and rice products. Nonetheless, these endpoint PCR approaches still rely on gel electrophoresis for amplicon visualization, making them less practical for rapid, field-based food testing.

Several qPCR assays targeting distinct *B. cereus* genes have been developed, enabling its detection across diverse food matrices. For example, qPCR-based assays have been developed to target *16S rRNA* of *Bacillus* spp. in fish (Fernandez-No et al., 2011), the *plc* gene of *B. cereus* in liquid egg and infant formula (Martinez-Blanch et al., 2009), and the *ces* gene of emetic *B. cereus* in rice and pasta (Fricker et al., 2007). Wehrle et al. (2010) developed a multiplex qPCR assay targeting *nheA*, *hblD*, *cytK1* and *ces* genes to detect *B. cereus* in rice, vegetable, and cereal samples. Similarly, Kwon et al. (2021) developed a multiplex qPCR assay capable of differentiating *B. cereus* from *Bacillus subtilis* (*B. subtilis*). The researchers designed specific primer-probe sets by comparing core genomes using a pan-genome analysis tool (panX). This resulted in a highly specific and sensitive assay, which was validated in milk samples. Although this genomics-based approach improved assay precision, it required advanced bioinformatics analysis for primer design, which may limit its accessibility for routine testing laboratories.

Similar to the PMA-mPCR method described above, Cattani et al. (2016) and Zhou et al. (2019) developed PMA-qPCR methods to detect the hemolysis gene of *B. cereus* and the *cesB* gene in emetic *B. cereus*. Both assays effectively reduce false-positive signals from dead cells while maintaining quantitative accuracy for viable cells.

PCR-based methods provide flexible framework for detecting *Clostridium* spp. and *Bacillus* spp., allowing either single-gene quantification or multiple toxin profiling. However, these methods still rely on thermal cyclers and multi-step workflows that can be resource-intensive for routine or on-site analysis. The applications of PCR-based methods for detecting spore-forming bacteria in food are shown in Table 1.

2.3. *Fluorescence in situ hybridization*

Fluorescence in situ hybridization (FISH) has been applied for the detection of *Clostridium* spp. and *Bacillus* spp. in food. The method relies on the hybridization of the target DNA sequence with the fluorescently labeled DNA probe through complementary base pairing within cells, allowing genus and species-level identification under a fluorescence microscope (Shakoori, 2017). FISH assays typically begin with fixation and permeabilization steps to preserve cell morphology while enabling probe access through the spore coat or cell wall.

2.3.1. *Clostridium spp.*

There was only one study that used the FISH method for the detection of *C. perfringens* in food samples. In this study, a combined FISH and filter cultivation (FISH-FC) was developed for the specific detection of culturable *C. perfringens*. In this approach, *C. perfringens* cells were first cultured on a filter membrane under anaerobic conditions, then hybridized with a *C. perfringens*-specific probe (CLP-180), targeting *16S rRNA* gene, labeled with 5′-carboxy-tetramethyl-rhodamine-N-hydroxysuccinimide ester (TAMRA; yellow fluorescence). A universal probe, EUB-338, labeled with fluorescein isothiocyanate (FITC; green fluorescence), served as a positive control. Following hybridization, yellow fluorescence indicated the presence of *C. perfringens*, enabling detection of 2 log CFU/g of *C. perfringens* in ground beef within 9 h (Shimizu et al., 2009). While the sensitivity of the FISH-FC method was comparable to that of standard plating methods, its application is limited by its endpoint detection, samples processing and reliance on fluorescence microscopy. Furthermore, the need for anaerobic incubation and specialized equipment restricts its feasibility for on-site or field-based detection of *Clostridium* spp. in complex food matrices.

2.3.2. *Bacillus spp.*

FISH-based methods have also been used to differentiate between closely related *Bacillus* spp. in food. For example, species-specific *16S rRNA* probes labeled with the rhodamine derivative Texas Red were used to distinguish *Bacillus polymyxa* (*B. polymyxa*) and *Bacillus macerans* (*B. macerans*). Fluorescence intensity, which reflects probe-target hybridization affinity, was used for species identification. Probe 1 showed 100% sequence identity with *B. polymyxa* and 60% with *B. macerans*, while Probe 2 had 100% identity with *B. macerans* and 86.6% with *B. polymyxa*, allowing species-level discrimination based on fluorescence signals (Jurtshuk et al., 1992). Similarly, Abella et al. (2000) developed fluorescence-labeled trinucleotide probes targeting strain-specific repeat sequences in the *16S rRNA* region to distinguish *B. subtilis* and *Bacillus fusiformis* (*B. fusiformis*) using FISH. Relatively short probes such as TTT (labeled with 5′-fluorescein), GGG (with FITC), and TAT (with 5′-tetrachlorofluorescein, TET) were used to detect these *Bacillus* spp. via synchronous fluorescence spectrometry. However, single nucleotide mutations in the target sequence can reduce hybridization efficiency, potentially resulting in false-negative signals with such short probes.

FISH-based methods have also been used to detect *Bacillus* spores, although the highly resistant spore coat limits probe accessibility. To address this, Filion et al. (2009) established species-specific protocols to permeabilize spores of *Bacillus megaterium* (*B. megaterium*), *Bacillus atrophaeus* (*B. atrophaeus*), and *B. cereus* using individual treatments with sodium dodecyl sulfate (SDS), 1,4-dithiothreitol (DTT), urea, and proteinase K under varying incubation conditions. After treatments, *Bacillus* spores were successfully detected within 1 h. However, these protocols have not been validated in food samples and remain complex,

limiting their routine application. An alternative strategy involves germinating spores into vegetative cells prior to FISH. Laflamme et al. (2009) optimized spore germination, permeabilization, and hybridization conditions for *B. cereus*, enabling detection of 10^3 CFU/mL in milk within 2 h. This highlighted that permeabilization and hybridization are critical steps in improving FISH-based detection of spores. However, FISH remains suboptimal for direct spore detection due to the necessity of a germination step. Once spores germinate, culture- or PCR-based methods generally provide more practical, standardized, and rapid detection of vegetative cells.

Nevertheless, FISH offers certain advantages for *Clostridium* and *Bacillus* detection: it requires only a single probe rather than multiple primers and thermal cycling steps as in PCR-based methods, and it can provide rapid results in certain contexts compared to PCR. However, its limited sensitivity, need for specialized equipment, and lower suitability for complex food matrices restrict its broader application for the detection of spore-forming bacteria in food. The applications of FISH-based methods for detecting spore-forming bacteria in food are summarized in Table 2.

2.4. Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) has been applied for the rapid detection of *Clostridium* spp. and *Bacillus* spp. in diverse food matrices. This technique is capable of rapidly amplifying the target DNA sequence at a constant temperature between 60-65 °C (Zhang et al., 2023). The LAMP technique uses a DNA polymerase with strong strand displacement activity, such as Bst polymerase (derived from *Bacillus stearothermophilus*). A set of four to six specifically designed primers that recognize six to eight distinct regions on the target DNA, is used to ensure high specificity. The inner primers initiate DNA synthesis and form loop structures, which facilitate continuous and exponential amplification through strand displacement. The outer primers also play a crucial role by initiating strand displacement and creating single-strand DNA, which served as templates for further amplification. This results in the accumulation of large amounts of DNA in a short time, often between 30-60 min. The whole reaction can be conducted in a simple water bath or heating block, making it accessible for low-resource settings. Fig. 1 illustrates the general workflow of a LAMP assay.

2.4.1. Clostridium spp.

Various LAMP-based assays have been developed for the rapid detection of *Clostridium* spp. in food that targeting toxin genes such as the *plc* toxin gene of *C. perfringens* in meat, and dairy products within 75 min (Priya et al., 2018; Radhika et al., 2016), the *neurotoxin* gene of *C. botulinum* in kimchi, meat, dairy products, canned fish and honey within 60 min (Chen et al., 2021; Sakuma et al., 2009), and the *BoNT/E* and *BoNT/F* genes of *C. botulinum* in canned fish within 30 min (Chu et al., 2024). No cross-reactivity was observed with any of the pathogens tested in these developed assays. The results indicate that LAMP-based assays enable rapid and specific detection of *Clostridium* spp. in diverse food matrices.

To enhance field applicability, two main strategies have been introduced; One involves combining LAMP with visual or equipment-free detection, in which colorimetric LAMP uses pH-sensitive or ion-reactive dyes that change color in response to DNA amplification. This is due to the formation of pyrophosphate and hydrogen ions as by-products during LAMP. In the presence of weakly buffered or non-buffered solutions, such by-products significantly lower the initial alkaline pH of the LAMP solution to a final acidic pH. This significant change in pH value offers the possibility of visually detecting DNA amplification with pH-sensitive dyes (Jaroenram et al., 2019). For example, Cecere et al. (2021) developed a Cresol Red-based LAMP assay to detect *C. tyrobutyricum* spores in milk, where a pink to yellow color change indicated positive amplification. However, this study required spore lysis at 130 °C, which may compromise DNA quality. The second is integrating lateral flow biosensor or strip (LFB/LFS) with LAMP for rapid and user-friendly diagnostics. In this approach, the 5' ends of the forward primers and probe are modified with biotin and FITC, separately. The primers were used to amplify the target region, and the biotin-labeled amplicons hybridize with the FITC-labeled probe. Detection is achieved by LFB/LFS coated with antibodies against biotin and FITC, allowing visual interpretation of colored lines with the naked eye within 10 min (Wang et al., 2022; Zasada et al., 2018). Sridapan et al. (2021) developed a LAMP-LFB assay targeting the *plc* gene of *C. perfringens* using digoxigenin-labeled loop primers and a FITC-labeled probe. The assay showed no cross-reactivity and achieved a detection limit of 10 CFU/g in chili paste, meat and gravy sauce after 16 h enrichment, comparable to qPCR. However, LAMP assays require multiple primers, which complicates assay design and limits simultaneous detection of multiple toxin genes in a single reaction. Nevertheless, the advantages of being rapid, specific and adaptable to visual or field-based readouts make it a valuable tool for detecting and monitoring pathogens like *Clostridium* in food safety.

2.4.2. Bacillus spp.

LAMP-based assays have also been developed to detect *Bacillus* spp. in food samples, including *Bacillus anthracis* (*B. anthracis*) spores in flour, baking soda and dried milk powder (Qiao et al., 2007), and *B. cereus* vegetative cells in milk samples (Liu et al., 2011). However, many of these early LAMP assays required agarose gel electrophoresis to visualize amplified products, making them time-consuming and not suitable for field detection or rapid detection.

Similar to the detection of *Clostridium* spp., various modifications have been incorporated to LAMP to develop different methods for detecting *Bacillus* spp. For example, Li et al. (2022) developed a real-time LAMP assay for detecting *B. cereus* in pasteurized milk. Roy et al. (2017) combined LAMP with a paper microchip and colorimetric detection to detect the *rpoB* gene of *B. subtilis* vegetative cells. In this method, crystal violet (CV), a triphenylmethane dye containing a *p*-quinoid group, acts as a chromophore, with high affinity for dsDNA. In aqueous solution, CV is violet in color, but the addition of substituents, such as sodium sulfite (Na_2SO_3), CV is converted into leuco crystal violet (LCV), which is colorless and unstable. When dsDNA is added, CV binds with dsDNA due to electrostatic interaction of the negatively charged

Table 2
The applications of FISH-based methods for detecting *Clostridium* spp. and *Bacillus* spp. in food

Strains	Methods	Genes	Targets		In food		References
			Spore	Vegetative	Food type	LODs	
C. perfringens	Culture and FISH	*16S rRNA*	-	+	Beef	2 log CFU/g	(Shimizu et al., 2009)
B. polymyxa and *B. macerans*	FISH	*16S rRNA*	-	+	-	-	(Jurtshuk et al., 1992)
B. subtilis and *B. fusiformis*	FISH	*16S rRNA*	-	+	-	-	(Abella et al., 2000)
B. cereus	FISH	*16S rRNA*	+	-	-	-	(Filion et al., 2009)
B. cereus	FISH	*16S rRNA*	+	-	Milk	10^3 CFU/mL	(Laflamme et al., 2009)

Target strains, detection methods, target genes, targets (spores or vegetative cells) were summarized. Validation for detection in food samples was also included. '-' indicates not applied in the study.

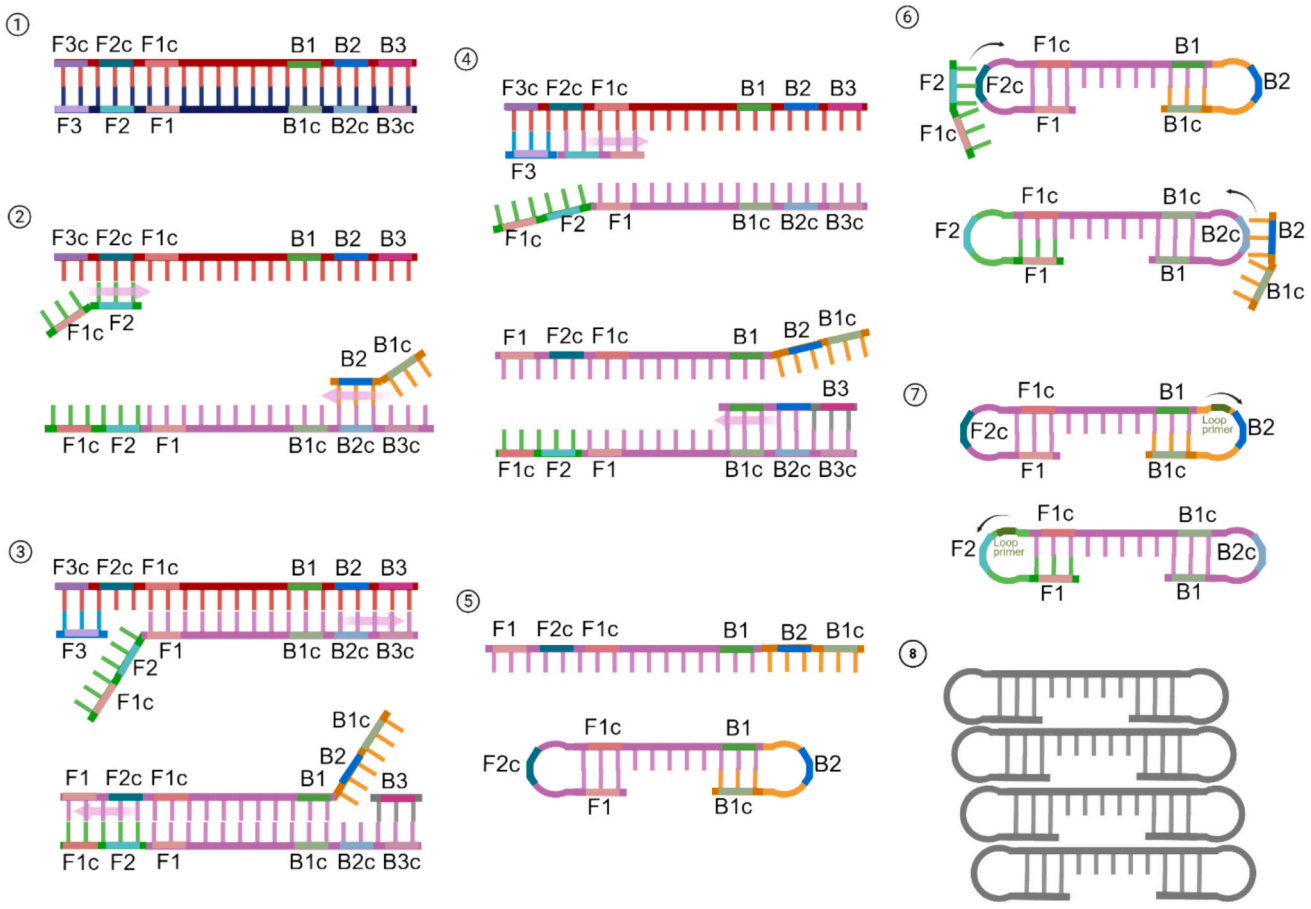

Fig. 1. The principle of the traditional LAMP method. Step 1, design of two inner primers (F2/F1c and B2/B1c) and two outer primers (F3 and B3) that target six distinct regions on the template DNA; Steps 2, the forward and reverse inner primer (F2/F1c and B2/B1c) initiates the DNA synthesis using DNA polymerase; Steps 3, the forward and reverse outer primer (F3 and B3) binds to target sequence; Steps 4, the outer primer (F3 and B3) displaces the newly synthesized DNA strand, resulting in single-stranded dumb-bell formed from the starting structure with loops at each end (steps 5); Steps 6, the dumbbell DNA structure is transformed into stem-loop DNA by self-primed DNA synthesis, and a complement of the structure is generated by DNA replacement of the primer strand and inner primer hybridization; Steps 7, additional loop primers can be added to increase the shape complexity of the amplicons; Steps 8, repeating these reactions achieves amplification of the product exponentially.

phosphate group of dsDNA, and the positively charged quinoid of CV. In this process, CV interacts with the major groove of dsDNA to form a CV-dsDNA complex. The stability of this complex prevents decoloration of the mixture and changes the color of the dye back to the original violet color. Consequently, the color changes from colorless (LCV) to violet (CV), indicating the presence of dsDNA and allowing visual detection with the naked eye. The LOD of the assay was 10 pg/μL of *B. subtilis* DNA (Roy et al., 2017). However, this assay was not validated in complex food samples, and in the colorimetric-based methods, the concentrations of each reagent and indicator should be precisely optimized.

To improve specificity for the detection of toxin-producing strains, Busch et al. (2022) developed a two-step LAMP assay targeting the *groEL* gene (present in all *B. cereus* group members) and the *nheB* gene (specific to non-hemolytic enterotoxin-producing strains). The assay was evaluated in meat and vegetable samples, with culture-based and qPCR methods as references. While the *groEL* assay showed 100% specificity for the *B. cereus* group, the *nheB* assay achieved 93.7% specificity. As reported, the *B. cereus*-group comprises numerous closely related species, which makes it challenging for the development of specific diagnostic assays (Ehling-Schulz et al., 2019). In this study, the one-step LAMP assay was unable to distinguish between two genetically closely related species.

These findings suggest that LAMP-based methods offer an easy-to-use, rapid, and highly accurate alternative to conventional PCR or FISH for detecting *Clostridium* spp. and *Bacillus* spp. in food. Its isothermal conditions eliminate the need for thermal cyclers and enable faster amplification. Compared to FISH, LAMP offers greater sensitivity due to nucleic acid amplification and has been validated in various food samples, making it more suitable for routine detection. The applications of LAMP-based methods for detecting spore-forming bacteria in food samples are summarized in Table 3.

2.5. Recombinase polymerase amplification

Recombinase polymerase amplification (RPA) is another isothermal amplification technique that enables rapid DNA amplification at a constant temperature (typically 37–42 °C) (Lobato & O'Sullivan, 2018). The principle of RPA relies on a recombinase enzyme that pairs primers with their complementary sequences in the target DNA, assisted by single-strand DNA-binding proteins (SSBs) that stabilize the displaced DNA strand. A strand-displacing DNA polymerase then extends the primers without the need for thermal cycling (Tan et al., 2022). RPA amplification can be completed within 20 min, making it potentially suited for rapid detection of foodborne pathogens including *Clostridium* spp. and *Bacillus* spp. The RPA amplification scheme is shown in Fig. 2.

Basic RPA products are usually visualized by agarose gel electrophoresis, which requires an additional purification step. This process is time-consuming, may cause cross-contamination, and limits its practical applicability. To overcome these limitations, the RPA has been adapted for real-time detection or end-point analysis using microfluidic devices,

Table 3
The applications of LAMP-based methods in detecting *Clostridium* spp. and *Bacillus* spp. in food samples

Strains	Methods	Genes	Analytical sensitivity		DNA isolation		In food	LODs	Refs.
			Spore	Vegetative	Spore	Vegetative	Food types		
C. perfringens	LAMP	*plc*	-	0.34 pg/μL	-	DNA extraction kit	Chevon	10^2 CFU/g after 6 h enrichment	(Priya et al., 2018)
C. botulinum	LAMP	*ntnh*	-	0.0001 pg/μL	-	Chelex method	Korean kimchi, pork, canned sardine, soybean paste, and dairy product	-	(Chen et al., 2021)
C. botulinum	LAMP	*BoNT/A* or *BoNT/B*	-	1 pg for *BoNT/A*; 10 pg for *BoNT/B*	-	DNA extraction kit	Canned fish and honey	1 CFU of cells for *BoNT/A*; 10 CFU of cells for *BoNT/B*;	(Sakuma et al., 2009)
C. botulinum	LAMP	*BoNT/A* or *BoNT/B*	-	-	Bead- beater + DNA extraction	-	Canned fish and honey	10 CFU of spores for *BoNT/A*; 10^3 CFU of spores for *BoNT/B*	(Sakuma et al., 2009)
C. perfringens	LAMP	*cpa*	-	10 CFU/ reaction	-	DNA extraction kit	Food: ham, sausage, beef, milk powder, hot pepper, soy sauce, oyster sauce, pepper paste, soybean paste, salad dressing, marinade, kimchi and Sunsik (dry grain food).	10^2 - 10^3 CFU/ reaction	(Hong, 2017)
C. botulinum	LAMP	*BoNT/E* and *BoNT/F*	-	10 copies/ reaction for *BoNT/E*; 10^2 copies/reaction for *BoNT/F*	-	DNA extraction kit	Canned fish		(Chu et al., 2024)
C. tyrobutyricum	coloric-LAMP	*pta*	2 spores/ mL	-	Heated to 130 °C	-	Milk	10 spores/ reaction	(Cecere et al., 2021)
C. perfringens	LAMP-LFB	*plc*	-	1 CFU/mL	-	Boiling	Chili paste, cured meat and gravy sauce	10 CFU/g after 16 h enrichment	(Sridapan et al., 2021)
B. anthracis	LAMP	*Ba813*, *pag*, and *capB*	10 spores for *pag* and *capB*; 100 spores for *Ba813*	-	Boiling	-	Fluor, baking soda, dried milk powder	100 spores for *pag* and *capB*; 1000 spores for *Ba813*	(Qiao et al., 2007)
B. cereus	two-step LAMP	*groEL* and *nheB*	-	0.5 pg for *groEL*, 1 pg for *nheB*	-	Boiling	Minced beef	0.1 pg/μL after 48 h enrichment	(Busch et al., 2022)
B. cereus	IMS-LAMP	*tetL*	-	11.6 CFU/mL	-	Boiling	Pasteurized milk	21.5 CFU/mL	(Li et al., 2022b)
B. cereus	LAMP	*16S rDNA* and *cesA*	-	1 CFU/mL for *16S rDNA*; 11 CFU/mL for *cesA*	-	Boiling	Directly from commercial fluid milk	-	(Liu et al., 2011)

Target strains, detection methods, target genes, analytical sensitivity, and DNA extraction approaches (from spores and vegetative cells using commercial kits or other methods) were summarized. Validation for detection in food samples was also included. '-' indicates not applied in the study.

LFS or LFB. Real-time RPA allows continuous monitoring of target sequence amplification and involves the use of an *exo* probe. This probe includes a tetrahydrofuran (THF) residue, flanked by a dT-fluorophore and a corresponding dT-quencher, along with a 3'-modification group. In the intact probe, the fluorophore signal is quenched by the nearby quencher. During amplification, in a double-stranded context, the THF residue serves as a cleavage site for Exonuclease III, which separates the fluorophore from the quencher, resulting in a detectable fluorescent signal (Wan Rasni et al., 2022).

2.5.1. *Clostridium spp.*

Several RPA-based assays have been explored for the rapid detection of *Clostridium* spp. and their toxin genes. For example, Bachmann et al. (2024) combined an RPA assay with a 3D printed microreactor device to detect the *tcdA* and *tcdB* genes of *Clostridium difficile* (*C. difficile*). Similarly, Tsaloglou et al. (2015) developed a real-time RPA method integrated with a microfluidic platform to detect the *tcdB* gene of *C. difficile*, achieving a detection limit of 1000 DNA copies within 20 min. These assays demonstrated that RPA can be adapted to detect distinct toxin gene targets of *C. difficile*, though their application to food has not been validated.

A few studies have used RPA-based methods for detecting *Clostridium* spp. and validated using food samples. For example, Xu et al. (2019) developed a real-time RPA assay targeting the *plc* gene of *C. perfringens*. The developed method allowed rapid identification of *C. perfringens* isolates from chicken samples. Compared with qPCR, the RPA assay detected as few as 10 copies/μL of *C. perfringens* DNA at 38 °C within 25 min. However, this method was still used in conjunction with culture-based techniques to confirm the identity of *C. perfringens*, which added to the overall processing time. Liu et al. (2020) further developed and compared both qPCR and real-time RPA methods, targeting the *plc* gene of *C. perfringens*. The developed methods were validated in milk powder and meat samples, with both methods demonstrating an LOD of 10^2 CFU/mL. These results showed that RPA can be optimized for the detection of *C. perfringens* from food-derived DNA and maintain sensitivity equivalent to qPCR while reducing assay time.

Tian et al. (2024) developed an RPA-LFB assay targeting the *plc* gene of *C. perfringens*, with an LOD of 100 pg/μL of purified DNA. The assay achieved detection limits of 10^4 CFU/mL in chicken and 10^3 CFU/mL in milk. While this demonstrates successful coupling of RPA with visual

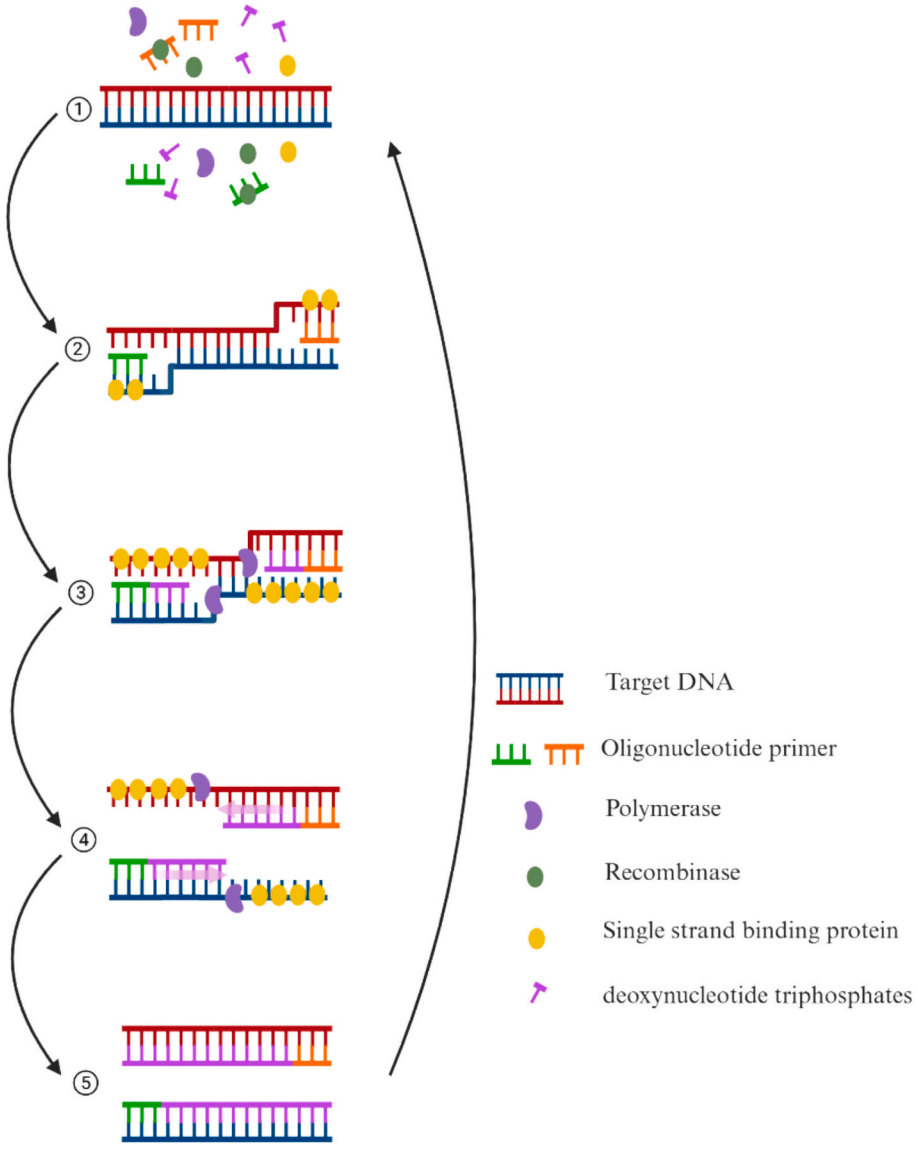

Fig. 2. The principle of RPA reaction. RPA reaction consists of recombinase and single strand binding proteins, DNA polymerase, and dNTPs. Step 1, the RPA reaction starts when the recombinase protein binds to primers, forming a recombinase-primer complex; Steps 2, this complex binds to the complementary sequence in the target sequence displacing the strands; Steps 3, the displaced DNA strand is stabilized by single-strand binding proteins to prevent the ejection of the inserted primer; Steps 4, the recombinase disassembles, and strand displacing DNA polymerase binds to the 3' end of the primers, elongating it in the presence of dNTPs; Steps 5, repeating this process continuously results in exponential amplification of the target sequence.

detection, the reduced sensitivity relative to qPCR and real-time RPA suggests further optimization is needed for complex food matrices. Furthermore, none of the RPA-based methods reported to date have demonstrated the ability to distinguish between different toxin types of *Clostridium* spp. This highlights a current limitation in the application of RPA methods for toxin typing and strain differentiation of *Clostridium* spp. in food safety.

2.5.2. *Bacillus spp.*

RPA-based methods have also been developed to detect *Bacillus* spp. with a focus on species- and gene-specific targets. Bentahir et al. (2018) developed four real-time RPA assays targeting three genes: the *BA_5345* chromosomal maker, *lef* and *capA* genes specific to *B. anthracis*, and the *adk* gene for the *B. cereus* group. The assays achieved a detection limit of 10^2 genomic copies of *B. anthracis*. No nonspecific signal was detected in the tested strains, and no cross-detection occurred with closely related strains, showing high specificity. However, because RPA alone cannot reliably differentiate between closely related genomic sequences, multiple assays were required to distinguish between *B. anthracis* and *B. cereus* group members. Wang et al. (2022) developed two RPA-LFS methods targeting the *gyrB* and *ces* genes, enabling specific detection of *B. cereus* and emetic *B. cereus*, respectively, and differentiating them from other foodborne pathogens. However, due to the exponential amplification process of RPA and the lack of a standardized fluorescence threshold, there is no direct correlation between the initial template concentration and the final signal output. This limits the ability of the RPA-based methods to achieve accurate quantification.

Despite these limitations, RPA offers several advantages over PCR and FISH. Both LAMP and RPA provide rapid, isothermal amplification without the need for complex equipment. Compared to LAMP, RPA is even more user-friendly, as it typically requires only one primer pair and a single probe, simplifying assay development. Moreover, the lower operating temperature of RPA (35-42 °C) compared to LAMP (60-65 °C) makes it more compatible with minimal instrumentation, enhancing its potential for the detection of foodborne pathogen, including *Clostridium* spp. and *Bacillus* spp. on-site and resource-limited settings. The Table 4

Table 4
The applications of RPA-based diagnostic methods for the detection of *Clostridium* spp. and *Bacillus* spp. from food samples

Strains	Methods	Genes	Analytical sensitivity		DNA isolation		In food		Reference
			Spore	Vegetative	Spore	Vegetative	Food types	LODs	
C. difficile	real-time RPA	*tcdB*	-	1 fg/reaction	-	DNA extraction kit	-	-	(Tsaloglou et al., 2015)
C. perfringens	real-time RPA	*plc*	-	1.3 pg/μL	-	DNA extraction kit	Milk powder, chicken, beef and lamb	10^2 CFU/mL	(Liu et al., 2020)
C. perfringens	real-time RPA	*plc*	-	10 copies/μL	-	DNA extraction kit	Chicken	-	(Xu et al., 2019)
C. perfringens	RPA-LFB	*plc*	-	100 pg/μL	-	DNA extraction kit	Chicken and milk	10^4 CFU/mL for chicken; 10^3 CFU/mL for milk	(Tian et al., 2024)
C. difficile	two-colour duplex RPA	*tcdA* and *tcdB*	-	119 molecules for *tcdB*; 1411 molecules for *tcdA*	-	DNA extraction kit	-	-	(Bachmann et al., 2024)
B. anthracis	real-time RPA	*BA_5345*, *lef* and *capA*	-	10^2 copies	DNA extraction kit	-	Cream, baking soda	-	(Bentahir et al., 2018)
B. cereus	RPA-LFS	*gyrB* and *ces*	-	10^2 CFU/mL	-	DNA extraction kit	Raw milk	10^2 CFU/mL	(Wang et al., 2022)
B. cereus	RPA-LFS	*gyrB* and *ces*	-	-	Enzymatic lysis and mechanical digestion before DNA extraction kit	-	Cream	10^3 CFU/mL	(Wang et al., 2022)

Target strains, detection methods, target genes, analytical sensitivity, and DNA extraction approaches (from spores and vegetative cells using commercial kits or other methods) were summarized. Validation for detection in food samples was also included. '-' indicates not applied in the study.

summarized the applications of RPA-based methods for detecting *Clostridium* spp. and *Bacillus* spp. in food.

2.6. Whole genome sequencing

Whole genome sequencing (WGS) has been used to characterize *Clostridium* spp. and *Bacillus* spp. in food safety contexts. By determining the complete DNA sequence of an organism, WGS provides a comprehensive view of its genetic content, including toxin genes, virulence factors, and strain-specific markers. Genomic DNA from the target bacterium is first extracted, fragmented, and sequenced using high-throughput platforms, after which the resulting reads are computationally assembled into contiguous sequences (Yang, 2020). The WGS workflow is described in Fig. 3.

2.6.1. Clostridium spp.

Yu et al. (2016) reported the complete genome of *Clostridium estertheticum* (*C. estertheticum*) DSM 8809 using single molecule real-time (SMRT) analysis. The sequencing results revealed a circular chromosome along with a single plasmid, and successfully identified genes associated with antibiotic resistance and virulence. Although this study provides valuable genomic information for risk assessment, WGS is typically performed on an organism. WGS-based approaches rely on culture-based methods to obtain the primary isolates responsible for foodborne outbreaks. Therefore, culture-based isolation remains a critical step for WGS applications in spore-forming bacteria in food safety.

For example, Abdelrahim et al. (2019) combined WGS phylogenomic with a qPCR-based virulence typing technique targeting 17 toxin-associated genes to differentiate toxin types of *C. perfringens* isolates from 42 foodborne outbreaks (FBOs) in France and other parts of Europe. All outbreaks associated strains carried the *cpe* gene encoding enterotoxin, but heterogeneity in other virulence genes highlighted the presence of genetically diverse strains or suggested potential gene transmission between strains (Abdelrahim et al., 2019).

WGS has also been used to explore the genetic diversity and evolutionary relationships of *Clostridium* spp. isolated from food samples. For example, genomic sequencing identified *C. difficile* isolates from sheep meat products (Mooyottu et al., 2015), *C. botulinum* isolates from salmon and fish samples (Weedmark et al., 2015), and *C. perfringens* isolates from chicken and pork (Li et al., 2020). These studies emphasize the utility of WGS in enhancing our understanding of pathogenicity, supporting biomarker discovery, and providing epidemiological insights by identifying genetic links between outbreaks and shared virulence profiles. However, the application of WGS for routine food diagnostics remains limited. In reported studies, bacterial isolates were obtained through culture-based methods, and purified genomic DNA was required. Additionally, sequencing data must be assembled, annotated, and interpreted using advanced bioinformatics tools, posing challenges

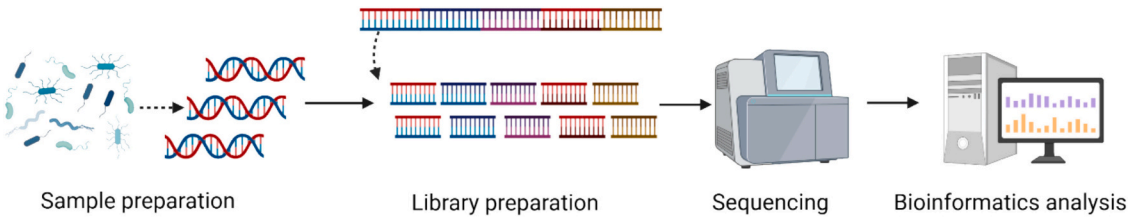

Fig. 3. The basic process of WGS. It generally involves four main steps: sample preparation, library preparation, sequencing and bioinformatics analysis. First, biological sample is isolated, and DNA is extracted; Second, primer pairs are deigned to cover the entire genome, and the genome is fragmented and amplified; Third, the amplified DNA fragments are sequenced using sequencing platform. Fourth, the resulting sequences are aligned and analyzed to reconstruct the complete genome sequence after bioinformatics analysis.

for rapid, routine, or point-of-care detection in food safety monitoring.

2.6.2. *Bacillus spp.*

WGS offers a powerful tool for providing comprehensive information for understanding strain-specific risks and identifying strains responsible for foodborne outbreaks. For example, Frentzel et al. (2022) investigated *B. cereus* group isolates from 73 food products using WGS. Zhang et al. (2025) reported that *Bacillus paranthracis* (*B. paranthracis*) isolated from uncooked rice was responsible for outbreaks in China in 2024. Nguyen and Tallent (2019) used WGS to expand the repertoire of detectable virulence genes in *B. cereus* isolated from various dehydrated foods, including infant formula, white chocolate pancake mix, whey powder, mashed potato mix, and cooked rice. These studies demonstrated the WGS has been successfully used to provide broad genomic coverage and detect multiple pathogenicity determinants in *Bacillus* spp.

WGS has also been used to differentiate closely related strains in complex food matrices. Bogaerts et al. (2023) characterized *B. cereus* isolates from commercial vitamin B2 feed and food additives using WGS. Following bacterial cultivation, two isolates per sample were analyzed. Sequence typing (ST), virulence gene profiles, antimicrobial resistance (AMR) genes, plasmid content, and phylogenomic relationships were assessed. Interestingly, isolates from the same product were often genetically distinct, emphasizing the depth and resolution of WGS in strain differentiation. Similarly, Li et al. (2019) compared 16S rDNA sequencing and WGS for spore-forming bacteria in skim milk powder, showing that WGS could accurately differentiate closely related species, such as *Bacillus licheniformis* (*B. licheniformis*) and *Bacillus paralicheniformis* (*B. paralicheniformis*), whereas 16S rDNA sequencing could not.

The utility of WGS extends to outbreak investigation and surveillance. Carroll et al. (2022) has also been successfully utilized WGS to analyze 25 *Bacillus cereus sensu lato* (*B. cereus s. l.*) strains from various meat and poultry product across South Africa. The study demonstrated that WGS could detect minimal genetic differences between strains, with some differing by zero to three single nucleotide polymorphisms (SNPs). It provided a powerful tool for differentiating species within *Bacillus* spp. Similarly, WGS has also been reported to differentiate and identify members of the *B. cereus* group: *Bacillus cereus sensu stricto* (*B. cereus s. s.*), *Bacillus mosaicus* (*B. mosaicus*) and *Bacillus thuringiensis* (*B. thuringiensis*) isolates from ice cream (Fraccalvieri et al., 2022) and *B. cereus s. s.*, *B. mosaicus*, *Bacillus toyonensis* (*B. toyonensis*) and *Bacillus mycoides* (*B. mycoides*) isolates from ready-to-eat food products (Kowalska et al., 2024). Notably, Carter et al. (2018) used WGS to identify *B. cereus* strains isolated from dried food including powder infant formula, dietary supplements, dairy and medicated fish feed. In this study, 13 out of 32 strains did not match any known sequence types, uncovering novel sequence types not presented in existing database.

These findings emphasize the unique capability of WGS to differentiate between closely related species and strains, as well as enabling identification of novel genetic sequence, an advantage not achievable with conventional nucleic acid-based methods (PCR, LAMP, FISH, RPA). However, the high cost, equipment requirements, and need for advanced bioinformatics analysis currently limit the widespread application of WGS in routine or field-based diagnostics. As a result, culture-based methods and targeted PCR assays remain the primary approaches for rapid detection and gene profiling in outbreak investigations. The

Table 5
The applications of WGS-based methods used for identification of *Clostridium* spp. or *Bacillus* spp. from food samples

Strains	Methods	Genes	Analytical sensitivity		DNA isolation		Applied in food		References
			Spore	Vegetative	Spore	Vegetative	Food types	LODs	
C. estertheticum	WGS	Whole genome	-	-	-	phenol–chloroform method for whole genome, alkali lysis method for plasmid genome	-	-	(Yu et al., 2016)
C. perfringens	Culture, WGS and qPCR	Whole genome	-	-	-	DNA extraction kit	Isolates from vegetables, poultry, pork and beef	-	(Abdelrahim et al., 2019)
C. difficile	Culture, WGS and mPCR	Whole genome	-	-	-	DNA extraction kit	Isolates from pork	-	(Mooyottu et al., 2015)
C. perfringens	Culture, WGS and mPCR	Whole genome	-	-	-	DNA extraction kit	Isolates from chicken and pig meat	-	(Li et al., 2020)
C. botulinum	Culture and WGS	Whole genome				DNA extraction kit	Isolates from foods	-	(Weedmark et al., 2015)
B. cereus	Culture, WGS, multiplex qPCR	Whole genome	-	-	-	DNA extraction kit	Isolates from food products	-	(Frentzel et al., 2022)
B. cereus s. l.	Culture, WGS	Whole genome	-	-	-	DNA extraction kit	Isolates from meat and poultry product	-	(Carroll et al., 2022)
B. licheniformis	Culture, WGS, and 16S sequencing	Whole genome	-	-	-	DNA extraction kit	Isolates from medium-heat skim milk powder	-	(Li et al., 2019)
B. cereus	Culture and WGS	Whole genome	-	-	-	DNA extraction kit	Isolates from vitamin B2 additive products	-	(Bogaerts et al., 2023)
B. cereus	Culture and WGS	Whole genome	-	-	-	DNA extraction kit	Isolates from ice cream	-	(Fraccalvieri et al., 2022)
B. cereus	Culture and WGS	Whole genome	-	-	-	DNA extraction kit	Isolates from infant formula powder	-	(Carter et al., 2018)
B. paranthracis	Culture, WGS and qPCR	Whole genome	-	-	-	DNA extraction kit	Isolates from uncooked rice	-	(Zhang et al., 2025)
B. cereus	Culture and WGS	Whole genome	-	-	-	DNA extraction kit	Isolates from ready-to-eat food	-	(Kowalska et al., 2024)
B. cereus	WGS	Whole genome	-	-	-	DNA extraction kit	Samples were directly extracted from bacterial growth in dehydrated infant formula, pancake mix, whey powder, and potatoes, condensed gravy and cooked rice.	-	(Nguyen & Tallent, 2019)

Target strains, detection methods, target genes, analytical sensitivity, and DNA extraction approaches (from spores and vegetative cells using commercial kits or other methods) were summarized. Validation for detection in food samples was also included. '-' indicates not applied in the study.

applications of WGS-based methods for detecting *Clostridium* spp. and *Bacillus* spp. in food are summarized in Table 5.

2.7. Clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein (Cas) system

CRISPR/Cas system is part of the adaptive immune system in archaea and bacteria (Jinek et al., 2012). This system has been adapted as a highly specific tool for detecting foodborne pathogens in food (Li et al., 2023). Guided and recognized by custom-designed CRISPR RNA (crRNA), the endonuclease activity of the Cas protein is activated to cleave the DNA or RNA, enabling precise detection of genes of interests (Freije et al., 2019; Makarova et al., 2011).

CRISPR/Cas systems are classified into 2 classes and 6 types (Xu & Li, 2020). For diagnostic applications, most CRISPR/Cas platforms utilize class 2 systems, including CRISPR/Cas9 and CRISPR/Cas12a targeting dsDNA, CRISPR/Cas13a targeting single stranded RNA (ssRNA) (Verma et al., 2022; Wang et al., 2020). The trans-cleavage (collateral cleavage) activity of Cas12a and Cas13a has been exploited to develop CRISPR/Cas-based systems for advanced diagnostics in foodborne pathogens.

The CRISPR/Cas12a system has been adapted for the detection of foodborne *Clostridium* spp. and *Bacillus* spp. owing to several unique features. Firstly, the CRISPR/Cas12a system is directly activated by mature crRNA and does not require trans-activating RNA (tracrRNA) to participate in the synthesis of guide RNA (compared to CRISPR/Cas9 system). Secondly, the CRISPR/Cas12a binary complex effectively binds to the target dsDNA by recognizing the PAM with a 5'T-rich short sequence (TTTN). Thirdly, after binding to the target DNA, the non-specific collateral cleavage activity of Cas12a is activated, resulting in efficient cutting of non-specific ssDNA (Chen et al., 2018). These features of CRISPR/Cas12a have been used to develop diagnostic assays using different readouts including a fluorescence-based detection or LFS for simple visualization (Sahel et al., 2024). The basic principle of CRISPR/Cas12a-based detection is shown in Fig. 4A.

CRISPR/Cas13a system has also been extensively explored for RNA-based detection due to its unique collateral cleavage activity. Cas13a from *Leptotrichia wadei* (LwaCas13a), used in the CRISPR/Cas13a system does not require any PAM site for the binding of the target region, making it highly flexible to target any region in the target RNA (Abudayyeh et al., 2017). Upon recognition of the target RNA sequence through base-pairing with the complementary crRNA, the collateral-cleavage property of Cas13a is activated, leading to cleavage of non-target ssRNA, shown in Fig. 4B. In bacterial detection, RNA-based targets offer an advantage for identifying viable cells, as RNA is rapidly degraded in non-viable organisms. However, the abundance of the target RNA could be low, limiting the sensitivity of the assay. Alternatively, extracted DNA can be transcribed into RNA in vitro through an added reverse transcription step, enhancing sensitivity while maintaining compatibility with one-pot detection formats conducted at 35-40 °C (An et al., 2021).

Other strategies to enhance sensitivity and applicability in food safety testing, CRISPR/Cas assays are often combined with amplification methods. For example, coupling CRISPR/Cas12a or CRISPR/Cas13a with isothermal amplification methods such as LAMP and RPA, allows rapid amplification of target genes from low abundance bacterial DNA or RNA, making the system suitable for point of care detection (Huang

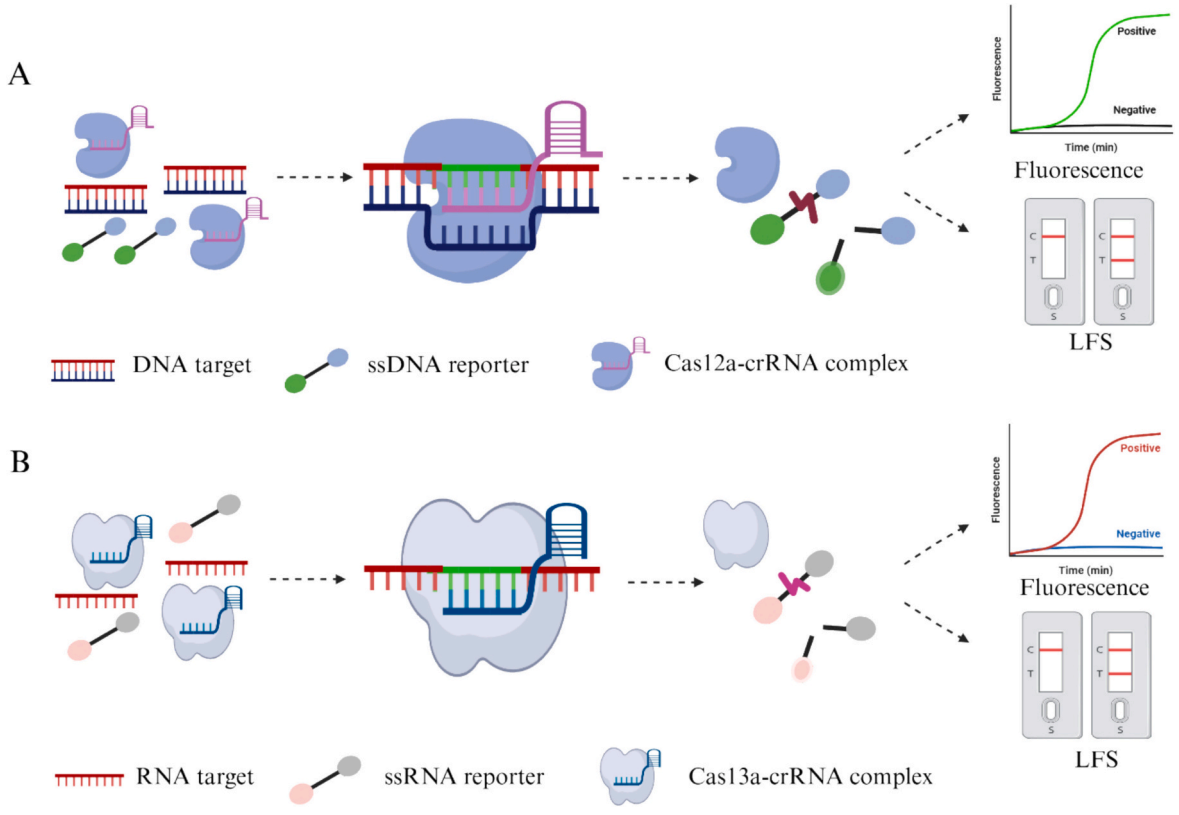

Fig. 4. The principle of CRISPR/Cas platforms. (A) CRISPR/Cas12a system: DNA target, ssDNA reporter probe and Cas12a-crRNA complex are incubated in the same reaction. The Cas12a-crRNA complex binds to the target sequence in dsDNA, which consists of 18-25 bp complementary sequence in the amplified product (highlighted in green). The trans-cleavage activity of Cas12a is then activated, cleaving the ssDNA reporter. The presence of the target DNA is subsequently detected using either fluorescence- or LFS-based methods. (B) RPA-CRISPR/Cas13a system: RNA target, ssRNA reporter probe and Cas13a-crRNA complex are incubated in the same reaction. The Cas13a-crRNA complex identifies the target sequence in ssRNA (highlighted in green), the trans-cleavage activity of Cas13a is then activated, cleaving the ssRNA reporter. The presence of the target RNA is subsequently detected using either fluorescence- or LFS-based methods. Since all components are incubated in the same tube for both Cas12a and Cas13a systems, these steps happen simultaneously.

et al., 2022; Kaminski et al., 2021). These adaptations have been used to detect species- and toxin-specific genes in foodborne spore-forming bacteria. CRISPR/Cas-based diagnostic systems combined with RPA are known as DNA endonuclease targeted CRISPR trans reporter (DETECTR) and Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) (Gootenberg et al., 2017). These systems have been adapted for bacterial diagnostics, however, their direct application to detecting *Clostridium* spp. or *Bacillus* spp. in food remains limited.

2.7.1. *Clostridium spp.*

CRISPR/Cas12a-based systems have been used to detect *Clostridium* spp., such as, targeting the *plc* gene of *C. perfringens* (Xiao et al., 2023), and the *tcdA* and *tcdB* genes of *C. difficile* (Jiang et al., 2023). However, these studies were validated in human or animal clinical samples rather than in food. These studies highlighted the potential of CRISPR/Cas12a as a programmable diagnostic platform. Recently, Wang et al. (2025) developed a multiplex RPA-CRISPR/Cas12a platform to detect six toxin-associated genes (*plc*, *cpb*, *etx*, *iap*, *cpe* and *netB*) of *C. perfringens*, enabling discrimination of the different toxin types. In this study, two three-plex RPA reactions amplified all six targets, and the resulting products were subsequently divided into separate reactions containing crRNAs complementary to each amplified region. Signals were detected via fluorescence signal or under UV. The assay achieved a LOD as low as 10 copies per target and was completed within 50 min. Validation in naturally contaminated chicken, beef and pork samples has been conducted (Wang et al., 2025). However, only plasmid DNA was used for assay development, genomic DNA from actual strains or food samples may differ, leaving the true detection limit uncertain. Furthermore, the multiplex detection with CRISPR/Cas12a remains constrained by the trans-cleavage activity of the activated Cas12a protein, which indiscriminately cuts all ssDNA reporters within a single reaction. Consequently, simultaneous detection of multiple targets still requires separate reactions to avoid cross-reactivity. Notably, CRISPR/Cas13a-based methods have yet to be applied for *Clostridium* spp. detection in food samples.

2.7.2. *Bacillus spp.*

Adaptation of CRISPR/Cas12a-based methods for detecting *Bacillus* spp. has also been validated in food. For instance, Meng et al. (2024) developed a one-tube RPA-assisted CRISPR/Cas12a assay targeting *cesB* gene specific to emetic *B. cereus*. The method achieved a detection limit of 10^2 CFU/mL of emetic *B. cereus* within 40 min and showed consistent performance across rice, milk and cooked meat samples. Li et al. (2024) developed six one-tube RPA-CRISPR/Cas12a assays targeting *nheA*, *nheB* and *nheC* for nonhemolytic enterotoxin, and *hblA*, *hblC* and *hblD* for hemolysin *B. cereus*. In one-tube assay, the RPA reaction was placed at the bottom of the tube, the CRISPR/Cas12a reaction in the inner side of the tube lid, and mineral oil covered the RPA reagents. Results were visualized via fluorescence and LFS assays, with detection limits of 10^1 to 10^2 CFU/mL of *B. cereus* in pure culture within 50 min using a portable device. The assays were further validated in spiked milk and rice samples. The detection of six enterotoxin genes contributed to the accuracy and reliability of the assay. These results indicate that RPA-CRISPR/Cas12a method enables rapid, user-friendly, and accurate detection of *B. cereus*, highlighting its applicability in the food industry.

Other approaches have combined WGS with RPA- or PCR-CRISPR/Cas12a assay to enhance target specificity. For example, Lyu et al. (2022) used WGS to identify SNPs specific to *B. anthracis* and Zhao et al. (2025) utilized genomic analysis to discover novel molecular markers for the *B. cereus s. l.*, which were subsequently used to develop an RPA-CRISPR/Cas12a assay. Genome sequences of *Bacillus* spp. were retrieved from database and analyzed to determine species-specific gene regions, which guided the design of primers for RPA- or PCR-based assays, ensuring reliable species identification. While these methods illustrated the potential of genomic informed CRISPR/Cas12a-based systems for the detection of *Bacillus* spp., most assays have yet to be validated in food samples. In addition, the PCR-based CRISPR/Cas12a methods remain less suitable for one-tube integration because of incompatible thermal requirements for and between PCR cycling and CRISPR/Cas12a activity. As a result, isothermal amplification methods, such as RPA and LAMP, are often preferred for integration with CRISPR/Cas systems due to their rapid, constant temperature operation and compatibility with portable diagnostic platforms.

One notable application of CRISPR/Cas13a for detecting *B. cereus* was developed by Zhang et al. (2021), who introduced a fluorescence-based method using a light-up RNA aptamer called Broccoli to detect RNA from *B. cereus*. In this method, the Broccoli RNA aptamer binds to the fluorophore DFHBI-1T (5'-difluoro-4-hydroxybenzylidene imidazolidinone, yellow), quenching its fluorescence and serves as the cleavage substrate for Cas13a. After recognition of the target RNA, the collateral activity of crRNA-Cas13a complex, is activated. This results in the cleavage of the Broccoli-DFHBI-1T complex, generating a detectable fluorescence signal. This assay achieved a sensitivity of 10 CFU of *B. cereus* after 24 h enrichment in spiked milk and rice samples without requiring preamplification or reverse transcription. This RNA-targeted approach offers advantages for selectively detecting viable cells, aligning with food safety needs where viable pathogens are of primary concern. However, some limitations remain. For instance, fluorescence intensity generated by CRISPR/Cas-based systems does not correlate linearly with initial target concentration, making accurate quantification difficult. Additionally, only a limited number of studies have investigated the use of CRISPR/Cas-based platform for detecting spore-forming bacteria. Thus, further research and validation are needed to establish the reliability, specificity, and practicality of this method in food safety settings.

Compared with other nucleic acid-based methods, CRISPR/Cas systems offer several advantages for spore-forming bacteria detection. Unlike PCR, CRISPR/Cas-based detection systems enable isothermal detection simplifies equipment needs. Compared to FISH and LAMP, CRISPR-based assays are generally more sensitive and specific, requiring only an 18-28 bp target sequence and are capable of distinguishing SNPs in the target. While RPA is an effective isothermal method, it lacks the ability to differentiate single-nucleotide differences, a key strength of CRISPR/Cas systems. Furthermore, in contrast to WGS, CRISPR/Cas-based methods do not require advanced bioinformatics analysis or expensive equipment, making them more suitable for rapid, on-site detection. CRISPR/Cas systems offer a highly adaptable and user-friendly platform that can be integrated with other nucleic acid amplification methods, such as RPA or LAMP, to enhance both sensitivity and specificity. The applications of CRISPR/Cas-based methods for detecting spore-forming bacteria in food are summarized in Table 6.

3. Conclusion and future perspectives

Spore-forming bacteria, particularly *Clostridium* spp. and *Bacillus* spp., are major contributors to food spoilage and foodborne illnesses due to their ability to form spores and survive extreme environmental conditions. Their presence in food poses both economic and public health concern (Adimpong et al., 2012; Scallan et al., 2011; Tirloni et al., 2022). Traditional culture-based and biochemical methods remain the gold standard for identification, but they are labor-intensive, time-consuming, and unsuitable for rapid, on-site diagnostics.

Nucleic acid-based detection methods have therefore been introduced for rapid, specific and sensitive identification and confirmation of spore-forming bacteria. PCR-, FISH-, LAMP-, RPA-, WGS-based assays have all been successfully applied to detect *Clostridium* spp. and *Bacillus* spp. in diverse food matrices, however, CRISPR/Cas-based systems still need further validation. These molecular tools not only reduce turnaround time but also enable gene-level differentiation, which is essential for distinguishing pathogenic from non-pathogenic strains.

Currently, plenty of opportunities and challenges exist in the nucleic acid based-detection of spore-forming bacteria in food. The structural

Table 6
The development and application of CRISPR/Cas-based systems to detect *Clostridium* spp. and *Bacillus* spp.

Strains	Methods	Genes	Analytical sensitivity		DNA isolation		Applied in food		References.
			Spore	Vegetative	Spore	Vegetative	Food types	LODs	
C. perfringens	Multiplex RPA-CRISPR/Cas12a	*cpa, cpb, iap, etx, cpe, netB*	-	1-10 copies	-	-	Chicken, pork, beef	-	(Wang et al., 2025)
B. anthracis	WGS + RPA-CRISPR/Cas12a	*CR5-2*, *CR5-1* and *Ba813*	-	5 aM	-	-	-	-	(Lyu et al., 2022)
B. cereus	RPA-CRISPR/Cas12a	*cesB*	-	0.01 ng/μL	-	DNA extraction kit	-	-	(Meng et al., 2024)
B. cereus	RPA-CRISPR/Cas12a	*cesB*	-	10^2 CFU/mL	-	Boiling method	Rice, milk and cooked meat	1 CFU/mL in rice, 10^2 CFU/mL in milk and meat	(Meng et al., 2024)
B. cereus s. l.	WGS + PCR-CRISPR/Cas12a	*NC2*, *ANN*, *NC3*, *group_4502*, *gabR*, *yloA* and *Group_8862*	-	10^3 CFU/mL	-	DNA extraction kit	-	-	(Zhao et al., 2025)
B. cereus	Light-up RNA aptamer CRISPR/Cas13a	*16S rRNA*	-	10 CFU	-	RNA extraction kit	Milk and rice	-	(Zhang et al., 2021)

Target strains, detection methods, target genes, analytical sensitivity, and DNA extraction approaches (from spores and vegetative cells using commercial kits or other methods) were summarized. Validation for detection in food samples was also included. '-' indicates not applied in the study.

complexity and resilience of spores limit DNA or RNA release, making efficient spore lysis a critical step. In addition, the presence of inhibitors such as fats, proteins, and polysaccharides in foods can impair amplification efficiency and assay sensitivity (Acharya et al., 2017; Moon et al., 2022; Sajali et al., 2018). Low bacterial abundance and uneven distribution within complex matrices further contribute to inconsistent detection. Genomic features, such as low GC content regions, repetitive sequences, and the need for multiplex amplification of toxin or virulence genes, also complicate primer and probe design (Galperin, 2013).

Performance differences are evident between genera and food types. *Clostridium* spp. as obligate anaerobes, often yield lower DNA recovery and less consistent amplification compared to *Bacillus* spp. Likewise, lipid- and protein-rich foods such as milk or meat tend to inhibit enzymatic reaction, whereas carbohydrate-rich matrices like rice or flour may interfere less but still pose extraction challenges. These differences underscore the need for method optimization tailored to both target species and food type. Summary of the advantages, limitations and technical challenges of nucleic acid-based detection methods for detecting *Clostridium* spp. and *Bacillus* spp. in food is provided in Table 7.

Therefore, advanced nucleic acid-based detection methods are essential to address these issues in identifying spore-forming bacteria in food. In particular, improving DNA extraction efficiency from resilient spores through optimized mechanical, chemical, enzymatic and germination-assisted lysis strategies will be critical to enhance assay sensitivity and reliability. Future studies should also focus on validation using real food samples and optimization across diverse food matrices to ensure reproducibility, scalability, and regulatory compliance within the food industry. Nucleic acid-based technologies remain a promising solution for *Clostridium* spp. and *Bacillus* spp., as they provides direct, sequence-specific identification of toxin and virulence genes that traditional culture-based methods cannot achieve. To enhance robustness and practicality, future work should integrate genomic insights and bioinformatics tools to refine target selection, assay design, and multiplex capability. WGS data can further support the development of more specific primers, probes, and crRNAs, improving accuracy and strain-level discrimination.

Table 7
Summary of nucleic acid-based detection methods for spore-forming bacteria in food: advantages and technical challenges.

Methods	Target genus	Food matrix	Cost	Detection Time	Field Detection	key advantages	Technical challenges
PCR	*Clostridium* spp./*Bacillus* spp.	Meat, milk seafood, vegetables and cheese	Moderate	2–4 h	Limited	High sensitivity and specificity, quantitative	Requires thermal cycler; PCR inhibitor
RPA	*Clostridium* spp./*Bacillus* spp.	Meat, milk, cream and baking soda	Moderate	~20–30 min	Excellent	Isothermal; rapid; portable; minimal equipment	Low GC/repetitive regions can affect amplification; cannot differentiate between two closely related strains;
FISH	*Clostridium* spp./*Bacillus* spp.	Beef, milk	High	Several hours	Poor	Visual confirmation of targets; no amplification needed	Lower sensitivity; expensive fluorescent probes; microscopy needed
LAMP	*Clostridium* spp./*Bacillus* spp.	Meat, milk, vegetables, seafood and condiments	Low–Moderate	~30–60 min	Good	Isothermal; rapid; simple setup; visual readouts possible	Complex primer design; limited multiplexing
WGS	*Clostridium* spp./*Bacillus* spp.	Meat, seafood, milk, ice cream, RTE, and rice	Very High	Days	No	Provides comprehensive genetic information; useful for strain typing	Expensive; time-consuming; requires bioinformatics
CRISPR/Cas-based methods	*Clostridium* spp./*Bacillus* spp.	Meat, milk, rice	Moderate	~30–60 min	Promising	Ultra-specific detection; programmable field deployment	Pre-amplification often needed; still being optimized for routine use

Integration of portable devices, such as microfluidics, biosensors and CRISPR/Cas systems, with nucleic acid-based methods will further expand in-field detection capability. Simplifying workflows, improving quantification, and incorporating automated sample processing and lyophilized reagents will make these tools more field-deployable and user-friendly. By addressing these challenges, CRISPR/Cas-based platforms coupled with genomic-driven assay design hold great potential for real-time, high specificity detection of spore-formers in complex food matrices.

Overall, integrating genomics, bioinformatics, and portable molecular detection technologies will be essential for overcoming current molecular and matrix-related barriers in the detection of *Clostridium* spp. and *Bacillus* spp. Continued progress toward sensitive, multiplexed, and ready-to-use nucleic acid-based assays will strengthen pathogen surveillance, enhance food safety management, and mitigate the public health and economic impacts of foodborne diseases.

CRediT authorship contribution statement

Chunyang Ma: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Nigel French:** Writing – review & editing, Supervision. **Xiyang Wu:** Writing – review & editing, Supervision. **Sandeep K. Gupta:** Writing – review & editing, Supervision, Resources. **Tanushree B. Gupta:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to improve language and readability, with caution. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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