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Development of fluorescence and colorimetric LAMP-based assays for rapid and sensitive detection of toxigenic *Bacillus cereus* group strains in plant-based products

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A B S T R A C T

Bacillus cereus represents a major concern for the microbiological safety of plant-based foods due to its ability to form heat-resistant spores and produce toxins associated with foodborne illness. Rapid, field-adapted tools for toxin gene detection are, therefore, needed to support surveillance and safety management. In this study, Loop-Mediated Isothermal Amplification (LAMP) assays targeting the emetic (*cesA*) and enterotoxigenic (*hblD*, *nheB*, *cytK*-1) genes of *B. cereus* were developed and their performance in plant-based products was evaluated.

LAMP primer sets using qPCR equipment (qLAMP) were assessed for sensitivity and specificity with a diverse strain panel. Analytical sensitivity was also tested in spiked plant-based matrices. Seventy-two commercial plant-based foods and supplements, including dairy, meat, and fish analogues, were screened by colorimetric assays, with inconclusive samples subsequently analysed via qLAMP.

No amplification occurred with non-*Bacillus* DNA. *hblD*, *nheB*, and *cytK*-1 assays showed 100% specificity to reference assignments, while *cesA* assay generated one apparent false positive. All assays successfully amplified target DNA in spiked matrices even at the lowest level of 10^1 spores × mL^{-1}. The colorimetric analysis of commercial products showed mostly clearly positive (yellow) or negative (red) results. Some intermediate (orange) signals were confirmed by qLAMP as very low-level positives, while others showed no amplification. Among detected genes, *nheB* was the most frequent, followed by *hblD*, *cytK*-1, and *cesA*.

Overall, LAMP assays provide rapid, sensitive, and specific detection of *B. cereus* toxin genes. The colorimetric method represents an effective first-line screening tool for plant-based foods, with qLAMP confirmation recommended for ambiguous results.

1. Introduction

Foodborne diseases continue to pose a significant global public health challenge, and plant-based products are not exempt from associated risks. Despite standard thermal processing, these products can be contaminated with bacterial pathogens, notably members of the *Bacillus cereus* group, which are recognized for their pathogenic potential and capacity to induce foodborne illness (World Health Organization [WHO], 2015). *B. cereus* is frequently associated with outbreaks due to its ability to form highly resilient spores and synthesize a broad spectrum of toxins responsible for both diarrheal and emetic syndromes (Dietrich et al., 2021; Ehling-Schulz et al., 2005, 2015; Fagerlund et al., 2008). *Bacillus* species are ubiquitous in the environment and particularly relevant in plant-derived products, given their natural association with soil and plants, and their capacity to survive standard thermal treatments through heat-resistant spores (Kyrylenko et al., 2023; Logan, 2012; Saxena et al., 2020). Several studies have documented the presence of *Bacillus* spp. in plant-based meat analogues (Bartula et al., 2023; Hai et al., 2024; Kyrylenko et al., 2023; Roch et al., 2024; Tóth et al., 2021); and plant-based beverages (Misiou et al., 2023), with

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approximately 5% of isolates identified as diarrheagenic *B. cereus* strains (Barmettler et al., 2025). Notably, a significant outbreak of *B. cereus* intoxication linked to oat-based plant milk was reported in 2022 (Rapid Alert System for Food and Feed [RASFF], 2022).

Among *B. cereus* virulence factors, the tripartite enterotoxins hemolysin BL (*hbl*) and non-hemolytic enterotoxin (*nhe*) disrupt host cell membranes through pore formation, leading to ionic imbalance and cytolysis (Dietrich et al., 2021; Fagerlund et al., 2008), while cytotoxin K (*cytK*), particularly the *cytK*-1 variant, exerts potent necrotic and cytotoxic effects (Castiaux et al., 2015; Guinebretière et al., 2006). The emetic toxin cereulide, encoded by the *ces* gene, is a heat- and acid-stable cyclic depsipeptide that interferes with mitochondrial function and triggers serotonin release in the gastrointestinal tract (Ehling-Schulz et al., 2005). Collectively, these toxins account for the principal pathogenic mechanisms of *B. cereus* in foodborne disease (Dietrich et al., 2021), highlighting the need for effective detection and control measures across diverse food products. While conventional microbiological methods are reliable, they are labor-intensive and time-consuming. Molecular techniques such as PCR and qPCR offer enhanced sensitivity and specificity; however, their reliance on thermocyclers, trained personnel, and complex workflows restricts their utility in field-based or resource-limited settings (Priyanka et al., 2016; Saravanan et al., 2021; Xiang et al., 2023).

Loop-mediated isothermal amplification (LAMP) has emerged as a rapid, robust, and user-friendly molecular diagnostic tool that enables nucleic acid amplification at a constant temperature, with results detectable through turbidity, colorimetric shifts, or fluorescence (Moon et al., 2022; Tanner et al., 2015). LAMP assays have been effectively applied to the detection of a wide range of pathogens, including *Bacillus anthracis*, *B. cereus*, and other toxin-producing or spoilage microorganisms in various food matrices (Busch et al., 2022; Lou et al., 2024; Zhang et al., 2019). However, despite notable advances in molecular diagnostics, only two previous studies have specifically addressed LAMP-based detection of *B. cereus* virulence-associated loci, namely *nheB* (Busch et al., 2022) and *ces*-related targets (Deng et al., 2019), and none of these studies investigated toxin genes in the emerging category of plant-based products.

Within this framework, the present study aimed to develop and analytically validate rapid LAMP-based assays targeting the principal emetic and enterotoxigenic determinants of the *B. cereus* group (*cesA*, *hblD*, *nheB*, and *cytK*-1), with specific application to plant-based food matrices. By addressing this expanding and heterogeneous product category, the work seeks to bridge an existing methodological gap in rapid detection strategies specifically adapted to non-traditional matrices. To ensure matrix representativeness and methodological robustness, a diverse panel of commercially available plant-based products (including milk alternatives, meat and fish-analogues, egg and cheese analogues, vegan pasta, and dietary supplements) was selected for method validation. A further objective was the development and optimization of a sensitive colorimetric detection format, enabling rapid, equipment-minimal result interpretation and facilitating on-site implementation by food business operators and control bodies. The innovative aspect of the proposed approach resides in the simultaneous targeting of multiple toxin-associated genes through specifically designed LAMP primer sets, expanding analytical coverage beyond conventional single-target assays and enabling a more comprehensive assessment of the toxigenic potential of the *B. cereus* group within a unified workflow.

2. Materials and methods

2.1. LAMP primers design and selection

To develop and validate LAMP assays for the detection of key *B. cereus* group toxin genes, new primer sets targeting *nheB*, *hblD*, *cytK*-1, and *cesA* were designed. The nucleotide sequences of the target genes *hblD*, *nheB*, *cesA*, and *cytK*-1 were retrieved from the National Center for Biotechnology Information (NCBI) database (genes IDs: 99619357, 9961802, 6382833 and 33896206 for *hblD*, *nheB*, *cytK*-1 and *cesA* genes, respectively). Each sequence was verified by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm gene identity, assess conservation across *B. cereus sensu lato* strains, and exclude significant homology with non-target organisms. Full-length gene sequences were then used as input for the free available software NEB® Primer Design Tool (New England Biolabs, https://www.neb.com/en/neb-primer-design-tools), which selected suitable conserved regions of approximately 200–300 bp. For each locus, six primers recognizing eight distinct regions were generated according to the standard LAMP architecture: primers F3/B3, FIP/BIP, LoopF/LoopB were created for all target genes using the quality parameters indicated in Table S1. Four thymine bases (TTTT) were added between F1c and F2, and between B1c and B2, in FIP and BIP primers respectively, as a linker to provide flexibility, prevent structural interference, and enhance the efficiency of the amplification reaction. Primer sequences were checked *in silico* for potential secondary structures and dimer formation (OligoAnalyzer) and were synthesized by Invitrogen™ (Thermo Fisher Scientific, Madrid, Spain).

The synthesized LAMP primer sets were then evaluated with quantitative loop-mediated isothermal amplification (qLAMP) fluorescence-based assays, using a QuantStudio™ 12 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Different concentrations (from 10 ng/μL to 1 fg/μL) of DNA from *B. thuringiensis* ATCC 10792^T for *hblD* and *nheB* genes, DNA from *B. cytotoxicus* DSM 22905^T for the *cytK*-1 gene, and DNA from *B. cereus* DSM 4312 for *cesA* gene were tested. These strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) and DNA extracted using the Master Pure Gram-Positive DNA purification kit (LGC Biosearch Technologies, USA).

2.2. Optimization of LAMP assays

Reaction parameters for the selected primer sets were optimized with the fluorescent assays on a QuantStudio™ 12 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), evaluating temperatures between 62 °C and 66 °C over a 30-min amplification period. The reactions were prepared according to the manufacturer's instructions in a total volume of 20 μL, consisting of 10 μL OptiGene Fast Master Mix ISO-004 (OptiGene, Horsham, UK), 0.3 μL of 50X CXR Reference Dye (Promega, Madison, WI, USA), 1 μL of 20 × LAMP primer mix (containing 16 μM FIP/BIP, 8 μM LF/LB, and 4 μM F3/B3, as recommended by the Master Mix manufacturer), 2 μL of template DNA, and 6.7 μL of nuclease-free water. Each condition was tested in triplicate across a range of DNA concentrations, from 10 ng/μL to 1 fg/μL.

2.3. Sensitivity and specificity assessment of LAMP reactions

The analytical sensitivity of the four targeted LAMP assays was evaluated using both fluorescence- and colorimetry-based detection. Genomic DNA templates were derived from *B. thuringiensis* ATCC 10792^T for the *hblD* and *nheB* genes, *B. cytotoxicus* DSM 22905^T for *cytK*-1, and *B. cereus* DSM 4312 for *cesA*. Serial tenfold dilutions of DNA were tested at the following concentrations from 10 ng/μL to 1 fg/μL. Fluorescence-based reactions were performed as described in Section 2.2. Once optimal conditions for the Real-Time assays were established, the same temperature and incubation time parameters were applied to the colorimetric assays to assess its sensitivity. The colorimetric LAMP reactions were performed in an Eppendorf Thermomixer® F1.5 (Eppendorf, Hamburg, Germany) using 1.5 mL tubes. Each 20 μL reaction contained 12.5 μL of WarmStart LAMP 2 × Master Mix (M1800; New England Biolabs; USA), 2.5 μL of 10X LAMP primer mix (16 μM FIP/BIP; 4 μM LF/LB and 2 μM F3/B3, according to Master Mix manufacturer's instructions), 2 μL of template DNA, and 8 μL of nuclease-free water.

Specificity was assessed using both Real-Time and colorimetric

assays on a panel of 45 bacterial strains (Table 1), including 27 *Bacillus* isolates (18 belonging to the *B. cereus* group) and 16 strains from other foodborne pathogenic genera. *Non-Bacillus* strains were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain), whereas foodborne *Bacillus* strains were sourced from the microbial collection of Università Cattolica del Sacro Cuore (Piacenza, Italy). All *B. cereus* group strains had been previously screened by end-point PCR for the presence of the four target genes, using primer sets and conditions described by Hansen and Hendriksen (2001) for *hblD* and *nheB*, Ehling-Schulz et al. (2005) for *cesA*, and Guinebretière et al. (2006) for *cytK*-1.

2.4. Artificial contamination of food matrices

The applicability of the LAMP assays for detecting *B. cereus* group toxin genes in food matrices was evaluated in artificially contaminated samples. Four products - a soymilk, a pea burger, a raspberry syrup and herbal pills - were first screened by Droplet Digital PCR (ddPCR) to quantify the presence of native *Bacillus* spp. in the products, as previously described (Bisaschi et al., 2025). Subsequently, the products were artificially contaminated with purified spore suspensions of five reference *Bacillus* strains to achieve final concentrations ranging from 1×10^1 to 1×10^5 spores $\times$ mL^{-1} (or g^{-1}) of products, following the methodology reported by Bisaschi et al. (2025). DNA was extracted from artificially contaminated products as reported by Bisaschi et al. (2025) and analysed using the qLAMP and the colorimetric LAMP assays for *hblD* and *nheB* genes, which were the only toxin genes present in the inoculated strains, under the same conditions outlined in Section 2.2.

Table 1
Bacterial strains used in this study for specificity tests.

Bacillus cereus group	Non-*Bacillus cereus* group	Non-*Bacillus* genera
Bacillus albus UC4402[a]	*Bacillus licheniformis* UC4420	*Enterococcus faecalis* CECT 481[b]
Bacillus albus UC4403	*Bacillus velezensis* UC4423	*Cryptococcus bhutanensis CECT 1085*
Bacillus proteolyticus UC4404	*Bacillus circulans* UC4424	*Streptococcus dysgalactiae* CECT 758
Bacillus thuringiensis UC4405	*Bacillus altitudinis* UC4425	*Escherichia coli* CECT 434
Bacillus albus UC4406	*Bacillus amyloliquefaciens* UC4426	*Staphylococcus epidermidis* CECT 231
Bacillus albus UC4407	*Bacillus megaterium* UC4427	*Salmonella enterica* subsp *enterica* CECT 4594
Bacillus cereus UC4408	*Bacillus agglomerans* UC4428	*Klebsiella pseumoniae* CECT 8453
Bacillus albus UC4409	*Bacillus subtilis* UC4429	*Staphylococcus aureus* subsp *aureus* CECT 59
Bacillus albus UC4410	*Bacillus vallismortis* UC4430	*Streptococcus agalactiae* CECT 183
Bacillus albus UC4411		*Clostridium celerecrescens* CECT 954
Bacillus cereus UC4412		*Listeria monocytogenes* CECT 934
Bacillus cereus UC4413		*Sagittula stellata* CECT 7782
Bacillus thuringiensis UC4414		*Klebsiella pneumoniae* subsp *pneumioniae* CECT 143
Bacillus cereus UC4415		*Klebsiella oxytoca* CECT 860
Bacillus cereus UC4416		*Klebsiella aerogenes* CECT 684
Bacillus cereus UC4417		*Klebsiella pneumoniae* CECT 142
Bacillus cereus UC4418		
Bacillus albus UC4419		
Bacillus thuringiensis UC4421		
Bacillus thuringiensis UC4422		

[a] UC = Microbial Culture Collection of the Università Cattolica del Sacro Cuore.
[b] CECT = Spanish Microbial culture collection.

2.5. Colorimetric analysis of plant-based products

The LAMP colorimetric assays were applied to a total of 72 commercially sourced plant-based products spanning multiple categories: 17 beverages, 17 meat and fish-analogues, 7 cheese and egg analogues, 4 ready-to-eat vegan pasta products, and 27 plant-derived food supplements (5 liquids and 22 solids). Table S2 summarizes the main characteristics of the products: in addition to belonging to different types of food, products were quite diverse in terms of composition (protein or plant source), heat treatment (*i.e.*, UHT, pasteurization) and formulation (*i.e.* pills, syrups, capsules, opercula). Total DNA was extracted from these products as previously explained (Bisaschi et al., 2025) and reaction conditions were the same as described in paragraph 2.2. For samples yielding ambiguous results in the colorimetric assays, qLAMP analysis was conducted as a confirmatory test, with the reaction time extended to 60 min to enable amplification of very low DNA concentrations.

3. Results

3.1. Primers selection

A total of 8 LAMP primer sets were designed, two sets for each target gene, and initially evaluated for amplification performance. Only three primer sets produced successful amplification with the target *Bacillus* strain DNA, characterized by strong fluorescence signal intensity, smooth sigmoidal amplification curves and single, sharp melt curve peaks. These features indicated both efficiency and assay reliability, making the corresponding primer sets suitable for subsequent experiments. The three effective sets (listed in Table 2), targeting *hblD*, *cytK*-1 and *cesA*, were therefore adopted in this study. In addition, a previously published LAMP primer set targeting *nheB* (Busch et al., 2022) was incorporated. Table 2 summarizes the LAMP primer sets employed in this work.

3.2. Optimization and sensitivity of LAMP reaction conditions

For the four selected primer sets, reaction conditions were optimized by assessing amplification temperatures over a gradient ranging from 62 °C to 66 °C, using 1 °C increments between trials. All assays were performed in triplicate across a dilution series of template DNA ranging from 10 ng/μL to 1 fg/μL. Detection time, amplification sensitivity, and melting curve profiles were assessed for each reaction. Although amplification curves were exported by the instrument software as acquisition cycles, each cycle corresponded to a 30 s acquisition interval under isothermal conditions. Therefore, Fig. 1 shows the time to threshold (T_t), defined as the time point at which fluorescence exceeded the threshold during the qLAMP reaction (Garrido-Maestu et al., 2018), expressed as acquisition cycles (30 s per cycle) obtained at each temperature for the corresponding DNA concentrations. T_t values decreased progressively with increasing DNA concentration for all toxin genes assays, reflecting the expected inverse relationship between template amount and amplification time (with the exception of the *hblD* and *cesA* assays at 10 ng/μL, where the reaction time was slightly longer than at 1 ng/μL). Overall, robust amplification was observed across the 60–66 °C temperature range, with only minor T_t values fluctuations at intermediate and high template concentrations (≥0.01 ng/μL). The *cytK*-1 assay exhibited a broader T_t distribution and greater variability at the lowest DNA concentrations (≤0.0001 ng/μL), indicating reduced amplification efficiency or higher temperature sensitivity. Conversely, *cesA* reactions were consistently faster, producing the lowest T_t values among the four assays at moderate template levels (0.01 – 0.1 ng/μL), and displaying stable amplification performance across all temperatures tested.

Table 2
List of primer sets used in this study.

Target gene	Primer name	Primer type	Sequence (5′→3′)	Reference
hblD	hblD-F3	Forward outer (F3)	TCCAGATGGGAAAGGTGG	This study
	hblD-B3	Backward outer (B3)	TACCGCCAGTTACAACAA	
	hblD-FIP	Forward inner (FIP)	CGAAGTTGCTCAATTTCAGCTTGAATTTTTTAACTGCAATATTAGCAGGTCA	
	hblD-BIP	Backward inner (BIP)	TGATGTATTAGCATGGTCAATTGGTTTTTTAACTACCGCTCCTCCAA	
	hblD-LF	Loop forward (LF)	GTTGTGGGATCGTTGCCTGT	
	hblD-LB	Loop backward (LB)	TGGATTGGGAGCAGCTATTTTAGTT	
nheB	nheB-F3	Forward outer (F3)	CTATTATGATACTTTAGTTGCTGC	Busch et al. (2022)
	nheB-B3	Backward outer (B3)	CGTTGTAATTTGATTTTGCAGAAG	
	nheB-FIP	Forward inner (FIP)	CTGATCCACTTGCGCTTTATTTTCATAAAGCGACTCTTACGAAAGG	
	nheB-BIP	Backward inner (BIP)	CCGAAATAAAATGACTTCGGATACGTCCTGCATCTTGACTAGC	
	nheB-LF	Loop forward (LF)	CTACTTGATAATCTTGTTAAG	
	nheB-LB	Loop backward (LB)	CAAAACTTCAAGGGTGAT	
cytK-1	cytK-F3	Forward outer (F3)	GCGGTTATTAATACAACAGGTAG	This study
	cytK-B3	Backward outer (B3)	GTCGTTTCTTCCACTGCA	
	cytK-FIP	Forward inner (FIP)	TTGCCCCTGGAATTGGATATCCTTTTTATGAAAGCAAACCCAACTC	
	cytK-BIP	Backward inner (BIP)	CACATTGCGCTATCCATCACAATATTTTTTTGTCGGTGCTACATGA	
	cytK-LF	Loop forward (LF)	CAGGTGCGTCACTAA	
	cytK-LB	Loop backward (LB)	CAAGATAATACGTCGCGATTCT	
cesA	cesA-F3	Forward outer (F3)	CATCACATTTCAATAGACGGTT	This study
	cesA-B3	Backward outer (B3)	TGTCTTACCTAATAAACTAGTTGG	
	cesA-FIP	Forward inner (FIP)	CCTGCATCAGGTAATGCTACTTTTCTTTTGGAGTATTTTCTTAGTGCTTAGC	
	cesA-BIP	Backward inner (BIP)	TCGTTCATTGAGTGGACAAATGATTTTGCTGTAAAACCTTTCATATACTCTT	
	cesA-LF	Loop forward (LF)	CAGTTGCTTATATGCTGCCACAAC	
	cesA-LB	Loop backward (LB)	ATCAAGCCAAGGCAAAGGCG	

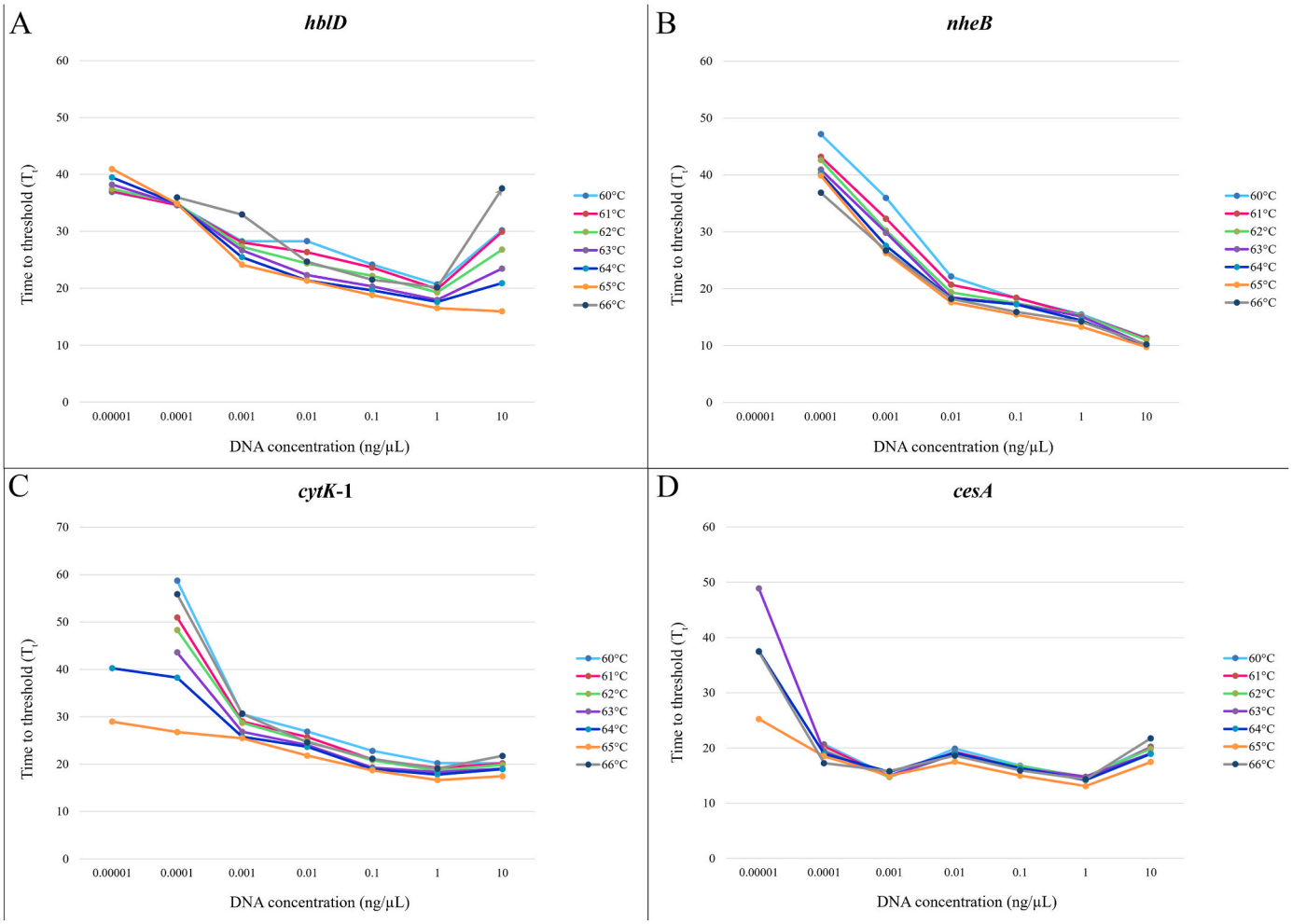

Fig. 1. Time to threshold (T_t), expressed as number of acquisition cycles (1 cycle = 30 s) as a function of template DNA concentration and amplification temperature for the four qLAMP assays targeting *hblD*, *nheB*, *cytK*-1, and *cesA* genes in the *B. cereus* group.

The shortest detection times were observed at 64 °C, 65 °C and 66 °C. At 64 °C, the *hblD*, *cytK*-1 and *cesA* assays produced the earliest amplification signals, while the *nheB* assay showed optimal detection times at 65 °C and 66 °C. No significant differences in specificity were detected between 64 °C and 65 °C. However, at 66 °C, amplification failed at the 10 fg/μL level for *hblD* and *cytK*-1, while at 60–62 °C no amplification was detected at 10 fg/μL for *nheB* and *cesA*.

Based on detection time, sensitivity and melt curve profiles (Table S3) across the tested temperatures, 65 °C was selected as the optimal reaction temperature for all four primer sets. Under these conditions, the *hblD*, *cesA*, and *cytK*-1 assays consistently detected template concentrations as low as 10 fg/μL. In contrast, the *nheB* assay did not amplify at 10 fg/μL, and amplification at 0.1 pg/μL was observed in only two out of three replicates. Therefore, the limit of detection for *nheB* was defined between 0.1 and 1 pg/μL.

Fig. 2 presents representative amplification curves (one out of three replicates is reported), obtained at 65 °C for the four assays, using DNA extracted from target *Bacillus* strains (*B. thuringiensis* ATCC 10792T for *hblD* and *nheB*, *B. cytotoxicus* DSM 22905^T for *cytK*-1, and *B. cereus* DSM 4312 for *cesA*) across the full dilution series (10 ng/μL to 1 fg/μL). As shown in Fig. 2, T_t values (expressed as number of acquisition cycles, 30 s each) increased progressively from 1 ng/μL down to 1 fg/μL; however, the highest concentration tested (10 ng/μL) did not conform to this pattern for any assay, exhibiting delayed amplification for *hblD* assay and flatter curves for *cytK*-1 and *cesA*, indicative of reduced reaction efficiency at elevated template loads. Additionally, amplification curves at higher DNA concentrations were highly reproducible across the three replicates, whereas lower concentrations resulted in increasingly heterogeneous curve profiles.

The colorimetric results of the LAMP assays targeting *hblD*, *nheB*, *cytK*-1, and *cesA* genes are presented in Fig. 3. Each assay was performed using a ten-fold serial dilution of *B. cereus* group DNA (from 10 ng/μL to 1 fg/μL). A clear color change from pink (negative) to yellow (positive) indicated successful amplification, whereas intermediate orange tones were classified as "suspect" results (de Oliveira Coelho et al., 2021; Tanner et al., 2015).

Positive amplification (yellow) was observed down to the 0.1 pg/μL sample for the *hblD* and *cytK*-1 assays, and down to the 1 pg/μL dilution for the *nheB* and *cesA* assays, corresponding to their analytical sensitivity limits. At the 10 fg/μL dilution (and at 0.1 pg/μL for the *nheB* and *cesA* assays) tubes of all genes displayed an orange coloration, interpreted as "suspect" results, likely resulting from borderline amplification near the detection threshold. No color change was detected at the highest dilution (1 fg/μL), confirming reaction specificity and the absence of false positives.

3.3. Specificity of LAMP assays

A total of 45 bacterial strains were tested to evaluate the specificity of the four primer sets, including 20 strains belonging to the *B. cereus* group. Specificity was evaluated using both fluorescence-based (qLAMP) and colorimetric detection methods. Overall, no amplification was observed with any of the primer sets when tested against non-*Bacillus* strains (data not shown). qLAMP results for the four genes tested with *Bacillus* strains are reported in Fig. 4. The *hblD* primer set demonstrated 100% specificity, successfully amplifying all *B. cereus* isolates carrying the target gene while showing no amplification in non-*B. cereus* group strains (Fig. 4A). Likewise, the *nheB* toxin gene was detected in all positive strains and was absent in all negative ones (Fig. 4B). The *cytK*-1 assay demonstrated complete specificity across both positive and negative strains (Fig. 4C). In contrast, the *cesA* assay yielded one false positive result (a strain previously confirmed as negative by conventional PCR, according to the protocol described by Ehling-Schulz et al., 2005), although it still exhibited 100% sensitivity for *cesA*-positive isolates (Fig. 4D).

Colorimetric assays corroborated these results, displaying the same specificity pattern: 100% specificity for *hblD*, *nheB*, and *cytK*-1, and again a single false positive for *cesA*, which according to conventional end-point PCR, should not harbour the gene (data not shown).

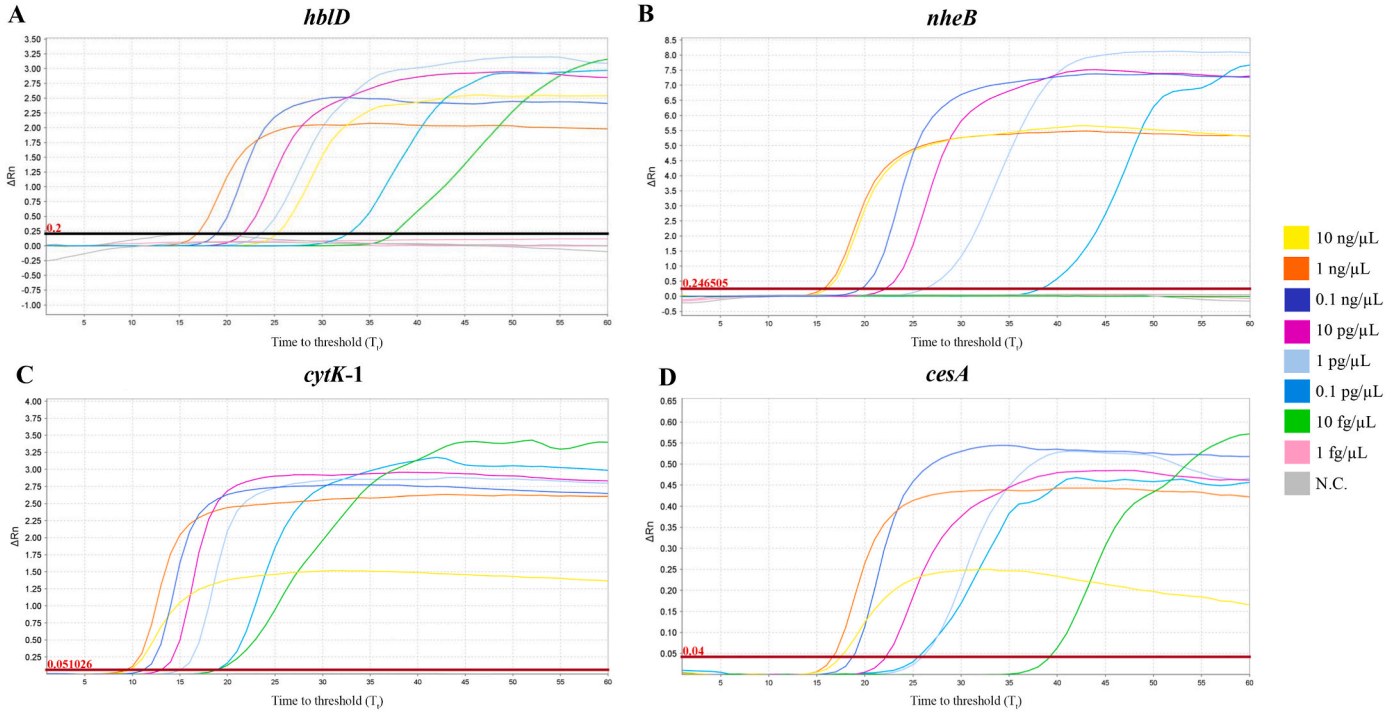

Fig. 2. Representative amplification curves for the four LAMP assays targeting *hblD* (Fig. 2A), *nheB* (Fig. 2B), *cytK*-1 (Fig. 2C) and *cesA* genes (Fig. 2D), at 65 °C for 30 min. X-axis reports time to threshold (T_t) values, expressed as acquisition cycles (1 cycle = 30 s).

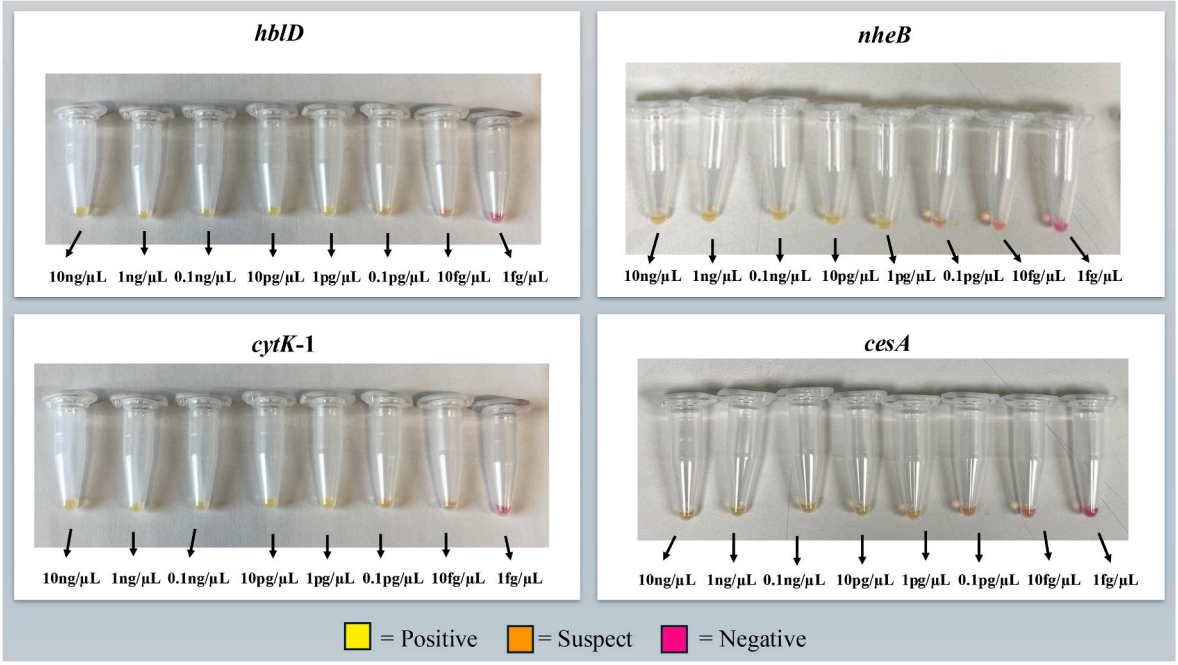

Fig. 3. Colorimetric LAMP assays outcomes for the *hblD, nheB*, *cytK*-1, and *cesA* genes. Tubes contain ten-fold serial dilutions of *B. cereus* group DNA from 10 ng/µL to 1 fg/µL). Positive reactions are indicated by a yellow color, suspect reactions by orange, and negative reactions by pink. The observed color change confirms gene-specific amplification and is consistent with the detection limits determined in quantitative assays. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Analysis of artificially contaminated products

Tests on artificially contaminated samples, spiked with purified spore suspensions, were conducted to evaluate primer efficiency and sensitivity of detecting *Bacillus* DNA extracted solely from spores, as well as the potential impact of sample matrices. For these experiments, 10 mL (or g) of a plant-based beverage, a plant-based burger, herbal tablets, and raspberry syrup were spiked to a final level of contamination from 10^1 spores × mL^{-1} (or g^{-1}) to 10^5 spores × mL^{-1} (or g^{-1}). Analysis with qLAMP amplification showed that T_t values were quite higher than in amplification of DNA from pure cultures: for this reason, reaction time was extended to 40 min (80 acquisition cycles, 30 s each). Table 3 reports average T_t values and melting temperature for the four products for *hblD* and *nheB* genes. The qLAMP analysis confirmed successful amplification at all five contamination levels from 10^1 to 10^5 spores × mL^{-1} (or g^{-1}) for both the *hblD* and *nheB* genes across all four food products. A clear inverse correlation was observed between spore concentration and T_t values for both targets in every matrix tested. As spore levels increased from 10^1 to 10^5 spores × mL^{-1} (or g^{-1}), T_t values decreased accordingly, reflecting the higher abundance of the target DNA. Melting temperature (T_m) values were consistent across all matrices, further confirming amplification specificity: *hblD* exhibited Tm values between 83.4 °C and 87.6 °C, while *nheB* ranged from 82.3 °C to 83.1 °C. Positive controls showed low T_t values and uniform melting profiles, while negative controls produced no amplification, confirming the absence of contamination and supporting the overall specificity of the assay.

Colorimetric analysis confirmed these results, showing clearly positive (yellow) reactions for both *hblD* and *nheB* across the contamination range from 10^3 to 10^5 spores × mL^{-1}. At lower contamination levels, the *hblD* assay yielded an intermediate (orange) coloration at 10^1 spores × mL^{-1} and a yellow at 10^2 spores × mL^{-1}, while the *nheB* assay produced intermediate (orange) signals at both 10^1 and 10^2 spores × mL^{-1} (data not shown).

3.5. Assessment of B. cereus toxin genes presence in commercial plant-based products

Following primer design, validation, and evaluation using artificially contaminated matrices, the colorimetric LAMP assays were employed to analyze 72 commercial plant-based products. Each sample was subjected to amplification with the four primer sets targeting *hblD*, *nheB*, *cytK*-1, and *cesA* genes according to the established colorimetric protocol. The outcomes of this screening are presented in Fig. 5.

Of the 72 plant-based products analysed, 30 samples tested positive for at least one virulence gene (3 beverages, 11 meat and fish-analogues, 3 cheese analogues, 2 vegan pasta and 11 supplements). Among these, the *nheB* locus exhibited the highest detection rate, with 18% of samples yielding clear positives (yellow colour change) and an additional 33% classified as suspicious. In contrast, *cesA* was detected least frequently, with 4% positives and 24% suspicious samples. Specifically, *hblD* was detected in 1 beverage, 3 meat analogues, and 5 dietary supplements; *nheB* was identified in 2 beverages, 4 meat and fish-analogues, 1 cheese analogue, and 5 supplements; *cytK*-1 was positive in 1 beverage, 3 meat analogues, and 1 supplement; whereas *cesA* was found in 2 meat analogues and 1 supplement. Across all four loci, a considerable proportion of samples generated suspicious signals (orange colour, indicative of partial colour change), particularly for *cesA* (24%) and *cytK*-1 (22%). When the results were stratified by product category, ambiguous colorimetric outcomes were not evenly distributed but occurred more frequently in certain matrices. Plant-derived dietary supplements—especially herbal infusions—accounted for the highest proportion of suspicious signals, representing 63% of the total supplements tested, compared with 51% of plant-based food products. Within the latter group, meat analogues exhibited the largest relative share of ambiguous results. Notably, most plant-based products showing suspicious colorimetric shifts were formulated with soy protein.

All samples producing orange signals were subsequently subjected to confirmatory fluorescence-based qLAMP. In this case, qLAMP reaction time was extended to 60 min (120 acquisition cycles, 30 s each) to enable amplification even from samples with the lowest DNA

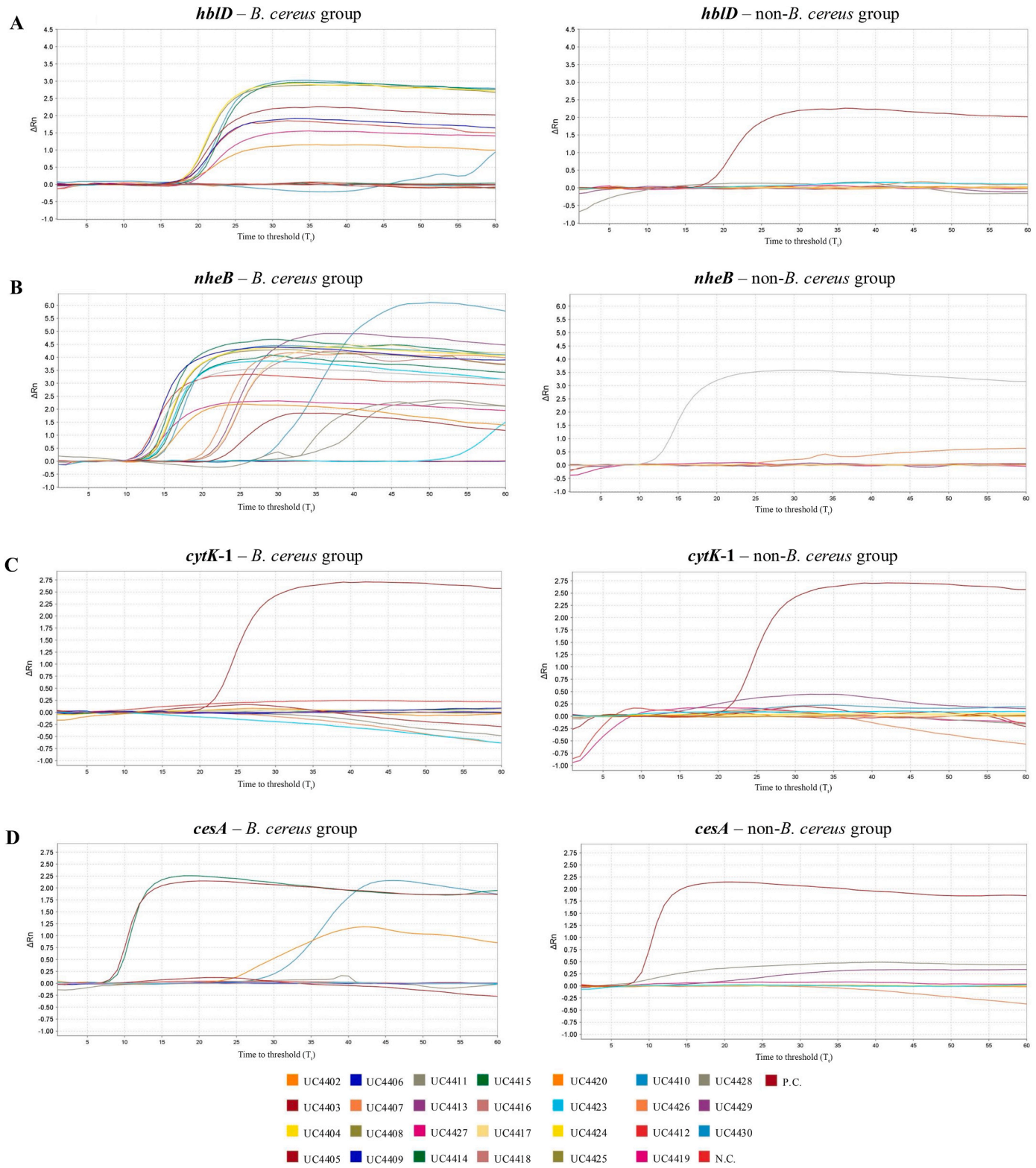

Fig. 4. Specificity tests of the LAMP assays though qLAMP, targeting *hblD* (Fig. 4A), *nheB* (Fig. 4B), *cytK*-1 (Fig. 4C) and *cesA* (Fig. 4D). Each assay was tested using a panel of 45 bacterial strains, including 27 *Bacillus* spp. isolates (18 of which belonging to *B. cereus* group) and 18 non-*Bacillus* controls. Amplification occurred exclusively in strains known to carry the corresponding toxin gene, except for the *cesA* assay, which yielded one false positive relative to conventional PCR. P.C.: positive control; N.C.: negative control. X-axis reports time to threshold (T_t) values, expressed as acquisition cycles (1 cycle = 30 s).

concentrations. Among these, a subset—ranging from 10% to 42% of the tested samples, depending on the target gene—produced amplification curves consistent with the presence of the respective genes, whereas the remaining samples showed no amplification and were classified as negative. Table 4 summarizes the colorimetric analysis results for all 72 products, including the re-analysis of suspicious orange samples by qLAMP.

A concise overview of the colorimetric results, along with the qLAMP

Table 3
Time to threshold (T_t) values and melting temperatures (T_m) obtained by qLAMP analysis of artificially contaminated plant-based products spiked with *B. cereus* spores at five contamination levels (10^1–10^5 spores × mL^{-1} or g^{-1}), for the *hblD* and *nheB* targets across four matrices (soy burger, soy milk, raspberry syrup, and herbal pills).

Spiked sample ID	*hblD*		*nheB*	
	Time to threshold (min)	Melting temperature (°C)	Time to threshold (min)	Melting temperature (°C)
Soy burger (10^1 spores × g^{-1})	23.5	83.5	17.1	82.9
Soy burger (10^2 spores × g^{-1})	19.7	83.8	15.3	82.9
Soy burger (10^3 spores × g^{-1})	17.5	83.9	13.7	83.1
Soy burger (10^4 spores × g^{-1})	14.4	83.8	12.5	83.0
Soy burger (10^5 spores × g^{-1})	11.9	83.7	11.2	82.9
Soy milk (10^1 spores × mL^{-1})	21.8	83.7	18.4	82.9
Soy milk (10^2 spores × mL^{-1})	21.2	84.3	16.7	82.8
Soy milk (10^3 spores × mL^{-1})	18.2	83.7	13.9	82.7
Soy milk (10^4 spores × mL^{-1})	14.7	83.6	12.7	82.3
Soy milk (10^5 spores × mL^{-1})	11.6	83.6	11.2	82.8
Raspberry syrup (10^1 spores × mL^{-1})	27.1	83.6	17.4	82.6
Raspberry syrup (10^2 spores × mL^{-1})	23.6	83.4	16.5	82.8
Raspberry syrup (10^3 spores × mL^{-1})	17.5	83.6	14.0	82.8
Raspberry syrup (10^4 spores × mL^{-1})	14.4	83.6	12.5	82.9
Raspberry syrup (10^5 spores × mL^{-1})	12.4	83.8	11.3	83.1
Herbal pills (10^1 spores × g^{-1})	27.2	84.6	16.8	83.1
Herbal pills (10^2 spores × g^{-1})	23.2	83.6	14.6	82.8
Herbal pills (10^3 spores × g^{-1})	17.1	83.7	14.25	82.7
Herbal pills (10^4 spores × g^{-1})	14.4	83.6	13.4	82.9
Herbal pills (10^5 spores × g^{-1})	11.7	83.6	11.5	82.9
Positive control	6.8	83.7	10.4	82.8
Negative control	No T_t	79.9	No T_t	80.1

amplification outcomes for "suspect" samples, is provided in Table S3. Among all tested products, 17 samples (1 beverage, 7 meat and fish-analogues, 2 cheese analogues, 2 vegan pasta and 5 supplements) showed positivity for a single target gene, most frequently *nheB,* while 9 products (2 beverages, 3 meat analogues, 1 cheese analogue and 3 supplements) carried two virulence-associated loci. A smaller subset of 3 samples was positive for three genes (2 meat analogues and 1 supplement). Finally, one product ("Chicken_fillets_pea") harboured all four target genes, representing the highest detected virulence gene load within the dataset.

4. Discussion

Given the increasing reports of *B. cereus* contamination in plant-based food products (Barmettler et al., 2025; Bartula et al., 2023; Hai et al., 2024; Pigłowski & Niewczas-Dobrowolska, 2024; Roch et al., 2024; Tóth et al., 2021), there is a growing need for reliable and rapid tools capable not only of detecting the presence of this microorganism but also of assessing its toxigenic potential. As plant-based products gain prevalence in the global food market, ensuring their microbiological safety requires methods that allow early detection of virulence factors such as enterotoxins and emetic toxins, thereby supporting more effective risk assessment and surveillance strategies (Gao et al., 2018; Kim et al., 2009; Moon et al., 2022).

In this study, we designed and validated four LAMP primer sets targeting key virulence genes of the *B. cereus* group (*cesA, hblD, nheB,* and *cytK*-1) and evaluated their performance on a diverse panel of *Bacillus* and non-*Bacillus* strains.

Regarding sensitivity, all four assays reliably detected DNA from reference pure cultures, in line with the high analytical sensitivity previously reported for LAMP (Thoraneenitiyan et al., 2022; Xiang et al., 2023; Zhang et al., 2019). Specifically, the detection limits were 10 fg/μL per reaction for *hblD*, *cesA*, and *cytK*-1. For the *nheB* assay, amplification was detected in two out of three replicates at 0.1 pg/μL, while all three replicates tested positive at 1 pg/μL, indicating a limit of detection between these two concentrations, consistent with previously reported results (Busch et al., 2022).

Notably, the highest DNA concentration tested (10 ng/μL) did not produce earlier amplification compared with lower concentrations; instead, it resulted in delayed amplification (in the *hblD* assay) and flattened curves (in *cesA* and *cytK*-1 assays). This pattern likely reflects inhibition phenomena commonly reported in nucleic acid amplification assays, where excess template or co-extracted compounds can interfere with primer annealing or polymerase activity, ultimately reducing amplification efficiency. Such inhibitory effects, including those related to reaction chemistry and template load, have been documented in LAMP assays (Sakatoku et al., 2024), as well as in PCR systems more broadly (Latham et al., 2023; Moon et al., 2022).

In spiked plant-based samples, the assays consistently achieved amplification of the target genes across all contamination levels, from 10^1 to 10^5 spores × mL^{-1}, confirming their applicability to practical food testing. The slight delay in T_t values observed across all products, compared with pure culture DNA, is likely attributable to inhibitory compounds naturally present in complex food matrices (de Oliveira Coelho et al., 2021; Moon et al., 2022; Saravanan et al., 2021). These

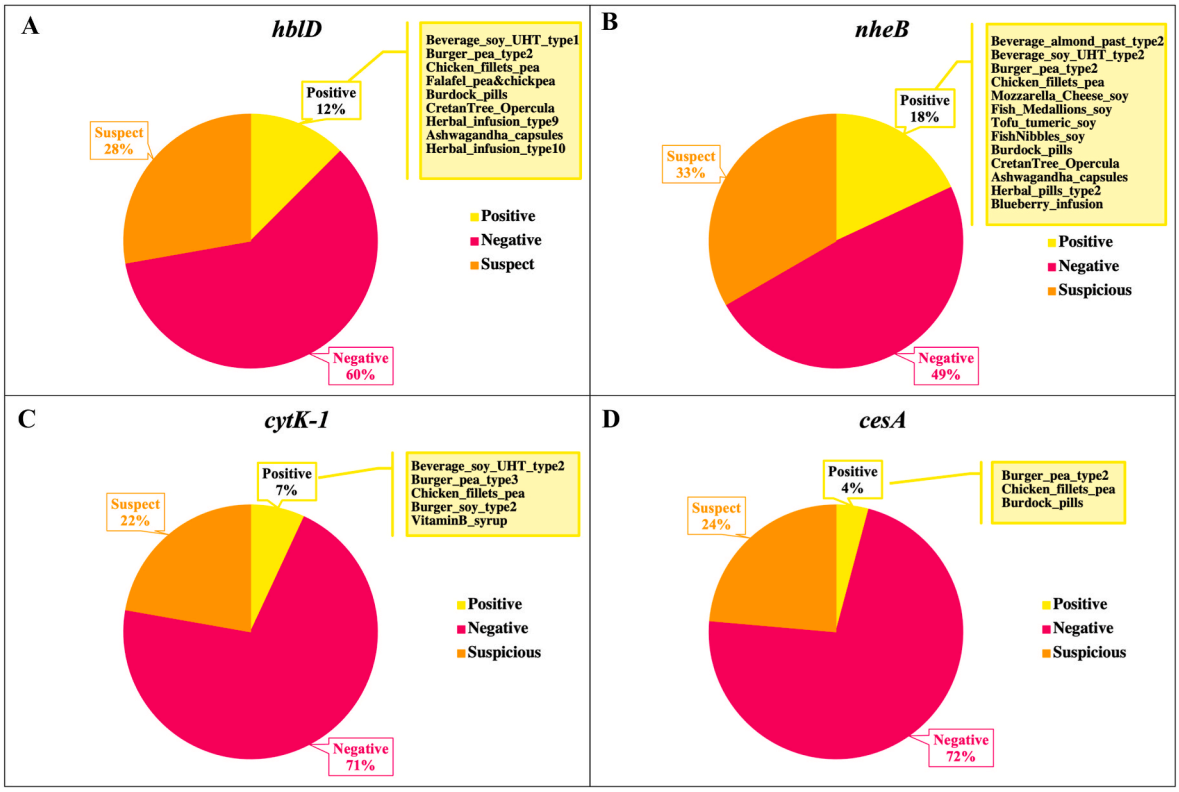

Fig. 5. Results of the colorimetric analysis of commercial plant-based samples for the *hblD* (Fig. 5A), *nheB* (Fig. 5B), *cytK*-1 (Fig. 5C), and *cesA* (Fig. 5D) genes.

Table 4
Colorimetric LAMP assay results for 72 commercial plant-based products and follow-up qLAMP analysis results of samples exhibiting suspicious signals.

Gene	Positive (%)	Negative (%)	Suspicious (%)	Suspicious confirmed by qLAMP (%)	Total confirmed positives (%)
hblD	12% (9)	60% (43)	28% (20)	10% (2 out of 20)	**15%** (11 out of 72)
nheB	18% (13)	49% (35)	33% (24)	42% (10 out of 24)	**32%** (23 out of 72)
cytK-1	7% (5)	71% (51)	22% (16)	25% (4 out of 16)	**13%** (9 out of 72)
cesA	4% (3)	72% (52)	24% (17)	12% (2 out of 17)	**7%** (5 out of 72)

matrix effects, which can interfere with primer annealing and polymerase function, underscore the importance of considering reaction kinetics and justify the need for extended amplification times to ensure reliable detection at the lowest DNA concentrations (Moon et al., 2022). Differences between analytical sensitivity and observed T_t values among the *hblD* and *nheB* assays are likely attributable to primer binding efficiency and reaction kinetics, as well as matrix-dependent DNA extraction efficiency, rather than to intrinsic limitations of assay performance (Bustin et al., 2009; Moon et al., 2022).

As for primers specificity, all primer sets delivered good analytical performance. No amplification was detected across the non-*Bacillus* panel, demonstrating high genus-level exclusivity. Within *Bacillus*, the *hblD, nheB,* and *cytK*-1 assays showed complete concordance with reference assignments (as obtained with conventional end-point PCR) in both fluorescence (qLAMP) and colorimetric chemistries, indicating robust target specificity and operational suitability for screening applications. The *cesA* assay represented the only notable deviation, yielding one false-positive result relative to the end-point PCR reference. Several mechanisms may plausibly explain this observation. The observed apparent false-positive result may be attributable to the inherently higher analytical sensitivity of LAMP compared with conventional end-point PCR, as previously noted by Bustin et al. (2009). LAMP assays can achieve lower detection limits due to their multi-primer design and strand-displacement amplification mechanism (Hardinge & Murray, 2019; Tanner et al., 2015). Additionally, the potential for primer cross-reactivity should be considered, as conserved sequence regions shared among closely related members of the *B. cereus sensu lato* group may promote non-specific primer annealing during LAMP amplification. Furthermore, *ces* is located on a plasmid-borne non-ribosomal peptide synthetase (NRPS) cluster containing modules with homology to other NRPS loci within the *B. cereus* sensu lato group (Castiaux et al., 2015; Ehling-Schulz et al., 2004), and partial sequence similarity may permit off-target priming in the LAMP reaction. Lastly, the multi-primer architecture of LAMP (six primers executing eight binding events) can occasionally tolerate mismatches and generate spurious late-stage amplification under permissive conditions (Hardinge & Murray, 2019; Novi et al., 2025). Two possible strategies to improve the assay accuracy involves re-targeting *ces* primers to a more discriminative sequence region (e.g., an inter-module junction or *cesB*), and/or adopting probe-based LAMP chemistries (e.g., strand-displacement probes or quenched-primer systems) to increase sequence stringency and mitigate late non-specific amplification.

Colorimetric LAMP analyses of commercial plant-based products confirmed, consistent with our previous findings (Bisaschi et al., 2025), the substantial and recurrent presence of *B. cereus* group contamination in plant-based products. Among the four loci investigated, *nheB* exhibited the highest prevalence (32% confirmed positives), followed by *hblD* (15%), *cytK*-1 (13%), and the comparatively low frequency of *cesA* (7%). These findings align with previous surveys of food products, including plant-based matrices such as rice, soy, and cereals, in which *nhe* and *hbl* represent the prevalent virulence determinants, while *ces* genes are detected infrequently (Busch et al., 2022; Oliveira et al., 2023). Comparison with our earlier study (Bisaschi et al., 2025) further

indicates that the LAMP assays targeting *B. cereus* toxin genes show strong overall agreement with both culture-based analysis and ONT sequencing. Multiple products displayed a clear concordance between toxin gene detection and the presence of *B. cereus* group species. In particular, "Beverage_soy_UHT_type1", "Burger_pea_type2", "Burger_pea_type3", "Cretan_tree_opercula", and "Chicken_fillets_pea" harbored multiple toxin loci (*hblD*, *nheB*, *cytK*-1, and/or *cesA*) and concurrently yielded *B. cereus*, *B. thuringiensis*, *B. paranthracis*, *B. albus*, or other *B. cereus sensu lato* taxa through culture or ONT-based profiling. Additional concordance was observed for "Herbal_infusion_type7" and "Burdock_pills", in which the presence of *nheB* or multiple toxin genes co-occurred with detection of *B. cereus* group or phylogenetically related *Bacillus* species. Collectively, these findings highlight the high analytical sensitivity and diagnostic utility of the LAMP assays for detecting potentially toxigenic *B. cereus* group DNA across a wide range of food matrices. In contrast, 9 samples that were positive only for *nheB* or for another single toxin gene (specifically, "Mozzarella_cheese_soy", "Fish_medallions_soy", "Fish_nibbles_soy", "Gyros_soy", "Tofu_tumeric_soy", "Aloe_syrup", "Blueberry_infusion", "Herbal_pills_type1" and "Herbal_pills_type2") did not yield *B. cereus* group species by either culture-based methods or ONT sequencing (Bisaschi et al., 2025). These discrepancies may reflect differences in analytical sensitivity among the methods used. LAMP was shown to detect very low DNA concentrations, whereas ONT sequencing requires substantially higher template loads to generate a positive result (10^2 spores $\times$ mL^{-1}, as previously demonstrated). Another plausible explanation is that these matrices contained trace amounts of extracellular or partially degraded DNA—originating from non-viable cells or from low-level environmental contamination—that remained amplifiable by LAMP but was insufficient for recovery through culture-based methods. Overall, integrating LAMP analysis with culture-based and ONT sequencing approaches provided complementary insights into the presence and toxigenic potential of B. cereus group bacteria.

Beyond these advantages, applying the method to plant-based foods highlighted both its strengths and practical considerations. Although colorimetric LAMP with phenol red enables instrument-free readout and rapid decision-making—an attractive feature for in-factory screening, due to the possibility for decentralized analysis—a notable proportion of samples produced "suspicious" intermediate (orange) colorimetric outcomes, especially for *cesA* and *cytK*-1. Only a subset of these ambiguous signals was confirmed by fluorescence-based qLAMP. In our experience, and in line with the known chemistry of phenol red–based LAMP, an "orange" endpoint can reflect (i) a low target load, where amplification occurs but proton release is insufficient within the predefined runtime to complete the pH transition from red (alkaline) to yellow (acidic) (de Oliveira Coelho et al., 2021; Novi et al., 2025; Tanner et al., 2015), or (ii) matrix-related interference, which may result in an incomplete colour shift (de Oliveira Coelho et al., 2021; Novi et al., 2025; Tanner et al., 2015). With respect to this latter effect, the complex chemical composition of certain products—including plant secondary metabolites and organic acids—can modulate the reaction environment and influence the clarity of the colorimetric transition by interacting with nucleic acids or affecting polymerase activity, thus impacting amplification efficiency (Moon et al., 2022; Sakatoku et al., 2024). The high frequency of ambiguous results observed in the colorimetric LAMP assays for dietary supplements could thereby be attributed to the complex phytochemical composition of herbal infusions, which can interfere with the visualization of a distinct color transition. In addition to the chemical composition, matrices with elevated acidity or strong buffering capacity may partially counteract the pH shift required for the red-to-yellow colour change, leading to incomplete or ambiguous visual signals (de Oliveira Coelho et al., 2021; Tanner et al., 2015). A similar effect may explain the higher frequency of suspicious results observed in soy-based meat analogues, as soy formulations—typically characterized by high protein content and a substantial proportion of ionizable amino acid residues—may contribute to enhanced buffering behaviour and protein-mediated modulation of pH dynamics during amplification, compared to other plant-based protein sources (Day, 2013; Mennah-Govela et al., 2019; Moon et al., 2022). Lastly, technological treatments such as pasteurization, UHT sterilization, and extrusion may further contribute to reduced assay performance by causing DNA fragmentation or promoting the release of PCR-inhibitory compounds from the food matrix, potentially limiting amplicon accumulation and thus attenuating the colorimetric readout (Latham et al., 2023; Moon et al., 2022).

Because all these factors can mimic weak positives, a confirmatory fluorescence-based test with higher discriminatory power proved essential: orange wells re-tested by qLAMP were either validated as true low-level positives or excluded as negatives. Operationally, we therefore recommend a two-tier workflow: colorimetric LAMP for primary screening (with a fixed endpoint time to avoid late non-specific signals), followed by qLAMP confirmation for any non-binary colour outcomes. Furthermore, it should be emphasized that the detection of toxin-encoding genes does not necessarily imply active toxin expression or an immediate food safety risk, as toxin production in the *B. cereus* group is strongly influenced by strain-specific regulation and environmental conditions. Accordingly, the proposed method should be regarded as a pre-screening tool for identifying potentially toxigenic samples, which, when positive, can be followed by conventional approaches aimed at confirming the presence of biologically active toxins. Remarkably, the multi-locus LAMP approach represents a rapid and sensitive method for direct detection of key virulence determinants in food matrices and may help address limitations associated with conventional culture-based assays, which can underestimate toxigenic *B. cereus* due to sporulation dynamics or sublethal cellular injury. While previous LAMP-based approaches have largely focused on single targets, such as *nheB* or emetic *ces*-related loci (Busch et al., 2022; Deng et al., 2019), the multi-target LAMP approach provides a more comprehensive and robust assessment of strain virulence, reducing the risk of false-negative interpretations that may arise when relying on a single genetic marker. This expanded analytical coverage represents a significant methodological advancement for rapid screening in diverse and heterogeneous food matrices, particularly plant-based products for which data on *B. cereus* toxigenic profiles remain limited.

In conclusion, this work advances the state of the art beyond single-locus assays by simultaneously targeting the major emetic and enterotoxigenic determinants of the *B. cereus* group. We report the design and validation of four LAMP assays for key *B. cereus* virulence genes, demonstrating high analytical sensitivity (down to 10 fg/μL per reaction) and reliable detection in spiked plant-based matrices (10^1 spores $\times$ mL^{-1} (or g^{-1})). Application to commercial products confirmed the frequent occurrence of diarrheal toxin genes—particularly *nheB* and *hblD*—whereas *cesA* remained uncommon, as expected for emetic strains. Despite one non-specific reactions observed for *cesA*, overall assay specificity was strong and can be further enhanced through primer refinement or adoption of probe-based LAMP chemistries. Colorimetric LAMP proved to be an effective rapid screening tool, and offers a practical, sensitive, and reliable workflow for detecting *B. cereus* toxin genes in plant-based foods, with the interesting possibility of enabling decentralized analysis across the supply chain.

CRediT authorship contribution statement

Marta Bisaschi: Writing – original draft, Investigation, Formal analysis, Data curation. **Alessandra Fontana:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Maria Luisa Callegari:** Writing – review & editing, Methodology. **Antonio Del Casale:** Writing – review & editing, Supervision. **Lorenzo Morelli:** Writing – review & editing, Supervision, Project administration. **Alexandre Lamas:** Writing – review & editing, Supervision, Conceptualization. **Marta Prado:** Writing – review & editing, Project administration, Conceptualization. **Vania Patrone:** Writing – review & editing, Supervision, Project administration, Conceptualization.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2026.112113.

Data availability

Data will be made available on request.

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