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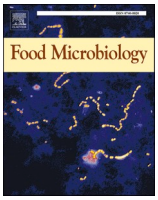

New insights into *Bacillus cytotoxicus* sources, screening, toxicity, and persistence in food production facilities

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ABSTRACT

Bacillus cytotoxicus is a thermotolerant member of the *Bacillus cereus* group. It has been linked to rare, but at times fatal cases of diarrheal disease and might be missed at routine diagnostic screening temperatures commonly used for the *B. cereus* group. The pathogen is mostly found on dehydrated foods containing potato starch or insects. How it enters the food chain or whether it persists in food producing environments is largely unknown. Increased consumption of insects and convenience foods in Europe and the lack of information on the persistence of *B. cytotoxicus* in food environments and its virulence demand for further characterization. In this study, we aimed to obtain a better understanding of i) the food sources of *B. cytotoxicus,* ii) screening temperatures needed for its isolation from food matrices, iii) cytotoxicity of the organism, and iv) its ecological niche and potential epidemiological links. To this end, 112 food samples were collected, with a focus on foods exhibiting low water activity. The samples were screened for *B. cytotoxicus* at 42 °C and at 50 °C. Presumptive isolates were characterized by *cytK-1* toxin gene PCR for differentiation of *B. cytotoxicus* from other *B. cereus* group members. Vero cell cytotoxicity assays were performed, and selected isolates were sequenced. Our results show that screening at 42 °C might be insufficient for detecting *B. cytotoxicus* in foods that harbor other less thermophilic *Bacillus* species. When screening at 50 °C, *B. cytotoxicus* was detected in 23% of the food samples (n = 26 isolates). The highest prevalence was detected in mashed potato products (82%) and potato flakes (67%). In contrast, a wide range of products not containing any potato ingredients did not yield *B. cytotoxicus* isolates. All *B. cytotoxicus* isolates exhibited either low or no detectable cytotoxicity. WGS analysis revealed that a highly toxic isolate is closely related to the French outbreak strain NVH 391-98. In addition, we could show that two isolates sampled 5 years apart from the same production facility only differed by seven SNPs, making it likely that *B. cytotoxicus* is able to persist in production facilities over a long time. Interestingly, the reoccurring strain possessed an additional plasmid and did not show cytotoxic potential when re-isolated after 5 years.

1. Introduction

B. cytotoxicus belongs to the *B. cereus* group that is ubiquitous in nature and is commonly found as part of the microflora of many agricultural products (Stenfors Arnesen et al., 2008). The hazardous potential of the members of this group varies substantially (Ehling-Schulz et al., 2019). Important group members include *B. anthracis*, the causative agent of anthrax (Kolstø et al., 2009). Another important member is the potential foodborne pathogen *B. cereus sensu stricto* for which some strains can cause both the diarrheal syndrome through formation of enterotoxins (Hbl, Nhe, CytK), and the emetic syndrome through formation of cereulide by cereulide synthetase activity (Ces) (Stenfors Arnesen et al., 2008). *B. thuringiensis* is another member of the *B. cereus* group that is widely utilized in biopesticide production, and some strains have recently been linked to several foodborne outbreaks (Biggel et al., 2021, 2022; Bonis et al., 2021). Other members are considered non-pathogenic, such as *B. mycoides*. A recent addition to the *B. cereus* group is *B. cytotoxicus* that was first isolated during an outbreak in France with three fatalities (Lund et al., 2000; Guinebretière et al., 2013). It forms a distinct phylogenetic cluster within the *B. cereus* group and can be distinguished by metabolic fingerprinting (Sorokin et al., 2006; Guinebretière et al., 2013; Bağcıoğlu et al., 2019; Stevens et al., 2019). It possesses a hydroxyphenylalanine operon, which might equip it with an evolutionary advantage and possesses plasmids that show its ability to exchange genetic content (Stevens et al., 2019; Fayad et al., 2021). Probably its most striking phenotypic feature is its high

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temperature tolerance allowing growth at temperatures up to 52 °C (Cairo et al., 2022). Standard screening protocols for *B. cereus s.l.* in foods employ incubation temperatures of 30 °C (International Organization for Standardization, 2004), which is at the lower end of permissive growth temperatures of *B. cytotoxicus*.

B. cytotoxicus (formerly designated *B. cereus*) has to date mainly been isolated from dehydrated potato products such as instant mashed potatoes or other dried foods (King et al., 2007; Contzen et al., 2014; Heini et al., 2018; Koné et al., 2019; Burtscher et al., 2021), demonstrating that this thermotolerant spore former can survive the heating and drying processes involved in the production of dehydrated foods. Recently, *B. cytotoxicus* was also found in food products consisting of or containing insects (Fasolato et al., 2018; Frentzel et al., 2022). The increasing consumption of insect products and convenience foods could potentially lead to a rise in *B. cytotoxicus* related disease incidents. Recently, new insect species such as *Alphitobius diaperinus* larvae (lesser mealworm) have been approved in the EU (Commission Implementing Regulation (EU), 2023). This harbors a risk for increased consumer exposure to *B. cytotoxicus*. Since there is only limited data about its virulence potential the associated risks are difficult to assess.

When *B. cytotoxicus* was originally isolated it was reported as being highly cytotoxic, which was mainly attributed to the *cytK-1* variant that it carries. This variant is believed to be more toxic than the *cytK-2* variant carried by *B. cereus* (Lund et al., 2000; Fagerlund et al., 2007). However, not all strains carrying the *cytK-1* gene result in high cytotoxicity in *in vitro* assays. In a comparison of 3 strains only 2 produced significant amounts of CytK-1 (Fagerlund et al., 2007). A recent study demonstrated that only a minor fraction of *B. cytotoxicus* strains isolated from dried potato products are highly cytotoxic in a Vero cell assay (Burtscher et al., 2021).

To date only limited data is available about the persistence of *B. cytotoxicus* in food producing environments, its distribution in different food products, cytotoxicity, and genetic relations amongst food isolates and outbreak strains. Here, we investigated the prevalence of *B. cytotoxicus* in a variety of food products, performed cytotoxicity assays to evaluate its virulence potential, and performed WGS on several isolates to investigate the population structure and potential epidemiological links.

2. Materials and methods

2.1. Food sampling

A total of 112 retail foods sold in Swiss supermarkets were sampled. Priority was given to foods low in water activity, with samples including instant mashed potato powder (n = 17), potato flakes (n = 3), instant sauce (n = 20), bakery mixes (n = 6), instant soup (n = 19), gnocchi (n = 5), insect-based products (n = 4), plant-based alternatives to meat and eggs (n = 3), and others (n = 35).

2.2. Screening for B. cytotoxicus

A screening approach described by (Contzen et al., 2014) was used and modified as follows: Ten g of food sample were mixed with 90 ml CGY medium (Beecher and Wong, 1994) for 30 s in a stomacher bag (Stomacher® 400 Circulator, Seward, Worthing, UK) and samples were subsequently incubated at 42 °C or 50 °C overnight. After overnight incubation, one loop of the overnight culture was streaked onto Mossel/MYP agar (mannitol-egg yolk polymyxin agar, BD Difco™ Columbia Blood Agar Base) and sheep blood agar and subsequently incubated at 37 °C overnight. For each plate, all single colonies exhibiting a phenotype consistent with the *B. cereus* group were restreaked to obtain pure cultures and were further characterized by colony PCR targeting the *cytK-1* variant of the cytotoxin K gene characteristic for *B. cytotoxicus* following a PCR protocol and using primer pair previously described (Guinebretiere et al., 2006). Pure cultures of *B. cytotoxicus* were conserved as glycerol stocks at −80 °C.

2.3. Cytotoxicity assay

Cytotoxicity assays were conducted as previously described by (Burtscher et al., 2021) using the WST-1 bioassay adapted from (Moravek et al., 2006). Briefly, cell-free culture supernatants were obtained in CGY. To this end, an overnight pre-culture of each isolate in 5 mL CGY was prepared and used to adjust a day culture of 30 ml CGY in an Erlenmeyer flask to an OD_{600} of 0.05. The day cultures were incubated at 30 °C (150 rpm shaking) until an OD of 7 was reached. Bacterial biomass was removed by centrifugation at 11.000 rpm for 10 min at 4 °C and filtration through 0.2 μm sterile filters. Aliquots of 1 ml supernatants were supplemented with 10 μL 0.1 M Na2 EDTA before storing them at −80 °C. Subsequently, we performed a Vero cell assay using WST-1 to determine the cytotoxicity of all isolated *B. cytotoxicus*. To this end, serial dilutions of cell-free sterile filtered supernatants were added to 96 well microtiter plates containing 10^4 Vero cells per well suspended in Dulbecco's modified Eagle medium supplemented with 1% FCS and 0.4% penicillin-streptomycin. We incubated the microtiter plates for 24 h (37 °C, 5% CO_2) before WST-1 was added and subsequently measured the extinction of the produced formazan dye at 450 nm and determined a dose-response curve that allows for calculation of reciprocal cytotoxicity titers as a measure of 50% loss of mitochondrial activity of viable cells. We performed three independent biological experiments for each isolate, each comprising two technical replicates of each dilution series per isolate. Strain NVH 0075–95 (CH_165), a *B. cereus sensu stricto* strain linked to foodborne diarrheal disease was used as a highly cytotoxic reference. We classified the isolates into toxicity levels (low, medium, high) according to (Jeßberger et al., 2015), with absolute cytotoxicity cutoffs being normalized based on the mean of the reference strain *B. cereus* s.l. NVH 0075–95 as previously published (Johler et al., 2018). Thus, absolute cytotoxicity values < 0.4 were being categorized as low, values from 0.4 to 0.8 as medium, and values > 0.8 as high.

2.4. Whole genome sequencing

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen).Libraries were prepared using the Nextera DNA Flex Library Preparation Kit (Illumina) and sequenced on the Illumina MiniSeq platform with 2 × 150 bp paired-end chemistries. Illumina read adapters and low-quality bases were trimmed with fastp (Chen et al., 2018) and draft genomes assembled using SPAdes 3.14.1 (Bankevich et al., 2012) implemented in shovill 1.1.03 (https://github.com/tseemann/shovill). Publicly available genome assemblies and read data of *B. cytotoxicus* were retrieved from NCBI Genbank and the NCBI Sequence Read Archive (SRA) (Sayers et al., 2022). Pre-assembled genomes were used for the analyses except for NVH-398-98, which was re-sequenced and assembled as described above. Assembly quality was assessed using QUAST 5.0.2 (Gurevich et al., 2013) and CheckM v1.1.3 (Parks et al., 2015).

Taxonomic classification was confirmed using BTyper3 v3.1.1 (Carroll et al., 2020) and sequence types determined with mlst 2.22.0 (https://github.com/tseemann/mlst). Assemblies were annotated using Bakta v 1.6.1 (Schwengers et al., 2021). A core genome alignment was generated using parsnp 1.5.3 (Treangen et al., 2014) with the assembly of strain NVH_391-98 as reference. Phages, IS elements, and repeat regions in the reference chromosome were identified with PHASTER (Arndt et al., 2016), ISEScan 1.7.2.3 (Xie and Tang, 2017), and NUCmer 3.1 (Marçais et al., 2018) and masked before the alignment. A maximum-likelihood phylogenetic tree was constructed from the core genome alignment using IQ-TREE 2.0.3 (Nguyen et al., 2015) with the generalized time-reversible (GTR) model with gamma distribution. The tree was visualized in iTOL v5 (Letunic and Bork, 2021). Pairwise cgSNP distances were determined with the CFSAN pipeline v2.2.1 for each sequence type separately. Whole genome SNPs were additionally

identified using snippy v4.6.0.

3. Results and discussion

3.1. Detection of B. cytotoxicus from food samples is improved at 50 °C

To compare isolation efficacy at different screening temperatures all food samples were incubated at 42 and 50 °C. At 42 °C of the total samples 2% (2/112) were positive for *B. cytotoxicus* via *cytK-1* screening (Fig. 1). In contrast, at 50 °C incubation temperature the number increased to 23% (26/112). This substantial increase in *B. cytotoxicus* growth indicates that *B. cytotoxicus* is outcompeted by other spore forming bacilli at temperatures permissive to mesophilic bacteria that grow between 20 °C and 45 °C. As in routine diagnostics food samples screened for *B. cereus* are generally incubated at 30 °C (International Organization for Standardization, 2004), *B. cytotoxicus* would likely go undetected. In our study *B. cytotoxicus* was most prevalent in mashed potatoes and dried potato flakes (Fig. 1). Interestingly, except for one soup powder all food samples positive for *B. cytotoxicus* contained at least one potato-based ingredient (raw/dried potato, potato starch etc.). In earlier studies a similar prevalence was found in potato products (Contzen et al., 2014; Koné et al., 2019; Burtscher et al., 2021). However, it remains unclear why *B. cytotoxicus* has been mostly associated with potato products. The source of contamination could be soil (Contzen et al., 2014) or insects (Fasolato et al., 2018; Frentzel et al., 2022) since *B. cytotoxicus* has been isolated from both matrices. The presence of an *hpa* operon in *B. cytotoxicus* could indicate an adaptation to the soil environment (Stevens et al., 2019). Once introduced, *B. cytotoxicus* might persist in production facilities and re-contaminate foods.

3.2. Cytotoxicity is low for all isolates

All 26 isolates that were obtained at 50 °C were subsequently characterized using Vero cell cytotoxicity assays. All investigated *B. cytotoxicus* isolates showed either low or mid-level cytotoxicity (Fig. 2). Only in three isolates (CH834, CH836, CH838) could we observe a trend towards mid cytotoxicity levels. These findings are in line with previous results (Heini et al., 2018; Burtscher et al., 2021) and reinforce the suggested hypothesis that the original concern of *B. cytotoxicus*' high cytotoxicity does not hold true for the majority of

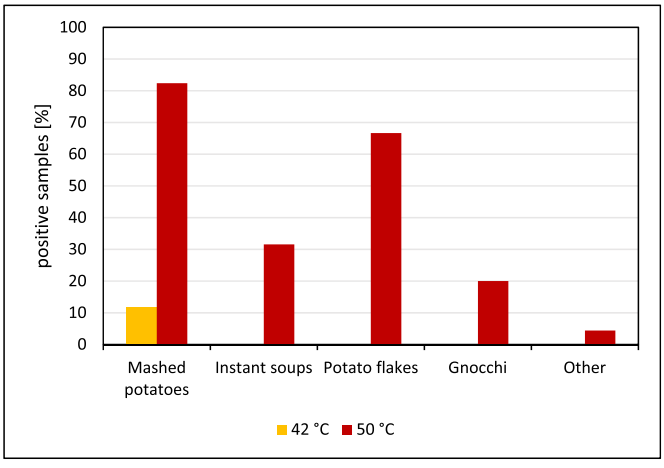

Fig. 1. Comparison of B. cytotoxicus detection in foods at different screening temperatures. 112 food items were both incubated at 42 °C (yellow) and 50 °C (red). At 42 °C incubation 2/17 mashed potato products were positive, all other products negative. At 50 °C 14/17 mashed potato products, 6/19 instant soups, 2/3 potato flakes, 1/3 gnocchi, and 3/68 other products were positive for B. cytotoxicus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

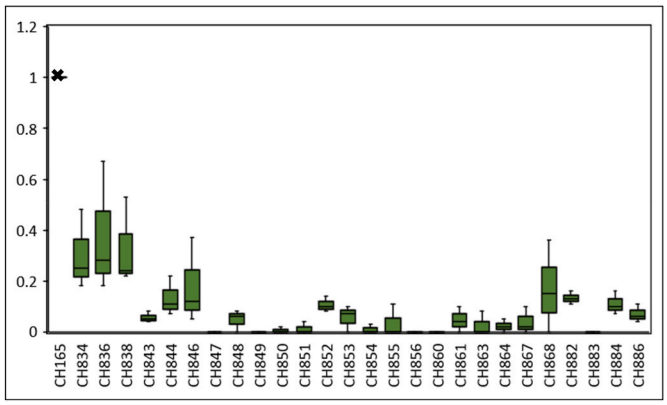

Fig. 2. Cytotoxicity assay of B. cytotoxicus isolates. Results of a WST-1 bioassay using Vero cells shown as normalized reciprocal cytotoxicity titers. Reference strain CH_165 (NVH 0075–95) indicated by **x**. Cytotoxicity values < 0.4 were being categorized as low, values from 0.4 to 0.8 as medium, and values > 0.8 as high.

strains. It remains unclear what renders some strains highly toxic while others present only mild toxicity. Since the presence of the *cytK-1* gene does not automatically lead to expression of CytK-1 and hence cytotoxicity, it is likely that differential gene expression plays a role. If expressed, CytK-1 does contribute to the bulk of the cytotoxic effect observed in *B. cytotoxicus* (Koné et al., 2021).

3.3. Phylogenetic analyses reveal persistence in food production facilities

We used Illumina whole genome sequencing to compare a selection of our isolated strains (n = 10) with all publicly available *B. cytotoxicus* genome assemblies (n = 29). The public data comprised predominantly assemblies of isolates from dehydrated potato products (n = 22), including 17 isolates recovered between 2014 and 2017 in Switzerland (Supplementary Table 1). Overall, the isolates showed limited diversity, with most (27/39) being assigned to ST2112, ST2116, or ST2121 (Fig. 3). To determine potential transmission clusters, high-quality SNP analyses were performed for 15 isolates that belonged to the three dominant sequence types and had read data available (Supplementary Table 1).

We found that the disease-associated strain NVH 391-98, associated with a fatal foodborne outbreak linked to vegetable puree in France in 1998 (Lund et al., 2000), was closely related (approx. 100 SNPs) to two isolates recovered in Switzerland in 2017 (isolate CH_213/M16) (Heini et al., 2018) and 2022 (isolate CH_834, this study) from two samples of the same mashed potato powder product. Despite being isolated 5 years later, CH_834 differed from CH_213 by only 7 SNPs, suggesting that this clone could have persisted in a food production facility, possibly in a dormancy state. CH_834 further differed from CH_213 by the presence of a putative plasmid (contig 21 [34 kb]; >99% sequence identity and coverage to *B. cereus* plasmid pCE3 [CP047088.1]), which was also absent in NVH 391-98. While CH_213 (Heini et al., 2018) and protein fractions of NVH 391-98 (Lund et al., 2000) were highly cytotoxic in Vero Cell assays, CH_834 exhibited low cytotoxicity (Heini et al., 2018). It remains unclear whether the loss in cytotoxicity is related to the observed plasmid acquisition, mutations, or differential gene expression. Missense or frameshift mutations CH_834 versus CH_213 were found in *rnjA* (ribonuclease J), *dppD* (peptide ABC transporter substrate-binding protein), and two genes encoding an integrase and a GntR family transcriptional regulator, respectively.

Among the ST2116 isolates, CH_134 and CH_214 were near-identical (4 cgSNP distance) and obtained in the same year (2017) but from different dehydrated potato products. Given their similarity, a shared origin from the same production facility is plausible. Similarly, CH_855 and CH_884 were obtained in 2022 from different dehydrated potato

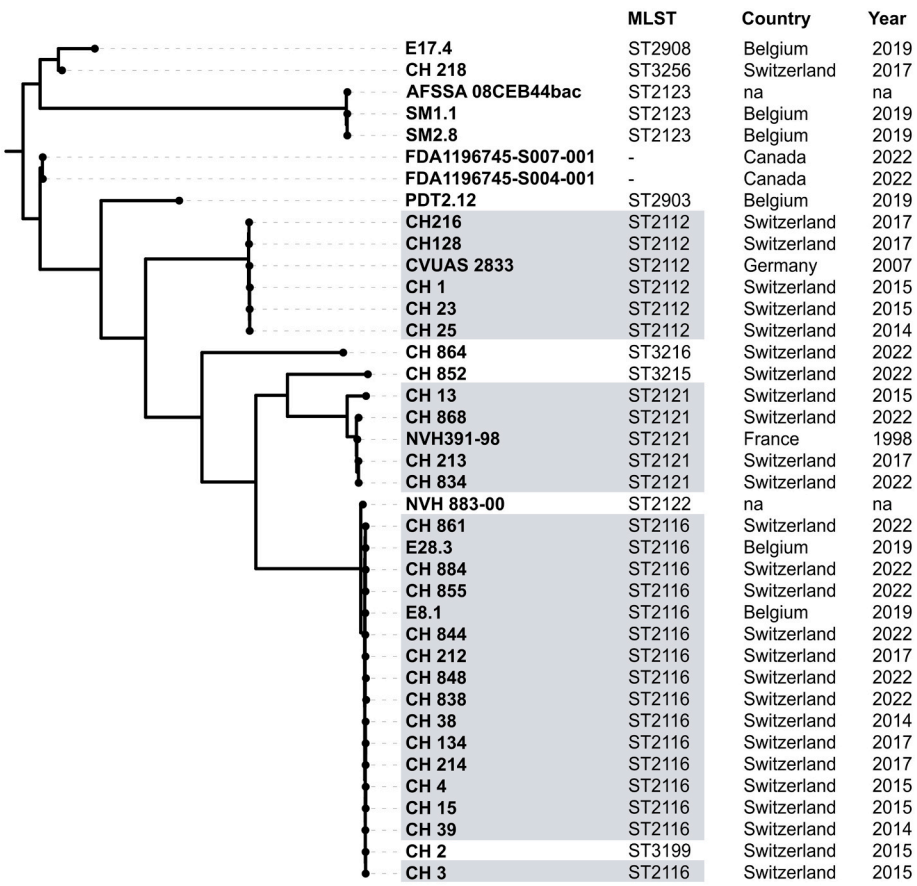

Fig. 3. Phylogenetic analysis of 39 B. cytotoxicus isolates. Strains isolated in this study were compared to all publicly available B. cytotoxicus genomes. The maximum likelihood phylogeny is based on 21,466 variable sites in a 3.1 Mbp core genome alignment and was visualized using iTOL v6 (Letunic and Bork, 2021). Isolates assigned to the dominant sequence types ST2112, ST2116, and ST2121 are shaded in grey.

products but differed by only 11 cgSNPs. The low mutation rates could indicate persistence within food production facilities in a state of dormancy as spores. The remaining five ST2116 isolates for which read data was available (CH_212, CH_838, CH_844, CH_848, CH_861) were more diverse with 33–99 pairwise SNPs compared to other ST2116 isolates. Of the six ST2112 isolates, read data was available for two isolates (CH_128 and CH_216) which differed by 13 SNPs.

4. Conclusion

In conclusion, our study shows that *B. cytotoxicus* is highly prevalent in foods containing potato ingredients. However, classical screening protocols at mesophilic temperatures (30–42 °C) fail to detect the majority of *B. cytotoxicus* present in foods. Frequent contamination of food products seems inevitable, due to the ubiquitous occurrence, resistance and persistence of *B. cereus* group organisms and the colonization of processing facilities with spores (Carlin, 2011). The presented data suggests that persistence of *B. cytotoxicus* in production facilities is possible. This is the first report of persistence of *B. cytotoxicus* in production environments, a factor that might play a significant role in recontamination of food products. Further research is needed to pinpoint the ecological niche of *B. cytotoxicus,* to identify bacterial and environmental factors favoring persistence in food production facilities and to determine suitable strategies to minimize contamination. In addition, the virulence potential of *B. cytotoxicus* seems to be highly variable. Most *B. cytotoxicus* strains exhibited no cytotoxic effects on Vero cells after generation of supernatants based on growth at 30 °C. Elevated growth temperatures and the use of enteric cell lines more closely mimicking conditions in the human host may result in higher toxicity levels. However, some strains are highly cytotoxic and have led to fatalities. It is not yet clear whether the differences in cytotoxicity are solely attributed to *cytK-1* expression, or stem from a combination of different enterotoxins. Our findings of *B. cytotoxicus* being ubiquitous in dehydrated potato products and persisting in food production environments with low mutation rates have important implications for the risk assessment and outbreak investigations.

Data availability

Sequencing data have been submitted under NCBI BioProject No. PRJNA817374. Accession numbers of all analyzed genomes are provided in Supplementary Table 1.

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Author contributions/ Credit statement

Danai Etter: Investigation, Supervision, Writing – original draft preparation, Reviewing, Editing. Michael Biggel: Software, Formal analysis, Data curation, Writing - Reviewing. Mariella Greutmann: Investigation, Formal analysis, Data curation. Nicole Cernela: Investigation. Sophia Johler: Conceptualization, Methodology, Resources, Investigation, Data curation, Supervision, Writing – original draft

preparation, Reviewing, Editing. All authors have read, revised, and approved the final draft of the manuscript.

Declaration of competing interest

None

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104399.

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