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Reliable delineation of *Bacillus cytotoxicus* from other members of the *Bacillus cereus* group by MALDI-TOF MS – An extensive validation study

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A B S T R A C T

Bacillus cytotoxicus is classified as a thermotolerant member of the *Bacillus cereus* group, producing the diarrhoea causing cytotoxin K1. This species came into the focus of food microbiologists when the type strain was isolated as the cause of a fatal food-borne outbreak. However, knowledge of the role of *B. cytotoxicus* as a food-borne microbe and data on its prevalence in food is still limited today. One reason for this is the lack of an easy to perform method of identification at the species level other than PCR, due to the highly similar phenotype of many *B. cereus* group representatives. Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) is widely used for the identification of microorganism species. This study describes the formal validation of a MALDI-TOF MS system for a reliable differentiation between *B. cytotoxicus* and other members of the *B. cereus* group. An extensive number of well-supported individual spectra from *B. cytotoxicus*, several other species of the *B. cereus* group, and 652 other microorganism species in a taxonomically wide range served as data sets. These were used to perform the validation for two database compilations: the commercial MALDI-Biotyper version K, and an extended compilation, including reference spectra from an open collection of spectra created by several users. Using both database combinations, the obtained values fulfil the formal criteria for the targeted identification of *B. cytotoxicus* in accordance with a national guideline for the validation of MALDI-TOF MS parameters in food analysis, with significant improvements due to the expanded database. By using 183 single spectra from 170 isolates, created in eleven different laboratories, the number of samples significantly exceeded the sample sizes for validation currently specified for ISO 16140–4:2020 for the validation of identification methods in food microbiology by single laboratories. Therefore, the method is suitable for routine application in an accredited laboratory context.

1. Introduction

Bacillus (B.) cytotoxicus was classified as a thermotolerant member of the *Bacillus cereus* group by Guinebretière et al. (2013). It is characterized by its growth at high temperatures (≤55 °C) as well as its production of the diarrhoea causing cytotoxin K1 (Cairo et al., 2022; Fagerlund et al., 2007). This species and its distinctive CytK-1 toxin are of special interest in food microbiology as the type strain was isolated as the cause of a fatal food-borne outbreak in France (Lund et al., 2000). To date, most isolates of *B. cytotoxicus* have been obtained from foods containing dried potato products or insects (Cairo et al., 2022; Contzen et al., 2014; Etter et al., 2024; Frentzel et al., 2022; Koné et al., 2019; Rau et al., 2009; Stevens et al., 2019).

In official food control laboratories, analyses of bacteria of the *B. cereus* group usually follow standardized procedures, unable to distinguish between members of the group. Several closely related species, such as *B. cytotoxicus* and *B. cereus sensu stricto*, will be aggregated as 'presumptive *Bacillus cereus*' or 'belonging to the *Bacillus cereus* group' (ISO 7932:2004/Amd 1:2020). Due to their varying degrees of pathogenicity, reliable species identification within the complex *B. cereus* group is of interest for risk assessment. Therefore, accepted validated methods that can be easily integrated into existing workflows, are required for routine microbiological food analysis. With the 2020 amendment, ISO 7932 features a number of additional methods for distinguishing members of the *B. cereus* group from each other, including PCR for the *cytK-1* gene of *B. cytotoxicus*, coding for the characteristic CytK-1. However, *B. cytotoxicus* shows relatively small colonies under standard cultivation conditions, which can easily be overlooked or sorted out as atypical (Cairo et al., 2022). Thus, special in-house methods apply higher growth temperatures (50–52 °C) to select for *B. cytotoxicus* as a thermotolerant species (Burtscher et al., 2021; Cavello et al., 2020; Contzen et al., 2014; Etter et al., 2024). Colonies can then be verified by the above mentioned detection of the specific *cytK-1* gene by PCR, by infrared spectroscopy, analysis of the 16s rDNA sequence, or by analysis of the whole genome (Bağcıoğlu et al., 2019; Burtscher et al., 2021; Cairo et al., 2022; Cavello et al., 2020;

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Contzen et al., 2014; Etter et al., 2024; Rau et al., 2009; Stevens et al., 2019).

There is still a lack of knowledge regarding the role of *B. cytotoxicus* as a foodborne pathogen, its prevalence in food, and its sources and routes of contamination. (Burtscher et al., 2021; Cairo et al., 2022; Etter et al., 2024). Glasset et al. (2016) found *cytK*-1 in 5 % of 564 strains associated with 140 strong-evidence food-borne outbreaks that had occurred in France. The *cytK*-1 gene is strictly associated with *B. cytotoxicus* (Guinebretière et al., 2013). However, apart from the aforementioned targeted *cytK*-1 specific PCR from ISO 7932:2004/Amd 1:2020 (Guinebretière et al., 2006), and a recently described colorimetric aptasensor system for *B. cytotoxicus* spores (Rizzotto et al., 2023), there is a lack of untargeted rapid identification methods for isolates of *B. cytotoxicus*, which can be broadly used in routine analysis and also pass accreditation in official laboratories.

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) systems are widely used for the identification of microorganism species in clinical microbiology, microbiological food control, and veterinary diagnostics (e.g. Malorny et al., 2020). Identification of bacterial isolates by pattern-based methods such as MALDI-TOF MS is directly dependent on the quality and quantity of available representative references in the database used for comparison. Therefore, species not included in the reference database cannot be identified correctly (Kostrzewa & Maier, 2017; Rau et al., 2019). The easy standard identification by MALDI-TOF MS is also hampered for taxonomically closely related species like members of the *Bacillus cereus* group (Chen et al., 2022; Fiedoruk et al., 2016; Manzulli et al., 2021; Pauker et al., 2018). This is a problem for several other species-groups that are relevant for food microbiology and veterinary diagnostics, such as *Escherichia coli/Shigella* sp., *Aeromonas* sp., *Vibrio (V.) cholerae/V. mimicus* and the *Streptococcus (S.) dysgalactiae/S. canis* separation (Dieckmann, Strauch, & Alter, 2009; Kitagawa et al., 2022; Nybakken et al., 2021; Paauw et al., 2015).

For accredited laboratories for food microbiology, strict requirements must be met for species identifications, also for in-house methods (ISO 16140 series). In view of this, a first guideline for the validation of species identifications using MALDI-TOF MS in a single laboratory or in laboratory networks were compiled by the MALDI-TOF MS working group (BVL-Working group MALDI) pursuant to § 64 of the German Foods, Commodities and Animal Feed Code (LFGB), coordinated by the Federal Office for Consumer Protection and Food Safety Berlin (BVL, 2022). Here, we transparently demonstrate the reliability of a MALDI-TOF MS method for the identification of *B. cytotoxicus* isolates from food, in accordance with this technical guideline. In this study the Bruker MALDI Biotyper system combined with the "research use only" (RUO) MBT_K database (revision K, 2022; Bruker Daltonics, Bremen, Germany), containing 11,897 database entries, was used in a first step as a reference method. As this commercial system allows for an extension of the database by adding user-made reference records, an increase in the identification rate was systematically achieved.

Because of the close taxonomic relatedness within the *B. cereus* group, its division into several ecotypes, and the continuous description of new species, the *B. cereus* group is highly complex and the identification of the members of this group on the species level is generally challenging (Carroll et al., 2020; Carroll et al., 2022; Chen et al., 2023; Guinebretière et al., 2008; Gupta et al., 2020; Jiménez et al., 2013; Liu et al., 2017; Makuwa et al., 2023; Miller et al., 2016; Tohya et al., 2020; Tourasse et al., 2023). Therefore, a clear differentiation of all members of the *B. cereus* group using MALDI-TOF MS has not yet been achieved, and will most likely always lag behind the quickly developing taxonomy of this group. Over the past years, there have been several efforts to differentiate the *B. cereus* group by MALDI-TOF MS with various evaluation methods but *B. cytotoxicus* was not included in any of these studies (Chen et al., 2022; Fiedoruk et al., 2016; Manzulli et al., 2021; Pauker et al., 2018; Takahashi et al., 2022). For the identification of unknown isolates using MALDI-TOF MS, sufficiently well stocked reference databases are a prerequisite. Even in the commercial version MBT_K, the number of reference spectra for members of the *B. cereus* group is relatively small: in the latest (as of February 2024) database version *B. cytotoxicus* was integrated for the first time with four reference entries in combination with only nine reference spectra of four further species of the *B. cereus* group (Table 1). As an add-on to the commercial database, other sources for a range of user-made reference entries have been provided to the public by the MALDI user platform MALDI-UP (https://maldi-up.ua-bw.de). For this study, a selection of such entries was used to complement the commercial database MBT_K (Table 1). The restricted Security-Relevant (SR) database extension (Bruker), including 23 reference spectra from *B. anthracis* is not used in routine food microbiology, therefore this specialized database is not available for many users and was not considered in this study. An extensive set of well-supported individual spectra from *B. cytotoxicus* (183 spectra from 170 isolates), and 659 other microorganism species from the MALDI-UP catalogue (1408 spectra) served as a validation set (Rau et al., 2016a). This set of spectra was used to perform a formal validation for two databases: the latest MBT_K, and the MBT_K database supplemented with user-made entries from MALDI-UP (MBT_K + MUP). The validation was consequently performed in line with the technical guidelines created by the BVL-Working group MALDI (BVL, 2022; Rau et al., 2022). Recently, this validation concept was also successfully used for fingerprinting applications besides MALDI-TOF MS, e.g. serotyping of *Listeria monocytogenes* via infrared spectroscopy (Oberreuter et al., 2023).

2. Materials and methods

2.1. Isolates/strains used

Including the type strain NVH 391-98 (= DSM 22905 = WDCM 00220) (Guinebretière et al., 2013), 170 isolates of *Bacillus (B.) cytotoxicus* were used in this study. From these, 74 *B. cytotoxicus* strains had been analysed in the context of prevalence studies, with a focus on potato-products and other foods (Contzen et al., 2014; Frentzel et al., 2022; Rau et al., 2009; Stevens et al., 2019). As far as is known, 34 isolates of the whole isolate set were obtained from food samples, taken for causal analysis of food-borne disease (Table S1). The set of bacteria from other species, which were used for comparison, comprised 281 well-defined isolates of the *Bacillus cereus* group, mainly isolated in daily routine food microbiology, as well as other members of the phylum *Bacillota*, represented by 389 bacteria from 198 species. Furthermore, a broad taxonomic selection of microorganisms not belonging to the phylum *Bacillota*, were also used in the validation process (Table S1).

2.2. Cultivation and sample preparation

Bacteria of the *B. cereus*-group and of other taxonomic categories were cultured according to their requirements. The individual agar/time/temperature combination for growth of all bacteria used in this study is available on MALDI-UP. An excerpt of these data is shown as Table S1. Two sample preparations were used for the MALDI-TOF mass reference spectra: the ethanol-formiate extraction protocol (EFEx) recommended by Bruker and the trifluoroacetic-acid-extraction protocol (TFA) according to Lasch et al. (2008), developed for highly pathogenic bacteria. For the set of individual spectra used for validation, both the direct transfer (DT) and the extended direct transfer extraction (eDT) protocols were used in addition, to reflect the routine procedures of identification in many laboratories (Table S1) (Pranada et al., 2016; Rau et al., 2016, 2016b).

2.3. Databases

Reference spectra from selected isolates were measured and calculated by trained personnel according to the manufacturer's manual and

Table 1
MALDI-TOF MS reference spectra of *B. cereus* group bacteria, available in the commercial MALDI Biotyper database revision K (MBT_K), and reference spectra listed on MALDI-UP used.

Species	isolate/strain	type strain	reference entry name (msp)	database	remarks
Bacillus (B.) cereus	DSM 31 =	yes	Bacillus cereus DSM 31T DSM	MBT_K	
	ATCC 14579 = WDCM 00218		Bacillus cereus DSM 31 CVUAS	MUP 0670	
			Bacillus cereus ATCC 14579 IGV	MUP 0334	
	4080		Bacillus cereus 4080 LBK	MBT_K	
	994000168		Bacillus cereus 994000168 LBK	MBT_K	
	CICC 23949		Bacillus cereus CICC 23949 CICC	MBT_K	
	ATCC 14893		Bacillus cereus ATCC 14893 CVUAS	MUP 0537	
	ATCC 11778 = WDCM 00001		Bacillus cereus ATCC 11778 IGV	MUP 0233	
	DSM 4312 = WDCM 00219		Bacillus cereus DSM 4312 IGV	MUP 0407	
	DSM 4313		Bacillus cereus DSM 4313 IGV	MUP 0406	
B. cytotoxicus	DSM 22905 =	yes	Bacillus cytotoxicus DSM 22905T DSM	MBT_K	
	NVH 391-98 = WDCM 00220		Bacillus cytotoxicus NVH 391-98 CVUAS	MUP 0002	
			Bacillus cytotoxicus DSM 22905 TUM 8699	MUP 0529	
	1Z46776_1e		Bacillus cytotoxicus 1Z46776_1e MVD	MBT_K	
	1Z46778_1e		Bacillus cytotoxicus 1Z46778_1e MVD	MBT_K	
	CVUAS 2833		Bacillus cytotoxicus 2833 CVUA	MBT_K	
			Bacillus cytotoxicus CVUAS 2833 CVUAS	MUP 0001	
			Bacillus cytotoxicus CVUAS 2833 RKI-CVUAS	MUP 0727	Raw spectra for MSP from Lasch et al., 2018
	CVUAS 6183		Bacillus cytotoxicus CVUAS 6183 CVUAS	MUP 0381	
	CVUAS 6661		Bacillus cytotoxicus CVUAS 6661 CVUAS	MUP 0542	
	IGV 003		Bacillus cytotoxicus IGV 003 IGV	MUP 0232	
B. mycoides	DSM 2048	yes	Bacillus mycoides DSM 2048T DSM	MBT_K	
			Bacillus mycoides DSM 2048 CVUAS	MUP 0671	
	DSM 299		Bacillus mycoides DSM 299 IGV	MUP 0409	
	DSM 11821 = WDCM 00222		Bacillus mycoides DSM 11821T DSM	MBT_K	Former type strain of *B. weihenstephanensis*. MSP from 2007
			Bacillus mycoides DSM 11821T DSM_2	MBT_K	MSP from 2017
	MK373681		Bacillus mycoides MK373681.1 RGL	MUP 1908	Fergusson et al. (2020)
B. pseudomycoides	DSM 12442	yes	Bacillus pseudomycoides DSM 12442T DSM	MBT_K	
			Bacillus pseudomycoides DSM 12442 CVUAS	MUP 0687	
	DSM 12443		Bacillus pseudomycoides DSM 12443 CVUAS	MUP 0688	
	CVUAS 7789		Bacillus pseudomycoides CVUAS 7789 CVUAS	MUP 0686	
B. thuringiensis	DSM 2046 = WDCM 00221	yes	Bacillus thuringiensis DSM 2046T DSM	MBT_K	
	DSM 6022		Bacillus thuringiensis israelensis DSM 6022 IGV	MUP 0408	
	BBA 848		Bacillus thuringiensis tenebrionensis BBA 848 CVUAS	MUP 0673	
	BBA 845		Bacillus thuringiensis kurstaki BBA 845 CVUAS	MUP 0674	
	BBA 847		Bacillus thuringiensis aizawai BBA 847 CVUAS	MUP 0675	
	BBA 846		Bacillus thuringiensis israelensis BBA 846 CVUAS	MUP 0676	
	CVUAS 9675		Bacillus thuringiensis israelensis CVUAS 9675 CVUAS	MUP 1098	

MUP: Custom made database entries selected from the MALDI-UP catalogue. For MUP, the catalogue number is given.

commentary (Kostrzewa & Maier, 2017), using FlexAnalysis version 3.4 and the MALDI Biotyper Compass Explorer software version 4.1 (both Bruker). Therefore, a minimum of 20 single spectra were used to calculate main spectra projections (MSP) used as a reference (Table 1). As an exception the reference entry "*Bacillus cytotoxicus* CVUAS 2833 RKI-CVUAS" was made from 10 single spectra (taken by an Autoflex MALDI-TOF-MS (Bruker)) and downloaded from the MALDI-TOF Mass Spectrometry Database of the RKI, freely accessible on Zenodo (version 3; Lasch et al., 2018). Subsequently, all custom MSPs were included in the project section of the Biotyper Compass Explorer software module (Bruker).

2.4. Validation spectra

Overall, 1591 single MALDI-TOF mass-spectra from all 1540 isolates were collected as a validation set. Of these, 884 spectra (from 840 isolates) belonged to the phylum *Bacillota*, including 483 (451) from *B. cereus*-group members, of which 183 (170) were taken from *B. cytotoxicus*. Further information on every individual spectrum and the reference spectra created outside the commercial MBT_K database can be found on MALDI-UP. This catalogue includes metadata of the isolates, cultivation conditions, the MALDI-System used (Bruker MALDI-TOF MS systems Autoflex, LT-microflex or Sirius), and the institute of the creator of the spectra (Table S1).

2.5. Validation procedure

To examine the effect of custom database additions on the identification results, two databases were compared using the validation spectra set: the latest commercial MBT_K database and the MBT_K database supplemented with 1086 user-made entries from MALDI-UP (MBT_K + MUP). These entries included 25 custom reference spectra of the *B. cereus* group (Table 1). In accordance with the manufacturer's notes, the first matching hits of the result list were assessed for the identification decision (Bruker). The first hit must have a score value of $\geq$2.0, and the second hit must show no contradictory species, likewise with a score value of $\geq$2.0. This simple rule was applied to the same extensive spectra set (n = 1591) for both database combinations. The evaluation of *B. cytotoxicus* as a targeted parameter was consequently performed in line with the guidelines created by the working group MALDI-TOF, pursuant to § 64 of the German Food and Feed Code (BVL, 2022; Rau et al., 2022), detailed in Tables S2 and S3 and summarized in Table 2.

3. Results and discussion

Food microbiology laboratories wishing to incorporate species-level identification of *B. cytotoxicus* by MALDI-TOF MS into their routine workflow must meet several requirements. On the one hand, a suitably large database is needed to feature enough reference spectra of the target organism. On the other hand, a formal validation for the

Table 2
Summarized validation report for the targeted identification of *Bacillus cytotoxicus* with MALDI Biotyper (Bruker) using two databases: MALDI Biotyper database revision K (MBT_K), and MBT_K extended by database entries from the MALDI-UP catalogue (MUP) (Tables S2 and S3). Criteria are aligned with the guidelines for validating species identifications using MALDI-TOF-MS in a single laboratory or in laboratory networks (BVL, 2022).

database:		MBT_K		MBT_K + MUP	
	requirement according to BVL guideline	n		n	
B. cytotoxicus	**n ≥ 20**	***183***		***183***	
identified		**177**		**182**	
identified rate	not specified		96.7 %		99.5 %
correct identification (true positive)		**177**		**182**	
true positive rate (inclusivity)	≥95.0 %		100 %		100 %
false negative		**0**		**0**	
false negative rate	≤1.0 %		0 %		0 %
non *B. cytotoxicus*	**n ≥ 30**	***1408***		***1408***	
Thereof spectra from					
B. cereus group bacteria		300		300	
other Bacillota		401		401	
further bacteria		707		707	
identified		**630**		**916**	
identified rate	not specified		44.7 %		65.1 %
correct identification (true negative)		**630**		**916**	
true negative rate (exclusivity)	≥99.0 %		100 %		100 %
false positive		**0**		**0**	
false positive rate	≤1.0 %		0 %		0 %
criteria for targeted identification fulfilled according to BVL (2022)			**yes**		**yes**

parameter itself as well as for non-target parameters is required for an accreditation of the workflow.

The commercial Bruker Biotyper database MBT_K contains only a small number of reference spectra for representatives of the *B. cereus* group (n = 13), including four reference entries for *B. cytotoxicus*. To improve the identification accuracy for *B. cereus* group members, we created and exchanged 25 custom reference entries for members of the *B. cereus* group, which have been made publicly available via the MALDI user platform, and which were combined with the current commercial database MBT_K (Table 1). Two options for analysis were created: either identification with the commercial database alone or with a combined database comprising the manufacturer's database and the in-house extension. Using the commercial MALDI Biotyper database (MBT_K), 177 of the 183 confirmed *B. cytotoxicus* isolate spectra (96.7 %) showed score values above 2.0 and were thus identified correctly (Table 2). The mean of the score-values for *B. cytotoxicus* using this set of individual spectra was 2.25 (Table S2). No false positive identification as *B. cytotoxicus* was observed, neither for other members of the *B. cereus* group, nor for any other species of *Bacillota* (n = 401) or even more distant microorganisms (n = 707) contained in the validation data set (Table 2). With the additional custom set of our own MSPs (MBT_K + MUP), the result was improved: 99.5 % of all spectra from verified *B. cytotoxicus* isolates were assigned correctly (182 of 183). The mean score value was increased to 2.37. Only one spectrum resulted in no identification, giving no assignment at all. Thus, *B. cytotoxicus* was distinctly separated by the MALDI-TOF MS system from all other members of the *B. cereus* group as well as from all other bacterial species used, and no false positive result was obtained. As shown in Table 2, validation for *B. cytotoxicus* fulfilled all formal criteria given in the BVL technical guidelines for targeted identifications by MALDI-TOF MS (BVL, 2022). Validation was successful for the commercial database MBT_K alone, as well as for the extended reference spectra collection (MBT_K + MUP). Due to the high number of well-documented individual spectra available, the number of samples significantly exceeded the sample sizes for validation currently specified for ISO 16140–4:2020 (100 target and 100 non-target strains for inclusivity and exclusivity testing, respectively). Having met all formal criteria of the BVL technical guidelines, the *B. cytotoxicus* parameter can be used in our laboratory for official food analysis under a flexible accreditation. An even more significant effect of the in-house database expansion was noted for the proportion of successful and correct identifications of the non-targeted bacteria species. For this taxonomically diverse isolate compilation only 44.7 % of all isolate spectra (n = 1408) were identified with MBT_K. In contrast, 65.1 % of these spectra were identified with the expanded database MBT_K + MUP. A closer look at the results for representatives of the *B. cereus* group other than *B. cytotoxicus* showed the difficulties of identification within this group using only the MBT_K database. Of the 300 spectra from this group, 168 (56.0 %) could be identified by using the minimum score value of 2.0, and all identified isolates were correctly assigned to the *B. cereus* group. The few amendments to the custom database as shown in Table 1 resulted in a remarkable increase in the number of identifications for this spectra set, up to 252 (84.0 %), with no new misclassification. The lower identification rate with MBT_K can probably be accounted for by the low number of reference spectra for the taxonomically complex *B. cereus* group available in the commercial database (Table 1). This issue is addressed in the manufacturer's database manual with specific instructions for the interpretation of such results (Bruker Biotyper rev. K manual). The problem has also been highlighted by the efforts made by several working groups to separate important species of this group using their own databases and special procedures (Chen et al., 2022; Doellinger et al., 2020; Lasch et al., 2009; Ulrich et al., 2019).

In addition to periodical updates of the commercial database, user driven exchange of custom entries focused on microorganism species of interest is helpful for extending the possibilities of in-house identification. With open catalogues providing a free download of spectra collections like the RKI database or inter-laboratory spectra exchange via the MALDI-UP list, user-made spectra or database entries are made available to the greater community (Lasch et al., 2018; CVUAS, 2015). The custom reference spectra of reliably assigned isolates of *B. cytotoxicus* and other members of the *B. cereus* group used in this study were independently created by several laboratories. The project section of the Bruker Biotyper software is an easy option for importing custom reference entries to improve identifications (Kostrzewa & Maier, 2017; Pranada et al., 2016). Apart from the technical knowledge necessary for MSP creation, the availability of defined and validly named isolates from public strain collections, own isolation or exchanges with other laboratories, is a prerequisite for having a sufficient number of addable reference spectra.

As shown in other examples, successful validation can be performed

based on a broad collection of well-documented MALDI-TOF MS single spectra (BVL, 2022; Rau et al., 2019). In addition to 841 spectra (93 for *B. cytotoxicus*, and 748 from other bacteria) from CVUA Stuttgart, 750 external spectra (46.9 %) were also integrated into this study (Table S2). This significantly reduced the workload for our individual laboratory. Moreover, with *B. cytotoxicus* as the parameter of interest, spectra of isolates from less common food sources (1 × semolina, 2 × camel milk, 5 × insect-based food, and 3 × instant pasta) were integrated into the compilation, in addition to the spectra of isolates from potato products, which are the most common source of isolates available in our laboratory (Contzen et al., 2014). The assembled single spectra used in our validation were generated on a total of eleven different Bruker MALDI-TOF MS systems. In addition to the common LT-Microflex instruments, spectra were also generated on Autoflex instruments and the newer Sirius series (all Bruker Daltonics, see Table S1). Individual spectra of *B. cytotoxicus,* generated over 15 years by six independent institutions, were combined, representing a variety of growth conditions and sample preparations. Within the framework of this study we could not find any of these combinations to be evidently detrimental to the quality of the generated spectra. However, a solid statistical evaluation of the effect of all applied combinations on spectra quality was not carried out. Nevertheless, the approach and its results demonstrate the robustness of the MALDI-TOF MS methodology in accordance with the guidance document used for validation (BVL, 2022). Use of this concept of validation will be systematically extended to other representatives of the *B. cereus* group. According to the MALDI-TOF MS results in our study, the parameters *B. mycoides* (incl. the former species "*B. weihenstephanensis*") or *B. anthracis* are potentially suitable for this approach. For the recently described number of new species of the *B. cereus* group, there are no commercial database entries available to date. In addition, the impact of these new species descriptions (e.g. Chen et al., 2023; Liu et al., 2017; Tohya et al., 2021) for the further development of international standards remains to be seen. Ultimately, food control is more oriented towards practical naming schemes that focus on food safety and less on the most up-to-date taxonomy. Transparently validated methods such as the one shown here for *B. cytotoxicus* can be very helpful in this context.

4. Conclusion

The accuracy of the identification of *Bacillus cytotoxicus* by means of MALDI-TOF MS was successfully improved. The validation of the method fulfils the criteria of the BVL technical guidelines as well as the sample sizes specified for ISO 16140-4, a prerequisite for accreditation of the procedure. The exchange of reference entries and validation spectra from several reliable sources provided a valuable input for conducting a transparent and robust validation study. This principle of validation can also be utilized for further applications.

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CRediT authorship contribution statement

Jörg Rau: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Danai Etter:** Writing – review & editing, Investigation, Funding acquisition. **Hendrik Frentzel:** Writing – review & editing, Investigation. **Peter Lasch:** Writing – review & editing, Investigation. **Matthias Contzen:** Writing – review & editing, Writing – original draft, Investigation.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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