



## Communication

# Discrimination of Bacteria Belonging to *Bacillus cereus* Group at Species Level by Fourier Transform Infrared Spectroscopy

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## Abstract

The *Bacillus cereus* group (*B. cereus* group) comprises several closely related species that share high genetic similarity but display markedly different phenotypic traits and pathogenic potential. Reliable and rapid discrimination at the species level remains challenging using conventional microbiological and molecular methods. In this study, Fourier Transform Infrared (FTIR) spectroscopy was evaluated as a rapid phenotypic approach to differentiate seven members of the *Bacillus cereus* sensu stricto (*B. cereus* s.s.), *Bacillus anthracis* (*B. anthracis*), *Bacillus thuringiensis* (*B. thuringiensis*), *Bacillus mycoides* (*B. mycoides*), *Bacillus toyonensis* (*B. toyonensis*), *Bacillus wiedmannii* (*B. wiedmannii*) and *Bacillus weihenstephanensis* (*B. weihenstephanensis*). A collection of 190 isolates previously characterized by whole genome sequencing was analyzed using the IR Biotyper system. Spectral data were processed through multivariate analyses, including principal component analysis and linear discriminant analysis, following a hierarchical classification strategy. FTIR spectroscopy enabled clear discrimination of *B. anthracis* from other members of the *B. cereus* group and allowed the separation of several additional species based on distinct spectral signatures. A further discrimination step permitted differentiation between *B. cereus* sensu stricto and *B. thuringiensis*, with minimal overlap. These findings demonstrate that FTIR spectroscopy represents a promising and rapid tool for species-level discrimination within the *B. cereus* group. While the results should be considered preliminary for species represented by a limited number of isolates, this approach shows strong potential as a complementary method to molecular techniques in routine diagnostics in food safety and veterinary microbiology.



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## 1. Introduction

The *Bacillus cereus* group is composed of about 22 closely related species of Gram-positive, rod-shaped and spore-forming bacteria [1]. These groups of microorganisms attract scientific interest not only because of their roles in human health, particularly foodborne illnesses and zoonotic infections, but also due to their ecological versatility and potential applications in biotechnology.

Accurate species-level discrimination within the *B. cereus* group is of critical importance in both clinical and epidemiological contexts, as different species are associated with markedly distinct risks and management strategies. In the field of food safety, the ability to distinguish *B. cereus* s.s. from closely related species is essential due to its well-established role in foodborne intoxications and infections mediated by emetic and diarrheal toxins. In veterinary and public health contexts, the rapid and reliable identification of *B. anthracis* remains a priority because of its high pathogenicity, zoonotic potential, and implications for biosafety and outbreak response. Moreover, other members of the *B. cereus* group, such as *B. thuringiensis*, *B. wiedmannii* and *B. toyonensis*, are increasingly recognized for their relevance in environmental monitoring, human health, and food production systems. Consequently, diagnostic approaches capable of resolving species-level differences within this group are essential to support risk assessment, surveillance, and appropriate control measures across diverse application areas.

However, the high genetic and phenotypic similarity of these microorganisms often makes discrimination challenging using traditional microbiological techniques.

Current methods for typing microbial strains often rely on labour-intensive, time-consuming, and expensive techniques such as whole genome sequencing (WGS) analysis, limiting their applicability in rapid-response scenarios. In this context, the IR Biotyper utilizing Fourier transform infrared (FTIR) spectroscopy, offers a novel approach, providing specific spectra for fast strain typing within approximately 3 h after bacterial growth, including sample preparation and spectra acquisition.

FTIR spectroscopy is a well-established and widely used analytical chemistry technique, recently introduced for the characterization of microorganisms relying on the strain-specific absorption patterns of the infrared radiation [2]. Technology leverages the principle of vibrational spectroscopy to detect subtle chemical differences between bacterial strains, facilitating species-specific discrimination [3]. Each microbial strain exhibits a unique IR radiation absorption spectrum, which represents the fingerprint obtained based on the biomolecular components of the cell. The FTIR-based IR Biotyper<sup>®</sup> system (IRBT—Bruker Daltonics GmbH & Co. KG, Bremen, Germany) proved to allow a promising novel approach in the field of microbial strain typing [4] with successful applications reported in different microbiology fields, namely food, veterinary and environmental microbiology [5–8], hospital hygiene [9–15] and probiotic production [16,17]. In this study, we evaluated the discriminatory power of IRBT to discriminate at the species level some members of the *B. cereus* group, investigating a cohort of animal and food-related isolates previously characterized with biomolecular methods. Definitively, the aim of this study was to demonstrate the possibility to use IRBT as a fast method for the identification and differentiation of seven species belonging to the *B. cereus* group, as an alternative to more common biomolecular analyses.

Compared to our previous study focusing on the discrimination between *B. anthracis* and *B. cereus* s.s. [18], the present work substantially expands the scope of FTIR spectroscopy application within the *B. cereus* group. Here, we extend the analysis from a binary species comparison to a multispecies framework, evaluating the discriminatory performance of the IR Biotyper across seven phylogenetically closely related species. Moreover, we introduce a hierarchical, multistep classification strategy that mirrors realistic diagnostic workflows, enabling progressive species differentiation within this complex group. This study therefore represents one of the first systematic assessments of FTIR spectroscopy for broad species-level discrimination within the *B. cereus* group, supporting its potential role as a complementary tool in food, veterinary, and public health microbiology.

## 2. Materials and Methods

A total of 190 *B. cereus* complex isolates were included in this study (n.51 strains of *B. cereus* s.s. isolated from various food, n.110 strains of *B. anthracis*, n.2 *B. thuringiensis*, n.1 *B. mycoides*, n.7 *B. toyonensis*, n.18 *B. wiedmannii*, n.1 *B. weihenstephanensis*), collected at the Anthrax Reference Center of Italy at the Istituto Zooprofilattico Sperimentale of Puglia and Basilicata. All used strains were previously typed by WGS analysis.

For IRBT analysis, strains were cultured on Tryptone Soy Agar (Liofilchem, Roseto degli Abruzzi, Italy) overnight at 37 °C. A 10 µL loopful of bacterial culture was resuspended in 100 µL of distilled sterile water and incubated at 98 °C for 30 min. Later, 50 µL were taken and added to 50 µL of 70% (vol/vol) ethanol in the tubes of the IR Biotyper kit (Bruker Daltonics, Bremen, Germany), which contain metal cylinders, spitting to obtain a homogeneous suspension. Finally, 15 µL of bacterial suspension was spotted into the IRBT silicon sample plate in three replicates and dried at room temperature. For each sample, three biological replicates (independent bacterial cultures on different days) were analyzed. Quality control was performed for every run using the Infrared Test Standards (IRTS 1 and 2) provided by the manufacturer (Bruker Daltonics, Bremen, Germany).

FTIR spectra were acquired, processed and analyzed by the IRBT system (Bruker Daltonics, Bremen, Germany), using the IRBT Opus and Client v3.1 software. Spectra were acquired in transmission mode in the spectral range of 4000–500 cm<sup>−1</sup> (mid-IR), vector-normalized using the Savitzky–Golay algorithm, and the second derivative over 9 datapoints was calculated.

Exploratory data analysis was performed using PCA (principal components analysis) and LDA (linear discriminant analysis), after spectra acquisition. The different wavenumber regions of the IR spectra, corresponding to the absorption of different classes of biomolecules, were investigated, to establish which wavenumbers provide the highest discriminatory power for the desired differentiation [19].

A multistep approach, using different wavenumber regions in each step, was found to be the optimal way to proceed, following a hierarchical approach. In the first step, a binary differentiation between *B. anthracis* and the other species was performed, as described by Manzulli et al. [18]. In the second step, the further separation of *B. cereus* s.s./*B. thuringiensis* from all the other species was achieved, analyzing the spectra in the wavenumber region corresponding to C-H bonds, amidic groups, carbohydrates and fingerprint (3000–2800, 1800–1700 and 1400–700 cm<sup>−1</sup>). In the third step, *B. cereus* s.s. was differentiated from *B. thuringiensis*, using the wavenumber region corresponding to carbohydrates and fingerprint.

In each step, LDA algorithm was used to create predictive models, to assess the suitability of the method as a potential tool to type unknown samples. As LDA is a supervised method, the assignment of a group identifier to define the classes to be differentiated was necessary, as well as the check for overfitting. Each LDA model was built using 50% of the strains (randomly selected) for its training, assigning the species as a group identifier (in this case, the species). The remaining 50% strains, not assigned to any group identifier (species), were used as testing set, to check the robustness of the model. An exception was made for *B. weihenstephanensis* and *B. mycoides*, for both of which the only available isolate was included in the training set.

## 3. Results

### 3.1. First Step

The IR spectral region between 1300 and 700 cm<sup>−1</sup>, corresponding to the absorption of polysaccharides and fingerprinting, proved to be the best for the discrimination of *B. anthracis*/*B. cereus*, in concordance with our previously published study [18]. Exploratory

data analysis performed with PCA/LDA showed that *B. anthracis* and the other species of the *B. cereus* group included in this study form two clearly resolved clusters (Figure 1).

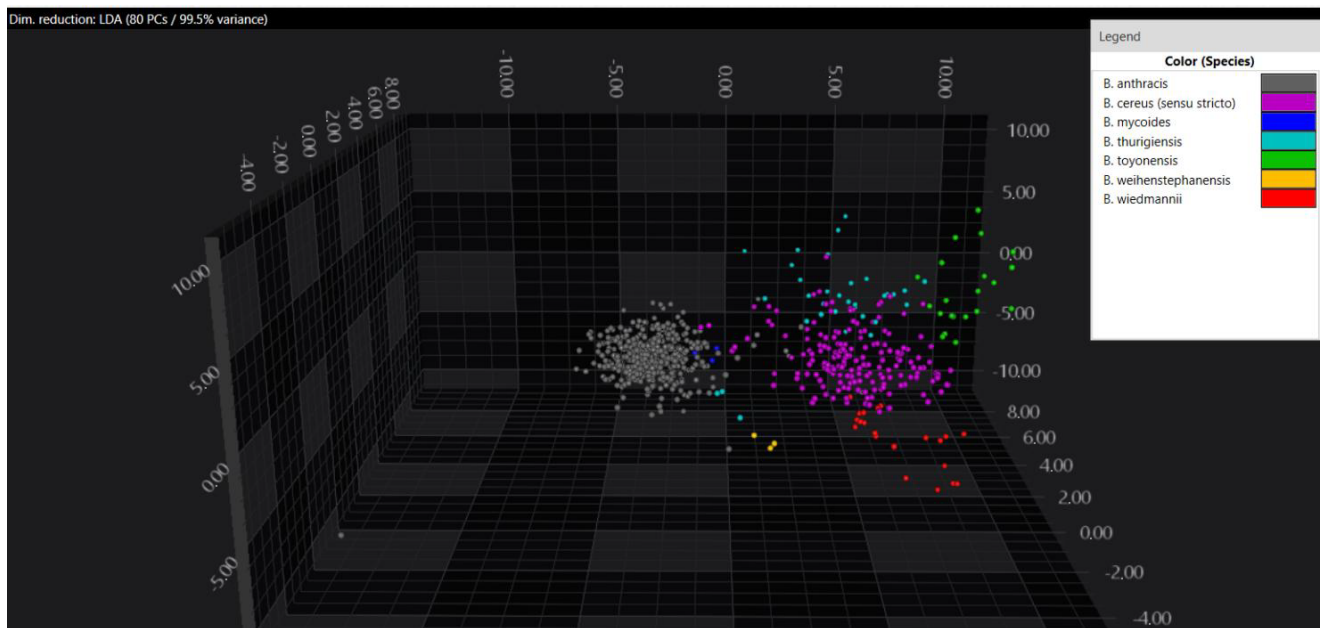


**Figure 1.** Three-dimensional LDA scatterplot showing the separation of *B. anthracis* isolates (in grey) from the other species (*B. cereus sensu stricto* in purple, *B. thuringiensis* in cyan, *B. wiedmannii* in red, *B. toyonensis* in blue, *B. weihenstephanensis* in yellow and *B. mycoides* in blue). Each geometrical form represents a spectrum. LDA was performed using 80 principal components, corresponding to 99.9% of the variance. Axis x, y and z represent LD1, LD2 and LD3, respectively.

The LDA model proved to be very robust and showed a very good separation between *B. anthracis* and all the other species. The test spectra (represented by crosses) clustered correctly in the group to which they belonged (Figure 2).

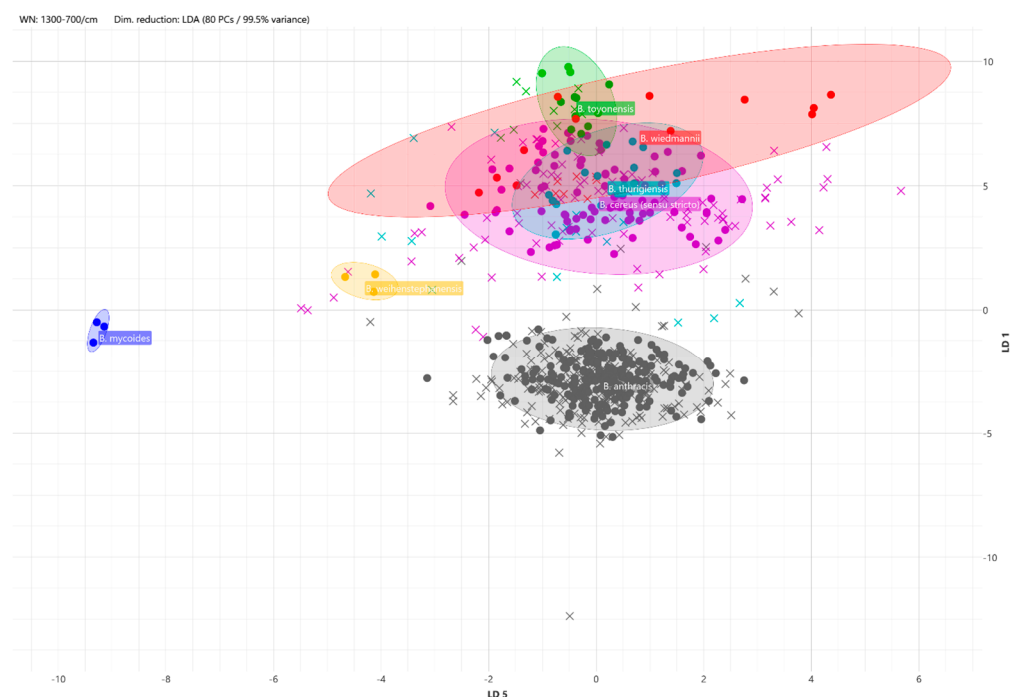


**Figure 2.** Two-dimensional LDA scatterplot showing the separation of *B. anthracis* isolates (in grey) from the other species (*B. cereus sensu stricto* in purple, *B. thuringiensis* in cyan, *B. wiedmannii* in red,

*B. toyonensis* in blue, *B. weihenstephanensis* in yellow and *B. mycoides* in blue). Each geometrical form represents a spectrum. The spectra belonging to the training set are depicted as spheres, while the spectra belonging to the testing set are depicted as crosses. LDA was performed using 80 principal components, corresponding to 99.9% of the variance. Axis x and y represent LD1 and LD2, respectively. The ellipses indicate the 95-confidence interval.

### 3.2. Second Step

The IR spectral region comprising C-H bonds ( $3000\text{--}2800\text{ cm}^{-1}$ ), amidic groups ( $1800\text{--}1700\text{ cm}^{-1}$ ), polysaccharides ( $1300\text{--}900\text{ cm}^{-1}$ ) and fingerprinting ( $900\text{--}700\text{ cm}^{-1}$ ) enabled the best discrimination of *B. cereus* s.s./*B. thuringiensis* from the other species. Exploratory data analysis performed with PCA/LDA showed that *B. anthracis* and the other species of the *B. cereus* group included in this study form two well-separated clusters (Figure 3).

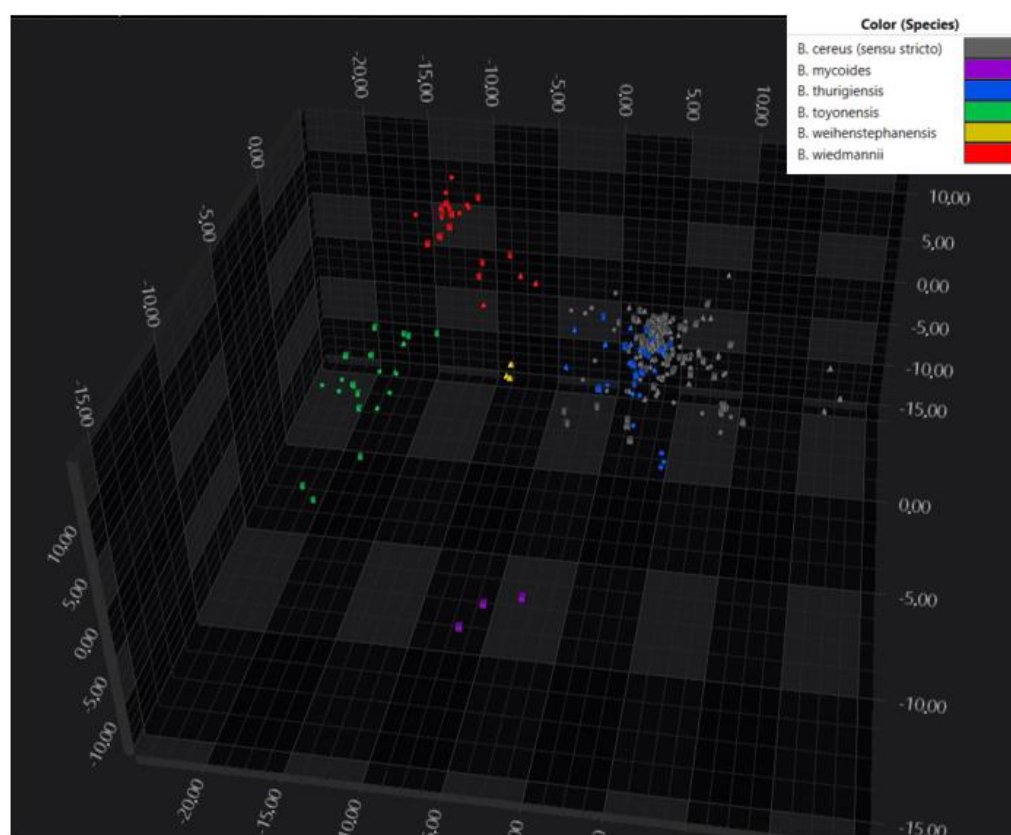


**Figure 3.** Three-dimensional LDA scatterplot showing the clusters of the different species of *B. cereus* group after the removal of *B. anthracis*. *B. wiedmannii* (red), *B. toyonensis* (green), *B. weihenstephanensis* (yellow), and *B. mycoides* (purple) are well differentiable from *B. cereus* s.s. (grey) and *B. thuringiensis* (blue), which appear indistinguishable. Each geometric form represents one spectrum. LDA was performed using 60 principal components, corresponding to 99.9% of the variance. Axis x, y and z represent LD1, LD2 and LD3, respectively.

The LDA model shows an equivalent clustering (Figure 4).

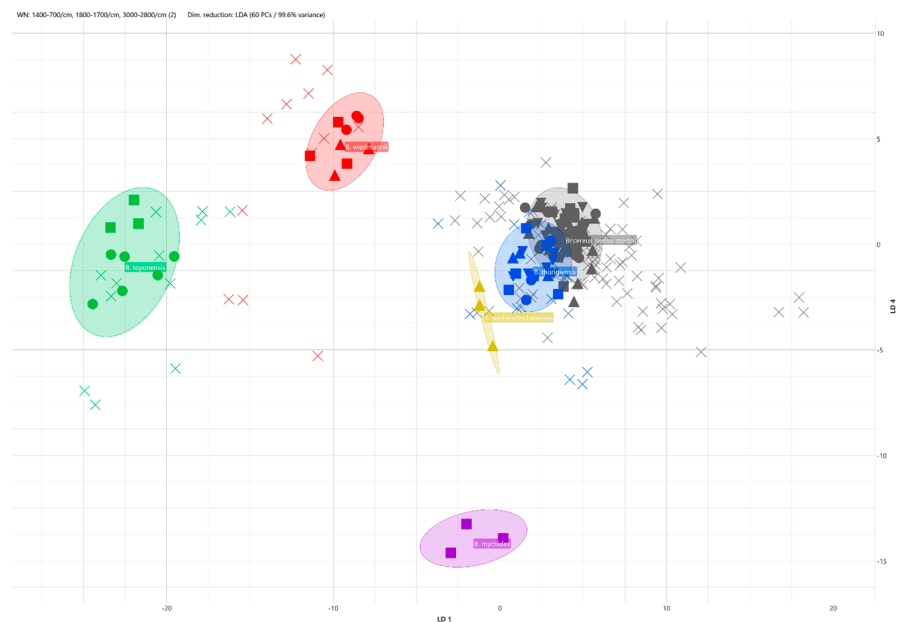


**Figure 4.** LDA model showing the clusters of the different species of *B. cereus* group after the removal of *B. anthracis*. *B. wiedmannii* (red), *B. toyonensis* (green), *B. weihenstephanensis* (yellow), and *B. mycoides* (purple) are well separated from *B. cereus* s.s. (grey) and *B. thuringiensis* (blue), which appear indistinguishable. Each geometric form represents one spectrum. LDA was performed using 60 principal components, corresponding to 99.9% of the variance. Axis x and y represent LD1 and LD2, respectively. The filled symbols represent spectra of training isolates (50% of the dataset). For each bacterial species, different shapes represent different isolates. Crosses represent the test spectra, which were not used to build the model. The ellipses correspond to the 95-confidence interval.

### 3.3. Third Step

The IR spectral region  $1300\text{--}800\text{ cm}^{-1}$  (corresponding to the carbohydrates) enabled the discrimination between *B. cereus* s.s. and *B. thuringiensis*, except for one *B. cereus* s.s. strain, which clustered in the *B. thuringiensis* group (Figures 5 and 6).

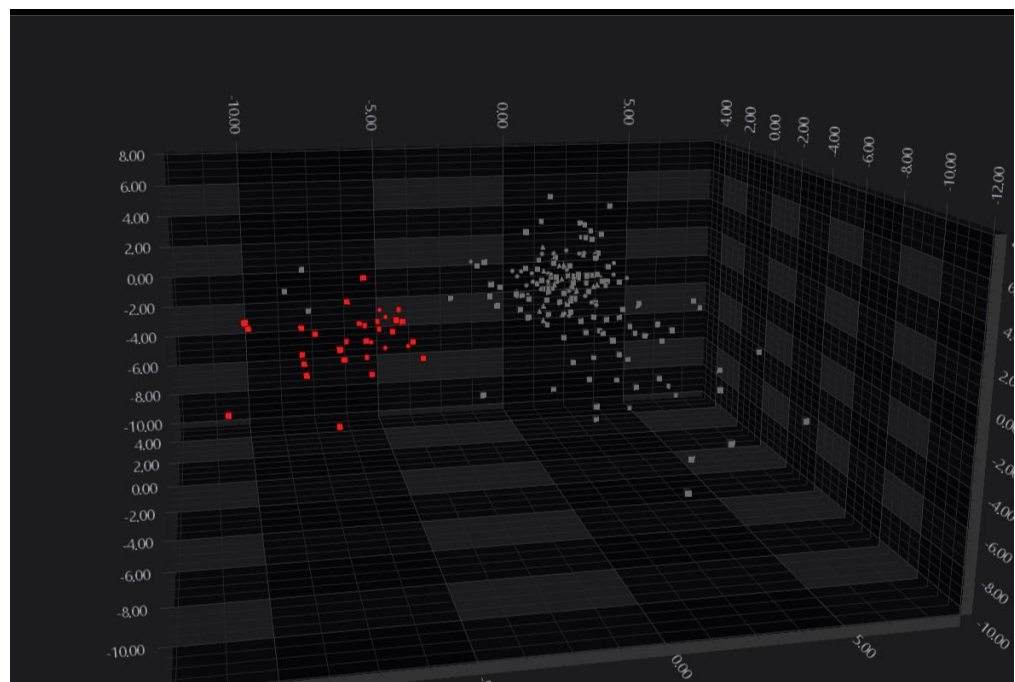


**Figure 5.** Three-dimensional LDA scatterplot showing the clustering of *B. cereus* s.s. (grey) and *B. thuringiensis* (red), after the removal of all the other species. Each square represents one spectrum.

LDA was performed using 60 principal components, corresponding to 99.7% of the variance. Axis x, y and z represent LD1, LD2 and LD3, respectively. The outlier *B. cereus* s.s. strain clustering with *B. thuringiensis* is represented by the three grey squares in the red cluster.

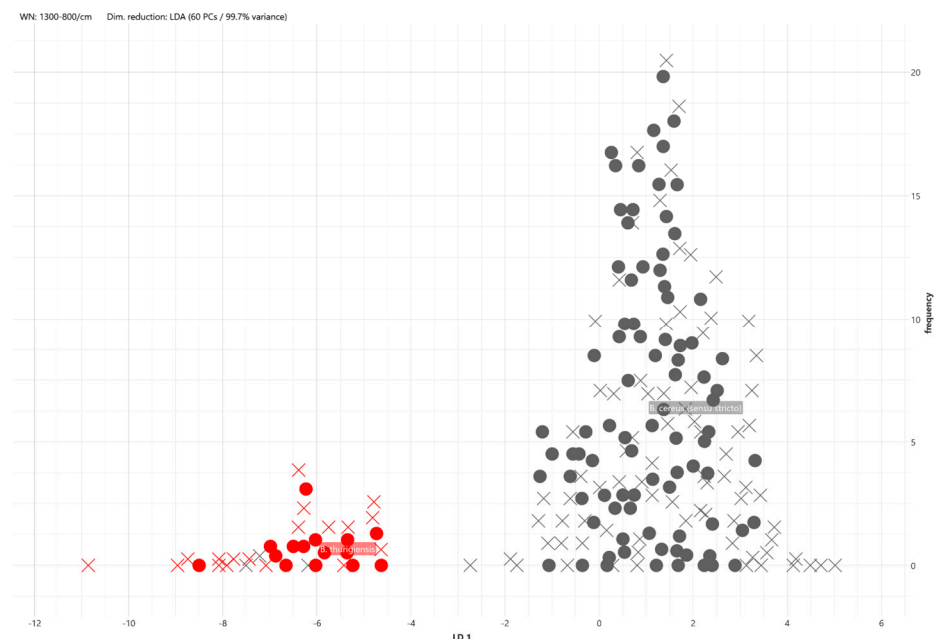


**Figure 6.** LDA model showing the separation between *B. cereus* s.s. (grey) and *B. thuringiensis* (red). Each geometric form represents one spectrum. LDA was performed using 60 principal components, corresponding to 99.9% of the variance. Axis x and y represent LD1 and LD2, respectively. The filled symbols depict spectra of training isolates (50% of the dataset), while the crosses depict the test spectra, which were not used to build the model. The outlier *B. cereus* s.s. strain clustering with *B. thuringiensis* is represented by the three grey crosses in the red cluster.

#### 4. Discussion

The *B. cereus* group comprises a complex of closely related bacterial species, which share a high degree of genomic similarity but exhibit remarkably diverse phenotypes and pathogenic behaviours [1]. Discriminating among these species remains a significant challenge in routine diagnostics due to overlapping biochemical profiles and the limitations of conventional assays. In this context, our study demonstrates the efficacy of FTIR spectroscopy as a rapid and reliable method for differentiating species within the *B. cereus* group. These findings align with previous studies supporting the utility of FTIR spectroscopy for correct and reliable bacterial typing [20].

In our study, multivariate analysis of the spectral data revealed reproducible clustering patterns that were species-specific, suggesting a strong correlation between spectral signatures and taxonomic identity.

Of relevance is the robust discrimination of *B. anthracis* from all other species included in the analysis. Given the clinical severity and biosafety implications associated with *B. anthracis*, the availability of a rapid, culture-based, and non-genomic screening method represents a valuable asset for reference and surveillance laboratories. These results are consistent with earlier findings and further support the reliability of FTIR spectroscopy for high-consequence pathogen differentiation.

The ability of FTIR to differentiate between *B. cereus* and *B. thuringiensis*, two species historically difficult to distinguish due to their nearly identical 16S rRNA gene sequences and shared ecological niches, is also significant. While whole genome sequencing (WGS) remains the gold standard for taxonomic resolution, it is time-consuming, costly, and not

always feasible in routine settings [21]. FTIR offers a complementary solution that is both rapid and more cost-effective for laboratories that are in possession of this instrument. Furthermore, the technique's high throughput potential and minimal sample preparation requirements make it suitable for integration into diagnostic workflows in clinical, veterinary, and food microbiology laboratories. As antimicrobial resistance and zoonotic threats continue to rise, the need for fast and accurate microbial identification methods becomes increasingly urgent [22]. In this light, FTIR could play a pivotal role in strengthening surveillance and early detection systems, especially for emerging or re-emerging *Bacillus* pathogens.

While our findings are promising, certain limitations must be acknowledged. FTIR analysis may be influenced by culture conditions, growth phase, and media composition, necessitating standardization across laboratories for reproducible inter-lab comparisons [20]. Additionally, the uneven distribution of isolates across species, particularly for *B. mycoides* and *B. weihenstephanensis*, restricts the generalizability of conclusions for these species. Despite this limitation, the hierarchical analytical strategy adopted in this study allowed us to explore the potential of FTIR spectroscopy as a rapid phenotypic screening tool within the *B. cereus* group. The results demonstrate that, even in the presence of an imbalanced dataset, FTIR spectroscopy can provide informative discriminatory signals, supporting its use as a complementary approach to genomic methods in routine diagnostic and surveillance settings. Future studies based on larger and more balanced strain collections will be essential to validate and extend these findings. Moreover, inter-laboratory validation will be necessary, integrating quantitative performance metrics to further substantiate the diagnostic value of this approach.

## 5. Conclusions

This study, together with a previously published work, represents a preliminary but significant step toward evaluating the IR Biotyper-based FTIR spectroscopy as a tool for species-level discrimination within the *B. cereus* group. The results demonstrate that this approach can reliably distinguish *B. anthracis* from closely related species and can further differentiate several other members of the group using a hierarchical analytical strategy.

Although the findings for species represented by limited numbers of isolates should be interpreted with caution, the overall results support the potential of FTIR spectroscopy as a rapid, cost-effective, and complementary method to molecular techniques such as whole genome sequencing. With further validation on larger and more diverse datasets, and with appropriate standardization, this methodology could contribute meaningfully to routine diagnostics, epidemiological surveillance, and outbreak investigations involving *Bacillus* species.

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