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Simple analytical approach to determine the microbiological profile of bee pollen by MALDI-TOF

Estela Prieto, Ana M. Ares, José Bernal, Silvia Valverde *

Analytical Chemistry Group (TESEA), I. U. CINQUIMA, Faculty of Sciences, University of Valladolid, 47011, Valladolid, Spain

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ABSTRACT

Bee pollen is a natural food supplement collected by honeybees that has significant nutritional value and therapeutic qualities, including antioxidant, antibacterial, and anticarcinogenic properties. Due to its hygroscopic nature, bee pollen readily promotes microbial growth; however, there is no specific legislation for bee pollen in the European Union. Microbiological contamination therefore stands out as a crucial factor in determining its quality. To address this, the study focused on isolating bacterial and fungal colonies from bee pollen using various culture media, followed by a protein extraction based on a lysis process. Identification was achieved through the Biotyper-MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization coupled to Time-of-Flight detector) system. The greenness and blueness of the methods were assessed using different tools, which classified them as environmentally friendly and practical. Thirty-two samples were analyzed using proposed sample treatment approaches. Data were processed using chemometric techniques based on linear models. The results revealed the presence of both beneficial and potentially pathogenic species, including key representatives such as *Bacillus licheniformis*, *Micrococcus luteus*, and *Aspergillus fumigatus*. These findings highlight the importance of microbiota characterization in bee pollen and demonstrate that MALDI-TOF is a rapid and effective tool for profiling culturable microbial communities in this matrix.

1. Introduction

Nowadays, there is a growing trend in the consumption of natural and organic food, driven by an increasing awareness of the direct relationship between diet and various associated diseases, pathological processes, and the immune system. Among beehive products valued for their bioactive properties, bee pollen stands out as a particularly sought-after natural supplement (Komosinska-Vassev et al., 2015). Bee pollen consists of a mixture of flower pollen grains collected by honeybees, agglutinated with nectar and enzymes secreted by their salivary glands, forming granules of approximately 2 mm in diameter (Komosinska-Vassev et al., 2015; Maruyama et al., 2010; Morais et al., 2011; Pascoal et al., 2014) that are transported in special baskets on their hind legs. From a nutritional perspective, bee pollen is considered a complete food supplement, containing all essential amino acids for humans, as well as carbohydrates (13–50 %), fiber (0.3–20 %), proteins (10–40 %), and lipids (1–10 %) and a rich mineral profile (Campos et al., 2008; Pascoal et al., 2014; Valverde et al., 2023). Moreover, it contains biologically active compounds such as flavonoids and polyphenols that have been linked to antioxidant, anti-inflammatory, and antibacterial properties.

Recently, researchers have shown increased interest in the microbiological composition of these bee products due to their direct relationship with bee health and consumer safety (Martín-González et al., 2023; Pełka et al., 2021; Uçar et al., 2022). Bee pollen microbiota can produce antimicrobial peptides, surfactants, siderophores, proteolytic enzymes, and cell wall-degrading enzymes. Among the beneficial microbial taxa, *Bacillus* spp. are of particular interest due to their well-documented ability to synthesize antimicrobial substances and enzymes with potential use in probiotics, plant growth promotion, and biodegradation. (Martín-González et al., 2023; Pełka et al., 2021). In addition to *Bacillus*, other genera such as *Pseudomonas*, *Paenibacillus*, and certain lactic acid bacteria (LAB) have also been reported in bee products and are being explored for applications in biocontrol, bioremediation, and functional food development. These microbial communities not only contribute to the preservation and quality of bee pollen but also represent a promising source of strains for biotechnological innovation.

The study of the microbiological composition of bee pollen has

* Corresponding author.
E-mail address: silvia.valverde@uva.es (S. Valverde).

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become a quality parameter, indicating the presence of contaminants and providing markers to establish relationships with product handling processes and hive health (Erler et al., 2022; Mauriello et al., 2017). Variations in microbial composition can reflect environmental conditions, botanical origin, seasonal changes, and contamination introduced during processing (Berg et al., 2020; Uçar et al., 2022). Therefore, microbiological profiling is a valuable tool for monitoring safety and traceability in bee pollen products.

Several analytical approaches have been employed to characterize the microbiota of bee pollen. Metagenomics based on polymerase chain reaction (PCR) remains the most widely used method for genomic-level analysis of bacterial and fungal communities (Bizzini et al., 2010; Friedle et al., 2021; Guo et al., 2024; Quero et al., 2016; Schäfer et al., 2014; Uçar et al., 2022). PCR has been the principal technique in cell identification due to its sensitivity and reproducibility. However, it presents some significant limitations such as its analysis time, which can take up to 3 days, and it requires highly qualified personnel. In addition to genomic techniques, proteomics has been applied to identify microorganisms through ribosomal protein profiling, offering species-level resolution (Bizzini et al., 2010; Quero et al., 2016; Schäfer et al., 2014). As shown in Table S1, most studies in recent years have relied primarily on PCR-based methods for identifying fungi and bacteria in bee pollen, often in combination with culture and sequencing approaches. This underscores the need for faster and equally accurate alternatives for microbial identification in food matrices.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) has emerged as a viable alternative to polymerase chain reaction as a highly promising method for microorganism identification (Jordana-Lluch et al., 2012). Unlike PCR, which targets genetic material, MALDI-TOF analyzes the mass spectra of highly conserved cellular proteins, primarily ribosomal proteins to generate a unique fingerprint for each microorganism. Compared with molecular approaches such as 16S rRNA or ITS sequencing, MALDI-TOF provides a faster and more cost-effective alternative for microbial identification. It allows high-throughput analysis with minimal sample preparation and reagent use, offering excellent reproducibility and sufficient taxonomic resolution for quality control applications (Ghyselinck et al., 2011; Lao et al., 2021; Seibold et al., 2010). These spectral profiles are then matched against reference libraries, such as the MALDI Biotyper v.3.1 (Bruker Daltonics, Bremen, Germany), enabling identification at the genus and species levels (Seng et al., 2009; Singhal et al., 2015). It is a technique that offers a rapid identification of isolated microorganisms, able to identify more than 90 microorganisms in 1 h (Calderaro and Chezzi, 2024). Despite the fact that the MALDI-TOF system has been used in numerous clinical microbiology laboratories (Bizzini et al., 2010; Deak et al., 2014; Gonzalo et al., 2021; Jordana-Lluch et al., 2012; Rodríguez-Sánchez et al., 2016; Xue et al., 2021), its utilization for the identification of bee pollen microorganisms is still underestimated. Given its potential to identify viable, culturable organisms with high taxonomic resolution, MALDI-TOF represents a promising tool for routine microbial profiling of bee products.

Although recent European and Spanish regulations emphasize the importance of preserving the natural constituents of apicultural products such as honey for authenticity and traceability purposes (BOE, 2003; BOE, 2025), no specific legislation currently addresses bee pollen. Some countries have established their own quality standards for bee pollen, such as Argentina through its Food Code (CODIGO ALIMENTARIO ARGENTINO, 1990). However, these regulations are generally limited in scope and provide little detail regarding microbiological criteria or safety requirements. To date, no internationally harmonized legislation exists for bee pollen. In this context, the microbiological characterization of bee pollen may provide a complementary tool to support product authentication and quality control. However, despite the emerging interest in the microbiological safety and bioactivity of food products, there is a lack of comprehensive studies applying MALDI-TOF to characterize culturable microorganisms, including both bacteria and fungi, in bee pollen samples. To our knowledge, this is one of the first studies to evaluate the feasibility of using MALDI-TOF for the identification of viable microbial species isolated from bee pollen.

Therefore, the aim of this study was to isolate and identify culturable bacteria and fungi from bee pollen samples collected from different botanical sources, using protein profiling and the Biotyper-MALDI-TOF system. This work contributes to a better understanding of the microbial composition of bee pollen and demonstrates the potential of this technique as a rapid and reliable tool for quality control and microbiological monitoring in beehive products. In addition, this study aimed to evaluate the proposed methodology from the perspective of environmental sustainability and practical applicability, in line with the principles of green and blue analytical chemistry. To this end, the method was assessed using Analytical Greenness Calculator (AGREE) (Pena-Pereira et al., 2020), Analytical Greenness Calculator for Sample Preparation (AGREEprep) (Wojnowski et al., 2022) and Modified Green Analytical Procedure Index (MoGAPI) (Mansour et al., 2024) tools, which are designed to measure the extent to which analytical procedures and sample preparation comply with the principles of Green Analytical Chemistry and Green Sample Preparation. The Blue Analytical Chemistry Index (BAGI; Manousi et al., 2023) was also applied to evaluate the practicality and operational feasibility of the method in routine laboratory conditions. It is worth noting that the use of these metrics is still not widespread in the field of microbiological analysis, despite their potential to provide a comprehensive assessment of methodological sustainability.

2. Materials and methods

2.1. Materials and reagents

EA-240 precision analytical balance (Mettler Toledo, Darmstadt, Germany); a Reax Control shaker (Heidolph, Schwabach, Germany); a Vevor MC-7S mini centrifuge (Vevor, Germany); an Ultra-Turrax homogenizer (Thermo Fisher Scientific, Waltham, MA, USA); inert homogenizing ceramics (Agilent Technologies, Santa Clara, CA, USA); and 2 mm borosilicate glass beads (Sigma–Aldrich, Steinheim, Germany), ultrasonic bath and an autoclave, both from J.P. Selecta (Barcelona, Spain), chloroform, methanol, ethanol, acetonitrile, and isopropanol (HPLC grade), all obtained from CARLO ERBA Reagents (Val-de-Reuil, France), formic acid and ammonium formate from Sigma–Aldrich (Steinheim, Germany) and sodium chloride from PanReac AppliChem (Barcelona, Spain) were used for sample treatment. PEG1000 calibrant (10 mg/mL); and the MALDI matrices HCCA (α-cyano-4-hydroxycinnamic acid), DHB (2,5-dihydroxybenzoic acid), DCTB (trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenyl]malononitrile), and DIT (dithranol), all purchased from Sigma–Aldrich (Steinheim, Germany) were used for the MALDI-TOF analysis. Ultrapure water was obtained using Milli-RO and Milli-Q purification units (Millipore, Bedford, MA, USA). For instrument calibration, Bacterial Test Standard calibrant (Bruker Daltonics, Bremen, Germany) was used. Culture media were prepared with Sabouraud dextrose agar (SDA) with chloramphenicol from Labkem (Premià de Dalt, Barcelona, Spain) and LB broth and bacteriological agar from Becton Dickinson (Sparks, MD, USA).

2.2. Samples

A total of 32 bee pollen samples were selected. Twelve samples were acquired from local stores, all of which were multifloral in origin and supplied in a dried state. An additional twenty samples were obtained from experimental beehives donated by the Apicultural and Agroenvironmental Research Center (CIAPA) in Marchamalo (Guadalajara, Spain). The botanical origin of the experimental samples was determined through melissopalynological analysis and is detailed in Table S2 (Supplementary Material). Before analysis, all samples were dried for 3 days at 45 °C under a relative humidity between 12 % and 20 % until

weight stabilization; commercial samples, already dehydrated at the time of purchase, lost less than 5 % (w/w) of their weight, while experimental samples showed an average moisture loss of around 10 % (w/w). After drying, samples were ground, homogenized, and stored in a desiccator until analysis.

2.3. Growth conditions and isolation

To isolate a wide range of microorganisms from bee pollen, the protocol was optimized by selecting suitable culture media, dilution factors, seeding volumes, and incubation conditions to enhance microbial growth and ensure well-separated colonies for individual isolation and identification by MALDI-TOF. Based on previous studies on bee products (Erkmen, 2021; Mauriello et al., 2017), Luria–Bertani (LB) agar was selected for bacterial growth and Sabouraud dextrose agar (SDA) supplemented with 0.05 g/L chloramphenicol was used to promote fungal growth while suppressing bacterial overgrowth (Bonnet et al., 2020). Both media were prepared in ultrapure water according to the manufacturer's instructions, sterilized at 121 °C for 20 min, and poured into sterile Petri dishes under laminar flow conditions at approximately 50 °C; once solidified, the plates were stored at 4 °C until use.

For seeding, bee pollen samples were diluted to 10^{-2} g/mL in sterile ultrapure water, and 20 μL of this suspension was spread onto the agar surface using a flame-sterilized glass spreader, with all procedures carried out inside a laminar flow cabinet to prevent contamination. This dilution was selected based on preliminary tests that showed it consistently produced colony-forming unit (CFU) counts within the optimal range of 20–200 colonies per plate (Jett et al., 2018), allowing for both reliable quantification and morphological differentiation. The inoculated LB plates were incubated at 30 °C for bacterial growth, while SDA plates were incubated at 35 °C to favor fungal development, both for periods ranging from 3 to 7 days depending on the growth rate of the microorganisms present. Following incubation, colonies with distinct morphologies were selected, subcultured to obtain pure isolates, and stored under refrigeration for subsequent protein extraction and identification by MALDI-TOF within 24 h.

2.4. Sample treatment of isolates

For bacterial isolates, protein extraction was performed directly on the MALDI target plate following standard protocols (Bizzini et al., 2010; Jordana-Lluch et al., 2012). A portion of each colony was collected using a sterile wooden stick and applied to the target spot. 1 μL of formic acid 70 % (v/v) was then added, and the sample was allowed to dry at room temperature before the matrix was applied.

In contrast, fungal colonies required a more elaborate protocol due to the rigidity of their cell walls, which hinders efficient extraction by formic acid alone. At this stage, alternative Bruker workflows such as the Liquid Cultivation and Extraction protocol were not evaluated. Instead, the procedure was adapted from the Bruker Biotyper protocol and previously reported methods (Lau et al., 2012;Luethy and Zelazny, 2018; Xu et al., 2021) and complemented with additional mechanical disruption steps to enhance protein release from filamentous fungi. Approximately a 5 mm-diameter portion from the center of each fungal colony, avoiding peripheral mycelium was suspended in 1 mL of ultrapure water. The central region was chosen to ensure sufficient biomass while reducing agar carry-over from the plate surface. After 1 min of agitation and centrifugation at 7000 rpm for 2 min, the supernatant was removed. The pellet was then mixed with 1.2 mL of a mixture of ethanol: water (3:1, v/v) for 10 min of sonication. After a second centrifugation (7000 rpm, 5 min) and the supernatant was discarded. To facilitate cell lysis and protein release, ceramic homogenizer beads were added to the pellet along with 80 μL of a mixture of acetonitrile and formic acid 70 % (1:1, v/v). This mixture was sonicated for 15 min at 35 °C, followed by centrifugation at 7000 rpm for 5 min. The resulting supernatant was stored at refrigeration (4 °C) temperature until analysis. The complete workflow for bacterial and fungal sample preparation is summarized in Fig. 1.

2.5. MALDI-TOF analysis

Microbial isolates were identified by MALDI-TOF using an Autoflex maX mass spectrometer (Bruker Daltonics, Bremen, Germany). For bacterial isolates, protein extraction was performed directly on the target plate as described in section 2.3, and 1 μL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (10 mg/mL in acetonitrile, water and trifluoroacetic acid mixture; 50:48:2, v/v/v) was added once the sample had dried. For fungal isolates, 1 μL of the protein extract was deposited on the MALDI target plate, and once dried, overlaid with 1 μL of the same HCCA matrix solution. Each isolate was prepared and analyzed in duplicate. Prior to analysis, the equipment was externally calibrated using the Bruker Bacterial Test Standard, composed of a protein extract of *Escherichia coli* DH5α spiked with RNase A and myoglobin, covering a mass range of 3.6–17 kDa. Spectra were acquired in positive linear mode within a mass range of 2–20 kDa, using a 1000 Hz laser at 335 nm, with the following operating parameters: ion source 1 at 19.62 kV, ion source 2 at 18.27 kV, lens voltage at 7.49 kV, and detector voltage at 3072 V. Data acquisition and analysis were conducted using the MALDI Biotyper Compass FlexSeries 1.4 software, including FlexControl 3.4 and FlexAnalysis 3.4. Spectral identification was performed with the MALDI Biotyper 3.1 software using the Bruker reference database (DB-6903), which includes 6903 reference spectra (6120 bacteria, 776 yeasts, and 7 archaea). Spectral similarities were expressed as log(score) values, automatically calculated by the Biotyper software. According to the manufacturer's guidelines, scores $\geq$2.300 are indicative of highly probable species-level identification, scores between 2.000 and 2.299 correspond to secure genus- and probable species-level identification, scores between 1.700 and 1.999 are consistent with probable genus-level identification, and scores $<$1.700 are considered unreliable. The main parameters for MALDI-TOF analysis are summarized in Table S3.

2.6. Statistical analysis

Pearson's correlation coefficients, principal component analysis (PCA), and hierarchical cluster analysis (HCA) were performed using IBM SPSS Statistics 29.0.2 software (SPSS Inc., Chicago, IL, USA). Heatmap representations of the correlation matrix were generated in Python 3.11.

3. Results and discussion

3.1. Culture method optimization

Four different culture media were initially tested: Luria–Bertani (LB) and Terrific Broth (TB) for bacteria, and SDA with chloramphenicol (with and without tartaric acid) for fungi and yeasts. LB agar provided higher colony counts and greater morphological diversity than TB, which yielded fewer and less varied colonies. Consequently, LB was selected as the optimal medium for bacterial growth. For fungal cultivation, acidification with tartaric acid proved problematic due to potential agar hydrolysis and poor solidification; thus, non-acidified SDA with chloramphenicol was retained as the preferred medium. To determine the best dilution and plating volume, serial dilutions from 10^{-1} to 10^{-6} g/mL of bee pollen samples in ultrapure water were plated in 20, 30, and 40 μL volumes. The results (see Table S4) showed that 10^{-2} g/mL combined with 20 μL produced CFU counts ranging from 52 to 67, falling within the optimal range (20–200 CFU/plate) for reliable enumeration and colony isolation. Higher volumes (30–40 μL) led to overcrowded plates, complicating CFU counting and colony selection, while 10^{-3} dilutions yielded too few colonies (6–10 CFU/plate). Therefore, the condition of 10^{-2} g/mL dilution and 20 μL plating volume

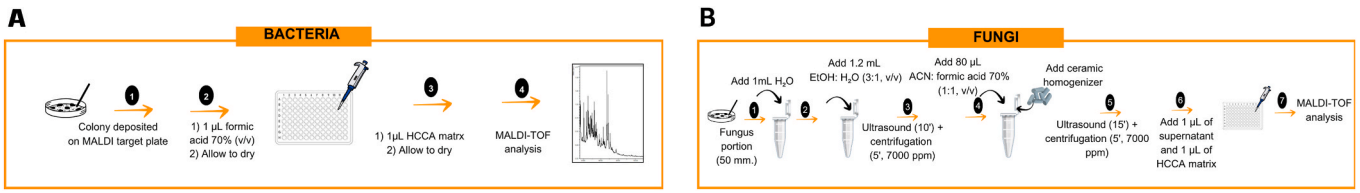

Fig. 1. Workflow of sample preparation protocols for the isolation and protein extraction of: **A)** bacterial and **B)** fungal isolates from bee pollen.

was selected as the standard for all subsequent analyses. Colonies were counted manually after incubation, and CFU values were expressed as colonies per gram of sample.

3.2. Protein extraction method optimization

For bacterial isolates, protein extraction was performed directly on the MALDI target plate using 1 µL of formic acid 70 % (v/v), followed by matrix addition (HCCA). This simple and widely used method proved effective for obtaining clear, high-intensity spectra that enabled reliable species-level identification using the Bruker Biotyper database. However, its application to filamentous fungi consistently resulted in low-intensity spectra that failed to match reference profiles in the database, indicating that the protein extraction was insufficient for reliable identification. Fungal cell walls are structurally complex and particularly resistant to chemical lysis, as they are composed of polysaccharides such as β-glucans and chitin (Liu et al., 2023).

The Bruker Biotyper protocol for filamentous fungi, which involves ethanol precipitation followed by extraction with a mixture of acetonitrile and formic acid 1:1(v/v), was initially applied. However, the resulting spectra were either too weak or too noisy, and no identifications were obtained. To investigate whether prolonged exposure to the extraction solvent could improve protein recovery, fungal pellets were incubated in the same mixture for 24 h, but this also failed to enhance the spectral intensity. To avoid compromising protein integrity by modifying the chemical composition of the extractant, we focused on mechanical disruption techniques to facilitate cell wall breakage. The first alternative tested involved homogenizing the fungal biomass directly in the extractant using an Ultraturrax® system. While this introduced intense shear forces, the spectra remained weak and did not allow identification. Then, a second approach was investigated: the addition of inert 2 mm borosilicate glass beads to the fungal suspension, followed by 15 min of sonication. This method aimed to exploit mechanical friction between beads and fungal cells under agitation. The result was a slight improvement in peak intensity, but the overall spectra remained insufficient for confident identification, only one or two peaks were distinguishable, with high background noise and low reproducibility. Finally, the use of ceramic homogenizer shavings combined with 15 min of sonication at 35 °C was evaluated. Unlike the smooth, spherical beads, ceramic particles are irregular and angular, providing higher mechanical impact during sonication. This treatment was clearly superior: it generated well-resolved, high-intensity spectra across the *2000–15000 m/z* range and enabled successful identifications using the MALDI Biotyper database. The spectra showed multiple sharp and reproducible peaks characteristic of ribosomal protein profiles. These results confirmed that the failure of previous methods was due to insufficient cell wall rupture, and that the ceramic-assisted sonication protocol was effective in overcoming the barrier imposed by fungal wall composition. The comparative performance of all four tested methods is shown in Fig. 2. Only the ceramic protocol resulted in a spectrum suitable for MALDI-TOF identification. This optimized procedure was adopted for all fungal isolates in this study and represents a key methodological innovation for analyzing culturable fungal communities associated with bee pollen and other food matrices. Although the Bruker liquid cultivation and extraction protocol was not evatuated in this study, our results indicate that combining ethanol precipitation with

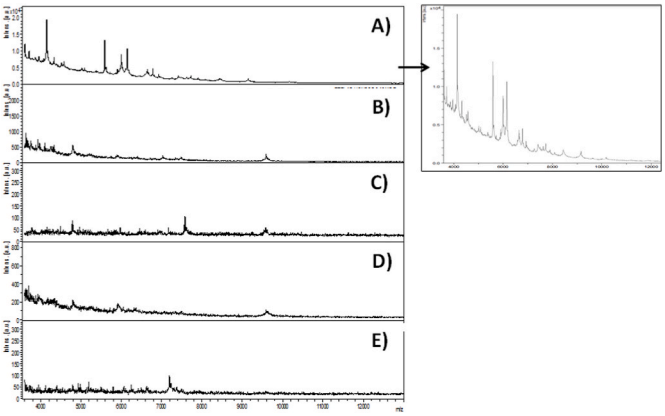

Fig. 2. MALDI-TOF analysis of fungal proteins extracts after different sample preparation methods: **A)** ceramic homogenizer shavings. The inset shows a magnified view of the *4000–12 000 m/z* region of the spectrum; **B)** borosilicate glass beads; **C)** Ultraturrax®; **D)** 24 h incubation in extractant solvent; **E)** standard Bruker protocol.

intensified mechanical disruption (ceramic beads and sonication) is sufficient to obtain robust MALDI-TOF spectra from filamentous fungi. While recent optimization studies and the Bruker reference protocol recommend sampling the advancing mycelial front, composed of younger hyphae, to maximize signal intensity (Dogan et al., 2025; Honsig et al., 2022), we chose to sample the central part of the colony to standardize biomass collection and reduce agar carryover, which likely contributed to the initially weak spectra obtained with the standard protocol. The enhanced mechanical disruption step effectively compensated for this limitation and enabled the acquisition of high-quality spectra from centrally sampled colonies. Future work could systematically compare liquid cultivation-based protocols with this optimized extraction workflow, as well as central versus marginal sampling strategies, to further refine fungal identification from bee pollen-derived isolates.

3.3. Greenness and blueness assessment

The greenness of both analytical methods was evaluated with four well-established greenness metrics: analytical greenness calculator (AGREE), AGREEprep, the modified green analytical procedure index (MoGAPI) and the *Blue Applicability Grade Index (BAGI)* (Manousi et al., 2023; Mansour et al., 2024; Pena-Pereira et al., 2020; Wojnowski et al., 2022). These tools were selected because they are widely used in recent analytical-chemistry studies, freely available and supported by clear user guides. AGREE offers an overall assessment of sustainability by converting the twelve principles of Green Analytical Chemistry (GAC) (Gałuszka et al., 2013) into a single score from zero to one. AGREEprep relies on the same scale but focuses on sample preparation, giving extra weight to how much solvent is used, how hazardous the reagents are, how efficiently the extraction works and how much waste is produced. MoGAPI extends the original GAPI concept by adding stricter values and factors such as energy use and sample throughput, providing a more detailed view of eco-efficiency. BAGI was designed to rate the

practicality and applicability of the method. Taken together, these four metrics give a balanced assessment (see Fig. 3).

The need for a sample treatment (parameter 1), at-line measurements (parameter 3) and the amount of waste (parameter 7) were penalized by the AGREE metric for both bacteria and fungi identification methods. However, most of the waste came from the culture medium used to incubate samples, which consists of biodegradable and innocuous components. For fungi, the higher number of sample preparation steps (parameter 4) was negatively weighted. In contrast, the rest of the parameters shown in green (sample amount, type of analysis, derivatization status, and number of analytes per hour, toxic reagents, and operator safety) were aligned with the GAC principles. Focusing on AGREEprep, the ex-situ sample preparation placement, the low reusability of materials, the amount of waste and the manual sample preparation were the parameters that had the most negative influence on the final score. As expected, the greenness scores calculated using both metrics were quite similar and indicated good compliance with the green principles. Regarding MoGAPI, multiple parameters were categorized as green, like reagents, solvents, compounds considering their health and safety hazard, amount of sample and energy consumption per sample (low heating during incubations of 30 samples per oven, and MALDI-TOF identification taking less than 5 s per sample). Conversely, sample preservation (incubation at 30–35 °C for 3–7 days) and the necessity of an extraction step were rated with a low degree of greenness. The nature of solvents/reagents and additional treatments were classified with a medium environmental impact for the fungal method. Specific considerations make some metrics genuinely challenging to measure and assess. In terms of practicability, both methods achieved high scores, positively influenced by the multi-analyte analysis (thousand of microorganism species can be identified by proteomic profiling), the high amount of samples prepared and analyzed per hour, the availability of material and reagents, the low amount of sample, and the absence of preconcentration steps. All the parameters of each metric are shown in the Supplementary Material (see Table S5–S12). The sample preparation and methods developed can be considered a promising approach for microbiological analysis not only in bee pollen, but in other food matrices as well, since they can be considered environmentally friendly methods aligned with the GAC principles. In addition, it should be noted that is the first study with these compounds and matrix supported by the AGREE, AGREEprep, MoGAPI and BAGI metrics which demonstrated a consistent correlation among the metrics.

3.4. MALDI-TOF identification

Microbial isolates from marketed and experimental bee pollen samples were identified using MALDI-TOF MS with the Biotyper Compass software. Spectral profiles were compared against the reference database, and identification confidence was expressed as log(score) values: scores ≥2.3 were considered highly reliable at the species level, scores of 2.0–2.299 as probable species-level or confident genus-level identifications, and scores <2.0 as low-confidence or ambiguous. Of the 32 bee pollen samples analyzed, 15 distinct microbial species were identified (see Table 1). Many spectra exhibited high signal intensity, indicating efficient protein extraction and acquisition from both bacterial and fungal isolates embedded in the pollen matrix. However, a subset of high-quality spectra could not be confidently matched to

Table 1
Tentative microorganisms isolated from bee pollen samples and their corresponding scores using the Biotyper MALDI-TOF system.

Ripening stage (days)	Marketed		Experimental	
	Microorganism	MALDI-TOF score	Microorganism	MALDI-TOF score
3	*Bacillus cereus*	2.340	*Bacillus licheniformis*	2.332
	Bacillus licheniformis	2.235	*Bacillus licheniformis*	2.215
	Bacillus licheniformis	2.125	*Bacillus licheniformis*	2.180
	Bacillus licheniformis	1.985	*Micrococcus luteus*	2.228
	Bacillus licheniformis	2.020	*Micrococcus luteus*	2.160
	Kocuria rhizophila	2.296		
	Micrococcus luteus	2.221		
4			*Bacillus licheniformis*	2.210
			Bacillus simplex	2.244
			Bacillus licheniformis	2.001
			Burkholderia cepacia	2.285
			Burkholderia cenocepacia	2.199
			Ralstonia insidiosa	2.231
			Streptomyces violaceoruber	2.188
5			*Dietzia cinnamea*	2.276
			Dietzia cinnamea	2.155
			Dietzia cinnamea	2.138
6	*Lactobacillus kunkeei*	2.014	*Aspergillus fumigatus*	2.258
	Fructobacillus fructosus	1.985	*Pantoea agglomerans*	2.263
			Pantoea agglomerans	2.115
			Staphylococcus xylosus	2.017
			Staphylococcus xylosus	2.195
7	*Zygosaccharomyces rouxii*	2.099	*Aspergillus fumigatus*	2.249
	Aspergillus fumigatus	2.080	*Aspergillus fumigatus*	2.160
	Cladosporium cladosporioides	2.215	*Zygosaccharomyces rouxii*	2.112
29			*Methylobacterium organophilum*	2.301
			Methylobacterium organophilum	2.250
			Methylobacterium organophilum	2.100
			Methylobacterium organophilum	1.980

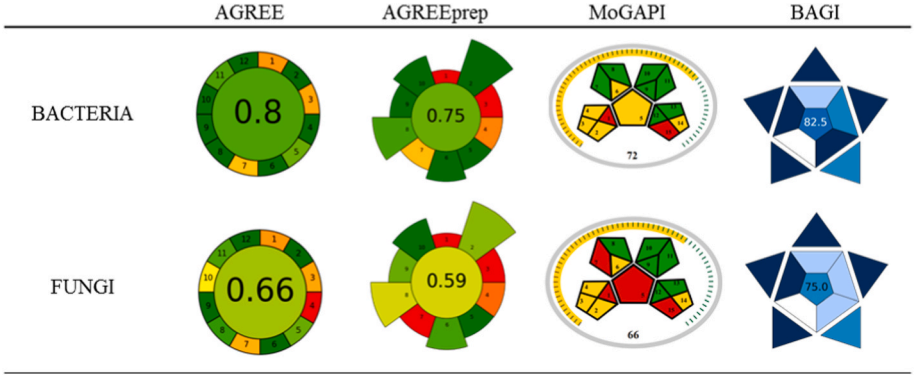

Fig. 3. Greenness and blueness evaluation using A) AGREE, B) AGREEprep, C) MoGAPI and D) BAGI metrics.

database entries, underscoring the limited coverage of environmental and pollen-associated taxa in the current Biotyper reference. As the database expands to include more environmental and food-related microorganisms, the identification success rate is expected to improve, reinforcing the applicability of MALDI-TOF in environmental microbiology and food quality control. Among the isolates, 72 % displayed scores ≥2.0, enabling confident genus- or species-level assignment, while 28 % fell below this threshold. The identified microbiota encompassed four main phyla: Firmicutes, Actinobacteriota, Pseudomonadota, and Ascomycota. Marketed bee pollen (Fig. 4A) was dominated by Pseudomonadota (40 %), followed by Firmicutes (27 %), Ascomycota (20 %), and Actinobacteriota (13 %). In contrast, bee pollen samples from experimental beehives (Fig. 4B) showed higher proportions of Firmicutes (34 %) and Ascomycota (33 %), with reduced representation of Pseudomonadota (22 %) and Actinobacteriota (11 %). These differences likely reflect distinct origins and handling: experimental bee pollen samples were collected directly from beehives without processing, favoring the persistence of environmental and fungal taxa, whereas marketed bee pollen underwent hygienic and storage treatments, enriching for more resilient bacterial species.

The identified microbial community included taxa with both beneficial and potentially harmful traits. Firmicutes, particularly *Bacillus spp*. (e.g., *B. cereus*, *B. licheniformis*), were the most frequent, corroborating previous reports of *Bacillus* as a core component of bee pollen microbiota (Pełka et al., 2025). Many *Bacillus* species are valued as plant growth-promoting rhizobacteria (PGPR), contributing to plant health through phytohormone production, nutrient solubilization, and pathogen suppression (Pandey et al., 2023, pp. 1–44). Actinobacteriota such as *Micrococcus luteus, Dietzia cinnamea*, and *Streptomyces violaceoruber* were detected less frequently, consistent with earlier studies describing Actinobacteria as minor yet metabolically versatile members of the hive microbiome, capable of producing antimicrobial compounds and contributing to colony health (Kwong & Moran, 2016; Grubbs et al., 2021). Among *Pseudomonadota, Burkholderia cepacia, Ralstonia insidiosa*, and *Methylobacterium organophilum* were more common in marketed bee pollen, a pattern previously observed in processed bee products (Tsadila et al., 2023). Notably, while *Burkholderia* and *Ralstonia* can act as PGPR in agriculture, they also include opportunistic human pathogens, emphasizing the dual relevance of these taxa for both plant productivity and food safety (James et al., 2022; Karthika et al., 2022). *Methylobacterium* species are known for their roles in biopolymer production and plant-associated interactions (Zúñiga et al., 2011). From a consumer safety perspective, the detection of *Bacillus cereus* in marketed bee pollen is noteworthy, as this species complex is a well-known cause of foodborne intoxications and spoilage in cereal- and spice-based products. Likewise, some members of the *Burkholderia cepacia* complex and *Ralstonia insidiosa* have been implicated in opportunistic infections, especially in immunocompromised patients or in nosocomial settings. Although our culture-based survey did not allow quantitative risk assessment, the presence of these taxa indicates that bee pollen can act as a vehicle for environmental microorganisms with potential clinical relevance. This highlights the importance of appropriate processing steps (drying, hygienic handling, controlled storage) and of defining microbiological specifications for bee pollen intended for human consumption.

Fungal isolates, mainly Ascomycota such as *Aspergillus fumigatus, Cladosporium cladosporioides*, and *Zygosaccharomyces rouxii*, were particularly abundant in experimental samples. This likely reflects the hygroscopicity of unprocessed pollen and storage under uncontrolled conditions, which promote fungal colonization (Krnjaja et al., 2019). While *Z. rouxii* is benign osmophilic yeast, *A. fumigatus* is a recognized opportunistic pathogen of both humans and bees, linked to respiratory disease and beehive collapse (Becchimanzi & Nicoletti, 2022). The detection of these fungi underscores the importance of appropriate drying and storage to minimize contamination and ensure product safety (Anjos et al., 2023).

Overall, the predominance of Firmicutes and Pseudomonadota in both sample types aligns with previous sequencing-based surveys of bee pollen microbiomes (Martins et al., 2021; Pandey et al., 2023, pp. 1–44). Although the taxonomic breadth here was narrower than in culture-independent studies, as expected from a culture-dependent method, MALDI-TOF enabled rapid, reproducible species-level identification of the cultivable fraction. This demonstrates its potential as a screening tool for monitoring the microbiological quality of bee pollen. In addition to their impact on bee health, airborne conidia of *A. fumigatus* and other storage fungi may pose a risk for sensitized consumers through inhalation or ingestion of contaminated bee pollen, particularly when products are consumed raw as dietary supplements. Previous surveys have reported the co-occurrence of toxigenic fungi and aflatoxins in commercial bee pollen (Deveza et al., 2015; Guo et al., 2024; Nuvoloni et al., 2020). Although MALDI-TOF MS does not directly detect mycotoxins, the rapid identification of mycotoxigenic species can support targeted chemical analyses and risk management strategies.

3.5. Chemometric analysis

3.5.1. Pearson correlation analysis

Pearson correlation coefficients among the microbial species isolated from bee pollen samples were calculated to explore potential patterns of co-occurrence and exclusion within the microbial community (see Fig. 5). Several significant positive and negative correlations were identified, indicating possible ecological interactions. Among the Firmicutes, *Bacillus cereus, B. licheniformis*, and *B. simplex* showed moderate to strong positive correlations ($r > 0.5$, $p < 0.05$), suggesting that these species tend to co-occur in processed bee pollen. This may reflect their similar environmental tolerance and frequent detection in dried or stored bee products, consistent with previous reports describing *Bacillus* as a core genus in bee pollen microbiota (Martins et al., 2021; Kamimura et al., 2019). In contrast, *Aspergillus fumigatus* was negatively correlated ($r < -0.3$, $p < 0.05$) with several bacterial taxa, including *Burkholderia cepacia, Micrococcus luteus,* and *Streptomyces violaceoruber*. This suggests possible competitive interactions between fungal and bacterial communities, likely influenced by their differing growth requirements and sensitivities to water activity. A notable positive correlation was

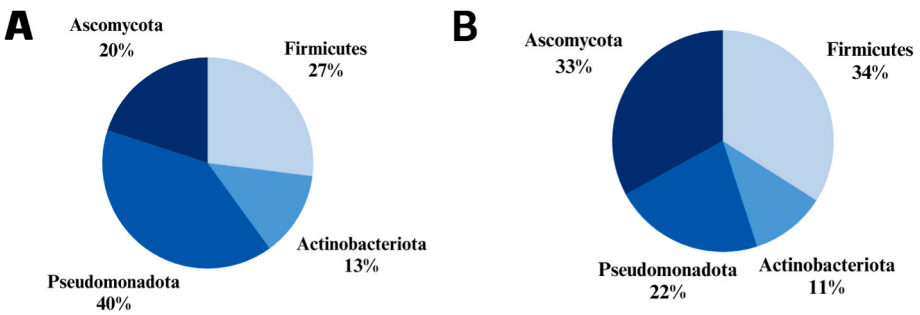

Fig. 4. Relative abundance of microbial phyla identified in bee pollen samples collected: **A)** from local markets; **B)** from experimental apiaries.

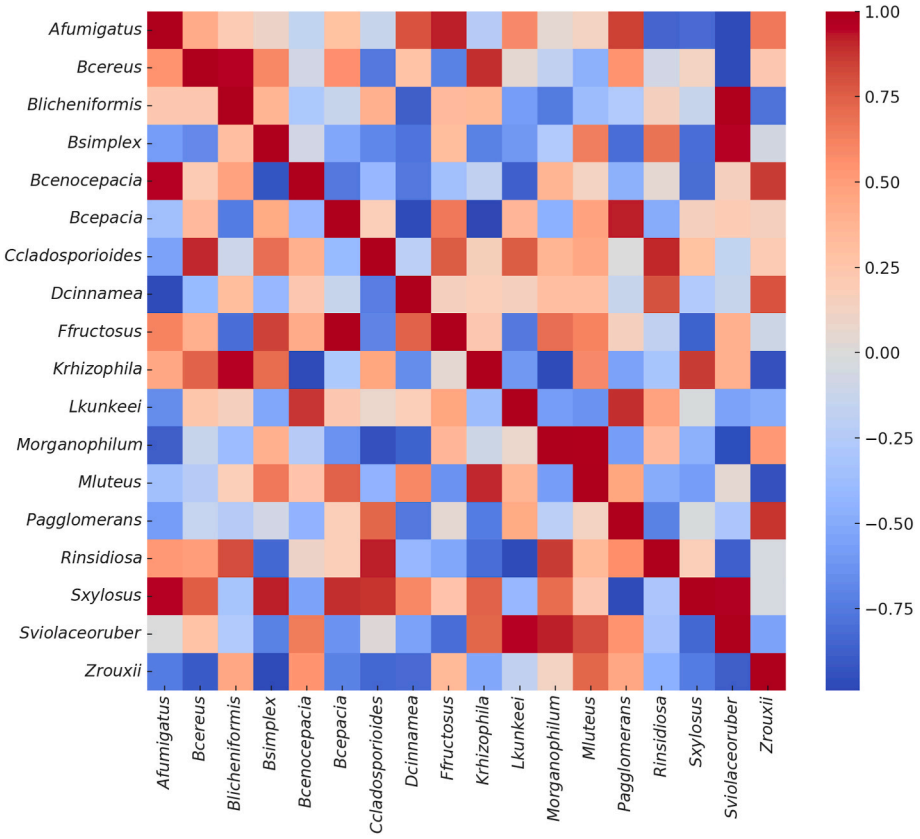

Fig. 5. Correlation analysis among the identified microbial genera based on Pearson's test.

observed *between Streptomyces violaceoruber* and *Zygosaccharomyces rouxii* ($r > 0.55$, $p < 0.01$), two species associated with high-sugar environments, as also reported in honey and bee pollen samples (Scognamiglio et al., 2019; Zhu et al., 2023). These results highlight the complex interactions within bee pollen microbiota, shaped by processing, storage, and botanical origin. Such findings can inform strategies for quality control and microbial risk assessment in bee products.

3.5.2. PCA and HCA analysis

The PCA was conducted using the relative presence/absence data of the identified microbial taxa in the samples. The first three principal components explained a cumulative variance of approximately 70 % (PC1: 37 %, PC2: 23 %, PC3: 10 %), supporting the use of a three-dimensional representation to capture the majority of the variability in the dataset. The Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy was computed at 0.87, confirming the suitability of the data for dimensionality reduction. The PCA plot revealed two main clusters of samples, highlighting clear differences in microbial composition among the bee pollen samples analyzed (see Fig. 6). The first cluster (Cluster I) encompassed almost all experimental samples reflecting their similar microbial profiles associated with direct collection and minimal post-harvest handling. Notably, sample E12 did not group within Cluster I and instead appeared as an outlier, suggesting a distinct microbial composition. The second cluster (Cluster II) included most marketed samples (e.g., M5-M11), which formed a well-defined group, indicating a consistent microbial signature possibly influenced by processing and storage practices. The remaining marketed samples (e.g., M1 and M3) were positioned away from the main clusters, suggesting higher variability or unique microbial profiles in these commercial products. The distances observed between clusters illustrate substantial differences in microbial communities at the group level, while the spread of points within each cluster indicates variability among individual samples. This separation emphasizes the influence of sample origin and handling on

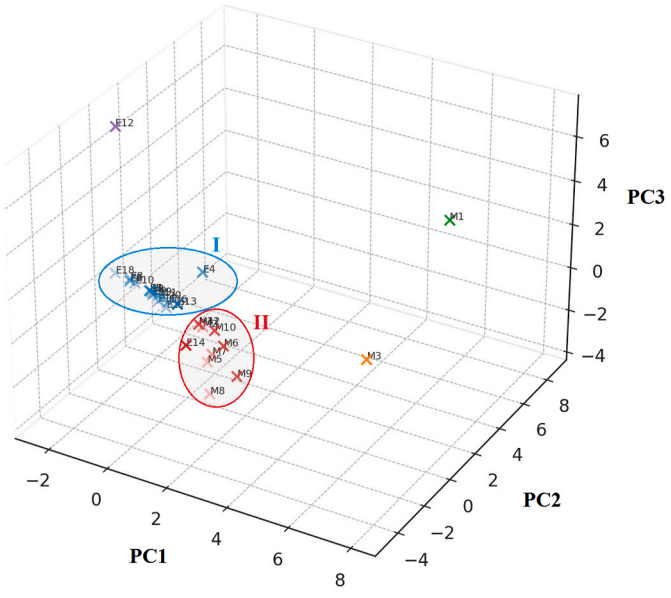

Fig. 6. Principal analysis component (PCA) of bee pollen samples.

the microbial composition of bee pollen and underlines the potential of PCA as a tool for distinguishing between experimental and marketed products based on their microbial signatures.

HCA was applied to classify the samples according to their similarities in microbial composition and taxonomic origin. The analysis was performed using Ward's method with squared Euclidean distances to evaluate similarities. Fig. 7 shows the dendrogram which separates bee pollen samples into two major clusters (I and II) at a distance level of 10.

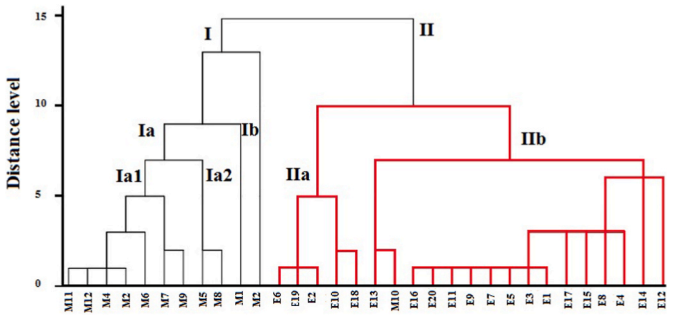

Fig. 7. Hierarchical cluster analysis (HCA) dendrogram of experimental (E) and commercial (M) bee pollen samples based on their microbial profiles.

Cluster I, composed entirely of commercial products, and Cluster II, predominantly consisting of experimental products. At a more stringent level (distance = 5), these main clusters subdivide into five distinct clades: Ia1, Ia2, Ib, IIa, and IIb, reflecting finer differences in microbial profiles and botanical origin. The marketed products in clusters I, Ia1, Ia2, and Ib shared microbial communities, dominated by *B.cereus*, *B. licheniformis*, and *S.xylosus* and shared the same botanical origins, multifloral and *Castanea* sources. Within these, subclade Ia1 was characterized by the presence of *B. simplex*, while subclade Ia2 showed an association with *P. agglomerans*. Clade Ib included two commercial samples distinguished by the occurrence of *M. luteus* and *S. violaceoruber*, which were not observed in other commercial clades. In contrast, the experimental samples grouped into clusters IIa and IIb displayed much greater diversity, both microbiologically and botanically. Cluster IIa was associated with taxons such as *Brassica,* Rosaceae, and *Retama*, and characterized by the presence of *B.cenocepacia, K.rhizophila*, and *R. insidiosa.* Cluster IIb, the largest, encompassed a broad array of experimental samples and one commercial product (M10), showing the highest microbial diversity, including *L.kunkeei, F.fructosus, Z.rouxii*, and rare occurrences of *C.cladosporioides* and *D.cinnamea.* These findings demonstrate that commercial products tend to group into uniform clusters with similar microbial and botanical profiles, while experimental products are distributed in more heterogeneous clusters. PCA analysis confirmed these trends, supporting the robustness of the HCA classification and highlighting a few outliers (M1, M3, E12) that deviated from the main groups.

4. Conclusions

This study demonstrates, for the first time, the feasibility of using MALDI-TOF as a rapid, practical, and environmentally sustainable tool for the identification of viable microorganisms, including both bacteria and fungi in bee pollen. Through optimized protein extraction protocols, especially for filamentous fungi, the methodological limitations related to cell wall disruption were successfully overcome, enabling the acquisition of high-quality spectra and reliable species-level identifications. The results revealed a diverse microbial community, with clear differences between commercial and experimental bee pollen samples. Commercial products were predominantly associated with resilient bacterial taxa such as *Bacillus spp.* and *Burkholderia spp.,* likely due to processing and storage practices. In contrast, experimental samples contained a broader range of environmental fungi and yeasts, such as *Aspergillus fumigatus* and *Zygosaccharomyces rouxii*, reflecting minimal handling and greater exposure to natural conditions. These differences underscore the impact of post-harvest practices on the microbial profile and highlight the need to balance hygiene with the preservation of beneficial microbiota.

In the absence of specific, internationally harmonized microbiological criteria for bee pollen, such information is essential for guiding good manufacturing practices and for informing future regulatory decisions. In this context, MALDI-TOF MS can support the establishment of evidence-based microbiological specifications by enabling rapid surveillance of viable microbial communities in bee pollen.

Environmental sustainability and practical applicability of the method were assessed using multiple green and blue analytical chemistry metrics (AGREE, AGREEprep, MoGAPI, and BAGI). The approach demonstrated strong compliance with sustainability principles, thanks to its minimal use of hazardous reagents, simple sample handling, and high-throughput capabilities. Finally, chemometric analyses (PCA and HCA) enabled clear discrimination of samples based on their microbial composition and origin, supporting the potential of MALDI-TOF as a tool for traceability and product authentication. The study also illustrates the value of MALDI-TOF as a proof-of-concept for high-throughput characterization of bee product microbiomes; with expected improvements in performance as reference databases expand.

CRediT authorship contribution statement

Estela Prieto: Writing – original draft, Investigation. **Ana M. Ares:** Supervision, Resources. **José Bernal:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Silvia Valverde:** Writing – review & editing, Validation, Supervision, Investigation.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2025.111939.

Data availability

Data will be made available on request.

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