MALDI Biotyping, an approach for deciphering and assessing the identity of the honeybee pathogen *Nosema*

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Complete List of Authors:	HOUDELET, Camille; Institute for Advanced Biosciences, Immunologie Analytique des Pathologies Chroniques; Platform BioPark Archamps BOCQUET, Michel; Michel Bocquet, Apimedia BULET, Philippe; Institute for Advanced Biosciences, Immunologie Analytique des Pathologies Chroniques; Platform BioPark Archamps,
Keywords:	MALDI Biotyping, Peptide profiling, Microsporidia, Flow cytometry, <i>Nosema</i> , <i>Apis mellifera</i>
Abstract:	<p>The microsporidia are obligate intracellular pathogenic fungi that parasitize a wide range of invertebrate and vertebrate hosts and have important impacts on health, food security and the economy. In this paper, we focus on <i>Nosema ceranae</i> and <i>N. apis</i>, which chronically infect the digestive tract of honeybees, altering their physiology and lifespan. We apply MALDI mass spectrometry for rapid molecular profiling of extracts of <i>Nosema</i> spores in order to identify the species and optionally the geographical origin and assess the viability status of <i>Nosema</i> microsporidia in conjunction with a flow cytometric approach. Pure solutions of spores were prepared for flow cytometry and MALDI profiling. A mechanical extraction of <i>Nosema</i> spores was conducted to obtain mass fingerprints of peptide/protein for samples of microsporidia from different geographical origins (MBO.NC01, MBO.NC02 and MBO.NA01), viable or heat killed. The present work demonstrates that MALDI-MS is a rapid, consumable cost-effective and simple method for identifying <i>Nosema</i> species. Moreover, a distinction in the peptide/protein profiles between two isolates with different geographical origins was observed. Mass fingerprints of viable and experimentally killed spores were also clearly distinguishable, regardless of <i>Nosema</i> species. Finally, using our computational models on the different <i>Nosema</i> species, we were able to classify five independent isolates of <i>Nosema</i> microsporidia. We demonstrated that MALDI Biotyping may represent a valuable tool for sanitary services and beekeepers to survey nosemosis in apiaries.</p>

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MALDI Biotyping, an approach for deciphering and assessing the identity of the honeybee pathogen *Nosema*

Camille HOUDELET^{a,b}, Michel BOCQUET^c, Philippe BULET^{a,b*}

^a Institute for Advanced Biosciences, CR Inserm U1209, CNRSUMR 5309, University of Grenoble-Alpes, Grenoble, France

^b Platform BioPark Archamps, Archamps, France

^c Michel Bocquet, Apimedia, Annecy, France

E-mails:

Camille HOUDELET: camille.houdelet@univ-grenoble-alpes.fr

Michel BOCQUET: apimedia@aol.com

Philippe BULET: philippe.bulet@univ-grenoble-alpes.fr

***Corresponding author**

Philippe BULET, Platform BioPark Archamps, 260 Avenue Marie Curie Archamps Technopole 74166 Saint Julien-en Genevois France

E-mail: philippe.bulet@univ-grenoble-alpes.fr

Phone number: +33 4 50 43 25 21

Abstract:

The microsporidia are obligate intracellular pathogenic fungi that parasitize a wide range of invertebrate and vertebrate hosts and have important impacts on health, food security and the economy. In this paper, we focus on *Nosema ceranae* and *N. apis*, which chronically infect the digestive tract of honeybees, altering their physiology and lifespan. We apply MALDI mass spectrometry for rapid molecular profiling of extracts of *Nosema* spores in order to identify the species and optionally the geographical origin and assess the viability status of *Nosema* microsporidia in conjunction with a flow cytometric approach. Pure solutions of spores were prepared for flow cytometry and MALDI profiling. A mechanical extraction of *Nosema* spores was conducted to obtain mass fingerprints of peptide/protein for samples of microsporidia from different geographical origins (MBO.NC01, MBO.NC02 and MBO.NA01), viable or heat killed. The present work demonstrates that MALDI-MS is a rapid, consumable cost-effective and simple method for identifying *Nosema* species. Moreover, a distinction in the peptide/protein profiles between two isolates with different geographical origins was observed. Mass fingerprints of viable and experimentally killed spores were also clearly distinguishable, regardless of *Nosema* species. Finally, using our computational models on the different *Nosema* species, we were able to classify five independent isolates of *Nosema* microsporidia. We demonstrated that MALDI Biotyping may represent a valuable tool for sanitary services and beekeepers to survey nosemosis in apiaries.

Keywords: *Apis mellifera*, Peptide profiling, MALDI Biotyping, Microsporidia, *Nosema*, Flow cytometry

1. Introduction

The microsporidia are obligate intracellular pathogenic fungi, which parasitize a wide range of invertebrate and vertebrate hosts. To date, two microsporidian species infect honeybees worldwide: *Nosema apis* and *N. ceranae*. Recently, the new species, *N. neumanii*, was discovered in Africa¹. European beekeepers have long faced hives with nosemosis due to *N. apis*, with an extreme mortality episode in 1904 in the south of England under the name of Isle of Wight disease². *N. ceranae*, first identified in *Apis cerana*, was found in *A. mellifera* in Taiwan in 2005 and in Europe in 2006³, and then in the Americas and Oceania^{4,5}, and finally identified worldwide^{6–10}.

Nosemosis reduces honeybee population size and causes significant losses in honey production. The impact of nosemosis at the individual level includes: changes in metabolism, especially in pathways involved in energy management^{11,12}; oxidative stress^{13,14}; inhibition of apoptosis of epithelial cells (avoiding host innate response to infection)^{15,16}; down regulation of bee immune genes^{17,18}; shortening of lifespan¹⁹; modifying the Vitellogenin/Juvenile Hormone equilibrium; accelerating the transition to forager activities²⁰ and changing the gut microbiota²¹. *Nosema* infections have complex interactions with other stressors. Considering biotic stressors, there is an unclear correlation with the mite *Varroa destructor*²², competition with some viruses (e.g. Deformed Wing Virus, DWV) or variable effects on other viruses, associated with extra mortality of the colonies²³. Additionally, frequent co-infections with the arthropod trypanosomatids *Crithidia mellificae*^{24,25} were observed. For abiotic stressors, a synergetic effect has been observed with pesticides, especially neonicotinoids, resulting in an increase in bee mortality and a reduction of learning capacity²⁶.

At the colony level, the impact of nosemosis is more difficult to demonstrate; correlation, but not causality, has been found between type C nosemosis (*N. ceranae*) and colony mortality²⁷. Nosemosis affects measures of colony resilience such as longevity, forager *versus* young bee equilibrium, pheromonal levels, ability of foragers to return to the colony, and behavioural changes²⁸. This predisposing factor may also increase the impact of pesticide treatment²⁹ and affects the efficiency of anti-*Varroa* medicines³⁰. Nosemosis may also be affected by renewal practices like changing of queens¹⁵. Globally, clinical signs of *N. ceranae* infection are not very clear and in some countries includes: modified population equilibrium, excess of brood *vs* nurses, depletion of foragers, and reduction of honey production³¹.

One challenge for scientists studying microsporidia is the multiplication of spores *in vitro*³². Microsporidia cannot grow or divide outside their host cell, but exactly how they interact and use resources from their host is only partially known³³. To date, there is no homologous effective system described in the literature for *in vitro* rearing and multiplying of *Nosema spp.* There is only one report of Gisder and colleagues³⁴ based on infection of an heterologous lepidopteran cell line IPL-LD-65Y³⁵. The authors described a new system based on lepidopteran cell line susceptible to *N. ceranae* and *N. apis* infection with a 15-30% and 5-10% infection rate, respectively. Using this

heterologous system Gisder and colleagues gave new insights into the intracellular life cycle of *N. ceranae* and *N. apis* spores. However, as microsporidia are of the most host-dependent parasites known developing homologous tools may represent an asset when fighting *Nosema* infections in *Apis mellifera*.

Regarding the pathogenic effect of nosemosis, their infectious capability or virulence (increased mortality rates in infected individuals) depends of the number of spores ingested by honeybees³⁶ and on temperature³⁷. *N. ceranae* spores better resist high temperatures (Mediterranean climate) than cold temperatures and complete their life cycle faster at higher temperatures. With regards to the physiology of the bee, the susceptibility or tolerance of bees to *Nosema* is age dependant: foragers are the most intensively infected but have a better ability to survive if challenged than younger bees³⁸. Nutritional status (i.e. pollen quality) and possibly genetic strain are also involved^{39,40}.

The absence of official treatment against nosemosis in Europe⁴¹ spurred investigations into better understanding the detailed mechanisms of infection and their variations, a key issue for better management of the disease, that requires development of the most appropriate experimental design. To date, microsporidian spores have been identified using morphological, immunological and molecular biological methods. In 2015, FDA clearance was announced for more than 200 species of micro-organisms using Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Two MALDI spectrometers are available on the market, MALDI Biotyper[®] and VITEK[®]^{42,43}. These MALDI-MS platforms perform by targeting the ribosomal proteins as biomarkers for the identification of clinical bacterial, fungal and yeast isolates (method referred to MALDI Biotyping^{44,45}). Proteins can be analyzed by mass spectrometry (MS); ionization of the proteins leads to measurement of the mass-to-charge (m/z) ratio, with the great benefit that the analysis is unbiased and a priori knowledge of the proteins is thus not necessary. Molecular mass fingerprints (MFP) of biological matrices by MALDI Biotyping is indeed a thriving approach, enabling the rapid detection of peptide/protein components that can provide comparative information.

In this work, we explore the potential of MALDI Biotyping for the identification of *Nosema* species and its potential to evaluate *Nosema* spore viability as its represents a challenge for monitoring the impact of *Nosema* on bee physiology. MALDI profiling and also flow cytometry allow to rapidly discriminate viable and non-viable spores, thanks to highly purified spore preparations. Finally, adapting MALDI Biotyping, a technology used for the identification of bacterial strains, to the identification of *Nosema* species, allowed us to obtain representative MFPs of the *Nosema* species (*N. apis* versus *N. ceranae*) and also of the geographical origin. This demonstrated that MALDI profiling represents an attractive and more robust alternative to optical observation and is more cost effective, compared to PCR analyses, for identifying *Nosema* species.

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126 **2. Materials and methods**

127 **2.1 Chemicals & reagents**

128 For sample preparation and analyses, acetonitrile (ACN) and trifluoroacetic acid
129 (TFA), both of LC-MS grade quality or higher, were obtained from Carlo Erba
130 Reagents (Val de Reuil, France). Protein Calibration Standard I (ProtMix) was
131 purchased from Bruker Daltonics (Germany). For all experiments, ultrapure water
132 (MilliQ water) was used (Billerica, MA, USA). For a calibration solution APISCAL,
133 the *A. mellifera* peptides (apidaecin, abaecin and melittin) were synthesized by Smart
134 Bioscience and the peptide ETD was a donation from Dr. Philippe Bulet. The Percoll®
135 and ammonium phosphate monobasic (NH₄H₂PO₄) were purchased from Sigma-Aldrich
136 (Missouri, USA). The two dyes, SYTO 16 and propidium iodide (PI) were obtained
137 from Invitrogen Corporation (Carlsbad, CA).

138 **2.2 Spore preparation**

139 The isolates of *N. ceranae* spores (MBO.NC01 from a non-European apiary and
140 MBO.NC02 from a French apiary) and *N. apis* (MBO.NA01) spores were generous
141 gifts from the collections of Michel Bocquet and Robert Paxton, respectively. For each
142 isolate, live spores were obtained after multiplication in honeybees and dead spores
143 after heat shock (20 min at 121°C).

144 The following method for purification of spores' solutions was inspired from the
145 recommendations of Fries and colleagues ⁴⁶. Briefly, abdomens of infected bees were
146 collected in 50 mL conical tubes (Falcon, Dominique Dutscher, France) containing
147 ultrapure water and strongly mixed manually using a spatula. The solution was filtered
148 with a 100 µm cell strainer (pluriSelect, Germany) and then centrifuged at 3,000 x g for
149 20 min at room temperature. After removing the supernatant, the residual pellet was
150 resuspended by adding 5 mL of ultrapure water. The spore suspension was filtered with
151 a 20 µm cell strainer (pluriSelect, Germany). The filtrate was centrifuged at 3,000 x g
152 for 1 hour and the residual pellet resuspended in 1 mL of Milli-Q water prior to
153 purification on a Percoll®-gradient. The second step of purification was performed on a
154 gradient of Percoll® from the bottom to the top: 100%, 75%, 50% and 25% of Percoll®
155 prepared in 0.25 mol/L of sucrose (Fluka, Germany). The spore solution (300 µL) was
156 centrifuged twice at 8,000 x g for 20 min at 6°C. The spore pellets were collected prior
157 to a second step of centrifuging at 16,000 x g for 30 min at 6°C. The supernatant was
158 removed and the spore pellet was resuspended in 1 mL of Milli-Q water and centrifuged
159 at 16,000 x g at 6°C. This step was repeated five times. The spore pellet obtained was
160 resuspended in Milli-Q water and spore concentration was estimated using a Malassez
161 cell counting chamber (Assistent®). The purity of the spore solution was estimated by
162 dividing the total number of spores by the number of spores and debris. Counting was
163 performed in triplicate. All spore solutions were kept at room temperature until use.

164 **2.3 Identification of *Nosema* spores by polymerase chain reaction (PCR)**

For PCR analysis, 200 μ L of each purified *N. ceranae* spores' solution (total 2×10^5 spores) were analysed in triplicate by the public agency ANSES (Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail) according to their internal method developed by Martín-Hernández and colleagues⁴⁷. The identity of the *N. apis* isolate was confirmed by PCR as described by Chen and colleagues in 2008⁴⁸.

2.4 MALDI profiling

For these analyses, all solutions were prepared at 2.5×10^6 spores/mL in triplicate.

2.4.1 Peptide/Protein extraction

To extract peptides and proteins from spores, three protocols were investigated using the fresh spore solution MBO.NC02: (P1) ultrasonic, (P2) stainless steel balls and (P3) zirconium oxide beads (Figure 1). For P1, the solution of spores was sonicated four times for 45 sec (15 sec ON and 30 sec OFF) with an amplitude of 50% (130 Watt, 20kHz, VibraCell™, Sonics®). The extract was centrifuged at $15,800 \times g$ for 4 min at 6°C before removing the supernatant and the pellet was vacuum-dried (Freezone Plus 2.5-liter freeze-dry-system; Labconco, Kansas City, MO, USA; Figure 1). For the two other extraction protocols (P2 and P3), solutions were centrifuged at $15,800 \times g$ for 4 min at 6°C before removing the supernatant and the pellets were vacuum-dried. The extraction step was executed using either stainless steel (SSB16, Next Advance, USA) or ceria stabilized zirconium oxide (ZrOB05, Next Advance, USA) beads for P2 and P3, respectively. The purified spores were disrupted using a Mill-Mix 20 system (Domel, Železniki, Slovenia). The spores were ground in 1.5 mL tubes (Eppendorf®, Germany) filled either with 1.6 mm stainless steel beads (no more than 3) or 0.5 mm zirconium oxide beads (less than 10) for 30 sec at 30 Hz. The dried extracts were either used immediately for MALDI analysis and then kept at -20°C.

2.4.2 Sample preparation

Prior to MALDI-MS analyses, dried pellets were suspended in 25 μ L of 0.1% TFA and centrifuged at $15,800 \times g$ for 2 min at 6°C. The cleared supernatant was transferred to a clean low-binding microcentrifuge tube (Eppendorf®, Germany). For MALDI analysis, an equivalent of 100,000 spores or one μ L of spore extract was spotted onto an MTP 384 polished steel MALDI target (Bruker Daltonics) in triplicate and vacuum-dried. A fresh solution of matrix (10 mg/mL α -cyano-4-hydroxycinnamic acid in 70% ACN, 2.5% TFA and 15mM $\text{NH}_4\text{H}_2\text{PO}_4$) was spotted onto the dried samples. The instrument was calibrated using a mixture of two sets of peptides and proteins (APISCAL, ProtMix) covering the dynamic range of interest. APISCAL is an in-house calibration solution composed of apidaecin and abaecin, two antimicrobial peptides from *Apis mellifera* (average m/z of 2,109 and 3,879, respectively), melittin, the major venom component, (average m/z of 2,847); and ETD, a recombinant peptide, (average m/z of 4,839). ProtMix (Protein Calibration Standard I, Bruker Daltonics) is a manufacturer-available mixture of four peptides and proteins (Insulin, Ubiquitin, Cytochrome C and Myoglobin at an average m/z of 5,734, 8,565, 12,360 and 16,952

respectively). A sample of APISCAL and ProtMix were also heat treated 20 min at 121°C to serve as control for the heat killed spores analysed by MALDI Biotyping. The sample spots were lightly vacuum-dried prior to analysis.

2.4.3 Sample analysis

Mass spectra were acquired on a MALDI AutoFlex III Smartbeam instrument (Bruker Daltonics) using the FlexControl 3.4 software (Bruker Daltonics) in positive linear mode. The instrument was set up with the following parameters: 200 Hz laser at a 40% global attenuation offset, 20 kV source voltage 1, 18.7 kV source voltage 2, 9.25 kV lens voltage, 1.906 kV linear detector voltage, 120 ns of pulsed ion extraction delay and 600 Da detector gating. MALDI-MS spectra were recorded at the mass range of 600 Da to 18 kDa by summing 2,000 laser shots. Data were previewed using the FlexAnalysis 3.4 software. For each individual spectrum baseline correction was performed with a TopHat algorithm, along with a smoothing according to the Savitzky-Golay algorithm (window size 5.0 m/z in one cycle). The peaks were automatically picked using a signal-to-noise (S/N) ratio of 3.0, a picking height of 80 and an application of baseline. Peak lists of each spectrum were extracted for data processing and statistical analyses.

2.5 Analyses of *Nosema* spores by flow cytometry

For flow cytometry analysis, solutions of purified spores (10^6 spores) were used in five and two replicates for *N. ceranae* and *N. apis* spores, respectively. Spore solutions were dyed with 6.25 μM of SYTO 16 (Invitrogen Corporation, Carlsbad, CA) and 12 μM of propidium iodide (PI, Invitrogen Corporation, Carlsbad, CA), based on Peng and colleagues' study⁴⁹. Spore solutions (1 mL) were incubated on ice, in the dark for 30 min. After centrifugation at 20,800 $\times g$ for 5 min at 21°C, 680 μL of supernatant were removed and the residual pellets were suspended in the remaining 320 μL . Flow cytometry analysis was conducted on a GUAVA® EasyCyte™ Plus Flow Cytometry System (Millipore) with the Cytosoft software. SYTO 16 and PI were excited using a 488 nm laser. Spore solutions were analysed at a high flow rate (0.59 $\mu\text{L/s}$). A threshold was established to exclude debris from spore events using Forward (FSC) and Side Scatter (SSC); counting was based on 10,000 events. A logarithmic amplification including FSC and SSC was applied. To establish settings, control solutions containing 50% dead spores were used.

Live spores were discriminated from dead spores using pulse height for PI and SYTO14 fluorescence, respectively. Compensations were made for *N. ceranae* (Green-%Yellow 54.9 and Yellow-%Green 27.4) and *N. apis* (Green-%Yellow 16.9 and Yellow-%Green 39.2). Parameters of acquisition were adjusted according to the species. Spore viability was assessed by two technical replicates.

2.6 Data processing and statistical analyses

Data processing and statistical analyses were performed using R studio with R 3.4.2 software. The data processing of spectra was conducted in five steps. Peak lists of each

replicate of each sample were extracted (step 1) and masses lower than 1,000 Da were suppressed (step 2). Peaks with an S/N of less than five were rejected (step 3). Peak lists of each replicate from the same sample were compared and only common peaks were conserved (step 4). Finally, potential double charged ions and modifications like oxidation or dehydration were suppressed (step 5). Principal component analysis (PCA) was performed on series of data including four variables for each individual spectrum: molecular ions, resolution, peak area and intensity.

To compare spore viability, data normality and homoscedasticity were evaluated. When these two conditions were not respected, a non-parametric Mann-Whitney test was applied; the confidence index was fixed at 0.95.

2.7. Generation of MALDI mass spectra profiles

A database of molecular profiles of the three isolates of *N. spores* (MBO.NC01, MBO.NC02 and MBO.NA01) was generated using the MALDI Biotyper® procedure (Bruker). For one average spectrum, each isolate was spotted eight times and each spot was acquired three times. The quality of the 24 spectra generated was evaluated following the Biotyper protocol and the main spectral profile was created using the MBT Compass Explorer v4.1.70. Five isolates of *Nosema* spores (s1 to s5), were collected from infected bees and analysed by MALDI Biotyping following the protocol described above and were compared to referenced mass spectra from our local database. For identification, a score was obtained depending of the confidence of the result. Three ranges of identification exist: 0.00 - 1.69, 1.70 - 1.99 and 2.00 - 3.00, corresponding to no organism identification, low confidence or high confidence, respectively.

3. Results and discussion

Nosema disease is quite complicated to diagnose without using laboratory equipment, as the clinical observation of the intestinal tract retrieved after pulling the last abdominal segment is not enough. A positive diagnosis is accomplished by visualizing the spores under a microscope. However, the presence of spores is not enough, other elements are necessary to affirm that we are in the presence of a *Nosema* disease. There are at least three elements to consider regarding spores of *Nosema*, first detection and counting, second identification of the species involved and third viability status, a factor often associated with virulence. In this study, we developed MALDI Biotyping method to serve these three elements and complemented this approach with flow cytometry analysis. The approach of MALDI Biotyping was applied to identify (i) the *Nosema* species, (ii) their geographical origins and (iii) their viability status. To validate our experimental procedure, we analysed two species of *Nosema* (*ceranae* and *apis*) and two isolates of *N. ceranae* from different geographical origins (one from France and one from a non-European country).

3.1 High degree of purification

Nosema spores were obtained from different bee matrices (faecal residues, dead bees or

hive residues), but whatever the method for *Nosema spp.* identification, the spore solution needs to be clarified and of the highest purity. For microscopic observation, a simple centrifugation is sufficient to visualise spores while for molecular identification, filtrated and centrifuged samples are requested for DNA extraction and PCR analysis (ANSES recommendation based on protocols developed by Martín-Hernández and colleagues)¹⁶.

In literature, three protocols were referenced for spore's suspension purification: centrifugation, triangulation and density gradient^{50,51}. With the two last protocols authors obtained percent of purification up to 92 %. In this study, we propose an optimized method derived from the Fries and colleagues (2013)⁴⁶ to fulfil the need of highly purified spore solutions for molecular analyses by combining the methods of filtration and Percoll[®]-gradient. The level of purity was estimated by counting the number of spores using a Malassez cell counting chamber as recommended by ANSES, following the recommendation of the World Organisation of Animal Health (ANSES/SOP/ANA-I1.MOA.09 - Version 6). The purity of MBO.NC02 was 96.7% (sd ± 0.2), the purity of MBO.NC01 was 99.2% (sd ± 0.3) and the purity of MBO.NA01 was 99.8 % (sd ± 0.04). Identities of the different spore solutions were confirmed by PCR prior to our analyses. MBO.NC01 and MBO.NC02 were identified as *Nosema ceranae* and MBO.NA01 as *N. apis*.

3.2 Differential analysis by MALDI profiling of various spore extracts

The first part of our study was to establish the optimal parameters (in terms of sensitivity, resolution, reproducibility and accuracy) of molecular ions between 1,000 and 18,000 m/z for recording the most representative MALDI peptide signatures/molecular mass fingerprints (MFPs) of the different *Nosema* spore extracts. We settled on extracts generated at a starting concentration of 2.5×10^6 fresh spores/mL. Three protocols of peptide/protein extracts from spores were tested on the MBO.NC02 isolate for its spore abundance: (P1) ultrasonic, (P2) stainless steel balls and (P3) zirconium oxide beads (P1, P2 and P3, see Fig. 1) and the extracts were analysed by MALDI-MS in triplicate (Figure 2). All measurements were done at the same initial spore concentration and using the same MALDI-MS parameters and sample dilution rate. Following data processing (5 successive steps, see details in Materials & methods section), P3 resulted in the most complex mass spectra (Fig. 2C) compared to P1 (Fig. 2A) and P2 (Fig. 2B) with 21, 17 and 5 significant detected peaks, respectively (Table 1).

The identification by MALDI profiling is a well-recognized method in clinical diagnostic microbiology but remains difficult for fungus, as the identification depends on the phenotype of the fungus⁵². Different studies have used the MALDI-MS approaches to study microsporidian spores but only two were based on the MALDI Biotyping workflow^{53,54}. Moura and colleagues used a physical extraction with glass beads while Kajiwarra and Murakami employed a chemical extraction with formic acid (70 %) and acetonitrile (100%). Other methods were focused on a target approach,

specifically to study proteins involved in spore maintenance or virulence (respectively Polar Tube Proteins or Spore Wall Proteins). In the case of the silkworm pathogen *Nosema bombycis*, the different studies show a complex protocol of extraction to examine protein extracts by standard 2D gel electrophoresis^{55–57}. For spore disruption and extraction, Wu and colleagues used a Sodium Dodecyl Sulfate (SDS) extraction buffer complemented with sucrose, β -mercaptoethanol, Tris and EDTA, whereas Cai and colleagues and Chen and colleagues, used another lysis buffer followed by the use of acid-washed glass beads. After migration on the 2D-gel, proteins spots were excised and prepared for MALDI-MS analyses. In our study, we developed and proposed a non-targeted approach to obtain spore extracts reliable for identification of species by MALDI profiling. Different protocols were tested including three mechanical molecular extractions using two models of beads (steel balls or zirconium beads) and an ultrasonic treatment. The most complex spectrum in terms of number of molecular ions detected was obtained from the extraction with zirconium beads (21 molecular ions, Fig 3.C and Table 1). This protocol was reproducible between replicates and an optical observation after treatment did not show an alteration of spore structure. The mature stage of *Nosema* presents a complex impermeable wall with a three layered-exospore, an endospore and an internal plasma membrane⁵⁰, which explains difficulties encountered in extracting proteins from the spores.

3.3 Statistical analyses for identification of spores by MALDI profiling

In order to evaluate the possibility to use MALDI profiling as an alternative to PCR to identify *Nosema* species (*N. ceranae* versus *N. apis*), geographical origin (European vs non-European country) and viability status., we performed a series of statistical analyses by principal component analysis (PCA, Figure 3).

From the six different sets of samples (MBO.NC01.V, MBO.NC02.V, MBO.NA01.V, MBO.NC01.NV, MBO.NC02.NV and MBO.NA01.NV) and the nine technical replicates generated per samples, the first two dimensions reflected 65.2 % of the variance (41.7% for Dim1 and 23.5% for Dim2, Fig. 3A) and were kept for analysis. Regarding the *Nosema* species determination from live spores, according to Dim1, *N. apis* (upper right, Fig. 3B) was clearly discriminated from *N. ceranae* (upper left, Fig. 3B). In addition, it seems that PCA data allowed to distinguish the two isolates of *N. ceranae* collected from the non-European country and France (MBO.NC01 and MBO.NC02, see Fig.3B). Finally, PCA revealed a good separation between live (above in Fig. 3B) and dead spores (below in Fig.3B) and a tendency to discriminate the dead spores of *N. ceranae* from the ones of *N. apis* (Fig. 3B, found on the rightmost side of the clustered data points).

In order to explain the clustering, we analysed molecular ions and their contribution to Dim1 and Dim2 (Figure 3C). Fifty-nine variables, corresponding to the 59 ion peaks found in samples, were considered (see peak list and colour code in Table 2). Among the 59 molecular ions, 11 were not discriminating (m1-2, m8, m12, m21, m26-27, m29-31 and m38, Fig. 3C, black labels in Table 2). Of the 48 discriminating peaks, 21

molecular ions (m3-4, m7, m10-11, m14, m16, m20, m23, m25, m32, m34, m36-37, m42, m45-47, m49 and m58-59) were highly correlated to Dim1 and had a high contribution to the discrimination of *N. apis* vs *N. ceranae* (green labels in Table 2). Fifteen molecular ions (m5-6, m9, m13, m15, m17, m22, m24, m43 and m50-55, red labels in Table 2) were negatively correlated to Dim1 and had a high contribution to the discrimination of *N. ceranae* vs *N. apis*. Considering Dim2, the following 12 molecular ions, m18-19, m28, m33, m35, m39-41, m44, m48 and m56-57, were negatively correlated to Dim2 and had a limited contribution in the discrimination of fresh vs dead spores regardless of *Nosema* species (Fig. 3C and blue labels in Table 2).

We visualised the average spectra (n=9) from technical replicates to first, highlight modifications of the peptide/protein composition according to the *Nosema* species (MBO.NC01 and .NC02 vs MBO.NA01, Figure 4A) and second, visualize a putative difference between geographically distant *Nosema* isolates (MBO.NC01 vs MBO.NC02, Fig. 4B). Between *N. apis* and *N. ceranae* isolates, the MALDI-MS spectra showed a series of overall differences in MFPs regardless of the *N. ceranae* isolate (see Fig. 4A1 and 2). Several ions within the 2,800-5,500 mass ranges displayed intensity variation according to the isolates considered (for enlarged mass area, see Fig. 4A3). Four major zones qualitatively discriminated the *Nosema* species, two in the mass range between 2,800 - 3,300 *m/z* and 4,200 - 4,600, more complex for *N. apis* than *N. ceranae*, and two others at *m/z* between 3,800 - 4,200 and 4,600 - 5,500 enriched in *N. ceranae* compared to *N. apis*. The most part of these differences were relevant in the statistical analyses (m14, m16, m36 and m46 for *N. apis* and m13, m17, m43 and m50-55 for *N. ceranae*; Fig. 4A3, Fig.3 and Table 2). These molecular ions that are not characterized in this study will be subjected to dedicated mass spectrometry approaches in further studies. These results suggest that MALDI-MS represents a rapid and simple method for assessing the identity of *Nosema* spore species.

As a next step, we analyzed two isolates of *N. ceranae* collected from two different geographical origins, MBO.NC01 from a non-European apiary and NBO.NC02 from a French apiary. Between the two *N. ceranae* isolates, the overall MALDI-MS spectra showed minor differences (see Fig. 4B). Several ions within the 1,300 - 3,600 mass ranges displayed intensity variation according to the origin of the isolate (enlarged mass area, Fig. 4B). Within this mass range, two qualitatively distinct areas discriminated the origin of the *Nosema* isolates, one in the mass range between 1,300 - 2,700 *m/z* with five molecular ions (*m/z* 1,342, 1,455, 2,028, 2,473, and 2,646) only detectable in the fresh extracts of the isolate MBO.NC02 (see Fig. 4B1). In contrast, the molecular ions at *m/z* 1,834 and 3,519 were specific to the non-European isolate MBO.NC01. These few differences explained the two distinct clusters within the PCA analyses (m1, m5, m13, m21 and m27 for French isolate, and m8, m38 for non-European isolate; Fig. 4B1, Fig.6 and Table 2). These results suggest that, in addition to an approach for the identification of *Nosema* species, MALDI-MS could be a promising method to classify *Nosema* spores according to their geographical origin. Using a molecular method, specifically working on the ITS region of rDNA of the parasite, Huang and colleagues,

2008, also observed a difference between isolates from Taiwan, France and Spain⁴. Our results confirm that MALDI profiling is a useful and adapted approach for identification of *Nosema* from different species and potentially from different geographical origins.

3.3 Statistical analyses for viability status of spores by MALDI profiling and flow cytometry

Another part of our study was the comparative evaluation of the viability of the spores by two methods: flow cytometry and MALDI profiling. We based the viability evaluation by flow cytometry of the three isolates (MBO.NC01, MBO.NC02 and MBO.NA01, Fig. 5) on the work of Peng and colleagues (2014). Using solutions at 10⁶ spores/mL labelled with PI (specific for dead cells) and SYTO 16 (nuclear staining), the following viability percentages were obtained: 82.4% (sd ± 4), 76.7% (sd ± 2,4) and 93.6% (sd ± 0.6) for MBO.NC01.V, MBO.NC02.V and MBO.NA01.V, respectively (Figure 5A). The difference in viability values was significant (Kruskal-Wallis, Chi square = 15, p-value < 0,001, df = 2). The percentage of viability in the solution containing dead spores (20 min at 121°C) decreased to 0%: 0.5% (sd ± 0.3), 0.2% (sd ± 0.06) and 0.2 % (sd ± 0.05) for MBO.NC01.NV, MBO.NC02.NV and MBO.NA01.NV, respectively (Figure 5B). According to Collado and colleagues, heat treated spores are dead and no longer infectious⁵⁸. Extracts from live and dead spores were analysed with MALDI-MS using the same procedure of peptide/protein extraction. Average spectra (n=9) were generated from technical replicates to visualize differences between fresh and heat killed spores for each spore isolate and species (MBO.NC01, MBO.NC02 and MBO.NA01; Figure 6). Within *N. apis*, the MALDI-MS spectra of live and dead spore extracts revealed strong differences (see Fig. 6A), while the MALDI-MS profiles of the control peptide/protein mixtures (APICAL and ProtMix) were not altered after the heat treatment (data not shown). Four ions within the 2,600 - 4,600 mass range (*m/z* 2,650, 3,583, 4,376 and 4,584) were specific to dead spores (see Fig. 6A1) and only three were common to both live and dead spores (*m/z* 2,588, 2,883 and 2,920, see Fig. 6A1). Within the two *N. ceranae* isolates, few distinct molecular ions were observed between live and dead extracts, depending on the isolates (see Fig. 6B1 and 6B2). For MBO.NC01, two molecular ions (*m/z* 3,599 and 3,630) were exclusively detectable in dead extracts (see Fig 6B1) whereas for MBO.NC02 three molecular ions at *m/z* 2,262, 5,669 and 5,784 were discriminant, (see Fig. 6B2). One molecular ion at *m/z* 3062 was shared by the two geographically distinct extracts (see Fig. 6B3). Spectra of dead spores were considerably less complex (*m*28, *m*39, *m*44 and *m*48 absent in dead extracts) than fresh spore extracts (Fig.3 and Fig.6). With MALDI-MS we could distinguish fresh and dead spores.

Measuring spore viability is important to evaluate the potential infectiousness of the pathogen. The link between viability and infectiousness is still unclear because three levels of information have to be considered: viability, extrusion rate of the polar tube and germination rate^{59,60}. Extrusion refers to spores that extrude their polar tubes but does not consider whether the sporoplasm of the spores will infect the host cell contrary to the germination⁵⁹. Even if viability cannot be equated to infectiousness, viability

profiling may represent an essential step to better study the model and the mechanistic of the infectious process. Two major studies worked on the longevity and viability of spores in various conditions of conservation^{49,58}. Nowadays, to evaluate the ability for spores to infect the host, scientists can infect bees with spore solutions and count the number of spores after a few days of infection and measure the mortality^{61,62}. Even if viability profiling does not reflect infectiousness^{59,60}, proteomic analyses could help to identify factors of infectiousness. For example, the polar tube proteins are well known for their involvement in invasion of the host cell^{63,64}. However, masses of these proteins are too high (up to 20 kDa) to be detected in our MALDI conditions.

Finally, between each extract (*N. apis* and the two *N. ceranae*, fresh and dead) analysed with MALDI-MS, we detected one common molecular ion at mass 2,588 *m/z* (Fig.4 and Fig.6), corresponding to the label peak m26 (see Table 2). We could not characterize this ion, but it is a potential biomarker of the genus *Nosema*.

3.4 Blind tests on different isolates of *Nosema* spores
Five spore isolates were freshly collected from infected bees. Each solution of spores was purified following our protocol. For their identification, the spore extracts were analyzed by MALDI Biotyping. The resulting spectra were compared to the *N. ceranae* and *N. apis* model we had generated. All isolates were identified as *N. ceranae* spores with a high confidence (score up to 2.00, Figure 7).

4. Conclusion
To conclude, for each individual spore isolate, we determined its mass fingerprint and its viability using the combinatorial approach of MALDI-MS and flow cytometry. Using this analytical strategy, we demonstrated the ability to discriminate *N. ceranae* extracts from *N. apis* extracts and potentially *N. ceranae* from two distinct geographical origins (France *versus* a non-European country). We also differentiated live spores from dead spores. Using flow cytometry these two spore solutions were segregated into two distinct clusters. For living spores, values averaged between 76 and 94%, while for dead spores less than 0.5% of living spores were detectable. Regarding the MALDI profiling, the statistical analyses of this set of spore solutions are clearly separated. Prognosis and/or Diagnosis have important implications for animal and patient care, research, and policy. When they are, accurate and made in a timely and cost-effective manner, the sanitary services and practitioners have the best opportunity for a positive health outcome. In addition, public policy decisions are often influenced by diagnostic information. The analytical strategy we are proposing has several advantages over microscopic and PCR analyses for monitoring nosemosis in honeybee, it is (i) cheaper in terms of consumables, (ii) less time consuming, (iii) requires less spores (by a factor of two) for a robust identification, and (iv) allows estimation of a viability status in contrast to PCR or direct microscopy. MALDI Biotyping may represent a valuable tool for sanitary services and beekeepers to survey nosemosis in apiaries.

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Table 1: Number of identified peaks in the peak lists of the three spore extracts prepared with three different protocols: (P1) ultrasonic, (P2) stainless steel balls and (P3) zirconium oxide beads. Each step corresponds to the data processing described in the Materials and methods section.

Sample Name	step 1	step 2	step 3	step 4	step 5
MBO.NC02.V (P1)	30	25	9	6	5
MBO.NC02.V (P2)	56	53	19	17	17
MBO.NC02.V (P3)	88	84	32	23	21

Table 2: List of molecular ions (*m/z*: mass/charge) and their corresponding labels. Peaks positively and negatively correlated to the Dim1 of the PCA are in green and red, respectively. For Dim2, the peaks in blue are negatively correlated. The peaks in black are not discriminating.

Labels	Mass (<i>m/z</i>)	Labels	Mass (<i>m/z</i>)
m1	1342.69	m31	2920.42
m2	1455.85	m32	2955.32
m3	1476.71	m33	3062.63
m4	1490.76	m34	3068.27
m5	1495.52	m35	3077.79
m6	1564.56	m36	3120.51
m7	1664.07	m37	3168.88
m8	1834.88	m38	3519.17
m9	1887.27	m39	3583.21
m10	1922.39	m40	3599.29
m11	1964.36	m41	3630.81

m12	1996.69	m42	3928.01
m13	2028.73	m43	4035.25
m14	2035.55	m44	4376.86
m15	2041.52	m45	4357.65
m16	2053.63	m46	4390.53
m17	2153.56	m47	4420.58
m18	2262.03	m48	4584.28
m19	2434.91	m49	4918.12
m20	2463.40	m50	4940.90
m21	2473.85	m51	4999.87
m22	2505.83	m52	5142.34
m23	2526.00	m53	5201.35
m24	2549.00	m54	5254.15
m25	2555.29	m55	5416.17
m26	2588.56	m56	5669.17
m27	2646.71	m57	5784.27
m28	2650.12	m58	6351.10
m29	2710.51	m59	6566.95
m30	2883.16		

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Captions

Figure 1: Experimental workflow from biological material preparation to MALDI-MS and flow cytometry analyses. *Nosema* spores are purified from infected honeybees (orange panel). Following purification, spores are counted and subjected to MALDI-MS (blue panel) and flow cytometry (green panel) analyses. For MALDI-MS, three alternative protocols (P1, P2 and P3) are used to generate molecular spore extracts. In P1, spore extracts are performed by sonication. In P2 and P3, spores are grounded using steel and zirconium beads, respectively. One μL of extract is used for MALDI sample preparation. For flow cytometry, spore solutions are dyed with SYTO 16 and propidium iodide (PI).

Figure 2: Flow cytometry analyses of *Nosema* spore isolates. The percentage of viability of [A] live spores (MBO.NA01.V (n=4), MBO.NC01.V (n=10) and MBO.NC02.V (n=10)) and [B] dead spores (MBO.NA01.NV (n = 4), MBO.NC01.NV (n=10), MBO.NC02.NV (n=10)) from the different isolates and their representative plots are reported. Letters (a, b and c) represent statistical differences between samples. The same letter means no difference whereas two different letters report a statistical difference. For each boxplot, from the bottom to the top, the first line represents the minimal value, the second line is the first quartile, the third is the median, the fourth is the third quartile and the last line is the maximal value. Points outside the boxplot are outliers. The boxplot *Nosema apis* is represented in grey, *N. ceranae* in white.

Figure 3: Differential MALDI-MS spectra of spore extracts obtained with P1, P2 or P3 protocols. Spore extracts prepared with protocols [A] P1 (ultrasonic), [B] P2 (stainless steel balls) and [C] P3 (zirconium oxide beads) are analysed by MALDI and represented in the mass range of 1,000 - 6000 m/z . Each spectrum is the average of three replicates per extract. a.i.: arbitrary intensity, m/z : mass/charge.

Figure 4: Differential MALDI-MS spectra between fresh spores of *Nosema* isolates. The different extracts, prepared from the three purified spore isolates MBO.NA01 from *N. apis* (blue spectra, above in panel A1 and A2), MBO.NC01 from *N. ceranae* (green spectra, below in panel A1 and above in panel B), MBO.NC02 from *N. ceranae* (red spectra, below in panel A2 and B) are compared. Each spectrum is the average of nine replicates per extract. For comparison between the two species (A1 and A2) the spectra are cropped in the range of 1,000 – 9,000 m/z and an enlarged area is defined between 2,800 - 5,500 m/z . Within this mass range, four major zones discriminate the *N. apis* and *N. ceranae* extracts (see right A3 for *N. apis* and left A3 for *N. ceranae*). For comparison between the two isolates of *N. ceranae* (B) the spectra are cropped in the range of 1,000 – 3,600 m/z . Within this mass range, seven molecular ions discriminate the *N. ceranae* MBO.NC01 and *N. ceranae* MBO.NC02 extracts (see right B1 for MBO.NC02 and left B1 for MBO.NC01). a.i.: arbitrary intensity, m/z : mass/charge. The spectral gel view shows the intensity of each of the peaks represented by their weight index and the m/z values (Da) found within the average spectrum of the extracts.

Figure 5: Differential MALDI-MS spectra between fresh and dead spores of *Nosema* isolates. The different extracts, prepared from the three purified spore isolates, MBO.NA01 (*N. apis*, panel A, blue), MBO.NC01 (*N. ceranae*, panel B1, green), and MBO.NC02 (*N. ceranae*, panel B2, red), are represented. For each panel, the upper spectrum represents fresh spores (V) and the lower spectrum shows dead spores (NV). Each spectrum, cropped in the range of 1,000 – 7,000 Da (A) or 1,000 - 6,000 Da (B1 and B2), is the average of nine replicates per extract. For MBO.NA01 (A) the different peaks discriminating between fresh and dead extracts are represented in A1. For MBO.NC01 (B1) and MBO.NC02 (B2) enlarged areas are represented for discriminating peaks and the common peak between the two dead spore extracts is in panel B3 (MBO.NC01 coloured in green and MBO.NC02 coloured in red). a.i.: arbitrary intensity, m/z : mass/charge. The spectral gel view shows the intensity of each of the peaks represented by their weight index and the m/z values (Da) found within the average spectrum of the extracts.

Figure 6: Differential PCA-based statistical analyses and hierarchical clustering of spore isolates from peak lists.

Panel A represents the percentage of explained variance by each dimension of the PCA analysis. Panel B depicts the distribution of samples on a 2D-PCA plot (Dim1 and Dim2). Differences between live (MBO.NA01.V (light blue), MBO.NC01.V (light green) and MBO.NC02.V (light red)) and dead (MBO.NA01.NV (dark blue), MBO.NC01.NV (dark green), MBO.NC02.NV (dark red)) spores are represented on the two dimensions Dim1 and Dim2. The ellipse represents the confidence of the results. For each sample nine points are represented. The distribution of samples is based on the molecular ions and their position on the correlation circle (C). Each arrow represents a variable and the colour indicates the contribution of each variable in the principal component (dimension). Representation of variation is explained by the two first dimensions (Dim1 and Dim2).

Figure 7: Differential MALDI-MS spectra of five spore solutions and identification scores. Five spore extracts (s1 to s5) prepared with protocols P3 (zirconium oxide beads) are analysed by MALDI-MS and represented in the mass range of 1,000 - 9000 m/z . For each individual spectrum the score of identification is associated. a.i.: arbitrary intensity, m/z : mass/charge.

Viability measurements with flow cytometry

Percentage of viability

Mass profiles generated with MALDI-MS

Principal Component Analysis