

1 A MALDI-MS biotyping-like method to address honey bee health status through
2 computational modelling

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16 Short title:

17 MALDI biotyping-like method to address bee health

18
19 **Abstract:**

20 Among pollinator insects, bees undoubtedly account for the most important species. They play a
21 critical role in boosting reproduction of wild and commercial plants and therefore contribute to the
22 maintenance of plant biodiversity and sustainability of food webs. In the last few decades,
23 domesticated and wild bees have been subjected to biotic and abiotic threats, alone or in combination,
24 causing various health disorders. Therefore, monitoring solutions to improve bee health are
25 increasingly necessary. MALDI mass spectrometry has emerged within this decade as a powerful
26 technology to biotype micro-organisms. This method is currently and routinely used in clinical
27 diagnosis where molecular mass fingerprints corresponding to major protein signatures are matched
28 against databases for real-time identification. Based on this strategy, we developed MALDI
29 BeeTyping as a proof of concept to monitor significant hemolymph molecular changes in honey bees
30 upon infection with a series of entomopathogenic Gram-positive and -negative bacteria. A *Serratia*
31 *marcescens* strain isolated from one “naturally” infected honey bee collected from the field was also
32 considered. We performed a series of individually recorded hemolymph molecular mass fingerprints
33 and built, to our knowledge, the first computational model made of nine molecular signatures with a
34 predictive score of 97.92%. Hence, we challenged our model by classifying a training set of individual
35 bees' hemolymph and obtained overall recognition of 91.93%. Through this work, we aimed at
36 introducing a novel, realistic, and time-saving high-throughput biotyping-like strategy that addresses
37 honey bee health in infectious conditions and on an individual scale through direct “blood tests”.

38

39 **Keywords:**

40 MALDI biotyping, Bee health, Immunity, *Apis mellifera*, Molecular mass fingerprint, Infection,
41 *Serratia marcescens*, Computational model, Antimicrobial peptides

42

43 **Significance Statement:**

44 Domesticated and wild bees worldwide represent the most active and valuable pollinators that ensure
45 plant biodiversity and the success of many crops. These pollinators and others are exposed to
46 deleterious pathogens and environmental stressors. Despite efforts to better understand how these
47 threats affect honey bee health status, solutions are still crucially needed to help beekeepers, scientists
48 and stakeholders in obtaining either a prognosis, an early diagnosis or a diagnosis of the health status
49 of the apiaries. In this study, we describe a new method to investigate honey bee health by a simple
50 “blood test” using fingerprints of some peptides/proteins as health status signatures. By computer
51 modelling, we automated the identification of infected bees with a predictive score of 97.92%.

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53

54 **Introduction**

55 Over several decades, an abnormal mortality of honey bees and other pollinators (bumblebees, solitary
56 bees) has been observed in all industrialized countries (1-4). This phenomenon has been particularly
57 recorded in honey bees (5). The global loss of honey bee colonies has detrimental consequences for
58 plant biodiversity, bee products, and negative economic and societal effects (6). As a result, many
59 scientific studies have been carried out to understand the mechanisms underlying phenomena such as
60 colony weakening or collapse and colony mortality observed in most of the countries practicing
61 intensive agriculture. Many reports concluded that biotic and abiotic factors are suspected to be
62 involved in this phenomenon, either alone or in combination (2, 5, 7-10). Potential causes are exposure
63 to (i) environmental and in-hive chemicals (11, 12), (ii) agricultural practices (13, 14), (iii) infection
64 by micro-organisms and predation by parasites (15-17) and (iv) nutritional factors (18-20), among
65 others, which lead to the transition from a health status qualified as normal to a health decline that
66 would contribute to the colony collapse (7). The expression of this pathological state may notably be
67 linked to a decrease in the immune capacities of the bee and/or the colony subjected to these
68 combinations of stressors (21-25). The complex underlying mechanisms of stressors (biotic and
69 abiotic) that affect bees and impact honey bee health status remain still partially understood. Both the
70 fundamental molecular mechanisms associated with the modifications of health status and the
71 development of solutions capable of rendering a prognosis, an early diagnosis or a diagnosis, remain
72 to be elucidated. This is a prerequisite for limiting colony losses and protecting honey bees, but the
73 tools and services to perform a clear sanitary diagnosis of beehives are currently lacking. Even if
74 visual and PCR analyses are available for surveillance of pathogen loads, prediction of the likely
75 impact on the colony remains an issue not satisfactorily addressed (26). Apart from typical methods
76 for honey bee colony health monitoring like polymerase chain reaction assays and sensor-based
77 devices (27-29), mass spectrometry (MS), which has been greatly improved in the past 20 years, may
78 play an essential role in the quest for innovative solutions in monitoring bee health. Among the
79 different MS approaches, Matrix-Assisted Laser Desorption-Ionization – Time of Flight Mass
80 Spectrometry (MALDI-TOF MS) has become increasingly popular for biological sample identification
81 in laboratory research and for clinical diagnostics in microbiology. The widespread interest of this
82 technology for analysing biological matrices is due to the generation of mostly monocharged ions
83 which satisfy the generation of simplified mass spectra when analysing complex biological samples
84 (30).

85 In the past ten years, MALDI-TOF MS has become a referenced system in microbiological
86 laboratories. Technological developments, making this analytical technique a robust, fast and widely
87 used commercial platform, paved the way for its use in routine clinical microbiology (31-33). In 2013,
88 two independent systems, the VITEK[®] mass spectrometer (bioMérieux clinical Diagnostics) and the
89 MALDI Biotyper[®] MicroFlex (Bruker Daltonics Inc.) received the US Food and Drug Administration
90 (FDA) clearance for the identification by biotyping of micro-organism species including yeasts and

91 aerobic / anaerobic bacteria. In 2015, FDA clearance was announced for 193 and over 280 species
92 using the VITEK[®] and the MALDI Biotyper[®] instruments, respectively (34, 35). These platforms
93 perform by targeting the ribosomal proteins as biomarkers for the identification of clinical bacterial,
94 fungal and yeast isolates (36). In early 2018, the Bruker MALDI Biotyper[®] solution received
95 international approvals as an official method of analysis for the food industry (source from Bruker
96 Corporation, related link <https://www.bruker.com>). Barcoding or molecular mass fingerprints (MFP)
97 of biological matrices by MALDI-TOF MS is indeed a thriving approach, enabling the rapid detection
98 of peptide/protein components that can provide comparative information.

99 Building on the concept of this MS-based MFP approach and on the demonstrated capability of
100 MALDI-TOF MS to decipher the molecular mechanisms of insect immunity in the *Drosophila* model
101 for various infections (37-39), we performed a peptidomics/proteomics-based mass fingerprinting of
102 honey bee hemolymph using MALDI MS to discriminate different models of bacterial infections.
103 Relying on previously published studies (39-42), we first developed and validated an experimental
104 model of challenged honey bees with Gram-positive and -negative bacteria (using notably a *Serratia*
105 *marcescens* strain isolated from honey bees). Then, we assessed the usefulness of MALDI-TOF MS to
106 fingerprint the peptides/proteins in honey bee hemolymph in order to build and validate a
107 computational model of bacterial recognition based on the molecular signatures within the molecular
108 mass range 2-20 kDa; a method we will refer to as BeeTyping. In addition, we determined the
109 performance of this computational model using a training set of challenged bees. Through this work,
110 we introduce BeeTyping as an effective method for monitoring honey bee health status by diagnosing
111 bacterial infections in young adult honey bees.

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113

114 **Results & Discussion**

115 **MALDI-TOF MS Biotyping successfully diagnosed a bacterial strain isolated from** 116 **honey bee hemolymph.**

117 In order to generate relevant biological models of honey bee infections, we used the bacteria
118 *Micrococcus luteus*, *Pectobacterium carotovorum* subsp. *carotovorum* and *Serratia entomophila*. *M.*
119 *luteus* is a Gram-positive bacterial strain frequently used when monitoring insect immunity (43) and
120 has been shown to colonize bee hives and gastrointestinal tracts of honey bees (44) while *P.*
121 *carotovorum* subsp. *carotovorum* (45) and *S. entomophila* (Institut Pasteur, CIP102919) are two
122 bacterial strains that trigger a systemic immune response in insects. We also performed an additional
123 model of infection using a *Serratia marcescens* strain (*Sm*BIOP160412, Lab. collection) isolated from
124 a naturally infected honey bee collected in the field. To certify the constructed biological models of
125 infection, the four bacterial strains were classified by MALDI MS biotyping (Figure 1). As shown, the
126 individual MFPs of the different strains detailed below and represented by the spectral gel views
127 passed the threshold score of identification with a significant score (reliability score ≥ 2) and
128 successfully matched the Bruker reference strains (see Fig. 1, *M. luteus* ref. 1270, *P. carotovorum*
129 subsp. *carotovorum* ref. 78398, and *S. entomophila* ref. 42906) of the MALDI BioTyper® database.
130 Moreover, applied to the bacterial strain *Sm*BIOP160412 isolated and cultured from an isolate
131 obtained from a naturally infected honey bee, MALDI MS biotyping demonstrated for the first time to
132 our knowledge, its ability to characterize a field bacterial infection in *Apis mellifera*. This identified
133 bacterial strain, namely *S. marcescens*, is known to be a widespread pathogen of adult honey bees
134 (46), and a virulent opportunist, taking advantage of disturbed microbiota to develop in honey bee guts
135 after exposure to the pesticide glyphosate (47).

136 **MALDI-TOF MS BeeTyping as a new approach to discriminate Gram-positive and** 137 **-negative biological models of infection directly from honey bee hemolymph**

138 A set of 64 MALDI MS spectra was recorded from individual hemolymph samples. These spectra
139 were obtained from 22 control honey bees and from 23 and 19 honey bees individually infected with
140 the Gram-positive *M. luteus* or the Gram-negative *P. carotovorum* subsp. *carotovorum*, respectively.
141 An averaged spectrum, containing 110 MALDI MS ion peaks (MFP, Table S1), was built for each of
142 the three biological models (Figure 2A). Statistical analysis based on Principal Component Analysis
143 (PCA) and performed on these MFPs clearly segregated the three biological models (Figure 2B). As
144 shown by the PCA plot score, the individual spectra were clustered in accordance with their
145 corresponding models and were segregated based on their mass fingerprints. The unsupervised
146 hierarchical clustering of hemolymph samples, classified almost all of the individual MFPs with
147 respect to their corresponding biological models (Figure 2C). Out of 64 normalized spectra used to
148 build the clustering dendrogram, four and three recorded mismatched spectra were observed,

149 corresponding to the lowest (70%) and highest (95%) limits of explained variance, respectively. The
150 mismatched spectra were further identified within the representation of the PCA plot score of the
151 hemolymph samples (Figure 2B, arrows). At the limit of explained variance of 70%, the four spectra
152 included one spectrum from the control condition and one from the *M. luteus* infection model, both
153 classified under the *P. carotovorum* subsp. *carotovorum* model, and two of this latter model,
154 mismatched to the control model (see asterisks in Fig. 2C). Regarding the three mismatched spectra
155 observed at 95% of the explained variance, one was from the *M. luteus* model and classified under the
156 *P. carotovorum* subsp. *carotovorum* model and two, from the *P. carotovorum* subsp. *carotovorum*
157 model, classified under the control model (see asterisks in Fig. 2C).

158 In order to assess the relationship between the MALDI-TOF MS MFPs of the biological models and
159 the honey bee's immune status, we correlated these MFPs with each of the four antimicrobial peptides
160 (AMP) defined from *Apis mellifera* (48): Apidaecin 1A (41) at m/z 2,107, Hymenoptaecin (49) at m/z
161 10,270, Abaecin (50) at m/z 3,878 and Defensin 1A (40) at m/z 5,519 (Figure 3). As shown, per-peak
162 fingerprint correlations with the antimicrobial peptides (AMPs) were scored based on the molecular
163 ion peak area and represented as heat maps through a colored scale intensity ranging from low
164 (minimum score of -1, in red) to high correlation (maximum score of 1, in green). Reported in relation
165 to the MFPs, four clades (A, B, C and D) described the positive and negative correlations of the MFPs
166 with each of the four AMPs (see Table S1) and segregated the three biological models (non-
167 experimentally infected as a control condition, *P. carotovorum* subsp. *carotovorum*. model and *M.*
168 *luteus* model, see Fig. 3).

169 Regarding the control condition, the four AMPs were found to be positively correlated with the
170 molecular ion markers of the hemolymph MFPs of clades A, C and D and negatively correlated with
171 markers of clade B. In the *P. carotovorum* subsp. *carotovorum* model, the same four AMPs were
172 positively correlated with the MFPs of clades B and C and negatively with clade A and D markers,
173 except for Hymenoptaecin, which exhibited positive and negative correlations with clade D. In the *M.*
174 *luteus* model of infection, each of the AMPs was predominantly positively correlated with the
175 molecular clades A and B, and negatively with clades C and D. These correlations show
176 complementary molecular signatures in the three experimental models. Discrete dynamic molecular
177 patterns are modulated and correlated to the immune status of the bees, allowing us to discriminate
178 infected from non-infected bees and the type of infection

179 **Machine-learning as the first reported computational model to recognize and** 180 **classify experimentally infected honey bees based on hemolymph MFPs**

181 Because the proteomic mass spectra of hemolymph samples reflect the immune status of
182 the honey bees, our next goal was to predict honey bee health status based on the bee
183 MALDI-TOF MS MFPs. For that purpose, we decided to build a molecular model based
184 on the MFPs of hemolymph samples, by using a machine-learning algorithm, the

185 Genetic Algorithm (GA). The GA classifier generated a set of discriminating peaks that
186 recognized and classified hemolymph according to the biological model (honey bees
187 challenged with *P. carotovorum carotovorum* or *M. luteus* and non-experimentally
188 infected honey bees). These discriminating peaks form a barcode model and define the
189 strength of this model through its recognition capability. The performance of the
190 classifier barcode model was evaluated through internal cross validation by iterative
191 reclassification of a set of spectra equal to half of the total number of spectra included
192 in the model. For each of the ten iterations performed, a new set of spectra was chosen
193 randomly through an automated internal process.

194 In an initial approach, we restricted our experimental infection to *M. luteus* as the Gram-positive strain
195 and to *P. carotovorum* subsp. *carotovorum*. While this is, to our knowledge, the first time such a
196 computational model has been applied to the classification of bacterial-infected honey bees, machine-
197 learning algorithms have been used previously in other biological subjects. For example, MALDI MS
198 has been successfully used to build a proteomic mass spectra database of different honeys and their
199 MFPs to identify their geographical origin (51). As another example of application, an experimental
200 model of male chicken fertility was designed to perform on-cell direct proteomic fingerprinting by
201 MALDI MS and demonstrated the capability of the GA classifier to build a predictive model to
202 classify chicken sperm fertility (52).

203 In the present study, using GA, based on the individual hemolymph spectra of a cohort of 22 controls
204 and 23 honey bees challenged with *M. luteus* or 20 honey bees challenged with *P. carotovorum* subsp.
205 *carotovorum*, we identified a set of nine best m/z molecular ions based on their capability to
206 discriminate the three biological models from each other (Figure 4). Further tests of recognition
207 capability and cross validation of the GA model were assessed by using the MFPs from the same
208 sample cohort. Considering the standard deviation and the 95% confidence interval of these nine
209 molecular ions, weight indexes were calculated to rank the nine molecular signatures from the most
210 discriminant molecular ion (m/z 3,348.17, weight of 6.24) to the least discriminant one (m/z 5,603.01,
211 weight of 1.97). Moreover, we rated the accuracy of the GA classifier model following two distinct
212 data processings. On the one hand, the classifier calculated the recognition capability by matching the
213 MALDI MS spectra described above against their respective biological models. Therefore, we were
214 able to re-assign hemolymph spectra derived from the control and the *M. luteus* biological models
215 (score of recognition 100%) and for the *P. carotovorum* subsp. *carotovorum* model (score of 93.75%).
216 Overall, performance recognition of the classifier reached 97.92%. On the other hand, internal cross
217 validation scores were calculated for each biological model. To perform this cross validation, the same
218 individual hemolymph spectra from each biological model were randomized and reassessed for
219 successful matching in a batch mode of analysis by the classifier using solely the set of the nine
220 molecular ion markers. The cross validations of the classifier were at 91.51%, 94.40% and 89.87% for

221 the control, *M. luteus* and *P. carotovorum* subsp. *carotovorum* biological models, respectively, giving
222 an overall validation of 91.93% (see Fig. 4).

223 A new set made of 26, 10 and 35 MALDI MS spectra of hemolymph from control, *M. luteus* and *P.*
224 *carotovorum* subsp. *carotovorum* biological models respectively was submitted for the to the GA
225 classifier and classified individually (Table 1 and Table S2). Among the 26 control spectra, 16 were
226 correctly classified, three were classified in *P. carotovorum* subsp. *carotovorum* and three in *M. luteus*
227 models. Four spectra were found as invalid spectra because of the recalibration step. This result was
228 caused by weaker intensities of the molecular fingerprints causing the ion mass recalibration to fail.

229 Regarding the *M. luteus* infectious model, 10 spectra were subjected to the classifier. Seven were
230 correctly classified, two were considered as control and one as belonging to the *P. carotovorum* subsp.
231 *carotovorum* model. No spectrum was deemed invalid. Considering the *P. carotovorum* subsp.
232 *carotovorum* biological model of infection, from the 37 spectra, 13 were correctly classified, one
233 matched to the control, three to the *M. luteus* biological models and 20 to the invalid spectra category.

234 These 20 spectra were qualified as invalid due to noisy mass spectra (intensities of the nine peaks not
235 sufficient to pass the classification) or to a failure in properly calibrating the mass spectra. Given these
236 results, we calculated the performance of the classifier for each of the biological model (see details in
237 Table 2). The GA algorithm achieved 80 % to 90 % accuracy discriminating thus the three biological
238 models. The sensitivity (true positive) and the specificity (true negative) of the GA classifier model
239 were calculated for the three biological models. The model scored at least 70% of sensitivity and at
240 least 84 % of specificity. As detailed in the Table 2, the highest sensitivity was observed for the *P.*
241 *carotovorum* subsp. *carotovorum* model (76.47 %) and the highest specificity for the control model
242 (95.16 %). Based on the sensitivity and the specificity, we calculated the informedness indexes and the
243 positive-negative stratum-specific likelihood ratio (abbreviated +LR, -LR) which inform about how
244 predictive the classifier model is and its performance as a diagnostic tool respectively. As reported in
245 the Table 2, the three biological models scored indexes within the range [-1 ;1] with -1 as incorrect
246 model predictions, 1 as maximum of correct predictions). The calculated informedness indexes for the
247 control, the *P. carotovorum* subsp. *carotovorum* scoring 0.68 and 0.64 respectively and for the *M.*
248 *luteus* (0.55) demonstrated the model was a good predictor. Regarding +LR and -LR, both parameters
249 were calculated. The +LR, which required scores over 1 to be significant were found equal to 15.02;
250 4.55 and 6.12 for the control, the *M. luteus* and the *P. carotovorum* subsp. *carotovorum* models
251 respectively (Table 2). This result demonstrated a good probability that our GA model classified
252 positively the spectra against the biological models. The -LR, which required scores as close as
253 possible to 0 to be significant were found equal to 0.28; 0.35 and 0.27 for the control, the *M. luteus*
254 and the *P. carotovorum* subsp. *carotovorum* models respectively (Table 2). This result demonstrated
255 the weak probability to misclassify the cohort of hemolymph spectra through the GA classifier. In
256 addition, we determined the false discovery rate (q-value) and the false positive rate (p-value) for each
257 of the three biological models. The lowest q-value was of 0.158 and concerned the control model

258 while *P. carotovorum* subsp. *carotovorum* and *M. luteus* models harbored highest values (0.235 and
259 0.461 respectively) denoting a better capability of the classifier to classify unknown spectra within the
260 control model followed by the *P. carotovorum* subsp. *carotovorum* and *M. luteus* models respectively.
261 Regarding the p-values, the control model harbored 0.0484 while *P. carotovorum* subsp. *carotovorum*
262 and *M. luteus* models scored 0.125 and 0.154. Based on this statistical parameter, the classifier shares
263 the same conclusion as obtained with the q-values regarding the algorithm's performance to classify
264 properly the spectra.

265

266 To summarize, the computational model significantly discriminated control from
267 infected honey bees and *M. luteus* from *P. carotovorum* subsp. *carotovorum* infection as well
268 on the basis of the hemolymph mass fingerprints.

269

270 **MALDI-TOF MS BeeTyping as an effective molecular method to discriminate** 271 **honey bees infected with different *Serratia* species**

272 We assessed the performance of MALDI BeeTyping to discriminate infection at the
273 species level, in particular between two *Serratia* species: *S. marcescens* isolated from a
274 naturally infected honey bee (*Sm*BIOP160412, Lab. collection) and a reference strain of
275 *S. entomophila* (see dendrogram, Figure 1). We found that the MFPs of the hemolymph
276 samples collected from honey bees infected by *S. entomophila* and *S. marcescens*
277 presented significant molecular differences (Figure 5A).

278 Moreover, by using the two best discriminant molecular ions (m/z 12,752.8 and m/z
279 7,186.95), revealed by the PCA analysis, we could differentiate the hemolymph spectra
280 of bees infected either with *S. entomophila* or *S. marcescens* *Sm*BIOP160412. In
281 contrast, the two weaker discriminant markers m/z 1,996.14 and m/z 6,113.68 failed to
282 discriminate the two types of spectra resulting from the two *Serratia* species (Figure
283 5B).

284 To further evaluate and rank the measured ion markers within the MFPs based on their
285 capability to discriminate the two *Serratia* species, we performed a receiver operating
286 curve (ROC) analysis to highlight eight m/z ion markers (12,752.8; 7,186.95; 7,688.27;
287 10,269.8 (Hymenoptaecin); 5,057.69; 5,160.28) with AUC scores between 0.8 and 1 in
288 sensitivity (Figure 5C). The Apidaecin, Abaecin and Defensin were also checked for
289 their capability to discriminate the two *Serratia* species (Figure S1). Based on the ROC
290 test, Abaecin and Defensin were also capable to discriminate, to some extent, the two
291 *Serratia* species (see Figure S1) (AUC of 0.739 and 0.639, respectively). However, like
292 the two markers m/z 1,996.14 and 6,613.68, Apidaecin (AUC=0.520) revealed to be a
293 poor discriminant (Figure 5D). Hence, based on these results, it seems possible to

294 discriminate infection by *S. marcescens* from *S. entomophila* in honey bees using
295 computational modelling. To our knowledge, this is the first report on the feasibility of
296 using MALDI MS as an MFP-based method capable of discriminating hemolymph
297 molecular response to systemic infections induced by two different bacterial species of
298 the same genus. *S. marcescens* is known to be a commensal bacterium present in low
299 abundance in the gut of honey bees. By studying the pathogenicity of different strains
300 of *S. marcescens* through two routes of *in vivo* exposure (oral and direct injection into
301 the hemolymph), Raymann et al (46) found that expression of the four honey bee AMPs
302 Abaecin, Defensin, Hymenoptaecin and Apidaecin did not differ between infected and
303 non-experimentally infected control honey bees. These results support the idea that
304 markers other than AMPs need to be identified and monitored to efficiently
305 discriminate bacterial infections in honey bee hemolymph. As we demonstrated, the
306 correlation of the molecular fingerprints and the AMPs in hemolymph allowed us to
307 discriminate the three different models. In order to determine how specific were the
308 nine markers of the classifier to the control, *M. luteus* and *P. carotovorum* subsp.
309 *carotovorum* biological models, we tested the classification of the hemolymph mass fingerprints of
310 honey bees infected using the two *Serratia* strains. We submitted 13 MALDI mass fingerprints of
311 hemolymph from infected bees with *S. entomophila* to the classifier. One was classified as control,
312 seven as fitting with the *P. carotovorum* subsp. *carotovorum* model, and one fitting with the *M.*
313 *luteus* model. The classifier excluded four hemolymphs' fingerprints because of noisy
314 spectra signal or invalid mass recalibration. We also submitted 19 MALDI mass fingerprint
315 of hemolymph from infected bees with *S. marcescens* to the classifier, which identified two as control,
316 five as fitting with the *P. carotovorum* subsp. *carotovorum* model, and four fitting with the *M. luteus*
317 model. The classifier excluded eight hemolymphs' fingerprints for the same reasons as
318 above. Interestingly, the majority of the classified spectra from both, the *S.*
319 *entomophila* and *S. marcescens* models matched with the *P. carotovorum* subsp.
320 *carotovorum* model. It is particularly interesting as these three bacteria are Gram-negative.
321 Nevertheless, some spectra matched against the control and the *M. luteus* models. Taking
322 altogether, these results suggest that the BeeTyping approach generates specific
323 molecular barcodes defined accordingly to biological models.

324

325

326 **Conclusion**

327 Along with most relevant, technically feasible and primary observation-based health status
328 indicators highlighted by EFSA's HEALTHY-B, MALDI-MS BeeTyping, a method derived

329 from the biotyping approach used routinely in clinical microbiology, analyze the downstream
330 responses to stressors through the matured effector molecules circulating in the hemolymph.
331 These effectors include the products of selected immune genes (i.e. genes coding for
332 Apidaecin, Defensin, Hymenoptaecin, and Abaecin) and other molecular mass fingerprints of
333 stress that we are under characterization through a proteomic approach. Our approach of
334 MFPs by MALDI-MS BeeTyping, is a cutting-edge analytic method that may complement
335 and address some limitations issued of the HEALTHY-B toolbox by establishing robust,
336 effective, sensitive and a comprehensive technology for profiling and deciphering, at the
337 individual level, the honeybee health parameters including its immunity stage with regards to
338 bacterial stressors. Moreover, as a robust and sensitive molecular approach, MALDI BeeTyping has
339 several advantages over other molecular biology techniques and visual observations, such as (i) the
340 use of a drop of hemolymph allowing to keep the rest of the body for complementary molecular
341 measurements such as PCR, (ii) a very simple and fast sample preparation, (iii) a short processing time
342 (data acquisition and processing), (iv) low consumable costs, and (v) a user friendly workflow that can
343 be standardized and automated for cost-effective high throughput use. We believe that future
344 developments of MALDI BeeTyping could improve monitoring of honey bee health upon
345 exposure to other biotic or abiotic stressors, the quality control and the origin traceability of
346 apiary products based on molecular markers fingerprinting. Based on specific proteomics
347 signatures, MALDI BeeTyping could bring out a novel analytical tool for early diagnosis of
348 honey bees parasited with *Nosema* species, *Varroa destructor* and infected or not with
349 deformed wing virus or acute bee paralysis virus. We aim at developing the BeeTyping
350 strategy for early diagnosis of honey bees health disorders.

351

352 **Material & Methods**

353 The BeeTyping strategy relies on a workflow divided into four major steps summarized
354 in Table S2 and described in this section.

355 **Biological models**

356 *Bacterial strains*

357 To generate biological models of infection, we used the Gram-negative strains
358 *Pectobacterium carotovorum* subsp. *carotovorum* 15 (formerly *Erwinia carotovora*
359 *carotovora* 15 CFBP2141, generous gift from Bruno Lemaitre, EPFL Switzerland),
360 *Serratia entomophila* (Institut Pasteur, CIP102919) and a *Serratia marcescens* strain
361 (*Sm*BIOP160412, our laboratory collection) isolated within the haemocoel from a
362 naturally infected *Apis mellifera* honey bee collected in the field, and the Gram-positive
363 *Micrococcus luteus* (ATCC 4698). Bacteria were cultured in Luria Bertani (LB) medium
364 overnight at 32°C.

365 *Bacterial strain identification by MALDI biotyping*

366 The *Pectobacterium carotovorum* subsp. *carotovorum*, *Serratia marcescens*, *S. entomophila* and
367 *Micrococcus luteus* strains were identified following Bruker's recommendations. Briefly,
368 one isolated colony of bacteria was spread onto a MALDI plate dedicated for
369 microorganism identifications (MALDI Biotarget 48 polished steel) and mixed with
370 1µL of Alpha-Cyano-4-hydroxycinnamic acid (4-HCCA) MALDI matrix. Spectra were
371 recorded using the MALDI-TOF MS AutoFlex III instrument and the associated materials,
372 chemicals and software package used for MALDI biotyping were all from Bruker Daltonik
373 (Germany) using the standard method (pre-processing step for which the lower mass
374 was set at 2,000, with a resolution of 5, and a compressing factor of 1). The smoothing
375 frame size was 20Da and the search window was 10Da with three runs for the baseline
376 subtraction. For the peak-picking, the maximum number of peaks was set at 200, with a
377 threshold of 0.0045. The method of peak-picking was based on peak fitting using the
378 Gauss profile. The recorded spectra were matched against the dedicated database MBT
379 Compass 4.1, build 70. The obtained gel spectra for the identified bacteria and their
380 corresponding dendrograms were built under MBT Compass Explorer 4.1 using the
381 standard method of identification. For external calibration of the mass spectrometer, a
382 mix of 1µL of bacterial test standard proteins (BTS) covering the entire mass range
383 (m/z 2,000-20,000) of the acquisition method was analyzed using the same protocol.

384 *Experimental infection of the honey bees*

385 Experimental infections were performed on newly-emerged honey bee workers (less
386 than 12h old). To design the computational analyses, a training set of spectra was built

387 using non experimentally infected (unpicked control) bees and bees infected with either
388 *Pectobacterium carotovorum* subsp. *carotovorum* 15, *M. luteus*, *S. entomophila* or the
389 isolated *Serratia marcescens* SmBIOP160412 strain. Infections were performed by
390 pricking honey bees individually in the anterior lateral thorax (spiracle) using a fine
391 needle (Fine Science Tools, Germany) dipped into a freshly concentrated culture pellet
392 of live bacteria. All honey bees (experimentally infected and controls) were placed for
393 24h at room temperature in dedicated small cages and fed *ad libitum* with sugar syrup
394 (Invertbee from SARL Isnard, France) containing fructose (36%), dextrose (30%),
395 saccharose (31%), maltose (1.5%) and other sugars (1.5%). Hemolymph was collected
396 from the dorsal side of the abdomen, using pulled glass capillaries (Sutter Instrument
397 Corp, Novato, California). The collected hemolymph was immediately transferred into a
398 chilled LoBind Protein microtube (Eppendorf, Germany) pre-coated with
399 Phenylthiourea and Phenylmethylsulfonyl fluoride (both from Sigma Aldrich, France)
400 to prevent melanization and proteolysis, respectively. The hemolymph samples were
401 stored at -20°C until use.

402 **Molecular mass fingerprints by MALDI MS**

403 *Data acquisition*

404 Each individual hemolymph sample was analyzed with the Bruker AutoFlex™ III. The
405 molecular mass fingerprints (MFP) were acquired following the Bruker Biotyper®
406 recommendations (matrix, method of sample deposition and detection) with minor
407 adjustments. Briefly, the hemolymph samples were 10-fold diluted in acidified water
408 (0.1% trifluoroacetic acid, Sigma Aldrich, France) and 0.5µL of a given sample was
409 mixed with 0.5µL of 4-HCCA (Sigma Aldrich, France) on a MALDI MTP 384 polished
410 ground steel plate (Bruker Daltonik). Following co-crystallization of the hemolymph
411 spots with the matrix droplet, MALDI MS spectra were recorded in a linear positive
412 mode and in an automatic data acquisition using FlexControl 4.0 software (Bruker
413 Daltonik). The following instrument settings were used: 1.5kV of electric potential
414 difference, dynamic range of detection of 600 to 18,000 Da, 69% of laser power, a
415 global attenuator offset of 46% with 200Hz laser frequency, and 2,000 accumulated
416 laser shots per hemolymph spectrum with a raster of random walk set to 50. The linear
417 detector gain was setup at 1.82kV with a suppression mass gate up to m/z 600 to
418 prevent detector saturation by clusters of the 4-HCCA matrix. The pseudo-molecular
419 ions desorbed from the hemolymph were accelerated under 1.5kV. An external
420 calibration of the mass spectrometer was performed using a standard mixture of
421 peptides and proteins (Peptide Standard Calibration II and Protein Standard Calibration
422 I, Bruker Daltonik) covering the dynamic range of analysis.

423 *Data post-processing and statistical analyses*

424 The MALDI-MS datasets were imported into the ClinProTools™ 2.2 Software (Bruker
425 Daltonik) for post-processing and statistical analyses. All of the recorded spectra were
426 processed with a baseline subtraction and spectral smoothing followed by an internal
427 recalibration step with exclusion of null and/or “non-recalibratable” spectra. The total
428 averaged spectra were calculated based on a signal over noise ratio equal to 5 for peak-
429 picking and area calculations. The irrelevant spectra that did not pass the required
430 signal intensity and resolution were excluded from any integration into the MALDI-MS
431 computational model designed to match the biological models of honey bee infections.
432 A post-processing step involving spectral normalization of all calculated peak area was
433 performed with ClinProTools™ software prior to statistical analysis (95% confidence
434 interval, standard deviation and Principal Component Analysis-PCA).

435 *Hierarchical Clustering, heat maps and ROC curves*

436 The total number of spectra used to design the computational models were normalized
437 and subjected to PCA and unsupervised hierarchical clustering analysis to measure
438 distances between spectra. This analysis was used to determine Euclidean distances
439 (based on PCA results with a reduced dimension limited to 70% and 95% of the total
440 explained variance). The molecular correlation between four antimicrobial peptides
441 (AMPs) known from the honey bee [Apidaecin 1A at m/z 2,107 (Uniprot entry
442 A0A088AIG0), Hymenoptaecin at m/z 10,270 (Uniprot entry Q10416), Abaecin at m/z
443 3,878 (Uniprot entry P15450) and Defensin 1A at m/z 5,519 (Uniprot entry P17722)];
444 and mass fingerprints (MFP) of the three biological models of infection were calculated
445 and represented with a heat map. The receiver operating characteristic (ROC) analyses
446 were built using the ClinProTools™ program and the heat maps, using the OMICs add-
447 on module provided by the XLSTAT program (interquartile threshold value of 0.25).

448 *Computational-based algorithm & machine learning model*

449 In the scope of delivering a barcode model capable of discriminating infected from
450 control honey bees, a training set of spectra was established by fingerprinting the
451 corresponding hemolymph samples using MALDI MS. Series of individual spectra were
452 recorded from 22 controls, 23 honey bees challenged with *M. luteus*, 20 with *P.*
453 *carotovorum* subsp. *carotovorum* 15, and an equal number of 15 spectra from bees
454 challenged with *S. entomophila* and *S. marcescens* SmBIOP160412. Data clustering
455 (optimal spectral separation combined with the determination of a fixed number of
456 peaks within the training set) was performed using the Genetic Algorithm (GA) with the

457 ClinProTools™ software. The GA parameters were as follow: a maximum of 10 peaks
458 harboring the greatest weight was selected and included in the model. A number of 50
459 generations (iterative algorithm searching) was chosen to achieve this maximum of
460 peaks. The k-nearest neighbor parameter, which is a key parameter of artificial
461 intelligence used in supervised machine learning, was set at 3.

462

463 *External validation of the barcode model and classification of unknown spectra*

464 In order to assess the capability of the GA classifier to recognize the infected bees from the control
465 group, a new set of hemolymph MS spectra, never processed in the classifier model, was used to
466 perform an external validation. This experimental set of honey bees included the three biological
467 models; 26 controls, 37 infected honey bees with *P. carotovorum* subsp. *carotovorum* 15 and 10 with
468 *M. luteus*. By submitting those hemolymph spectra to the classifier resulted in counting the correctly
469 classified spectra, and also the mismatched and the invalid ones. In order to assess the performance of
470 our classifier model, accuracy, sensitivity, specificity, informedness, specific-positive and negative
471 likelihood ratios, false discovery rate (q-value) and false positive rate (p-value) were calculated. The
472 accuracy, which informs on how efficient the model is, was calculated according to Wang *et al.* (53).
473 Sensitivity scores real positive cases that are correctly predicted positive by the model and the
474 specificity scores the opposite i.e. the real negative cases that are correctly predicted negative.
475 Informedness scores the probability that a prediction (e.g. result of a machine-learning model to
476 classify one condition against the others) is informed regarding to the tested condition *versus* odds.
477 Informedness helps to make diagnosis decision. Sensitivity, specificity and informedness were
478 determined as previously described (54). The specific-positive and -negative likelihood ratios
479 (abbreviated +LR and -LR) classically used in diagnostic testing with multiple classes informs
480 on how likely the results from the classifier model will match the condition. +LR gives the
481 change in the odds of satisfying the condition (fitting to the biological models), given a
482 positive test result and -LR, the change in the odds of satisfying the condition when the test
483 comes negative. +LR ranges from zero to infinity. With +LR values between zero and one,
484 there is a weak probability that the test matches the condition. If the ratio equals to one, then
485 the test lacks diagnostic value and if the ratio is >1 , then the test increases the probability to
486 match correctly with the condition. Regarding -LR, the closer to zero the value is, the more
487 informative the test is (55).

488

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496 References

- 497 1. Biesmeijer JC, et al. (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* 313(5785):351-354.
- 498 2. Potts SG, et al. (2010) Global pollinator declines: trends, impacts and drivers. *Trends in ecology & evolution* 25(6):345-353.
- 499 3. AJ V (2013) Threats to an ecosystem service: pressures on pollinators. *Front Ecol Environ* 11(5):251-259.
- 500 4. Koh I, et al. (2016) Modeling the status, trends, and impacts of wild bee abundance in the United States. *Proceedings of the National Academy of Sciences of the United States of America* 113(1):140-145.
- 501 5. Goulson D, Nicholls E, Botias C, & Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* 347(6229):1255-957.
- 502 6. Breeze TD, et al. (2014) Agricultural policies exacerbate honeybee pollination service supply-demand mismatches across Europe. *PLoS one* 9(1):e82996.
- 503 7. Smith KM, et al. (2013) Pathogens, pests, and economics: drivers of honey bee colony declines and losses. *EcoHealth* 10(4):434-445.
- 504 8. Dainat B, Vanengelsdorp D, & Neumann P (2012) Colony collapse disorder in Europe. *Environmental microbiology reports* 4(1):123-125.
- 505 9. Neumann P. CN (2010) Honey bee colony losses. *Journal of apicultural research* 49:1-6.
- 506 10. Steinhauer N, et al. (2018) Drivers of colony losses. *Current opinion in insect science* 26:142-148.
- 507 11. Mullin CA, et al. (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS one* 5(3):e9754.
- 508 12. Wu JY, Smart MD, Anelli CM, & Sheppard WS (2012) Honey bees (*Apis mellifera*) reared in brood combs containing high levels of pesticide residues exhibit increased susceptibility to *Nosema* (Microsporidia) infection. *Journal of invertebrate pathology* 109(3):326-329.
- 509 13. Clermont A, Eickermann M, Kraus F, Hoffmann L, & Beyer M (2015) Correlations between land covers and honey bee colony losses in a country with industrialized and rural regions. *The Science of the total environment* 532:1-13.
- 510 14. Smart M, Pettis J, Rice N, Browning Z, & Spivak M (2016) Linking Measures of Colony and Individual Honey Bee Health to Survival among Apiaries Exposed to Varying Agricultural Land Use. *PLoS one* 11(3):e0152685.
- 511 15. Beyer M, et al. (2018) Winter honey bee colony losses, *Varroa destructor* control strategies, and the role of weather conditions: Results from a survey among beekeepers. *Research in veterinary science* 118:52-60.
- 512 16. Traver BE, Feazel-Orr HK, Catalfamo KM, Brewster CC, & Fell RD (2018) Seasonal Effects and the Impact of In-Hive Pesticide Treatments on Parasite, Pathogens, and Health of Honey Bees. *Journal of economic entomology* 111(2):517-527.
- 513 17. Desai SD & Currie RW (2016) Effects of Wintering Environment and Parasite-Pathogen Interactions on Honey Bee Colony Loss in North Temperate Regions. *PLoS one* 11(7):e0159615.
- 514 18. Di Pasquale G, et al. (2013) Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? *PLoS one* 8(8):e72016.
- 515 19. Alaux C, Dantec C, Parrinello H, & Le Conte Y (2011) Nutrigenomics in honey bees: digital gene expression analysis of pollen's nutritive effects on healthy and *varroa*-parasitized bees. *BMC genomics* 12:496.
- 516 20. Dolezal AG & Toth AL (2018) Feedbacks between nutrition and disease in honey bee health. *Current opinion in insect science* 26:114-119.
- 517 21. Sinpoo C, Paxton RJ, Disayathanoowat T, Krongdang S, & Chantawannakul P (2018) Impact of *Nosema ceranae* and *Nosema apis* on individual worker bees of the two host species (*Apis cerana* and *Apis mellifera*) and regulation of host immune response. *Journal of insect physiology* 105:1-8.
- 518 22. Nazzi F, Annoscia D, Caprio E, Di Prisco G, & Pennacchio F (2014) Honeybee immunity and colony losses. *Entomologia* 2(203):80-86.
- 519 23. Flenniken ML & Andino R (2013) Non-specific dsRNA-mediated antiviral response in the honey bee. *PLoS One* 8(10):e77263.
- 520 24. Zhang Y, Liu X, Zhang W, & Han R (2010) Differential gene expression of the honey bees *Apis mellifera* and *A. cerana* induced by *Varroa destructor* infection. *Journal of insect physiology* 56(9):1207-1218.
- 521 25. Di Prisco G, et al. (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences of the United States of America* 110(46):18466-18471.
- 522 26. (2016) E (2016) Assessing the health status of managed honeybee colonies (HEALTHY-B): a tool box to facilitate harmonised data collection. *EFSA Journal* 14(10):1-248.
- 523 27. Meikle W, Holst N (2015) Application of continuous monitoring of honeybee colonies. *Apidologie* 46:10-22.
- 524 28. Gil-Lebrero S, et al. (2016) Honey Bee Colonies Remote Monitoring System. *Sensors* 17(1).
- 525 29. Becher MA, et al. (2018) Bumble-BEEHAVE: A systems model for exploring multifactorial causes of bumblebee decline at individual, colony, population and community level. *The Journal of applied ecology* 55(6):2790-2801.
- 526 30. Karas M (2012) *A history of european mass spectrometry* (IM Publications LLP).
- 527 31. Spinali S, et al. (2015) Microbial typing by matrix-assisted laser desorption ionization-time of flight mass spectrometry: do we need guidance for data interpretation? *Journal of clinical microbiology* 53(3):760-765.
- 528 32. De Respinis S, et al. (2014) Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry using the Vitek MS system for rapid and accurate identification of dermatophytes on solid cultures. *Journal of clinical microbiology* 52(12):4286-4292.
- 529 33. van Belkum A, et al. (2013) Rapid clinical bacteriology and its future impact. *Annals of laboratory medicine* 33(1):14-27.
- 530 34. Senan S, et al. (2015) Geriatric Respondents and Non-Respondents to Probiotic Intervention Can be Differentiated by Inherent Gut Microbiome Composition. *Frontiers in microbiology* 6:944.
- 531 35. Bourassa L, Susan M, & Butler-Wu (2015) Current and Emerging Technologies for the Diagnosis of Microbial Infections. *Method in Microbiology*:37-87.
- 532 36. Clark AE, Kaleta EJ, Arora A, & Wolk DM (2013) Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clinical microbiology reviews* 26(3):547-603.
- 533 37. Sabatier L, et al. (2003) Pherokine-2 and -3. *European journal of biochemistry* 270(16):3398-3407.
- 534 38. Boulanger N, et al. (2001) Immune response of *Drosophila melanogaster* to infection with the flagellate parasite *Crithidia* spp. *Insect biochemistry and molecular biology* 31(2):129-137.

- 567 39. Uttenweiler-Joseph S, et al. (1998) Differential display of peptides induced during the immune response of *Drosophila*: a matrix-
568 assisted laser desorption ionization time-of-flight mass spectrometry study. *Proceedings of the National Academy of Sciences of*
569 *the United States of America* 95(19):11342-11347.
- 570 40. Casteels-Josson K, Zhang W, Capaci T, Casteels P, & Tempst P (1994) Acute transcriptional response of the honeybee peptide-
571 antibiotics gene repertoire and required post-translational conversion of the precursor structures. *The Journal of biological*
572 *chemistry* 269(46):28569-28575.
- 573 41. Casteels P, Ampe C, Jacobs F, Vaecq M, & Tempst P (1989) Apidaecins: antibacterial peptides from honeybees. *The EMBO*
574 *journal* 8(8):2387-2391.
- 575 42. Ishii K, Hamamoto H, & Sekimizu K (2014) Establishment of a bacterial infection model using the European honeybee, *Apis*
576 *mellifera* L. *PLoS one* 9(2):e89917.
- 577 43. Hetru C & Bulet P (1997) Strategies for the isolation and characterization of antimicrobial peptides of invertebrates. *Methods in*
578 *molecular biology* 78:35-49.
- 579 44. Pomastowski P, et al. (2019) Analysis of bacteria associated with honeys of different geographical and botanical origin using two
580 different identification approaches: MALDI-TOF MS and 16S rDNA PCR technique. *PLoS one* 14(5):e0217078.
- 581 45. Acosta Muniz C, Jaillard D, Lemaitre B, & Boccard F (2007) *Erwinia carotovora* Evt antagonizes the elimination of bacteria in the
582 gut of *Drosophila* larvae. *Cellular microbiology* 9(1):106-119.
- 583 46. Raymann K, Coon KL, Shaffer Z, Salisbury S, & Moran NA (2018) Pathogenicity of *Serratia marcescens* Strains in Honey Bees.
584 *mBio* 9(5).
- 585 47. Motta EVS, Raymann K, & Moran NA (2018) Glyphosate perturbs the gut microbiota of honey bees. *Proceedings of the National*
586 *Academy of Sciences of the United States of America* 115(41):10305-10310.
- 587 48. Jifí Danihlik KAMP (2015) Antimicrobial peptides: a key component of honey
588 bee innate immunity *Journal of apicultural research* 54(2):123-136.
- 589 49. Casteels P, Ampe C, Jacobs F, & Tempst P (1993) Functional and chemical characterization of Hymenoptaecin, an antibacterial
590 polypeptide that is infection-inducible in the honeybee (*Apis mellifera*). *The Journal of biological chemistry* 268(10):7044-7054.
- 591 50. Casteels P, et al. (1990) Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis*
592 *mellifera*). *European journal of biochemistry* 187(2):381-386.
- 593 51. Wang J, et al. (2009) Rapid determination of the geographical origin of honey based on protein fingerprinting and barcoding
594 using MALDI TOF MS. *Journal of agricultural and food chemistry* 57(21):10081-10088.
- 595 52. Soler L, et al. (2016) Intact Cell MALDI-TOF MS on Sperm: A Molecular Test For Male Fertility Diagnosis. *Molecular & cellular*
596 *proteomics : MCP* 15(6):1998-2010.
- 597 53. Wang H.Y., et al. (2018) Application of a MALDI-TOF analysis platform (ClinProTools) for rapid and preliminary report of MRSA
598 sequence types in Taiwan. *PeerJ*. 7(6):e5784
- 599 54. Powers, D.M.W. (2011) Evaluation: From precision, recall and F-measure to ROC, informedness, markedness & correlation.
600 *Journal of Machine Learning Technologies*. 2(1):37-63
- 601 55. McGee, S. (2011) Simplifying Likelihood Ratios. *J Gen Intern Med*. 17(8):647-650

602 **Figure legends**

603 **Figure 1:** Classification by MALDI-MS biotyping of *Micrococcus luteus* (*M. l.*) and *Pectobacterium*
604 *caratovororum* subsp. *caratovororum*, (*P. c. c.*), *Serratia entomophila*, (*S. e.*) bacteria including the
605 *Serratia marcescens* (*S. m.*) isolated from a naturally infected bee.

606 The strains used to build the infectious model (*M. l.* and *P. c.c.*) and to assess the computational
607 capability to discriminate proteomic fingerprints from the same bacterial species (*S. m.* and *S. e.*).
608 were analyzed in order to confirm their identity based on their molecular profiles by matching the
609 MALDI MS spectra (mass range m/z 2,000 to 11,000) to the reference strains of the Bruker database
610 containing 6,903 MSP. The BioTyper parameters to validate the identifications of strains, *i.e.* the
611 scores and the matching strains (references and library), were obtained for each biotyped bacterial
612 sample in addition to the MALDI MS spectral gel-view. The dendrogram was built from the Main
613 Spectra Projection (MSP) statistical mode of calculation, which is used to identify, analyze and
614 classify the MALDI MS spectra. These bacteria were identified by their scores and classified
615 according to their distance level (MSP Dendrogram) in comparison to reference strains from the
616 database MBT Compass 4.1, build 70 (*M. l.* IMET 11249HKJ, *P. c. ssp odoriferum* NB 1892
617 PAH, *S. m.* 13103_1 CHB and *S. e.* DSM 12358T DSM). The mass spectra (m/z) were transformed
618 into gel views where the grey scale bar and thickness of the lines refer to the m/z peak intensities.
619 Classically, a high confidence identification is obtained with a score between 2.00 and 3.00, a low
620 confidence identification with a score between 1.70 and 1.99 and a failed identification with a score
621 strictly below 1.70. The mass spectra (m/z) were transformed into gel views where the grey scale bar
622 and thickness of the lines refer to the m/z peak intensities.

623

624 **Figure 2:** Differential PCA-based statistical analyses and hierarchical clustering of individual
625 hemolymph samples from the biological models.

626 Total averaged spectra were fingerprinted by MALDI MS from the infected and control individuals
627 (A). The individual spectra were subjected to PCA analysis, which discriminated the hemolymph
628 molecular mass fingerprints of *Micrococcus luteus* (*M. l.* in blue), *Pectobacterium caratovororum* subsp.
629 *caratovororum* 15 (*P. c. c.* in green) and control (red) groups (B); arrows mark the mismatched outliers.
630 An unsupervised hierarchical clustering based on the PCA results classified the individual spectra
631 according to the lowest (70% left panel C) and highest (95%, right panel C) limits of explained
632 variances.

633 **Figure 3:** Heat-map of four antimicrobial peptides (AMPs) from *Apis mellifera*, Apidaecin 1A,
634 Abaecin, Defensin 1A and Hymenoptaecin correlating with the MALDI MS fingerprints (102 m/z) of
635 the hemolymph samples.

636 Per-peak MALDI MS correlation in standard mode between the AMP molecular ions of Apidaecin 1A
637 (m/z 2,107), Abaecin (m/z 3,878), Defensin 1A (m/z 5,519) and Hymenoptaecin (m/z 10,270), and the
638 MALDI MS fingerprints of the biological model following an experimental infection with either

639 *Micrococcus luteus* (*M. l.*) in blue, or *Pectobacterium carotovorum* subs. *carotovorum* (*P. c. c.*) in
640 green, and the control experiment (non-experimentally infected bees, in red). Each rectangle in the
641 heat map dendrogram represents the abundance level (scale from -1 to +1 from the lowest in red to the
642 highest in green, respectively) of the area of each AMP cross-related with each molecular ion from the
643 fingerprint.

644 **Figure 4:** Genetic Algorithm-based classifier used to discriminate non-experimentally infected
645 (Control, red) bees from experimentally infected ones with *Micrococcus luteus* (*M. l.*, in blue) or
646 *Pectobacterium carotovorum* subsp. *carotovorum* (*P. c. c.*, in green).

647 Nine molecular ion peaks determined by the computational model and ranked according to their
648 weight indexes were found as the best discriminative features of the hemolymph samples on the basis
649 of statistical criteria (Standard deviation determined for each curve representing the molecular ions of
650 the model and the box plots showing the first and third interquartile range with line denoting the
651 median and whiskers encompassing 95% of the individuals). The spectral gel view represents the
652 intensity of each of the discriminative peaks represented by their weight index and the *m/z* values (Da)
653 found within the individual spectra of the biological models.

654 **Figure 5:** Differential PCA-based analysis of hemolymph fingerprints following infection with two
655 *Serratia* strains, *Serratia marcescens* (*S. m.*) isolated from a naturally infected *Apis mellifera* and *S.*
656 *entomophila* (*S. e.*) and statistical relevance of predictive markers.

657 The differential fingerprinting and PCA analysis discriminated the non-experimentally infected bees
658 (control in black) and the experimentally infected groups (*S. e.* in red or *S. m.* in green) with n=13 bees
659 per group (A). The detected peaks in the differential analysis with the highest and lowest discriminant
660 scores were assessed statistically by measuring the standard deviation and the 95% confidence
661 interval. (B). The most interesting peaks classified through the Receiver Operating Characteristics
662 (ROC) curves are shown using the Area Under Curve (AUC) calculation (C). The biological model
663 used as the positive class was the experimental *S. m.* infection and the sensibility (True Positives) and
664 specificity (False Positives) parameters were determined for all calculated peaks. Eight markers
665 defined by their *m/z* values (Da) were found as the best predictive markers (AUC>0.8) for
666 discriminating honey bee infections (*S. e.* or *S. m.*). Conversely, the two least discriminant peaks had
667 an irrelevant AUC (≤ 0.5) with ROC curves fitting the non-discriminant line of the statistical test (D).

668 Table 1: External validation of the genetic algorithm classifier model using a new set of hemolymph
669 spectra.

670 Fifty-seven hemolymph samples were collected individually from the biological models *M. l.*, *P. c. c.*
671 and control prior to being fingerprinted. The spectra were submitted to the GA-based computational
672 model in order to assess classifier performance.

673

674 Table 2: Assessment of the Genetic Algorithm classifier performance.

675 Based on the result of the external validation, the performance of the GA-based classifier model was
676 assessed for each of the biological models by calculating the accuracy, the sensitivity, the specificity,
677 the specific-positive and -negative likelihood ratios (all five expressed as percentage), informedness,
678 p-value and q-value.

679

680 Table S1: Peak-to-peak correlation scores of the four Antimicrobial peptides (abaecin, apidaecin,
681 defensin and hymenoptaecin) with the mass fingerprint of hemolymph across the three biological
682 models (non-experimentally infected/control, *M. l.* for *Micrococcus luteus* infection and *P. c. c.* for
683 *Pectobacterium carotovorum* subsp. *carotovorum* 15 infection)

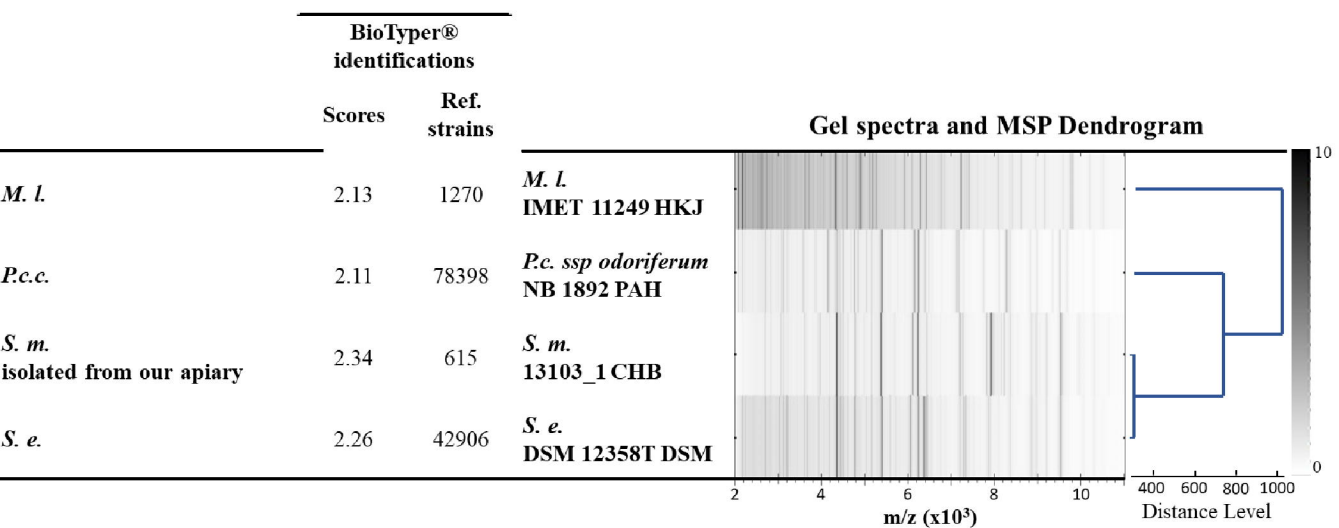
684 Table S2: Results for the external validation of the genetic algorithm-based classifier

685 Figure S1: Assessment of ROC curves of Apidaecin, Abaecin and Defensin to discriminate *S.*
686 *marcescens*- from *S. entomophila*-infected honey bees.

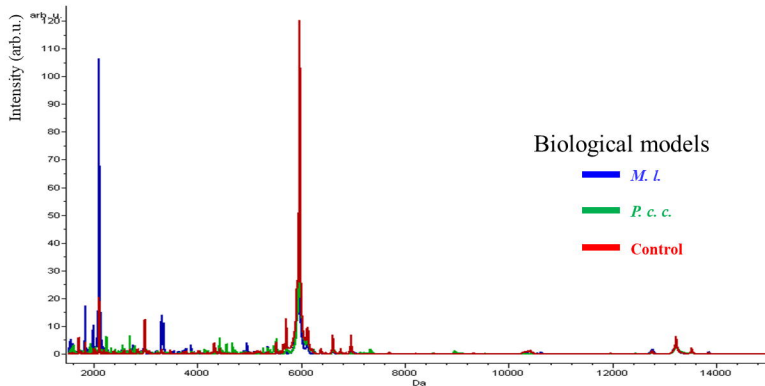
687 Figure S2: BeeTyping workflow for machine learning data-driven analysis of honey bee infections.

688 The methodological approach relied on four main steps addressing major tasks.

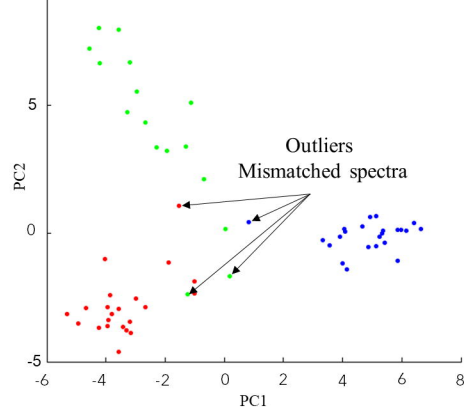
689 Step 1: Sampling of unchallenged bees (controls) and experimental infection obtained by pricking
690 honey bees with live strains of *P. c. c.* and *M. l.*. Step 2: Individual hemolymph collections followed
691 by MALDI-TOF MS molecular mass fingerprinting, and strain identification by MALDI biotyping.
692 Step 3: Multi-stage processing of MALDI MS fingerprints including recalibration, peak picking,
693 normalization and statistical calculation of individual MS spectra through Principal Component
694 Analysis (PCA) for revealing differential molecular patterns across infection groups. Step 4: Genetic
695 algorithm-based computational model for recognition and classification of honey bee infection using
696 PCA discriminant analysis. Barcodes were built following the molecular fingerprints that discriminate
697 control bees from bees infected either with *M. l.* or with *P. c. c.*.



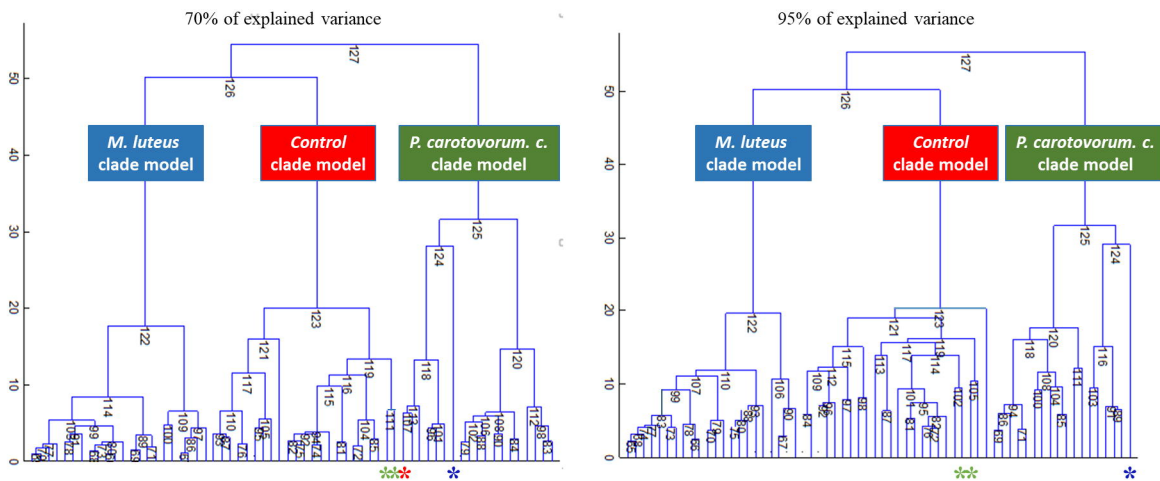
A/ Averaged MALDI MS spectra of the haemolymphs from the biological models



B/ PCA plot score discriminating the individual hemolymphs spectra



C/ Unsupervised Hierarchical Clustering dendrograms based on PCA



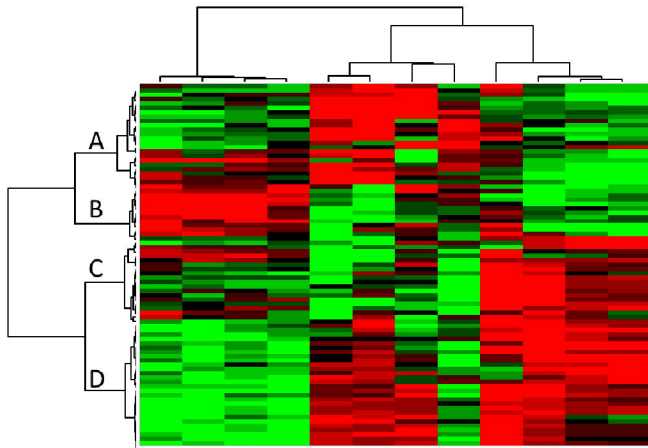
PCA results and Data reduction

Unsupervised hierarchical clustering

Distance Method	Spectra datasets	% of Explained Variance	Number (N) of found mismatches out of a total set of 64 spectra
Euclidean	Normalized	70 (lowest limit)	N=4 i.e. 1 Control (*) in <i>P. c. c.</i> ; 1 <i>M. l.</i> (*) in <i>P. c. c.</i> ; 2 <i>P. c. c.</i> (**) in Controls
	Normalized	95 (highest limit)	N=3 i.e. 1 <i>M. l.</i> (*) in <i>P. c. c.</i> ; 2 <i>P. c. c.</i> (**) in Controls

AMPs DENDROGRAM

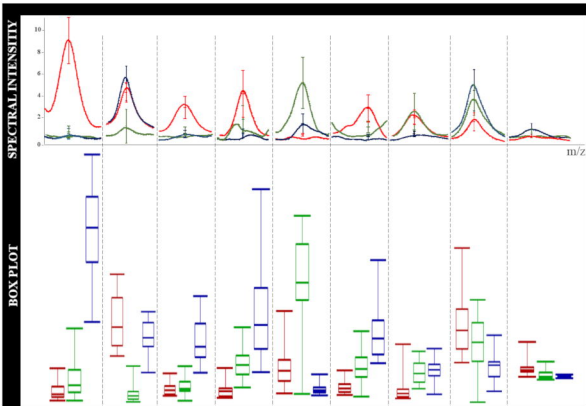
MOLECULAR MASS FINGERPRINT DENDROGRAM



m/z 2,107 Apidaecin IA control
m/z 3,878 Abaecin control
m/z 5,519 Defensin IA control
m/z 10,270 Hymenoptaecin control
m/z 3,878 Abaecin *P.c.c.* model
m/z 2,107 Apidaecin IA *P.c.c.* model
m/z 5,519 Defensin IA *P.c.c.* model
m/z 10,270 Hymenoptaecin *P.c.c.* model
m/z 2,107 Apidaecin IA *M.I.* model
m/z 3,878 Abaecin IA *M.I.* model
m/z 5,519 Defensin IA *M.I.* model
m/z 10,270 Hymenoptaecin *M.I.* model

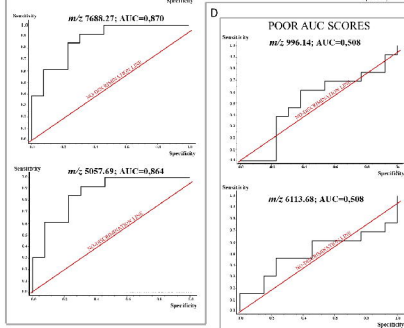
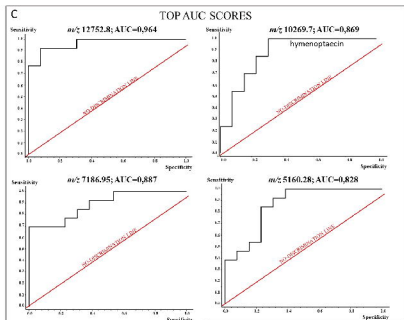
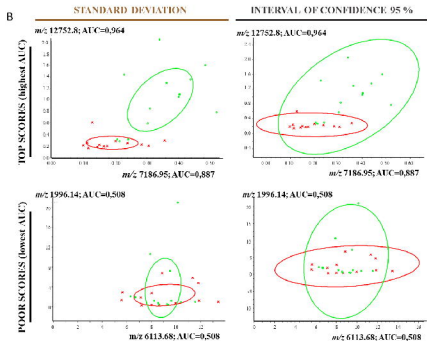
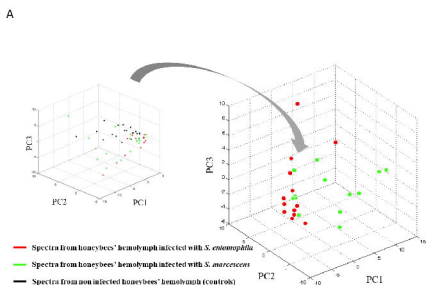
Scale intensity
of cross-correlating
ion peaks





Best Discriminant peaks	m/z ions peaks	3348.17	6959.43	4954.72	1567.63	2701.04	1610.44	5354.11	6608.12	5603.01
	Weight index	6.24	4.29	3.71	3.23	3.16	2.87	2.43	1.98	1.97
Gel View	<i>M. l.</i> infection									
	<i>P. c. c.</i> infection									
	Controls									

Classifier Model Scoring					
Biological model	Classifier	Recognition capability (%)	Overall recognition (%)	Cross validation (%)	Overall validation (%)
Unchallenged (controls)	Genetic Algorithm	100	97.92	91.51	91.93
<i>M.l.</i> infection		100		94.40	
<i>P.c.c.</i> infection		93.75		89.87	



0.5<AUC<1 ; positive test made with "honey bees are infected by *Smarcescens*."