Report on comparative studies and use of BeeTyping® in a sanitary plan

Deliverable D9.15

25 May 2023

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PoshBee

Pan-european assessment, monitoring, and mitigation of stressors on the health of bees

Prepared under contract from the European Commission

Grant agreement No. 773921
EU Horizon 2020 Research and Innovation action

Project acronym:	**PoshBee**
Project full title:	**Pan-european assessment, monitoring, and mitigation of stressors on the health of bee**
Start of the project:	June 2018
Duration:	60 months
Project coordinator:	Professor Mark Brown Royal Holloway and Bedford New College www.poshbee.eu
Deliverable title:	Report on comparative studies and use of BeeTyping® in a sanitary plan
Deliverable n°:	D9.15
Nature of the deliverable:	Demonstrator
Dissemination level:	Public
WP responsible:	WP9
Lead beneficiary:	CNRS
Citation:	Bulet, P., Bocquet, M, Askri, D., Arafah, K.(2023). *Report on comparative studies and use of BeeTyping® in a sanitary plan* Deliverable D9.15 EU Horizon 2020 PoshBee Project, Grant agreement No. 773921.
Due date of deliverable:	Month n°60
Actual submission date:	Month n°60

Deliverable status:

Version	Status	Date	Author(s)
1.0	Draft	25 May 2023	Philippe Bulet, Michel Bocquet, Dalel Askri, & Karim Arafah CNRS, BIOPARK ARCHAMPS

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Preface

The identification of micro-organisms (bacteria, viruses, fungi and other eukaryotic parasites) responsible for infections is the main role of medical microbiology laboratories. For bacterial, and recently for fungal and parasite identification, conventional techniques using the biochemical (Api gallery for Gram negative bacteria or automated biochemical identification systems) and genetic (qPCR reactions based on 16S-ribosomal RNA) characteristics of strains have been replaced by matrix-assisted laser desorption ionisation mass spectrometry referred to as MALDI Biotyping. There are two major actors in this field of MALDI Biotyping, Bruker from Germany with its Microflex and BioMérieux (France) with the Vitek-MS. This technology is also used in veterinary laboratories and certain agri-food laboratories (research and development, quality control for the detection of food pathogens). Several years ago, the approach of MALDI was successfully developed and applied by partner 11-CNRS to decipher the molecular impact of pathogens and microbial infections on the genetically amenable model system that the fruit fly *Drosophila melanogaster* represents. Thanks to this work, we have adapted and used this approach on honey bees, bumble bees, the solitary bee *Osmia* and some additional wild bees as a proof of concept that this approach may serve a direct tool to follow the health status of bees facing stress conditions or as a source of biomarker identification to develop new prognosis and/or diagnosis tools.

1. Introduction

The development of soft ionisation mass spectrometry techniques such as MALDI has allowed the analysis of a large panel of biomarkers and the growth of this technology. In parallel, less technically complex (linear mode) and less expensive mass spectrometers have been developed, facilitating their diffusion beyond the research laboratories. Initially reserved for the research field, MALDI MS has recently made its appearance in microbiology laboratories. The current enthusiasm for the routine use of MALDI MS in microbiology (MALDI Biotyping) is linked to its high accuracy and speed (identification of a micro-organism for example requires only a few minutes), its ease of use and the simplicity of its routine integration in laboratories, as well as the low cost (less than 0.20-0.30€ per analysis, not including human power and the cost of the equipment (<200 k€ for acquisition)). In addition, this approach can be used to directly identify microorganisms from body fluids (i.e., blood, urine), ultimately allowing for the optimisation of patient management. An interesting mini review led by Jenna Rychert lists the Pros and Cons of MALDI Biotyping for the identification of microorganisms (Rychert, 2019). We took advantage of MALDI mass spectrometry to develop what will be referred to as "MALDI-BeeTyping®", in order to (i) generate molecular mass fingerprints as indicators of health dysfunction in response to abiotic and biotic stresses, (ii) identify molecular markers for prognosis and diagnosis of a stress status of bees, and (iii) develop potential additional enzymatic and immunological assays to survey bee health through individual blood tests. The tools proposed are not based on molecular biology techniques (transcriptomics and genomics) since we are looking for non-identified molecules. In fact, the techniques of molecular biology, even if essential, are mostly usable when you can design probes to identify the presence of molecules/markers that you are looking for. In contrast, MALDI-BeeTyping® is a non-supervised approach that proposes innovative solutions to evaluate the impact of stressors on bee health. We will detail these objectives in a step-by-step workflow.

2. MALDI-BeeTyping® as an individual blood test for monitoring bee health

2.1 An individual blood test to track the health status of bees, based on a tissue that carries key information on bee immunity

In health care, a blood test is done to check how organisms (animal and human) cope, for example, with infection, medication or pathology. If the blood test results are abnormal, it gives indications of how to treat or prevent future problems.

The use of a blood test to monitor insect health had not been developed until the pioneering work of Bulet and collaborators on the fruit fly *Drosophila melanogaster* (Uttenweiler-Joseph et al., 1998), an approach which remains a reference for monitoring *Drosophila* host defence against infections (Xu et al., 2022). *D. melanogaster* represents a genetically amenable model system that is well-suited to study infections and innate immunity as there is no vertebrate-like adaptive immunity. Like other insects, honey bees have evolved defence mechanisms against pathogens that help them survive under abiotic and biotic stresses. The mechanisms of the insect immune system rely mostly on both humoral and cellular responses. As with other insects, honey bees have an open circulatory system that contains a transparent or light yellowish haemolymph for molecular transport, providing a readout of the humoral immune defences, for example, synthesis of antimicrobial peptides (AMPs) by the fat body and the hemocytes (blood cells). Honey bee haemolymph contains high concentrations of inorganic ions, amino acids, sugars, and proteins compared to vertebrate blood. In addition to the synthesis of AMPs such as defensins, abaecin, apidaecins, and hymenoptaecin, the humoral immune response is also associated with melanisation mediated by the prophenoloxidase pathway and vitellogenin. Most of these molecules and more precisely AMPs and other small peptides/proteins below a molecular mass of 18 kDa can be easily detected and mapped by matrix assisted laser desorption ionisation (MALDI) mass spectrometry (MS). For bees, a "blood/haemolymph test" performed by mass spectrometry (MALDI-BeeTyping®) produces a record of molecular mass fingerprints (MFPs) of peptides and proteins (<18 kDa) circulating in bee haemolymph that are representative of a bee's physiology, in the same way that a biometric fingerprint is exclusive to one human and can be used both for identification and authentication (Figure 1).

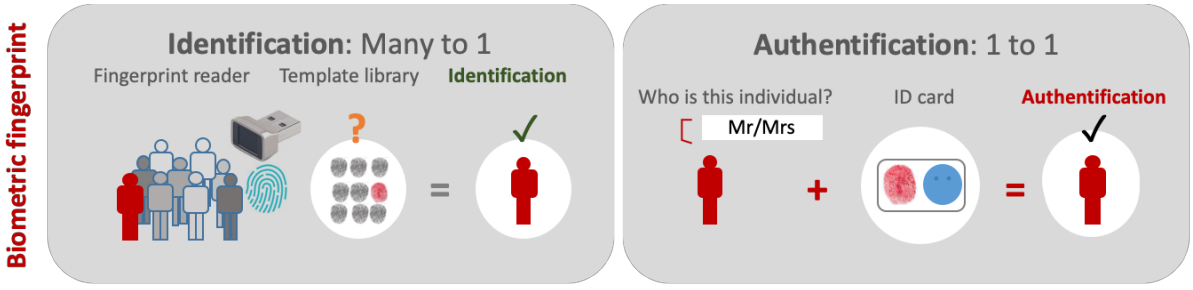

Figure 1: The fingerprint is the most suitable way to identify a person among a group (left). The fingerprint is unique to a person (right).

In a related project initiated before PoshBee, BioPark and Philippe Bulet (CNRS) extended this technique to *Apis mellifera* (Arafah et al., 2019) and proposed the name of MALDI-BeeTyping®. For PoshBee, BioPark and CNRS partners optimised the experimental MALDI-BeeTyping® workflow including designing dedicated and adapted haemolymph kits to extract blood from the three main bee species (honey bee, bumble bee and a solitary wild bee). Sources of information can be obtained in Arafah et al., (2019) and Houdelet (2020).

Recently, the MALDI-BeeTyping® tool/prototype (see video: https://poshbee.eu/media/1578) was also successfully applied to the novel wild species investigated within Work Package (WP) 4.

2.2 How to track the impact of a stressor on bee health?

A molecular fingerprint is generated and compared to a library of reference MFPs according to an experimental workflow composed of a few steps summarised in figure 2. These MFPs are obtained from haemolymph samples collected from individual bees under different stress conditions (e.g., bacteria, parasites, bad nutrition, pesticides).

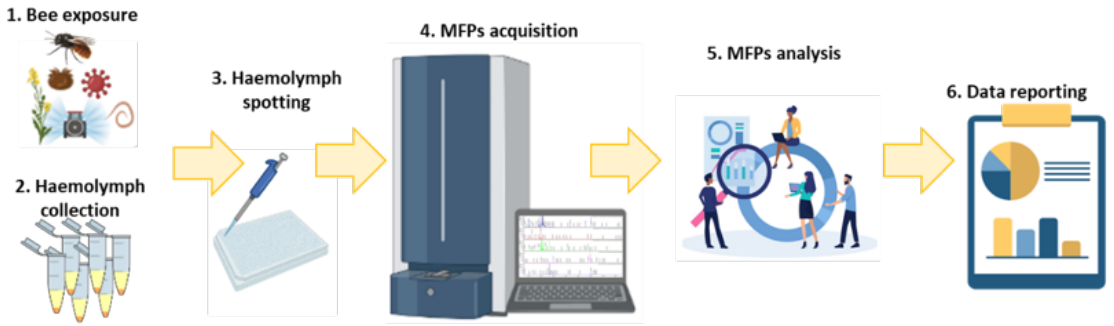

Figure 2: General workflow used to generate molecular mass fingerprints (MFPs) from bees exposed to stress conditions (step1), after haemolymph collection (step 2), sample preparation (step 3) MFP acquisition by MALDI MS (step 4); data analysis (step 5) and reporting (step 6)

MALDI-BeeTyping® enables classification of bees according to their responses to stressors and gives the immune status of the bee (Figure 3).

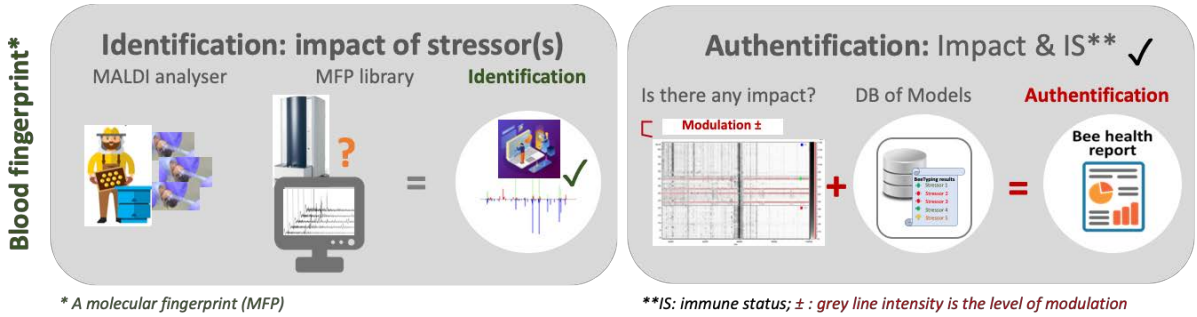

Figure 3: As compared to the unicity of a fingerprint for a person, the molecular mass fingerprint (MFP) recorded from an individual sample of haemolymph (insect blood) is representative of the impact of stressors.

Our main goal is to transform the individual MFP of the individual haemolymph test recorded by MALDI-BeeTyping® into a series of impact scores that indicate how closely a haemolymph MFP profile matches to a library of referenced MFP profiles. The library was built on MFPs acquired during the holistic analyses we conducted in experimental conditions in the laboratory (WPs 3-6), semi-field environments (WP7), and in the field (WP1 and 7) (a brief overview of PoshBee WPs can be found via the PoshBee webpage: https://poshbee.eu/about). The overall impact level and the immune status of the bee will be represented by an appropriate “traffic light” colour code: green, yellow and red for low, medium and high impact, respectively. This MALDI-BeeTyping® approach is designed as a user-friendly read-out of bee health status.

The report summarising the results of the analysis by MALDI-BeeTyping® will be generated in a format that can be interpreted by the beekeeper or bee veterinary services, who can integrate it with complementary analytical measurements (detection/identification of viruses, residues of chemicals, etc.) and field observations of bee hive health (strength of the colony, brood conformation, pathogens presence, behaviour, other symptoms).

3. Comparative study between MALDI-BeeTyping® and other diagnostic techniques

3.1 Present status on tools available

Visual diagnosis is largely used in honey bee surveys, in the case of pathogens such as *Varroa* that are easily recognisable, or like Deformed Wing Virus (DWV) that produce clear pathogenic phenotypes. Nevertheless, a large set of stressors do not present visual signatures, for instance some viruses or the microsporidian parasite *Nosema*, especially at low levels of infection. The diagnosis of stressors in bees uses numerous methods and tools as recommended/reported in the Coloss BeeBook volumes I and II (https://coloss.org/activities/coreprojects/beebook/), including molecular approaches. The main molecular tools used in the field samples are limited to (i) pesticide residue monitoring by chromatography coupled to mass spectrometry analysis, which requires large size samples for robustness and reliability, (ii) PCR analysis for the detection of viruses and microorganisms and (iii) enzymatic assays (e.g., measurement of the prophenoloxidase activity and detection of vitellogenin as indicators of dysregulation of honey bee health).

Table 1: Comparative advantages/disadvantages of MALDI-BeeTyping® relatively to PCR, immunological tests and enzymatic kits.

	MALDI-BeeTyping®	**PCR**	**Immunological tests Enzymatic kits**
Usable by untrained operators	No	No	yes
Usable by trained operators (S short and H high training levels)	S	H	S
Usable directly in the field	No	No	Yes
Unitary cost	10-25€	30-80€	Between 10-50€
Need to send a sample to a dedicated service	Yes	Yes	Yes/no
Minimum number of bees necessary/analysis	One individual	One pool	Not evaluated yet
Need for cold storage of samples	Yes	better	No
Type of results	MFPs confronted to a MFP library composed of reference spectra from 5 different stress conditions. Semi-quantitative values on a set of immune molecular ions, mainly AMPs.	Identification of suspected stressors such as viruses	Quantification of the targeted indicator (e.g., enzyme, inhibitor of enzyme, antigen
Prerequisites	Needs a library of models covering a large set of impacted molecular responses to stressors	Needs specific primers to target and amplify DNA of searched stressors	Analysis and/or quantification of an identified marker such as an antigen or a protease or protease inhibitor activity

3.2 An unprecedented tool for bee health monitoring: MALDI-BeeTyping®

The MALDI-BeeTyping® approach is both innovative and unprecedented compared to the tools used today to monitor bee health. Nevertheless, an evaluation of pros and cons can be summarised as follows. As already mentioned, compared to other molecular approaches that look at gene expression (genomics and transcriptomics data), the MALDI-BeeTyping® is fast (< 5 min), reliable (only one individual is sufficient) and cost-effective (less than 0.20-0.30€/individual haemolymph sample without equipment and personnel costs included), and thus could provide a valuable tool for bee health. The cost of such analysis per sample includes all the consumables, equipment and man power, a cost that decreases as the number of samples increases. The two main current limitations of MALDI-BeeTyping® are (i) this technique requires the acquisition of equipment averaging 250-300 k€, a laboratory facility and technical training, and (ii) it may be challenging to process enough bee samples and train the algorithm to generate the MFP of stressor impact or to reach the limit of detection of the MALDI beetyping®.

3.3 Currently used approaches: molecular biology tools (i.e. qPCR, RT-PCR)

In bee diagnosis, PCR is frequently used, for example, in the case of American Foulbrood to confirm field diagnosis by veterinarians and launch the official procedures of control. It is also used to identify *Nosema* species (*N. ceranae vs N. apis*), and for virus identification. PCR is a molecular diagnostic method that amplifies specific DNA/RNA sequences from a biological sample (Figure 4).

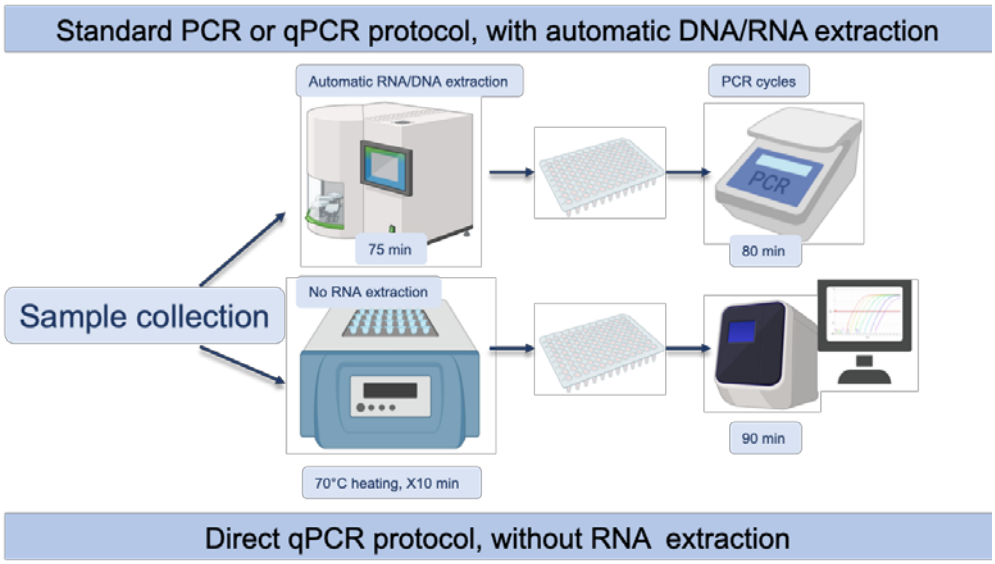

Figure 4: Typical workflow for PCR and qPCR analysis

It is very sensitive and specific, but it requires a DNA/RNA purification step and subsequent analysis of PCR products for the identification of microorganisms. PCR is also more expensive per sample compared to MALDI mass spectrometry. For viruses, PCR identification remains the most common technique. To support this exercise of comparison between PCR and MALDI MS, we would like to illustrate it by presenting analyses we conducted in parallel with PoshBee within the PhD program of Camille Houdelet (not financed by PoshBee; Houdelet 2020). In 2021, we applied MALDI MS for rapid molecular profiling of extracts of *Nosema* spores in order to identify the species and the geographical origin (Houdelet et al., 2021; Figure 5). A difference 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. Finally, using our computational models on the different *Nosema* species, we were able to classify five independent isolates of *Nosema* microsporidia. In conclusion, adapting MALDI Biotyping to the identification of *Nosema* species allowed us to obtain representative MFPs of the *Nosema* species (*N. apis* vs *N. ceranae*) and of their geographical origin. This demonstrated that MALDI MS profiling represents an attractive and more robust alternative to optical observation and is more cost-effective than polymer chain reaction (PCR) analysis for identifying *Nosema* species. For comparison of both techniques, we ordered through the PoshBee project PCR analysis of the same spore populations to the public agency ANSES (Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail) according to their internal method (https://www.anses.fr/fr/system/files/ANSES_SOP_ANA-I1-MOA-11_V4.pdf). *N. ceranae* spore solutions (total 2×10^5 spores *versus* 10^4 for MALDI Biotyping) were analysed in triplicate for a cost of 320€ without VAT for six samples, meaning 53€ per sample without VAT. This demonstrates that MALDI MS could represent a valuable surveillance tool of nosemosis in apiaries for sanitary services and beekeepers.

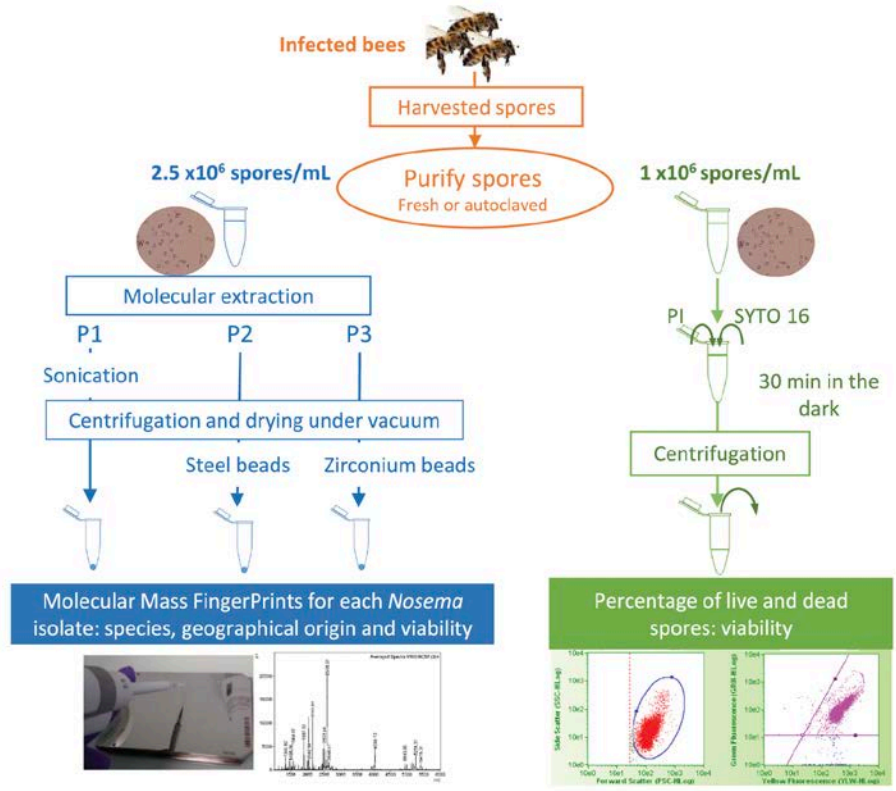

Figure 5: Experimental workflow for biological material preparation for MALDI Biotyping. Purified *Nosema* spores from infected honey bees (orange panel) were counted and subjected to MALDI-MS (blue panel) and flow cytometry (green panel) analyses. For MALDI Biotyping, three alternative protocols (P1, P2 and P3) were used to generate molecular spore extracts. In P1, spore extracts were performed by sonication. In P2 and P3, spores were ground using steel and zirconium beads, respectively. For MALDI Biotyping 10^4 spores were used (Houdelet et al., 2021).

PCR has advantages and disadvantages over microscopic or microscopic observations, or other analytical methods such as colorimetric tests. PCR is appropriate when you know which organism you are looking for and is accurate/highly specific if the probes are properly selected and designed, as it can distinguish DNA sequences by just one nucleotide. It is also sensitive, since in general it allows the detection of even a single copy of a specific DNA template. The PCR technique is versatile and can be used for various applications such as genetic testing, identification of viruses and other micro-organisms when such probes are available. PCR can efficiently and rapidly amplify a small amount of DNA to a million copies in a few hours. However, PCR technology has several disadvantages, including (i) it cannot detect novel sequences if primers are lacking, (ii) it is expensive and requires expert knowledge for high-throughput projects.

4. Enzymatic kits and immunological tests as tools for prognosis and diagnosis of infections, diseases and molecular dysregulations due to stress

During the PoshBee project, we proposed, to complement the MALDI-BeeTyping®, two additional tools. The first one is an enzymatic test based on the identification by MALDI-BeeTyping® of a protease inhibitor as an indicator of dysregulated health status in honey bees, bumble bees and the solitary

wild bee *Osmia bicornis*. The second one is an ELISA test, a test based on the recognition of an antigen (in our case a peptide or a protein by an antibody, which will be itself recognised by a second antibody to allow identification and quantification of the presence of the antigen). Omics investigation (e.g. genomics, transcriptomics and proteomics), are frequently used in clinical and veterinary researches but are not economically transposable to a sanitary plan for beekeeping due to the high cost implied for large sample sizes. In health care (animal and human), modern approaches in the discovery of protein biomarkers have hugely contributed to improving the prognosis and diagnosis of diseases by veterinarians and doctors. The discovery of blood protein biomarkers through proteomics is one of the existing tools. Biomarker discovery research based on proteomics is advanced in different human and animal diseases such as infections, cancer and cardiovascular disorders, while providing opportunities to work with limited to non-invasive methods by the use of blood samples. Blood analysis can indicate if certain elements circulating in the blood stream are, or are not in a normal range, thus enabling the prediction and detection of pathologies. The figure 6 below shows a conventional workflow for protein biomarker discovery, which bridges the gap between visual examination and targeted molecular analyses**.**

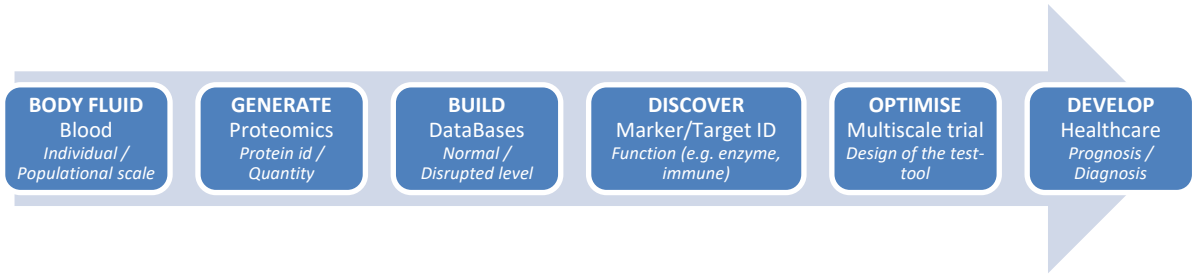

Figure 6: Conventional workflow in the discovery of a protein biomarker from the tissue collection to the development of a prognosis/diagnosis marker for application in healthcare

As mentioned previously, omics refers to high-throughput analyses, for example of metabolites (metabolomics), proteins (proteomics), or genes (genomics/transcriptomics) in a biological system, enabling comprehensive studies of the roles, relationships, and actions of various types of molecules in an organism. These system-based approaches can unravel stressor-related processes and are important for biomarker discovery in different contexts (i.e., disease, environmental exposure, reproduction, infection and behaviour) and interestingly in our project in bee health monitoring. This issue is preliminary to any development of tools for health monitoring and evaluation of treatments. We reviewed the literature through a formal literature search performed exclusively on scientific manuscripts published between 2018 and 2022 (data reported in deliverable D10.5), the period covered by the PoshBee project. Even though we observed that the number of omics studies (10% were proteomics studies) performed with the aim of understanding the effects of pesticides and/or pathogens and/or climate change on bees had increased substantially over the past decade, proteomics studies performed on haemolymph were scarce. In omics, the highest number of published papers was in genomics (n=557), then metabolomics (n=389), transcriptomics (n=176) and proteomics (116), and mass spectrometry was mentioned in 505 scientific documents, split between metabolomics (389) and proteomics (116). Based on our bibliometric analysis, the proteomic markers that have been most studied belong to the Cytochrome P450 family. The peptides/proteins Vitellogenin, Defensin, Hymenoptaecin, Abaecin, and Apidaecin (those last four peptides are key players in the bee immune defences) were mostly reported in *Apis* with a very few studies on *Bombus*.

Within PoshBee, these markers were also observed by MALDI-BeeTyping® in *Apis*, *Bombus* and *Osmia* as key markers. Interestingly, thanks to our innovative approach of MALDI-BeeTyping®, an additional marker was identified in these three species, namely the protease inhibitor *Apis mellifera* Chymotrypsin Inhibitor (AMCI). Having in hand AMPs such as Apidaecin and the newly identified AMCI, we started to develop an antigenic test to follow Apidaecin in bee haemolymph and an enzymatic test to quantify the activity of this inhibitor in response to different stress conditions.

For enzymatic kits and immunological/antigenic tests (e.g. ELISA tests) the monitoring report is based on the molecular results observed by colour intensity evaluation. In an enzymatic kit, a colour intensity reflects the activity level of the enzyme or an inhibitor of an enzymatic activity. In an antigenic test, for example the SARS-CoV-2 antigenic test, one band at the area of "C" (Figure 7) is the control, and an additional band at T reflects the presence of the antigen (Figure 7). The report can be interpreted by the beekeeper or bee veterinary services, who can integrate it with complementary analytical measurements (detection of viruses, residues of chemicals, etc.) and field observations of bee hive health. Compared to other molecular approaches looking at gene expression in bees, colorimetric kits and strip-based lateral flow assays (i) have cost-effective advantages, (ii) can be user-friendly and applicable from laboratories to the real-world for prognosis and diagnosis of health problems, (iii) are already available for beekeepers for American foulbrood (AFB) and European foulbrood (EFB), two diagnostic kits proposed by Vita bee health, and (iv) when appropriate, can be developed for transportable devices interfaced with smartphone applications for in field monitoring.

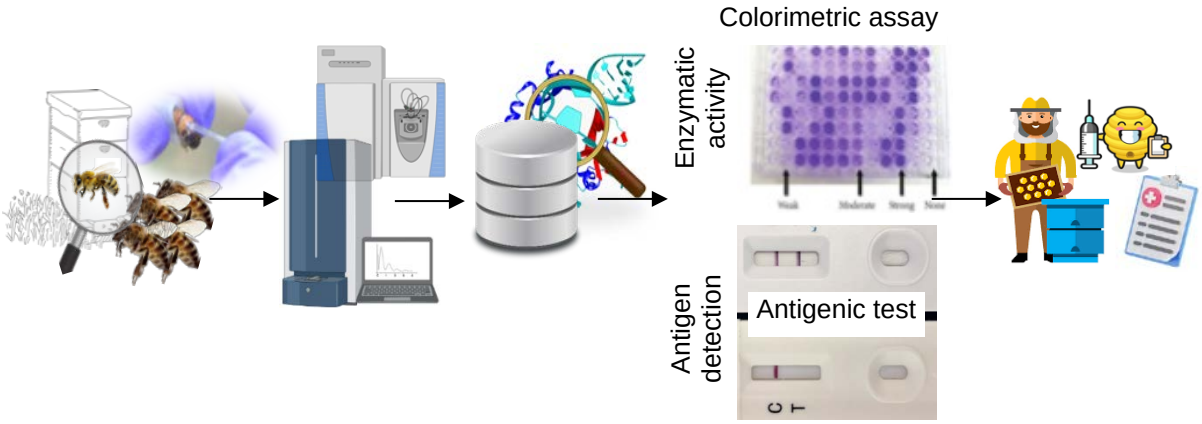

Figure 7: Workflow used to identify biomarkers and to develop kits/tests for bee health monitoring. The workflow involved (from the left to the right), haemolymph collection, analysis by mass spectrometry (MALDI-BeeTyping® and proteomics), data processing for marker identification, development of kits/tests (a kit to follow an enzymatic activity by colorimetry and an antigenic test to quantify an antigen), and finally providing a user-friendly report for bee health management.

5. Potential use of MALDI-BeeTyping® in sanitary plans

5.1 MALDI-BeeTyping® and sanitary plans

The Animal Health Law (LSA), that came into force on April 21, 2021, harmonises the sanitary measures applicable to beekeeping **in the European Union** (https://food.ec.europa.eu/animals/animal-health/animal-health-law_en). This law classifies bee diseases into five categories according to their

severity and management. The diseases with higher sanitary and economic impacts are under the responsibility of member states, while the others are the responsibility of professional organisations. Plans for surveillance, control or eradication can be developed to help to fight against each disease and adapted in each country. **At the local level**, this policy is applied in a plan for sanitary inspection of apiaries. This sanitary inspection is carried out by veterinarians or sanitary inspectors specialised in beekeeping, or bee experts in charge of the detection, follow-up and eradication of diseases in apiaries. The sanitary inspection may sometimes include the control of bee products such as honey and/or wax quality and the protection of bees against toxic substances.

In daily practice, **the beekeeper themself** follows the health status of their colonies, mainly in managing colonies to be strong and productive, and caring for the weakest colonies (e.g. food complementation, queen renewal, additional population introduction). At this scale the sanitary plan includes general management of *Varroa*, continuous diagnosis of the colonies, and the following of good beekeeping practices (GBPs). At each scale, the need for an appropriate sanitary plan is required to anticipate the risks and to react in an appropriate manner in case of problems. The usual approach, based on individual pathogen diagnosis, is nevertheless limited in the case of apiculture:

- Pathogens are not the only drivers of bee health; others biotic and abiotic factors contribute to the health status of the colony. Of these, various factors may act in synergy in the same colony at the same time or successively.
- The impact of the stressors may not be rapidly and directly visible. For instance, an acute intoxication has an immediate effect on foragers, while a disease like *Nosema* needs time to impact the colony.
- Additionally, social immunity or nutritional conditions may strongly change the impact of a stressor on the colony.

All together these reasons explain why the diagnosis techniques used are not totally accurate:

- Visual inspection is relatively limited and can only detect general deterioration of the colony or identify pathogens known to have morphological or behavioural impacts.
- PCR analysis is more adequate for the determination of the species for *Nosema* or viruses for instance, or to confirm a first visual diagnosis of foulbrood. A first hypothesis on the potential stressor is necessary to apply a good PCR test. This approach is only applicable when you know what you are looking for and is not applicable in a blind strategy.
- Other laboratory techniques can be used for pesticide measurement (based on chromatography coupled to MS), global genetic survey for pathogens and microbiota (e.g. genomics and transcriptomics), but their cost is often higher than the value of the colony.

All these techniques are extremely useful and adapted to confirm a diagnosis, to identify a stressor or to certify the presence of a stressor. However, they give little information on the stressor impact on the colony health. We established within PoshBee that our approach of MALDI-BeeTyping® brings pertinent molecular information for a more global diagnosis of the health of individual bees within colonies:

- probabilistic analysis of the presence of different stressors.
- the presence or absence of AMPs (presence or absence) are relevant markers of the impact of the stressors on the bee.

- an indication of how important the identified stressors are for current bee health
- sensitive early detection of impact

Importantly, MALDI-BeeTyping® complies with the health indicator attributes described in WP8:

- **Variability**: The indicator values should vary over a reasonable range of detection: we observe a large variation in the intensity/area of usually more than 100 molecular ions by MFP of one individual bee.
- **Measurability**: It should be possible to measure the indicator reliably and accurately: The technique is approved by FDA and other agencies, who verified this point.
- **Feasibility**: It should be feasible to measure the indicator in practical situations: we did tens of thousands of measures from field, semi-field and laboratory samples.
- **Validity**: The indicator should measure what it intends to measure. This point has been verified by authorities and sanitary agencies such as FDA.
- **Timeliness**: The results of the measurement should be available fast enough to allow for useful intervention. When the sample arrives at the lab, it can be processed rapidly, within the day of receipt.
- **Robustness:** The indicator should match the health status stably under all conditions. There are evidently some individual variations among bees, but our results show statistically consistent results among the different replicates in the experiments.
- **Replicability**: The indicator measurement should be similar between technical operators. We produced a SOP indicating different cases of exclusion of some samples (cloudy or brown haemolymph) but as for human blood sampling, the operator does not influence the composition of the haemolymph.
- **Sustainability**: The indicator should be useful over a long period of time: For this point we can refer to the importance of blood analyses in human health.
- **Relevance and importance**: The indicator should ideally be associated with an actionable mitigating intervention and not just of academic interest. For instance, low immunity profile could be corrected by a good nutrition or inserting an emerging brood frame in the colony.
- **Comprehensibility**: The indicator should be understood by practitioners so that they can use the information correctly and profitably: We are working on this point to deliver a very simple message, such as traffic light colours.
- **Independence**: The indicator should ideally be minimally affected by other indicators. The majority of individual MFP are independent of each other, and this can be confirmed by correlation analyses.
- **Universality**: The indicator should be applicable to as many bee species as possible. We used this technique successfully for all the species studied within the framework of PoshBee.

One of the main advantages of MALDI-BeeTyping® is its capacity to measure the impact of a stressor. In the case of sanitary plans, it gives a useful tool to determine accurate thresholds, to open the road to the establishment of real IPM (Integrated Pest Management) at the scale of the colony, of the apiary, or even at regional or national level. Additionally, it meets the main conditions defined by the stakeholders in WP10 in terms of cost and benefits.

MALDI-BeeTyping® will not resolve all the needs for good diagnosis in apiculture. The accuracy and precision of PCR will be necessary to clearly identify some pathogens, especially the viruses. The

enzymatic assays may be very useful in the field for their immediate response. All these tools will be necessary to complete the colony health card.

5.2 Case studies within PoshBee (WP1 and WP7)

In the frame of PoshBee, we used MALDI-BeeTyping® for samples collected under laboratory conditions. We proved the high value of MALDI-BeeTyping® in experiments with multiple factors in interaction. This technique is able to discriminate small differences between experimental conditions, even where mortality is not able to discriminate these conditions. We also applied MALDI-BeeTyping® to experiments conducted under semi-field conditions, for instance in the UK for honey bees we identified a clear specific profile of the colonies before and after the introduction of stressors inside the semi-field cages. We also applied MALDI-BeeTyping® to honey bee, bumble bee and solitary wild bee samples collected from a continent-wide field experiment (WP1, WP7) across 128 agricultural sites, and two different crop systems [oilseed rape (OSR) and apples (APP)]. Molecular signatures of haemolymph and the presence/absence of molecular-related ions of three markers of immunity, the three AMPs Apidaecin, Abaecin and Defensin-1, allowed discrimination of bee responses by country, crop type and site. A machine learning model was developed to discriminate the haemolymph of bees from APP and OSR sites. The model was 90.6% accurate in identifying the crop type from the samples used to build the model. A second field experiment (WP7) was performed in Spain and Germany, in field conditions with or without Glyphosate spray on some parcels of the foraging area.

The individual collection of tissues allows us to explore all dimensions, from molecules to the ecological level of the “onion layers” of the PoshBee bee health concept (WP8):

- Individual bee level gives a good idea of the variability of the impact of a stressor in a bee population, the best dimension for research and laboratory uses of MALDI-BeeTyping®.
- Colony level: we get good differentiation among experimental conditions usually using few bees. For experimental purpose, we use 30 bees by condition, but in field assays, 10 bees gave results to highlight differences among colonies.
- Territory level: we can discriminate by MALDI-BeeTyping® the MFPs of different territories, leading to the possibility to conduct longitudinal studies during the year. We are already running case studies.

5.3 One example of an external case-study based on a two-years survey of a field honey bee colony by MALDI-BeeTyping®

In the framework of the thesis of Camille’s thesis (Houdelet 2020), unpublished data, personal communication by Philippe Bulet 11-CNRS partner), we did a long-term study of a colony about every month for two years. We observed the natural presence of two pathogens, *Nosema* spp. and *Varroa destructor*, which allowed us to evaluate the analytical approach of MALDI BeeTyping® at the scale of a field colony and to assess what could be the best sampling to gauge the health of this colony and more globally of any given colony. This mirrors the scale that interests a beekeeper in the management of their apiary. Tracking the prevalence of the two pathogens, *Nosema* and *Varroa*, allowed us to highlight certain periods where the parasites are likely to weaken the colony. During each collection, we also noted any unusual elements, such as white haemolymph samples, a bee with a hole in the cuticle, or reinforced hygienic behaviour. As already mentioned, the objective of this experiment was not only to evaluate the presence of certain pathogens, but to show that haemolymph sampling and its analysis by the MALDI BeeTyping® method would represent both a useful alternative to conventional PCR approaches to evaluate the health status of a colony, and an important complement

to the often subjective or incomplete visual observations made by beekeepers. This study is one of the relatively few that have attempted to monitor colonies under natural conditions over the long term. The question of sampling remains an open question in the scientific community to which we have brought some answers. We have considered the need to have a representative sampling of the hive, which does not impact the survival of the colony and which is relatively fast in order to limit the costs associated with this kind of analysis. The global approach that we propose here has the advantage of enabling, based on 30 foragers and 30 indoor bees, assessment of the level of infection by *Nosema* and of the general health of the colony. In addition to monitoring *Nosema*, we estimated the number of *Varroa* in the colony, but for this we assessed the recommended global number of 300 bees (indoor bees). Our counting methods allowed us to identify the most sensitive periods of our experimental hive to two visible stress factors (*Varroa* and *Nosema*). In a second step, we showed the potential of the MALDI BeeTyping® approach to assess the general health of the colony, from this sample size (for statistical reasons 30 indoor bees and 30 foragers). We linked particular MFPs, for example some peaks corresponding to PAMs of abnormally high intensity, to a period of increased infection by *Varroa* and *Nosema*. We were also able to confirm the importance of monitoring the presence of immune indicators (antimicrobial peptides such as Apidaecin, Abaecin and Defensin) in bee haemolymph as they represent good markers of the physiological state of the bees. We have identified new potential markers associated with age, sex and health status of the individual bee from its natural environment. Additionally, we were able to distinguish clearly wintering bees from summer bees.

All the information collected during this experiment seems to us essential to help beekeepers to understand the dynamics of infection of their hives and thus to apply the right actions at the right time. Indeed, much more than a diagnosis of the infection, the beekeeper needs a prognosis and to avoid waiting for a point of no return for the survival of their hive.

7. Conclusion

During the PoshBee Project, we showed that MALDI-BeeTyping® was a very valuable technique, at reasonable cost, to distinguish the profiles of stressed/not stressed bees, when other methods concluded no impact, especially for pesticide experiments. We have also identified, through MALDI-BeeTyping®, two peptide markers (Apidaecin and the *Apis mellifera* Chymotrypsin Inhibitor AMCI). Apidaecin is an AMP that circulates in bee haemolymph when the systemic immune defences are activated, meaning this peptide may serve as an antigen to develop an antigenic test. Regarding AMCI, its characterisation by our approach of off-gel bottom-up proteomics, allowed us to design an enzymatic assay to quantify its inhibitory activity in response to any type of stress condition. To apply the method as a recognized method we need some additional steps, as the application to the field is more difficult. In order to simplify what we did, where we are and where we are planning to go, we propose to position our three tools (MALDI-BeeTyping®, Antigenic test against Apidaecin, and our Enzymatic test to monitor the AMCI activity) using the method for evaluating the technical maturity of a technology during the course of its development referred as Technology Readiness Level (TRL, Figure 8). We reached TRL 4 for the enzymatic assay on AMCI, and for the antigenic test against Apidaecin (not on but through an ELISA test before investigating the possibility to develop an antigenic test on haemolymph). We reached TRL 7 for the MALDI-BeeTyping® approach as we are already running experiments at a small-scale in a local project on a limited number of hives, and we are discussing with the Region Champagne (France) for a large-scale application.

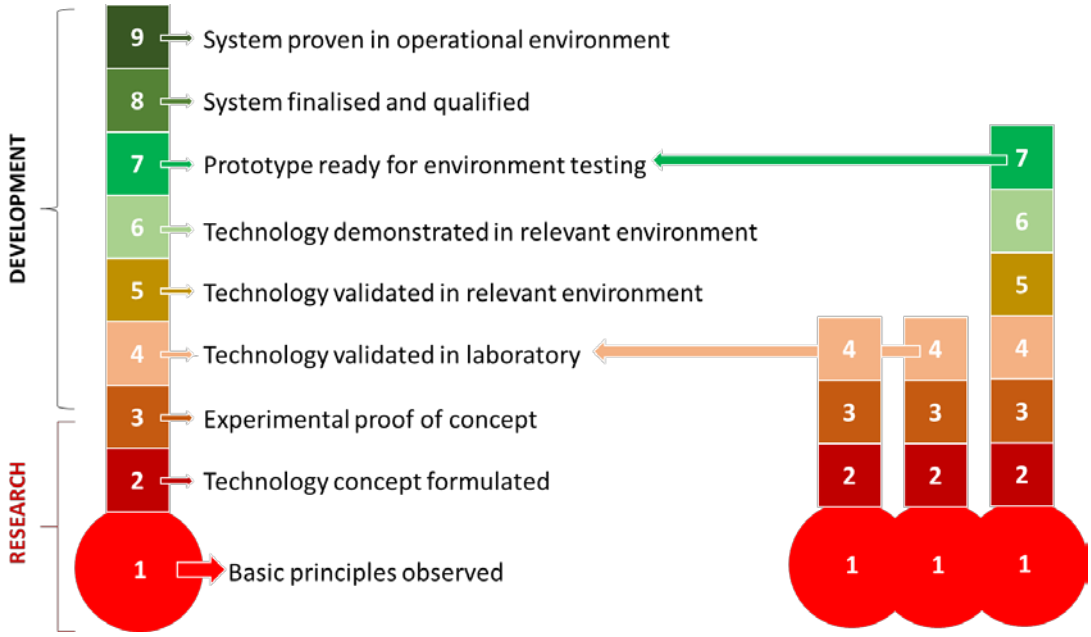

Figure 8: Positioning of the three tools developed within PoshBee: (1) MALDI -BeeTyping® (TRL 7), the enzymatic assay for AMCI (TRL 4) and the antigenic test against Apidaecin (TRL 4).

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