Manuscripts on omics research for model bee II

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PoshBee

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

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Preface

Pollinators, including the buff-tailed bumblebee *Bombus terrestris*, are of crucial importance for maintaining biodiversity in ecosystems and for agriculture. Health declines in pollinator populations are thought to be linked to various abiotic and biotic stressors. In the hope of protecting these populations, deciphering their immune response to stress conditions is a critical issue. To assess this metric, we analysed bumble bee haemolymph, as a readout of the immune status. Here we show, in laboratory conditions, that bacterial infections with two entomopathogenic strains and a well-recognised inducer of defence reactions in insects impact the systemic immune response. Analysis of the haemolymph was carried out using a two-stage approach of mass spectrometry that combines MALDI molecular mass fingerprinting (MALDI MFP or MALDI-BeeTyping®) for its effectiveness in assessing the immune status of the bumble bee through a basic "blood" test, and LC-ESI-MS/MS to measure the impact of our infection models on the "haemoproteome". In this manuscript on the second PoshBee model bee, *B. terrestris*, we:

- Developed and validated an experimental model of challenged bumble bees with Gram-positive (G+) and -negative (G-) bacterial strains using a *Serratia marcescens* strain that we isolated from a naturally infected honey bee.
- Evaluated survival rates in response to the bacterial strain used for the experimental infections.
- Assessed the usefulness of MALDI profiling, our referred MALDI-BeeTyping® approach, to acquire molecular fingerprints of the haemolymph peptidome.
- Observed distinctions in the molecular profiles between different infection treatments and an uninfected control.
- Provided bottom-up proteomics results showing alterations in different pathways under the pressure of bacterial infections.
- Introduced a bimodal mass spectrometry approach (MALDI-BeeTyping® + bottom-up proteomics) to study the immune responses of the bumble bee to biotic stressors and to serve as models for bumble bee health monitoring.
- Implemented BOMDBase-1.0, a database listing the *Bombus* proteins identified during the proteomics analysis of the bee haemolymph samples provided by the PoshBee consortium.

Summary

Among pollinating insects, the buff-tailed bumble bee *Bombus terrestris* represents one of the most important commercially available pollinators. By carrying out three different types of bacterial infections, we observed that the bumble bee reacts in a specific way to bacterial attacks. Indeed, bacteria impact survival and stimulate an immune response in infected individuals that is visible through changes in the molecular composition of their haemolymph. The characterisation and label-free quantification of proteins involved in specific signalling pathways in bumble bees by bottom-up proteomics (off-gel digestion and LC-ESI-MS/MS) revealed differences in the protein expression between the non-experimentally infected and the infected bumble bees. Our results highlight differences in the molecular composition of their haemolymph to distinguish these different infections from non-experimentally infected bumble bees, and alteration of different pathways involved in immunity and defence reactions, stress and energetic metabolism. To conclude, we established bioinformatic models based on molecular markers reflecting the health status of these pollinators to enable diagnosis/prognosis at the population level in response to environmental stress.

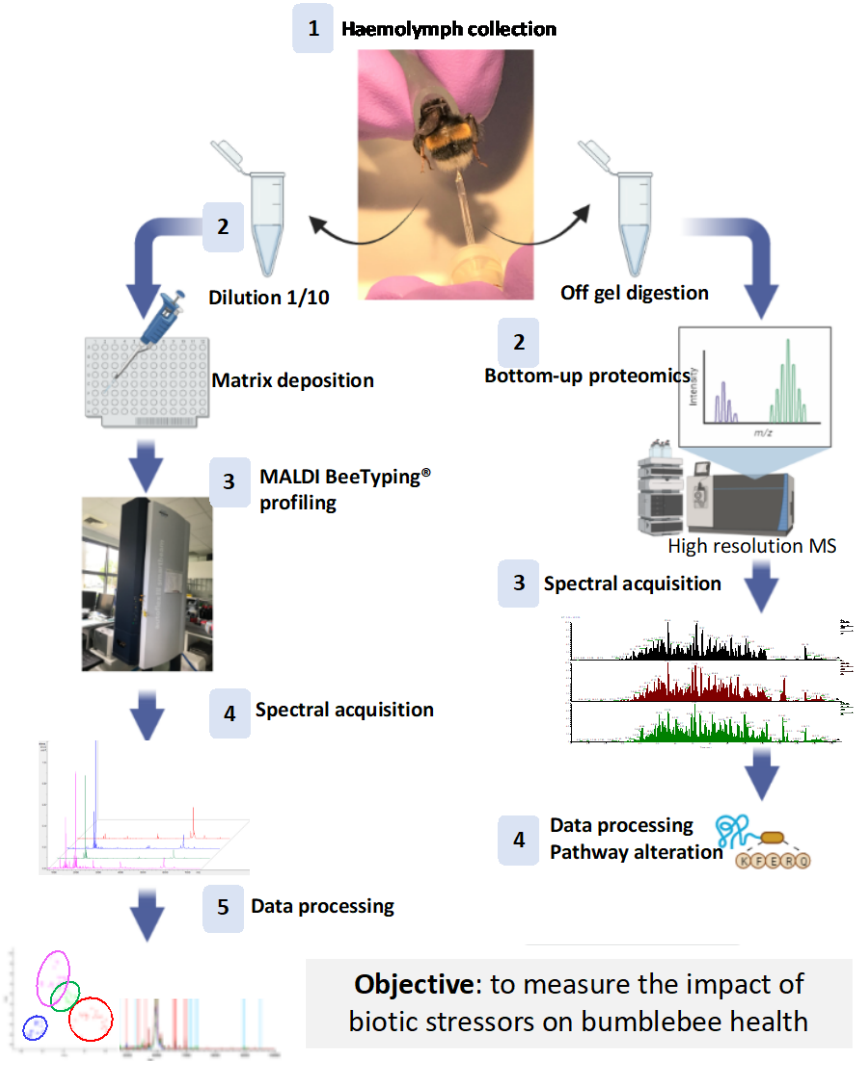


Figure 1: Summary of the omics strategy developed to follow the impact of biotic stressors (bacteria) on *Bombus terrestris* health status

1. Introduction

Bees are fundamental insects in agroecosystems, mainly due to their role in pollination. They are of crucial importance for the diversity of ecosystems and for agriculture. In general, more than one third (35%) of the world's food production is pollinated by bees, including bumble bees among others [1]. According to the report of the Intergovernmental science policy platform on biodiversity and ecosystem services [IPBES, 2], this represents between 5 and 8% of the value of world food production. Unfortunately, since 2006 declines in pollinator populations have been observed, and it has been suggested that 40% of the insect species in the world could disappear in the upcoming decades, which will lead to a global loss of biodiversity on a planetary scale [3]. While the use of chemical molecules, such as insecticides, herbicides, fungicides and acaricides, has contributed to advances in world agriculture [4], their potential negative impact on pollinators needs to be considered.

After honey bees, bumble bees are the second-most economically important bee pollinator species worldwide. The bumble bee *Bombus terrestris* is one of the most common wild Eurasian pollinators. Its ability to forage at low temperatures and its technique of vibrating flowers make it a very efficient pollinator, even more than other bees [5]. This technique is particularly suited to flowers of the *Solanaceae* family, such as tomatoes. Their pollinating efficiency has led to significant commercial breeding of these populations, especially for the production of dozens of greenhouse crops [6]. Thus, the significant decline of these populations is of concern and the causes are diverse, including the reduction of their natural habitats and nutritional resources, global warming, the use of agricultural pesticides, and the presence of infectious agents (e.g., viruses, bacteria, fungi, parasites) naturally present in the environment [7,8]. To fight these pathogens, bumble bees, as all metazoans, have developed defence mechanisms that rely to a large degree on innate immune responses [9,10].

Innate immunity, which represents the first line of defence shared by all living organisms (plants, invertebrates, and vertebrates) against harmful stressors and pathogenic invaders, encompass virtually all tissues [for a review see 11]. Across these tissues, plant sap, vertebrate blood, and insect haemolymph represent centres of the humoral immune response, which involves clotting, melanin production, and synthesis of immune effectors, such as the antimicrobial peptides (AMPs), that target pathogens [12,13]. Insects, and particularly the fruit fly (*Drosophila melanogaster* being a genetic work-horse of biology), have been key to deciphering innate immunity [10]. A key fact that has emerged since the 2000s is the observation that AMP production is more exclusive than initially considered and varies depending on the specific infection [11,14].

Regarding Hymenoptera, despite a fragmented scientific understanding of their innate immune responses, several studies have led to a better characterization of the antimicrobial response of *Apis mellifera* and of the bumble bee *B. terrestris* thanks to genome sequencing programs. The *A. mellifera* immune system has similarities to that of *D. melanogaster* and *Anopheles* mosquitoes, with one third of the immune genes shared by the fruit fly and the mosquito [8,9]. Interestingly, as a social insect, *A. mellifera* has more genes for olfactory receptors and genes involved in the regulation of the collection of pollen and nectar [15]. Insects secrete AMPs in their haemolymph that are involved in the activated innate immune response to microbial infections [16]. Bees like bumble bees mainly secrete four AMPs (Apidaecin, Abaecin, Defensin and Hymenoptaecin) that provide a broad-spectrum antibacterial defence [17]. The molecular understanding of the innate immune response and its primary component, the AMPs, remains sparsely documented. At the individual level, bacterial infection can

induce metabolic deregulations, particularly in biological pathways involved in energy management, stress response or defence mechanisms [18].

For several years, much work has been done on *A. mellifera* but, in contrast, few studies have focused on the bumble bee. It is now well documented that several immune defence reactions take place in the haemolymph of bees such as phagocytosis, melanization, coagulation or the secretion of AMPs produced by haemocytes and the fat body (functional equivalent of the mammalian liver) [19]. The main goal of our work was to trigger an effective immune response of the bumble bee to different bacterial stressors to assess the impact of those biotic stressor on the health status of the bumble bee at a molecular level. This study followed a similar study performed on *A. mellifera* using different bacterial strains that may represent challenging strains for bees [20]. We focused on the Gram positive bacterial species *Micrococcus luteus* which is widely distributed in the environment and in insects, and two Gram negative entomopathogenic species, *Pectobacterium carotovorum* subsp. *carotovorum* [20] and the opportunistic pathogen of bees *Serratia marcescens* [21]. We observed an increasing mortality of bumble bees, giving a gradual response to the bacterial aggression. Using complementary mass spectrometry (MS) approaches (Matrix-assisted laser desorption ionization-time of flight) and high-performance liquid chromatography coupled to high-resolution tandem electrospray MS (LC-ESI-MS/MS), we observed different molecular mass profiles of the bumble bee haemolymph in response to the different bacterial strains used as biotic stressors and evidenced differences in the physiological pathways impacted by the challenging bacterial strain considered.

2. Experimental section

The experimental workflow for Omics on the bumble bee model, from experimental infection to proteomics studies (MALD-MS profiling & Bottom-up proteomics), is divided into seven major steps described in this section and summarised in Figure 2.

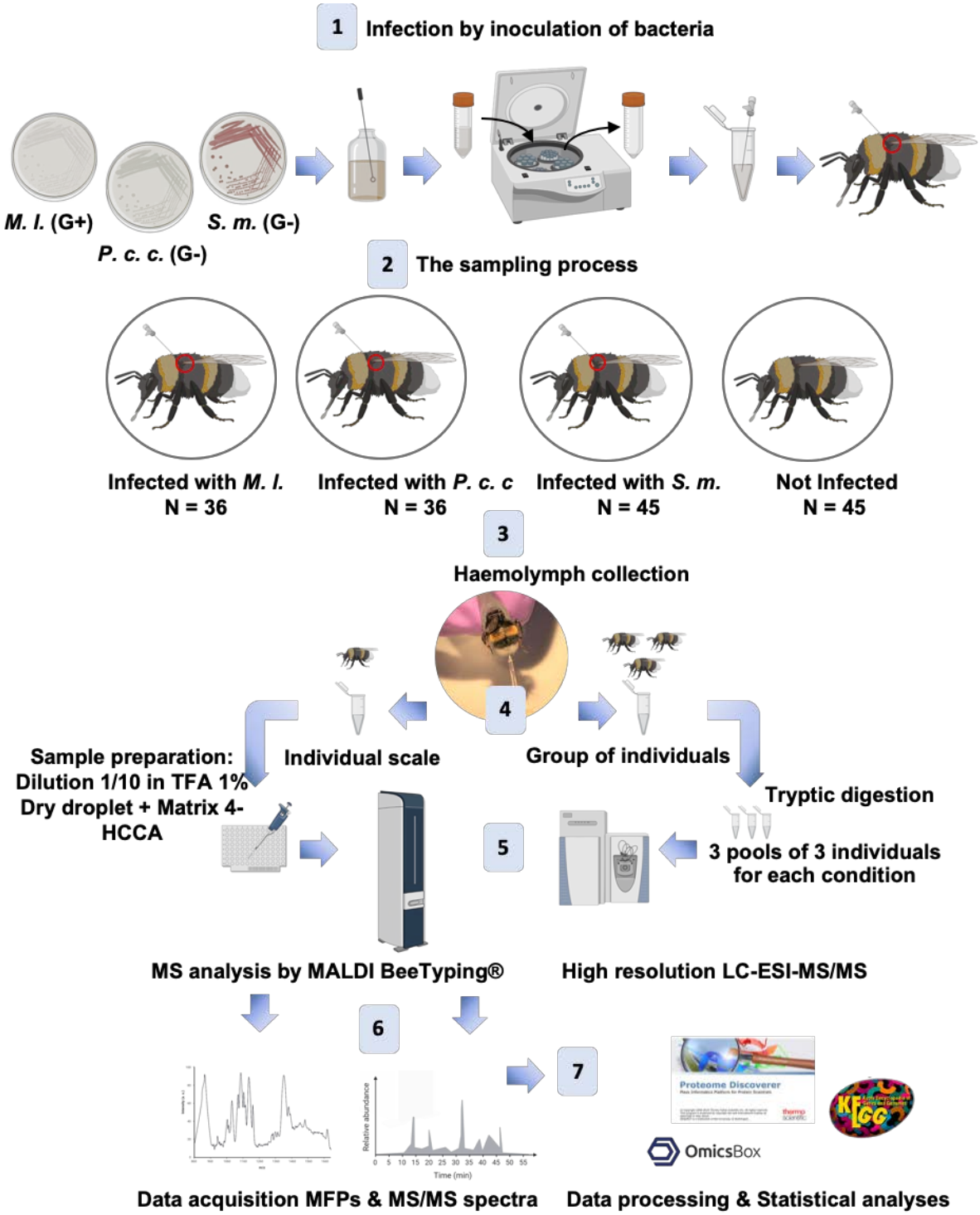


Figure 2: Omics workflow (from experimental infection to data processing) applied to *Bombus terrestris*

2.1 Materials

2.1.1 Bacterial strains used for the experimental infections

To generate biological models of bacterial infection, two entomopathogenic Gram-negative strains (1) *Pectobacterium carotovorum* subsp. *carotovorum* 15 (formerly *Erwinia carotovora carotovora* 15

CFBP2141, generous gift from Bruno Lemaitre, EPFL Switzerland) and (2) *Serratia marcescens* (*Sm*BIOP160412, from our own collection), were used. *Sm*BIOP160412 is an isolate from the haemocoel of a naturally infected *Apis mellifera* collected in the field. Bumble bees were also challenged with the Gram-positive *Micrococcus luteus* (ATCC 4698), an opportunistic pathogen that is widespread in nature. Bacteria were cultured in Luria Bertani medium overnight at 32°C (see Fig. 2 step 1).

2.1.2 Bumble bees, infection experiments and haemolymph collection

Bumble bee colonies

Three *Bombus terrestris* colonies were obtained by HELIOGREEN SAS (Fillinges, France) and placed under artificial conditions, maintained at room temperature (24°C) and continuously in the dark.

Bacterial infections (see Fig. 2 step 2, sampling process)

Experimental infections were performed according to the procedure detailed in Arafah and colleagues [20] with minor adjustments. Briefly, a group of 45 non-experimentally infected individuals as control and three groups of individuals infected with the bacteria were used. All individuals had approximately the same body size. Infections were performed by pricking the bumble bees individually in the thorax at the anterior lateral level under the wing with a fine needle (Fine Science Tools, Germany). The needle was dipped into a concentrated pellet of live bacteria. Thirty-six individuals were infected with *M. luteu*s, 36 with *P. carotovorum* subsp. *carotovorum* and 45 with *S. marcescens*. All bumble bees belonging to the same group (experimentally infected and controls) were placed for 24h at room temperature (RT) in small cages and fed *ad libitum* with sugar syrup (Invertbee from SARL Isnard, France).

Haemolymph collection (see Fig. 2 step 3, haemolymph collection)

Haemolymph was collected from the dorsal side of the abdomen, using pulled glass capillaries (Sutter Instrument Corp, Novato, California) following the procedure reported in Arafah et al [20]. After collection, the haemolymph was immediately transferred into a chilled LoBind Protein microtube (Eppendorf, Germany) pre-coated with phenylthiourea (PTU) and phenylmethylsulphonyl fluoride (PMSF) to prevent melanization and proteolysis, respectively. The haemolymph samples were frozen and stored at -20°C until use.

2.2 Survival rate assessment

The effectiveness of the experimental infection was evaluated by recording mortality. Mortality assessments were conducted before and at several time points post experimental infections: at 6, 12, 24, and 48 hours post experimental infection for the three types of infections, up to 72 hours for the infection to *P. carotovorum* subsp. *carotovorum*, and finally up to day 6 for the infection with *M. luteus* and for the control samples.

2.3 MALDI-MS profiling

2.3.1 Sample preparation

Before MALDI-TOF analysis, the samples were thawed on ice and then centrifuged for a few seconds to push them to the bottom of the tube. A tenfold dilution was performed by adding 0.5 µL of bumble bee haemolymph to 4.5 µL of a 1% solution of TFA, in a 0.5 mL LoBind Eppendorf® tube. For MALDI-MS analysis, the procedure used follows Houdelet et al [22] with minor modification. Briefly, the 10x diluted samples were deposited in three replicates on a MALDI plate (MTP 384 target plate polished

steel BC, Bruker Daltonics) and vacuum-dried for 10 minutes. Once dried, the samples were covered with 1 µL of a fresh solution of matrix (15 mg/mL 4-HCCA in 70% ACN, 2.5% TFA). Finally, the sample spots were lightly vacuum-dried prior to analysis. The calibration was carried out using 0.5 µL of APISCAL and 0.5 µL of Pepmix (Peptide Calibration Standard II, 700-3,200 Da, Bruker Daltonics). APISCAL is an in-house calibration solution composed of two antimicrobial peptides from *Apis mellifera*, namely Apidaecin (average *m/z* of 2,109) and Abaecin (average m/z of 3,879), Melittin (average *m/z* of 2,847), the major venom component; and ETD (average *m/z* of 4,839), a recombinant peptide. After drying under vacuum, the calibrators are covered with 1 µL of matrix. The plate is further dried before MALDI analysis.

2.3.2 Sample analysis and data acquisition

Mass spectra were acquired in triplicates on a MALDI AutoFlex III Smartbeam instrument (Bruker Daltonics) using the FlexControl 4.0 software (Bruker Daltonics) in an automatic positive linear mode. The instrument was set up with the following parameters: 200 Hz laser at a 50% global attenuation offset, 20 kV source voltage 1, 18.7 kV source voltage 2, 9.25 kV lens voltage, 1.906 kV linear detector voltage, 120 ns of pulsed ion extraction delay and 600 Da detector gating. MALDI-MS spectra were recorded at the mass range of 600-18,000 in *m/z* by summing 1,000 laser shots. Data were previewed using the FlexAnalysis 3.4 software.

2.3.3 MALDI data processing

MALDI data were imported into ClinProTools ™ 2.2 software (Bruker Daltonics) for reprocessing. All spectra have undergone baseline correction performed with a TopHat baseline algorithm, along with a smoothing according to the Savitzky-Golay algorithm (window size 2.0 *m/z* in 5 cycles). The total average of the spectra was calculated based on a signal-to-noise threshold of 3 for the selection of the peaks, a picking height of 80 and an application of baseline. Peak lists (maximum peak number of 100) of each spectrum were extracted for data processing and statistical analyses. Comparative analyses were carried out between the different experimental conditions depending on the intensity of the selected peaks. The software normalizes the spectra before performing Statistical Principal Component Analyses (PCA). We referred to two characterized molecules specific for immunity in the bumblebee, Apidaecin (accession number C0HKX3, ANRPVYIPPPRPPHPRL, average *m/z* of 1,978) and Abaecin (accession numbers A0A6P3D796_BOMTE and D2XR04, FVPYNPPRPGQSKPFPTFPGHGPFNPKTQWPYPLPNPGH, average *m/z* 4,397), and a Chymotrypsin inhibitor (accession numbers K7WRE1_BOMTE and A0A6P5I1S5, QQCGLNEEFKSCGSCEPTCAKPRVTICTMECKIGCQCKSGYLRNGEGTCVLPEKC, average *m/z* of 5,938 considering cysteine pairing and a N-terminal pyroglutamic acid).

2.4 Bottom-up proteomics

2.4.1 Sample preparation

Before proteomic analysis, haemolymph samples collected at 24 hours post-infection (24h p.i.) were grouped into 3 pools of 3 individuals for the control condition and the *M. luteus* and *P. c. c*. infections and into 3 pools of 4 individuals for the *S. marcescens* infection. The samples were then dried out by centrifugation under vacuum before being analysed by a bottom-up proteomic approach following Houdelet et al [22]. Briefly, haemolymph samples were suspended in 0.1% RapiGest surfactant in 50 mM ammonium bicarbonate. After reduction with DTT at 56°C for 30 min in the dark and alkylation with 4-VP at room temperature for 30 min in the dark, samples were digested overnight at 37°C with

0.5 µg of trypsin. Digested samples were acidified with 5 µL of 20% ACN/10% TFA to stop enzymatic digestion. After 45 min of incubation at 37°C, samples were centrifuged for 10 min at 15,000g and transferred into inserts for high-performance liquid chromatography (HPLC) autosampler vials for injection.

2.4.2 Nano-LC-MS/MS Analysis

Nano-LC-MS/MS was carried out using an Ultimate 3000 nano-HPLC (Thermo Scientific, Germany) to separate the tryptic-digested peptides according to the protocol established by Masson et al [23]. The separation was performed on a reversed-phase column (3 µm, 75 µm × 250 mm) Acclaim C18 PepMap 100 from Thermo Fischer Scientific, using a biphasic linear gradient (water/ACN, each supplemented with 0.1 % formic acid) from 2% to 32% ACN in 100 min and from 32% to 65% ACN in 5 min. The Q-Exactive mass spectrometer, equipped with a nanospray ion source, was used in positive mode and data-dependent acquisition. The voltage applied to the nanotips was adjusted to produce 0.3 µA and the entrance capillary was maintained at 320°C. The Q-Exactive Orbitrap acquired a full-range scan from 380 to 2000 *m/z* (70,000 resolution, automatic gain control (AGC) target 3 × 106, maximum ion trap time (IT) 200 ms) and then fragmented the top ten-peptide ions in each cycle (17,500 resolution, AGC target 2 × 105, maximum IT 100 ms, intensity threshold 4×10^4, excluding charge-unassigned ions, Normalized Collision Energy of 30). Parent ions were excluded from MS/MS for the next 15 s. The software Chromeleon Xpress and Xcalibur 2.2 were used to control the HPLC and the mass spectrometer, respectively.

2.4.3 Database searching, Protein identification and Functional annotation

The Sequest HT search algorithm was run by Proteome Discoverer™ 2.5 (Thermo Fisher Scientific, Bremen, GmBH) to match the acquired MS/MS spectra to a database consisting of the protein sequences of the Order Hymenoptera (including *B. terrestris*), a selection of bee pathogens, and the three bacteria used in the experiment downloaded from UniProtKB, on April 1st 2022. The sequences of common protein contaminants (e.g., human keratins) were also added to this database. The following parameters were used: trypsin digest with three maximum missed cleavages; six and 150 amino acids as minimum and maximum peptide lengths, respectively; a tolerance of 10 ppm/0.02 Da for precursors and fragment ions, respectively; cysteine pyridyl-ethylation was set as a fixed modification; C-terminal protein amidation, methionine and tryptophan oxidation were set as variable modifications. The MS features were extracted from the chromatographic timefram between 20 min. to 132 min and the min./max. precursor masses were selected at 350/500 Da, respectively. The identification confidence was set at a false discovery rate of 1%. The target/decoy selection was based on concatenated mode and validation made on q-Value.

Regarding the Peptide Validation node, a retention time shift of 10 min and a mass tolerance of 10 ppm and coarse mode were used for parameter tuning. Label-free quantification was performed using Proteome Discoverer 2.5. Chromatographic alignment by the Minora Feature Detector node was achieved with a maximum trace length of 5, a signal to noise ratio of 3 and a maximum ΔRT of isotope pattern multiplets of 0.2 min. The precursor ions of unique and razor peptides were used to determine protein abundance, and normalization was performed using the total peptide amount. Statistical significance was determined using an ANOVA (individual proteins) test on summed abundances with Top 3 and a max. value of fold change set to 100. P-values were calculated and found significant when below 0.05. Protein ratios < 0.5 or > 2 in the different conditions relative to the not-experimentally-infected controls were considered significantly differentially expressed (DEPs). To complete missing

protein identity information, TBlastN was used. For functional annotation of the lists of DEPs generated from the LC-ESI-MS/MS analyses, the bioinformatic software OmicBox (v2.1.14, https://www.biobam.com) was used. To get the most complete annotation labels, the analyses were performed using the four cloud-powered algorithms (Blast, InterProScan, GO Mapping, GO slim). Separate lists of DEPs of the pairwise comparisons (different bacteria vs control) were loaded to investigate the biological processes and the protein functions following bacterial challenge. Combined pathway analysis was performed on the annotated sequences (proteins) joining Reactome and KEGG to identify the enriched pathways.

2.5 Statistical analyses

Statistical analyses were performed using RStudio 1.4 software. The normality and homogeneity of the variances of the data were tested are indicated by a p-value <0.05 *, p-value <0.01 ** and p-value <0.001 ***.

3. Results and Discussion

Deciphering the overall humoral immune responses of the bumble bee *Bombus terrestris* at the molecular level is essential for a comprehensive understanding of how bumble bees are impacted by stressors (abiotic and/or biotic). We designed an experimental infection workflow to evaluate by mass spectrometry the biochemical/physiological changes occurring in bumblebee haemolymph collected after experimental infections with opportunistic entomopathogenic bacteria (*Pectobacterium carotovorum* subsp. *carotovorum* 15, an isolate of *Serratia marcescens* from the haemocoel of a naturally infected *Apis mellifera* collected in the field) and *Micrococcus luteus*, an opportunistic pathogen spread in nature.

3.1 Mortality rate of experimentally infected bumblebees versus control

Our results showed an effect on bumble bees whatever the bacterial strain used in our experimental infections (Figure 3). Statistical analyses performed on the mortality rate of *B. terrestris* after infections with different bacterial strains showed that both entomopathogenic Gram negative bacteria used for experimental infections were pathogenic for bumble bees, *Pectobacterium c. c.* strain (Test log-rank; $\chi2 = 49.94$; p-value < 0.0001) and *S. marcescens* strain (Test log-rank; $\chi2 = 50.25$; p-value < 0.0001). *S. marcescens* induced slightly greater mortality than *P. carotovorum carotovorum* (Test log-rank; $\chi2 = 7.47$; p-value < 0.01). The experimental methodology, with infection being performed by pricking the bumble bee with a fine needle dipped into a bacterial pellet of *S. marcescens*, which relies on an uncontrolled number of injected bacteria, must be taken into consideration when interpreting these results. It would be interesting to perform a titration of the number of bacteria inoculated rather than simply pricking individuals with a septic needle. As *S. marcescens* is lethal to bees [21], a larger number of infected individuals would be required. In contrast, infection with the Gram-positive bacteria *M. luteus* did not cause mortality in bumble bees when compared to the control condition where bumble bees were not experimentally infected. As previously observed for *Apis mellifera* (personal observation P Bulet), *M. luteus* appears to be of low pathogenicity for *B. terrestris*. These first results indicate that *P. carotovorum carotovorum* and *S. marcescen*s impact the health status of *B. terrestris* and that *M. luteus* is not generating mortality of bumble bees during the time course of our experiment (up to 5 days). This contrasts with our observations that using pathogenic microorganisms such as *P. carotovorum carotovorum* profoundly affected bumblebee survival, suggesting that the immune system is rapidly overtaken by the infectious events.

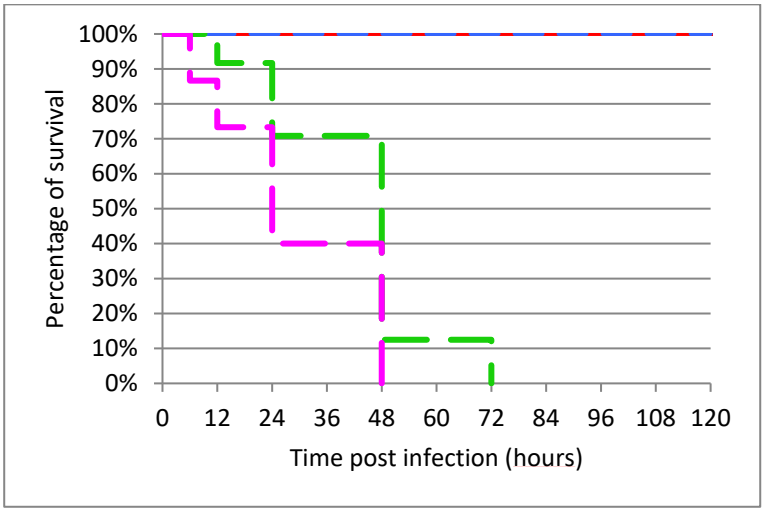


Figure 3: Survival of *Bombus terrestris* after infections with *Micrococcus luteus* (blue); *Pectobacterium carotovorum carotovorum* (green) and *Serratia marcescens* (magenta). The control samples (non-experimentally infected bees) are reported in red. Survival analysis was performed using the Kaplan-Meier estimator statistical method and the distribution of survival curves was analysed using a log-rank statistical test. G+ and G- stand for Gram positive and Gram negative, respectively.

To evaluate the impact of experimental infections at the peptidomic/proteomic level, individual haemolymph samples were collected at different times post experimental infection (24h, 48h, up to 5 days and 15 days when possible) and the molecular mass fingerprints by MALDI mass spectrometry (MALDI MFP or MALDI BeeTyping®) established between a mass range of 600-18,000 in *m/z*.

3.2 Molecular haemolymph signatures by MALDI BeeTyping® to follow the impact of bacterial infections in *B. terrestris*

MALDI MFP is a commonly used strategy to elucidate molecular signatures of complex biological matrices such as, for example, extracts of tissues or direct body fluids under different physiological conditions (e.g., diseases, response to treatments or stressors). So far to our knowledge this approach has not been used to follow the immune status of the bumble bee *B. terrestris*, despite it already being used to monitor the immune responses of other insects (e.g., *Drosophila,* [24]) to different biotic stressors. The use of MALDI MFP was pioneered on the insect model, the fruit fly *Drosophila melanogaster*, at the end of the 1990s [24-26]. Through MALDI MFP analyses, the authors showed that this method applied at the individual scale provided reliable molecular mass signatures of immune-induced peptides of 24 *Drosophila* immune-induced molecules (DIMs) including four AMPs (two glycosylated forms of Drosocins, Metchnikowin and the antifungal Drosomycin) detectable in the mass range considered 1.5 to 11 kDa [26]. Interestingly, in addition to the AMPs identified by their molecular masses, a series of highly induced peptides after infection, the *Drosophila*-specific Bomanin peptides (Boms former DIMS 1-4), were recognized as critical for resistance against pathogens [27,28].

In this study, MFPs were used to follow the impact of experimental microbial infections in bumble bee haemolymph according to a procedure applied to the honey bee, *Apis mellifera* [20,22] and the honey bee pathogen *Nosema* [28].

3.2.1 Global analysis of MFPs discriminates between different bacterial infections

To generate MFP models, an average haemolymph spectrum was recorded as the signature of this tissue in response to each pathogen used (*M. luteus*, *P. carotovorum carotovorum*, or *S. marcescens*). Statistical Principal Component Analysis (PCA) (Figure 4) showed that the three infected groups differed from the control group 24 hours post experimental infection in the mass range of 600-18,000 in *m/z*. Over time, the group infected with the Gram-positive strain *M. luteus* appears to be less distinguishable from the control group, unlike the group infected with *P. carotovorum carotovorum* which can still clearly be distinguished at 48 hours post-infection (48h p.i.). As already evidenced by our survival follow-up, none of the experimentally infected bumblebee could survive pricking with a fine needle dipped into a bacterial pellet of *S. marcescens*. After a 5-days post experimental infection (5d p.i.), the group infected with *M. luteus* tends to overlap with the control group while after 15 days the *M. luteus* infected group was no longer distinguishable from the control one.

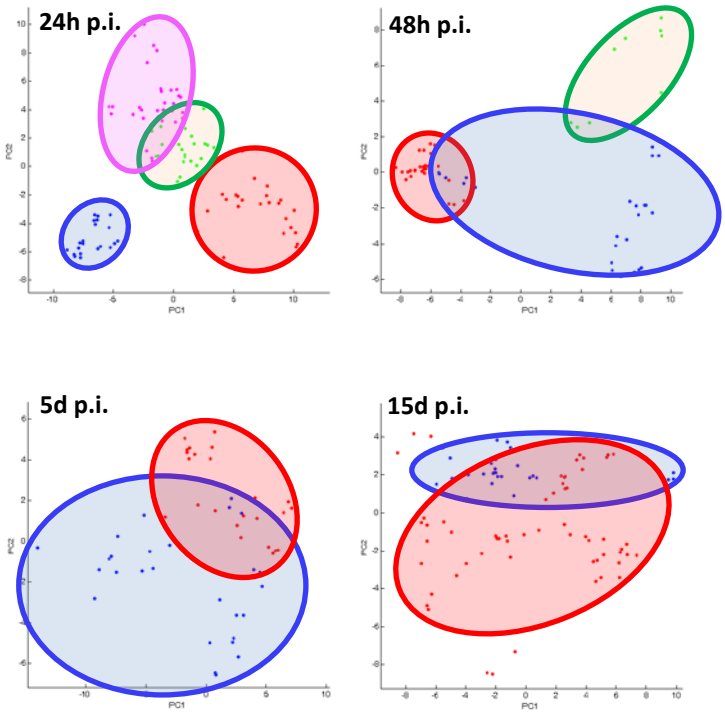


Figure 4: **Principal Component Analysis (PCA) of the MALDI BeeTyping® spectra of the haemolymph of bumblebees infected with different bacteria strains (for different exposure times, 24h post experimental infection (24h p.i.); 48 hours (24h p.i.); 5 days (5d p.i.) and 15 days (15d p.i.)).** Each individual was analysed in triplicates, i.e., three spectra represented by three points. The spectra of each experimental condition were subjected to PCA to test for discrimination between the control group (red) and the different bacterial infections: *Micrococcus luteus* (blue), *Pectobacterium carotovorum carotovorum* (green) and *Serratia marcescens* (magenta).

Overall, this analysis showed that within 24h p.i, MFPs enabled the impact of the different strains tested, from a non-pathogenic widely distributed strain (*M. luteus*) to an opportunistic strain with a high pathogenicity such as *S. marcescens*, to be discriminated. This reflects the impact of the strains on the molecular complexity of the haemolymph, and represents a read-out of the humoral immune response of the bumble bee.

3.2.2 The immune response to microbial infections

To go deeper in the molecular understanding of the defence responses of bumble bees to microbial infections, we looked for the presence of antimicrobial peptides (AMPs) in the haemolymph of the bees in response to the bacterial infections. In bumble bees, several AMPs have already been characterized, notably Apidaecin (accession number C0HKX3, average *m/z* of 1,978) and Abaecin (Abaecin (accession numbers D2XR04 or A0A6P3D796_BOMTE, average *m/z* 4,397). The intensity of Apidaecin (figure 5A) and Abaecin (Fig. 5B) peaks was measured 48h p.i. in bumble bees infected with *M. luteus* and *P. carotovorum carotovorum* (p-value < 0.0001). A significant (p-value < 0.0001) increase in intensity of both peaks is visible only 24 hours after infection with *M. l*. In contrast, the intensity of expression of the Chymotrypsin inhibitor (accession numbers K7WRE1_BOMTE and A0A6P5I1S5, average *m/z* of 5,938 in Fig. 5C) tends to be down-regulated during the different infections at 24 and 48h p.i.. But a very low stimulation of the immune system is observed during an infection with *S. marcescens*. We suggest that the virulence of this bacterial strain is too strong for the bumble bees to defend themselves and activate their immunity. According to these results, an infection with *M. luteus* triggers an immune response more rapidly than an infection with *P. carotovorum carotovorum*, which would enable the bumble bees to defend themselves effectively against this bacterium. Indeed, these differences are linked to the pathogenicity of the bacteria because *P. carotovorum carotovorum.* strongly stimulates the immune system of the bumblebees but too late, which would explain the relatively high mortality rate observed previously (Fig. 3).

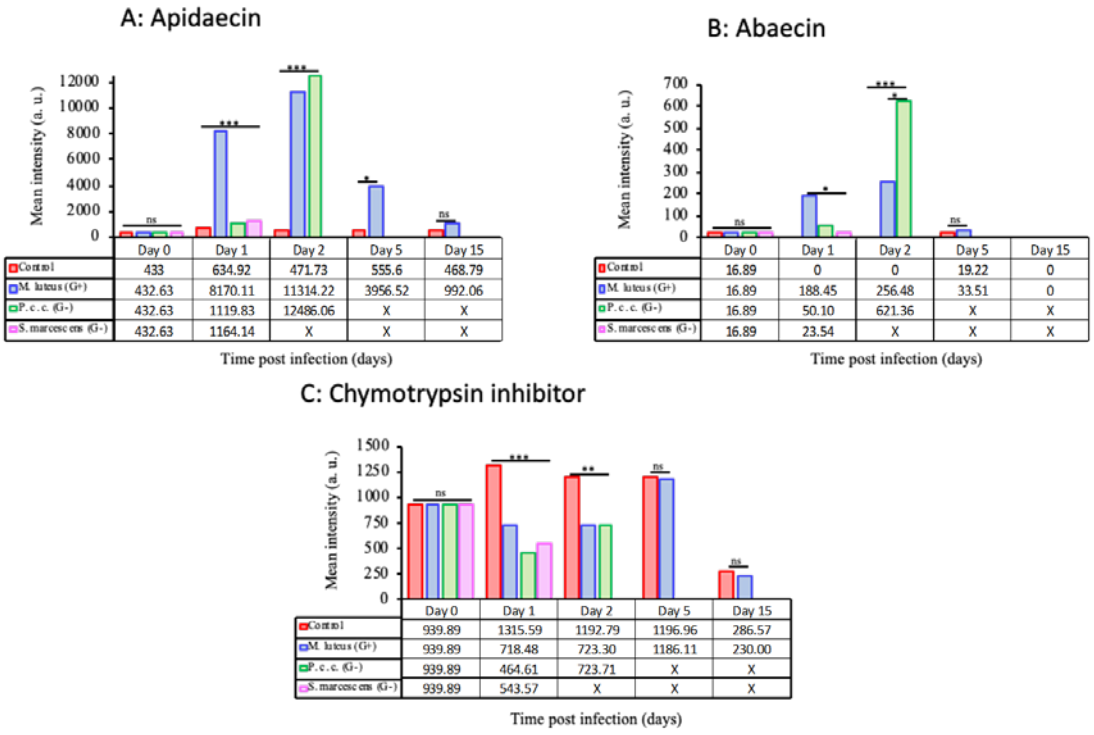
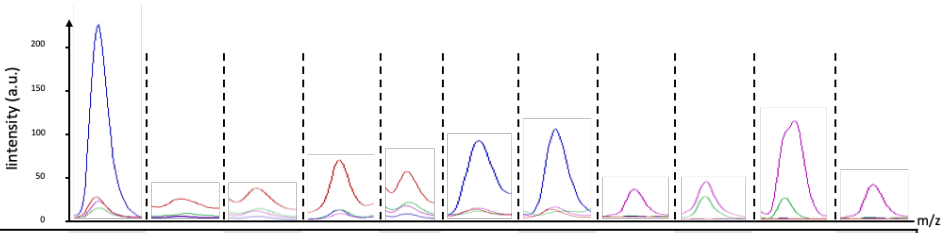


Figure 5: Average intensity of some immune molecules in bumblebee haemolymph, Apidaecin (A), Abaecin (B) and Chymotrypsin inhibitor (C). The intensity averages were analysed using Kruskal-Wallis statistical tests with significant differences at p-value < 0.05 (*), highly significant at p-value < 0.01 (**) and very highly significant at p-value < 0.001 (***). The samples of control or experimentally infected bumble bees were classified using a set of eleven molecular ions to discriminate the different biological models (Figure 6), From the MALDI MFPs, the models recognize with 100% accuracy each condition. A cross-revaluation of the

different individual haemolymph spectra allowed us to obtain a 100% match for the control model, *M. luteus* and *P. carotovorum c carotovorum* and 97.44% for the *S. marcescens* experimental infection model (Fig. 6). The most discriminating molecular ion (*m/z* 1977.78) is Apidaecin (theoretical *m/z* = 1978) which is in agreement with the results found in Figure 5.



Best discrimination peaks	*m/z* ions	1977.78	5839.26	5995.45	5801.24	5974	2015.85	1999.57	4218.81	1092.48	1277.34	3997.7
	Weight index	13.29	8.22	6.96	6.87	6.20	5.26	5.20	5.19	5.08	4.68	3.97

Classifier Model Scoring

Infectious model	Classifier	Recognition capability (%)	Overall recognition (%)	Cross validation (%)	Overall validation (%)
Control	Genetic Algorithm	100 %	100 %	100 %	99.36 %
M. l.		100 %		100 %	
P. c. c.		100 %		100 %	
S. m.		100 %		97.44 %	

Figure 6: Biological models for classification of uninfected or experimentally infected bumblebees. These models were generated from eleven molecular ion peaks selected according to their importance index as having the best discriminating characteristics of haemolymph samples. They were obtained by an algorithmic program of the ClinProTools™ software configured with a maximum of 50 generations and a maximum selection of 15 peaks. Only the spectra obtained at 24h p.i. were used for the creation of the models. Genetic Algorithm-based classifier was used to discriminate the control group (non-experimentally infected, red) bees from experimentally infected ones *Micrococcus luteus* (*M.l.*, blue), *Pectobacterium carotovorum carotovorum* (*P.c.c.*, green) and *Serratia marcescens* (*S.m.*, magenta).

These MALDI-MS analyses revealed the impact of bacterial infections on the molecular composition of bumble bee haemolymph, particularly on certain immune peptides. Unfortunately, the use of this technique limits the analysis and interpretation of such results. As a matter of fact, the MALDI BeeTyping® approach is not detecting or only slightly detects proteins over 12-15 kDa. This is why we performed bottom-up proteomic analyses using high-performance liquid chromatography coupled to high-resolution tandem electrospray MS or LC-ESI-MS/MS.

3.3 Proteomics analysis of the haemolymph of *B. terrestris* in the context of experimental bacterial infections

From bottom-up proteomics analysis by LC-ESI-MS/MS on bumble bee haemolymph samples (Figure 7), we identified a total of 568 proteins in the control condition (Fig. 7A), 735 during the experimental infection with *M. luteus* (Fig. 7B), 766 upon infection with *P. carotovora carotovora* (Fig. 7C) and 1610 upon infection with the entomopathogenic *S. marcescens* (Fig. 7D). The majority of these proteins have been attributed to the genus *Bombus*. Among these proteins, some have been identified and still classified as uncharacterised (Fig. 7A-D; values expressed in %) due to the fact that annotation of the genome of *B. terrestris* needs to be improved [18]. During infection with *P. carotovora carotovora* and *S. marcescens,* a significant amount of bacterial proteins was identified in the haemolymph samples, precisely 189 and 795 bacterial proteins after *P. carotovora carotovora* and *S. marcescens*

experimental infection, respectively. This contrasts with the number of bacterial proteins detected in the haemolymph samples of bumble bees infected with *M. luteus.* (8 proteins, Fig.7B), a number similar to that detected in the control experiment (10 proteins, Fig 7A). In addition to these proteins of bacterial origin a limited number of proteins, originating from parasites (3 to 13 proteins) and fungi (5 to 12), were identified irrespective of sample type (Fig7A-D). Finally, all proteins identified in this study were loaded in BOMDBase-1.0 (Deliverable D9.9). They represent 53% of the total accessions recorded in BOMDBase-1.0.

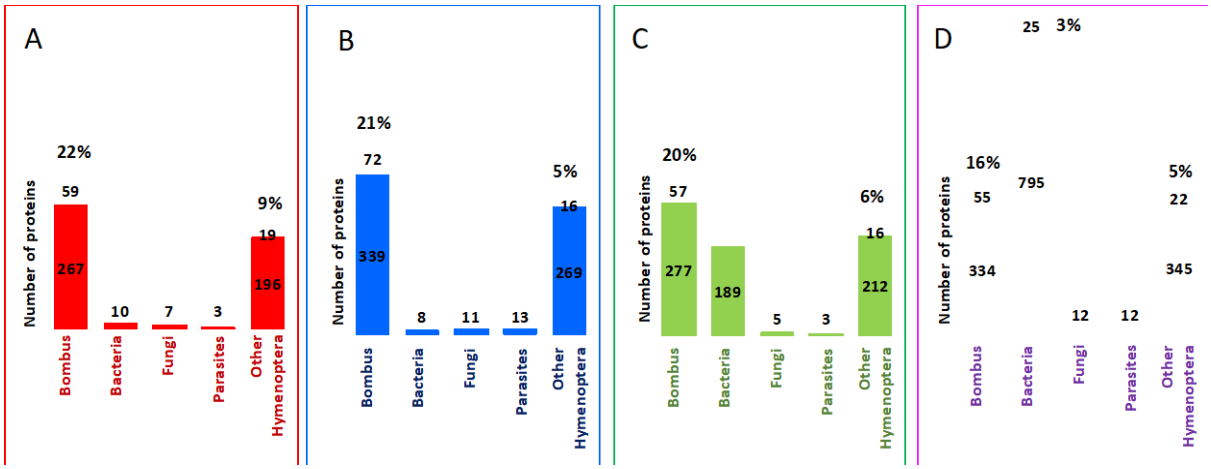


Figure 7: Overall number of proteins identified in the haemolymph of *Bombus terrestris* by LC-ESI-MS/MS at 24 hours post-infection. All the identified proteins obtained after the proteomic analysis of the different experimental conditions, Control (A), bumblebees infected with *Micrococcus luteus* (B), *Pectobacterium carotovorum carotovorum* (C) and *Serratia marcescens* (D). For each condition, the proportions of identifications of proteins from the genus *Bombus*, bacteria, fungi, parasites and other Hymenoptera are plotted in the bar charts. The lighter colours at the top of the bars represent uncharacterized proteins and the values above represent the percentage of these proteins.

Following bacterial challenge with either *M. luteus*, *P. carotovorum carotovorum* or *S. marcescens*, label-free quantitative (LFQ) proteomics were conducted on haemolymph pools using LC-ESI-MS/MS, and bottom-up strategy and fold-change ratios for 538 proteins were calculated as a measurement of the protein regulation in the infected over the control conditions. Amongst the 12 proteins that were found to be significantly up-regulated in infected bumble bees with *M. luteus*, the two antimicrobial peptides Abaecin (accession D2XR04, FC = 6.01) and Apidaecin (accession C0HKX3, FC = 6.64) were those with the highest fold change (Figure 8). One protein was found to be significantly down-regulated under *M. luteus* challenge (Figure 8).

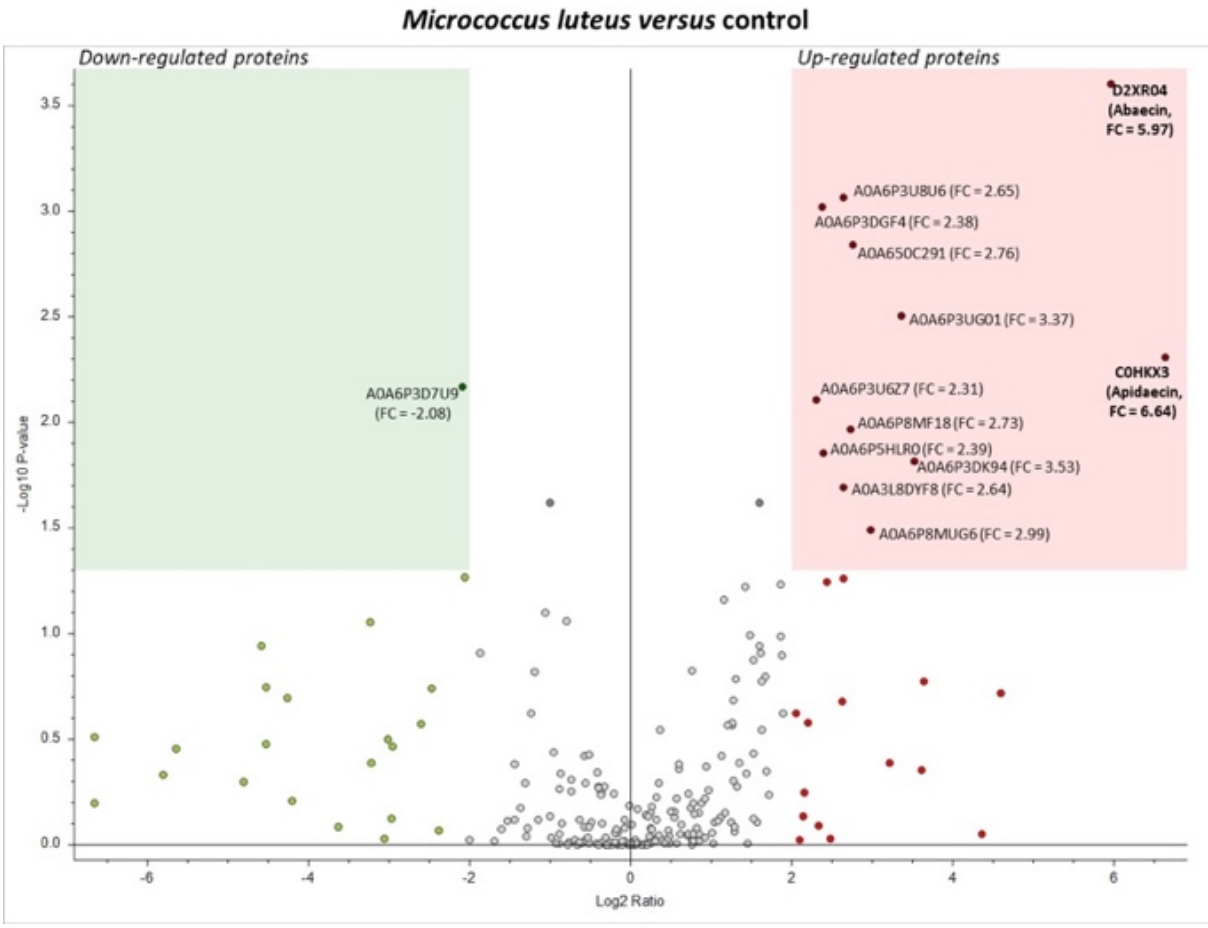


Figure 8: Up and down regulated proteins in bumblebee haemolymph following an experimental bacterial challenge with *Micrococcus luteus versus* the control experiment (non-experimentally infected bumblebees). The coloured dots in green represent the proteins with a fold change >2 (up-regulation threshold) while the ones in red are for a fold change <-2 (down-regulation). Dots in the red and green boxes represent the proteins (denoted with their UniProtKB accession number) that were found to be significantly up-regulated (Fold Change FC >2, p-value < 0.05) and down-regulated (FC <-2, p-value < 0.05) according to treatment, respectively. The grey dots represent all the remaining proteins which did not pass the different thresholds (-2>FC>2 and p-value <0.05).

Twenty-two and two proteins were found to be significantly up and down regulated respectively, in bumble bees infected with *P. carotovorum carotovorum.* (Figure 9). The protein ratio of Abaecin (accession D2XR04, FC = 5.53) and Defensin 1 (accession D2XR05, FC = 6.01) was found as up-regulated following the bacterial challenge.

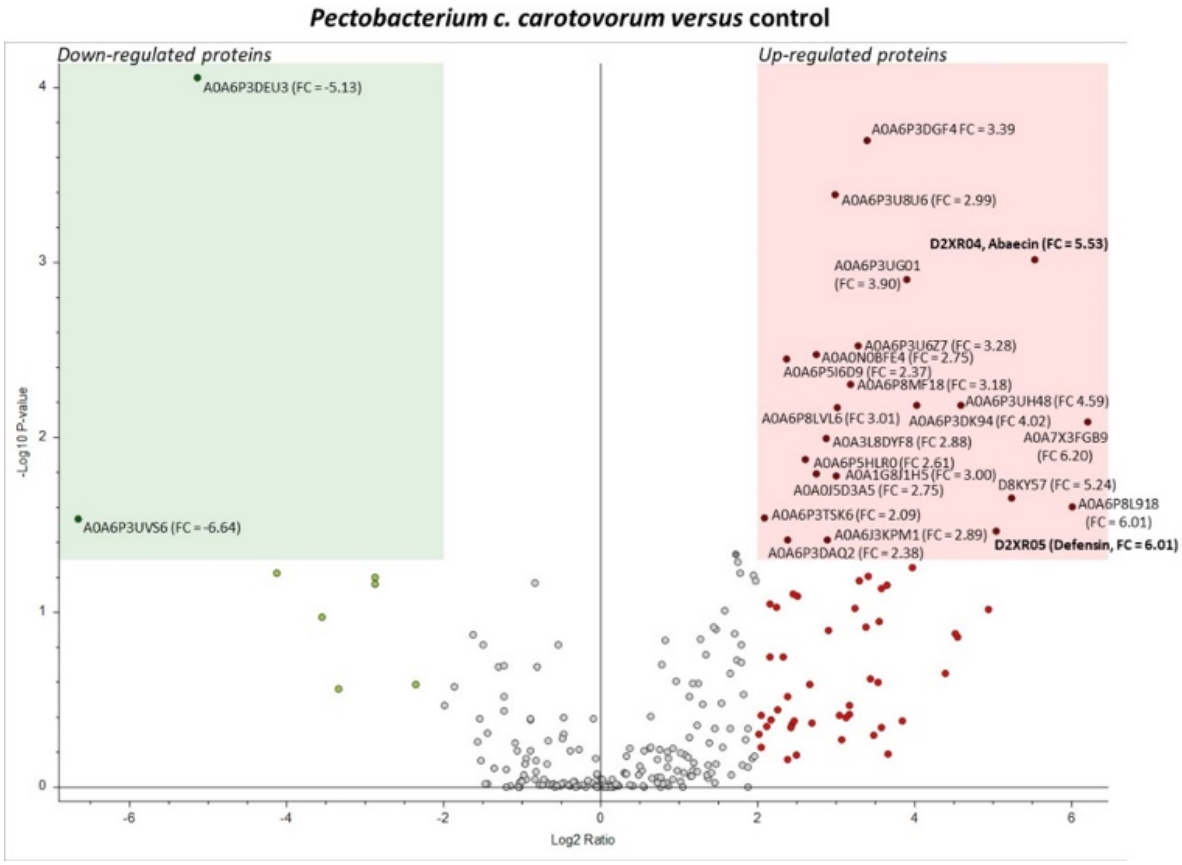


Figure 9: Up and down regulated proteins in bumble bee haemolymph following bacterial challenge with *Pectobacterium carotovorum carotovorum* versus the control (non-experimentally infected bumblebees. The coloured dots in green represent the proteins with a fold change >2 (up-regulation threshold) while the ones in red are for a fold change <-2 (down-regulation). Dots in the red and green boxes represent the proteins (denoted with their UniProtKB accession number) that were found to be significantly up-regulated (Fold Change FC >2, p-value < 0.05) and down-regulated (FC <-2, p-value < 0.05) according to treatment, respectively. The grey dots represent all the remaining proteins which did not pass the different thresholds (-2>FC>2 and p-value <0.05).

Infection of bumble bees with the entomopathogenic bacterial strain *S. marcescens* resulted in significant protein disregulation. Forty-five proteins were up-regulated and six down-regulated, including the Chymotrypsin inhibitor (K7WRE1_BOMTE, accession A0A6P5I1S5, FC = -4.16).

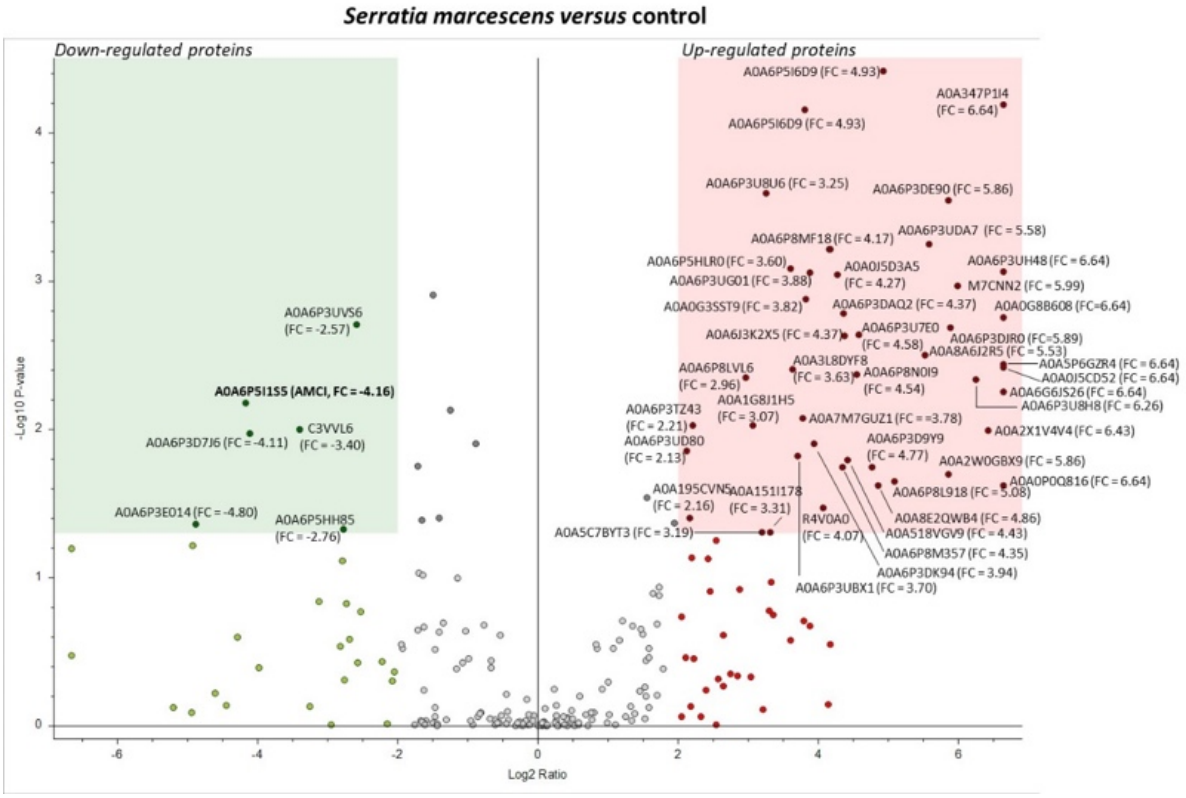


Figure 10: Up and down regulated proteins in bumble bee haemolymph following bacterial challenge with *S. marcescens* versus the control (non-experimentally infected bumblebees. The coloured dots in green represent the proteins with a fold change >2 (up-regulation threshold) while the ones in red are for a fold change <-2 (down-regulation). Dots in the red and green boxes represent the proteins (denoted with their UniProtKB accession number) that were found to be significantly up-regulated (Fold Change FC >2, p-value < 0.05) and down-regulated (FC <-2, p-value < 0.05) according to the treatment, respectively. The grey dots represent all the remaining proteins which did not pass the different thresholds (-2>FC>2 and p-value <0.05).

The lists of DEPs following bacterial infection with *M. luteus*, *P. carotovorum carotovorum* and *S. marcescens* were analysed with OmicsBox software; the results obtained regarding the biological processes and the molecular function of these proteins are shown below (Figure 11). Several processes (mainly metabolism) and molecular functions of the dysregulated proteins were shared between different bacteria investigated in this study.

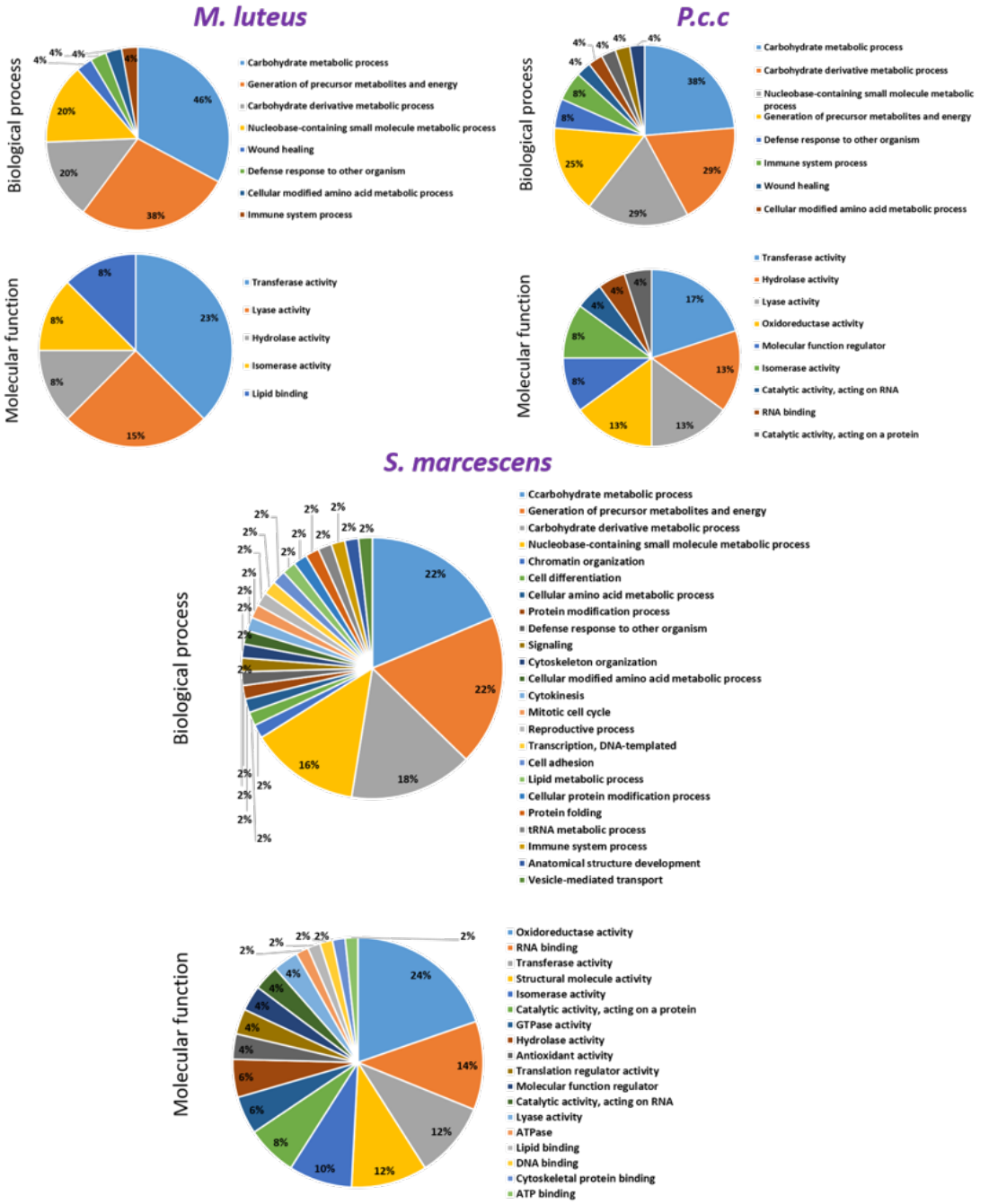


Figure 11: Functional distribution of DEPs following bacterial infection according to biological processes and molecular functions. Assignments were made with OmicsBox

To continue the functional annotations of the DEPs, we investigated the pathways in which the DEPs are involved and thus could be impacted by the bacterial infection. A total of 231 pathways were identified to be impacted, based on the involvement of at least one protein. From these pathways, 50 are common to all of the three bacteria, 41 are common between two bacteria, and 140 are specific to a given bacterium (five for *M. luteus*, 97 for *P. carotovorum carotovorum* and 38 for *S. marcescens*).

The top 15 pathways identified in each bacterium are presented in Table 1:

Table 1: Top 15 pathways impacted following bacterial infection by *M. luteus* (*M.l*), *P. carotovorum* carotovorum (*P.c.c*).*c* and *S. marcescens (*S.m). #Seqs means the number of proteins

Bacteria	Pathway	Pathway ID	#Seqs
M.l	Glycolysis / Gluconeogenesis	ko00010	6
M.l	Gluconeogenesis	R-DME-70263	4
M.l	Glycolysis	R-DME-70171	4
M.l	Methane metabolism	ko00680	4
M.l	Carbon fixation in photosynthetic organisms	ko00710	3
M.l	Glucagon signaling pathway	ko04922	3
M.l	HIF-1 signaling pathway	ko04066	3
M.l	Neutrophil degranulation	R-DME-6798695	3
M.l	Central carbon metabolism in cancer	ko05230	2
M.l	Fructose and mannose metabolism	ko00051	2
M.l	Pentose phosphate pathway	ko00030	2
M.l	Adherens junction	ko04520	1
M.l	AMPK signaling pathway	ko04152	1
M.l	Amyotrophic lateral sclerosis	ko05014	1
M.l	Anther and pollen development	R-SIT-8986768	1
P.c.c	Glycolysis / Gluconeogenesis	ko00010	7
P.c.c	Gluconeogenesis	R-DME-70263	4
P.c.c	Glycolysis	R-DME-70171	4
P.c.c	Neutrophil degranulation	R-DME-6798695	4
P.c.c	Methane metabolism	ko00680	4
P.c.c	HIF-1 signaling pathway	ko04066	4
P.c.c	Carbon fixation in photosynthetic organisms	ko00710	4
P.c.c	Glucagon signaling pathway	ko04922	3
P.c.c	Fructose and mannose metabolism	ko00051	3
P.c.c	Proteoglycans in cancer	ko05205	3
P.c.c	Degradation of the extracellular matrix	R-DME-1474228	2
P.c.c	Platelet degranulation	R-DME-114608	2
P.c.c	Type I hemidesmosome assembly	R-DME-446107	2
P.c.c	Laminin interactions	R-DME-3000157	2
P.c.c	Prion disease	ko05020	2
P.c.c	Amoebiasis	ko05146	2
S.m	Glycolysis / Gluconeogenesis	ko00010	10
S.m	Carbon fixation in photosynthetic organisms	ko00710	7
S.m	Salmonella infection	ko05132	6
S.m	Methane metabolism	ko00680	6
S.m	Pyruvate metabolism	ko00620	5
S.m	Fructose and mannose metabolism	ko00051	5
S.m	Citrate cycle (TCA cycle)	ko00020	5
S.m	Ribosome	ko03010	5
S.m	Fluid shear stress and atherosclerosis	ko05418	4
S.m	Glyoxylate and dicarboxylate metabolism	ko00630	4

S.m	Proteoglycans in cancer	ko05205	4
S.m	HIF-1 signaling pathway	ko04066	4
S.m	Platelet activation	ko04611	3
S.m	Neutrophil extracellular trap formation	ko04613	3
S.m	Pathogenic Escherichia coli infection	ko05130	3

Among these pathways, the glycolysis/gluconeogenesis pathway seemed to be highly impacted by the different types of experimental bacterial infections. Here we present the pathway with the involved DEPs according to each pairwise comparison to the control (Figure 12).

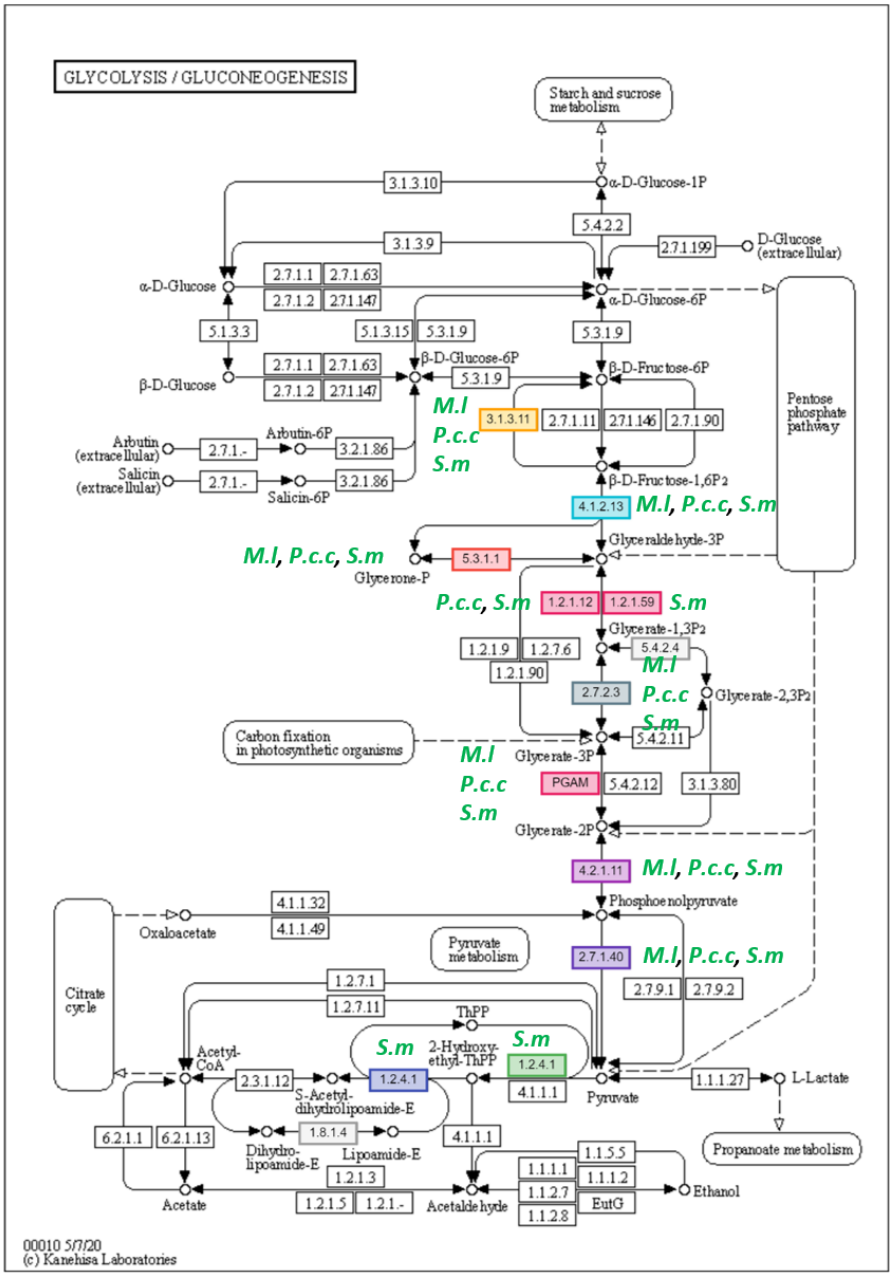


Figure 12: Glycolysis/Gluconeogenesis pathway with the involved up-regulated proteins following bacterial infection with *Micrococcus luteus* (*M.l*); *Pectobacterium carotovorum carotovorum* (*P.c.c*) and *Serratia marcescens* (*S.m*)

As we mentioned above, *P. carotovorum carotovorum* strongly stimulates the immune response. As pathways involved in this process, Toll and Imd pathways were identified to be impacted following *P. carotovorum carotovorum* infection by the up-regulation of Defensin 1 (accession D2XR05, FC = 5.05) (Figure 13).

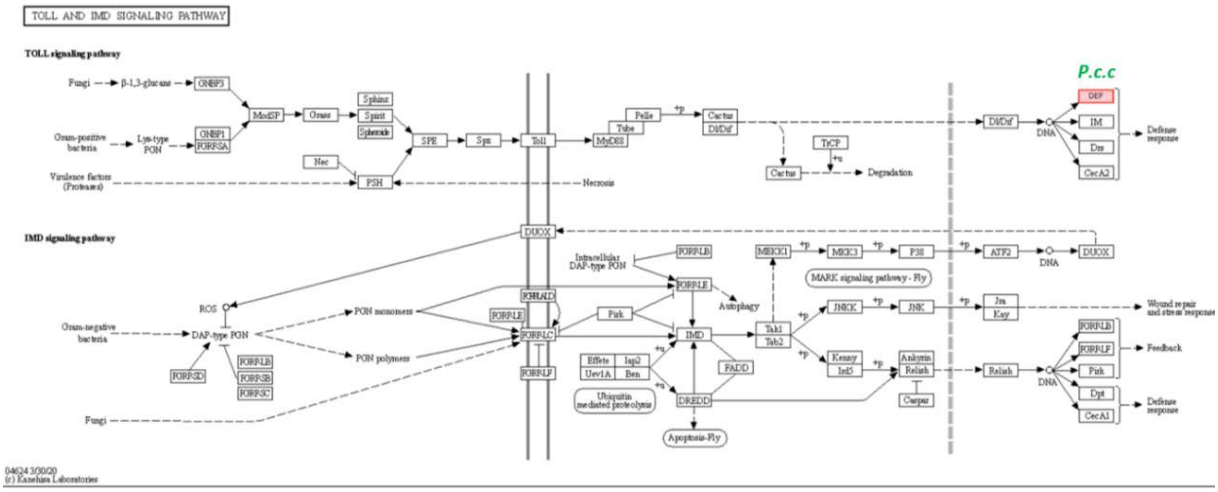


Figure 13: Toll and Imd signalling pathway impacted by *Pectobacterium carotovorum carotovorum* (*P.c.c*) infection showing the Defensin 1 (DEF)

From our analysis, we observed that *S. marcescens* has more impact on the bumble bees than the others. For this reason, we were interested in the specific pathways linked to this bacterium (Table 2):

Table 2: Specific pathways impacted by *S. marcescens*.

Aminoacyl-tRNA biosynthesis	NOD-like receptor signaling pathway
Carbon fixation pathways in prokaryotes	Oocyte meiosis
Cell cycle	Pantothenate and CoA biosynthesis
Chemical carcinogenesis - receptor activation	Pathways in cancer
Citrate cycle (TCA cycle)	Peroxisome
Complement and coagulation cascades	Phenylpropanoid biosynthesis
Coronavirus disease - COVID-19	PI3K-Akt signaling pathway
Cysteine and methionine metabolism	Plant-pathogen interaction
Flagellar assembly	Progesterone-mediated oocyte maturation
Glycerophospholipid metabolism	Propanoate metabolism
Glyoxylate and dicarboxylate metabolism	Prostate cancer
Hepatitis C	Proximal tubule bicarbonate reclamation
IL-17 signaling pathway	Ribosome
Inositol phosphate metabolism	RNA polymerase
Longevity regulating pathway - worm	Th17 cell differentiation
Lysine degradation	Tryptophan metabolism
MAPK signaling pathway - fly	Two-component system
MAPK signaling pathway - yeast	Valine, leucine and isoleucine biosynthesis
Necroptosis	Valine, leucine and isoleucine degradation

4. Conclusions

The lack of tools to monitor the host immune status in pollinators has prompted us to develop mass spectrometry approaches on pollinator insect models such as *Apis mellifera* [20,22, D9.11] and *Bombus terrestris* (this Deliverable D9.12). The combination of MALDI-BeeTyping® to generate molecular mass fingerprints (MFPs)/peptidome profiling and Off-Gel bottom-up proteomics have allowed us to study the impact of bacterial pathogens on bumble bee haemolymph. This body fluid is equivalent in its biological functions to vertebrate blood, making it a collector of many biomolecules with a potential to be markers of pathologies, diseases or stress indicators. Vertebrate blood is a recognised biofluid to identify individual biomarkers [29,30]. MALDI MS is a powerful tool that is currently spreading in the clinical setting of microbiological biotyping (MALDI BioTyping) and in biomedical research for the study of complex biological diseases including sepsis [31,32]. MALDI BeeTyping® technique is derived from MALDI BioTyping and we have already successfully applied this methodology to follow the impact of stressors in *A. mellifera* bees to differentiate bacterial infections [20,22, D9.11].

In this work, we focused on another biological model, the bumble bee *B. terrestris*. We infected them with different bacterial strains in order to activate the different activation pathways (for example Toll and Imd pathways known in bees [33]). We used the reference Gram positive bacterial strain *Micrococcus luteus* and two entomopathogenic Gram negative bacterial strains *Pectobacterium carotovorum* subsp*. carotovora* and *Serratia marcescens*. These bacteria have been shown to trigger a systemic immune response in insects [21,34]. The strain *S. marcescens* is an opportunistic bacterium that is very virulent for bees [21]. Recorded post-infection mortality showed us that the entomopathogenic bacterial strains *P. carotovorum* subsp*. carotovora* and *S. marcescens* are highly pathogenic for the bumble bees as a relatively high mortality rate was noted (see Fig. 3). Conversely, the Gram-positive *M. luteus* did not cause mortality in bumble bees when compared to the control condition where bumble bees were not experimentally infected. This has been already demonstrated for the *Drosophila* [19] and *A. mellifera* models [20]. Bumble bees are therefore able to cope with *M. luteus* by developing an effective immune response, which is not the case for the two entomopathogenic Gram negative strains *P. carotovorum* subsp*. carotovora* and *S. marcescens* with mortality rates of 82% and 100% at 48 hours post-infection, respectively. Regarding the experimental infection with these two bacterial strains, the results on the survival rate of these insects need to be improved by carrying out infections with a titration of the number of inoculated bacteria. This improvement might allow the bumble bees infected with *P. carotovorum* subsp*. carotovora* and *S. marcescens* to survive longer for a better understanding of the impact of such infections on the immune peptidomes and proteomes of *B. terrestris*.

This is the first time that MALDI MS profiling (MALDI BeeTyping®) to address bumble bee health status under bacterial challenge has been applied. Mass molecular fingerprints (MFPs) or peptidomics profiling of haemolymph allowed us to differentiate control individuals from infected individuals. According to our results, bacteria have an impact on the molecular composition of the haemolymph of *B. terrestris*. (see Fig. 4). More specifically, we observed that these three bacteria did not impact bumble bees in the same way and demonstrate that the MFPs analysed make it possible to clearly differentiate these three infections from one another. Nevertheless, the molecular composition of the haemolymph of bumble bees infected with the non-pathogenic widely distribute dGram positive strain *M. luteus* seems very similar to the one of the control condition 15 days after infection. This

would suggest a successful defence against this bacterium in connection with the absence of mortality mentioned above.

Finally, we would like to mention that to establish a more in-depth analysis, we were interested in their defence mechanisms and in particular the activation/establishment of an effective and powerful immune response capable of fighting off the "intruder" that potentially may impact the health status of the host. The average intensities of certain molecular peaks corresponding to immunity molecules were analysed and compared between the different experimental conditions. These are Apidaecin [35, accession number for *B. terrestris* Apidaecin, C0HKX3], an active AMP known to be more involved in cases of infection by Gram negative bacteria, Abaecin [36, accession number for B. terrestris Abaecin, A0A6P3D796_BOMTE] which is also an active AMP against bacteria and a promising marker to follow the impact of stressors in bees (honey bees and bumble bees) and the chymotrypsin inhibitor AMCI [37, accession number for the AMCI-like from *B. terrestris*, K7WRE1_BOMTE] involved in the regulation of physiological processes such as the blocking of cellular potassium channels, blood coagulation or inflammation [37]. Regarding Apidaecin and Abaecin, under our experimental conditions, an immunity peak appears 48 hours post-infection in *B. terrestris* infected with *M. luteus* and *P. carotovorum* subsp*. carotovora.* Conversely, the intensity of expression of the chymotrypsin inhibitor tends to be deregulated downwards in any experimental infection. A very weak stimulation of the immune system is observed during infection by *S. marcescens* supporting the hypothesis that the virulence of this bacterium is too strong to allow bumble bees to defend themselves and activate an effective immunity to fight off this bacterium. In contrast, infection with *M. luteus* triggers an immune response more rapidly than an infection with *P. carotovorum* subsp*. carotovora*. which would allow bumblebees to defend themselves effectively against this bacterium. Indeed, these differences in reactions can be linked to the pathogenicity of the entomopathogenic bacterial strains for bumble bees, which would explain the relatively high mortality rate observed.

The results obtained on the peptidome profiling (MFPs) revealed the potential of haemolymph fingerprints analysis by MALDI-BeeTyping® mass spectrometry as a simple, speedy and readily transferrable method for infected bumble bee stratification that could contribute to follow the impact of stressors on bees and for a realistic application to assess the health status of bees in response to stressors based on their immunological status. However, this technique on its own is not sufficient to characterise the pathways that are deregulated in response to the bacterial infections considered in this study. Some molecules are not or are barely detectable in the mass range studied by MALDI BeeTyping® (1 to 18 kDa). Complementary proteomics analyses were used to characterise proteins, quantify them and finally appreciate the physiological and biological processes impacted by stressors. However, in the classical approaches in marker discovery, working in a complex matrix such as vertebrate blood and in our case haemolymph from bees, samples need to be decomplexified by combining liquid chromatography and high resolution mass spectrometry. To overcome these difficulties, we proposed a bottom-up approach, allowing us to confirm that the immunity of bumble bees was altered, but so were other metabolic and cellular processes. Many carbohydrate metabolism (energy storage) proteins were deregulated following bacterial infection as a common response, but to a variable degree according to the bacterium.

All the data presented in this deliverable were acquired by combining MALDI proteome profiling and bottom-up proteomics, confirming that it is possible to assess the general health status of *B. terrestris* by a simple "blood test" as already demonstrated for example on *Drosophila* [25,26], through our

pioneered project HematoBeeTest [20], and more recently in the frame of PoshBee on *A. mellifera* in a context of Nosemosis [22]. In order to define molecular markers reflecting a state of stress, we generated a predictive model by an algorithmic program of the ClinProTools™ software from the mass spectra of molecular ions obtained by MALDI BeeTyping®. Depending on the parameters assigned to it, it selects a set of discriminating peaks from the haemolymph samples. Consequently, the results obtained confirm our initial hypothesis that it would be possible to establish a "map" of bumble bee health through a “blood test”. Thus, we aimed to create a readily transferrable method (MALDI BeeTyping®). Our approach was designed to be as simple as possible and individual haemolymph samples were directly read by MALDI MS. Improvements and validation with other haemolymph samples prepared/collected (lab, semi-field and field experiments) by the different partners involved in *B. terrestris* experiments (EMU, UMONS, RHUL) of the PoshBee project will consolidate our results on experimentally infected bumble bees with our different models of bacterial infections.

5. Perspectives

This study allowed us to demonstrate that bumble bees react in a specific way to environmental stress such as experimental infection by opportunistic and entomopathogenic bacteria. The molecular modifications of the haemolymph following a bacterial attack allow us to establish distinctions between “healthy” individuals (not experimentally infected) and experimentally infected ones. Several immunity peptides and the chymotrypsin inhibitor AMCI-like from *B. terrestris* were detected and quantified as effective markers for assessing the health status of the bumble bees from a simple "blood" test using the innovative MALDI BeeTyping® technique. These results allowed us to create predictive models capable of establishing the state of health of bumble bees following an experimental biotic stress with bacteria as already observed for *A. mellifera* [20]. Our Off-Gel high-resolution bottom-up proteomics study (derived from the one used on *A. mellifera*, [22]; POSHBEE Deliverable D9.11) allowed us to characterise for the first time the proteomics profiles of haemolymph of the buff-tailed bumble bee *Bombus terrestris* subjected to different experimental infections with different bacteria known to be promoters of a mild up to a strong or deleterious response to such infections. Using a label-free quantification proteomic approach, we showed that bacterial infection significantly modulates several other proteins in addition to immune proteins, for example proteins involved in insect metabolism, response to stimulus, and energetic pathways. Having in-hand this omics workflow on the pollinator model *Bombus terrestris*, we are running all the haemolymph samples collected within the frame of PoshBee from lab to field experiments.

6. Associated information

A manuscript presenting this work will be submitted shortly to Journal of Proteome Research. The title is “Unravelling the *Bombus terrestris* haemolymph, a readout of the immune response to microbial infections, through complementary mass spectrometry approaches” with the proposed authors: Lorène Bournonville, Sébastien N. Voisin, Karim Arafah, Dalel Askri, Michel Bocquet and Philippe Bulet. The mass spectrometry proteomics data have already been deposited in the online open access repository ProteomeXchange, with identifier PXD035224. In addition, the list of proteins identified through this work have been included in the BOMDBase-1.0 which is listing *Bombus* proteins identified during the proteomics analysis of the bee haemolymph samples. BOMDBase-1.0 has been made public. BOMDBase-1.0 is intended to be mined by any researcher looking for specific proteins or interested in cross-referencing their findings with ours. BOMDBase-1.0 will be further implemented

and updated as more samples are provided by the PoshBee partners, analysed by 10-BIOP and validated by 11-CNRS.

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