



OPEN Unravelling pollen diet and microbiome influence on honey bee health

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In the last decade, drought has been identified as one of the most relevant climate change factors affecting ecosystem integrity across countries. It can severely affect plant growth in agroecosystems, leading to changes in the trophic potential of nectar and pollen. As a cascade effect, a deficit in the nutritional composition of pollen can weaken pollinators, triggering additional threats to ecosystem stability. In this scenario, understanding the impact of trophic sources on honey bee health remains a significant gap that needs to be addressed. This study aims to correlate pollen of different botanical and geographical origins, and therefore of different trophic potential, with selected honey bee markers: the abundance of core microbial taxa and proteins involved in the immune response detectable in the haemolymph. A comprehensive proteomic approach based on MALDI BeeTyping[®] and SDS-PAGE profiles, together with qPCR for the quantification of target microorganisms, was used to elucidate these interactions in bees fed with pollen deriving from 8 botanical families. Our results show that different pollens do not significantly affect the concentration and the total amount of small and large haemolymph proteins but do significantly affect the core gut microbiome composition. Furthermore, the effect of different diets on the microbiome suggests an indirect effect on the immune system response by modulating and influencing the synthesis of some immune-related peptides. This research confirms the importance of the gut microbiome in honey bee health and may also help to understand the honey bee response to climate changes in a scenario of compromised trophic resources.

Keywords Nutrition, Vitellogenin, MALDI BeeTyping, SDS-PAGE, Immunity, Proteomics, Core microbiota

Intensive agricultural practices, anthropogenic pollutants, and climate change increasingly impact the distribution and growth of plants in the agroecosystems^{1,2}, as well as soil health and ecosystem stability^{3,4}. Among climate-driven factors, drought has emerged over the last decade as one of the most disruptive to ecosystem integrity across Europe⁵. In high-biodiversity countries like France, Italy and Spain⁶, the effects of climate change and human activities are expected to intensify⁷, reducing vegetation both in diversity and biomass⁸, and depleting soil organic carbon^{9,10}. Drought and high temperatures impact plant metabolic functions, and pollen's nutritional characteristic¹¹ resulting in a lower pollen and nectar quality and quantity¹². This can lead to nutritional deficits in pollinators, reducing their activity, altering their spatial distribution, and introducing new risks to the ecosystem stability¹³. For example, declines in honey bee colonies due to drought, rising temperatures, and scarce food resources are increasingly reported worldwide¹⁴, prompting beekeepers to supplement feed for colonies^{15,16}. Reduced pollen diversity may negatively affect honey bee survival, metabolism¹⁷, immune response and stress resistance^{18,19}, pathogen tolerance^{20,21}, and sensitivity to agrochemicals^{22,23}. Additionally, limited amino acid availability can hinder immune cell diversity²⁴, the development of the fat body, ovaries, and hypopharyngeal glands²⁵, and the formation of a balanced gut microbiota²⁶.

The gut microbiota supports many essential functions in honey bees. Specific bacterial are specialized in nutrient absorption; for instance, *Gilliamella* is supposed to facilitate pollen cell wall digestion through specialized

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metabolic pathways²⁷, although, it has not been confirmed that the bee host benefits of any pollen digested by-products or content release. Moreover, other members of the gut core family, such as Lactobacillaceae, are crucial for maintaining the gut-brain axis²⁸. The social bee gut microbiota involvement in pathogens defence is partially demonstrated in pollinators such as bumble bees and honey bees^{29–31}. Consequently, the support of a well-developed microbial community is essential for the immune system activation and stimulation^{26,32,33}. Therefore, a balanced core microbiome is vital for pollinator health²⁶.

The effect of pollen's nutritional profile on honey bee health and gut microbiome balance has not been extensively studied. Research shows a positive correlation between the quality and quantity of pollen proteins and honey bee haemolymph protein profiles³⁴, highlighting the importance of trophic resources available for pollinator wellbeing, hereafter defined as “trophic potential”. Pollen nutrition is supposed to influence the synthesis of key storage proteins, such as Vitellogenin and Hexamerins, in the fat body, which are then secreted into the haemolymph¹³. Hexamerins are particularly abundant in larvae and support functions like gonad development, egg production, and foraging³⁵. Vitellogenin, a phosphoglycolipoprotein whose synthesis is known to depend on the nutrient availability of protein and lipids sources for honey bees³⁶, effectively contributes to honey bee fitness by regulating honey bee aging and modulating immunity and stress resilience³⁷. Other proteins, like Apolipoproteins and Transferrins, contribute to innate immunity^{38,39} and oxidative stress reduction^{40,41}. These proteins can be separated and quantified using SDS-PAGE electrophoresis and provide a useful measure of honey bee health at the colony level⁴². Additionally, smaller proteins relevant to honey bee immunity include the *Apis mellifera* Cathepsin G/Chymotrypsin inhibitor (AMCI)⁴³ and antimicrobial peptides (AMPs) such as Apidaecins, Abaecin, Defensins and Hymenoptaecin⁴⁴. Recently, matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS) has been applied to analyse the peptide content of honey bee haemolymph, leading to the development of MALDI BeeTyping^{45–47}.

Due to climate-driven pollen shortages, assessing nectar and pollen availability has become critical in many European countries. However, a significant knowledge gap remains on how these food sources impact honey bees immune system and health. This study addresses this gap by correlating pollen supplementation from various botanical and geographical origins with selected honey bee markers, focusing on the presence and abundance of core microbial taxa and immune-related haemolymph proteins. Using a comprehensive proteomic approach, based on MALDI BeeTyping⁴⁵, SDS-PAGE profiles, together with qPCR for the quantification of target microorganisms, this research presents the first integrated analysis of pollen's nutritional effects on honey bee gut microbiome, immunity, and overall health.

Materials and methods

Pollen collection and palynological analysis

Pollens from various botanical species were collected by local beekeepers across five Italian regions: Avellino (Campania), Nuoro (Sardinia), Palermo (Sicily), Ledro and Ampola (Trentino-Alto Adige), and Grosseto (Tuscany). Pollen samples were collected every two weeks from mid-April to mid-June 2023 using wooden pollen traps (LEGA, Faenza, Italy). The collected polyfloral pollen was immediately stored at $-20\text{ }^{\circ}\text{C}$ and then shipped in dry ice to the Department of Agricultural and Food Sciences (DISTAL) of the University of Bologna for further analysis. A portion of each polyfloral pollen sample was analysed, monofloral pollen was separated by morphological characteristics (e.g. colour and shape), and classified taxonomically via palynological analysis performed by Piana Ricerca S.r.l according to⁴⁸ to obtain monofloral samples. The polyfloral and the monofloral samples obtained were used in the cage experiments.

Extraction and quantification of total proteins and lipids from pollen

Protein extraction followed a modified version of the method described by⁴⁹. Briefly, 250 mg of pollen were homogenised in SDS buffer and sonicated for 30 min. Proteins were precipitated using 20% tri-chloroacetic acid (TCA)/acetone. The obtained pellet was washed with acetone and resuspended in Tris HCl 0.5 M pH 8. The extracted proteins were quantified with the Bradford method. Lipid extraction was performed according to⁵⁰. Briefly, 500 mg of pollen was mixed with chloroform:methanol 2:1, shaken, centrifuged at 10,000 rpm for 10 min, and the supernatant filtered in a Büchner funnel using Whatman 1 filter papers (diameter 42.5 mm and pore size 11 μm). Crude lipids were calculated by comparing the difference in weight between extracted pollen and the amount obtained after solvent extraction and overnight evaporation.

Honey bees and pollen diets

Three brood frames from healthy Italian honey bee colonies (*Apis mellifera ligustica*) located in the Bologna district (Farneto and Castello di Serravalle) were collected weekly starting from mid-June for six weeks, and incubated at $32 \pm 2\text{ }^{\circ}\text{C}$ with 60% relative humidity (RH) until honey bee emergence. Due to climatic constraints (peak of the dry season) and brood scarcity, 14 different colonies (hereafter referred as “source colony”) with sister queens, where used as brood donors. Every experimental replicate was built up with bees deriving from a specific colony. Groups of 50 newly emerged honey bees were collected and placed in transparent, ventilated plastic cages (12 cm \times 8 cm \times 6 cm)⁵¹. One-week experiments were conducted over six weeks (June to August 2023) at DISTAL facilities to progressively test the collected pollens. For each monofloral pollen (P) and Pollen Mixture (Mix) three cages were set up. For each cage, 1 g of pollen was suspended in 10 mL of 2:1 sucrose:water solution (w:w), with 0.5 mL of this mixture administered daily for four days using gravity feeders. Control cages received only sugar syrup. Cages were maintained at an average temperature of $29 \pm 2\text{ }^{\circ}\text{C}$ and 60% relative humidity (RH). Detailed experimental conditions are outlined in Table 1, Tables S1 and S2.

Each cage (n) received either a polyfloral pollen mixture or monofloral pollen samples (as described in Table 1) based on the following group classification: Cistaceae (n=12), Asteraceae (n=17), Cornaceae (n=5), Boraginaceae (n=12), Fagaceae (n=14), Leguminosae (n=15), Rosaceae (n=23), Salicaceae (n=3), mixed

Code	Pollen origin	Botanical family	Protein content	Lipid content
P11	Sardinia	Cistaceae	17.1%	7.6%
P12	Sardinia	Boraginaceae	12.4%	9.6%
P13	Sardinia	Cistaceae	12.5%	8.0%
Mix1	Sardinia	Mix	n.a.	n.a.
P14	Campania	Leguminosae	7.4%	10.8%
P15	Campania	Cornaceae	11.9%	7.6%
Mix2	Campania	Mix	n.a.	n.a.
P16	Tuscany	Asteraceae	5.3%	12.0%
P18	Tuscany	Fagaceae	6.9%	9.6%
Mix3	Tuscany	Mix	n.a.	n.a.
P21	Tuscany	Salicaceae	12.1%	13.6%
P22	Sicily	Asteraceae	16.6%	12.0%
P23	Sicily	Leguminosae	11.5%	12.4%
P25	Sicily	Fagaceae	14.1%	9.2%
P26	Sicily	Asteraceae	6.6%	n.a.
P27	Sicily	Rosaceae	17.6%	8.8%
P28	Sicily	Rosaceae	4.6%	11.2%
Mix4	Sicily	Mix	n.a.	n.a.
P31	Sardinia	Boraginaceae	5.9%	9.6%
P32	Sardinia	Boraginaceae	14.6%	10.8%
P33	Sardinia	Fagaceae	16%	10.0%
Mix5	Sardinia	Mix	n.a.	n.a.
P34	Campania	Fagaceae	26.2%	8.4%
P35	Campania	Rosaceae	13%	10.0%
Mix6	Campania	Mix	n.a.	n.a.
P36	Trentino	Asteraceae	12.6%	15.2%
P37	Trentino	Salicaceae	10.3%	16.4%
Mix7	Trentino	Mix	n.a.	n.a.
Mix8	Tuscany	Mix	n.a.	n.a.
Mix9	Campania	Mix	n.a.	n.a.
Mix10	Tuscany	Mix	n.a.	n.a.
Mix11	Tuscany	Mix	n.a.	n.a.
Mix12	Sicily	Mix	n.a.	n.a.
Mix13	Sicily	Mix	n.a.	n.a.
P51	Sardinia	Rosaceae	32.7%	9.6%
P52	Trentino	Rosaceae	2%	6.8%
P53	Trentino	Cistaceae	0.1%	8.8%
P54	Trentino	Fagaceae	4.4%	9.2%
P56	Trentino	Leguminosae	3.9%	9.2%
P57	Trentino	Rosaceae	0.5%	17.2%
P58	Trentino	Asteraceae	6%	14.8%
P59_1	Sardinia	Rosaceae	18%	10.0%
P61	Sardinia	Leguminosae	6.4%	9.6%
P62	Sardinia	Cistaceae	3.7%	11.2%
P63	Sardinia	Fagaceae	10.9%	8.8%
P64	Campania	Boraginaceae	2.8%	6.8%
P65	Campania	Cornaceae	32.2%	7.2%
P69_1	Sardinia	Leguminosae	77.2%	5.2%
P69_2	Sardinia	Asteraceae	18.4%	9.2%

Table 1. Pollen collection area and palynological analysis according to Braglia et al.⁴⁷. The protein and lipid content in pollen samples are expressed in percentage. Botanical identifications that result from the palynological analysis are also reported. *P* monofloral, *Mix* multifloral, *n.a* data not available, *Mix* mixture of different botanical families.

pollen sources ($n = 13$), and a negative control with no pollen (NEG-CTR, $n = 10$). The monofloral pollen tested derived from the same pollen mixture used.

Haemolymph sampling

Honey bees were anaesthetised on ice, and haemolymph was collected from the dorsal aorta using glass microcapillaries, as described by⁵². Haemolymph samples from ten honey bees per cage were pooled, mixed, and divided into two identical aliquots: one for SDS-PAGE analysis and one for MALDI BeeTyping[®]. Haemolymph aliquots were transferred to a chilled Eppendorf LoBind Protein pre-coated microtube (Eppendorf, Germany) as described in^{45,53,54}. The pre-coating consisted of 40 μM PTU (1-Phenyl-2-Thiourea) and 10 mM PMSF (Phenylmethylsulfonyl Fluoride) to minimise proteolysis and melanisation, respectively. The extraction process was conducted on ice to prevent degradation, and haemolymph samples were subsequently stored at $-80\text{ }^{\circ}\text{C}$.

DNA extraction from honey bee gut

DNA was extracted from the midgut and rectum of six honey bees per cage, using the PureLink™ Genomic DNA Mini Kit (K182002; Invitrogen) with slight modifications. These honey bees were pooled, manually macerated with plastic micro pestles, and homogenised with glass beads (0.2 mm) in a rotovortex (50 Hz) for 10 min following lysis buffer addition. DNA quantification was performed using a Qubit Flex Fluorometer (Thermo Fisher Scientific, Milan, Italy) within the DNA BroadRange Assay kit (Thermo Fisher Scientific, Milan, Italy), and samples were stored at $-20\text{ }^{\circ}\text{C}$ until further analysis.

Separation and quantification of haemolymph proteins using SDS-PAGE

Total protein determination

The total protein (TP) concentration in haemolymph was measured using the Bradford method (Bradford Reagent, Sigma-Aldrich, MO, USA). Bovine serum albumin (Sigma-Aldrich, MO, USA) was used as a standard for the calibration curve. Absorbance was measured at 590 nm using an Infinite F50 ELISA Plate Reader (Tecan Trading AG, Männedorf, Switzerland).

Protein separation and quantification using SDS-PAGE

One-dimensional SDS-PAGE was used to separate haemolymph proteins. The samples were diluted to 3 μg of total proteins for loading onto 4–12% Bis-Tris polyacrylamide gels (NuPage/Thermo Fisher Scientific, Waltham, MA, USA). Electrophoresis was conducted using an Xcell SureLock Mini-Cell with 2-(N-morpholino) propane sulfonic acid (MOPS) buffer (NuPage/Thermo Fisher Scientific, Waltham, MA, USA) at pH 7.3 containing sodium dodecyl sulphate (SDS). Standard proteins of known molecular mass (SeeBlue™ Plus2 Pre-stained Protein Standard, Thermo Fisher Scientific, Waltham, MA, USA) were used for molecular mass calibration. The electrophoresis system was connected to a power supply (Power Pack Basic—Bio-Rad, Hercules, CA, USA) with a constant voltage of 200 V for 40 min. Following electrophoresis, gels were stained with Coomassie G250, digitised using ChemiDocMP (BioRad, Hercules, CA, USA), and pherograms were obtained using ImageLab 5.2.1 software (BioRad, Hercules, CA, USA). Each sample included 1 μg of lactate dehydrogenase (LDH-B, 1 $\mu\text{g}/\mu\text{L}$), (Sigma-Aldrich/Merck KGaA, Darmstadt, Germany) as an internal standard, and protein band volumes were compared to this standard for quantification, as described by⁴².

MALDI BeeTyping[®] analysis

Haemolymph preparation

Haemolymph samples from each cage were analysed by MALDI BeeTyping[®] following minor modifications to the method by⁴⁵. Each sample was diluted 1:100 in ultrapure water (MilliQ water, Millipore, Billerica, USA) acidified with 1% trifluoroacetic acid (TFA, Sigma-Aldrich, St. Louis, USA), with 1 μL of the diluted sample spotted in triplicate on a MALDI MTP 384 polished steel plate (Bruker Daltonics, Germany) and dried under mild vacuum for 25 min. A 1 μL layer of 15 mg/mL alpha-cyano-4-hydroxycinnamic acid (4-CHCA, Sigma-Aldrich, St. Louis, USA) matrix solution in 70% acetonitrile ACN and 2.5% TFA was added to each spot and dried for 15 min, until the complete co-crystallisation of haemolymph and matrix droplet.

MALDI-MS data acquisition

The spectral acquisition was performed using an AutoFlex III Smartbeam[®] MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). An external mass spectrometer calibration was performed according to⁴⁶ using two protein sets: APISCAL, with molecular ions in the m/z range of 2100 to 4893, and ProtMix, covering 5734 to 16,952 Da. MALDI-MS spectra were recorded in positive automatic linear mode using FlexControl v4.0 Software (Bruker Daltonics, Germany) with a dynamic range of detection of 600–18,000 Da. Settings included a 60% global attenuator offset, 27% attenuator range, 200 Hz laser frequency, 70% laser power, and 1000 accumulated laser shots per haemolymph spectrum. A potential difference of 1.5 kV and linear analogue offset of 50.0 mV were applied, with a suppression gate up to m/z 600 to prevent detector saturation. Data were visualised in FlexAnalysis v3.4 Software (Bruker Daltonics, Germany), with peaks identified based on m/z values.

Gut Microbiome core microorganism quantification

The honey bee core microbial taxa *Bartonella*, *Bifidobacterium*, *Bombilactobacillus* (Firm-4), *Frischella*, *Gilliamella*, *Lactobacillus* (Firm-5), *Snodgrassella*, and total bacteria were quantified with qPCR (QuantStudio[™]5 Real-Time PCR System, Applied Biosystems) in control and treated samples. Specific primers targeting the 16 S rRNA gene are listed in Table S3, along with final primer concentration in the reaction, annealing temperature and time, and amplicon melting temperature. Reactions were carried out with PowerUp SYBR Green Master Mix (Applied Biosystems) in triplicate, according to manufacturer instructions. Quantification was based on a

standard curve obtained with serial dilutions (10^4 to 10^8 copies) of the target amplicon, and final regression lines calculated⁵⁰. Total bacterial counts were expressed as Log 16 S rRNA copies/intestine.

Data analysis

Pollen samples representing unique botanical families found only once were excluded from statistical analysis. Therefore, the experiment was based on eight botanical families well represented among the different tests, for a total of 36 monofloral pollens and 13 mixtures, for a total of 10 comparisons for the correction for multiple testing considering the control (honey bees fed with sugar syrup only).

MALDI BeeTyping[®] machine learning model

MALDI-MS data were post-processed and analysed using ClinProTools™v2.2 Software (Bruker Daltonics, Germany). Spectral smoothing and baseline subtraction were applied, followed by total averaged spectra calculation based on area calculations with a signal-to-noise ratio (S/N) of five for peak picking. Spectra that did not meet the required intensity and signal resolution thresholds were excluded. A post-processing step (spectral normalisation of all calculated peak area) was performed using ClinProTools™v2.2 Software before generating the principal component analysis (PCA). All recorded molecular ions were manually selected and clustered for model generation and statistical interpretation. Known molecular ions corresponding to honey bee immune peptides were isolated and excluded from the model clustering. Manually corrected data were used to generate a model able to discriminate honey bees fed with or without pollen. ClinProTools™ v2.2 Software Genetic Algorithm (GA) was used for data clustering by determining a fixed number of molecular ions within training sets to optimise spectra separation.

Statistical analysis

Statistical analyses were conducted using R 4.3.3 (R foundation for Statistical Computing; Vienna, Austria), with results reported as median, mean \pm SD (standard deviation) and significance set at p -value < 0.05 . Data distribution and homoscedasticity of haemolymph proteins analysed with both proteomic techniques (SDS-PAGE and MALDI-MS) and the microbiome composition were assessed using the Shapiro-Wilk⁵⁵ test and the Levene test⁵⁶.

The three main datasets obtained (SDS-PAGE, MALDI BeeTyping[®] and Microbiome) were firstly analysed separately with the most appropriate statistical methods. Honey bees One-way ANOVA with Tukey HSD post-hoc testing^{57,58} was applied for normally distributed data with homoscedasticity relying on package `car` (v3.1-3), `agricole` (v1.3-7), `lme4` (v1.1-35.2), `Rmisc` (v1.5.1), `stats4` (v4.3.3), `PairedData` (v1.1.1) and `foreign` (v0.8-86). In contrast, for data with non-normal distribution Kruskal–Wallis test was used to compare peak intensity, with pairwise comparisons conducted using the Dunnett post-hoc test from the `dunn.test` (v1.3.5) package for Dunn's multiple comparisons⁵⁹.

Correlations among total haemolymph proteins vs. total pollen proteins, and MALDI BeeTyping[®] detected AMPs intensity vs. microbiome, were carried out using Spearman's correlation analyses based on `ggpubr` (v0.6.0), `devtools` (v4.3.3), `dplyr` (v1.1.4), `Hmisc` (v5.1-2) and `rstatix` (v0.7.2) packages.

Where necessary, the p -value was corrected with Bonferroni correction, accounting for 32 comparisons for analysis on pollen proteins and lipids, while 45 comparisons were considered in tests where also CTR and pollen mixtures were investigated (CTR vs. pollens of all the botanical families vs. pollen mixture).

Linear Mixed-Effects Model (`lmer`) was performed on all the datasets obtained using the R packages `lme4` (v1.1-35.2), `ggplot2` (v3.5.1), `lmerTest` (3.1-3), `dplyr` (v1.1.4), and `tidyr` (v1.3.1). The following `lmer` models were applied:

- Model without interaction (Botanical_Family + (1 | Colony_Source)). This model considers only the main effects of botanical family and treats the different honey bee source colonies as a random effect. The random effect is additive, as the source colony is included as an independent random effect (added) to the model structure.
- Model with random interaction (Botanical_Family + (1 + Botanical_Family | Colony_Source)). This model allows testing whether the effect of botanical family varies randomly among different bee colonies. This model structure is again additive and does not include interactions between fixed and random effects, but rather modulates the effect of the fixed variable at the level of the random effect.
- Model with full interaction (Botanical_Family * Colony_Source + (1 | Colony_Source)). The model considers the interaction between botanical family and the different honey bee source colonies as part of the fixed effects while also including a random effect associated with source colony. With this model the effect of botanical family changes depending on honey bee source colony as a fixed effect, and not as part of the random structure, where the effect of botanical family on honeybees is strictly linked to the source colony.

ANOVA was used to compare each model (Botanical_Family + (1 | Colony_Source) and Botanical_Family * Colony_Source + (1 | Colony_Source)). The “c” model was chosen as the most appropriate because it was reliable and robust considering the output of calculated residual variance, Log-likelihood and ANOVA significance (see Supplementary Materials file). However, results for the models “a” and “b” were also reported (see supplementary materials file for models “a” and “b”).

In the `lmer` model the response variables were identified in the microbiome core taxa, SDS-PAGE large size proteins, MALDI BeeTyping[®] data on small size proteins. Moreover, in a further analysis focalized only on the impact of the botanical families, the CTR was used as a reference condition within the model, and only in this case the p -value was Bonferroni corrected considering 9 comparisons (eight botanical families and the control).

The R packages *stat* (v4.3.3), *dplyr* (v1.1.4), and *ggplot2* (v3.5.1) were used for the correlation matrix, involving microbiome core taxa, SDS-PAGE large size proteins, MALDI BeeTyping[®] data on small size proteins. Microbiome data were heteroscedastic and therefore not normal, while SDS-PAGE and MALDI data were normal and homoscedastic, therefore Spearman correlation was relied on to generate the correlation matrix. The *p*-values of the correlations were extrapolated with the *ppcor* function (*ppcor* v.1.1), with a cut-off of 0.01.

Results and discussion

Understanding how trophic resources affect honey bee health remains a significant knowledge gap, limiting the development of strategies to protect bees from climate change. Analysing and correlating gut microbiome composition with immune-related proteins are key aspects that may contribute to filling this gap.

Pollen identification and protein and lipid contents

In total, 36 monofloral and 13 polyfloral (i.e. composed of more than one species) pollens were analysed in this study. Pollen identification details are presented in Table 1 (see Tables S1 and S2 for full information) while the taxonomy of the pollens used has been fully reported in Braglia et al.⁴⁷. Briefly, nineteen botanical genera across 8 botanical families were identified: Cistaceae (*Cistus* sp. and *Helianthemum* sp.), Asteraceae (*Centaurea* sp., compositae T and S), Cornaceae (*Cornus* sp.), Boraginaceae (*Borago* sp. and *Echium* sp.), Fagaceae (*Quercus* sp. and *Castanea* sp.), Leguminosae (*Hedysarum* sp., *Genista* sp., and *Trifolium* sp.), Rosaceae (*Rubus* sp., *Crataegus* sp., *Malus* sp., *Pyrus* sp., and *Sorbus* sp.) and Salicaceae (*Salix* sp.). Protein content across pollens showed no significant differences (Fig. S1), aligning with the reported average pollen protein content range 10–40%⁶⁰. Lipid content varied, however not significantly, by botanical species (Fig. S2), consistent with prior findings for Asteraceae and Rosaceae⁶¹, Cistaceae^{61–63}, Boraginaceae^{62,64}, Salicaceae, and Leguminosae^{61,62}.

Influence of geographical location on pollen protein and lipid content

Pollen and lipid content varied significantly within pollen of the same genus collected in different regions. For example, *Quercus* pollen protein content ranged from 17.15 mg/g in Tuscany to 65.52 mg/g in Campania ($p < 0.01$) (Fig. S3A), while lipid content remained consistent, with values from 0.21 mg/g in Campania to 0.22, 0.24, 0.23 mg/g in Sardinia, Tuscany and Sicily, respectively ($p < 0.05$). Similarly, *Rubus* pollen protein level ranged from 1.19 mg/g in Trentino Alto Adige to 45.10 mg/g in Sardinia ($p < 0.05$) (Figure S3B), with significant lipid differences among regions as well ($p < 0.05$). Differences in pollen protein content within the same botanical families or given by the geographical origin have been previously reported^{65,66}.

Effects of different pollens on haemolymph protein concentrations

Total protein concentration in haemolymph is a valuable indicator of honey bee nutritional status. Our results (Table S4) showed no significant differences in total haemolymph proteins with different pollen diets. Cistaceae pollen yielded the highest concentration (13.77 ± 6.40 mg/mL) while Fagaceae pollen resulted in the lowest (9.93 ± 3.19 mg/mL). The results are reported in Fig. S4.

Control bees fed only sugar syrup had a mean haemolymph protein concentration of 15.07 ± 9.87 mg/mL, lower than values reported by Isani et al.⁴². The lower protein concentration might be attributed to the younger age of the bees, as haemolymph protein levels vary with colony role (e.g., nurses or foragers)³⁵. Dietary variation and social interactions can further influence protein accumulation.

The haemolymph protein profiles from SDS-PAGE of control samples matched typical profiles for summer bees, consistent with findings from Chan et al.⁶⁷, Cabbri et al.³⁴, and Isani et al.⁴². As an example, a gel is shown in Fig. S5.

The concentration of Apolipoprotein-I and II, Vitellogenin, Transferrin and Hexamerin 70a in the haemolymph varied by plant family, though differences were not statistically significant. Overall, bees fed Cistaceae pollen had the highest concentrations of Apolipoprotein-I, Vitellogenin, Transferrin, and Hexamerin 70a, while those fed Salicaceae pollen had the lowest. Notably, Vitellogenin concentration was 8.7 times higher in bees fed with Cistaceae pollen compared to Salicaceae pollen. Cistaceae and Asteraceae pollens supported higher protein concentrations. However, due to high within family variability of these proteins, these differences were not statistically significant. Di Pasquale et al.¹³ found that Vitellogenin levels in bees fed with *Rubus* pollen did not significantly differ from those fed with *Erica* pollen, despite differences in total proteins.

Finally, our analysis showed that there was no significant correlation between the concentration of total proteins, Vitellogenin, Transferrin, and Hexamerin in haemolymph and the protein content in pollen, except for pollen of the Cistaceae family; in this case we found a significant positive correlation between pollen protein content and Apolipoprotein-I (Pearson correlation 0.950, $p = 0.05$), Transferrin (Pearson correlation 0.954, $p = 0.05$), and Hexamerin 70a (Pearson correlation 0.956, $p = 0.04$).

MALDI BeeTyping mass spectrometry analysis output

MALDI-MS recorded 71,000 molecular ions across the 186 samples, within a mass range of 600–18,000 Da. Key molecular ions associated with honey bee antimicrobial peptides (AMPs) were manually isolated and clustered for further analysis, specifically, AMCI-1 (m/z 5962), AMCI-2 (m/z 5981), Apidaecin (m/z 2110), Abaecin (m/z 3878), Defensin-1 (m/z 5518), and Hymenoptaecin (m/z 10,286). Similarly, peaks-of-interest (POI) generated by the Genetic Algorithm (GA) model were isolated, as described below. A comprehensive AMP identification and variance analysis is available as Supplementary Materials (Appendix 1).

Effect of pollen botanical families on AMPs intensity

MALDI-MS results indicated that honey bee haemolymph contained AMCI protease inhibitors and AMPs, with intensity varying by pollen family (Fig. S6).

AMCI protease inhibitors, involved in phagocytic pathways that deactivate proteins involved in the degradation of foreign organisms, dead tissues, or antigen-antibody complexes linked to inflammatory responses⁴⁴, showed a significant reduction when honey bees consumed Salicaceae pollen ($p < 0.05$ for both AMCI-1 and -2) and Cornaceae pollen ($p < 0.05$ for AMCI-1 only) compared to the control (NEG-CTR, Figure S6A and S6B). This reduction may indicate potential effects of these pollens on the regulation of the activity of this protease inhibitor, as AMCI deregulation has been linked to pathogen infections like *Nosema ceranae*, *Serratia marcescens*, *Micrococcus luteus*, and *Pectobacterium* sp., and stress conditions in honey bees and bumblebees^{66–69}.

As for AMPs, Apidaecin peptides are effective against a wide range of plant-associated Gram-negative bacteria in adult honey bees⁶⁹, while Abaecin is active against Gram-positive bacteria in both larvae and adult honey bees⁷⁰. Increased Apidaecin and Abaecin levels typically reflect an active immune response against pathogens^{71–73}. Although pollen diet has been shown to influence AMP gene expression¹⁷, our results revealed only a modest Apidaecin increase in honey bees fed pollen mixtures ($p < 0.1$, Fig. S6C), and a decrease in Abaecin after Salicaceae pollen consumption ($p < 0.05$, Fig. S6D), when compared to the NEG-CTR.

Defensin-1 (m/z 5,518), an antimicrobial peptide found in honey bee haemolymph, showed no significant variation across pollen diets (Fig. S6E). This peptide differs from its isoform, Defensin-1 (Royalisin, m/z 5,526), which is detectable only in royal jelly⁴⁵. Defensin-2, an antimicrobial peptide up-regulated in response to bacterial lipopolysaccharides⁷⁴, was absent in our MALDI-MS profiles, in agreement with the findings of Klaudiny et al.⁷⁵. Similarly, Hymenoptaecin, which inhibits both Gram-negative and Gram-positive bacterial growth, was not detected in any haemolymph sample. This absence is noteworthy, as Hymenoptaecin was previously observed by Arafah et al.⁴⁵ following a bacterial challenge test, making this result unique to our study conditions.

The genetic algorithm model to differentiate feeding conditions

This study also aimed to assess whether MALDI-MS profiles could differentiate between honey bees fed with or without pollen. A Genetic Algorithm (GA) model was developed using spectra from control bees fed glucose syrup (Class 1) and bees fed pollen mixtures (Class 2).

After the manual selection of 49 molecular ions, the GA model was able to detect 10 potential markers for pollen consumption, achieving a 64.83% cross-validation accuracy and a 84.72% cross-capability. Specificity for Class 1 was 77.77%, while for Class 2 it reached 91.67%. The mean mass range and intensity of each POI are detailed in Table S5.

Statistical analysis of the detected POIs revealed significant differences in the intensities of three molecular ions (Figure S7). Molecular mass analysis identified average molecular ions 47 at m/z 2132, 43 at m/z 8190, and 45 at m/z 13,515 as unknown peptides of interest. The ion at m/z 8190 was associated with a diet without pollen ($p < 0.05$, Figure S7B), while ions at average m/z 2132 and 13,515 were linked to pollen consumption ($p < 0.05$ and $p < 0.1$, respectively, Figure S7A and S7C). Further validation, detailed in Appendix 2 of the supplementary materials, involved the analysis of pollen mixtures from five different Italian regions (Campania, Sicily, Sardinia, Tuscany and Trentino).

Impact of pollen diet on core gut microbiota

Recent studies indicate that different pollen diets may not significantly affect core microbial diversity in some pollinators (*Megachile rotundata* and *Bombus impatiens*)⁷⁶. However, Castelli et al.²¹ observed increased *Bartonella* and *Parasaccaribacter* in honey bees fed *Eucalyptus grandis* monofloral pollen, suggesting that *Apis mellifera* may be more responsive to dietary changes. Moreover, Ricigliano and Anderson⁷⁷ also reported elevated absolute abundance of core microorganisms when honey bees were fed with pollen supplementations.

Our study confirms that monofloral pollen sources can influence the abundance of the honey bee core gut microbiota (Fig. 1 and Fig. S8). Asteraceae pollen, for example, significantly reduced *Bartonella* and *Snodgrassella* when compared to the controls ($p < 0.05$, Fig. 1B and G). Boraginaceae pollen significantly decreased *Bartonella* and *Bifidobacterium* (from 5.17 ± 2.09 in control to 3.30 ± 3.09 with Boraginaceae, $p < 0.05$, Fig. 1A and B), while significantly increasing *Lactobacillus* and total bacterial counts (eubacteria) (Fig. 1F and H). Notably, Cistaceae pollen significantly raised *Bombilactobacillus* (from 2.88 ± 1.45 in control to 4.17 ± 1.48 with Cistaceae, $p < 0.05$, Figure S8C) and *Lactobacillus* ($p < 0.05$, Fig. 1F).

Similarly, Cornaceae pollen significantly decreased *Snodgrassella* and *Gilliamella* ($p < 0.05$ both, Fig. 1E and G), consistent with previous observations of diet-dependent reduction of *Snodgrassella* and *Gilliamella* reported by Ludvigsen et al.⁷⁸. Fagaceae pollen lowered the absolute abundance of *Bifidobacterium* and *Frischella* ($p < 0.05$, Fig. 1A and D) but significantly increased total bacterial counts (from 8.37 ± 1.85 in controls to 8.75 ± 1.63 with Fagaceae, $p < 0.05$, Fig. 1H). Leguminosae pollen increased nearly all core microbial taxa: *Bartonella*, *Bifidobacterium*, *Bombilactobacillus* ($p < 0.05$, Fig. 1A–C) and *Lactobacillus* ($p < 0.01$, Fig. 1F) without affecting total bacterial counts, suggesting resilience of core microbiota to the growth of environmental opportunistic taxa (Fig. 1H).

Rosaceae pollen reduced the total *Bifidobacterium* levels ($p < 0.05$, Fig. 1A) without significantly altering the other taxa of core microbiota, while Salicaceae pollen significantly decreased the absolute abundance of all core microorganisms (compared to the control), except *Snodgrassella* (Fig. 1G). *Bartonella*, *Bifidobacterium* and *Bombilactobacillus* (Fig. 1A–C) were the most affected core organisms, with *Bartonella* abundance declining from $\text{Log } 5.72 \pm 1.83$ in controls to 3.54 ± 1.03 ($p < 0.05$, Fig. 1B), when honey bees were fed with Salicaceae pollen, and *Bifidobacterium* from $\text{Log } 5.17 \pm 2.09$ in controls to $\text{Log } 1.92 \pm 2.07$ ($p < 0.05$, Fig. 1A). Similarly, *Bombilactobacillus* levels decreased from 2.88 ± 1.45 to 1.66 ± 1.83 (Fig. 1C). However, the total bacterial counts in honey bees fed Salicaceae pollen were similar to the controls, possibly indicating opportunistic environmental bacterial proliferation (Fig. 1H), which tend to over proliferate under stress conditions^{79,80}.

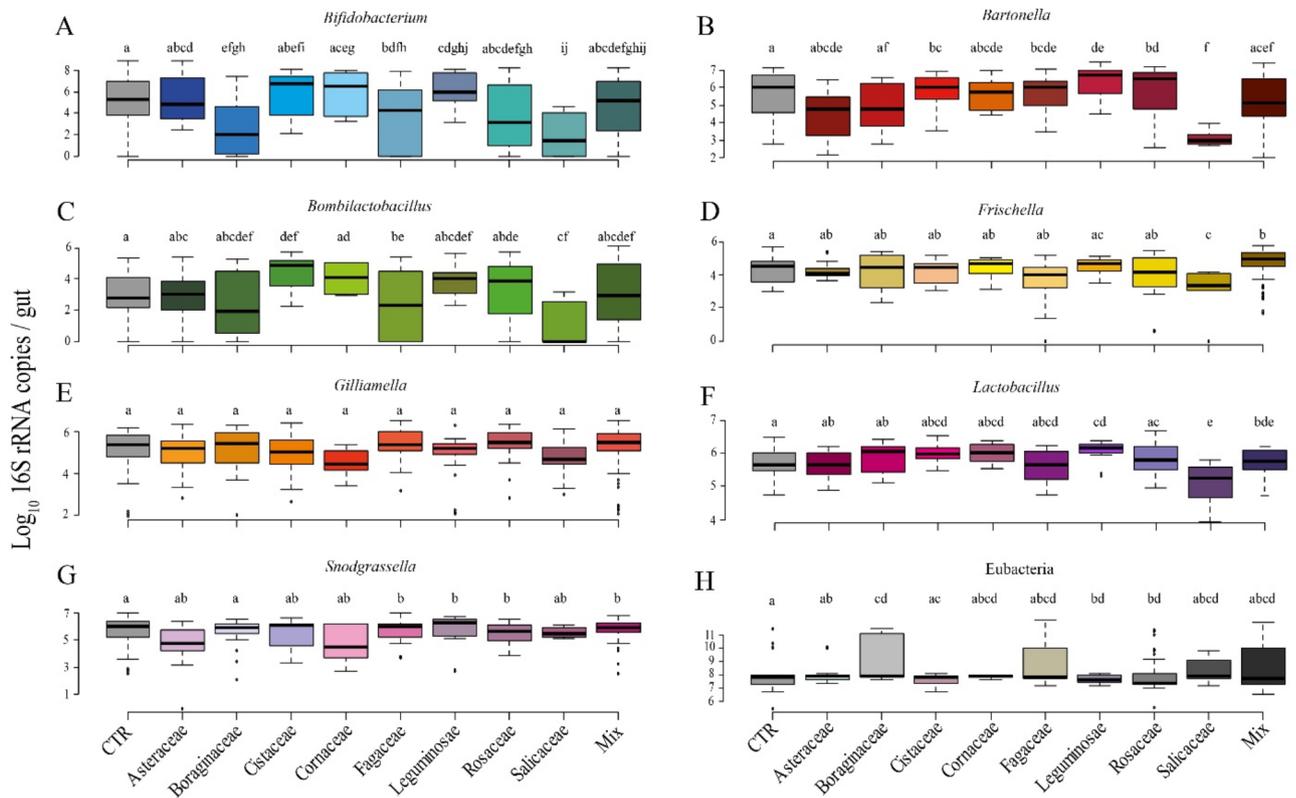


Fig. 1. Absolute abundance of core gut microbial taxa assessed with qPCR. The boxplots show the absolute abundance expressed in Log₁₀ of gut microorganisms in honey bees feed with pollen of different botanical families. The qPCR analysis focalized on (A) *Bifidobacterium*; (B) *Bartonella*; (C) *Bombilactobacillus*; (D) *Frischella*; (E) *Gilliamella*; (F) *Lactobacillus*; (G) *Snodgrassella* and (H) *Eubacteria*.

Finally, pollen mixtures had minimal impact on core microorganisms, with the exception in *Frischella*, which slightly increased ($p < 0.05$, Fig. 1D). Full absolute abundances and standard deviations for core microbiota with different pollen supplements are presented in Table S6.

Our results showed that total bacteria absolute abundance did not vary among the honey bees fed with the different pollen botanical families. This pattern suggests that environmental microorganisms associated with pollen may play a role, as suggested by^{81,82} for the bacterial genera *Morganella*, *Citrobacter*, *Pseudomonas* and *Pantoea*.

The interplay between trophic sources, immune response and microbiome

The lack of an analytical technique that can simultaneously detect all immune-related proteins (both low and high molecular weight) remains a constraint in analysing factors influencing honey bee health. Developing indicators that correlate immune-related proteins, core microbiome composition, and pollen protein content could lead to rapid, easy-to-use tools for assessing honey bee health. In this study the lmer analysis was attempted such an omic-approach, with significant results highlighted in the complex interplay between feed sources, microbiome and immune response. Therefore we proceeded with pairwise correlations to investigate potential relationships among these different factors.

The pollen botanical family and the source colony affect the gut microbiome composition and the pollinator immune response

The Linear Mixed-Effects Model (lmer) revealed an influence of the botanical family of the supplied pollen on the gut microbiome composition (Table 2). Almost all the core microbial taxa were significantly affected by the different diets except for *Gilliamella* and the total bacteria populating the gut. This result is consistent with previous works^{83,84} describing this genus as associated with pollen digestion. Our results show that the botanical origin of pollen does not directly influence the development of *Gilliamella*.

Interestingly, the source colony did not significantly influence the gut microbiome composition alone; although few significant contributions were highlighted when considering the interaction between the botanical family and the source colony. Microbial diversity within the honey bee gut microbiome differs among honey bees populations according to geographic origin⁸⁴. Recently, a first approach of the effect of the environment on the honey bee gut microbiome has been carried out by Gaggia et al.⁸⁵, showing a relevant difference in the microbiome of two different honey bee subspecies collected from different environments. However, in the same work it was not possible to address the specific contribution of environmental trophic potential to

	All treatment vs. all treatment			CTR vs. treatment
	Botanical family	Source colony	Botanical family × source colony	Botanical family*
<i>Bifidobacterium</i>	1.15E-05	0.528	0.005	1.73E-04
<i>Bombilactobacillus</i>	0.019	0.278	0.253	0.052
<i>Lactobacillus</i>	1.97E-07	0.951	0.0005	1.65E-04
<i>Frischella</i>	0.003	0.888	0.032	6.23E-04
<i>Bartonella</i>	0.0001	0.895	0.062	1.62E-06
<i>Gilliamella</i>	0.700	0.293	0.859	1.000
<i>Snodgrassella</i>	0.012	0.172	0.644	0.078
Eubacteria	0.093	0.731	0.025	0.061
Apolipoporphin-I	0.044	0.049	0.003	0.106
Vitellogenin	0.948	0.003	0.0002	0.014
Apolipoporphin-II	0.0006	0.035	0.001	0.041
Transferrin	0.113	0.001	0.009	0.611
Hexamerin	0.043	0.017	1.43E-05	1.000
Total protein hemolymph	0.083	0.018	0.742	0.008
Apidaecin	0.0001	0.0007	0.0004	0.172
Abaecin	0.025	0.001	0.003	1.000
Defensin	0.217	0.01	0.023	1.000
AMCI-1	0.191	0.277	0.253	1.000
AMCI-2	0.232	0.012	0.002	0.405

Table 2. Linear mixed-effects model results (p -value) of the influence and the interaction of botanical family and source colony on core gut Microbiome composition and immune system response. Significant values are in bold. The test on the reliability of the Linear Mixed-Effects Model, including data dispersion, ANOVA, Residual Variance, and Log-Likelihood are reported in Figs. S9 and S10 and Tables S7, S8 and S9. *Bonferroni correction applied.

the different gut microbiomes. Our results suggest that different botanical species of pollen can contribute to the modifications of specific core taxa abundance, providing specific nutrients or microbial growth inhibitors targeting specific taxa. Moreover, pollen might be a source of novel taxa or may provide antagonist taxa that suppresses specific honey bee core microbes, acting as biotic filter.

On the other hand, the source colony strongly influenced the immune system response, being enhanced by the diet. The source colonies showed a significant influence on the synthesis of immune-related peptides. It is also interesting to note how the synergy between colony source and diet, represented by the botanical family of pollen, contributed even more to the modulation of the immune system of the bee.

The results obtained in models “a” (baseline model without interaction) and “b” (model with random interaction) also confirm a significant impact of the pollen botanical families on the core gut microbiome (Table S10 and S11). Conversely, model “b” did not show a relevant effect of the pollen botanical families on the honeybee immune system if compared to model “a” and “c” (model with full interaction).

Haemolymph total protein concentration is correlated to the pollen protein content

Correlation matrix analysis (Fig. 2) showed a significant negative correlation between the total protein concentration in haemolymph and the protein content in pollen (Spearman correlation -0.33 , $p < 0.1$, Fig. 2) whether no significative correlations were highlighted across all botanical families (Spearman correlation -0.271 , $p = 0.1$) or within individual families. In our experimental conditions, Vitellogenin levels also did not correlate with the content of total proteins in pollen consistent with Di Pasquale et al.¹³, who observed no change in Vitellogenin levels when honey bees were fed with pollens of varying protein content. This trend was also observed for Apolipoporphin-I, Apolipoporphin-II, and Transferrin, with the exception of Hexamerin that showed a positive correlation (Spearman correlation 0.29 , $p < 0.1$, Fig. 2). However, considering the different pollen botanical families, in the Cistaceae family, we found a significant positive correlation between pollen protein content and Apolipoporphin-I (Pearson correlation 0.950 , $p = 0.05$), Transferrin (Pearson correlation 0.954 , $p = 0.05$), and Hexamerin 70a (Pearson correlation 0.956 , $p = 0.04$).

Correlations in core microbial taxa and immune system response

Correlation matrix highlighted few significant correlations between the large-size proteins detected in SDS-PAGE; however, it is worth to noting a positive correlation between Apolipoporphin-I and Apolipoporphin-II (Spearman correlation 0.73 , $p < 0.01$; Fig. 2) Considering the antimicrobial peptides (AMPs), a significant correlation was detected between Apidaecin and Abaecin (Spearman correlation 0.87 , $p < 0.01$, Fig. 2), and Vitellogenin and Transferrin (Spearman correlation 0.39 , $p < 0.01$, Fig. 2). Considering the microbiome composition, correlation matrix revealed both positive and negative correlations. Through the different taxa investigated, *Bifidobacterium* showed a positive correlation with *Bombilactobacillus* and *Lactobacillus* (Spearman correlation 0.76 and 0.60 , respectively, $p < 0.01$, Fig. 2). *Bombilactobacillus* highlighted a positive correlation with *Lactobacillus*, *Frischella*

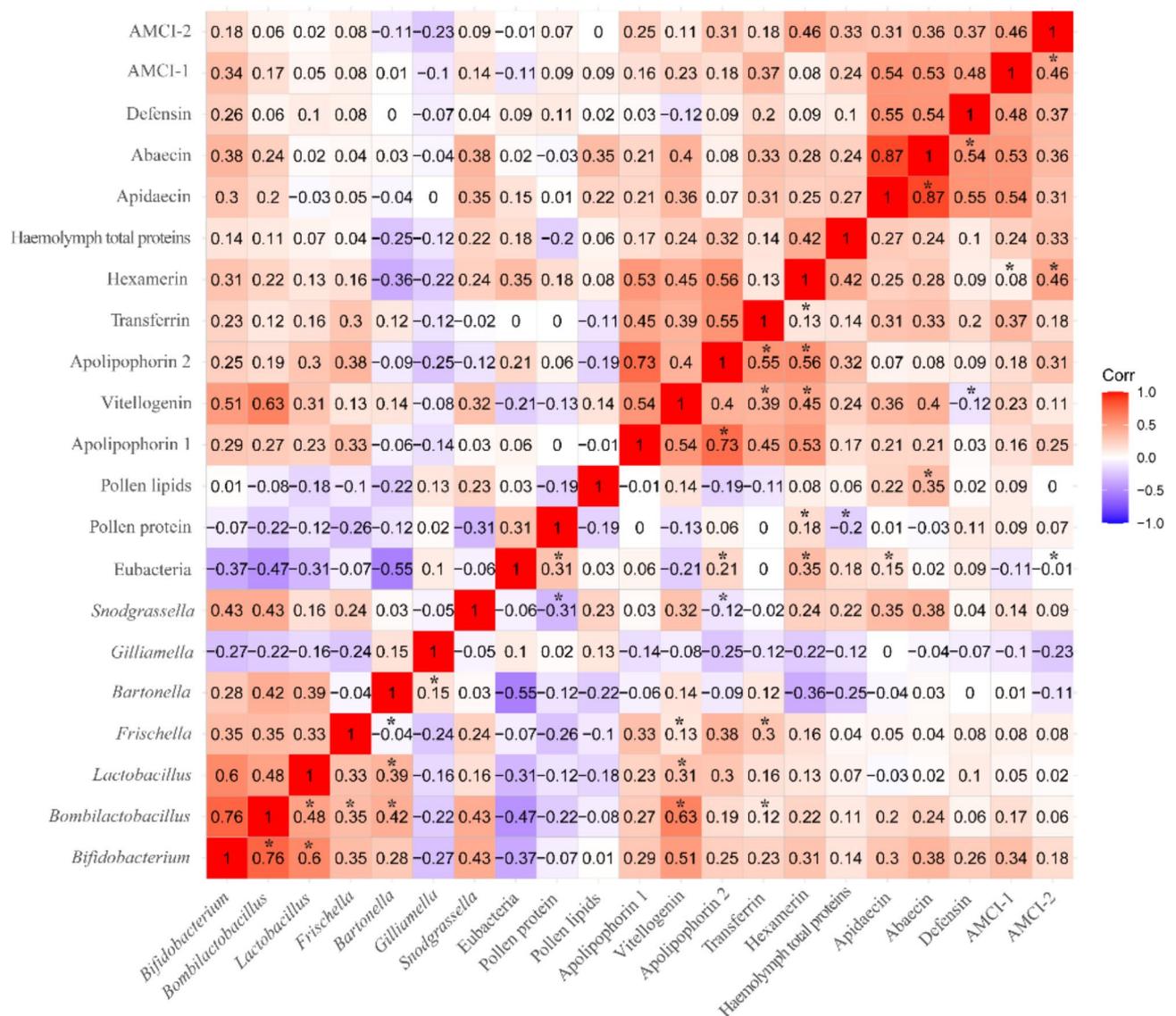


Fig. 2. Correlation matrix. This heatmap illustrates the correlation matrix derived from the correlation analysis between two sets of variables: immune system peptides from honey bees, the core microbiota components, the feed nutritional properties, and the total proteins in the honey bee haemolymph. Each cell in the matrix represents the strength and direction of the correlation between the variables. The colour gradient ranges from blue (negative correlations) to red (positive correlations). Asterisk (*) highlight significant data ($p < 0.01$).

and *Bartonella* (Spearman correlation 0.48, 0.35 and 0.42, respectively, $p < 0.01$, Fig. 2). Moreover, a positive correlation of *Bartonella* with *Lactobacillus* (Spearman correlation 0.39, $p < 0.01$, Fig. 2) were detected. Finally, a positive correlation between *Gilliamella* and *Bartonella* was highlighted (Spearman correlation 0.15, $p < 0.01$, Fig. 2). Considering the whole dataset, interesting data were highlighted, especially when microbiome taxa were correlated to the immune system proteins. Vitellogenin resulted significantly correlated with the absolute abundance of *Bombilactobacillus* and *Lactobacillus* (Spearman correlation 0.63 and 0.31, respectively, $p < 0.01$, Fig. 2). No significant correlations were highlighted when microbial taxa were compared with AMPs, with the sole exception of total bacteria and Apidaecin (Spearman correlation 0.15, $p < 0.01$, Fig. 2).

Conclusion

This study addresses the impact of different pollen sources on honey bee health by analysing haemolymph proteins and core microbial taxa as potential biomarkers. Our results indicate that the pollen type does not significantly affect the total amount of immune-related proteins in the haemolymph. This suggests a resilience in protein synthesis despite dietary variations. However, pollen type leads to significant changes in the gut microbiome composition, with core taxa significantly altered according to the pollen diet which, in turn, impacts the peptides of the immune system. Our results, also suggest an indirect dietary effect on the immune system response, via the gut microbiome modulation. In addition, our results indicate that the colony origin has a significant impact

on the immune response of honey bees. This study comprehensively examined the three-way interaction among pollens of different botanical origin, the gut microbiome and the honey bee immune system response, expanding the important role of the gut bacterial symbionts in honey bee health. These results may also provide a new insight in understanding the complex adaptation of honey bees to compromised trophic resource conditions due to climate change, indicating the gut microbiome and some immune peptides (Abaecin, Apidaecin and Vitellogenin) as the main biomarkers. Moreover, this work, while expanding the knowledge of the interaction between honey bees and environmental trophic sources, may help in the application of mitigation measures against climate change, urbanization and intensive agriculture, such as the improvement of plant biodiversity strategies able to support pollinators or microbial resource management strategies at the colony level.

Data availability

Data on MALDI mass spectrometry and qPCR are available on AMS-Acta repository (repository of the University of Bologna) at the following DOI:<https://doi.org/10.6092/unibo/amsacta/7905>.

Code availability

ImageLab 5.2.1 Software, Bio-Rad. Free License. URL link: <https://www.bio-rad.com/it-it/product/image-lab-5.2.1-software?ID=KRE6P5E8Z> (last accessed 10/02/2025); ClinProTools™ v2.2 Software, Bruker Daltonics. License number: 496203CC00BL1R3, URL link: <https://www.bruker.com/en/landingpages/bdal.html> (last accessed 10/02/2025); FlexAnalysis™ v3.4 Software, Bruker Daltonics. License number: 2698364AC04DW9C7, URL link: <http://www.bruker.com> (last accessed 10/02/2025).

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Author contributions

D.A., C.B. and A.G., conceptualisation. G.I., D.A., P.B. methodology. C.B., A.T., C.R. and M.B. formal analysis, investigation and validation. C.B. and C.R., data curation. A.G., D.A., G.I., D.D.G., P.B. funding acquisition. D.A. supervision. C.B., D.A., C.R. visualisation. C.B., D.A., and C.R. writing—original draft. D.D.G., G.I., A.G. and P.B. writing—review and editing.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

Plant ethics The collected plant samples (pollen grains) originated mainly from wild plants. Piana Ricerca S.r.l. provided taxonomical identification of pollens as an outsourced service, and by the authors Daniele Alberoni and Chiara Braglia. The collected pollens were not deposited in a publicly available herbarium. The authors confirm that no plant samples (pollens) were collected from protected areas or endangered species to our knowledge. **Animal ethics** Ethical review and approval were waived for this study, because the Italian law does

not require an ethical approval for tests performed on arthropods with exceptions of cephalopods according to the Italian D.L. 4 March 2014 n. 26, and Italian implementing decree following the European regulation 2010/63/UE.

Additional information

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