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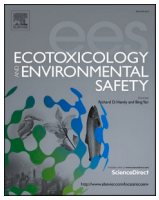

Beyond pesticides: Evaluating the role of botanical origin and nutritional composition in shaping honey bee stress responses

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ABSTRACT

The western honey bee (*Apis mellifera* L.) is increasingly affected by chronic dietary exposure to pesticide-contaminated pollen. This study investigates the long-term effects of *Malus domestica*, *Phacelia tanacetifolia*, and *Taraxacum* sp. pollen collected from orchard and alpine habitats alongside a commercial feed additive (Promotor-L Apis) on honey bee survival, physiology, and gut microbiota. Multiresidue analysis revealed distinct pesticide and heavy metal profiles across pollens, while compositional analyses showed variation in amino acids, flavonoids, and phenolamides. Despite high contamination, *Malus* pollen with elevated flavonoid content promoted the highest vitellogenin accumulation and did not totally inhibit survival. *Phacelia* pollen from organic vineyards, though low in pesticides, had high copper levels and showed high mortality. *Taraxacum* pollen from apple orchards, though moderately contaminated, supported high survival. Unexpectedly, alpine *Taraxacum* pollen with elevated histidine content caused the highest mortality and microbial disruption, despite no pesticide residues. Promotor-L improved survival but did not increase vitellogenin. Pollen-fed bees generally exhibited higher gut microbiota abundance, while pathogen levels (including *Nosema ceranae* and *Serratia marcescens*) were specifically elevated under *Taraxacum*-based diets. These findings highlight that the impact of pollen nutrition on bee health is multifactorial, governed not only by pesticide exposure but also by botanical origin, nutritional traits, and secondary metabolites.

1. Introduction

Along with many wild bee species, the western honey bee, *Apis mellifera* L., serves as one of the most important pollinators for both cultivated crops and wild plants, contributing to both food security and plant biodiversity (Klein et al., 2018). Pesticides are among the multiple stressors that honey bees regularly encounter in agroecosystems (Sgolastra et al., 2019). Exposure can occur through direct spray drift and residues in pollen and nectar, but also via contaminated water, soil dust, and airborne particles within agricultural landscapes (Gradish et al., 2019). Among commonly used insecticides, neonicotinoids such as acetamiprid remain widely used in agricultural systems and are considered environmentally relevant for pollinator exposure (EFSA et al., 2022). Pollen is an essential protein source for honey bees, and it also provides carbohydrates, lipids, vitamins, and minerals needed for their growth and development (Roulston and Cane, 2000). Beyond

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nutrition, pollen plays a crucial role in supporting the honey bee microbiome development and immune function (Braglia et al., 2025), while also enhancing resilience against pesticide exposure (Castle et al., 2023). A systematic literature review by Zioga et al. (2020) examining studies from the past five decades (1968–2018) identified pollen as the most analysed matrix for plant protection products (PPPs), with residues of most assessed compounds being detected in pollen samples. In addition to pesticides, pollen can act as a carrier of trace metals and metalloids such as copper, cadmium, and lead, originating from agricultural practices, atmospheric deposition, and contaminated soils, which can accumulate in floral resources and be transferred to pollinators through foraging, potentially contributing to sublethal physiological stress (Gekière et al., 2023). Therefore, the safety of pollen directly impacts honey bee health, as it represents not only a key nutritional resource but also a major route of exposure to xenobiotics, making it essential to understand how chemically contaminated pollen affects honey bee health and survival.

Pollen nutrition produces high protein levels in the hemolymph and often determines the levels of storage proteins such as vitellogenin (Di Pasquale et al., 2013). Vitellogenin is a phosphoglycoprotein synthesized by the fat body, which plays a crucial role in honey bee physiology by regulating aging through its antioxidant potential and being involved in immune functions (Alaux et al., 2010; Sagona et al., 2023). For decades, it has been known that its synthesis depends on the honey bee nutritional status and especially on the pollen quality (Bitondi and Simoes, 1996). Changes in vitellogenin levels have been shown to respond sensitively to diet quality and chronic sublethal stress, making it a suitable endpoint for evaluating physiological trade-offs under long-term dietary exposure scenarios (Christen et al., 2016). In this context, quantifying vitellogenin provides an integrative measure of how pollen nutritional quality and chemical contamination jointly influence honey bee physiological condition under chronic dietary exposure. Moreover, honey bees possess a specialized gut microbiota comprising a cluster of a few dominant bacterial species that contribute significantly to host nutrition, immunity, and overall health (Raymann and Moran, 2018). Diet composition, particularly pollen quality and diversity, strongly influences microbiota structure and function, while exposure to pesticides can disrupt microbial balance and impair host health (Alberoni et al., 2021; Braglia et al., 2025; Rouzé et al., 2019). Assessing microbiota responses therefore provides important insight into the interaction between nutritional and chemical stressors at the host–microbe interface.

Previous studies have demonstrated that pollen diet composition strongly influences honey bee development, physiology, and survival. In particular, pollen from some botanical origins may represent nutritionally suboptimal diets when provided alone. For example, *Taraxacum* pollen has been shown to have low nutritional value and to impair larval and adult development when offered as a monofloral diet (Barraud et al., 2022). Beyond direct nutritional effects, pollen quality can also modulate honey bee sensitivity to pesticides. Experimental evidence shows that nutritionally rich pollen diets can partially buffer pesticide toxicity by enhancing detoxification capacity and physiological resilience, whereas poorer-quality pollen exacerbates pesticide-induced mortality (Barascou et al., 2021). These findings highlight that pollen nutritional traits, botanical origin, and chemical composition interact to shape bee health outcomes and motivate the simultaneous assessment of nutritional parameters, pesticide residues, and other contaminants such as trace metals in pollen.

In our study, we examined the chronic impacts of feeding pollens with varying contamination levels on honey bee survival and physiological responses. Pollen from three plant species, *Phacelia tanacetifolia*, *Malus domestica,* and *Taraxacum* sp., collected by honey bees either from pesticide-treated agroecosystems or from an uncontaminated meadow was used. This design enabled us to analyze the ecological implications of long-term exposure to pesticides from the diet. A commercial feed additive (Promotor L Apis) was also included as a standard dietary control. Survival analysis was complemented by measurements of vitellogenin protein levels as a key physiological indicator, together with assessments of changes in the core gut microbiota. This integrative approach provides a comprehensive view of the sublethal impacts of pesticide-contaminated pollen on honey bee health.

2. Materials and methods

2.1. Pollen collection, separation, and identification

Pollen from three plant species, lacy phacelia (*Phacelia tanacetifolia*), apple (*Malus domestica*), and dandelion (*Taraxacum* sp.), was utilized in this study. Pollen traps were installed in 3 colonies at each site to collect corbicular pollen from forager honey bees. Pollen loads were initially sorted by their color and tentatively identified according to Kirk (1994). To confirm the botanical origin, palynological analysis was performed using light microscopy and morphological comparison with reference images in the PalDat database (Halbritter, 2020) (www.paldat.org). To further assess botanical origin, pollen DNA was extracted and amplified targeting an ~1600 bp region of the *18S rRNA* gene using primers developed in-house (unpublished data). Lacy phacelia pollen was collected from bee colonies placed in organically managed vineyards in the municipality of Trento (TN, Italy; ~194 m a.s.l.), where *Phacelia tanacetifolia* is a non-native species intentionally sown as a green manure crop to improve soil fertility. Whereas apple and dandelion pollens were collected at the same time from bee colonies placed in apple orchards intensively cultivated with IPM methods in the municipality of Caldes (TN, Italy; ~689 m a.s.l.). An additional sample of dandelion pollen was collected from bee colonies placed in meadows in the municipality of Vermiglio (TN, Italy; ~1261 m a.s.l.), which was presumed to be uncontaminated or very little contaminated by pesticides.

2.2. Pesticide residue analysis

After pollen loads separation, the monofloral pollen samples were analyzed by pH Laboratories (TÜV Italia S.r.l., Barberino Tavarnelle, Fl, Italy), an Accredia-accredited laboratory (license no. 0069), operating under the certified method UNI CEI EN ISO/IEC 17025:2018. For each pollen type, residue analysis was conducted on a pooled monofloral sample with the aim of characterizing contamination profiles. The analysis comprised a comprehensive multi-residue screening of pesticides (including insecticides, fungicides, and herbicides) and their relevant metabolites, together with targeted determination of trace metals (Cu, Cd, Pb). Detailed analytical procedures are reported in the Supplementary Materials (Appendix 1, section A).

2.3. Analysis of crude protein content, free amino acids, lipids, phenolic and phenolamides of pollen samples

These analyses were conducted on pooled pollen samples with multiple analytical replicates. The crude protein (CP) content estimation was performed through Kjeldahl nitrogen determination using a copper catalyst in 1 g of each sample (Aylanc et al., 2023) in a Kjeldahl steam distillation unit (Pro-Nitro A, Selecta, Spain) following the AOAC 920.87 method. For the conversion of nitrogen levels to protein, the factor 6.25 was used.

To assess the free amino acid composition, a Vanquish UHPLC system coupled to an Orbitrap™ Exploris 120 mass spectrometer via an H-ESI source was used. Separation was achieved on a bioZen Glycan LC column. The system enabled precise detection of target compounds. Data analysis was performed using Xcalibur® software. Free amino acids were analyzed as a readily bioavailable nutritional fraction, complementing crude protein measurements and allowing assessment of compounds potentially relevant for bee physiology and gut microbiota.

For lipid extraction, 500 mg of pollen was extracted with chloroform: methanol (2:1), and the obtained supernatant was filtered through a

Büchner funnel. Crude lipid content was determined by comparing the weight difference before and after solvent extraction (Folch et al., 1957), which is a widely accepted reference procedure for total lipid extraction in biological samples, including pollen.

The phenolic and phenolamide compounds were extracted using ultrasound-assisted extraction (UAE) according to the method described previously (Aylanc et al., 2022). Pollen was sonicated, and the extracts were then lyophilized and stored at −20°C until further analysis. For chromatographic analysis, 20 mg of each extract was mixed with 2 mL of 80% EtOH and processed in the liquid chromatography (LC) following the protocol described in Larbi et al. (2023).

Detailed protocols for pollen crude protein, free amino acids, and phenolic and phenolamide analysis are reported in the Supplementary materials: Appendix 1, section b.

2.4. Honey bee feeding experimental design

This study relied on a local Italian genotype of honey bees (*Apis mellifera ligustica*) maintained in the Bologna district (Castello di Serravalle) apiary. Brood frames with capped cells from two different colonies, expected to hatch on the same day, were incubated at 32 ± 2°C and 60% RH until honey bee eclosion. Upon hatching, over 1300 one-day-old honey bees were marked with different colored pencils (Uniposca, Mitsubishi) to indicate their colony of origin and then returned to their respective colonies for 2 days to allow the gut microbiome acquisition (Figure S1). On the third day, 25 marked honey bees, consisting of a mix of individuals from both hives, were placed in each plastic cage (12 cm × 8 cm × 6 cm), with a total of 6 cages per experimental condition (Braglia et al., 2021). Four of these cages were used for mortality data collection, and two for hemolymph sampling (total protein and vitellogenin quantification) and gut extraction for analysis of gut microbiome composition. Additionally, four extra cages without honey bees were maintained to measure evaporation losses. A chronic oral toxicity test was conducted according to OECD guideline 245 (OECD, 2017). This guideline recommends the use of dimethoate as a reference substance due to its high and well-documented toxicity to bees. However, in this study, acetamiprid was selected as the positive control, as dimethoate is no longer authorized for use in many EU countries and was not available for testing due to regulatory restrictions. Acetamiprid, a neonicotinoid insecticide with moderate toxicity to bees, was used at a dose sufficient to validate test sensitivity. The commercial formulation EPIK SL (4.67% or 50 g/L a.i., Sipcam) was diluted to a concentration of 67.6 μg a.i./g in a 50% (w/v) sucrose solution, providing a targeted exposure of 0.081 μg a.i./μl based on an estimated consumption rate of 20 μl per bee per day. This corresponds to a daily intake of 1.6 μg a.i. per bee, equivalent to one-fifth of the oral LD_{50} for Apis mellifera (8.85 μg a.s./bee; EFSA et al., 2022). The experiment lasted 31 days, as 100% mortality was reached in positive control by then. A total of eight experimental groups were set up as shown in Table 1.

2.5. Food consumption and mortality data collection

To ensure sufficient supply, 750 mg of pollen was provided freshly every 3 days per replicate of 25 bees (10 mg/bee/day), aligning with daily intake estimates of one nurse bee as 6.5 mg (Rortais et al., 2005) to 9.5 mg of pollen per day (Crailsheim et al., 1992). The pollen samples were provided only for the first ten days of the test, directly as corbicular pollen. It was supplied in 2 mL Eppendorf tubes with tips cut for easy access and was refreshed every three days to prevent desiccation and microbial contamination, as recommended by Williams et al. (2013). The tubes with pollen were weighed before placing them in cages and reweighed after 3 days (4 days for the last period) to calculate the consumption, and were then replaced with new pollen. The total intake of each active ingredient via pollen consumption was calculated based on the cumulative pollen intake per honey bee over the 10-day exposure period (Supplementary materials: Appendix 2). A risk index (RI) to calculate the dietary risk from pollen was performed following the protocol of Sgolastra et al. (2024). The 50% sucrose solution was supplied daily in 2.5 mL plastic syringes, with consumption assessed by weighing the syringes each day. Evaporation losses were estimated using four replicate cages without bees, each containing sucrose-filled syringes under identical experimental conditions, and the mean daily weight loss due to evaporation was subtracted from the measured consumption values. The 31-day experiment included daily mortality assessments at a consistent time. From day 21 onwards, mortality and feed consumption data were recorded only on days 21, 24, 26, 28, and 31.

Table 1
Schematic description of the experimental setup and origin of pollen samples.

Experimental groups	Treatment description	Origin
CTR (negative control)	50% sucrose solution (w/v)	
ACE (positive control)	a.i. Acetamiprid 67.6 μg/g in 50% sucrose solution	
ACE+PL	a.i. Acetamiprid 67.6 μg/g and Promotor L Apis* 20.85 μg/g in 50% sucrose solution	
PL	Promotor L Apis* 20.85 μg/g in 50% sucrose solution	
ML	Pesticide-contaminated apple (pollen of *Malus domestica*)	Apple orchards, Caldes (TN), Italy
PH	Pesticide-contaminated lacy phacelia (pollen of *Phacelia tanacetifolia*)	Vineyards, Trento (TN), Italy
CTAX	Pesticide-contaminated dandelion (pollen of *Taraxacum* sp.)	Apple orchards, Caldes (TN), Italy
UTAX	Uncontaminated dandelion (pollen of *Taraxacum* sp.)	Meadows, Vermiglio (TN), Italy

* Analytical components: crude protein > 13.5%; crude fat 0%; crude fibre 0%; ash < 2%; methionine 0.18%; lysine 0.76%; Ca, Na, P, Mg 0%. Additives per litre: vitamins, provitamins and analogous substances: niacin (3a315) 16.25 g; D-panthenol (3a842) 7.5 g; vitamin B1 (3a820) 1.75 g; vitamin B2 2.5 g; vitamin B6 (3a831) 1.125 g; biotin (3a880) 0.001 g. Amino acids and their salts: DL-methionine (3c301) 0.18%; lysine (3.2.3) 0.76%. Composition: hydrolysed yeast 470 mL/1000 mL; dextrose 0.1 g/1000 mL. Hydrolysed yeast amino acid profile: alanine 7.4 g; arginine 8.6 g; aspartic acid 10.0 g; phenylalanine 5.8 g; cystine 4.6 g; glutamic acid 17.8 g; glycine 10.3 g; histidine 1.5 g; isoleucine 5.6 g; leucine 8.7 g; lysine 7.6 g; methionine 1.8 g; proline 11.8 g; serine 12.0 g; threonine 6.2 g; tyrosine 3.1 g; valine 9.3 g.

2.6. Haemolymph extraction, total protein, and vitellogenin analysis

On day 6 of the test, eight bees, each 9 days old, were sampled from each of the last two replicate cages. This time point was selected to capture early physiological and microbiota responses directly linked to pollen consumption, prior to the cessation of pollen feeding (day 10) and before mortality-related secondary effects could confound physiological measurements. Haemolymph was collected from the dorsal aorta using glass microcapillaries after anaesthetizing the honey bees on ice, as described by Braglia et al. (2025). Briefly, eight honey bees for each experimental condition were sacrificed to extract 3 μL haemolymph from each honey bee. Haemolymph samples were stored separately in dedicated tubes and immediately stored at −80°C until further analysis.

The total protein concentration in haemolymph was determined by the Bradford method, with a calibration curve generated from known concentrations of bovine serum albumin (BSA) (Braglia et al., 2025). The absorbance was measured at 590 nm with a plate reader (Tecan Spark®, Tecan, Männedorf, Switzerland).

Haemolymph proteins were separated using one-dimensional SDS-PAGE according to Braglia et al. (2025) with some modifications. A prominent protein band migrating at approximately 180–200 kDa, corresponding to the reported molecular weight of honey bee vitellogenin (Cabbri et al., 2018), was consistently observed across samples (Figure S2) and was therefore identified as vitellogenin. Band intensity

was quantified for comparative analyses among dietary treatments. This approach was used to assess relative differences in vitellogenin abundance rather than absolute protein quantification. Detailed procedure is reported in Supplementary materials (Appendix 1, section c).

2.7. Gut DNA extraction and qPCR analysis on microbiome

On the same day (day 6), midguts and rectums were dissected from the same bees used for haemolymph extraction, and DNA was subsequently extracted for gut microbiome analysis. DNA was isolated using the PureLink™ Genomic DNA Mini Kit (K182002; Invitrogen, Milan, Italy) following the manufacturer's protocol with minor modifications. Briefly, guts were singularly macerated manually with metal micro pestles, mixed with 0.2 mm glass beads, and homogenized with a rotovortex (50 Hz) for 10 min after lysis buffer addition. DNA quantification was performed using a Qubit Flex Fluorometer (Thermo Fisher Scientific, Milan, Italy) and, finally, stored at −20°C until further analysis.

Honey bee gut core microbial genera and the pathogens *Serratia marcescens* and *Nosema ceranae* were quantified in qPCR (QuantStudio®5 Real-Time PCR System, Applied Biosystems). *Bartonella*, *Bifidobacterium*, *Bombilactobacillus*, *Frischella*, *Gilliamella*, *Lactobacillus*, *Snodgrassella*, and total bacteria were analyzed according to Favaro et al. (2023), while *S. marcescens* and *N. ceranae* according to Braglia et al. (2024) and Braglia et al. (2021), respectively. Specific primers targeting the 16S rRNA gene are reported in Table S1. Briefly, a standard curve-based quantification, obtained with serial dilution of the target amplicon (10^4–10^8 copies), was carried out for each microbial genus using PowerUp SYBR Green Master Mix (Applied Biosystems) in a final volume of 10 μL. Data were converted according to the rRNA copy number to obtain the number of microorganisms as Log CFU/intestine, Log spores/intestine for *N. ceranae*, or Log 16S rRNA copies/intestine for the total bacteria.

2.8. Data analysis

The data analyses were conducted using R statistical software (v4.3.3). Kaplan-Meier survival analysis was performed with the survfit function from the *survival* package, and pairwise comparisons were carried out using the log-rank test with Bonferroni correction for multiple comparisons (*pairwise_survdiff* function). Survival curves were visualized using the *survminer* package, with significance groupings for treatments annotated using the compact letter display (CLD) groupings. Pollen and sugar solution consumption data were analyzed by calculating the mean and standard error for each treatment group using the *dplyr* package. Differences among treatments were assessed using Analysis of Variance (ANOVA), followed by Tukey's HSD test for pairwise comparisons. Significant differences between treatment groups were visually represented with annotations generated using the CLD groupings. Vitellogenin concentrations and absolute abundances of individual bacterial taxa were analyzed across treatments using non-parametric Kruskal–Wallis tests, followed by Dunn's post hoc tests with Bonferroni correction. For gut microbial community structure, bee-level abundances of the seven core taxa were converted into Bray–Curtis dissimilarities. Treatment effects were tested with PERMANOVA (999 permutations), and homogeneity of multivariate dispersion was evaluated with PERMDISP. Principal coordinates analysis (PCoA) was used to visualize treatment-level clustering. To control for multiple comparisons in per-taxon analyses, false discovery rates were adjusted using the Benjamini–Hochberg procedure. To further evaluate potential influences of pollen chemistry on vitellogenin and gut microbiota, generalized linear models in an ANCOVA framework (GLM/ANCOVA) with robust (HC3) standard errors were applied to pollen-fed treatment groups, incorporating histidine, copper, and total flavonoid intake as continuous covariates alongside treatment. Covariate selection was intentionally restricted to a limited number of biologically meaningful intake variables to avoid overfitting and instability of model estimates. Because intake values were measured at the cage level and shared by all bees within a cage, the effective number of independent replicates was limited. Including a larger number of correlated dietary variables (*e.g.*, total amino acids, crude protein, lipids, pesticides, or multiple phytochemicals) would have increased multicollinearity and reduced model interpretability. Covariate intake values were calculated from cumulative pollen consumption up to day 6, corresponding to the sampling point for guts and haemolymph.

3. Results

3.1. Pollens identification

18S rRNA sequencing confirmed assignment of both CTAX and UTAX samples to the genus *Taraxacum*, with multiple species showing 100% identity, consistent with the known taxonomic complexity of the *Taraxacum officinale* aggregate. Species-level discrimination was therefore not possible. Malus pollen (ML) was confirmed at the genus level by sequence similarity and was attributed to *Malus domestica* based on field collection from apple orchards during peak bloom and corresponding palynological features. *Phacelia* pollen (PH) was confirmed to belong to the species *Phacelia tanacetifolia*.

3.2. Pesticide residue analysis of pollen samples

The pesticide analysis, including concentrations of each detected substance, is summarized in Table 2. Distinct contamination profiles were observed among the pollens collected from different landscapes. Apple (ML) and dandelion (CTAX) pollens from intensively managed apple orchards in Caldes exhibited substantial pesticide residues, with high concentrations of tau-fluvalinate and flonicamid. In contrast, dandelion pollen (UTAX) from the meadows of Vermiglio, a high altitude natural grassland, and lacy phacelia pollen (PH) from organic vineyards in Trento showed minimal insecticide residues. Trace levels of fosetyl-Al and cadmium were detected in UTAX, while PH pollen showed the highest copper contamination (22,800 ppb) among all samples. Risk index (RI) calculations confirmed that dietary risk was lowest for UTAX (RI = 0.0003) and highest for ML (RI = 0.0086), with PH showing a similarly elevated value (RI = 0.0071), while CTAX (RI = 0.0015) displayed an intermediate risk level (Table S2). Although all RI values remained well below 1, indicating low acute hazard relative to established LD_{50} thresholds.

3.3. Crude protein, free amino acid, and lipid analysis of pollen samples

Crude protein and lipid content analysis revealed clear differences in the composition of the tested pollens. PH pollen showed the highest crude protein content, whereas CTAX and UTAX pollens were comparatively poorer in protein but richer in lipids. Specifically, PH pollen contains 261,710 ± 1394 mg/kg protein and 166,000 ± 800 mg/kg lipids (P:L = 1.58); ML pollen contains 221,191 ± 2812 mg/kg protein and 158,000 ± 900 mg/kg lipids (P:L = 1.40); CTAX pollen contains 157,870 ± 7159 mg/kg protein and 269,000 ± 1400 mg/kg lipids (P:L = 0.59); and UTAX pollen contains 162,756 ± 6518 mg/kg protein and 261,000 ± 1300 mg/kg lipids (P:L = 0.62). The free amino acid composition along with the crude protein and lipid content of pollen are reported in Table S4.

Clear variation was observed in free amino acid profiles across pollen types (Table S4). ML pollen showed the lowest total free amino acid content (≈ 630 mg/kg) and the most limited profile, with only modest amounts of proline (314.71 mg/kg), valine (267.86 mg/kg), and threonine (7.01 mg/kg). PH pollen exhibited the highest total free amino acid content (sum of detected free amino acids; approximately 8367 mg/kg), characterized by high levels of several essential amino acids for honey bees, including valine (3545.20 mg/kg), leucine (191.81 mg/kg), isoleucine (100.36 mg/kg), threonine (32.43 mg/kg), phenylalanine

Table 2
Results from the multi-residue pesticide analysis of pooled monofloral pollen samples with concentrations of the contaminants in ppb. Bold values indicate residues in apple pollen (ML) and dandelion pollen (CTAX) exceeding MRLs for apple fruit and in lacy phacelia pollen (PH) exceeding MRLs for grapefruit. Dandelion pollen from meadows (UTAX), collected at high altitude in the absence of nearby orchards, was not compared to any crop-specific MRLs due to the lack of applicable reference values.

	Class	ML	PH	CTAX	UTAX
Etofenprox	Pyrethroid	27			
Flonicamid (Tot)*	Pyridine	117		154	
tau-Fluvalinate	Pyrethroid	1140		92	
Bupirimate	Pyrimidine	16			
Fosetil-Al (Tot)**	Organophosphate	483	583	902	23
Cyflufenamid	Amide/Amidoxine	90		33	
Cyprodinil	Anilinopyrimidine	28		13	
Dimethomorph	Morpholine		34		
Dithianon	Quinone	493			
Dithiocarbamates (Tot)	Fungicide complex	3040	55	120	
Dodine	Guanidine	130		69	
Fluazinam	Pyridine	388	79	61	
Fluxapyroxad	Pyrazole	1140		55	
Pyrimethanil	Anilinopyrimidine	900		239	
Tetraconazole	Triazole; Conazole	62		17	
Glyphosate	Organophosphate, Phosphonoglycine			84	
6-Benzylaminopurine	Plant hormone	50		11	
Cadmium	Heavy metal	82	35	33	23
Lead	Heavy metal	54	50	23	
Copper	Heavy metal	15500	22800	7650	173
Total detected substances		18	8	17	3

Maximum Residue Limits (MRLs) for apples and grapes were obtained from the EU Pesticide Database and converted from mg/kg (ppm) to ppb for comparison with residue levels detected in pollen (see Table S3). No official MRLs were available for pollen. * Expressed as the sum of the insecticide Flonicamid (Pyridine) and TFNA (Flonicamid metabolite). ** Of the Fosetyl-Al sum, residues were detected primarily as the metabolite phosphonic acid (363 ppb in mL, 678 ppb in CTAX, 17 ppb in UTAX, and 438 ppb in PH), while parent fosetyl was below the limit of quantification (<10 ppb) in all samples.

(106.03 mg/kg), and tryptophan (19.75 mg/kg), together with elevated proline and glutamic acid. CTAX pollen displayed a substantially lower total free amino acid content (≈ 1224 mg/kg) than PH but higher than ML, with moderate levels of proline (531.77 mg/kg) and valine (608.71 mg/kg) but comparatively low concentrations of several essential amino acids, including threonine (6.65 mg/kg) and leucine (14.16 mg/kg). In contrast, UTAX pollen showed the second-highest total free amino acid content (≈ 6843 mg/kg) and a distinct profile dominated by exceptionally high histidine (315.87 mg/kg), alongside elevated levels of essential amino acids such as valine (2988.65 mg/kg), leucine (222.07 mg/kg), isoleucine (119.03 mg/kg), and phenylalanine (89.13 mg/kg). Free amino acid concentrations are presented without correction for moisture content and are therefore used for comparative evaluation among pollen types.

3.4. Phenolic and Phenolamide analysis of pollen samples

The analysis identified a diverse array of flavonoids and phenolamides across the four tested monofloral pollens, with marked differences in compound identity and abundance (Table 3). *Malus* (ML) and *Phacelia* (PH) pollens exhibited a broad spectrum of glycosylated flavonoids. Notably, ML pollen was uniquely enriched in methylherbacetin derivatives, such as methylherbacetin-O-dihexoside and methylherbacetin-malonyl-dihexoside, which were absent in all other pollen types. PH pollen displayed a distinctive profile, with numerous flavonoids, including kaempferol-, quercetin-, and isorhamnetin-based compounds, that were either exclusive to or most abundant in this pollen type. In contrast, both *Taraxacum*-derived pollens (CTAX and UTAX) were dominated by phenolamides, particularly isomers of N1, N5,N10-tri-p-coumaroylspermidine and tetracoumaroyl spermine. While CTAX and UTAX shared similar total amounts of phenolamides and flavonoids, their specific compound profiles differed. For example, UTAX contained quercetin-3-O-rhamnoside, quercetin-O-arabinoglucoside, arabinoglucoside, and kaempferol-O-dihexoside, which are not seen in other pollen.

Total flavonoids and total phenolamides were calculated as the sum of individually identified and quantified compounds (expressed in mg/g). Compounds reported only as peak areas due to the lack of calibration standards were excluded from total concentration calculations.

3.5. Feed consumption and survival analysis

Pollen consumption patterns during the 10-day feeding period are summarized in Table S5. Average consumption varied significantly among the pollen types. Bees fed with ML and PH pollen showed the highest intake, with average values of 2.44 ± 0.21 mg/bee/day and 2.66 ± 0.37 mg/bee/day, respectively, and did not differ significantly from each other ($p > 0.05$). In contrast, bees consuming CTAX pollen had the lowest intake (1.21 ± 0.18 mg/bee/day), which was significantly lower than the other treatments ($p < 0.05$). Bees fed with UTAX exhibited an intermediate consumption level (1.69 ± 0.19 mg/bee/day), not significantly different from ML and PH, but also not from CTAX.

The overall mean daily sucrose solution consumption differed significantly among experimental conditions (one-way ANOVA, $F(7, 984) = 40.29$, $p < 0.001$), indicating a clear treatment effect on feeding behavior (Fig. 1). The negative control (CTR) showed the highest consumption (45.2 ± 1.39 mg/bee/day). Intermediate consumption levels were observed for the pollen-fed groups ML (38.0 ± 1.17 mg/bee/day), PL (33.7 ± 1.30 mg/bee/day), PH (35.6 ± 1.48 mg/bee/day), and CTAX (37.3 ± 1.23 mg/bee/day), which did not differ significantly from each other. The UTAX and ACE treatments exhibited similarly reduced consumption (28.0 ± 1.51 and 23.9 ± 1.37 mg/bee/day, respectively), with no significant difference between them. The lowest consumption was recorded in the ACE+PL group (20.1 ± 0.76 mg/bee/day). Post-hoc Tukey HSD tests confirmed significant differences among treatments, as indicated by distinct letter groupings in Fig. 1.

Table 3
Identification and quantification of the phenolic and phenolamide compounds in different pollen samples, expressed as mg/g ± SD, obtained using LC/DAD/ESI-MSn. ML = pesticide-contaminated apple pollen; PH = pesticide-contaminated lacy phacelia pollen; CTAX = pesticide-contaminated dandelion pollen; UTAX = uncontaminated dandelion pollen. Where "-" is displayed, no compound was detected.

No.	tR (min)	Compound	Class	ML	PH	CTAX	UTAX
1	5.31	Quercetin-O-hexosyl-O-rutinoside[a,b]	Flavonoid	-	-	0.72 ± 0.01	0.70 ± 0.03
2	6.39	Quercetin-O-deoxyhexosyl-O-hexosyl-deoxyhexoside[a,c]	Flavonoid	-	0.33 ± 0.01	-	-
3	6.61	Quercetin-O-diglucoside[a,d]	Flavonoid	0.32 ± 0.0005	-	-	-
4	6.70	Kaempferol-O-dihexoside[a,b]	Flavonoid	-	-	-	0.31 ± 0.01
5	7.03	Isorhamnetin-O-gentiobioside[a,c]	Flavonoid	-	-	0.71 ± 0.01	0.77 ± 0.02
6	7.11	Methyl herbacetin-O-dihexoside[a,d]	Flavonoid	1.12 ± 0.01	-	-	-
7	7.53	Kaempferol-O-deoxyhexosyl-O-hexosyl-deoxyhexoside[a,c]	Flavonoid	-	1.03 ± 0.03	-	-
8	7.56	Quercetin-O-arabinoglucoside[a,c]	Flavonoid	-	-	-	0.24 ± 0.01
9	7.68	Isorhamnetin-O-deoxyhexosyl-O-hexosyl-deoxyhexoside[a,d]	Flavonoid	-	1.26 ± 0.01	-	-
10	8.01	Isorhamnetin-O-malonyl-dihexoside[a,c]	Flavonoid	-	-	0.38 ± 0.01	0.35 ± 0.002
11	8.02	Methylherbacetin-malonyl-dihexoside[a,d]	Flavonoid	1.14 ± 0.03	-	-	-
12	8.45	Kampferol-O-rutinoside-O-glucoside[a,c]	Flavonoid	1.22 ± 0.01	-	0.52 ± 0.01	-
13	8.98	Kaempferol-3-O-rutinoside[a,d]	Flavonoid	0.28 ± 0.01	1.47 ± 0.03	-	-
14	9.14	Isorhamnetin-3-O-hexosyl-deoxyhexoside[a,d]	Flavonoid	-	0.55 ± 0.0004	-	-
15	9.49	Methyl herbacetin-O-malonyl-hexosyl-deoxyhexoside[a,d]	Flavonoid	0.28 ± 0.04	-	-	-
16	9.60	Quercetin-3-O-glucoside[a,d]	Flavonoid	-	0.35 ± 0.02	-	-
17	10.72	Methylherbacetin-O-hexoside[a,d]	Flavonoid	0.47 ± 0.001	-	-	-
18	10.82	Isorhamnetin-3-O-hexosyl-deoxyhexoside (isomer)[a,d]	Flavonoid	-	0.40 ± 0.0001	-	-
19	11.51	Kaempferol-O-hexoside[a,d]	Flavonoid	-	1.86 ± 0.04	-	-
20	11.89	Isorhamnetin-3-O-glucoside[a,d]	Flavonoid	-	0.21 ± 0.01	-	-
21	12.06	Isorhamnetin-O-malonyl-dihexoside[a,c]	Flavonoid	7.08 ± 0.04	-	-	-
22	12.71	Syringetin-O-malonylhexoside[a,c]	Flavonoid	2.33 ± 0.04	-	-	-
23	13.02	N(Z), N'(Z)-di-p-coumaroyl putrescine[a,c]	Phenolamide	-	0.43 ± 0.06	-	-
24	13.41	Isorhamnetin-O-malonyl-dihexoside[a,c]	Flavonoid	-	-	0.30 ± 0.02	0.28 ± 0.01
25	13.45	N-p-coumaroyl-N'-caffeoyl putrescine[a,c]	Phenolamide	-	0.63 ± 0.02	-	-
26	13.81	N10,N5,N10-tricaffeoyl spermidine (isomer)[a,d]	Phenolamide	0.07 ± 0.01	-	-	-
27	14.81	N(Z), N'(Z)-di-p-coumaroyl putrescine (isomer)[a,c]	Phenolamide	-	0.65 ± 0.02	-	-
28	14.83	N10,N5,N10-tricaffeoyl spermidine (isomer)[a,d]	Phenolamide	0.35 ± 0.002	-	0.03 ± 0.005	-
29	14.99	N10,N5,N10-tricaffeoyl spermidine (isomer)[a,d]	Phenolamide	0.24 ± 0.05	-	-	-
30	16.60	Quercetin-O-rhamnoside[a,d]	Flavonoid	3.84 ± 0.01	-	-	-
31	16.60	N(Z), N'(Z)-di-p-coumaroyl putrescine (isomer)[a,c]	Phenolamide	-	3.72 ± 0.001	-	-
32	17.04	Quercetin-3-O-rhamnoside (isomer)[a,d]	Flavonoid	-	-	-	0.36 ± 0.01
33	17.81	N1-p-coumaroyl-N5,N10-dicaffeoyl spermidine[a,d]	Phenolamide	0.01 ± 0.01	0.25 ± 0.02	-	-
34	18.14	N1,N5-di-p-coumaroyl-N10-caffeoyl spermidine[a,d]	Phenolamide	-	0.22 ± 0.01	-	-
35	19.63	N1, N5, N10-tri-p-coumaroyl spermidine[a,d]	Phenolamide	0.31 ± 0.01	1.21 ± 0.06	0.07 ± 0.01	-
36	21.35	N1, N5, N10-tri-p-coumaroyl spermidine (isomer)[a,d]	Phenolamide	1.18 ± 0.001	4.02 ± 0.05	0.15 ± 0.002	0.13 ± 0.01
37	22.91	N1, N5, N10-tri-p-coumaroyl spermidine (isomer)[a,d]	Phenolamide	1.05 ± 0.04	1.52 ± 0.04	0.09 ± 0.01	0.07 ± 0.01
38	23.91	N1, N5, N10-tri-p-coumaroyl spermidine (isomer)[a,d]	Phenolamide	0.31 ± 0.002	0.41 ± 0.04	0.03 ± 0.01	-
39	25.51	N1, N5, N10-tri-p-coumaroyl spermidine[a,d]	Phenolamide	3.65 ± 0.10	6.88 ± 0.02	-	-
40	25.89	Tetracoumaroyl spermidine[a,d]	Phenolamide	-	-	1.42 ± 0.01	1.37 ± 0.05
41	26.55	Feruloyldicoumaroyl spermidine[a,d]	Phenolamide	0.05 ± 0.03	0.08 ± 0.01	-	-
42	27.67	Tetracoumaroyl spermine (isomer)[a,d]	Phenolamide	-	-	2.42 ± 0.10	2.45 ± 0.05
43	28.35	Tetracoumaroyl spermine (isomer)[a,d]	Phenolamide	-	-	0.25 ± 0.02	0.23 ± 0.03
44	29.56	Tetracoumaroyl spermine (isomer)[a,d]	Phenolamide	-	-	2.52 ± 0.05	2.49 ± 0.08
45	31.32	Tetracoumaroyl spermine (isomer)[a,d]	Phenolamide	-	-	1.36 ± 0.02	1.55 ± 0.04
46	32.28	Tetracoumaroyl spermine (isomer)[a,d]	Phenolamide	-	-	0.14 ± 0.01	0.20 ± 0.02
47	33.52	N1, N5, N10-tri-p-coumaroyl-N14-hydroxyferuloyl spermine[a,c]	Phenolamide	-	-	1.55 ± 0.004	1.99 ± 0.04
				mean area ± SD	mean area ± SD	mean area ± SD	mean area ± SD
48	48.7	Not identified*	Unknown	242116.7 ± 2072.7	216747.4 ± 4881.4	222041.8 ± 2470.5	524184.2 ± 2459.5
		Total Flavonoids		18.08 ± 0.11	7.45 ± 0.06	2.62 ± 0.03	3.02 ± 0.04
		Total Phenolamides		7.23 ± 0.12	20.88 ± 0.13	10.04 ± 0.12	10.48 ± 0.13

Identification confirmed with: [a] (MS^n fragmentation); references: [b] (Ertosun et al., 2023), [c] (Qiao et al., 2023), [d] (Larbi et al., 2023)
* Tentatively a sesquiterpenoid derivative based on UV match (λmax: 236 nm). Due to the absence of an appropriate calibration standard, no mg/g quantification was performed. Only peak area values are reported for comparison.

The survival analysis revealed significant differences across the treatment groups (Fig. 2). The control and PL groups demonstrated the highest survival rate (above 79%), and therefore no median survival time was calculated, and no significant differences were detected ($p = 1$) when compared to the control. Similarly, the CTAX group maintained a high survival rate of 77%, while the ML group exhibited intermediate survival with a 55% probability and no median survival time. In stark contrast, the ACE group, which accumulated a total oral intake of 49.98 ± 5.36 ng a.i./bee through feeding over the course of the experiment, experienced 100% mortality by the end of the test and was

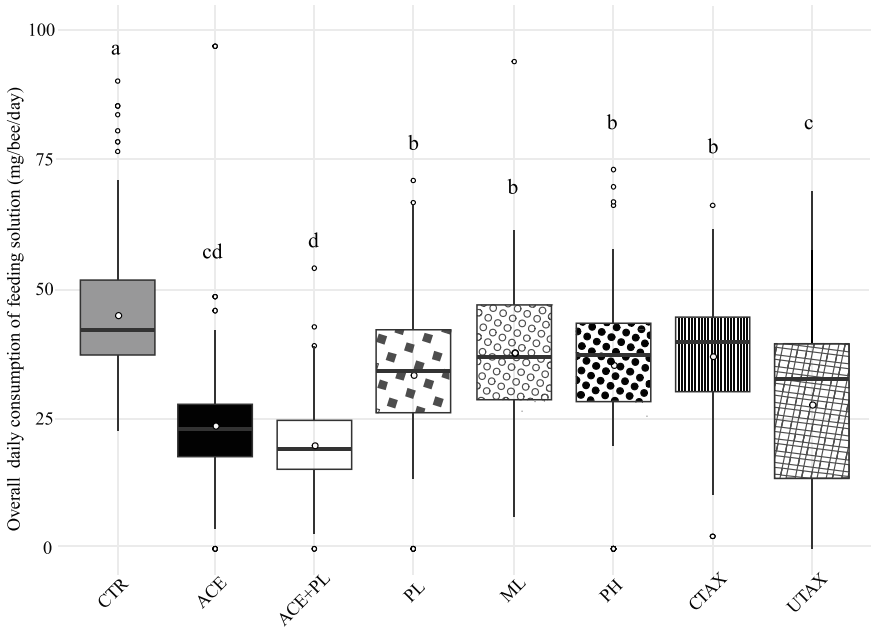

Fig. 1. Dietary consumption. Impact of different experimental conditions on the daily consumption of feeding solution (mg/bee/day) over 31 days (n = 4 cages per group with 25 bees per cage). Groups sharing the same letters did not differ significantly ($p < 0.05$, one-way ANOVA followed by Tukey's HSD test). The box plot displays the mean (white dot), the maximum (excluding outliers), the 75th, 50th (median), and 25th percentiles, the minimum (excluding outliers), and any identified outliers. CTR = negative control (50% sucrose solution); ACE = positive control (a.i. acetamiprid + 50% sucrose solution); PL = Promotor L Apis; ACE+PL = a.i. acetamiprid + Promotor L Apis; ML = pesticide-contaminated apple pollen; PH = pesticide-contaminated lacy phacelia pollen; CTAX = pesticide-contaminated dandelion pollen; UTAX = uncontaminated dandelion pollen.

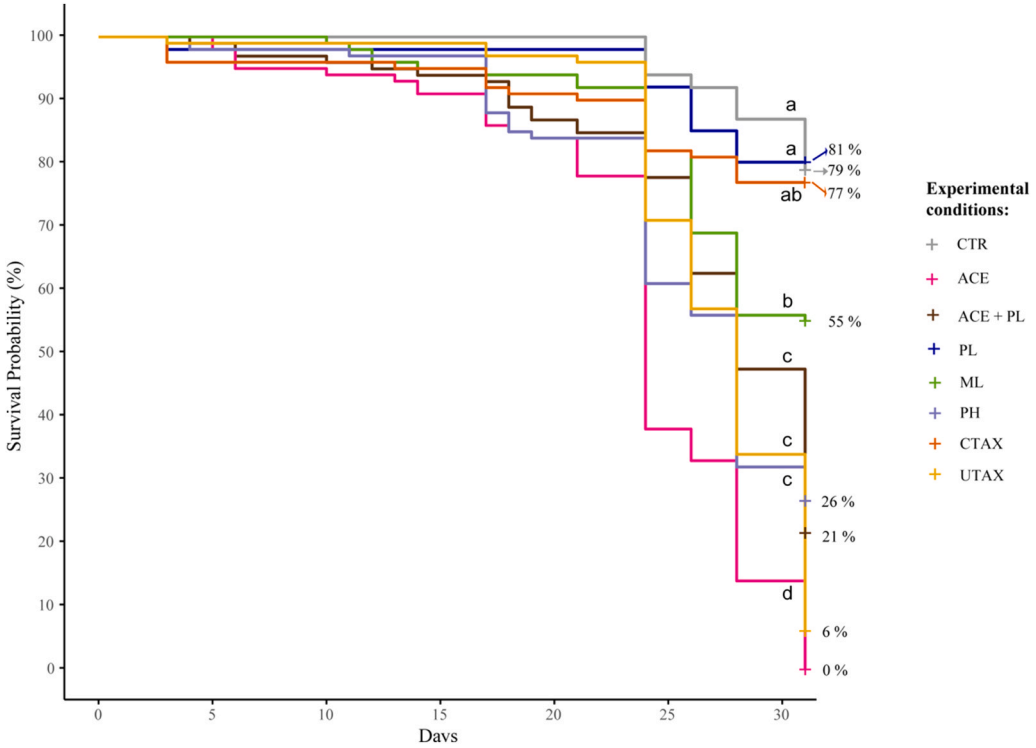

Fig. 2. Kaplan–Meier survival curves show the survival probability (%) over the test period for each experimental condition ($n = 100$ per group). Kaplan–Meier survival curves show the survival probability (%) over the test period for each experimental condition (n = 4 cages per group with 25 bees per cage). Colored lines represent different groups. Groups sharing the same letters did not differ significantly ($p < 0.05$, log-rank test with Bonferroni correction for pairwise comparisons). CTR = negative control (50% sucrose solution); ACE = positive control (a.i. acetamiprid + 50% sucrose solution); ACE+PL = a.i. acetamiprid + Promotor L Apis; PL = Promotor L Apis; ML= pesticide-contaminated apple pollen; PH= pesticide-contaminated lacy phacelia pollen; CTAX= pesticide-contaminated dandelion pollen; UTAX= uncontaminated dandelion pollen.

significantly different from all other groups, followed by UTAX with only 6% survival, showing no significant differences from the PH and

ACE+PL groups, which had survival probabilities of 26% and 21%, respectively. Notably, the three groups UTAX, PH, and ACE+PL shared a median survival time of 28 days, underscoring their comparably lower survival outcomes.

3.6. Vitellogenin quantification

Vitellogenin (Vg) quantification in honey bee hemolymph (Fig. 3) revealed significant differences among treatment groups. Pollen-fed bees exhibited higher Vg levels than pollen-free treatments, with pesticide-contaminated apple pollen (ML) showing the highest mean concentration (3.59 ± 2.82 mg/mL). No significant differences were detected among pollen-fed groups (ML, PH, CTAX, UTAX).

Bees fed the acetamiprid + Promotor L Apis combination (ACE+PL) showed the lowest Vg levels (0.25 ± 0.21 mg/mL), which were significantly lower than those observed in all pollen-fed groups, including ML (3.59 ± 2.82 mg/mL), PH (2.50 ± 1.83 mg/mL), CTAX (1.31 ± 1.08 mg/mL), and UTAX (1.53 ± 0.89 mg/mL). ACE+PL did not differ significantly from acetamiprid alone (ACE: 0.56 ± 0.25 mg/mL) or PL alone (PL: 0.69 ± 0.58 mg/mL).

PL alone did not result in significantly higher Vg levels compared to the control group (CTR: 1.06 ± 0.75 mg/mL). Full statistical details are provided in Supplementary File 1.

3.7. qPCR results on the gut core microbial taxa, the total bacteria, and the pathogens S. marcescens and N. cearanae

qPCR analysis (Figure S3) revealed that all pollen-based diets significantly enhanced total bacterial abundance compared to the control (CTR, $p < 0.01$), whereas no such increase was observed in the ACE treatment. Core taxa, including *Bifidobacterium*, *Frischella*, *Bartonella*, and *Gilliamella,* showed significant increases across all pollen treatments ($p < 0.01$ vs. CTR). *Bombilactobacillus* abundance significantly increased with ML ($p < 0.05$), PH ($p < 0.01$), and CTAX ($p < 0.01$ - Figure S3B), while *Lactobacillus* increased under PH ($p < 0.01$) and UTAX ($p < 0.05$ - Figure S3C). *Snodgrassella* abundance was also significantly higher in ML, PH, and CTAX treatments (all $p < 0.01$ - Figure S3G), whereas *Bombilactobacillus* showed a significant decrease with UTAX ($p < 0.01$). ACE+PL significantly increased the abundance of *Bifidobacterium*, *Bombilactobacillus*, *Fischella*, Bartonella, and *Gilliamella* (all $p < 0.01$) taxa, and total bacteria ($p < 0.05$) when compared to CTR. Finally, PL significantly enhanced all taxa ($p < 0.01$) except *Lactobacillus*.

Pathogen analysis revealed that *N. ceranae* (Figure S3I) spore loads increased significantly in bees fed PH, CTAX, and UTAX (all $p < 0.01$). *S. marcescens* was significantly elevated in CTAX and UTAX ($p < 0.05$) compared to the CTR (Figure S3L). No significant pathogen proliferation occurred in the ACE+PL and PL groups. The absolute abundance log data, standard deviations, and melting temperatures are reported in Table S6 and Supplementary File 2.

Beyond single-taxon changes, overall gut community composition differed markedly among treatments (PERMANOVA, Bray–Curtis: $F_{7,98} = 13.44$, $R^2 = 0.490$, $p = 0.001$). However, group dispersions were also heterogeneous (PERMDISP: $F = 7.20$, $p < 0.001$), indicating that part of the treatment effect reflected unequal within-group variability. Restricting the analysis to control plus pollen diets confirmed strong compositional differences (PERMANOVA: $F_{4,60} = 19.42$, $R^2 = 0.564$, $p = 0.001$). Dispersion was higher in the control group than in any pollen group (PERMDISP Tukey tests, $p \leq 0.001$), whereas pollens did not differ significantly (all $p > 0.74$). PCoA illustrates treatment-level clustering of pollen groups and control (Figure S4).

3.8. Influence of pollen chemistry on microbiota and vitellogenin

To evaluate whether dietary chemistry helped explain variation beyond treatment-level differences, we tested histidine, copper, and total flavonoid intake as covariates because these compounds were enriched in UTAX, PH, and ML pollen, respectively, representing distinct dietary features. For vitellogenin, treatment effects were not significant once chemistry was included (GLM/ANCOVA, Treatment: $F_{3,50} = 1.38$, $p = 0.26$). None of the covariates explained variation in vitellogenin (histidine: $F_{1,50} = 0.32$, $p = 0.57$; copper: $F_{1,50} = 0.001$, $p = 0.97$; flavonoids: $F_{1,50} = 3.04$, $p = 0.087$). In contrast, gut bacterial community

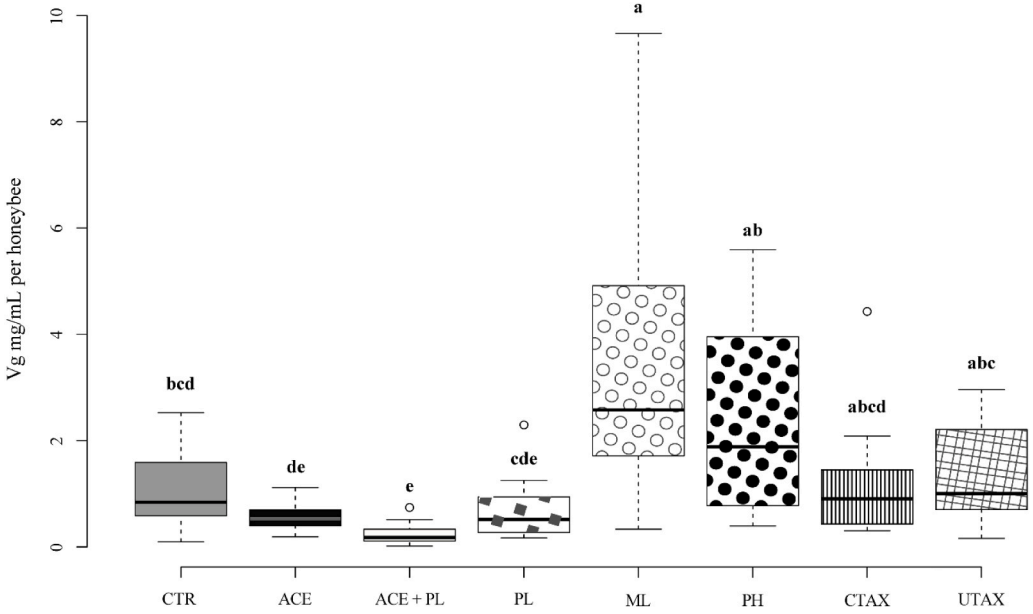

Fig. 3. Vitellogenin quantification in honey bee haemolymph using SDS-PAGE Vitellogenin quantification in honey bee haemolymph. The boxplot shows the vitellogenin content in 9-day-old honey bee haemolymph expressed in mg/mL/bee (n = 16 bees per group). The horizontal line indicates the median, boxes the interquartile range (IQR), whiskers extend to 1.5 × IQR, and points represent outliers. Groups sharing the same letters did not differ significantly ($p < 0.05$, Kruskal–Wallis test followed by Dunn's test with Bonferroni correction). CTR = negative control (50% sucrose solution); ACE = positive control (acetamiprid + 50% sucrose solution); PL = Promotor L Apis; ACE+PL = acetamiprid + Promotor L Apis; ML = pesticide-contaminated apple pollen; PH = pesticide-contaminated lacy phacelia pollen; CTAX = pesticide-contaminated dandelion pollen; UTAX = uncontaminated dandelion pollen.

composition in pollen-fed bees was shaped primarily by treatment, with no significant effects of histidine, copper, or flavonoids (PERMANOVA, all $p > 0.22$). Per-taxon ANCOVAs (BH–FDR) identified *Bombilactobacillus* as significantly treatment-sensitive, whereas no robust associations were detected for other taxa (Table S7).

4. Discussion

4.1. Landscape-driven pesticide exposure and pollen contamination

The regions of South Tyrol and Trentino in the north of Italy are intensively cultivated with apples and vineyards, resulting in high pesticide contamination (Favaro et al., 2019). Our multiresidue analysis revealed widespread pesticide presence in bee-collected pollen. Among insecticides, tau-fluvalinate, etofenprox, and flonicamid reflected typical pest management practices in apple orchards, as per the official production guidelines for pome fruit cultivation in South Tyrol (Agrios, 2020). Fungicides, like dithiocarbamates, were more prevalent, raising concerns due to their persistence and toxicity. ML pollen showed high levels of dithianon and fluxapyroxad, both of which are widely applied against apple scab. Fosetyl-Al and its metabolite phosphonic acid were found in all sites, including PH pollen, which lacked insecticides but showed moderate fungicide and high copper levels, reflecting organic vineyard practices (Burandt et al., 2024). UTAX pollen from alpine meadows contained no pesticides, and the detected traces of phosphonic acid could have stemmed from environmental sources rather than fosetyl-Al degradation (del Mar Gómez-Ramos et al., 2020). EFSA et al., (2023) underscores the necessity of evaluating the chronic risks of pesticide exposure in bees by considering long-term dietary intake. Responding to this need, our study investigates the effects of pesticide-contaminated pollen at environmentally realistic concentrations, thereby offering a more ecologically relevant assessment of their consequences on honey bee survival and physiology.

4.2. Effects of pollen origin and environment on feeding and survival

In agricultural landscapes, pesticide exposure is a well-documented stressor for pollinators and has been shown to negatively affect bee survival (Sgolastra et al., 2019). Large-scale analyses across European agroecosystems have demonstrated that cumulative pesticide exposure from floral resources is widespread and biologically relevant for bees (Nicholson et al., 2024). In the present study, pesticide residues detected in apple (ML) and orchard-derived dandelion (CTAX) pollen were consistent with this landscape-level exposure scenario. Although pesticide residues in ML and CTAX pollen were substantial, their effects on survival appeared modulated by pollen nutritional properties and botanical origin, rather than pesticide load alone.

PH pollen, despite being less contaminated than ML, supported similar feed intake but resulted in higher mortality. The elevated copper content in PH pollen led to an intake of approximately 0.60 μg Cu per bee over the 10-day feeding period. This exposure is well below reported acute oral LD_{50} values for copper sulphate in honey bees (40 μg bee^{-1}; PPDB), and the observed mortality here likely reflects chronic, diet-borne exposure within a complex matrix, where copper bioavailability, nutritional background, and co-occurring fungicides may modulate toxicity. More broadly, trace metal toxicity in bees is shaped by interactions among exposure matrix, co-stressors, and diet quality (Gekière et al., 2023), which may explain why copper-rich pollen diets resulted in elevated mortality despite relatively low pesticide residues.

The contrasting survival outcomes between CTAX and UTAX pollens further indicate that factors beyond contamination contribute to dietary risk. Although both pollens originated from *Taraxacum* sp., they differed markedly in their effects on honey bee survival. Such variability may partly reflect environmental influences on pollen quality, as CTAX was sourced from intensively cultivated apple orchards at 697 m a.s.l., whereas UTAX originated from alpine meadows at 1261 m a.s.l. Climatic stress associated with high-altitude environments has been shown to alter pollen nutritional quality and amino acid composition in several plant species, with potential consequences for pollinator nutrition and health (Descamps et al., 2021). Similar regional variation in pollen composition has been reported for *Rubus* and *Quercus* pollens across contrasting environments (Braglia et al., 2025).

4.3. Asteraceae pollen traits and their impact on feeding behavior

Beyond contamination levels, pollen botanical origin and taxonomic traits can significantly affect honey bee nutrition, digestion, and survival. The so called “Asteraceae paradox” describes how pollen from Asteraceae species like *Taraxacum,* exhibit protective traits that may limit its suitability for generalist bees despite its widespread availability (Mueller and Kuhlmann, 2008). These traits include thick pollen multilayer walls that hinder digestion, the presence of specific sterols and secondary metabolites, and low nutrient content (Vanderplanck et al., 2018). Such characteristics can lead to nutrient deficiencies in unspecialized bee species unless physiological adaptations are present. Under natural foraging conditions, polylectic bees mitigate these limitations by collecting diverse pollen sources, thereby balancing nutritional intake (Eckhardt et al., 2014). In contrast, the monofloral diets imposed in the present study likely amplified the inherent limitations of *Taraxacum* pollen, particularly for UTAX-fed bees.

In the present study, pollen consumption differed among diets, with CTAX pollen consumed significantly less than ML and PH, while UTAX showed intermediate intake and did not differ significantly from the other pollen treatments (Table S5). This pattern is consistent with previous findings that Asteraceae-rich pollen diets may be less readily consumed by honey bees, potentially due to reduced palatability or digestibility constraints (Frias et al., 2016). Interestingly, reduced pollen consumption did not consistently predict adverse outcomes, as CTAX pollen supported high survival despite lower intake, whereas UTAX pollen showed high mortality at intermediate consumption levels, indicating that feeding behavior alone cannot explain the observed survival patterns. In addition, the contrasting responses between CTAX and UTAX pollens, despite both originating from *Taraxacum*, may reflect species-level/ecotype diversity within this taxonomically complex genus, which comprises numerous morphologically similar but ecologically distinct taxa (*e.g.,* Wolanin et al., 2023). *Taraxacum* pollen has been reported to be nutritionally limiting when provided as a monofloral diet (Barraud et al., 2022), and such limitations may be exacerbated under specific environmental contexts, including high-altitude conditions as at the UTAX site. Together, these factors may contribute to the observed differences in feeding behavior and downstream physiological responses.

4.4. Amino acid balance as a driver of honeybee responses

Despite only moderate differences in crude protein content, the four pollens differed substantially in the composition and relative abundance of free amino acids. PH and UTAX showed higher total free amino acid concentrations but contrasting amino acid balance, whereas CTAX and ML exhibited lower and more constrained profiles. These differences are interpreted descriptively, as normalization to dry matter was not possible due to the lack of moisture content measurements. Consistent with broader nutritional frameworks emphasizing amino acid balance over crude protein concentration (Bryś and Strachecka, 2024), our results based on free amino acid profiles indicate substantial qualitative differences among pollens. UTAX pollen contained a remarkably high amount of histidine. The enrichment likely reflects adaptive metabolic responses of high-altitude *Taraxacum* (1261 m a.s.l.), as plants from high-altitudes under oxidative and UV stress often upregulate histidine biosynthesis (Kumar et al., 2023). UTAX pollen characterized by an unusually high histidine content was also associated with microbial dysbiosis. However, when included as a covariate in our models,

histidine intake did not explain variation in either vitellogenin or gut microbiota, suggesting that its effects cannot be disentangled from broader treatment-level differences. Thus, histidine may represent a distinctive chemical feature of UTAX pollen rather than a direct driver of the observed responses. While our analyses do not support a causal or explanatory role of individual amino acids in this dataset, the marked differences in free amino acid composition across pollens are consistent with broader evidence that amino acid balance can influence diet–physiological interactions in honey bees.

4.5. Influence of pollen-derived phenolics and phenolamides

Flavonoids and phenolamides represent important bioactive compounds that can influence honeybee health beyond basic nutrition. While altitude has been positively correlated with total flavonoid content in honey (Grassi et al., 2025), our two *Taraxacum* pollens (CTAX and UTAX), collected at markedly different elevations (~689 vs. 1261 m a.s.l.), exhibited similar levels. ML pollen stood out with exceptionally high flavonoid content, consistent with the naturally polyphenol-rich profile of *Malus domestica* pollen (Fotirić Akšić et al., 2024). This is in agreement with improved survival despite heavy pesticide load, supporting earlier work showing links between flavonoids, antioxidant activity, and reduced pesticide-induced mortality (Hýbl et al., 2021). Although ML pollen was characterized by high total flavonoid content, flavonoid intake did not show a significant association with either vitellogenin or microbiota composition in our analysis. Wu et al. (2024) showed that very high concentrations of quercetin (75–151 mg/L in sugar solution) reduced bacterial load in *Apis cerana,* though without affecting survival. In contrast, quercetin derivatives in our study were detected at far lower, environmentally relevant concentrations (0.68–4.16 mg/g across pollen types), limiting direct comparison with experimentally elevated exposures. Finally, an unidentified compound (tR: 48.73 min) was detected in all pollen types but was particularly abundant in UTAX. Its UV absorbance (λmax: 236 nm) resembled sesquiterpenoids such as Germacrene D (Theerthavathy et al., 2019), a volatile compound identified in *Taraxacum officinale* tissues (Fan et al., 2023). Considering that its identity could not be confirmed, its prevalence in UTAX alongside high mortality, and the effect on microbial taxa highlight the need for further characterization of this compound.

4.6. Vitellogenin dynamics

In our study, ML showed the highest mean vitellogenin concentration and was significantly higher than CTR and the pesticide/supplement treatments (ACE, ACE+PL, PL). Among the pollen diets, PH, UTAX, and CTAX did not differ significantly from ML or from each other, indicating broadly comparable Vg responses across pollen-fed groups. Previous studies have reported constraints in the digestibility of *Taraxacum* pollen and associated reductions in vitellogenin synthesis under specific experimental conditions (Frias et al., 2016). However, in the present study, *Taraxacum*-based diets did not result in reduced Vg levels relative to other pollen treatments. Importantly, although UTAX bees consumed less pollen, their vitellogenin levels were not significantly different from those of other pollen-fed groups, indicating that reduced intake did not translate into diminished Vg synthesis. By contrast, Promotor L Apis alone (PL) did not significantly increase vitellogenin levels relative to the control group, indicating that this artificial diet was insufficient to stimulate vitellogenin synthesis. Although vitellogenin is a lipoprotein, lipid availability per se is unlikely to be the limiting factor, as honey bees are capable of de novo lipid synthesis from carbohydrates and proteins. Recent work shows that fat body lipogenic capacity is primarily regulated by dietary protein and worker physiological state rather than direct lipid intake (Scofield and Amdam, 2024). The limited Vg response under PL, therefore, likely reflects the absence of pollen-derived nutritional and physiological cues required to activate vitellogenin synthesis. Notably, survival remained high under PL feeding, further indicating that vitellogenin levels and survival outcomes are not necessarily coupled. However, ACE+PL did not differ significantly from acetamiprid alone (ACE), indicating that the dietary supplement did not mitigate the suppressive effect of acetamiprid on vitellogenin expression.

4.7. Gut microbiota composition and pathogen load

Diet composition exerted a strong influence on gut microbiota structure, with protein availability and pollen origin emerging as key drivers of bacterial abundance and taxon-specific responses. Bees fed only sugar syrup (CTR) exhibited a poorly developed gut microbiota, characterized by low abundance of several core taxa, confirming previous evidence that protein deprivation constrains microbial establishment and maintenance in honey bees (Alberoni et al., 2023; Anderson et al., 2023). In contrast, pollen-fed groups generally supported higher bacterial loads, although responses differed markedly among taxa and pollen types. Notably, *Bombilactobacillus*, a taxon known to be sensitive to environmental and nutritional stressors (Favaro et al., 2023), showed a pronounced reduction in UTAX-fed bees, consistent with strong diet-associated stress under this pollen regime. In the ACE group, several core gut taxa, including *Bifidobacterium*, *Frischella*, *Bartonella*, and *Gilliamella*, were significantly higher than in control bees, whereas total bacterial abundance did not show a clear increase relative to CTR, indicating selective taxon-level responses rather than generalized microbiota expansion. In the ACE+PL treatment, total bacterial abundance was significantly higher than CTR, while PL under acetamiprid exposure was associated with taxon-specific shifts rather than a uniform microbiota-wide change. Similar limited microbiota responses to acetamiprid exposure have been reported previously, with (Cuesta-Maté et al., 2021) observing only minor gut community changes even at relatively high doses. In contrast, more pronounced microbiota alterations have been documented for other compounds, such as glyphosate, under controlled exposure scenarios (Castelli et al., 2021). In the present study, bees ingested only trace levels of glyphosate from pollen (1.02 ng/bee from CTAX pollen), and its contribution to the observed microbiota patterns cannot be isolated from the broader dietary context. Finally, pathogen abundance differed in a diet-specific manner. *N. ceranae* and *S. marcescens* levels were significantly higher in bees fed *Taraxacum*-based diets (CTAX and UTAX) compared to the control, whereas PL and ML did not differ from CTR (Figure S2I, L). This consistent pattern across both *Taraxacum* sources suggests that dietary context associated with this pollen is linked to increased pathogen proliferation. However, because pathogen loads in pollen were not assessed, these differences likely reflect post-ingestion host–microbiota–pathogen interactions rather than direct dietary intake of pathogens.

5. Conclusion

In conclusion, our results demonstrate that honey bee responses to monofloral pollen diets arise from multiple, interacting factors, and that within this experimental context, pesticide contamination levels alone did not fully explain observed survival and physiological outcomes. High mortality was observed both in nutritionally imbalanced monofloral diets (UTAX) and in pollen characterized by elevated metal exposure (PH), despite marked differences in pesticide contamination and amino acid abundance. Conversely, pollen with substantial pesticide loads but without pronounced nutritional or metal imbalances (ML and CTAX) supported comparatively better survival. Together, these results indicate that botanical origin, nutritional imbalance, specific toxic co-stressors such as heavy metals, and chemical contamination jointly shaped honey bee health responses across pollen types. Overall, these findings emphasize the need for integrated assessments of pollen quality that consider multiple stressors when evaluating risks to honey bees.

CRediT authorship contribution statement

Daniele Alberoni: Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition. **Diana Di Gioia:** Writing – review & editing, Supervision, Resources, Project administration. **Abdulrahim T. Alkassab:** Writing – review & editing, Supervision, Conceptualization. **Soraia I. Falcão:** Methodology, Investigation, Formal analysis, Data curation. **Sergio Angeli:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Chiara Braglia:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Bhanu Janam:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Ethics approval

Ethical review and approval were waived, because the Italian law does not require and ethical approval for tests performed on arthropods with the exceptions of cephalopods.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT to improve English quality and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Diana Di Gioia reports financial support and equipment, drugs, or supplies were provided by University of Bologna. Daniele Alberoni reports financial support was provided by University of Bologna. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.120010.

Data availability

Data will be made available on request.

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