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Combined effect of a neonicotinoid insecticide and a fungicide on honeybee gut epithelium and microbiota, adult survival, colony strength and foraging preferences

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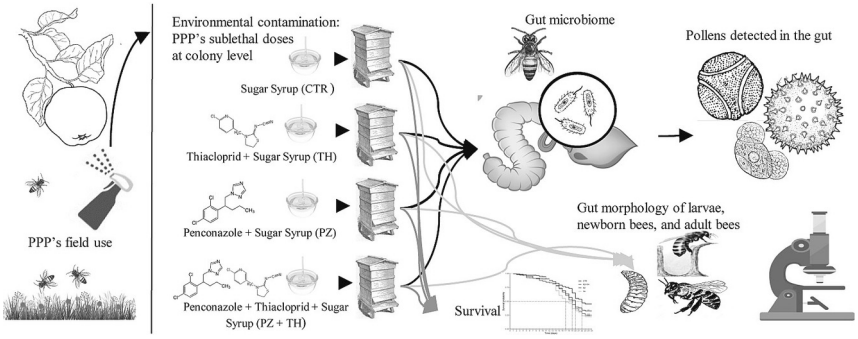
HIGHLIGHTS

- The synergic effect of two agrochemicals on honeybees was studied for the first time.
- Honeybees exposed to thiacloprid and penconazole showed reduced lifespan.
- Morphological changes on the gut epithelium were not observed upon exposure.
- Both pesticides alter gut microbial taxa linked with nutrition but not yeast community.
- Exposure to penconazole alter the honeybee foraging preference for pollen.

GRAPHICAL ABSTRACT



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ABSTRACT

Fungicides, insecticides and herbicides are widely used in agriculture to counteract pathogens and pests. Several of these molecules are toxic to non-target organisms such as pollinators and their lethal dose can be lowered if applied as a mixture. They can cause large and unpredictable problems, spanning from behavioural changes to alterations in the gut.

The present work aimed at understanding the synergistic effects on honeybees of a combined in-hive exposure to sub-lethal doses of the insecticide thiacloprid and the fungicide penconazole. A multidisciplinary approach was used: honeybee mortality upon exposure was initially tested in cage, and the colonies development

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Pesticides
Pollen

monitored. Morphological and ultrastructural analyses *via* light and transmission electron microscopy were carried out on the gut of larvae and forager honeybees. Moreover, the main pollen foraging sources and the fungal gut microbiota were studied using Next Generation Sequencing; the gut core bacterial taxa were quantified *via* qPCR.

The mortality test showed a negative effect on honeybee survival when exposed to agrochemicals and their mixture in cage but not confirmed at colony level. Microscopy analyses on the gut epithelium indicated no appreciable morphological changes in larvae, newly emerged and forager honeybees exposed in field to the agrochemicals. Nevertheless, the gut microbial profile showed a reduction of *Bombilactobacillus* and an increase of *Lactobacillus* and total fungi upon mixture application. Finally, we highlighted for the first time a significant honeybee diet change after pesticide exposure: penconazole, alone or in mixture, significantly altered the pollen foraging preference, with honeybees preferring *Hedera* pollen.

Overall, our in-hive results showed no severe effects upon administration of sublethal doses of thiacloprid and penconazole but indicate a change in honeybees foraging preference. A possible explanation can be that the different nutritional profile of the pollen may offer better recovery chances to honeybees.

1. Introduction

Intensive agriculture relies on plant protection products (PPPs) such as fungicides, insecticides, and herbicides, widely used to counteract pathogens and pest species, thus increasing crop yield. PPPs represent a serious environmental risk because they can also act on non-target organisms (Gomes et al., 2021; Urionabarrenetxea et al., 2022), such as beneficial insects like pollinators (Leska et al., 2021). Alessandrini et al. (2023) and Sgolastra et al. (2017), carried out a study on the toxicity of several single active substances used in agriculture towards non-target organisms, determining the LD_{50}, LD_{10} and LD_{90} for pollinators. Other studies have highlighted that the lethal dose of PPPs to non-target organisms, such as bees, can be further lowered if a mixture of agrochemicals is applied on the same organism (Mullin et al., 2015; Wang et al., 2020; Lv et al., 2023). Thiacloprid, a chloronicotinyl insecticide, has previously been discovered to present an advantageously low toxicity risk to the honeybee (Iwasa et al., 2004). As observed with pyrethroid molecules, monooxygenases are involved in the metabolism of chloronicotinyl compounds (Schmuck et al., 2003). However, some fungicides known as ergosterol biosynthesis inhibitors (EBI), if applied alongside these insecticides prevent the chloronicotinyl compounds metabolization because of the inhibition of monooxygenases. Chloronicotinoids (thiacloprid and acetamiprid) and EBI fungicides (the class of conazoles) emerged in previous studies (Favaro et al., 2019; Favaro et al., 2022) as ubiquitarian molecules in the honeybee environment as revealed by polled load analysis. Mixtures of different PPPs at sublethal doses may have negative effects that are often difficult to measure. The understanding of these complex interactions on non-target organisms such as wild pollinators and honeybees, the development of accurately designed, albeit difficult, represents the new frontier of toxicology (EFSA, 2022; Sanchez-Bayo and Goka, 2014; Stanley and Preetha, 2016).

Exposure to PPP has been demonstrated to alter several physiological pathways related to behaviour, immunity, nutrition, and detoxification in worker honeybees (Schmehl et al., 2014; Lv et al., 2023). Among the different honeybee behavioural changes caused by neonicotinoids, the alteration of the antennal signals (Straub et al., 2021; Favaro et al., 2022) and the cognitive working memory during differential olfactory learning was highlighted by Mustard et al. (2020a). This may alter the ability of these insects to forage quality resources present in the environment (Mustard et al., 2020b) and is also energetically costly (Arrese and Soulages, 2010). Moreover, PPPs can disrupt the honeybee fat body, as recently demonstrated by exposing them to pyriproxyfen and spirodiclofen pesticides (Elizabeth Deeter et al., 2023). Interestingly, in the same study changes in the lipid profile of the fat body were directly correlated to modifications in the foraging attitude (Elizabeth Deeter et al., 2023). Despite the difference in the honeybee diets, nutrient uptake depends on the integrity of the gut epithelium as well as of the gut microbial community. According to the literature, both epithelial cells and the gut microbiota seem to be damaged by PPPs. For instance, azoxystrobin, clorpyrifos, imidacloprid, spiromesifen, λ-cyalotrine, and acaricides for the control of varroosis like amitraz were found to affect the epithelial gut cells (de Castro et al., 2020; Gregorc and Bowen, 2000; Pervez and Manzoor, 2021; Serra et al., 2023; Serra and Hengge, 2021). PPPs were also found to alter the composition of the gut microbiome which (reviewed by Hotchkiss et al., 2022), in healthy honeybees, is composed of 7–8 core microorganisms (Zheng et al., 2017). Recent studies have shown that anthropic activities can change the proportion of the core microbial taxa, possiblyresulting in functionality changes (as reviewed by Raymann and Moran, 2018), also in synergy with pathogens such as *Nosema ceranae* (Alberoni et al., 2022). The fungicide chlorothalonil, for example, is able to modify the gut microbiota (Wu et al., 2022) and its functional potential in different pathways such as phosphorylation and sugar metabolism (Kakumanu et al., 2016) altering the honeybee nutrition efficiency and lifespan (O'Neal et al., 2019). Studies on the gut microbiome and PPP interaction in honeybees have been carried out both in cage and in field conditions, with different results depending on the target molecule and experimental conditions. Glyphosate was demonstrated to be lethal for some microbial taxa, deeply shaping the gut microbiome of honeybees (Motta et al., 2018; Motta et al., 2020), but other molecules, like imidacloprid, showed contradictory results on their impact on the gut microbial communities (Raymann et al., 2018; Alberoni et al., 2021a). Indeed, gut microbes represent a fundamental barrier for the protection of honeybees from PPP toxic effects, whereas the presence of gut dysbiosis in honeybees was reported as a factor that increases the toxicity of pesticides (Almasri et al., 2022). Indeed, the proliferation of gut pathogens is a well-known driver of changes in the gut microbial community of honeybees and wild bees (Alberoni et al., 2022; Rubanov et al., 2019; Rouzé et al., 2019; Fernandez de Landa et al., 2023). In addition, the exposure of honeybees to PPPs and acaricides for the control of varroosis was found to increase the level of some pathogens such as *N. ceranae* (Pettis et al., 2012), *Serratia* (Raymann et al., 2018), and viruses (O'Neal et al., 2017; Di Prisco et al., 2013), and this synergy increased honeybee mortality (Vidau et al., 2011; Raymann et al., 2018).

The present work aimed at understanding the synergistic effects of a combined exposure to the EBI fungicide penconazole and the insecticide thiacloprid on the honeybee biological cycle, feeding behaviour, and digestive system, both in larvae and adults. Specifically, a multidisciplinary approach was used to assess the health status of bees in their colonies, focusing on parameters such as honeybee population and colony development. A realistic field-dose combination of PPPs was provided to simulate a contaminated nectar flow. Survival of newly emerged bees which were exposed in-hive to the PPPs was monitored. Morphological and ultrastructural analyses were carried out on the gut of young larvae exposed to contaminated sugar syrup, as well as on newly emerged and forager bees. In addition, the main pollen foraging sources and the fungal gut microbiota of the honeybees were determined using Next Generation Sequencing (NGS) approach. Finally, the honeybees gut core microbial members were quantified through qPCR, and

community variations among the different experimental conditions assessed.

2. Materials and methods

2.1. Experimental design of the field test

The colonies of western honeybee *Apis mellifera* subsp. *carnica* (Pollmann) were kept in the experimental apiary of the Free University of Bolzano in the locality Altenburg (46°23′12.6″N 11°13′57.5″E, Bolzano, Italy). The apiary was located in the forest, while vineyards and apple orchards were apart in the lower valley, at 1 km flight distance. The trials were carried out at the end of summer, a moment of low nectar flow from the environment but where a normal colony development could still be expected. At the end of July 2019, twelve new colonies were created with the "shook swarm" method (Baffoni et al., 2021) from healthy colonies managed according to good beekeeping practice. The swarms of 1 kg bees were placed in regular 10-frames Dadant hives for nomadic beekeeping with five frames of organic wax foundation (Il Pungiglione Soc. Coop., Mulazzo, Italy). The swarms were provided with new *A. m. carnica* sister-queens and sugar syrup (Apiinvert®, Südzucker, Germany) and the hives were arranged in four rows, one for each treatment, placed few meters away from each other. After seven days the new colonies were treated with 50 mL of 3.5 % (*w/v*) oxalic acid dihydrate sucrose solution tickled in between frames against the parasitic mite *Varroa destructor* (Anderson and Trueman). In the following weeks, the development of each colony was supported with 6 L of sugar syrup (Apiinvert®). All the queens were accepted, and the colonies' development was assessed weekly. The brood was spread on at least four frames at the trial start (August 17th). Twice a week, each colony received 500 mL (680 g) of sugar syrup (Apiinvert®) through a rapid top feeder (Il Pungiglione Soc. Coop.).

To achieve the oral exposition of the honeybees to the chemicals, 0.588 mg/L (0.8 ppm) of penconazole (CAS 66246–88-6, Sigma-Aldrich, Milan, Italy) and 0.14 mg/L (0.2 ppm) thiacloprid (CAS 111988–49-9, Sigma-Aldrich, Milan, Italy) were dissolved in acetone and then diluted in distilled water at a final concentration of 0.005 % of acetone. Aliquots were stored at −80 °C. The colonies were subjected to four different treatments provided with the sugar syrup (3 colonies for each treatment, Fig. 1): [PZ] Penconazole dissolved in sugar syrup at a final concentration of 0.588 mg/L (0.8 ppm), [TH] Thiacloprid dissolved in sugar syrup at a final concentration of 0.14 mg/L (0.2 ppm), a combination of the two molecules at the same final concentration of chemicals previously indicated [PZ + TH], and the control [CTR] (sugar syrup with a final concentration of 0.005 % acetone, only). Penconazole concentration was chosen based on the maximum residual amount found in the study of Favaro et al. (2019), whereas Thiacloprid concentration was chosen according to the trials performed by Siede et al. (2017). Treatment syrups were prepared fresh on the day of administration and provided in the evening. All colonies finished the syrup poured in the feeder within the following morning. The treatment was provided once a week for five weeks. Then, brood frames were collected from the colonies for laboratory tests (Section 2.2) and forager honeybees and 5th instar larvae were sampled for molecular (Sections 2.4–2.6) and electron microscopy analyses (Section 2.7).

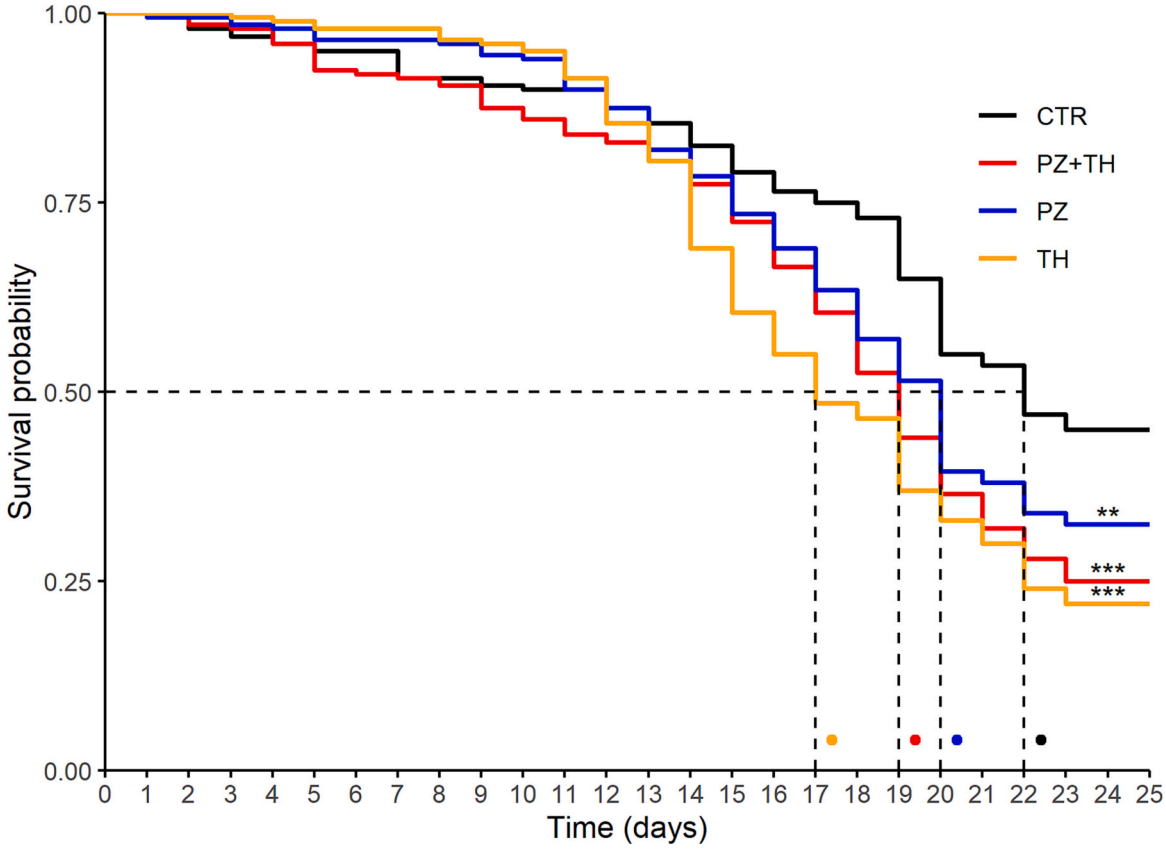

Fig. 1. Kaplan-Meier survival probability curve of adult bees. Mean proportion of surviving bees after confinement in hoarding cages ($n = 10$ cages, 20 bees per cage). The bees were exposed at larval stage during in-hive administration of contaminated syrups [TH] = Thiacloprid 0.2 ppm, [PZ] = Penconazole 0.8 ppm, their combination [PZ + TH] or control treatment [CTR] = control with no pesticide treatment) but fed at adult stage with only pure syrup *ad libitum*. Dashed lines indicate the day when 50 % of population mortality was reached. Asterisks report statistical significance of the treatment comparing with control (** = $p < 0.01$, *** = $p < 0.001$).

2.2. Survival test in laboratory conditions

Collection of the newly emerged honeybees: two frames containing closed brood were collected from each colony, to have six frames per experimental condition. The frames were marked, placed in four polystyrene containers, and kept in an incubator (ST5P, BioApp, Italy) at 34.5 °C. The relative humidity was regulated by placing a tray filled with water at the bottom of the incubator (Williams et al., 2013). An internal ventilation system was running at minimum to ensure air homogenization. During the two following days we collected the newly emerged bees, and they were sacrificed for the gut dissection.

Mortality assessment: From the same frames newly emerged bees were collected for the mortality trial. Gently handled with plastic forceps, 20 individuals were collected from the polystyrene boxes and placed in plexiglass hoarding cages (160 × 110 mm). The cages per treatment were 10, the newly emerged bees per treatment were 200. The frames with the remaining brood were returned to the source colonies. The cages were marked and then randomly assigned to the incubator shelves. The honeybees were provided with a sugar-water solution (50 % *w/v*) through a disposable syringe inserted in the cage top (Williams et al., 2013). The incubator temperature was lowered to 30 °C to match the needs of newly emerged bees (Williams et al., 2013), and a small opening on the side (5 mm diameter) allowed the air exchange. During each of the following 24 days, at 9 a.m., the dead bees were counted and removed from the cages and the syringes changed with sterile ones. The sugar-water solution (1:1 *w/v*) was replaced every morning.

2.3. Adult bee population assessment in hive

The number of adult honeybees in the [CTR] and the [PZ + TH] colonies was estimated (Delaplane et al., 2013) at the start and at the end of the trial. The colonies were opened at dawn, when there was no flight, and the frames were photographed at each side (Fernandez Ferrari et al., 2020). [PZ] and [TH] hives were not evaluated due to time constrains. The pictures were analysed by using ImageJ software (Alberoni et al., 2018; Rueden et al., 2017) Fiji version (Schindelin et al., 2012), drawing the outline of the surface occupied by adult bees. The honeybee colonies survival was monitored through the winter and the following spring for possible long-term lethal effects of the treatments.

2.4. DNA extraction

For molecular analysis, 84 gut samples (21 single guts (midgut+rectum) per experimental condition) were processed. Forager bees carrying loads were collected at the hive entrance and their gut extracted with a forceps, placed in an Eppendorf vial and immediately in ice. Each vial was filled with five guts. Samples were then stored in −80 °C freezer upon processing. DNA extraction from honeybee guts was performed with a PureLink™ Genomic DNA Mini Kit template preparation kit (K182002; Invitrogen, Milan, Italy) following manufacturer instructions with few modifications. Briefly, midgut and rectum were manually macerated with plastic micro pestles, mixed with glass beads (0.2 mm), and homogenised with Rotovortex (50 Hz) for 10 min after lysis buffer addition. Each DNA sample was quantified using a fluorometric approach performed with Qubit Flex Fluorometer (Thermo Fisher Scientific, Milan, Italy). Finally, samples were stored at −20 °C until further analysis.

2.5. Quantitative PCR

Absolute quantifications of target microbial groups were carried out by qPCR according to Baffoni et al. (2021) on 20 samples for every experimental condition (a total of 80 honeybees individually analysed) and for each target microbial group. Briefly, standard curves were constructed using PCR products of target genes, purified, and serially diluted to obtain standards ranging from 10^4 to 10^8 copies. Quantification was performed using FastSYBR™ Green Master Mix (Applied Biosystems, Milan, Italy) on a 10 μL reaction. The total number of bacteria (Eubacteria) in the honeybee gut was determined with primers Eub338-F and Eub518-R according to Lane (1991). The honeybee core genera (*Bartonella*, *Bifidobacterium*, *Bombilactobacillus*, *Frischella*, *Gilliamella, Lactobacillus* and *Snodgrassella*) were quantified with specific primers targeting the 16S rRNA gene as described by Kešnerová et al. (2017), Rinttilä et al. (2004) and Kešnerová et al. (2020). *Commensalibacter intestini* was quantified with primers reported in Fink et al. (2013). Total fungi were determined using primer targeting the 28S rRNA region according to Vollmer et al. (2008). The list of primers is reported in Table S1.

2.6. ITS2 next generation sequencing and bioinformatic analysis

Samples used in qPCR analyses were also prepared for NGS sequencing for yeast and pollen identification. Sample preparation was performed according to Alberoni et al. (2021a). Briefly, the amplified region for total fungi was ITS2, based on ITS3-Mi and ITS4-Mi primers according to Kim et al. (2019), as reported in Table S1. Samples were barcoded and sequenced on the MiSeq Illumina platform 2x300bp V3 chemistry on a dedicated flow cell according to the protocol of Baffoni et al. (2021). Raw reads were analysed with Qiime 1 (Caporaso et al., 2010) and chimera detection performed with Userach61 (Edgar, 2010). The script pick_outs.py was performed with 0.99 similarity. The file with representative sequences contained 60,196 sequences. assign_taxonomy.py script was implemented using UNITE database (Abarenkov et al., 2021), sortmerna as assignment method (with a coverage of 0.75) and 0.7 for similarity option. The obtained out_table contained 1,197,332 sequences with a mean of 66,518 per sample. Core diversity analysis was performed with nonphylogenetic_diversity option and rarefaction at 31843.

The obtained rarefied biom_table were used for summarize_taxa.py script in order to obtain files at different taxonomic levels for further file parsing and data elaboration.

2.7. Light and transmission electron microscopy

Guts for microscopy analysis were collected from larvae, newly emerged and forager bees. Fifth instar larvae were collected from capped cells on the same day the brood frames were collected to obtain newly emerged bees. 10 larvae were collected from each colony. On the same days the gut for the genetic analysis were collected, also the guts of 10 newly emerged bees were taken for each treatment condition from the polystyrene hives, and for each colony, 5 forager bee guts were collected. After isolation from larvae, newly emerged and forager bees, midgut samples were fixed in 4 % glutaraldehyde (in 0.1 M Na-cacodylate buffer, pH 7.4) overnight at 4 °C. They were postfixed in 1 % osmium tetroxide for 1 h at room temperature in the dark, dehydrated in an increasing ethanol series, and then embedded in epoxy resin (Epon/Araldite 812 mixture). Midgut sections were obtained with Leica Reichert Ultracut S (Leica, Wetzlar, Germany). Semi-thin sections (600-nm-thickness) were stained with crystal violet and basic fuchsin and then analysed with Eclipse Ni-U microscope (Nikon, Tokyo, Japan) equipped with TrueChrome II S digital camera system (Tucsen Photonics, Fuzhou, China). Ultra-thin sections (60-nm-thickness) were stained with lead citrate and uranyl acetate and analysed with JEM-1010 transmission electron microscope (Jeol, Tokyo, Japan) equipped with Morada digital camera (Olympus, Tokyo, Japan) - Centro Grandi Attrezzature, University of Insubria. At least 3 specimens per developmental stage were analysed.

2.8. Statistical analysis

Statistical analysis of qPCR and NGS data was carried out with R software (R Core Team, 2022), according to Alberoni et al. (2021b).

Data normality and homoscedasticity were assessed prior to the application of specific statistical models. GLM procedure was used for non-normal data with normal distribution of residuals and Kuskal-Wallis (Dunn-test post-hoc analysis) test for non-normal data. Bonferroni's correction was applied, considering 5 comparisons among the different experimental conditions [CTR] *vs* [PZ], [TH], [PZ + TH]; [PZ] *vs* [PZ + TH]; [TH] *vs* [PZ + TH]. Total fungi α and β diversity indexes were calculated with QIIME 1. The areas of adult bees in the colonies prior and after the treatments were compared with paired *t*-tests. The effect of treatment on mortality was tested with Kaplan-Meier survival analysis ("survival" package, Therneau, 2022) and a pairwise comparison with Bonferroni correction performed with the package "survminer" (Kassambara et al., 2021). The figures were created with "survminer" package and "ggplot2" package (Wickham, 2016).

3. Results

3.1. Adult bee' mortality

The effect of the in-hive treatments on the survival of newly emerged adult honeybees was tested in hoarding cages. Sixty nine percent (551 of the 800) of honeybees died before the end of the trial. Honeybees flickered from treated colonies showed a higher mortality rate than those from control colonies (log-rank, df = 3, $\chi^2 = 32.06$, $p < 0.001$), with [TH] (z = 5.33, p < 0.001), [PZ + TH] (z = 4.29, p < 0.001) and [PZ] (z = 2.78, $p = 0.005$). Pairwise comparison revealed significant difference between the survival of the [PZ] group and the [TH] group ($p = 0.013$), but not between [PZ] and [PZ + TH] ($p = 0.14$) and between [TH] and [PZ + TH] ($p = 0.27$), suggesting no synergistic effect co-administering the compounds. < The 50 % mortality in each group was reached after 17, 19, 20, and 22 days for [TH], [PZ + TH], [PZ] and [CTR], respectively (Fig. 1). At the end of the trial, the bees still alive in the hoarding cages were 7.8 ± 4.3 in the [CTR] group, 4.1 ± 3.1 in the [TH] group, 4.2 ± 4.1 in the [PZ + TH] group, and 5.6 ± 3.3 in the [PZ] group. The status of the colonies monitored over next spring showed no increased mortality.

3.2. Adult bee population assessment

The number of adult bees in [CTR] and [PZ + TH] colonies was compared at the beginning and the end of the treatment period (5 weeks). The three control colonies showed a variation of +45.1 %, +44.1 % and + 3.6 % in the estimated number of adult bees, while the treated colonies of −1.9 %, +27.4 % and − 6.3 % (Fig. 2). The statistical analysis revealed no effect of [PZ + TH], but the control colonies showed an increasing population trend ($p = 0.15$), while [PZ + TH] treated colonies did not show variations in the population comparing both times ($p = 0.64$).

3.3. Morphological analysis of the midgut

To investigate a possible effect of [PZ], [TH], and a mix of [PZ + TH] on the midgut, we performed a morphological analysis by comparing samples from treated larvae (Figs. 3D, G, J), newly emerged bees (Figs. 3E, H, K), and forager bees (Figs. 3F, I, L) with control insects [CTR] (Figs. 3A, B, C). For all the developmental stages analysed, no alteration in the general organization of the epithelium was identified as result of the exposure to the treatments. In detail, enterocytes showed a typical structure, with euchromatic nucleus, regular basal lamina, and apical membrane characterised by an intact, well-developed brush border. No features attributable to cell death processes were detected. In addition, stem cells were organised in compact nests at the base of the epithelium and no evidence of proliferation was observed. An ultrastructural analysis was performed to verify the absence of negative effects on gut tissues after the administration of [PZ], [TH], or [PZ + TH]. TEM analysis confirmed the integrity of the gut epithelium and no alteration of cell organelles was observed. Specific features of enterocytes, such as long and intact microvilli (Figs. 4A-D) and organised cytoplasm with developed rough endoplasmic reticulum (Figs. 4E-H), were comparable in control and treated insects. Moreover, an intact basal lamina (Figs. 4M-P) and stem cell nests localised in the basal region of the epithelium (Figs. 4I-L) were present in all the samples analysed.

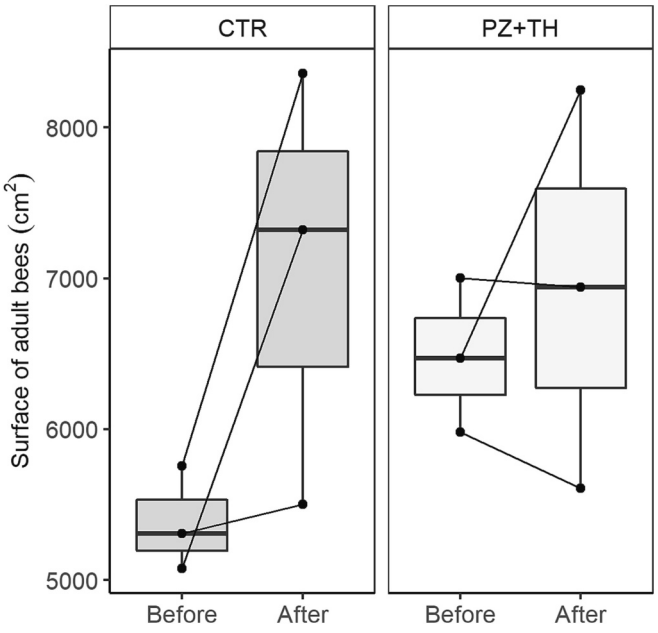

Fig. 2. Surface of adult bees. Surface occupied by bees in the control ([CTR], *n* = 3) and in the Penconazole Thiacloprid treated hives ([PZ] + TH, n = 3) before and 5 weeks after the administration of the contaminated syrup. The area was calculated from pictures of the frames taken at dawn.

3.4. NGS results on pollen sources

A total of 64 gut (midgut + rectum) samples (18 for [CTR], 12 for [PZ], 16 for [TH] and 18 for [PZ + TH]) were sequenced on an Illumina MiSeq platform after amplification with primers targeted to the ITS2 sequence. 10,139,848 million raw reads were obtained from sequencing, with an average of 65 k paired sequences per sample. All analysed samples were rarefied at 31,840 reads, beyond the plateau curve. Elaborated data on the relative abundance at class, family and genera taxonomical level are reported in Tables S4-S6 for fungi and in Tables S7-S9. The biodiversity of the analysed fungi and plants was evaluated through α- and β-diversity indexes. α-diversity metrics (Chao1 and observed OTUs) did not show any significant difference among experimental conditions. Regarding β-diversity, the Bray Curtis analysis showed significant differences among experimental conditions. In particular, [CTR] *vs* [PZ] and [CTR] *vs* [PZ + TH] resulted significant for both parametric and non-parametric analysis ($p < 0.05$). About 85 % of the obtained ITS sequences belonged to fungi, whereas the remaining were classified as plants, allowing the identification of the pollen sources. Concerning plants, the phylum Eudicotiledonae 65.81 % and Streptophyta 3.38 % were the most representative although a considerable fraction of plants was unidentified (unidentified_plants 30.81 %). Families belonging to Eudicotiledonae were mainly Araliaceae (51.71 %), Asteraceae (11.52 %), and other_Eudicotiledonae (36.77 %). The identified genera were *Hedera sp.* (45.25 %) and *Artemisia sp.* (8.08 %). In [PZ] and [PZ + TH] the total number of *Hedera sp.* reads significantly increased if compared to [CTR] from 34.9 % to 68.2 % in [PZ] and 54.9 % in [PZ + TH] ($p < 0.05$) (Fig. 5A). Artemisia and all the other identified plant species did not show any significant difference among

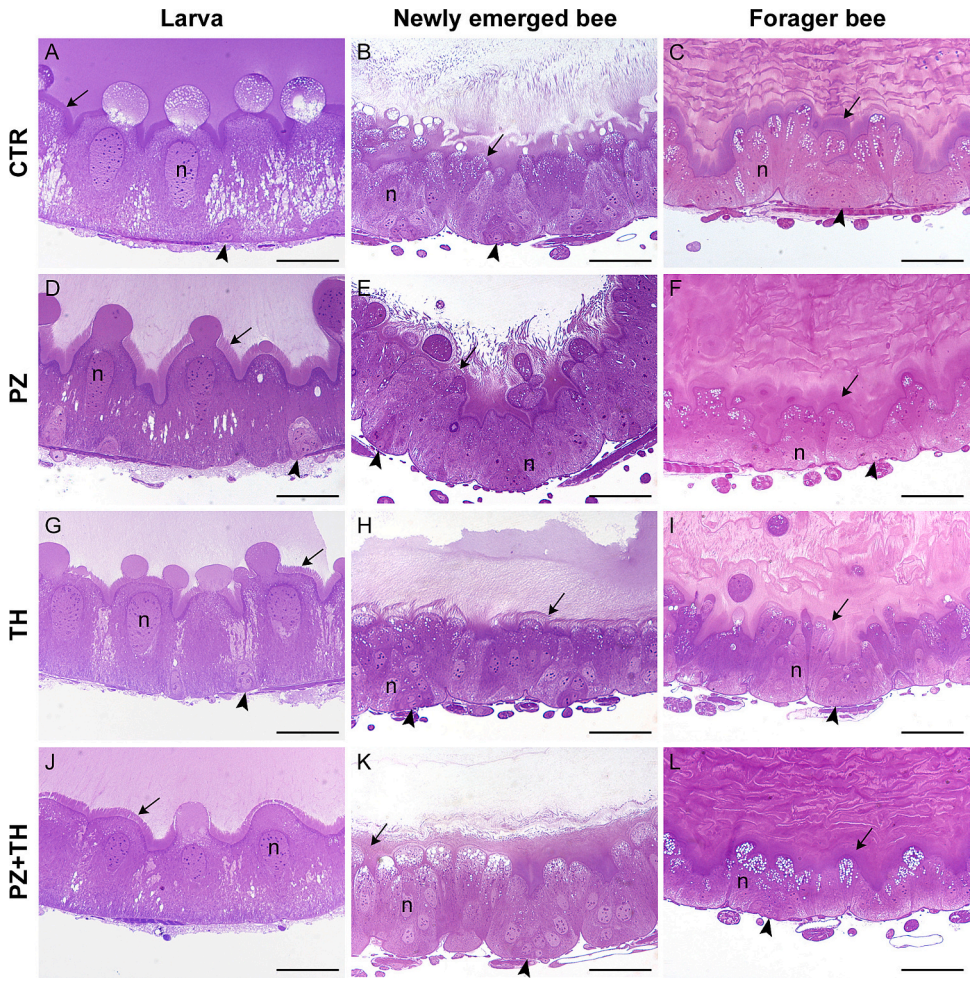

Fig. 3. Morphological analysis - Optical microscopy. Cross sections of midgut epithelium from larvae (A, D, G, J), newly emerged bees (B, E, H, K) and forager bees (C, F, I, L) exposed to penconazole [PZ] (D, E, F), thiacloprid [TH] (G, H, I) and a mix of both chemicals [PZ + TH] (J, K, L). Arrows: brush border; arrowheads: stem cell nests; [CTR]: control insects; n: nucleus. Bars: 50 μm.

experimental groups (Figs. 5B-C).

3.5. The gut microbiome: Absolute quantification of core bacteria with qPCR

Total bacteria amount was Log 8.23 ± 0.24, 8.03 ± 0.35, 8.20 ± 0.30 and 8.31 ± 0.35 rRNA copies/intestine for [CTR], [PZ], [TH] and [PZ + TH], respectively, and no significant variations were detected among experimental conditions (Fig. 6A). The main parameters, *i.e.* average slope, intercept, R^2, and melting temperatures (T_m °C), regarding qPCR analyses of total bacteria, yeasts and core microbial taxa are reported in Table S2.

At genus level, *Bartonella* (Fig. 7D) showed a decreasing trend in [TH] and [PZ] *vs* [CTR], but only the comparison of [TH] *vs* [CTR] was significant ($p < 0.05$) with a decrease of about 0.5 Log in the [TH] experimental conditions. Surprisingly, the combination of [PZ + TH] increased *Bartonella* amount although data were not significant when Bonferroni's correction was applied. Consequently, the comparisons [TH] *vs* [PZ + TH] and [PZ] *vs* [PZ + TH] resulted significant ($p < 0.01$).

Bombilactobacillus (Fig. 7B) showed a non-significant reduction in [TH] with average Log 5.89 ± 0.57 CFU/intestine when compared to the [CTR] (average Log 6.09 ± 0.53 CFU/intestine). [PZ] was not significantly different compared to [CTR], as well as the comparison [PZ + TH] *vs* [CTR], but [TH] showed a significant increase of *Bombilactobacillus* to Log 6.17 ± 0.57 CFU/intestine ($p < 0.01$) when compared *vs* [PZ + TH]. A significant increase in the *Lactobacillus* (Fig. 7C) load in the experimental conditions treated with the fungicide penconazole was detected ([PZ] *vs* [CTR] $p < 0.05$ and [PZ + TH] *vs* [CTR] $p < 0.1$, respectively). [PZ] showed an average count of Log 6.31 ± 0.40 CFU/intestine and [PZ] + TH] a log 6.27 ± 0.37 CFU/intestine whereas the [CTR] showed an average of Log 6.14 ± 0.24 CFU/intestine. [TH] reduced the *Lactobacillus* count per gut to Log 5.97 ± 0.58, even if not significant when compared to the [CTR]. However, the comparison [TH] *vs* [PZ + TH] was also significant ($p = 0.05$). *Bifidobacteria, Commensalibacter, Frischella, Gilliamella*, and *Snodgrassella* (Figs. 7, E, F, G, H) did not show significant variation among experimental groups; average values and standard deviations are reported in Table S3.

3.6. The gut microbiome: Fungal analysis with qPCR and NGS

qPCR quantification of total yeasts in the honeybee gut (midgut+recutum) collected from honeybees colonies, (Fig. 8) showed a higher content of yeasts comparing [CTR] *vs* [PZ] (from 7.08 ± 0.43 of [CTR] to 7.17 ± 0.27 of [PZ], $p < 0.05$) and [CTR] *vs* [PZ] (from 7.08 ± 0.43 to 7.22 ± 0.32 in [CTR] and [PZ + TH], respectively, $p < 0.05$).

NGS results identified 4 major fungal Classes: Saccaromycetes (95.53 %), Microbotriomycetes (2.51 %), Dothideomicetes (0.79 %), Leothiomicetes (0.43 %), Class_other (0.74 %), see Fig. S1. Among these the yeast families belonging to Saccaromycetes were Saccaromycodaceae (86.86 %, Fig. 9), Metschnikowiaceae (0.58 %, Fig. 9D) and Family_Others (12.56 %). In Microbotriomycetes the most abundant family was Microbotriaceae (4.53 %, Fig. 9B). Among microbial genera,

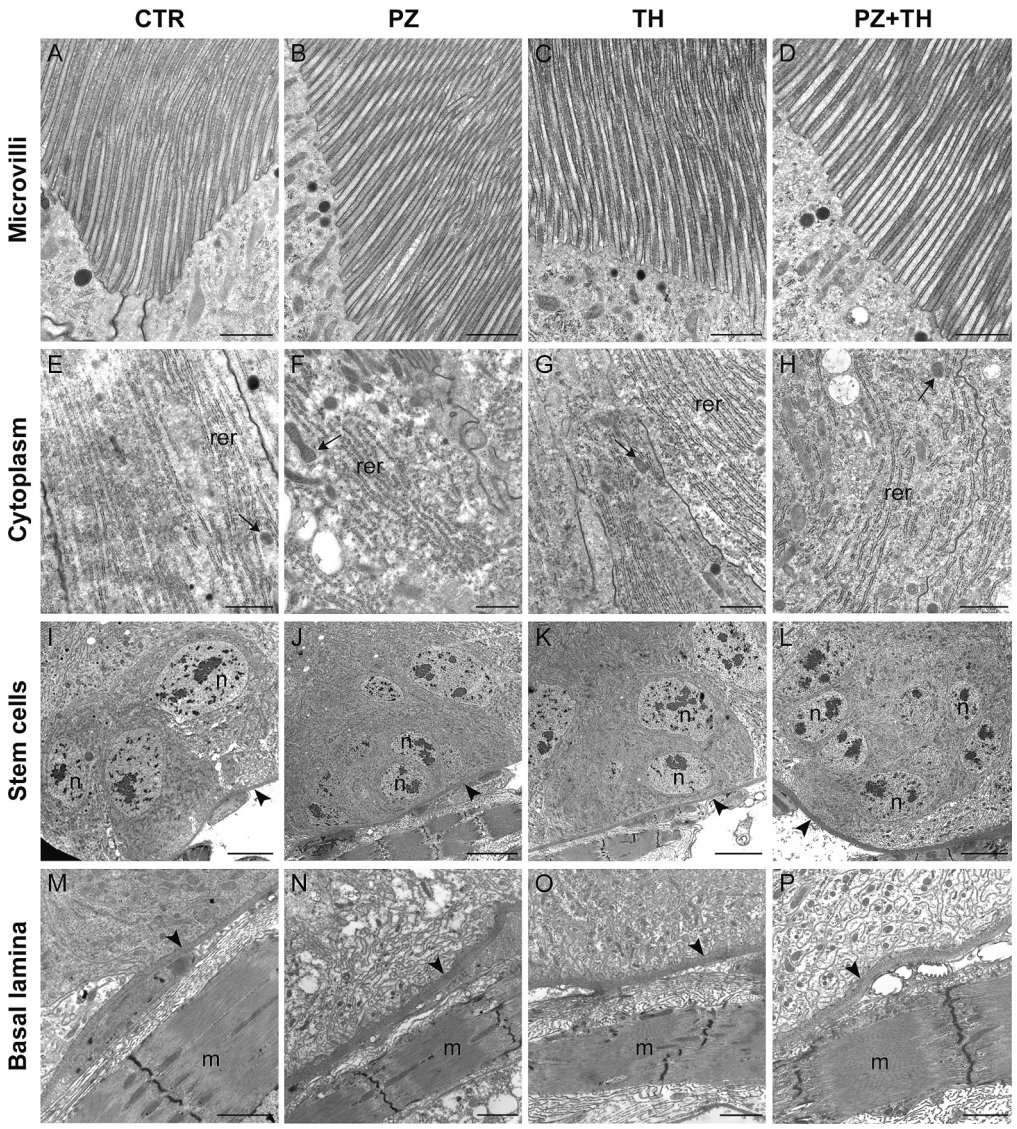

Fig. 4. Morphological analysis - Transmission electron microscopy. Ultrastructure of microvilli (A-D), cytoplasm (E-H), stem cells (I-L), and basal lamina (M-P) in midgut samples of control bees (A, E, I, M) and bees exposed to [PZ] (B, F, J, N), [TH] (C, G, K, O), and [PZ + TH] (D, H, L, P). Forager bees are shown as a representative stage for TEM analysis. Arrows: mitochondria; arrowheads: basal lamina; m: muscle; n: nucleus; rer: rough endoplasmic reticulum. Bars: 1 μm (A-D, F, G, J), 2 μm (E, H, M-P), 5 μm (I, K, L).

Hanseniaspora represented 86.23 % of total sequences, *Microbotriom* 4.51 %, *Botrytis* 0.88 %, *Metschnikowia* 0.57 %. The Other_genus cluster was represented at 7.81 %. No statistically significant differences among the experimental groups were evidenced at any taxonomical level, showing that the tested compounds did not affect fungi and yeast community in the honeybee gut.

4. Discussion

Contamination of nectar and pollen sources from pesticides is a widespread phenomenon (David et al., 2016; Favaro et al., 2019). However, the interaction between different PPP categories on honeybees is still being explored (Schuhmann et al., 2022). Toxicity tests on xenobiotic molecules are mostly carried out in laboratory cage conditions, which may alter the results, since they do not allow interactions within colony mates and structure, as well as with the external environment (Baffoni et al., 2021). In this study, a field realistic approach was used to investigate the effects of a fungicide, an insecticide, and the mixture of them on honeybee survival and foraging preference, and on the gut ecosystem (epithelium morphology and microbiota composition).

At colony level, no effect of thiacloprid [TH] in combination with penconazole [PZ] on the total number of honeybees was detected. However, colonies exposed to the pesticide mixture showed lower population growth compared to non-exposed colonies. This is in partial agreement with other studies (Alberoni et al., 2021b), reporting negative effects on the adult honeybee's growth following exposure to pesticides. The effect on the total number of honeybees is clear, although differences in the number of individuals of the assayed populations were detected at the beginning of the experiment. Therefore, the exposure to the pesticides may have influenced the colony growth dynamic. It is worth noting that all the colonies survived the trial and the winter, and no drawback effect was reported on the following spring. The pesticide effect on the mortality was much more evident when single honeybees were studied. Toxicity assessment usually involves the administration of contaminated feed to healthy honeybees in cage (Medrzycki et al., 2013), to study potential effects of single pesticide or mixture (Sgolastra et al., 2017; Yao et al., 2018; Wang et al., 2020; Pal et al., 2022). The results of these studies are however not consistent with those of the field trials. The numerous attempts made thus far to evaluate the potential

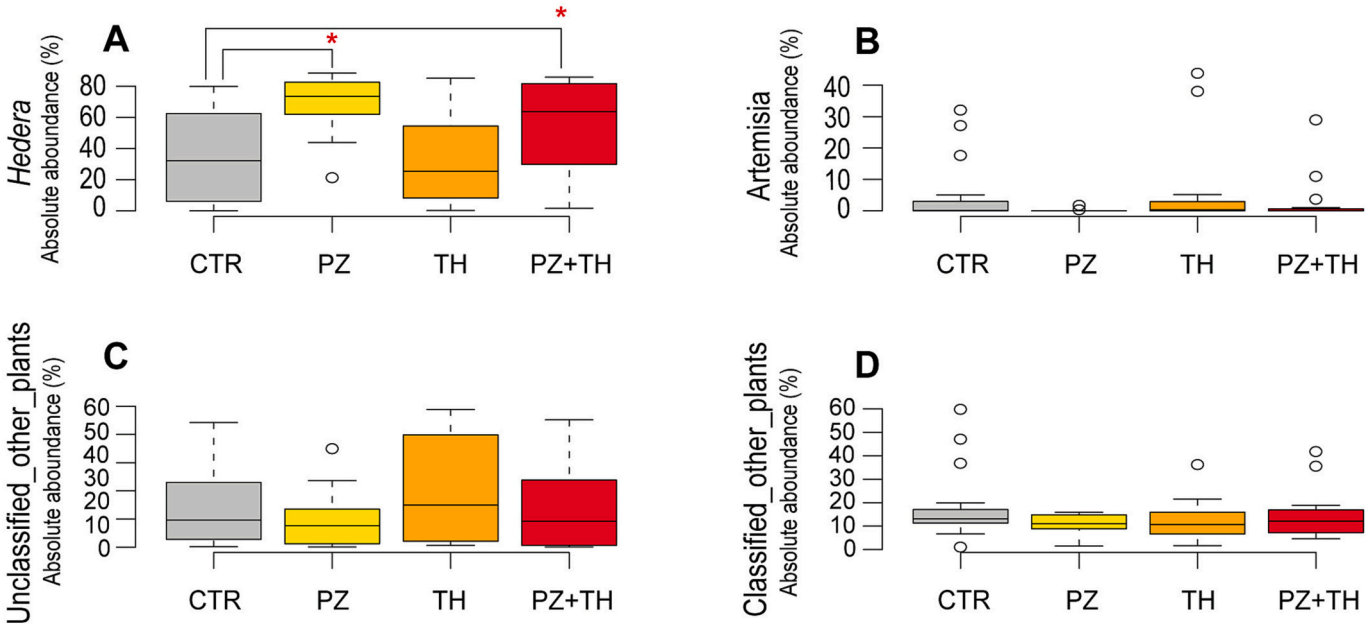

Fig. 5. Box plots reporting the major plant genera found in the pollen ingested by honeybees and expressed for their relative abundance in %, and in relation to experimental conditions. Plant taxa described: (A) *Hedera sp.*; (B) *Artemisia sp.*; (C) Unclassified_other_plants; (D) Classified_other_plants (below 1 % relative abundance); Experimental conditions: [CTR] control, [PZ] penconazole, [TH] thiacloprid and [PZ + TH] penconazole + thiacloprid. Asterisks report statistical significance of the treatment comparing with control ($*p < 0.05$).

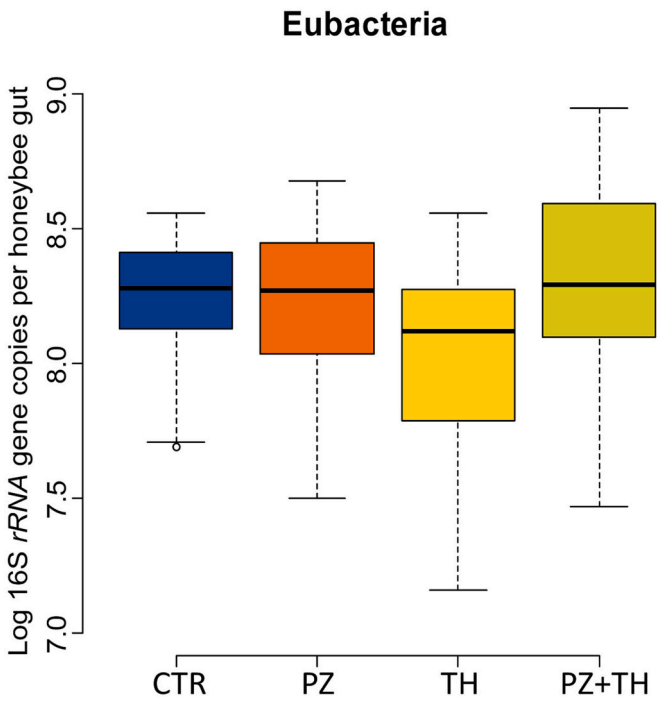

Fig. 6. Total bacteria (Eubacteria) expressed as Log 16S rRNA copies/intestine; Experimental conditions: [CTR] control, [PZ] penconazole, [TH] thiacloprid and [PZ + TH] penconazole + thiacloprid.

effects of honeybee exposure under field scenarios have not detected any appreciable changes in colony performance, and the outcomes of toxicity evaluations in the laboratory and in the field diverge (Henry et al., 2015). Various reasons have been proposed to explain such a discrepancy, but it seems that honeybee colonies' resilience brought on by systems for population control and honey storage may offset effects in the field. The key appears to be the group homeostasis. Lattorff (2022) showed that once caged, the individual honeybees experience higher stress. Workers in cages displayed higher heat shock response expression levels and lower trehalose-related haemolymph titres. These findings show that the absence of a social environment, such as a queen or a large enough group, causes stress in caged bees. This stress may interact with other stressors, such as pesticides, causing the higher mortality typically seen in the wild (Lattorff, 2022). Furthermore, it has been proposed that the quality of the diet can influence the ability of bees to metabolize certain pesticides and withstand their detrimental effects (Barascou et al., 2021a). Although additional parameters, such as behavioural and reproductive endpoints are proposed to be integrated in the regulatory toxicological bioassays (Barascou et al., 2021b), the mortality results here presented are still a significant indication of the pesticides effect on the adult honeybees. Remarkably, these bees were exposed only at the larval stage, but it seems to be enough to provide a substantial impairment. Whether early lethal effects might have affected the brood already in the honeybee colony prior to adult emergence has not been investigated. However, only considering the adult bees, our findings are in line with the few other studies that assessed the adult survival after a larval stage exposure. Shi et al. (2020) observed a lower adult survival after a continuous exposure to acetamiprid (the other chloronicotinyl insecticide) from the larval stage. A synergistic interaction of thiamethoxam exposure at larval stage and *Nosema* resulted in higher adult mortality rate in Tesovnik et al. (2020). In Tadei et al. (2019), the repeated larval exposure to the insecticide clothianidin and the fungicide pyraclostrobin reduced the adult longevity of Africanised *Apis mellifera*. Therefore, the validation of this field-realistic methodology for PPPs exposure to honeybees represents a remarkable tool to complement laboratory assays.

A variety of damages and morphological alterations in the midgut epithelium of honeybees following the exposure to different PPPs were previously reported (de Castro et al., 2020; Pervez and Manzoor, 2021; Serra et al., 2023). For example, an increased vacuolization of the cytoplasm, a high number of cellular protrusions, autophagosome formation, and disorganised microvilli were described after the exposure of honeybees to carbaryl, imidacloprid, iprodione, and chlorpyrifos in cage conditions (Carneiro et al., 2020, 2022; Pervez and Manzoor, 2021). Differently, in our field test, no appreciable changes of midgut

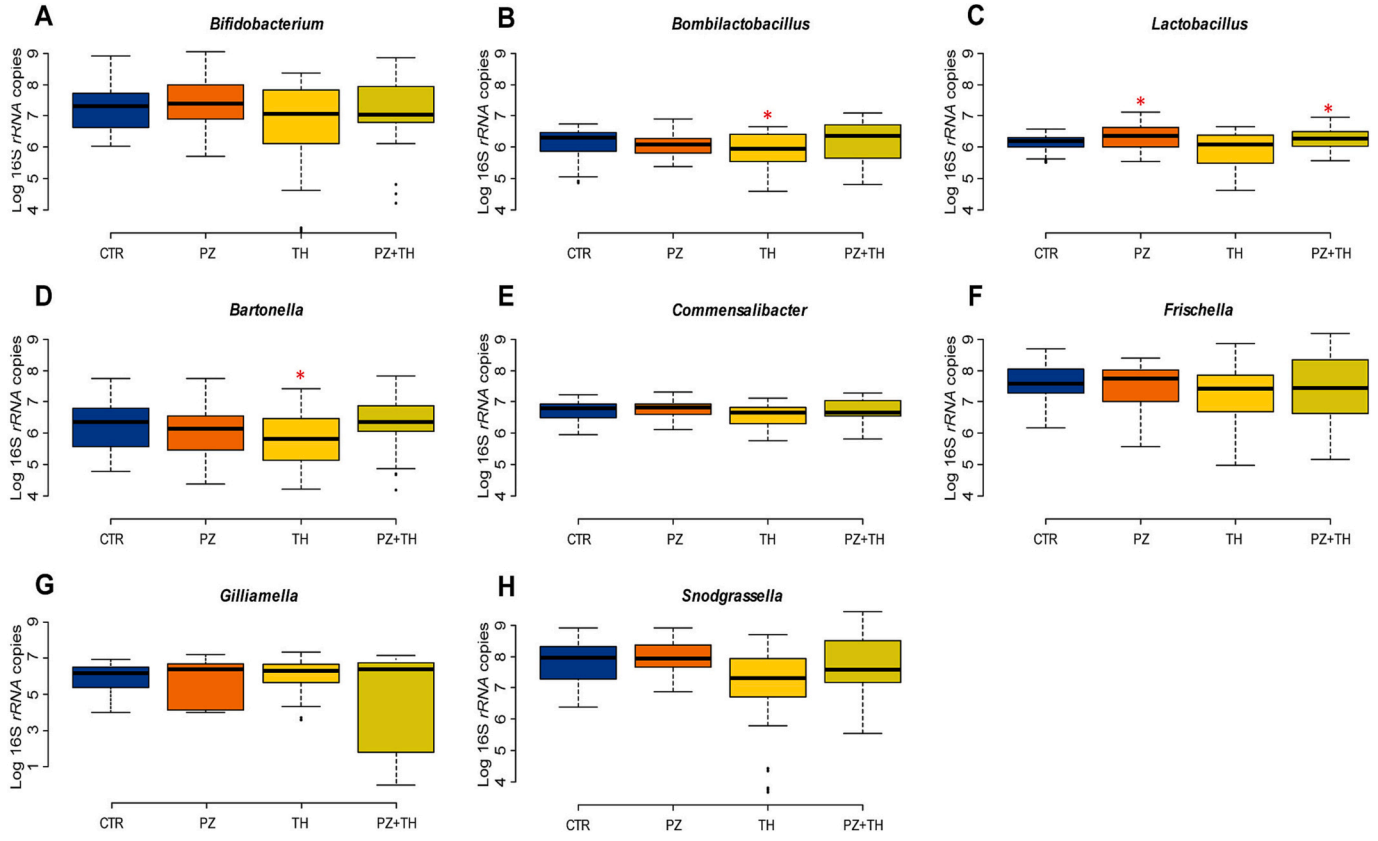

Fig. 7. Box plots reporting the major microbial genera analysed in qPCR expressed for their absolute abundance of the 16S rRNA gene per bee gut (expressed in Log), and in relation to experimental conditions (significant pairwise comparisons *p < 0.05; ***p < 0.01). Microbial taxa described: (A) *Bartonella spp.*; (B) *Bombilactobacillus spp.*; (C) *Lactobacillus spp.*; (D) *Bartonella spp.*; (E) *Commensalibacter spp.*; (F) *Frischella spp.*; (G) *Gilliamella spp.* and (H) *Snodgrassella spp.* Experimental conditions: [CTR] control, [PZ] penconazole, [TH] thiacloprid and [PZ + TH] penconazole + thiacloprid.

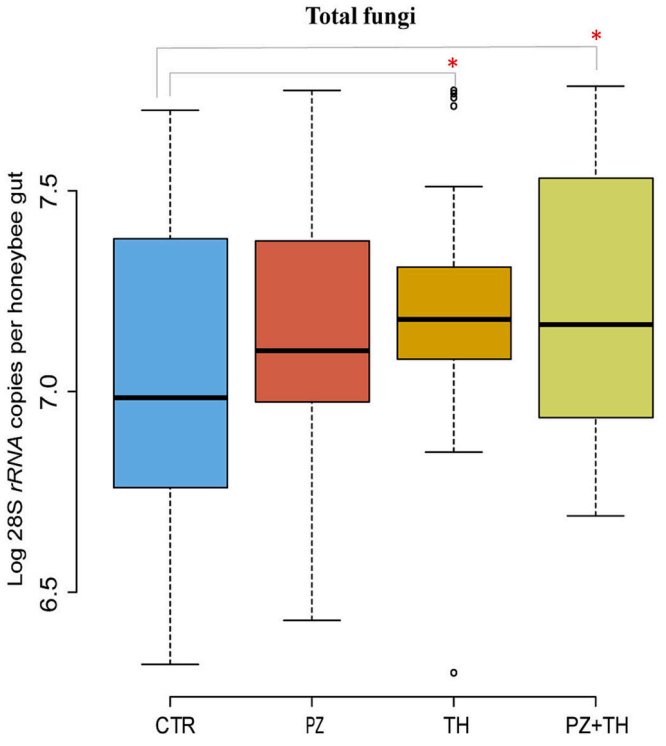

Fig. 8. Total yeasts expressed as Log ITS2 copies/intestine; Experimental conditions: [CTR] control, [PZ] penconazole, [TH] thiacloprid and [PZ + TH] penconazole + thiacloprid. * $p < 0.05$.

epithelium from larvae, newly emerged honeybees, and forager honeybees exposed to the tested PPPs were detected. In detail, the fine morphological analysis performed herein, which combined optical and electron microscopy, definitely confirmed all the features of a healthy epithelium and excluded alterations in this tissue. In particular, the proliferation of stem cells, which usually indicates the occurrence of repairing mechanisms to restore the integrity of the gut epithelium if damaged (Caccia et al., 2019), was excluded. Although a proper comparison with the current literature is not easy since some previous studies have adopted inadequate processing methods of this organ, likely leading to technical artifacts, the lack of effects on the morphology of the epithelium demonstrated in our study could be due to: i) the specific action of PPPs. Insecticides can elicit lethal and sublethal effects on honeybees by affecting molecular targets related to processes involved in cognitive functions, behaviour, or maintenance of physiological functions (Bonnafé et al., 2017), such as thermoregulation and muscle activity (Belzunces et al., 2012). In particular, thiacloprid was found to affect the expression of genes involved in detoxification metabolism, development, and immunity in newly-emerged adult honeybees, with the consequent reduction of survival and delay of honeybee development (Li et al., 2022; Lv et al., 2023). In addition, a dose dependent effect on gut microbiota (and on insect survival) of middle-aged honeybees by this neonicotinoid was reported (Liu et al., 2020). Accordingly, the midgut epithelium of honeybees might not be a specific target of [TH] and [PZ]; ii) the ability of honeybees to self-medicate from damages by changing the diet (Simone-Finstrom and Spivak, 2012; Abbott, 2014). An example of diet change was described by Elizabeth Deeter et al. (2023), who showed how honeybees exposed to pesticides shifted their foraging activity from pollen with a lower fat content to pollen with a different nutritional profile to restore lipid

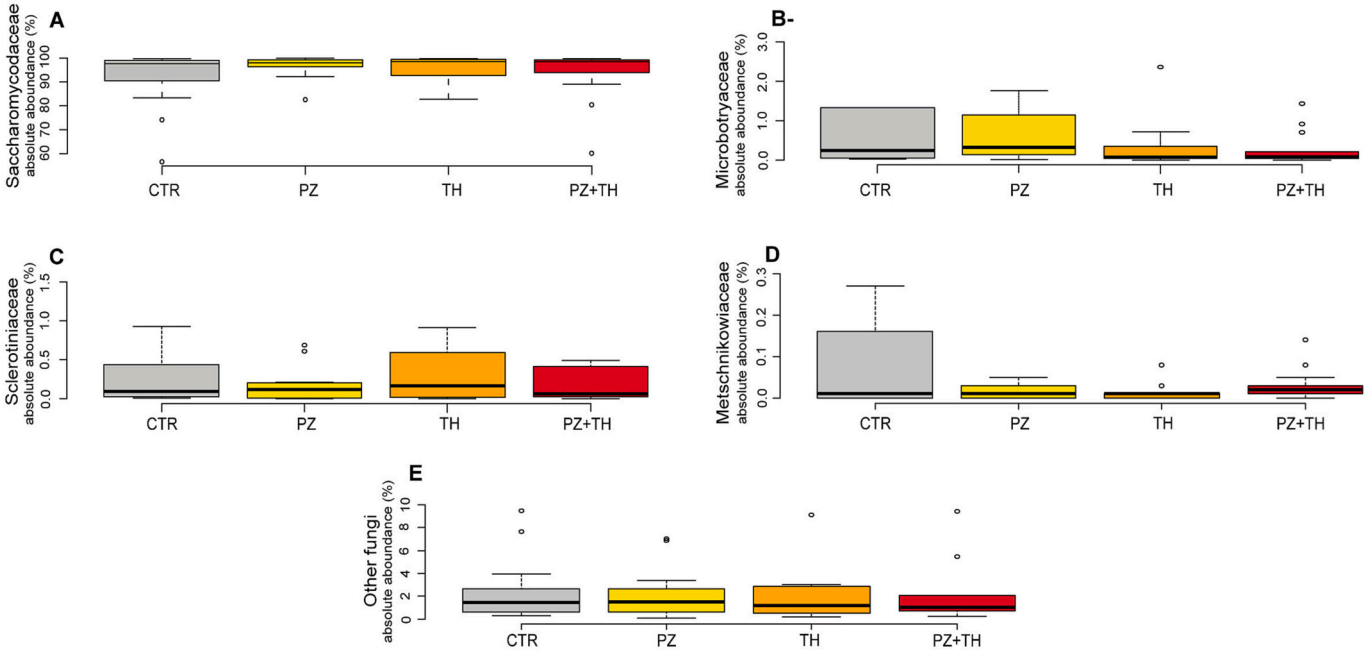

Fig. 9. Box plots reporting the major microbial genera expressed for their relative abundance in %, and in relation to experimental conditions. Outliers were removed in the figure plotting, for a full data view see Table S5. Microbial taxa described: (A) Saccharomycodaceae; (B) Microbotryaceae; (C) Sclerotiniaceae; (D) Metschnikowiaceae; (E) Other fungi unclassified; Experimental conditions: [CTR] control, [PZ] penconazole, [TH] thiacloprid, and [PZ + TH] penconazole + thiacloprid.

homeostasis after PPP exposure. This confirms that some PPPs can directly or indirectly modify the foraging attitude of honeybees. Pollinators in general have adapted to assess resource quality, especially the protein-lipid ratio to balance nutritional deficiencies (Vaudo et al., 2016; Vaudo et al., 2020; Elizabeth Deeter et al., 2023). However, to the best of our knowledge, this aspect has never been verified on self-restoring of epithelial damages in honeybees, with an exception for the exoskeleton that was restored with nectar containing a higher content of abscisic acid (Negri et al., 2019; Ramirez et al., 2017). The change of diet observed in the present study is significant, as shown by NSG results, which allowed us to determine the botanical origin of the pollen ingested by honeybees. Penconazole, alone or in combination with thiacloprid, significantly altered the pollen foraging preference, with honeybees that preferred *Hedera* pollen. According to Sagona et al., 2017, *Hedera* pollen showed the highest content of Fatty Acids (FAs) if compered to pollen with known high nutritional values such as Cestnut (*Castanea setiva*) and *Rubus,* analysed in the same work. In particular, *Hedera* pollen showed the highest tricosanoic acid content (52.5 g/kg of total FAs) when compared to the other pollens analysed, and α-linolenic acid as the main FA (270.9 g/kg of total FAs) (Sagona et al., 2017). The obtained data are supported by the findings of Elizabeth Deeter et al. (2023), where intoxicated honeybees shifted their diet to pollens with a higher lipids content. The nutritional profile of the pollen offers different recovery chances to honeybees, as already demonstrated for *Rubus* and *Chestnut* pollens in the containment of mortality due to infection of *N. ceranae* by Di Pasquale et al., 2013. Therefore, the role of pollen in the recovery of honeybees exposed to PPPs should be taken into account in the pesticides risk assessment performed by researchers and governmental agencies. Also, a possible explanation of the foraging and diet change might be found in the altered olfactory perception of natural resources, as it was shown for neonicotinoid pesticides (Li et al., 2015; Favaro et al., 2022).

The different feeding may also have consequences on the gut microbiome profile. In this study, the absolute abundance of bacteria assessed in qPCR confirmed the results obtained in Alberoni et al. (2021a), showing a general decrease in bacteria in honeybees treated with thiacloprid. The same field work showed that imidacloprid can severely affect the gut microbiome of honeybees, significantly decreasing *Bartonella*, bifidobacteria and *Lactobacillus*, whereas thiacloprid showed a milder impact when compared to the control group at the same sampling time. In the present study, we relied on qPCR to obtain an absolute quantification of the core microbial members. This analysis showed a significant reduction of *Bartonella* and *Bombilactobacillus* which are involved in biochemical processes related to nitrogen metabolism (*e.g.*, urea degradation) and polysaccharide digestion (Alberoni et al., 2022). *Bombilactobacillus* has already been highlighted as a particularly sensitive strain towards exposure to xenobiotics and antibiotics (Motta et al., 2018; Baffoni et al., 2021). Similar mild chronic exposure to other PPPs such as imidacloprid, glyphosate, and difenoconazole did not perturb the core gut microbiome of cage honeybees (Almasri et al., 2022; Raymann et al., 2018). The acaricide chlorothalonil, used against *Varroa destructor*, was also found to alter the composition of the gut microbiota (Kakumanu et al., 2016); however, the same authors also highlighted a reduction of few transient non-core bacterial species (*e.g. Serratia*, *Acetobacter* and *Klebsiella*) and the protective role of the gut microbiome against pesticides damages to honeybees. The fungicide penconazole, alone or in combination with thiacloprid, significantly increased *Lactobacillus*, probably due to resistance mechanisms, as already observed with the fungicide mixture pyraclostrobin and boscalid (DeGrandi-Hoffman et al., 2017). Moreover, some *Lactobacillus* strains are known to degrade some PPP molecules, gaining additional carbon sources from them (Kumral et al., 2020). Overall, the combined effect of penconazole and thiacloprid on gut microbiome was not as severe as expected. Moreover, as discussed above for the epithelium morphology, the self-restoring ability of honeybees can be postulated to positively influence the gut microbiome as well.

Surprisingly, our work detected a significant increase of the total fungi count in honeybees treated with the insecticide thiacloprid, also in combination with the fungicide penconazole. There is limited information on the effects of thiacloprid on fungi in the literature, apart from its degradation by a *Rodotorula* strain *in vitro* and in soil (Dai et al., 2010), although not related to honeybee metabolism. Also, there is evidence of resistance to penconazole in *Saccharomyces* strains (Jawich et al., 2006), or even an increase of fungal amount and virulence in the presence of

fungicides, which has been defined as "the fungal paradox" (Rohr et al., 2017). In agreement with these studies, the most represented fungal group is Saccharomycodaceae. Indeed, forager honeybees collected in all the experimental conditions were found to be mainly colonised by *Hanseniaspora* (Saccharomycodaceae), showing a very low yeast diversity. The high *Hanseniaspora* population is consistent with the findings of Callegari et al. (2021) on honeybees collected in Italy and *Metschnikowia* population is consistent with the findings of (Gaggìa et al., 2023), on honeybees collected in Malta. In these honeybees, a prevalence of other yeasts communities, among which *Hanseniaspora, Starmerella* and *Metschnikowia.* Conversely, differently from Yun et al. (2018), neither *Saccharomyces* nor *Zygosaccharomyces* were found.

5. Conclusions

Overall, the present study performed in-hive showed no particularly severe effects on honeybees upon administration of sublethal doses of thiacloprid and penconazole, apart from some significant alterations within the core bacterial groups and total fungi counts. However, our results indicated that honeybees may change their pollen foraging preference in the presence of agrochemicals. Whether this happens because of an altered olfactory perception, as already shown for neonicotinoid pesticides, or because of improved recovery potential due to the different nutritional profile of the pollen still has to be validated by specific tests. Our work shows the importance of the pollen nutritional profile in the evaluation of the pesticides risk assessment, especially at field conditions. Finally, our results support the fundamental role of field studies to obtain reliable results on the effects of synthetic agrochemicals on honeybees.

CRediT authorship contribution statement

RF carried out the field investigation. DB and GT morphological analysis. CB and PMG extracted the DNA from honeybees samples and analysed the gut microbiome in qPCR. DA and CB prepared the library for NGS sequencing. DA and LB carried out bioinformatics on the microbiome NGS raw data. CB analysed qPCR data. CB, LB and RF were involved in statistical analysis. RF, SA, GT and DDG were involved in the research design. DA, CB, RF, DB wrote the manuscript. SA, PMG, MPP, GT and DDG were involved in founding acquisition. All authors read and approved the final manuscript.

Ethic statement

The presented research work comply with the ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments, but also with Italian law that does not require and 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. All the used honeybees were female workers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

ITS sequence data have been submitted to the NCBI repository Sequence Read Archive (SRA) under the Bio project no PRJNA655184.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.167277.

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