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Neonicotinoids in the agroecosystem: In-field long-term assessment on honeybee colony strength and microbiome

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## Neonicotinoids in the agroecosystem: In-field long-term assessment on honeybee colony strength and microbiome

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### ABSTRACT

Bees can be severely affected by various plant protection products (PPP). Among these, neonicotinoid insecticides are of concern as they have been shown to be responsible for extensive honeybee colonies death when released into the environment. Also, sublethal neonicotinoid doses contaminating single honeybees and their colonies (e.g. through contaminated pollen) are responsible for honeybees physiological alterations with probable implication also on microbiome functionality. Honeybees show symbiotic interactions with specific gut bacteria that can enhance the adult host performances. Among the known mechanisms, the modulation of the immune system, the degradation of recalcitrant secondary plant metabolites, pollen digestion, and hormonal signaling, are the most important functional benefits for the host honeybee. To date, few research efforts have aimed at revealing the impact of PPP on the gut microbial community of managed and wild honeybees. The majority of the existing literature relays on cage or semifield tests of short duration for research investigating neonicotinoids-gut microbiome interactions. This research wanted to unravel the impact of two neonicotinoids (*i.e.* imidacloprid and thiacloprid) in natural field conditions up to 5 weeks of exposure. A long-term impact of neonicotinoids on gut microbial community of honeybees was observed. The alterations affected several microbial genera and species such as *Frischella* spp., lactobacilli and bifidobacteria, whose shifting is implicated in intestinal dysbiosis. Long-term impact leading to dysbiosis was detected in case of exposure to imidacloprid, whereas thiacloprid exposure stimulated temporary dysbiosis. Moreover, the microbial diversity was significantly reduced in neonicotinoid-treated groups. Overall, the reported results support a compromised functionality of the gut microbial community, that might reflect a lower efficiency in the ecosystemic functionality of honeybees.

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### 1. Introduction

The European honeybee, *Apis mellifera*, is an important pollinator of several plant species and provides a crucial ecological and ecosystem service both for agricultural food production and for wild plant diversity and conservation (Saunders et al., 2018). In the last decade, the occurrence of high colony losses has raised the attention of the scientific community on bee health. The parasitic mite *Varroa destructor*, together with various viruses (e.g. deformed wing virus, acute bee paralysis virus), bacteria (e.g. *Paenibacillus larvae*, *Melissococcus plutonius*, *Serratia* spp.) and the microsporidian *Nosema ceranae* certainly contribute to colony collapse. However, there is a consensus among scientists concerning the combination of biological and environmental factors that

leads to premature colony mortality by adversely affecting the colony health and its lifespan, considering each colony as a superorganism (Shi et al., 2020). Abiotic factors include climate change, habitat loss, pesticides and genetically modified crops (Alberoni et al., 2016; Blacquière et al., 2012). Within abiotic factors, much attention has been addressed to agrochemicals that are applied for pest management in a variety of agricultural crops (Favaro et al., 2019; Hladik et al., 2016; Van der Sluijs et al., 2013). Agrochemicals may be responsible for weakening honeybees making them more susceptible to diseases, adverse climate conditions and nutritional stresses, or for affecting their learning ability (Qi et al., 2020; Brandt et al., 2016; Blacquière et al., 2012; Decourtye et al., 2003). Neonicotinoids are synthetic insecticides developed in the 1990s with a chemical structure similar to nicotine, a naturally occurring insecticide that targets nicotinic acetylcholine receptors in the insect nervous system. They are effective against a wide range of sucking and chewing insects and can be applied either as foliar spray or seed coating, showing a systemic capacity. Among them, imidacloprid is most widely used insecticide in the world, being registered in early 1990s for cotton, rice, cereals,

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peanuts, potatoes, vegetables, pome fruits, pecans and turf, while thiacloprid was registered in 2003 for use on cotton and pome fruits (Fairbrother et al., 2014; Maxim and van der Sluijs, 2013; Wang et al., 2008). Of particular concern is the potential exposure of pollinators, considering that their distribution throughout the plant, including pollen, nectar, and guttation fluids (Hrynko et al., 2019; Fairbrother et al., 2014; Girolami et al., 2009). In 2013, the European Food Safety Authority (EFSA) has released reports assessing the risks to honeybees for 3 neonicotinoid insecticides, including imidacloprid (EFSA, 2013). The European Union, in the same year, placed a 2-year moratorium on the use of neonicotinoids while waiting for a more accurate characterization of risks related to the use of these chemicals (Auteri et al., 2017). In 2018, the European Parliament prohibited the use of imidacloprid, clothianidin and thiamethoxam, except for use in greenhouses.

Most of the studies on the toxicity of neonicotinoids on honeybees have been performed in laboratory conditions. However, several works have indirectly highlighted that physiological responses, when tested in a laboratory condition, can give conflicting results compared to natural field conditions. This also applies for pesticides risk assessment: some studies have failed to detect noteworthy change in colony performances at field level post neonicotinoids exposure (Cutler and Scott-Dupree, 2007; Pilling et al., 2013; Cutler et al., 2014). As an example, the neonicotinoid thiamethoxam was reported as extremely deleterious on honeybees at trace levels in laboratory trials whereas it did not decrease the performance of honeybee colonies near treated fields (Pilling et al., 2013). Similar observations were outlined by De Smet et al. (2017) with tests carried out both in cage and in field conditions exposing honeybees to imidacloprid dosages and testing immune response obtaining opposite results. Therefore, more studies in field effective conditions are envisaged to reconcile the conflicting laboratory and field assessments (Colin et al., 2019; Henry et al., 2015), regardless of what physiological effect or ecological niche is under study.

The importance of the gut microbial community for bee health has been highlighted only recently. The gut microorganisms display important functions for the host related to nutritional support, stimulation of the immune system and protection from pathogens (Baffoni et al., 2016; Alberoni et al., 2018). The relationship between microbial composition and host functionality has been outlined in several studies. *Acetobacteraceae*, *Lactobacillus* spp., *Bifidobacterium* spp., *Bartonella* spp., *Gilliamella* spp., *Snodgrassella* spp., *Frischella* spp. were shown to be involved in the digestion of monosaccharides (mannose, xylose, rhamnose, arabinose), polysaccharides, gut protection, weight gain and hormonal signaling (Engel et al., 2012; Kwong et al., 2017; Zheng et al., 2017; Zheng et al., 2019). The homeostasis of the insect microbial gut community can be compromised by environmental chemicals, including agrochemicals, leading to a reduced ability to process food and a reduced protection against pathogens. An example of gut microbial community perturbation by agrochemicals was recently reported by Motta et al. (2018), who highlighted that the weed killer glyphosate can decrease the abundance of *Snodgrassella* spp., lactic acid bacteria and bifidobacteria. Moreover, glyphosate can have a bacteriostatic effect on the honeybee gut microbial community, with a presumptive negative effect on commensal bacteria colonization of newly emerged honeybees which are therefore more susceptible to gut bacterial pathogens such as *Serratia marcescens* (Motta et al., 2018; Raymann et al., 2018a). Organophosphates and pyrethroids insecticides showed changes in the honeybee gut microbiome in the in-field study of Kakumanu et al. (2016). Concerning neonicotinoids, Di Prisco et al. (2013) showed an adverse effect of the clothianidin on honeybee antiviral defences leading to an uncontrolled proliferation of viral agents. Neonicotinoid exposure (imidacloprid, thiacloprid and fipronil) can also increase honeybees' susceptibility to gut pathogens, as for example *N. ceranae* (Vidau et al., 2011; Pettis et al., 2012), directly affecting microbial taxa populating the honeybee gut such as *Bifidobacterium* spp. (Zhang et al., 2019) or *Gilliamella* spp. (Rubanov et al.,

2019). Finally, imidacloprid has shown to directly modify microbial communities in other insects (*i.e. Drosophila melanogaster*) significantly increasing the abundance of specific taxa (*i.e. Acetobacter* spp. and *Lactobacillus* spp.) (Daisley et al., 2017).

Jia et al. (2015) tested the impact of imidacloprid in laboratory conditions at 3 different doses (0.005, 0.015 and 0.045 mg/l), reporting no significant differences on the gut microbiota composition. Liu et al. (2020) tested the impact of 4 different doses (0, 0.2, 0.6 and 2.0 mg/l) of thiacloprid in a cage assay, bees were sampled for 13 days and results showed that the gut microbiota, which was affected at the beginning of the experiment, was capable of recovering at day 13. Raymann et al. (2018b) designed a semi-field assay (partially performed in laboratory and partially in field), using imidacloprid at a dose of 500 µg/l. The neonicotinoid was administered once through sucrose syrup, then the honeybees were released in the experimental honeybee colony and sampled 3 and 5 days after exposure. With this experimental approach imidacloprid showed little or no impact on the size or composition of the gut microbiome of adult worker bees with already established gut communities, but imidacloprid confirmed its ability to shorten bees' life.

The present work aimed at studying the effect of two commonly used neonicotinoids, imidacloprid and thiacloprid, on the worker honeybee gut microbiota in open field conditions after acute and chronic exposure. To the best of our knowledge, this the first work investigating a long-term impact (up to 5 weeks) of neonicotinoids on the gut microbial community.

## 2. Materials and methods

### 2.1. Environmental condition description and experimental colonies set up

The field experiment took place 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 experiment was performed at the end of July 2017, two weeks after the end of chestnut trees blossoms, (the latest relevant nectar flow of the year in the area). That is the time when local beekeepers begin to provide supplementary sugar syrup to sustain colonies. The apiary was located in the forest, and orchards (mainly vineyards and apples) are apart in the lower valley, at 1 km flight distance. Suitable conditions to perform a field trial with no external interferences were therefore assured by distance and seasonality: in late summer, the main pesticide treatments in orchards are fungicides and not anymore insecticides. Moreover, the local regulation imposes a continuous mowing in orchards, to avoid pollinators attraction and consequent poisoning. Fifteen honeybee (*Apis mellifera* subsp. *carinica* (Pollmann)) colonies were created by the shook swarm method in May 2017 from healthy colonies managed according to good beekeeping practice and were subjected to regular sanitary treatments against the parasitic mite *Varroa destructor* (Anderson and Trueman). 1.5 kg of adult bees were transferred in standard 10-frames Dadant-Blatt beehives for nomadic beekeeping with organic wax foundation (Il Pungiglione Soc. Coop., Mulazzo, Italy) and provided with new sister-queens. The colonies were sorted in three treatment group rows, each placed at 10 m distance one the other. After five days, they were treated with 50 ml of 3.5% oxalic acid dihydrate sucrose solution tickled in between frames for the control of *Varroa destructor* mites. Each colony was fed for three weeks with 6 l of sugar syrup (Apiinvert®). All the queens were accepted and the colonies development was assessed weekly. At the trial start (July 25, 2017), the brood was spread at least over four frames in each beehive. Each colony was provided daily with 500 ml (680 g) sugar syrup (Apiinvert®, Südcuker, Germany) through a rapid feeder (Il Pungiglione Soc. Coop.) for seven days. Insecticides were at first dissolved in acetone and then further diluted in water. Aliquots were stored at -80 °C. The two groups of colonies treated with neonicotinoids received imidacloprid 50 ppb (Sigma-Aldrich, CAS 138261-41-3) and thiacloprid 4500 ppb (Sigma-Aldrich, CAS 111988-49-9) contaminated syrup with 0.005% acetone.

Thiacloprid concentration was chosen based on the study of Tison et al. (2016), whereas Imidacloprid concentration was chosen according to Dively et al. (2015) and Meikle et al. (2016).

Control colonies were administered with sugar syrup also containing 0.005% of acetone. Treatment syrups were prepared fresh every day and administered in the evening. All colonies finished the syrup poured in the feeder within the following morning. The experimental hives were also equipped with scales for the weight monitoring using the Melixa hive monitoring system (Melixa srl, Italy), to assess the consumption and the storage.

## 2.2. Colonies strength assessment

The colonies strength was recorded on July 19, *i.e.* 6 days before the beginning of the neonicotinoid trial (T0) and on August 4, 3 days after one week of treatment (T1) according to the standard method in the BEEBOOK (Delaplane et al., 2013). The hives were opened at dawn, when there was no flight, and the frames were photographed at each side. The pictures were later analysed by using the software ImageJ (Rueden et al., 2017) Fiji version (Schindelin et al., 2012). The surface of adult bees, capped brood and open brood was considered for the strength evaluation (Fernandez Ferrari et al., 2020). Standard under baskets were placed in front of the hives to monitor the number of dead bees expelled by the colonies, and their number was recorded daily during the week of neonicotinoid administration (Human et al., 2013).

## 2.3. Honeybee gut sampling and DNA extraction

Honeybee guts were sampled at T0 beginning of the trial, after 10 days (T1), and after 5 weeks (T2) from forager honeybees carrying pollen. For each hive, 10 honeybees were sacrificed, and their midgut and rectum extracted and pooled. Moreover, a sixth pool for every these and time was picked harvesting two guts per hive belonging to the same treatment. 54 honeybee gut pools were obtained (18 per sampling time), corresponding to 540 honeybees sacrificed. Pools were conserved on ice and then stored at  $-80^{\circ}\text{C}$ . Samples were named with a code according to the picking time (T0, T1 and T2), and the treatment ([CTR] Control; [THIAC] Thiacloprid; [IMID] Imidacloprid), finally followed by the number of the hive in the experimental group, from 1 to 5 (*e.g.* T0\_THIAC\_5).

Microbial gut DNA was extracted with Quick-DNA™ Faecal/Soil Microbe Miniprep Kit (ZYMO Research), with some modifications of the manufacture protocol: gut pools were smashed with micropestles and then lysed with a mixture of different size glass beads (0.1–0.5 mm). Extracted DNA was eluted in Tris-HCl 10 mM and quantified according to Alberoni et al. (2018).

## 2.4. Quantification of target microbial groups

Total bacteria, *Lactobacillus* spp., and *Bifidobacterium* spp. were quantified with qPCR (StepOne™ Real-Time PCR System, Applied Biosystems) in control and treated samples. Specific primers, targeting 16S rRNA gene, for total bacteria Eub338F and Eub518R (Muyzer et al., 1993), *Lactobacillus* spp. Lac-F and Lac-R (Castillo et al., 2006) and *Bifidobacterium* spp. and BifTOT-R (Rinttilä et al., 2004) were used to amplify target regions in each sample. Triplicate 20  $\mu\text{l}$  reactions were carried out with Fast SYBR® Green Master Mix (Applied Biosystems). The quantification was based on standard curve obtained with serial dilutions ( $10^4$  to  $10^8$  copies) of the target amplicon and final regression lines calculated according to Lee et al. (2009).

Data were transformed to obtain the number of microorganism as Log CFU/intestine according to the rRNA copy number (Lee et al., 2009) for *Lactobacillus* spp., and *Bifidobacterium* spp. calculated according to rrnDB database (Stoddard et al., 2015). For total bacteria data were expressed as Log 16S rRNA copies/intestine.

## 2.5. NGS analysis of honeybee microbiota

16S rDNA was amplified with primers targeting the V3-V4 regions according to the Illumina protocol (Illumina, 2013) using PCR-BIO HiFi Polymerase (PCR-Biosystems). Libraries were prepared with Nextera XT indexing kit, purified with Agecount AMPure XP magnetic beads (Beckman Coulter), quantified with Qubit dsDNA HS Assay kit (Thermo Scientific) and sequenced on Illumina MiSeq platform. Bioinformatic analyses were performed with Qiime1, and representative OTUs blasted against the SILVA 132 database.

The phylogenetic tree was generated using `make_phylogen.py` (`fasttree`). Diversity analyses were performed with the script `core_diversity_analysis.py`.  $\alpha$ -Diversity was evaluated using Chao1, Observed OTU e PD whole tree metrics;  $\beta$ -diversity was evaluated using both weighted and unweighted UniFrac. The obtained rarefied biom table was then used to provide summary information of the representation of taxonomic groups within each sample at all taxonomic levels as relative abundances. OTUs having less than 0.1% abundance were removed.

## 2.6. Statistical analysis

The colony strength data were analysed using the Analysis of Variance (ANOVA) and linear mixed model (`lmer`) with package `lme4`. Data were reported as mean  $\pm$  standard deviation, unless otherwise indicated.

Statistical analysis for qPCR and NGS data was performed with R software (R Core Team, 2018) and packages `car`, `agricole`, `mulcomp`, `multcompView`, `lsmeans` and `dunn.test`. The analysis was performed considering the data normality and homoscedasticity, using ANOVA analysis for normal data and GLM procedure for non-normal data with normal distribution of residuals. Post-hoc test among different groups was carried and Bonferroni's correction was applied. The *post hoc* analysis of data took into consideration 9 comparisons considering the impact of each treatment over time. The control was considered as a further treatment to monitor and evaluate the normal gut microbial community evolution upon the interaction of honeybees with the environment. The packages `ggplot2` and `ggpubr` were used for graphs.

## 3. Results

### 3.1. Colony strength and weight analysis

The colonies were created by shook swarm method two months before the experiment. Even though, one week before the neonicotinoid administration the colonies displayed a certain variability (Fig. 1), but no difference was detected with ANOVA analysis between the groups in the total surface of adult bees, capped brood and open brood. At T0, the mean values for the surface of adult bees were  $8973 \pm 2223$ ,  $7631 \pm 1190$  and  $10,160 \pm 2738 \text{ cm}^2$  for CTR, IMID and THIAC, respectively. At the same time, the surface of capped brood was  $2218 \pm 640$ ,  $2109 \pm 581$  and  $2177 \pm 816 \text{ cm}^2$ , while the open brood was  $804 \pm 366$ ,  $870 \pm 241$  and  $1047 \pm 634 \text{ cm}^2$  (CTR, IMID and THIAC respectively).

Significant differences were reported after the treatment, as the THIAC group showed a reduced number of adult bees  $8291 \pm 2761 \text{ cm}^2$  ( $p < 0.01$ ), while none was detected in the CTR and IMID groups ( $7895 \pm 2073$  and  $7900 \pm 1568 \text{ cm}^2$ ). The IMID group had an overall increase of open brood  $1455 \pm 498 \text{ cm}^2$  ( $p < 0.05$ ), while in the CTR ( $929 \pm 337 \text{ cm}^2$ ) and THIAC groups ( $1481 \pm 498 \text{ cm}^2$ ) the variation was not significant. The capped brood did not vary after the treatment ( $2014 \pm 505$ ,  $1749 \pm 441$  and  $1686 \pm 699 \text{ cm}^2$  for CTR, IMID and THIAC).

The daily number of dead bees collected in the under-baskets did not differ between groups as it was similar for CTR ( $38.8 \pm 12.3$ ), IMID ( $32.5 \pm 8.7$ ) and THIAC colonies ( $37.4 \pm 5.2$ ). All experimental colonies survived, and no failure was observed in the following weeks.

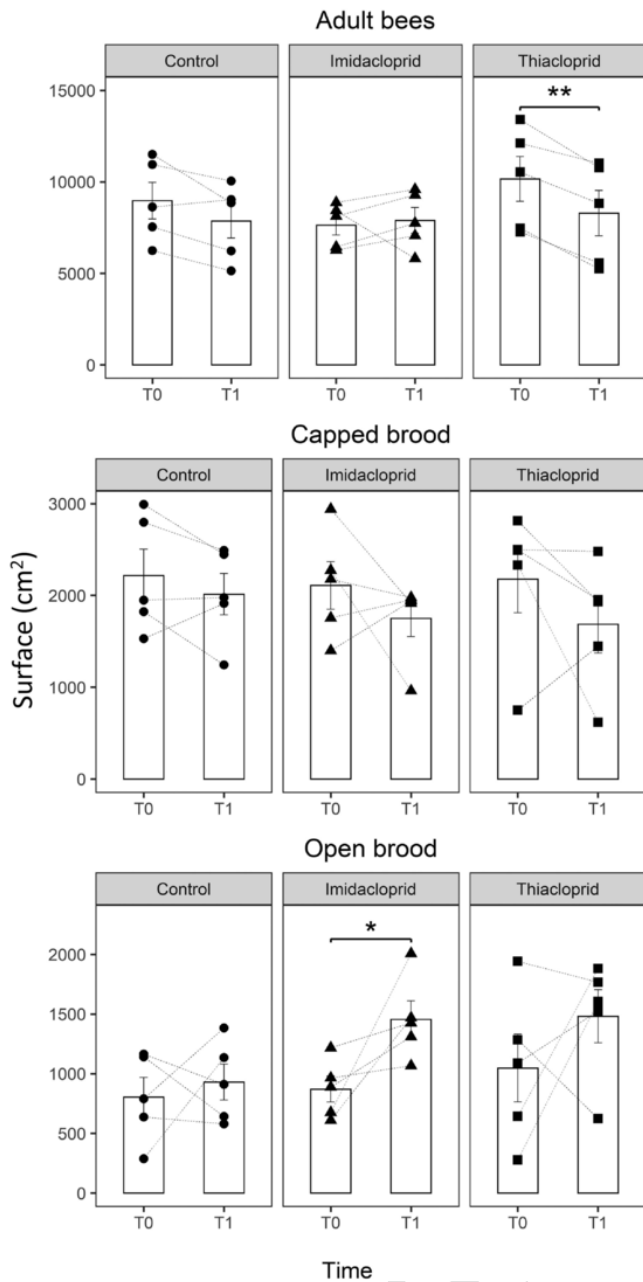


Fig. 1. Surface (cm<sup>2</sup>) of adult bees, capped brood and open brood recorded from the experimental colonies ( $n = 15$ ) one week before the experiment start (T0) and after one week of treatment administration (T1).

Focusing on sugar syrup consumption during the field assay, before and after the sugar syrup administration, the colonies showed a decreasing weight trend (Fig. 2a), probably due to the lack of nectar flow from the environment. Each hive was provided with 4760 g (3.5 l syrup) during the treatment week, and overall the colonies gained an average of  $2737 \pm 753$  g, thus showing a consumption of about half the total amount provided and the storage of the other half. Fig. 2b shows a single hive monitoring, with the observation of sudden increases of 680 g due to the 500 ml of sugar syrup. The weight then decreased strongly of about 50%, thus indicating a consistent consumption of sugar syrup.

### 3.2. qPCR results

Total bacteria (Eubacteria) were in the range 8.3–8.5 Log 16S rRNA copies/intestine at T0 (Fig. 3a). Statistical analysis on qPCR data evidence the presence of significant interactions between treatment and T2 with a general decrease in all treatments, but the decrease was statistically significant ( $p < 0.01$ ) comparing T0 vs T2 only for IMID and THIAC and also comparing T1 vs T2 for IMID (Fig. 3b,c).

*Bifidobacterium* spp. counts showed a significant decrease comparing T0 vs T1 sampling times for IMID group ( $p < 0.05$ ). THIAC also showed a decreasing trend over time that resulted significant comparing T1 vs T2 ( $p < 0.05$ ). The CTR group registered also a significant decrease of bifidobacteria comparing T0 vs T2 and T1 vs T2 sampling times ( $p < 0.05$  and  $p < 0.01$ ) showing a natural decreasing trend. However, the bifidobacteria decrease is significant in CTR after 5 weeks and not after one week as registered in IMID treatment.

*Lactobacillus* spp. analysis showed a significant decrease comparing T0 vs T2 and T1 vs T2 sampling times in the IMID group ( $p < 0.01$ ) and no significant decrease in THIAC group. However, also the CTR group showed a significant decrease of lactobacilli count comparing T1 vs T2 ( $p < 0.01$ ).

### 3.3. Bee gut microbiota analysis: NGS

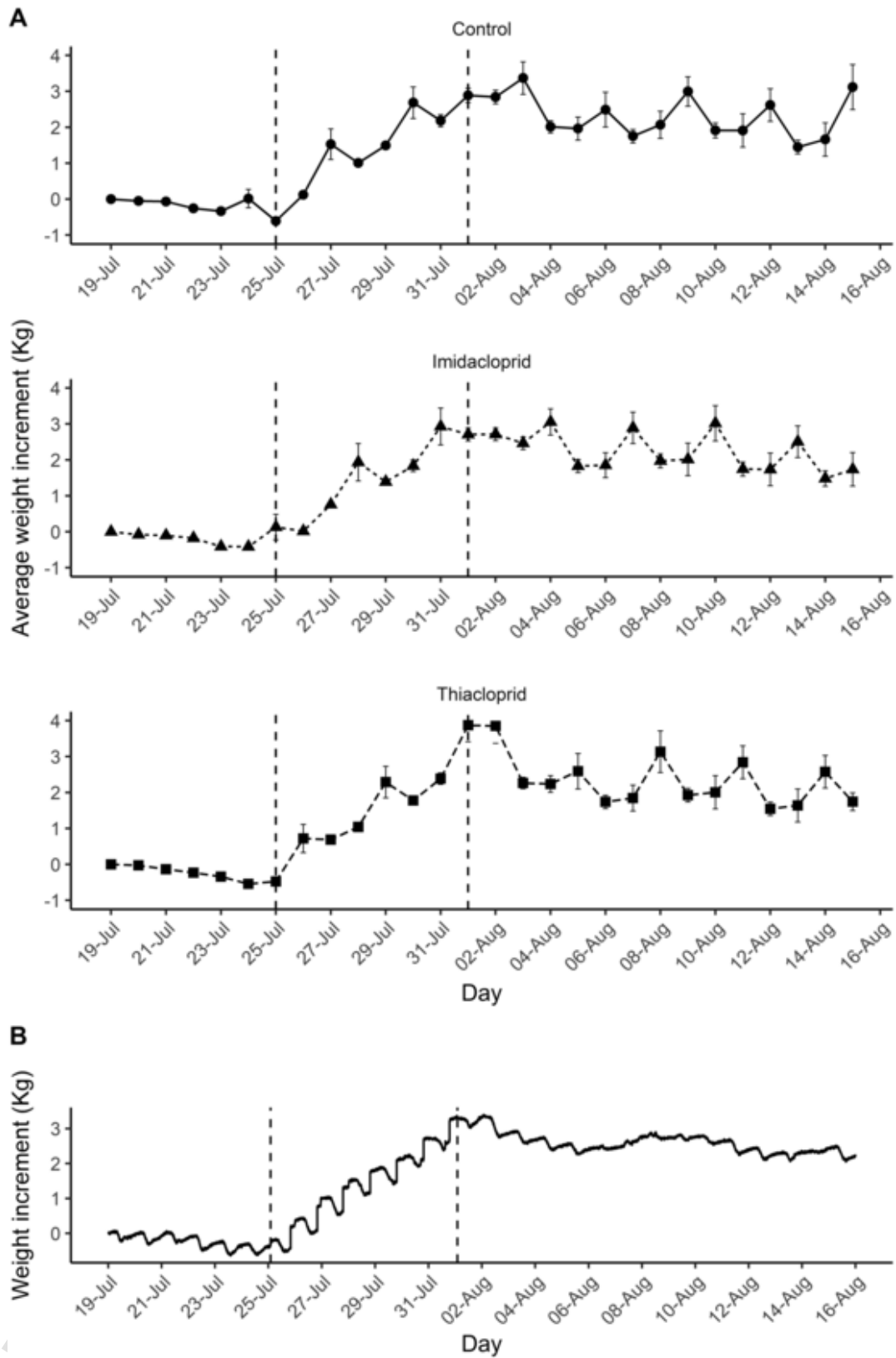
A total of 54 samples (6 samples per theses for the 3 picking times: T0, T1 and T2) were run on an NGS Illumina MiSeq sequencer. The total amount of reads after pairing and cleaning for low quality reads, chimeras and unaligned sequences was 7347144. Therefore, a total of 3673572 of ~450 bp final reads were obtained, with an average of 69312 sequences per sample. Taxonomical assignment based on SILVA 132 database indicated 4 samples as outliers due to an abnormal deviation from all the other samples for the obtained OTUs and taxonomical assignments. Consequently, samples T0\_THIAC\_5, T0\_THIAC\_6, T2\_THIAC\_5 and T2\_THIAC\_6 were discarded. Samples were rarefied at 47400 reads.

$\alpha$ -Diversity indexes did not show significant differences in the CTR group over time, the number of OTUs, the abundance of taxa and taxonomy did not vary considerably (Fig. 4). On the other hand, the data collected from bees treated with pesticides showed significant variations over time. IMID treatment resulted in a constant decrease of all indexes till reaching the lowest values at T2, while in THIAC lower values were registered at T1 with a subsequent increase at T2 of all indexes.

In detail, Chao1 index decreased significantly in THIAC comparing T0 vs T1 ( $p < 0.01$ ), while the decrease is significant in IMID comparing T0 vs T2 and T1 vs T2 ( $p < 0.01$ ). The index has later registered an increase for THIAC comparing T1 vs T2, reaching again the starting value. Observed OUT index showed the same trend as Chao1 (Fig. 4). PD whole tree index, on the other end, registered a significant decrease both in THIAC and IMID comparing T0 vs T1 ( $p < 0.01$  and  $p < 0.05$ , respectively); in IMID treatment this decrease proceeded till T2 with significant comparisons both between T1-T2 and T0-T2 ( $p < 0.01$ ), while for THIAC, after the sudden decrease, the value registered an increase comparing T1-T2 as for all the other indexes ( $p < 0.01$ ).

The analysis of  $\beta$ -diversity (Table 1) underlined no differences comparing the intra-variability of the different time points for THIAC and IMID treatments, while CTR showed significant variation comparing T0 vs T1 and T1 vs T2. However, comparing the variability along time in treatments a significant difference was reported only between CTR group vs THIAC group.

Results of taxonomical assignment are summarized in Figs. 5a and 6a through histograms at both genus and species levels. The taxonomical assignment of the 45 samples produced 20,518 OTUs at 97% similarity picking, represented by 10 phyla, 3 of which (Firmicutes 58.65%,



**Fig. 2.** Weight increment of the experimental hives recorded during the trial. Average daily values (kg ± SD) are reported for the three treatment groups (a). Detailed view of values recorded every 5 min for a representative hive (n° 5, CTR group) (b). The increment values are the difference from the starting point (July, 19th). The values were recorded for four weeks. Dotted lines indicate the start and the end of the treatment week.

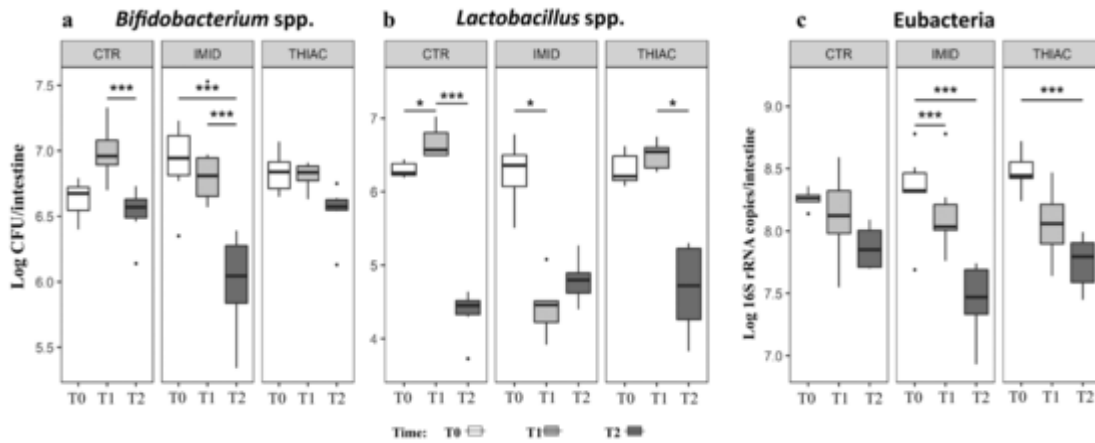


Fig. 3. a–c Real time data on total bacteria (Eubacteria), *Bifidobacterium* spp. and *Lactobacillus* spp. Data are expressed in Log CFU/intestine for *Bifidobacterium* spp. and *Lactobacillus* spp. whereas for Eubacteria data are expressed as Log 16S rRNA copies/intestine.

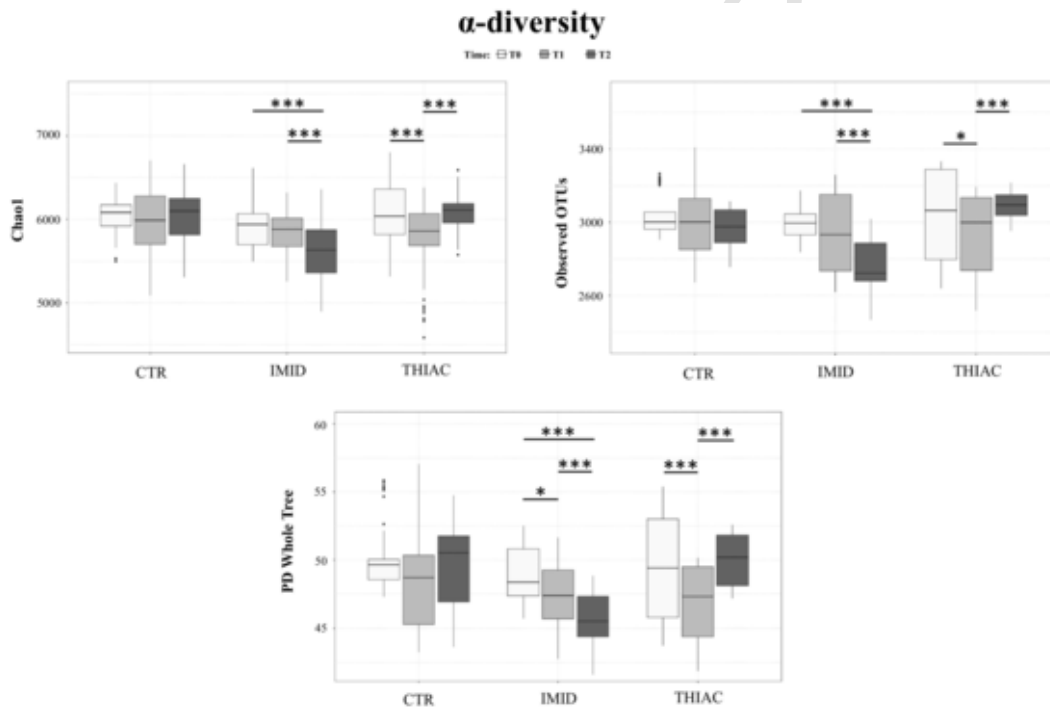


Fig. 4.  $\alpha$ -Diversity indexes analysis: a) Chao1; b) Observed OTUs; c) and PD Whole Tree (significant pairwise comparisons \* $p < 0.05$ ; \*\*\* $p < 0.01$ ).

Table 1

Significance values for  $\beta$ -diversity in all compared these adjusted with Bonferroni correction for 12 comparisons (only groups significant before Bonferroni correction are listed).

Group 1	Group 2	p-Value	p-Value corr
Comparison of variability in time points within each treatment			
T0_CTR vs T0_CTR	T1_CTR vs T1_CTR	0.001	0.012
T0_CTR vs T0_CTR	T5_CTR vs T5_CTR	0.786	>1.000
T1_CTR vs T1_CTR	T5_CTR vs T5_CTR	0.002	0.024
Comparison of variability between time point among treatments			
T0_CTR vs T1_CTR	T0_IMID vs T1_IMID	0.019	0.228
T0_CTR vs T5_CTR	T0_IMID vs T5_IMID	0.050	0.600
T1_CTR vs T5_CTR	T1_IMID vs T5_IMID	0.044	0.528
T0_CTR vs T1_CTR	T0_THIAC vs T1_THIAC	0.002	0.024
T0_CTR vs T5_CTR	T0_THIAC vs T5_THIAC	0.008	0.096
T1_CTR vs T5_CTR	T1_THIAC vs T5_THIAC	0.323	>1.000

Proteobacteria 36.13%, Actinobacteria 4.8%) accounting for 99.6% of the total reads.

The elaboration of the NGS data showed a non-significant variation of the main honeybee gut microbial phyla in both imidacloprid and thiacloprid treated groups, at any sampling time. On the other hand, control samples showed a significant decrease of Proteobacteria (T0 vs T1  $p < 0.05$ ) while Firmicutes increased (T0 vs T1  $p < 0.01$  and T0 vs T2  $p < 0.05$ ).

At the family level, the gut microbial community was dominated by members of Lactobacillaceae (58.65%), followed by Orbaceae (24.53%), Neisseriaceae (9.26%), Bifidobacteriaceae (4.8%), Acetobacteraceae (1.27%), Bartonellaceae (0.53%) and Enterobacteriaceae (0.29%). The statistical analysis carried out at the family level showed a significant increase of Lactobacillaceae in CTR group over time (T0 vs T1, from 47% to 62%) and a decrease of this family upon thiacloprid treatment (T0 vs T2, from 63% to 52%), although not significant after the Bonferroni correction. Orbaceae significantly decreased in CTR group (T0 vs T1  $p < 0.05$  and T0 vs T2  $p < 0.01$ ) and increased mar-

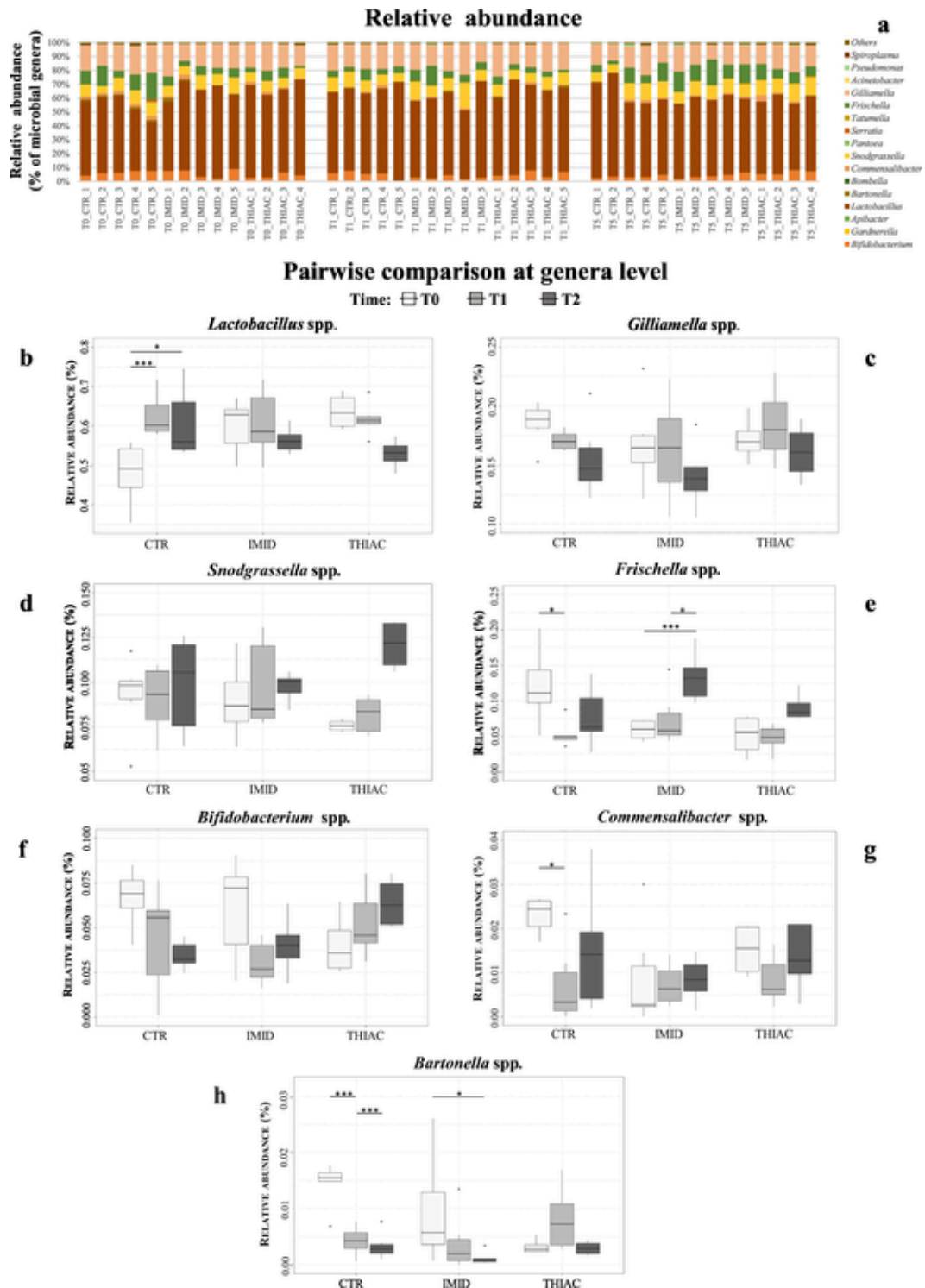


Fig. 5. a-h Histograms of microbial genera expressed for their relative abundance (a). Relative abundance expressed in % of the major bacterial genera in relation to experimental these (significant pairwise comparisons \*p < 0.05; \*\*\*p < 0.01) (b-e).

ginally in the imidacloprid and thiacloprid treated groups but not significantly. Neisseriaceae did not show significant variations in CTR and imidacloprid at any sampling time, whereas the comparison T0 vs T2 showed a significant increase ( $p < 0.05$ ) in THIAC (from 7% to 12%). Acetobacteraceae and Bifidobacteriaceae decreased significantly only in the CTR group (T0 vs T1 and T0 vs T2 respectively,  $p < 0.05$ ), Bifidobacteriaceae decreased after imidacloprid treatment comparing T0 vs T1 but not significantly after statistical correction. Bartonellaceae fam-

ily showed a significant decrease in CTR comparing T0 vs T1 and T0 vs T2 ( $p < 0.01$ ); they showed a decreasing trend also in IMID, non-significant comparing T0 vs T1 after the Bonferroni correction but significant when comparing T0 vs T2 ( $p < 0.05$ ). Finally, Enterobacteriaceae, Yersiniaceae and Erwiniaceae did not show any significant variation.

At genus level, the same trend of Lactobacillaceae was found for *Lactobacillus spp.* for both abundance and statistical results considering that *Lactobacillus spp.* represented 99.99% of Lactobacillaceae family,



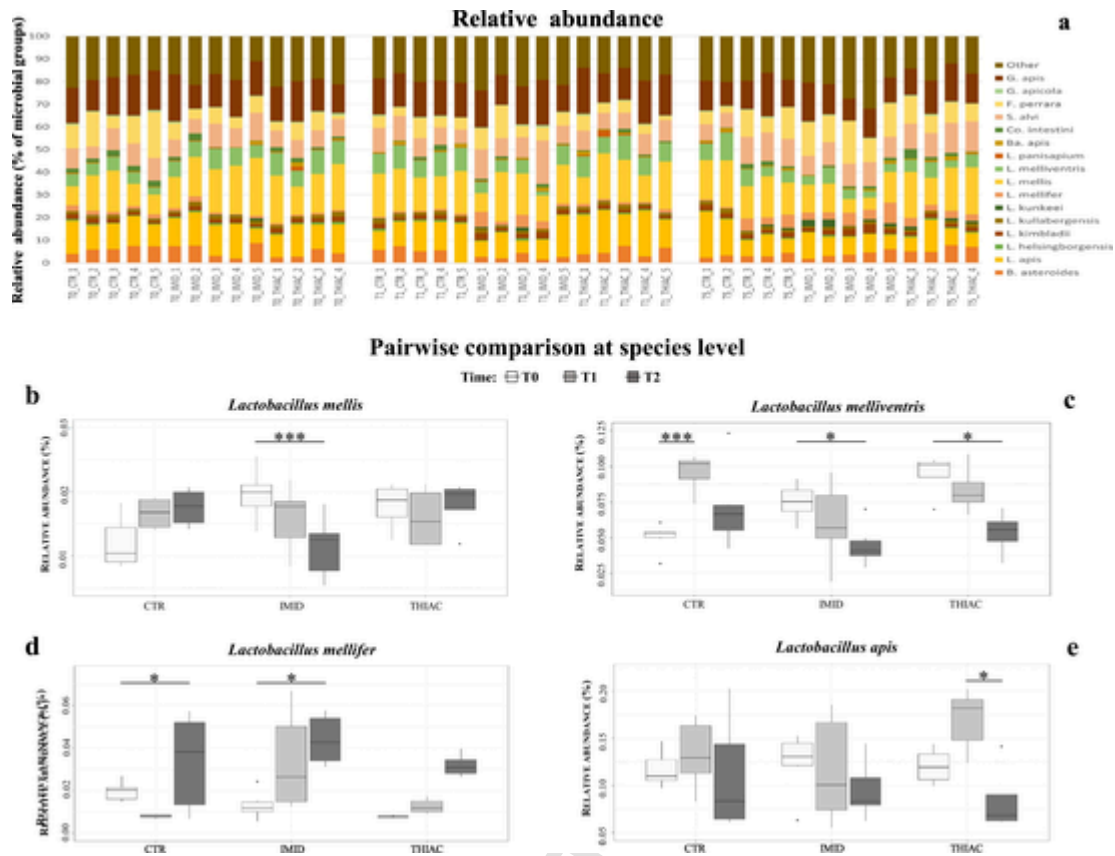


Fig. 6. a–e Histograms of microbial species expressed for their relative abundance (a). Relative abundance expressed in % of the major bacterial species in relation to experimental these (significant pairwise comparisons \* $p < 0.05$ ; \*\*\* $p < 0.01$ ) (b–e).

confirming this genus as the most abundant with 58.64% of reads. *Lactobacillus* spp. are then followed in relative abundance by *Gilliamella* spp. (16.81%), *Snodgrassella* spp. (9.22%), *Frischella* spp. (7.29%), *Bifidobacterium* spp. (4.76%), *Commensalibacter* spp. (1.24%), *Bartonella* spp. (0.53%), *Serratia* spp. (0.05%), *Apibacter* spp. (0.027%) and *Citrobacter* spp. (0.001%). Statistical results did not evidence any significant variation for *Gilliamella* spp., *Snodgrassella* spp., *Serratia* spp., *Apibacter* spp., and *Citrobacter* spp. (Fig. 5b–h).

*Lactobacillus* spp. increased significantly in CTR group comparing T0 vs T1 ( $p < 0.01$ ) and T0 vs T2 ( $p < 0.05$ ), while no differences were observed for THIAC and IMID treatments. *Frischella* spp. decreased significantly in CTR group comparing T0 vs T1 ( $p < 0.05$ ) but increased significantly in IMID treatment comparing T0 vs T2 ( $p < 0.01$ ) and T1 vs T2 ( $p < 0.05$ ). *Bifidobacterium* spp. showed a decreasing trend over time in CTR and IMID but not significantly. *Commensalibacter* spp. decreased significantly in CTR group comparing T0 vs T1 ( $p < 0.05$ ) but remained unvaried over time in IMID and THIAC treatments. *Bartonella* spp. showed the same decreasing trend of *Commensalibacter* spp. in CTR group comparing both T0 vs T1 and T0 vs T2 ( $p < 0.01$ ), but also the IMID thesis registered a significant drop comparing T0 vs T2 ( $p < 0.05$ ) (Fig. 5h).

Other Acetobacteraceae (Acetobacteraceae\_other), not represented by *Commensalibacter* spp., showed a different trend. While remaining stable in CTR group, the taxon increased in IMID group significantly comparing T0 vs T2 and T1 vs T2 ( $p < 0.01$ ).

The species *Lactobacillus mellis* was the most represented not only within the dominant genus *Lactobacillus* spp. but also among all other genera, resulting in 15.92% of reads assigned to this taxon. *L. mellis* is immediately followed by *Lactobacillus apis* (12.00%), *Lactobacillus melliventris* (6.94%), *Lactobacillus mellifer* (2.36%), *Lactobacillus kimbladii* (1.94%), *Lactobacillus kullabergensis* 1.36%, *L. helsingborgensis* (0.61%), *L. kunkei* (0.5%) and *L. panisapium* (0.36%).

Statistical analysis focused on *Lactobacillus* species showed that *L. mellis* increased constantly in CTR colonies over time even if not significantly after the Bonferroni correction (Fig. 6b). In contrast, *L. mellis* significantly decreased from 19.6% to 11.4% in IMID group comparing T0 vs T2 ( $p < 0.01$ ) whereas in THIAC the same species remained stable over time. *L. apis* did not show any significant changes in CTR and IMID groups, whereas it strongly increased in THIAC at T1 (from 12.0% to 17.0%), even if not significantly, and subsequently declined significantly from 17% to 8.5% comparing T1 vs T2 ( $p < 0.05$ ) reaching the abundances of CTR and IMID theses (Fig. 6e). *L. melliventris* increased significantly in CTR comparing T0 vs T1 ( $p < 0.01$ ), but both IMID and THIAC showed a constant drop over time (higher than 3% at T2) that is significant comparing T0 vs T2 ( $p = 0.05$  and  $p < 0.05$  respectively) (Fig. 6c). *L. mellifer* showed an increase in CTR and IMID groups over time comparing T0 vs T2 ( $p < 0.01$ ) (Fig. 6d). *L. kimbladii*, *L. kullabergensis*, *L. helsingborgensis* and *L. panisapium* did not show any significant variation in all the treatments. *L. kunkei* increased significantly when exposed to IMID comparing T0 vs T2 ( $p < 0.01$ ; increasing 4.7 folds the number of reads) and T1 vs T2 ( $p < 0.01$ ). The same species did not show any difference over time in CTR and THIAC. The taxonomical classification based on SILVA 132 database was unable to assign at the species level 18.95% of totals reads that were ascribed to the genus *Lactobacillus* spp. Statistical analysis on this grouped taxon showed no differences. It is just noteworthy to evidence how IMID increased the proportion of this group from T0 to T2 even if not statistically significantly (from 14% to 20%).

The genus *Gilliamella* is mostly represented by *Gilliamella apis* with 14.78% of the reads, followed by *Gilliamella apicola* (0.55%). Both species did not show significant variations over time in any experimental group. *Snodgrassella alvi*, *Frischella perrara*, *Bifidobacterium asteroides* and *Corvinia intestini* were identified with respectively as 9.48%, 7.90%,

4.59% and 1.19% of the total sequences. *Bartonella apis* was surprisingly poorly represented, with a 0.58% of total sequences. The change in relative abundance of *S. alvi* exposed to thiacloprid comparing T0 vs T2 is noteworthy, although non-significant ( $p = 0.07$ ), the other theses did not show significant differences.

*F. perrara* significantly dropped in CTR comparing T0 vs T1 ( $p < 0.01$ ) then slightly increased. In contrast, *F. perrara* significantly increased in IMID comparing T0 vs T1 ( $p < 0.05$ ) and T0 vs T2 ( $p < 0.01$ ), while in THIAc it showed no significant variation. *B. asteroides* in CTR decreased over time with a non-significant variation between T0 and T1 and a significant variation comparing T0 vs T2 ( $p < 0.05$ ). On the other hand, imidacloprid showed a negative effect on *B. asteroides* that decreased from 5,8 to 2,9% comparing T0 vs T1 (not significant). Finally, *B. apis* significantly dropped in both CTR (1.4% to 0.3%) and IMID (0.9% to 0.1%) comparing T0 vs T2 ( $p < 0.01$  and  $p < 0.05$ , respectively). Interestingly, *B. apis* increased from 0.3 to 0.8% in THIAc comparing T0 vs T1 even if not significantly.

#### 4. Discussion

It is well established that plant protection products (PPP) and herbicides can affect the ecosystem, interfering with the functionality of living organisms, and that their effect is not only on the target organisms for which they have been designed and marketed (EFSA, 2015; Kohlschmid and Ruf, 2016; Cullen et al., 2019). A relevant case of concern for the beekeeping sector is glyphosate, which has a significant impact on the intestinal microbiota of honeybees (Motta et al., 2018), thus demonstrating a clear side effect in the environment. In the same way, many other PPPs are used in agriculture, and, among these, neonicotinoids are considered among co-factors responsible for the honeybee decline (Cresswell et al., 2012; Tsvetkov et al., 2017).

Therefore, this work aimed at evaluating the effects of the neonicotinoids imidacloprid and thiacloprid on hives and on the honeybee gut microbiota at field level on experimental honeybee colonies placed on a mountain forest in the North of Italy. To the best of our knowledge, this study is the first one monitoring neonicotinoids effects at field level on honey bee microbial composition. Cage tests may contribute, in fact, to altering the intestinal microbiota due to artificial and forced environmental conditions (Anderson and Ricigliano, 2017; Taylor et al., 2019) and do not allow to monitor the colony strength parameters. On the other hand, the choice of a forest environment, with respect to agroecosystems, may contribute to a greater stability of the gut microbiota at the beginning of the experiment, excluding further perturbation due to environmental contaminants. Moreover, the study relayed onto two sampling times post exposure to IMID and THIAc, aiming at unravelling not only gut microbiome acquisition, development and evolution post direct exposure, but assessing also two different types of exposure: samples picked at T1, assessed the impact on the gut microbiome of a direct exposure to a strong contamination (i.e. adult foragers feeding on a contaminated nectar source), T2 allowed to observe the impact of the neonicotinoids residues on the next generation of honeybees. Since the life span of an adult bee in summer is about 4 weeks (Remolina et al., 2007), after 5 weeks, it is assumed that the forager bees present in the hive belonged to a new generation of bees that did not experienced direct contamination coming from outside the hive, but rather a late chronic exposure to residual contaminants already present in the hive.

Melixa hive scale measured indeed weight gain and loss, before and after the syrup administration, suggesting that the natural nectar flow in the weeks of the field experiment was very little and not enough to allow a storage. Data showed that the colonies consumed half of the sugar syrup provided during the treatment in all experimental theses. The other half was immediately stored, but consumed in the following weeks. This amount of contaminated syrup entering the hive matrix allowed observing and evaluating chronic exposure to sub-lethal doses of neonicotinoids. The measured colony strength parameters revealed

alteration of the colony structure after exposure to a week of contaminated feed. The reduction of the number of adult bees in the THIAc group may find an explanation with the forager loss phenomenon as also observed in another study (Tison et al., 2016). Considering that the capped brood surface did not change significantly and the amount of dead bees found in the THIAc underbaskets was as much as the other two groups, it is possible that the bees missing from the THIAc colonies might have gone lost outside the hive. At the same concentration we used (4500 ppb), Tison et al. (2016) reported an impairment of homing success (the percentage of foragers returning back to the hive) after the bees were chronically exposed to thiacloprid in the field for weeks. These bees required a longer time to return to the hive, and the homing success was 91.76% for the control and 76% for the treated group, thus supporting the idea of a neuronal impairment of the synaptic transmission involved in the navigation. Interestingly, when the difference between these two percentages (15.76%) is subtracted to the mean surface of adult bees in THIAc at T0 (10160), the result (8559) approximates the mean surface of adult bees at T1 (8291) recorded in the present study.

It has been pointed out (Guedes and Cutler, 2014) that the dose of a stressor is not linearly proportional to its response, in a way that a low dose of a pesticide might indeed stimulate the biological response of an organism. This hormesis phenomenon has also been observed by other honeybee researches (Cutler and Rix, 2015; Cutler and Guedes, 2017), suggesting that honeybees can cope with pesticide-induced stress through stimulatory responses. In this respect, the increased surface of open brood in the IMID colonies observed in the study might be a consequence of this hormetic response. This result matches with previous observation by Faucon et al. (2005), who described an increase of capped brood in honeybee colonies in the weeks following successive administrations of imidacloprid 5  $\mu\text{g}/\text{l}$  of sugar syrup, the same concentration used in the present study.

The present work wants also to study the microbial dynamic occurring when the entire hive's superorganism is taken into consideration, simulating a neonicotinoid contaminated nectar flow coming from treated crops. In the CTR, significant changes were found over time, as shown by the decreased proportions of most of the microbial genera except *Lactobacillus* spp. The large variations observed in the CTR make comparisons between experimental theses more complex. This phenomenon could however be explained by the seasonal and caste variations of the microbiota also described by Ellegaard and Engel (2019) and Kešnerová et al. (2020). Instead, IMID and THIAc did not reflect the trend of CTR honeybee colonies and recorded highly significant changes. IMID theses showed a significant reduction of  $\alpha$ -diversity from T0 to T2. The reduction in the number of different OTUs, mostly representing strains belonging to the same species, even when clustering with an identity percentage of 97%, is also accompanied by a microbial population drop detected in qPCR total bacterial count. This data is different with any other obtained on the topic. In fact, previous studies have considered a short period of time post exposure to IMID (5 days) in Raymann et al. (2018b) in order to evaluate gut microbial community changes. Probably IMID effects on the gut microbial balance are evident only after a long-term exposure, in the present study significant decrease in  $\alpha$ -diversity indexes were obtained comparing starting values with data collected after 5 weeks. On the contrary THIAc was reported to significantly decrease microbial diversity during the week of the treatment (T0-T1) but also surprisingly increasing it after 4 weeks (from T1 to T2). This observation can be described as a resilience effect after the gut microbial community perturbation, as evidenced by Liu et al. (2020) in a laboratory test after 13 days of exposure. It is therefore evident that the effect of THIAc can be much more incisive than IMID in the short-term but milder in the long term. Also,  $\beta$ -diversity analysis showed a significant difference between CTR group and THIAc soon after treatment.

The impact of tested chemicals on gut microbiome could be explained by both positive and negative interactions of these molecules

with specific microbial taxa:

- i) neonicotinoids can be used as a carbon and energy source by some bacteria as it was reported for the nitrogen fixing bacteria *Ensifer adhaerens* for the neonicotinoid thiamethoxam (Zhou et al., 2013) or *Pigmentiphaga* spp. for acetamiprid (Wang et al., 2013). Therefore, neonicotinoids can alter the microbiota composition by advantaging specific microbial taxa, altering the microbiome gene pool that sustain the gut metabolic function of the host.
- ii) neonicotinoids can be toxic and alter metabolic processes, exhibiting a bacteriostatic effect slowing down bacteria growth and proliferation. This was assessed in soil microbial communities by Wang et al. (2014), testing inhibition of both proliferation and metabolism after soil exposure to acetamiprid and imidacloprid. They discovered that, after a short-term exposure, a significant downregulation of urease and dehydrogenase activity was detectable. Thus, inhibition of metabolic function of sensitive bacterial strains in the gut microbial community of honeybees could be hypothesized and be a driver in the change of microbial proportions inside the honeybee gut. In other words, some ecological niches remained empty by sensitive bacteria are then replaced by less susceptible bacteria;
- iii) some bacterial products such as SCFA and exopolysaccharides in humans (Hidalgo-Cantabrana et al., 2014) and insects (Jones et al., 2018; Liberti and Engel, 2020) interact with the immune response.

Little is known about insects with regards to exopolysaccharides. Pesticides can alter the secretion of EPS from bacteria: for example, a decrease was reported for microorganisms inhabiting the soil matrix (Niewiadomska and Klama, 2005; Zhou et al., 2013) but an opposite effect was reported by Ahemad and Khan (2012). It is therefore likely that the interaction between microorganisms and hosts is inhibited, thus affecting the normal crosstalk and evolution of symbionts with the host. This interaction can be only hypothesized and transcriptomic studies are necessary for clarification.

- iv) honeybee guts contain biofilms (bacteria adherent to the host epithelium producing extra polymeric substances), mostly in the ileum, that plays a role connecting host physiology and environment having a role in the information exchange. Changes in proportion of the core and non-core species can displace other important core bacteria, diminishing or altering biofilm function and leading to dysbiosis and sensitivity to infections (Daisley et al., 2020). This is reported for *F. perrara* with help of hive opportunistic bacteria (*L. kunkeei* and *Parasaccharibacter apium*) at the expenses of *S. alvi* (Engel et al., 2015; Maes et al., 2016). Interestingly both *F. perrara* and *L. kunkeei* increased significantly in IMID at T1 vs T2 and T0 vs T2, thus contributing to gut dysbiosis and the missed recovery of IMID treated hives at T2, otherwise observed in THIAC at T2.

The data showed a decrease in  $\alpha$ -diversity indexes in neonicotinoids treated colonies, with a reduction of OTUs number and/or their abundance, that is primarily linked to a reduction of number of OTUs belonging to the same species. As reported for *S. alvi*, honeybees symbionts can show a high gene diversity among strains up to 20% (Powell et al., 2016) and this applies also for genera and species inhabiting the honeybee gut. Therefore, a decrease in  $\alpha$ -diversity can be explained in a PPPs driven selection of core symbionts resistant strains, as already observed for antibiotics, that reduces the microbiome genetic pool leading to a lack of functional capabilities reducing the benefit for host nutrition (Daisley et al., 2020). In addition, a strong microbial symbionts selection is indirectly performed by the host, through mechanisms such as epithelial receptors, gut proteins and growth-promoting secretions (Bonilla-Rosso and Engel, 2018), contributing to modeling the bee gut microbiota. The implication is that young honeybees

acquiring the gut microbiota, might benefit from a wider microbial diversity to find the most compatible microbial strains capable of boosting their activity. In this light, the decrease in microbial diversity observed in IMID and THIAC, could have strong repercussions on new generations of bees, debilitating and compromising the functions of the hive, already significantly altered by the direct effects of these pesticides.

Differences between treated groups and CTR group are present at species level when taking into consideration clades that dominate the posterior part of the hindgut as for example *Lactobacillus* Firm-4 with *L. mellis* and *L. mellifer* and *Lactobacillus* Firm-5 with *L. apis* and *L. melliventris*. These groups increased significantly in CTR over time but decreased drastically in treated groups (*L. mellis* and *L. melliventris* with a 8.2% and 3.1% decrease in IMID, while *L. apis* and *L. melliventris* with a 8.5% and a 4.1% decrease in THIAC), probably reflecting the species-specific sensitivity to toxic substances (and also probably strain-specific sensitivity as evidenced above).

It is already known that *Lactobacillus* Firm-5 and Firm-4 (together with *Bifidobacterium*) functions consist in the use of various pollen coat-derived compounds including flavonoids (that are deglycosylated and sugars fermented), phenolamides and  $\omega$ -hydroxy acids in order to promote gut health and nutrient digestibility (Bonilla-Rosso and Engel, 2018). The impoverishment of the variability of these clades and especially of the available gene pool at intestinal level could affect general bee health. Moreover, data underlined a significant decrease of environmental/opportunistic species (i.e. *F. perrara* and *L. kunkeei*) in CTR bees along time while IMID treatment showed an increase of these taxa (+7.4% *F. perrara*, +1.4% *L. kunkeei*) that coupled with the previously evidenced decrease of lactobacilli may reflect a condition of dysbiosis linked to the decrease of important taxa in favour of environmental/opportunistic bacteria (Anderson and Ricigliano, 2017). These are well tolerated in no-stress conditions but following perturbations, hive residents may displace the native gut taxa.

Also, *Bifidobacterium* spp. was found to be significantly inhibited by IMID treatment as evidenced at genus level by qPCR analysis and partially in NGS relative abundance for *B. asteroides*. Literature evidenced that *Bifidobacterium* strains are important degraders of hemicellulose and pectin for bees with an extensive strain-level diversity in gene repertoires linked to polysaccharide digestion, and a particular enrichment of such genes have been found in strains isolated from honeybees compared to other bee species (Zheng et al., 2019). Although, the proportion of bifidobacteria is not high in the honeybees rectum, the high number of glycosyl-hydrolases they produce play a fundamental function for the honeybee metabolism that lactobacillus are unable to carry out. Bifidobacteria number naturally decrease along time in control foragers, after a month, but IMID exposed honeybee lost 2 Log soon after treatment, evidencing a sensitivity of this taxon to the pesticide.

On the other hand, differences between treated groups and CTR group are present at species level when taking into consideration important *Lactobacillus* species like *L. mellis* that account in average ~16% of total reads, and *L. melliventris* that account for ~7%. These groups increased significantly in CTR over time but decreased drastically in treated groups (comparing T0 vs T2, from 19% to 11% in IMID for *L. mellis*, and from 7% to 5% in THIAC for *L. melliventris*), showing the species-specific sensitivity to toxic substances (and also strain-specific sensitivity).

## 5. Conclusions

The impact of the two studied neonicotinoids on the honeybee microbiota at field level seems to be targeted to specific microbial taxa, with a supposed functional dysbiosis. An effect of the treatments was observed also at colony level in a change of reproductive physiology. By comparing the IMID and THIAC treatments with the microbial population trends of the control hives, a counter-trend is evidenced with a slowly and significant decrease of important species in *Lactobacillus*

Firm-4 and Firm-5 and an increase in opportunistic/environmental taxa with a consequent loss of functionality related to the nutrition and host defense. The differences, evidenced in the microbiota gut composition between control and treated groups, showed that it is necessary an in-depth study of the effects of neonicotinoids at field level also at transcriptomic level for moth microbial and host responses in order to better understand whether the reduction and modulation of gut microbial biodiversity can cause a depletion of gut functionalities and be the potential driver of the negative impact of pesticides on bee health. Finally, this study evidence the discrepancy between results achieved in laboratory or in field conditions, taking in consideration that also a long-term impact must be considered.

EFSA, 2013

#### CRedit authorship contribution statement

**D. Alberoni:** Formal analysis, Investigation, Data curation, Resources, Visualization, Supervision, Writing – original draft. **R. Favaro:** Conceptualization, Software, Formal analysis, Data curation, Resources, Visualization, Writing – original draft. **L. Baffoni:** Software, Formal analysis, Data curation, Writing – original draft. **S. Angeli:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **D. Di Gioia:** Resources, Supervision, Funding acquisition, Writing – review & editing.

#### 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.

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#### Data availability statement

Raw sequencing data are deposited in NCBI repository: NCBI Bioprocess and Sequence Read Archive (SRA) n° PRJNA655184, Biosample n° SAMN15728541-SAMN15728593.

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