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ORIGINAL PAPER

Colonies under dysbiosis benefit from oxalic acid application: the role of landscape and beekeeping practices in microbiota response to treatment

June Gorrochategui-Ortega[1] · Marta Muñoz-Colmenero[1,2] · Egoitz Galartza[3] · Andone Estonba[1] · Iratxe Zarraonaindia[1,4]

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Abstract

The *Varroa destructor* mite causes severe losses of *Apis mellifera* colonies, requiring recurring treatments. One such treatment is oxalic acid (OA), considered ecological. However, it is unclear whether OA affects the honey bee gut microbiota or other hive-associated microbiotas. Herein, we studied the effect of three OA treatments (trickling at 2.1% or 4.2%, and sublimation through Varrox®) upon microbial communities associated with workers' gut, hive bee bread and pupae, sampled from conventionally or ecologically managed colonies under different anthropization levels (located in urban, rural or mountainous landscapes). We hypothesized that treatment with OA would impact the diversity and composition of bacteria and/or eukaryotic communities, and that the effect would be dose-dependent and specific to the beehive niche. Results showed that the microbiomes of apiaries under different anthropization levels and management strategies differed prior to OA application. Neither the bacterial nor the fungal communities of bee bread and pupae shifted due to OA treatment. Independent of the dosage and the application method (trickling or sublimation), OA induced slight compositional changes in the bacterial profiles of honeybee guts. Those changes were stronger the higher the anthropization (in colonies from urban areas under conventional management). OA treatment reduced the relative abundance of several pathogens, such as *Nosema ceranae,* and decreased the overall bacterial diversity down to values found in less anthropized colonies. Thus, our results suggest that, aside from managing Varroa infestations, OA could have beneficial effects for stressed colonies while not impairing honey bee resilience from a microbial point of view.

Keywords *Varroa destructor* · Oxalic acid · Microbiome · Apibiome · Honey bee

Key message

- Microbiota associated with honey bees are involved in their health status.
- There are scarce data on how oxalic acid (OA) treatment affects honey bee microbiotas.
- OA-derived shifts are driven by landscape and management, not by dosage or application method.
- OA impact on the gastrointestinal bacteria is more uniform and stronger in anthropized colonies.
- OA reduces pathogen abundance and enriches beneficial bacteria in anthropized colonies.

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✉ June Gorrochategui-Ortega
june.gorrochategui@ehu.eus

[1] Department of Genetics, Physical Anthropology and Animal Physiology, Applied Genomics and Bioinformatics, University of the Basque Country (UPV/EHU), Leioa, Spain

[2] Department of Genetics, Physiology and Microbiology, Complutense University of Madrid (UCM), Madrid, Spain

[3] ERBEL, Iberian Bee (Erle Beltz) Breeding Association, Zaldibia, Spain

[4] IKERBASQUE, Basque Foundation for Science, Bilbao, Spain

Introduction

The controlled use of pesticides for pest management has expanded to beekeeping practices, in order to avoid the loss of pollinators. Oxalic acid (OA) is a common acaricide, with proven effect against *Varroa destructor* mites in honey bees (*Apis mellifera*) (Gregorc et al. 2017; Rademacher and Harz 2006; Rademacher et al. 2017). OA is usually applied as oxalic acid dihydrate (OAD) by sublimating, trickling or spraying after mixing with syrup (water plus sugar). It is applied during broodless periods (Rademacher and Harz 2006), as OA cannot enter capped brood cells (Jack et al. 2020) and *Varroa* mites can only reproduce during caped pupae stages (Rosenkranz et al. 2010)

Oxalic acid is considered ecological as it is naturally produced and consumed by organisms, including fungi and bacteria (Palmieri et al., 2019), and can be found as either free acid or oxalate (Nanetti et al. 2015) in foods such as honey (Bogdanov et al. 2002). Despite its natural origin and extended use, OA treatments have several limitations and drawbacks. Firstly, oxalic acid application does not guarantee uniform effect within hives; as distribution of the product can be unequal with certain areas receiving higher doses, OA might accumulate unequally or the bees themselves might re-distribute OA by trophallaxis (Rademacher et al. 2017). Secondly, bee tolerance to OA treatments is climate dependent, with warmer climates tolerating higher doses and concentrations (Rademacher and Harz 2006). Indeed, for a 30–50 ml/hive dose bees in southern Europe tolerate concentrations of 6% (weight/volume, w/v), while bees in central Europe tolerate only up to 3.5% (w/v) (Rademacher and Harz 2006). Higher dosages can reduce treatment efficacy (Rademacher and Harz 2006) as well as harm bee physiology by altering bee behaviour, reducing bee longevity and activity, and irreparably damaging the epithelium of the bee digestive system (Martín-Hernández et al. 2007a; Rademacher et al. 2017). Even exposure to approved doses can affect bee behaviour and lifespan (Schneider et al. 2012) or alter the bee gastrointestinal and hemolymph pH (Rademacher et al. 2017). Jack et al. (2020) also detected higher mortality of bees when OA treatment was combined with other common anti-Varroa practices (e.g. brood interruption).

Despite the extensive knowledge regarding oxalic acid effect in bee health, little is known about the bee microbiome response. Honey bees present a stable core microbiome in their gut, composed of approximately 8 taxa (Ellegaard and Engel 2019; Kešnerová et al. 2020) partaking in nutrition, detoxification and immunity against pathogens (Kešnerová et al. 2017; Kwong and Moran 2016; Raymann and Moran 2018). This microbial community is conserved across landscapes and hives (Ellegaard and Engel 2019), yet multiple factors are capable of stressing or altering its diversity or composition, both alone (Castelli et al. 2020, 2021; Hotchkiss et al. 2022; Rayman et al. 2017) or in synergistic interaction (Goulson et al. 2015). Anthropization (i.e. the human impact on the environment) is the starting point of most of those stressors. Human activities can reduce the floral diversity of landscapes, causing nutritional stress on bees (DeGrandi-Hoffman et al. 2016) and impacting the honey bee gut microbiota (Castelli et al. 2020). Anthropization also results in environmental pooling of chemicals due to agriculture or beekeeping practices, which can alter the microbial communities of honey bee guts, as reviewed by Hotchkiss et al. (2022).

While OA effectiveness as a varroasis control is known, its effect in microbial communities has been scarcely studied. Bacteria can consume OA (Palmieri et al. 2019), although oxalotrophy (i.e. OA degradation and consumption) is not often their sole carbon and energy source as it yields low amounts of energy (Palmieri et al. 2019). The Actinobacteria, Firmicutes and Proteobacteria phyla possess oxalotrophic ability (Hervé et al. 2016) and are thus capable of using OA for decarboxylation and energy production, or for reduction in anabolic reactions, glyoxylate oxidation and the citric acid cycle (TCA cycle) (Palmieri et al. 2019). Considering that several genera found in honey bee gut belong to those bacterial groups (e.g. the Proteobacteria *Gilliamella*, the Firmicutes *Lactobacillus* or the Actinobacteria *Bifidobacterium*) (Kešnerová et al. 2020), we would expect the application of OA to affect their abundances and favour their growth. However, no consensus effect has been detected after OA treatments in bee microbiota. Contrary to the expected trend, inhibition of several *Lactobacillus* species present in bee midguts was confirmed 7 days after OA consumption (Diaz et al. 2019). Cuesta-Maté et al. (2021) also demonstrated inhibition of five honeybee core gut bacteria (*Bifidobacterium asteroides, Frischella perrara*, *Gilliamella apicola, Lactobacillus kunkeei* and *Snodgrassella alvi*) after in vitro application of medium doses of OA. However, the in vivo experiment of the same study did not support in vitro results: although OA treatment depleted both *L. kunkeei* and *Bombella* genus, it also resulted in the enrichment of *Bifidobacterium, Frischella*, *Gilliamella* and *Snodgrassella* genera (Cuesta-Maté et al. 2021).

Thus, further trials must be conducted to ascertain the impact of OA treatment on the bee gut microbiome. Additionally, OA treatment might also affect other microbial communities present within hives (such as bee bread communities), or have a cumulative effect over time, seeing as OA (in its oxalic acid dihydrate form) can remain within hives after treatment (Rademacher et al. 2017). Which, to our knowledge, has not yet been studied.

To fill this knowledge gap regarding the impact of OA treatment on the microbiota of different hive niches (the apibiome) (Gorrochategui-Ortega et al. 2022), we examine the mid-term effect (i.e. one month post-treatment) that different OA dosages and application methods (trickling and sublimation) have on the diversity and composition of bacterial and fungal communities of managed honey bees. To unravel whether the environment can synergistically influence oxalic acid treatment, colonies were chosen from apiaries located under an anthropization gradient (urban, rural and mountainous landscapes) and under different management strategies (ecological versus conventional).

Materials and methods

Apiary and hive characterization: management and human impact

A total of 21 hives of *Apis mellifera iberiensis* were sampled across 3 apiaries: AP1 (6 hives), AP2 (6 hives) and AP3 (8 hives) Fig. 1. All hives were established previous to the experiment and were under beekeeping management for commercial purposes. Two different management strategies were accounted for. Ecological beekeeping practices were in use in apiaries AP1 and AP2 (henceforth named ecological apiaries when grouped together), while AP3 was managed through conventional strategies (hereafter called conventional apiary).

Apiaries were located in 2 adjacent regions of northeast Spain. Apiary AP1 was located within the Chartered Community of Navarre (42°40′41.7″N, 2°03′27.5″W), whereas AP2 (42°48′16.1″N, 2°19′36.4″W) and AP3 (43°11′12.”N, 2°03″36.3″W) apiaries were located in the Basque Autonomous Community. While AP1 was situated in a rural landscape, AP2 was located in the mountainous area of Opakua, and AP3 was situated in an urban-horticultural landscape within the industrial town of Villabona.

Thus, a landscape anthropization gradient was present in the study, with ecological and natural conditions in AP2, ecological and low anthropization conditions (rural area with a population below 200) in AP1, and conventional and anthropized conditions (industrialized urban area with approximately 5800 inhabitants) in AP3 Fig. 1. For ease of reading, during this paper the term anthropized colony refers to the colonies located in anthropized landscapes, as opposed to colonies located in natural (non-anthropized) landscapes (i.e. AP2 colonies).

Application of oxalic acid treatments and sampling of apiaries

As colony survival was to be encouraged for future exploitation of beehives, oxalic acid (OA) treatments were coupled with queen caging. Queen caging before OA application increases OA treatment efficacy without endangering the colony (Gregorc et al. 2017). Hives were sampled pre- and post-treatment. Queen caging and pre-treatment sampling

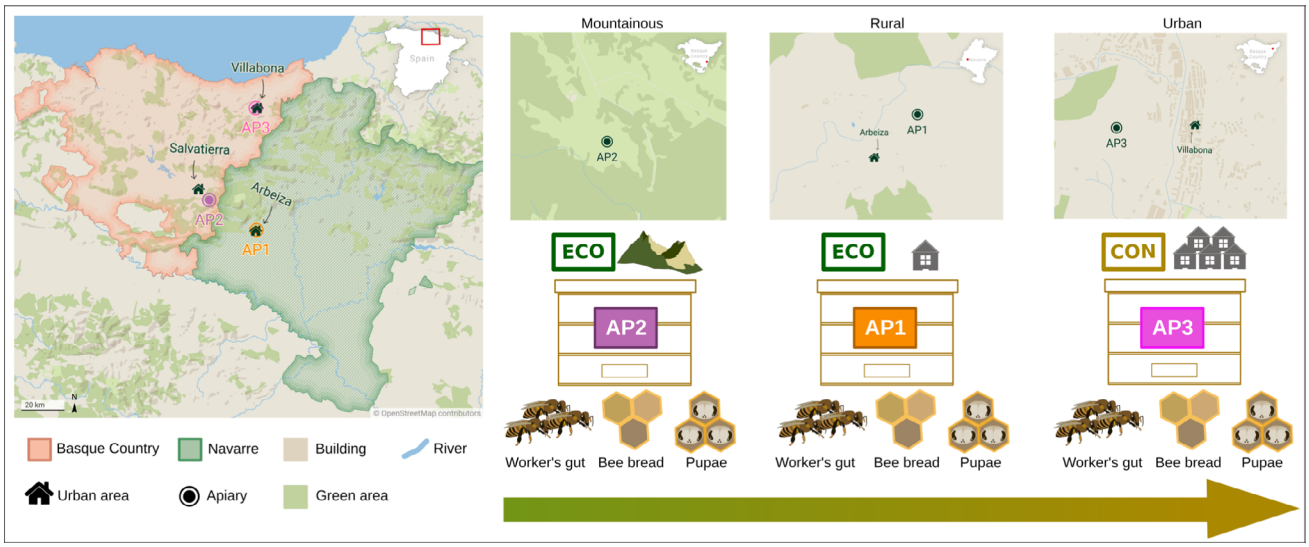


Fig. 1 Map and scheme of sampling sites. Apiaries were under a management (from ecological to conventional) and anthropization (from natural to urban) gradient. AP2 was located in the mountain pass of Opakua, AP1 was located in a rural area containing a few houses, and AP3 was situated in an urban area within the municipality of an industrialized village. Abbreviations: ECO for ecological management, CON for conventional management

were done simultaneously on 28-30th August 2017, to reduce tampering of hives. After 24 days queens were liberated and hives were treated (1 unique application, as recommended for broodless periods) (Rademacher and Harz 2006). Post-treatment sampling was performed approximately 20 days later (middle of October 2017) during development of the first batch of brood laid after treatment. Hives were treated and sampled by collaborators of the "Asociación de Apicultores de Gipuzkoa" (GEE). Materials were sterilized with ethanol and ultraviolet light prior to samplings. No other treatment was applied during this experiment.

Three OA treatments were studied, by applying different dosages and using different application methods: liquid trickling at 4.2% (OA4.2), liquid trickling at 2.1% (OA2.1) and sublimation (VAX). The 2.1% concentration was selected to evaluate low-dosage effects, while 4.2% is the most common dosage used in the Basque Autonomous Community (personal communication from beekeepers). For both 4.2% and 2.1% (*w/v*) concentrations, oxalic acid dihydrate (OAD, Oxuvar®) was dissolved in 50% sucrose solution (*w/w*) (https://www.theapiarist.org/oxalic-acid-api-bioxal-preparation/) and 5 mL of this OA solution was applied per comb seam (30–50 mL per hive depending on size) (Adjlane et al. 2016). The second method applied, sublimation, was done using Varrox® vaporizers (Andermatt Group AG), with 2 g of OAD and following the manufacturer's recommendations.

Each apiary received two treatment types. In ecological apiaries (AP1 and AP2), 3 hives received the OA4.2 treatment and another 3 hives OA2.1. In the conventional apiary (AP3), VAX was applied in 5 hives, while the remaining 3 hives received the OA4.2 treatment. Three samples were collected per hive: adult honey bees taken from brood frames (most likely nurses) whose gut was dissected (G, gut sample), 5 cm^2 of bee bread comb (BB, beebread sample) and 5 cm^2 of brood cells for both black-eyed pupae obtention (P, pupae sample). Low microbial loads were expected in brood, and thus pupae of 3 hives treated with OA4.2 (one hive per apiary) were sampled in triplicate. Samples were shipped to and stored in the Genetics Laboratory of the University of the Basque Country (UPV/EHU), maintaining the cold chain (-20ºC) until their analysis. Considering the three treatments (OA4.2, OA2.1, VAX), two timepoints (pre- and post-treatment) and 3 sample types (G, BB, P), the total number of samples studied equalled 138.

Sample processing and DNA extraction

Each G sample ($n=42$) was obtained by pooling 10 adult honey bee worker guts extracted by dissection. Hindgut and midgut regions were studied together, while crop was excluded. Processing and DNA extraction was performed following the previously established protocol by Muñoz-Colmenero et al. (2020). Briefly, 200 µL of the supernatant obtained after centrifuging a homogenate of 10 guts and 1 mL of 1xPBS (phosphate-buffered saline) were utilized for DNA extraction with the QIAamp® DNA Mini Kit (Qiagen).

For each BB sample ($n=42$), 10 pollen cells were randomly retrieved and pooled in a 15-mL falcon tube with 1 mL of 1xPBS. Cells were selected from different corners of both sides of combs. After sample homogenizing by vortexing, 200 µL was transferred to a 2-mL tube. Cell lysis and DNA extraction were performed following the protocol established in Muñoz-Colmenero et al. (2020) that combines a phenol:chloroform:isoamyl alcohol cleaning step followed by QIAamp® DNA Mini Kit (Qiagen). One post-treatment BB sample could not be pooled, limiting the final sample count to 41.

Regarding P samples ($n=54$), 6–8 black eyed pupae were pooled together when possible. Each pupa was removed from its caped cell, cleaned thrice using a 70% ethanol-soaked cotton swab, beheaded to avoid the PCR inhibiting proteins present in pupae eyes (Muñoz-Colmenero et al. 2020), and introduced in an Interscience© BagFilter® P filtered bag together with 500 µl of cold 1xPBS. After pooling 6–8 pupae, the bag was frozen overnight at -25ºC. The frozen pupae-PBS mixture was then manually homogenized on ice by carefully pressing bags using the BIOREBA AG© (Art. No. 400010) hand model homogenizer. The resulting liquid mixture was passed through the bag filter and transferred into a 15-mL falcon tube. 400 µl was taken for DNA extraction, mixed with 400 µl of AL lysis buffer and 40 µl of proteinase K, vortexed and incubated at 56ºC and 600 rpm for 1 h. Then, 400 µl of ethanol was added. The DNA-ethanol mixture was vortexed and 700 µl of it was applied to the QIAamp® Mini spin column (Qiagen) to perform the DNA extraction as indicated in the manufacturer's protocol. Eight brood frames had between 0 and 2 black eyed pupae and thus their pupae were not processed, limiting the final sample count to 129. One extraction blank (negative control) containing only 1xPBS (no bee material) was processed for each sample type extraction protocol.

16S rRNA and ITS2 gene amplification and sequencing

Bacteriome characterization was performed by amplifying the 4th hypervariable region (V4) of the 16S ribosomal RNA (16S rRNA) gene, using the 515F/806R primer set (Earth Microbiome Project, http://www.earthmicrobiome.org/) containing Illumina® sequencing adaptors and barcode sequences (12 bp) bound to the forward primer. Amplification was performed with the Illumina Amplicon Protocol,

using previously described conditions (Muñoz-Colmenero et al. 2020). PCR products were visualized using a 1.5% agarose gel stained with RedSafe™ (iNtRON Biotechnology). Samples were sequenced using Illumina® MiSeq technology in two separated runs: one run for G samples and another one for BB and P. The RUN kit v2 PE 2 × 150 bp (300 cycles, 15 M reads) was used for G samples and the kit v2 PE 2 × 250 bp (500 cycles, 15 M reads) for BB and P samples. 10% of PhiX were added in both cases. At least one library blank (water sample) was included in each sequencing run, as the negative control of amplification. DNA purification, library preparation and paired-end sequencing were performed at the Sequencing and Genotyping Unit of the University of the Basque Country (SGIKER).

The fungal community was characterized by amplifying the Internal Transcribed Spacer 2 region (ITS2), using the fITS7/ITS4R primer set and the Nextera XT v2 dual index adapters of the Nextera XT assay. Amplification was performed with the KAPA HiFi DNA Polymerase at recommended concentrations using a PCR mix of 12.5 µl KAPA HiFi, 1 µl of each primer (at 10 µM), 8.5 µl H2O and 2 µl DNA. The PCR cycling protocol consisted of: 95ºC for 5 min, 95ºC for 20 s plus 56ºC for 30 s and 72ºC for 1.30 min (× 37 cycles), and a final cycle of 72ºC for 7 min. DNA purification, library preparation and paired-end sequencing were performed at the University of the Basque Country, in SGIKER (Sequencing and Genotyping Unit). Samples were sequenced in two separated runs using the Illumina® MiSeq RUN kit v2 PE 2 × 250 bp (500 cycles, 15 M reads). 25% of PhiX were added to prevent the low diversity typical of ITS sequencing. DNA extraction controls and library blanks were added to both runs.

Quality checking and processing of sequences

In-depth data quality control, treatment and analysis were performed using Qiime2 (v2-2021.4) (Bolyen et al. 2019), and statistical data were analysed using R v4.1.0 unless otherwise stated. Statistical significance was considered when p values ≤ 0.05.

All 4 runs were separately imported. Bacterial datasets were imported and demultiplexed using the EMP paired-end pipeline with no Golay error correction, while using the Casava 1.8 paired-end protocol for pre-demultiplexed fungal sequences. Denoising of all runs was performed using the DADA2 method (Callahan et al. 2016) and applying the same trimming parameters for both runs of each amplicon (16S or ITS2), as recommended. Runs were merged to obtain one 16S dataset and one ITS2 dataset. Two Naive Bayes taxonomic classifiers were trained through f*eature-classifier fit-classifier-naive-bayes* and *feature-classifier classify-sklearn*. For bacterial data, a dataset containing full length 16S rRNA gene sequences from BEExact (Daisley and Reid 2023) was trimmed to the V4 region. The ITS2 classifier was trained using UNITE v10.05.2021 (Abarenkov et al. 2021). Singleton amplicon sequence variants (ASVs), and ASVs of mitochondrial/chloroplast origin, were filtered out from the 16S data, thus resulting in the loss of one pupae sample. While singletons were also removed from the ITS2 dataset, mitochondrial and chloroplast features were kept. Phylogenetic trees were created using mafft and fasttree alignment. The output rooted trees served to determine adequate sampling depths for diversity analyses. The potential contaminants among the ASVs were identified (but not filtered out because the small group size might confound results) through *decontam v1.6.0* (Davis et al. 2018), using feature counts and the prevalence method and default thresholds Tables S1, S2.

Microbial community richness, diversity and characterization

ASVs were split into sample-type specific datasets (G, BB and P). All diversity analyses were conducted at the same sequencing depth cutoff (2200 reads for 16S-derived ASVs and 6999 for ITS-derived ASVs) for all datasets Fig. S1. Alpha rarefaction curves were obtained and microbial community alpha diversity was determined through Shannon's diversity index (H) using *diversity core-metrics-phylogenetic*. Significant differences were tested based on pairwise Kruskal–Wallis tests with Benjamini & Hochberg False Discovery Rate (BH-FDR) correction and visualized through *ggplot2*.

Microbial structure differences between sample types were visualized within Qiime2 at appropriate sampling depths using Principal Coordinate Analysis (PCoA) based on Bray–Curtis distances, which were also used to calculate the homogeneity of dispersion of the tested variables through *betadisper* and *permutest* from *vegan (v2.5.7)* (Oksanen et al. 2020). A Permutational Multivariate Analysis of Variance Using Distance Matrices was done on the rarefied Bray–Curtis distances through *qiime diversity adonis,* to determine whether microbial communities composition significantly differed. Both PCoAs and adonis analyses were performed independently for each sample type and utilized to compare: (1) apiaries (AP1, AP2, AP3) in both the pre-treatment samples and the complete set of samples (pre- and post-treatment), (2) treatment stages (pre and post for OA2.1, OA4.2 and VAX) and 3) interactions among the variables (i.e. determine the effect of apiary on treatment stage). Pairwise comparisons among treatment stages were performed per apiary using permutational multivariate analysis of variance (PERMANOVA) analyses, after dispersion testing via permdisp.

Relative abundances of the bacterial and fungal genera presenting > 1% abundance in 33% of samples (for bacteria) or 20% of samples (for the more variable fungal communities) were represented per hive niche via barplots using *ggplot2* (Wickham 2016), *microbiome* (Lahti et al. 2017) and *phyloseq* (McMurdie and Holmes 2013). Same cutoff values and packages were used to represent bacterial and fungal species grouped by apiary.

Variations in the relative abundances of bacterial and eukaryotic species were identified through Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) v1.4.0 (Lin and Peddada 2020), by comparing between: i) pre-treatment samples, to determine the apiary effect (a combination of landscape and management strategies), and ii) pre- and post-treatment samples, to determine treatment effect. Analyses were only done on niches presenting significant values in diversity and composition. Differences between treatment stages were calculated per apiary. False Discovery Rate (FDR) correction was used for pairwise comparisons. Features (ASVs) with significant ANCOM-BC results were agglomerated to species levels and the resulting ASVs (species or highest taxonomic level identified) were plotted after compositional (for boxplots) or logarithmic (log10, for heatmap) transformation of their relative abundances, using the R packages *ggplot2*, *microbiome* and *phyloseq*. For bacteria or fungi of interest (significant ANCOM-BC results combined with the literature), niche-specific datasets were filtered and taxa plotted using also *ggplot2, microbiome* and *phyloseq*.

Results

Pre-treatment microbiota status: "apiary" effect

Eukaryotic diversity did not differ between apiaries prior to OA treatment for any hive niche Figs. 2a, S2a, S3a. In contrast, there were significant differences on the bacterial diversity of pre-treatment gut and pupae samples (*Kruskal–Wallis*, $p < 0.05$) Figs. 2a, S2a, S3a. Gut samples belonging to colonies with ecological management and located in a rural or mountainous landscape (AP1 and AP2, respectively) had more homogeneous diversities, while AP3 colonies, under conventional management and located in an urban area, presented higher diversity values and higher heterogeneity between colonies.

Differences in bacterial composition between apiaries were detectable at the apibiome level. The composition of both microbial fractions (bacterial and fungal) had homogeneous dispersion in all sample types when considering the variable apiary (*betadisper*, $p > 0.05$), except for the eukaryotic community of gut (*betadisper* by apiary pre-treatment, $p = 0.001$). However, that was likely due to a few outlier colonies, as revealed by the PCoA. Notably, pre-treatment samples from AP3 clustered apart from those of ecologically managed and less anthropized apiaries (AP1 and AP2), in all sample types (gut, bee bread and pupae) Figs. 2b, S2b, S3b.

Overall, pre-treatment gut samples from the urban and conventionally managed apiary of AP3 had an enrichment of species scarcely present or absent in the ecological apiaries, such as *Hafnia alvei* (*ANCOM-BC,* $W = 126.64$, $p = 0.000$), *Bartonella apis* (*ANCOM-BC,* $W = 31.72$, $p = 0.000$), or the *Enterobacter* (*ANCOM-BC,* $W = 487.39$, $p = 0.000$) and *Serratia* (*ANCOM-BC,* $W = 24.91$, $p = 0.000$) genera Fig. 2c. In addition, bacteria present in pre-treatment guts from both AP1 and AP2 were in general less abundant in AP2, including the *Hafnia* genus, and the *Hafnia alvei* and *Nosema ceranae* species. The eukaryotes of guts were less influenced by location and management (*adonis*, $R2 = 0.088$, $p < 0.01$) Fig. 2b, and abundance variations only occurred in the plants *Erica terminalis* (more abundant in AP2, *ANCOM-BC* $W = 91.22$, $p > 0.001$) and the fungal genus *Cladosporium* (more abundant in AP3, *ANCOM-BC* $W = 26.68$, $p > 0.001$) Fig. 2c.

Pre-treatment bee bread samples presented higher differences between apiaries than bee guts in both bacterial and eukaryotic composition, with clear sample clustering by apiary along the axes of PCoAs, and significant adonis results for the apiary factor ($R^2 = 0.357$ plus $p = 0.001$ for bacteria, and $R^2 = 0.335$ plus $p = 0.001$ for eukaryotes). Compositional differences were larger between AP1 and AP3, clustering at opposite ends of Axis 1, while AP2 hives separated along Axis 2 Fig. S2b. AP3 had the most differentiated microbial profiles, with increased relative abundances of bacterial species from genera such as *Pseudomonas, Fructobacillus* or *Asai*, and lower relative abundances of eukaryotic genera such as *Ramularia* or *Didymella*. The fungal species *Thecaphora semienis-convolvuli* (*ANCOM-BC*, $W = 66.60$, $p < 0.001$), as well as the plant species *Ulex parviflorus* (*ANCOM-BC*, $W = 116.45$, $p < 0.001$), *Erica cinerea* (*ANCOM-BC*, $W = 11.82$, $p = 0.037$), *Angelica sylvestris* (*ANCOM-BC*, $W = 12.16$, $p = 0.035$) and *Origanum majorana* (*ANCOM-BC*, $W = 37.20$, $p < 0.001$) were exclusive to AP3 Fig. S2c.

The eukaryotic composition of pupae samples Fig. S3b did not differ between apiaries, although there was significant variation in two fungal species Fig. S3c. In concordance with the compositional differences observed in the rest of the apibiome (G and BB samples), bacteria varied more than eukaryotes. Bacterial profiles were similar in the apiaries with ecological management and less anthropized landscapes (AP1 and AP2), while samples from AP3 had enrichment of several bacterial species, such as *Pseudomonas fildesensis* or *Delftua acidovorans* Fig. S3c.

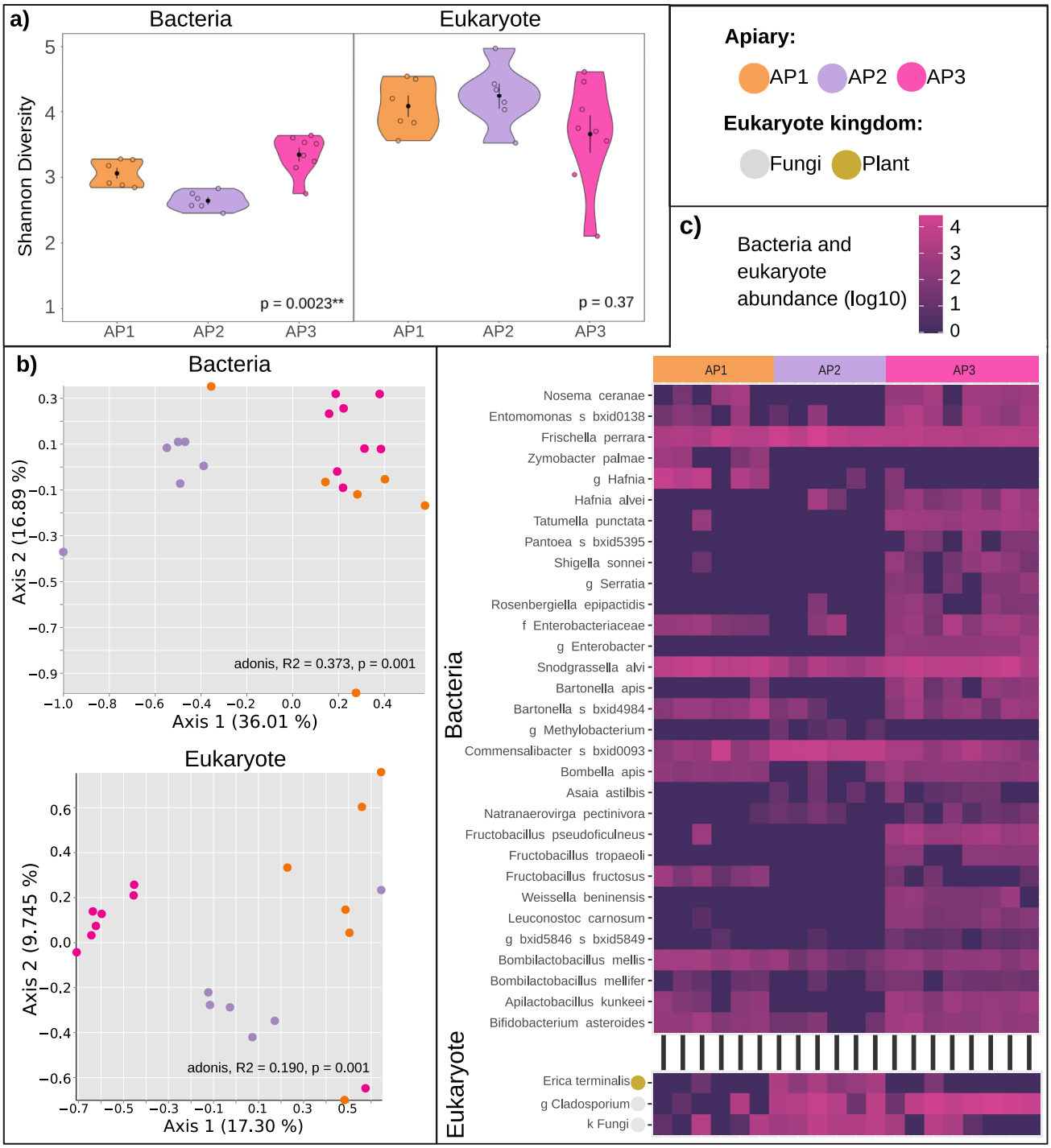


Fig. 2 Apiary effect on bacterial and eukaryotic communities of pre-treatment gut samples. **a)** Bacterial and eukaryotic community diversity of gut samples per apiary, as determined through Shannon's index. Bottom p values refer to global Kruskal–Wallis comparisons. **b)** Distribution of colonies according to their apiary, visualized via Principal Coordinate Analysis (PCoA) based on Bray–Curtis distances. **c)** Heatmaps of the log10 transformed relative abundances of the microbial species presenting different relative abundances across apiaries, according to ANCOM-BC analyses.

Oxalic acid impact on the apibiome: "treatment" effect

No differences were detected in bacteria and eukaryotes when comparing community diversities and composition of pre- and post-treatment colonies by dosage (OA2.1% and OA4.2%) or application method (tricking OA and Varrox) (Table 1). Therefore, the comparison between pre- and post-treatment stages was made without accounting for those factors.

Oxalic acid sparsely influenced bee bread and pupae microbiotas, with no changes in their community diversity Figs. S4a, S4b and no clustering of pre-/post-samples in the PCoAs for either bacteria or eukaryote Figs. S4c, S4d.

On the contrary, OA treatment significantly decreased the diversity of bacteria in the honey bee guts from AP3 colonies, and the diversity of eukaryotes in bee guts from AP2 (*pairwise Kruskal–Wallis*, $p = 0.001$, $p = 0.037$, respectively) Figs. 3a, 3b. Bacterial composition had heterogeneous dispersion when considering treatment stages (*betadisper*, $p = 0.009$), likely due to a few outlier colonies from post-treatment which appeared in each apiary, as revealed by the PCoAs Fig. 3c.

Gut bacterial composition was also significantly influenced by OA application (*PERMANOVA,* $p < 0.05$ for all apiaries) Fig. 3c Table 2. However, most detected changes were apiary and colony specific, as seen in the ANCOM-BC results Figs. 3d, 3e, S5. For instance, enrichments of the Firmicutes *Lactobacillus kimbladii* ($W = 3.85$, $p = 0.001$) and the Actinobacteria *Bifidobacterium indicum* ($W = 2.73$, $p = 0.032$) were observed only in some AP1 colonies Fig. S5a, while the core bacteria *Bombella apis* only significantly increased its abundance ($W = 2.92$, $p = 0.024$) in particular hives of AP2 Fig. S5b. Nevertheless, honey bee guts from the conventionally managed apiary of AP3, located at the urban landscape, showed consistent changes in abundances after treatment, with similar microbial shifts occurring for all or most colonies. In these AP3 colonies OA depleted the relative abundances of Gammaproteobacteria and Bacilli species. Reduction occurred for most Bacilli, such as *Serratia* ($W = 2.51$, $p = 0.043$), as well as for some Proteobacteria such as the *Enterobacter* genus ($W = 6.68$, $p = 0.000$). Increases in the relative abundances of bacteria, across all colonies, were only detected for the Proteobacteria *Salmonella sp. bxid5188* ($W = 4.17$, $p = 0.000$) and *Gilliamella* ($W = 4.28$, $p = 0.0001$) Figs. 3d, 3e, S5c. Interestingly, most of these bacteria mainly appeared at low relative abundances, with mean relative abundances being overall lower than 0.02, except for *Gilliamella* and *Entomomonas sp bxid0138* Figs. 3d, 3e, S5c.

Importantly, pathogen reduction after OA treatment was taxa, apiary and colony specific. *Frischella perrara* showed a decreasing trend in all apiaries but AP3 Fig. 3e. The *Hafnia* genus was completely depleted in AP1 after treatment, although its pathogenic species *H. alvei* increased its abundance in most colonies from AP2 (*ANCOM-BC,* $W = 3.44$, $p = 0.007$) and some from AP3 (*ANCOM-BC*, $p > 0.05$) Figs. 3e, S5. Interestingly, the microsporidia *Nosema ceranae*, when present in the pre-treatment stage (in AP1 and AP3 apiaries), was significantly reduced after OA treatment in all apiaries Fig. 3e.

Table 1 Effect of application methods and treatment dosages in oxalic acid treatment, for all sample types

Domain	Sample type	Adonis		
		Comparison	R^2	*p*
Bacteria	Gut	Treatment stage x OA dosage	0.0197	0.269
	Bee bread		0.0295	0.046*
	Pupae		0.0177	0.351
	Gut	Treatment stage x Application method	0.0083	0.864
	Bee bread		0.0148	0.439
	Pupae		0.0294	0.103
Eukaryote	Gut	Treatment stage x OA dosage	0.0293	0.106
	Bee bread		0.0145	0.745
	Pupae		0.0210	0.603
	Gut	Treatment stage x Application method	0.0225	0.563
	Bee bread		0.0169	0.624
	Pupae		0.0258	0.102

Community compositions were compared through adonis. Significance (marked with *) was considered when $p < 0.05$ for both cases, and *p* values correspond to Benjamini–Hochberg FDR corrected values

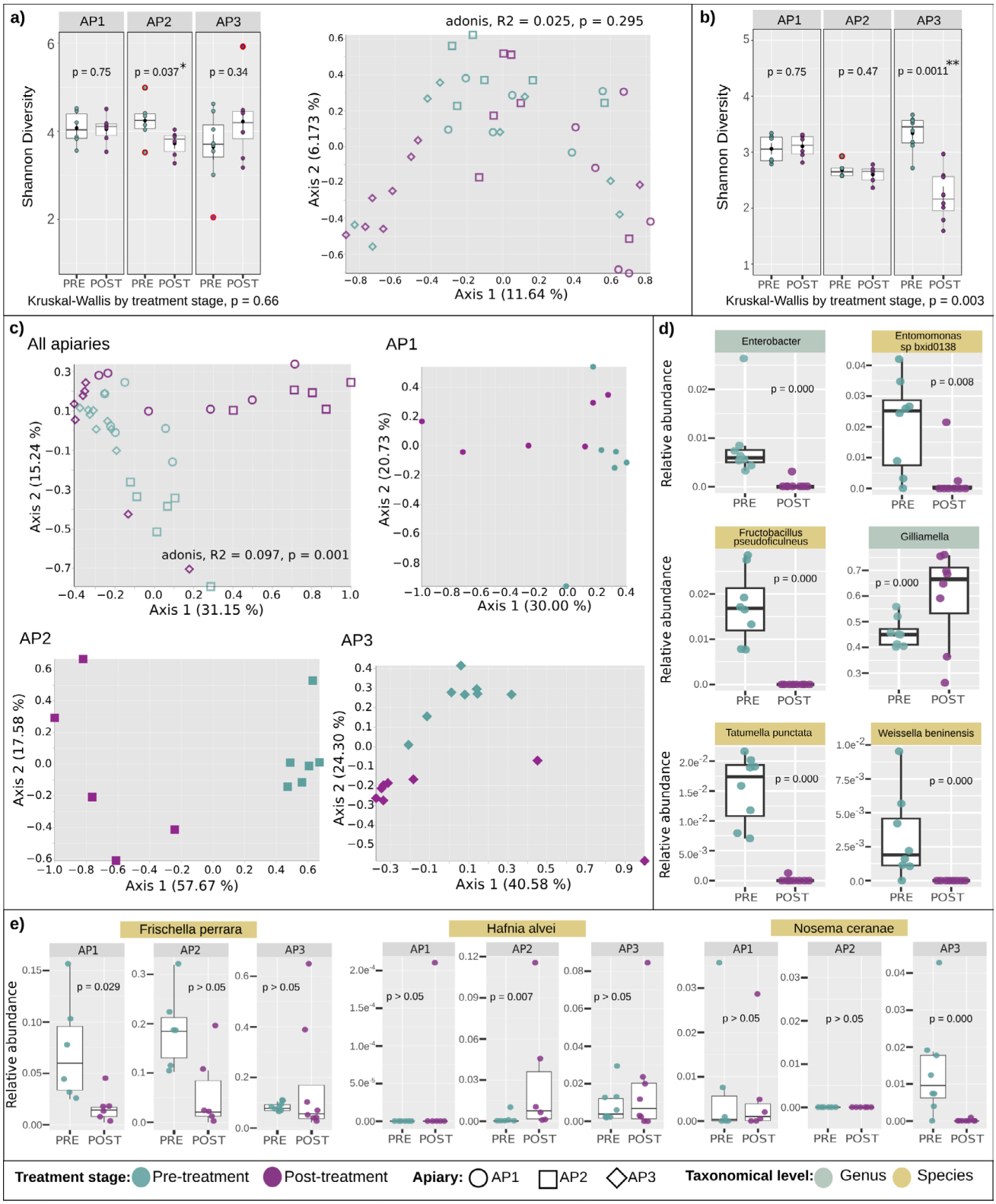
Discussion

The response of the microbiota to oxalic acid (OA) treatment was complex for the studied hives. Overall, Proteobacteria genera and species were the most sensitive, with abundance changes in gut core bacteria (Kešnerová et al. 2020) such as *Bombella, Gilliamella, Commensalibacter, Serratia, Enterobacter* and *Frischella.* However, shifts were not uniform across colonies, even showing opposite trends in abundance changes in some cases. This complexity was in concordance with a previous study performed by Cuesta-Mate et al. (2021), where variability was observed both between apiaries and between experimental setups (in vivo *versus* in vitro experiment). Within the apibiome (the combination of the multiple microbiomes present within hives), OA mostly impacted the bacterial communities of honey bee guts, while the shifts occurring in the other niches (bee bread and pupae) and in the eukaryotic communities were less prominent. In addition, results showed that a month after the application, the exposure to different doses (OA2.1% and OA4.2%) and application methods (tricking OA and Varrox) induced no perceptible differences in the shifts seen at the microbial diversity and community composition level.

The three apiaries we employed in the present study had contrasting anthropic pressure determined by two factors: beekeeping practices and nutritional landscape. The management strategies of the studied beehives were either conventional (AP3) or ecological (AP1, AP2), while the nutritional landscape of the colonies was overall different across all locations, yet had more similar flora in the mountainous and rural landscape of AP1 and AP2, in contrast to the urban and horticultural landscape of AP3. These environmental differences were reflected on the microbial diversity and composition of the pre-treatment stage and likely gave rise to the different response trends to treatment detected between apiaries. For instance, the bee bread samples of AP3 had comparably higher abundances of *Ulex parviflorus* and *Thecaphora seminis-convolvuli,* as well as of the bacteria *Pseudomonas oryzihabitans*, reflecting the more urbanized surroundings of the AP3 apiary. *Ulex parviflorus* is a shrub which flowers from mid-autumn to winter (Nescolarde-Selva et al. 2020), which is local to the Mediterranean area (Sutton 2022) and is often pollinated by *Apis mellifera* (Nescolarde-Selva et al. 2020). The fungi *Thecaphora seminis-convolvuli* is usually found in the seeds and flowers of *Convolvulus* plants (Woods et al. 2018), some of which are horticultural, while *P. oryzihabitans* is a pathogen of warm-blooded animals found in various plant environments not native to the sampled landscapes (Choi et al. 2019; Li et al. 2021). The flowering shrub *Erica cinerea* and *Daboecia cantabrica*, both enriched in AP2, are native to the natural landscapes of northern Spain (GBIF Backbone Taxonomy 2023a, 2023b). Similarly, guts from AP2 showed an enrichment of *Erica terminalis*, not native to northern Spain (GBIF Backbone Taxonomy 2023c).

Besides the singularities derived from the pollen sources, the bacterial profile of honey bee guts from the more anthropized apiary AP3 (situated in an urban landscape and conventionally managed) was characterized by higher bacterial diversity and higher loads of pathogens such as *Nosema ceranae*, *Hafnia alvei* and *Serratia.* These characteristics suggested a dysbiotic state (homeostasis loss) prior to treatment. Stressors linked to anthropization (e.g. pesticides) are known to induce microbial dysbiosis (Hotchkiss et al. 2022) and to increase community diversity (i.e. increased number of distinct taxa) (Castelli et al. 2021), despite the limited number of conserved phylotypes usually found in honey bee guts (Kešnerová et al. 2020). That increase is likely due to the dysbiosis facilitating the growth of opportunistic bacteria in honey bee guts, as previously hypothesized (Blot et al. 2019). Increased microbial stress or dysbiosis can also prompt increased pathogen loads (Castelli et al. 2020; Dosch et al. 2021), and while the presence of bee pathogens was overall low in our colonies, some pathogens appeared at varied abundances across apiaries, being in general the least abundant in AP2, the most ecological and natural environment, which in pre-treatment had the lowest relative abundances of the *Hafnia* genus, *Hafnia alvei* and *Nosema ceranae*. In contrast, the abundances of those pathogens, as well as of the *Serratia* genus, were overall highest in the most anthropized apiary of AP3. *N. ceranae* enrichment has been linked to bee populations with poor diets (Castelli et al. 2020). *H. alvei* is enriched in American Foulbrood-affected colonies, inducing septicemia in worker guts (Erban et al. 2017; Grabowski and Klein 2017), and acts as an inflammatory agent of gut mucosa in bees suffering microbial dysbiosis (Lang et al. 2022). *Serratia marcescens*, the *Serratia* species occasionally found at low abundance in worker's guts, is an opportunistic pathogen (Raymann et al. 2018) which is considered a marker of dysbiosis (Kakumanu et al. 2017; Moran et al. 2012; Raymann et al. 2017).

Higher levels of anthropization in AP3 colonies would also explain the increased *Enterobacteriaceae* (both at family and at genus level, for genera such as *Shigella* or *Serratia*) and decreased *Commensalibacter* relative abundances in pre-treatment guts from that location, as both shifts have been previously linked to landscape anthropization after intensive agricultural practices (Gorrochategui-Ortega et al. 2022). Admittedly, the less anthropized apiary (AP2), located in a mountainous landscape, had the highest abundances of *F. perrara*, an ubiquitous species of honey bee gut which causes the scab phenotype (Engel et al. 2015) and



is linked to developmental problems after poor diet (Maes et al. 2016).

Importantly, the results presented herein suggest that the state of the initial microbiome (i.e. the microbiome present in the colony prior to treatment, which is closely related to the landscape and management type of the apiary) conditions the microbial response to OA treatment. The changes seen after treatment in the bacterial abundances of ecological

◂**Fig. 3** Effect of oxalic acid treatment on the eukaryotic and bacterial communities of gut samples. **a** Eukaryote diversity and composition, visualized via boxplots of Shannon diversity values (bottom *p* value refers to global Kruskal–Wallis comparisons) and Principal Coordinate Analysis (PCoA) of Bray–Curtis dissimilarities (*p* value refers to adonis analyses by treatment stage). **b** Alpha diversity values for bacteria in the gut, calculated via Shannon diversity. Bottom *p* values refer to global Kruskal–Wallis comparisons. **c** Distribution of the bacterial communities of guts according to their treatment stage, while considering all apiaries together or individually. PCoAs are based on Bray–Curtis dissimilarities and adonis results show the differences between treatment stages for all apiaries. **d** Boxplots of the relative abundances of the bacteria showing varied abundances across treatment stages, for taxa with relative abundances equal or bigger than 0.01, in the urban and conventional apiary (AP3). Values within plots correspond to corrected *p* values (Bonferroni) from ANCOM-BC. **e)** Relative abundances of two pathogenic bacteria (*F. perrara* and *H. alvei*) and one pathogenic fungi (*N. ceranae*) across apiaries and treatment stages. *N. ceranae*, despite being a microsporidia, is shown with bacterial taxa as it was detected through 16S amplicon sequencing. The scales of the vertical axes (x axes) vary across apiaries and microorganisms

apiaries located in natural or rural landscapes (AP1 and AP2) were more heterogeneous and varied between colonies, while uniform microbial shifts across colonies only occurred in the most anthropized apiary of AP3. Stressors can have synergistic effects in bee gut microbiomes, exacerbating gut microbial disruptions, while healthier communities are more capable of withstanding them (Dosch et al. 2021; Motta et al. 2018; Paris et al. 2020). Therefore, we hypothesize that the microbiomes of anthropized apiaries could be more susceptible to additional stressors (e.g. pesticides), while healthier communities could more readily withstand chemical application. This would explain the stronger impact of OA treatment in the more diverse yet potentially more unbalanced gut bacterial communities of AP3. The abundance changes in those AP3 colonies, mostly decreases, occurred in low abundant bacterial groups most likely acting as opportunistic bacteria, with shifts only occurring on three common representatives of honey bee guts (*Gilliamella, Serratia* and *Enterobacter*) (Kešnerová et al. 2020). In addition, OA treatment in the conventional AP3 not only affected specific taxa, but also induced a reduction in the diversity and evenness of their gastrointestinal bacteria, which has been previously reported in response to other pesticides (DeGrandi-Hoffman et al. 2017; Liu et al. 2020). The diversity values observed after OA treatment left AP3 post-treatment gut samples with diversity values that resembled those observed in the pre-treatment guts of AP1 and AP2 (less anthropized), suggesting that OA is able to diminish the presence of opportunistic bacteria (causing the higher bacterial diversity), which if not halted could lead to dysbiosis of the microbiome. The response of pathogens to OA treatment, in concordance with the bulk of bacteria in this study, was not uniform but relied on the apiary and colony of origin, as seen for *Hafnia alvei* and *Frischella perrara*. Although treatment did not significantly reduce *F. perrara* abundance in the guts of AP2 bees, where it was most abundant, OA did decrease it in colonies from AP1. This result contradicts the findings of Cuesta-Maté et al. (2021), who showed an enrichment of *F. perrara* after OA application. In contrast, the reduction of *Nosema ceranae* abundances after OA application was general across colonies and apiaries. *N. ceranae* infects the midgut of honey bees, negatively impacting bee lifespan (Alaux et al. 2010; Martín-Hernández et al. 2018; Schwarz and Evans 2013), and causing nutritional and energetic stress after modifying bee carbohydrate reserves in favour of itself (reviewed by Martín-Hernández et al., 2018). Some studies have reported a synergistic effect of *N. ceranae* and pesticides, with neonicotinoid applications increasing bee mortality and altering bee behaviours associated with social immunity (Alaux et al. 2010; Doublet et al. 2015; Pettis et al. 2012, 2013; Vidau et al. 2011). *N. ceranae* prevalence in non-temperate climates has seasonal tendencies, with peak infections during spring–autumn in Europe (Meixner et al. 2014). However, as no seasonal changes have been reported for *N. ceranae* abundances in Spain (Higes et al. 2010; Martín-Hernández et al. 2007b) and other temperate regions (Tapaszti et al. 2009), the depletion detected here is likely due to OA treatment. In concordance, Nanetti et al. (2015) confirmed decreased (compared to controls) prevalence of *N. ceranae* spores in colonies after in-laboratory administration of 0.25 M OA. As we did not observe an enrichment of *N. ceranae* in bee bread samples, bee infection might have occurred by nestmate transmission of spores through trophallaxis, as seen in experimental conditions for queens (Higes et al. 2009) and as hypothesized in other works (Meixner et al. 2014).

Of particular interest was also the significant enrichment of *Gilliamella* abundances in post-treatment samples from AP3, which matched the results previously reported by Cuesta-Maté et al. (2021). *Gilliamella* species, which present great functional diversity at strain level (Engel et al. 2012), digest recalcitrant polysaccharides from the bee gut (Zheng et al. 2019) and participate in pathogen clearance (Lang et al. 2022). Their enrichment in post-treatment guts from AP3 could benefit honey bees, as they would fill in the niches previously occupied by opportunistic bacteria, cleared by OA, and thus prevent future colonisations and avoid dysbiosis. In summary, in colonies located in an urban landscape and under conventional beekeeping practices OA treatment can: i) reduce the abundances of opportunistic bacteria that could induce microbial dysbiosis, ii) reduce pathogen loads and iii) increase the relative abundances of some beneficial groups, such as the *Gilliamella* genus. In contrast, in colonies located in less anthropized landscapes OA treatment did not alter microbial diversity, and only induced bacterial shifts in a few colonies with no general trend across apiaries. However, it is important to remember

Table 2 Pairwise PERMDISP and PERMANOVA results for comparison of the microbial communities present in gut samples at pre- and post-treatment stages, at each apiary

Domain	Apiary	Comparison	PERMDISP		PERMANOVA	
			pseudo-F	*p*	*pseudo-F*	*p*
Bacteria	AP1	Pre-treatment Vs Post-treatment	0.226	0.831	1.983	0.036*
	AP2		0.819	0.512	10.779	0.007*
	AP3		0.672	0.806	3.482	0.007*
Eukaryote	AP1	Pre-treatment Vs Post-treatment	0.545	0.546	1.102	0.432
	AP2		0.062	0.741	1.053	0.482
	AP3		2.563	0.396	1.096	0.432

PERMDISP test measures dissimilarity matrix dispersion for pairwise analyses, where significant p values (<0.05) indicate heterogeneous dispersion. Significance is marked by (*), and *p* values correspond to Benjamini–Hochberg FDR corrected values

that these changes reflect relative abundances, so quantification of both bacterial and fungal load should be considered for further studies.

Conclusion

The results presented herein highlight the role that landscape and beekeeping practices play on the response of *Apis mellifera iberiensis* microbiomes to oxalic acid treatment. Both factors shaped microbial community fitness. Bees from urbanized locations and under conventional management (anthropized colonies) suffered microbial dysbiosis in their gut, characterized by higher bacterial diversity, enrichment of opportunistic bacteria and higher loads of pathogens such as *Nosema ceranae* and *Hafnia alvei*. Less anthropized hives had a less diverse microbiome and more sparse pathogens. Oxalic acid treatment of colonies in a dysbiotic state reduced the presence of opportunistic bacteria (decreasing bacterial diversity), decreased the abundances of pathogens (e.g. *N. ceranae)* and enriched for beneficial bacteria (e.g. *Gilliamella*) in the gut, after approximately one month (middle-term reactions). In comparison, colonies from a more natural landscape and ecological management presented changes in the abundances of specific bacteria only in a few colonies after OA application. Therefore, aside from the proven effectiveness of oxalic acid for varroosis control, our findings suggest that OA could have additional benefits for dysbiotic colonies, while only slightly altering the microbiota of colonies in more balanced states. However, a more extensive risk assessment will be needed to advocate for OA safeness by expanding the number of colonies and landscapes under study, while assessing the long-term effects of oxalic acid on honeybees microbiota and its health.

Author contributions

M.MC., I.Z., A.E. and E.G. designed the experiment and conceptualized the study. E.G. performed field experiments and sampling. J.GO. realized the laboratory experiments. J.GO. analysed and interpreted the data with inputs from M.MC., I.Z. and A.E. J.GO. wrote the manuscript with major contributions from M.MC., I.Z. and A.E. All authors read and approved the manuscript.

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Data availability The 16S rRNA and ITS2 sequences supporting the conclusions of this article are available in the Qiita database with the ID 15168 (https://qiita.ucsd.edu/public/?study_id=15168) and the EMBL-EBI accession number PRJEB66205 (https://www.ebi.ac.uk/ena/browser/view/PRJEB66205).

Declarations

Conflict of interest The authors declare no competing interests.

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