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Beehive products as bioindicators of antimicrobial resistance contamination in the environment

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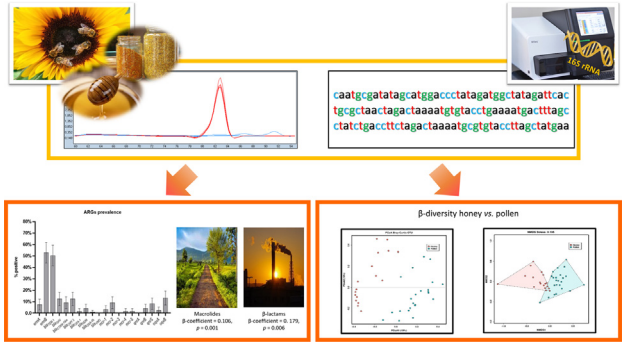
HIGHLIGHTS

- Honey and pollen show similar ARG profiles.
- Beehive products harbour ARGs of environmental origin.
- β-Lactam and macrolide ARGs correlate with anthropogenic environments.
- Honey and pollen represent reliable bioindicators of environmental AMR.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of antimicrobials in agricultural, veterinary and medical practice exerts selective pressure on environmental microbiota, promoting the emergence and spread of antimicrobial resistance (AMR), a global concern for the One Health Initiative Task Force (OHITF). Honeybees have been studied as bioindicators of AMR in the environment, but little is known about beehive products like honey and pollen. The aim of this study was to assess the prevalence of AMR genes (ARGs) in beehive products and investigated their origins. Specifically, possible associations between ARGs, microbiota and other characteristics of different honey and pollen samples, including country of origin, flower type, type of commercial distribution and environmental factors, such as land use, weather and composition of the environment surrounding the beehives were investigated. We found that beehive products harboured ARGs conferring resistance to β-lactams, macrolides, (fluoro)quinolones and polymyxins. Most samples possessed resistance to multiple antimicrobial classes, with honey and pollen showing similar ARG profiles. Even if *Lactobacillus* and *Acinetobacter* genera were common in the microbial communities of both honey and pollen, *Bacillus*, *Clostridium*, and *Bombella* defined honey microbiota, while *Pseudomonas* and *Vibrio* were enriched in pollen. *ErmB* and bla_{TEM-1} co-occurred with *Lactobacillus* and *Fructobacillus*, while positive associations between β-lactams and macrolides and anthropogenic environments (i.e. industrial and commercial areas and non-irrigated arable lands) were found. Altogether, our findings suggest that ARGs in honey and pollen might originate from the honeybee foraging environment, and that the beehive products can be used as bioindicators of the AMR environmental contamination.

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1. Introduction

The spread of antimicrobial resistance (AMR) in the environment is a matter of great concern for public health, since it can be transferred to humans and animals through dispersion in waterways, surface runoff and soil drainage, or by entering into the food chain (Berendonk et al., 2015; Hruby et al., 2016; Marti et al., 2013; Pruden et al., 2012). Wildlife and insects have been investigated as potential indicators of environmental dissemination of AMR, as they are not usually intentionally exposed to antimicrobials for clinical purposes (Martinez, 2009; Zurek and Ghosh, 2014). In recent years, studies have proposed honeybees as indicator of AMR in the environment (Cenci-Goga et al., 2020; Piva et al., 2020). Indeed, during their usual foraging activities, honeybees are able to cover wide areas in which agricultural, industrial, and other anthropogenic activities occur, and therefore are likely to be exposed to contaminated (environmental) sources, such as pollen, nectar and water (Bargańska et al., 2016). Furthermore, since no maximum residue limits have been established for antimicrobials in honey (European Parliament and the Council of the European Union, 2010), the use of antimicrobials in beekeeping is not permitted in the European Union (EU). However, the potentiality of beehive products, such as honey and pollen, to act as indicators of AMR dissemination into the environment has yet to be explored (Bezirtzoglou, 2016).

In the present study, we investigated the prevalence of AMR genes (ARGs) for several antimicrobial classes of public health interest that are commonly used in conventional animal farming (i.e. β-lactams, macrolides, (fluoro)quinolones and polymyxins) in honey and pollen samples collected from either Italian local beekeepers and purchased from the retail markets or online stores. Despite the ban in the EU, antimicrobials are approved and used in beekeeping in many countries all over the world (Bonerba et al., 2021; European Parliament and the Council of the European Union, 2010; Savarino et al., 2020); therefore, beehive products produced outside the EU were also included in the study. Moreover, we investigated the microbial community composition of beehive products and the potential effects of the environment surrounding the beehives. This latter analysis was performed on a subset of georeferenced beehives located in Northeast Italy, considering a radius around the beehives corresponding to the average honeybee's foraging range. Therefore, the main purpose of the study was to assess the ARG profiles in beehive products of different origins in light of honeybees being a potential indicator of AMR dissemination in the environment, as well as to indirectly assess the potential risk for (beehive product) consumers' health. Furthermore, we aimed to identify potential correlates (i.e. land use, microbial community composition, country of origin, flower type, and type of commercial distribution) of ARG occurrence in beehive products.

2. Materials and methods

2.1. Samples

A total of 97 honey and 24 pollen samples were included in the present study. The complete list of samples by type (i.e. honey or pollen), year of production (2017–2019), type of flower (i.e. mono- or multifloral), country of origin (i.e. Italy or outside Italy) and type of commercial distribution (i.e. local, large-scale market, or e-commerce), is reported in Supplementary material 1.

2.1.1. Honey samples

Honey samples from local beekeepers were collected from producers located in the north-eastern part of Italy (n = 65), except for one sample of Romanian origin, while the remaining samples (n = 31) were purchased from the large-scale market (LSM). According to the label, the samples were produced in 2017 (n = 60), 2018 (n = 18), and 2019 (n = 19). The large-scale market samples were either of Italian (n = 14) or non-Italian (n = 17) origin.

2.1.2. Study area of local beekeepers

Of the 66 local honey samples, 54 were obtained from beehives located in Belluno, a province characterized by a predominant mountainous environment, in Northeast Italy. According to the Italian Institute of Statistic (ISTAT), this province has a surface of 3610.20 km^2, and a population density of 55.42 people/km^2. For 26 out of 54 samples, the coordinates of the beehive locations were available.

2.1.3. Pollen samples

Pollen samples (n = 24) were all obtained from on-line retailers. Samples were collected in 2018 (n = 5) and 2019 (n = 19). According to the label, eight samples were of Italian origin, while the remaining 16 samples were of non-Italian origin.

2.2. DNA extraction

Ten grams (g) of honey were processed following the protocol optimized by Balzan et al. (2020) and DNA was extracted from 250 mg of the resulting pellet using the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany). DNA extraction from pollen samples was carried out using the same commercial kit. Briefly, 250 mg of pollen were placed directly into PowerBead Pro Tubes, added with lysis solution and shacked in a TissueLyser (Qiagen) for 30 s four times. After this first step, the isolation was performed following the manufacturer's instructions. DNA purity and quantity were assessed using a UV–Vis spectrophotometer NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, United States).

2.3. 16S rRNA gene amplification, sequencing and data analysis

Amplification of the V3-V4 regions of the 16S rRNA gene and NGS libraries preparation were carried out for honey samples collected in 2019 (n = 19) and for all pollen samples (n = 24), as previously described Balzan et al. (2020). Libraries were then sequenced using the Illumina MiSeq sequencing platform (San Diego, California, USA) with a 2 × 300 bp paired-end approach.

Within the Quantitative Insights into Microbial Ecology 2 (QIIME2 version 2019.4) software, the DADA2 package was used for 16S rRNA data analysis (Bolyen et al., 2019; Callahan et al., 2016). Taxa assignment was carried out by using SILVA- Naive Bayes sklearn trained database (Yilmaz et al., 2014). Raw reads have been deposited in the NCBI Short Read Archive under the accession number PRJNA751468. To assess α- and β-diversity statistics of the microbial communities, the on-line based software Calypso (http://cgenome.net/wiki/index.php/Calypso) was employed, with default parameters data filtering, and by adopting total sum normalization (TSS) and SquareRoot for data transformation (Zakrzewski et al., 2017). The microbial community composition was visualized using heatmap. To quantify the microbiome diversity within honey and pollen samples, Shannon and Simpson indexes were employed. To assess differences in microbiome composition between the two beehive products, the permutational multivariable analysis of variance (PERMANOVA) based on the Bray-Curtis dissimilar measure for significance testing using the Adonis function, was used. For β-diversity visualization, principal coordinate analysis (PCoA) plots and nonmetric multidimensional scaling (NMDS) were used. The linear discriminant analysis (LDA) effect size method (LEfSe) was used to identify the taxa most likely to explain differences between classes.

2.4. Real-time PCR analysis of antimicrobial resistance genes (ARGs)

A total of 97 honey samples and 24 pollen samples were tested for the presence of ARGs by real-time polymerase chain reaction (Real-time PCR), employing the protocols described by Laconi et al. (2021). In detail, gene-specific SYBR® Green assays paired with melting curve analysis were used for detecting the following ARGs: bla_{TEM-1}, bla_{SHV}, $bla_{CTX-M-1like}$, bla_{CMY-2}, bla_{OXA-1}, bla_{OXA-48}, bla_{VIM-2}, bla_{NDM}, *ermA*, *ermB*,

oqxA, *oqxB*, *qnrS*, *qnrA*, *qnrB*, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*. All real-time PCRs were performed using PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific) in a LightCycler®480 Roche (Roche, Basel, Switzerland) real-time platform.

2.5. Environmental variables

The environmental variables included in the analysis were computed within a buffer radius of 3 km around each of the 26 Italian beehives geolocalized from the longitude and latitude coordinates acquired by GPS. The percentage of land coverage in the radius was calculated for each of the 44 land cover classes defined by the Corine Land Cover (CLC) 2018 in vector format. Since CLC classes include urban, industrial, and agricultural areas, as well as forests, wetlands, and water bodies, they were used to investigate the effects of both anthropogenic and natural areas on the occurrence of ARGs in honey samples. The density of farms (farms/km^2) for poultry, pigs, cattle and other livestock (i.e. sheep, goats, horses, rabbit, and aquaculture) in the radius was also calculated using the georeferenced farms in the 2017, 2018, 2019 generated by information registered in the Italian National Beekeeping Registry (BDNA) and stored in the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) Data warehouse. The number of rainy days (>0.1 mm of water) and the mm/day of rain over the foraging period (three months) were obtained from the IZSVe Environmental data for Veterinary Epidemiology system (a system dedicated to process and store data derived from satellite products such as MODIS, Sentinel-2), processing the original data published by Environmental Prevention and Protection Agency of the Veneto Region (ARPAV).

2.6. Statistical analysis

Statistical analysis was performed according to Laconi et al. (2021). To test the associations between the explanatory variables (i.e. type of samples, year of production, type of flower, foreign origin, and type of commercial distribution) a hierarchical clustering was generated based on the presence/absence of each ARG using pHeatmap package version 1.0.12 in R (version 3.6.3) (https://www.r-project.org/). Differences in each ARG occurrence (binary outcome variable) over the aforementioned explanatory variables (covariates) were tested for significance using multivariable logistic regression analysis. The same analysis was performed also with the outcome variable being the presence/absence of at least one ARG per antimicrobial class (i.e. β-lactams, macrolides, (fluoro)quinolones, polymyxins). Differences in single ARG-level multi-resistance (i.e. sum of all ARGs detected in a sample), as well as antimicrobial class-level multi-resistance (i.e. sum of one ARG per antibiotic class detected in a sample) over the explanatory variables were tested for significance using multivariable ordinal logistic regression. A sub-analysis considering only the honey samples was also performed. To assess the association between the relative abundance of the main microbial taxa at genus level (≥25% prevalence over all samples) and the ARGs detected at a minimum prevalence level of 5% in the samples, multivariate regression analysis with several dependent variables (i.e. log-transformed relative abundances) was used to jointly regress on the same independent variables (i.e. presence/absence of the different ARGs), while adjusting for the other explanatory variables using bias-corrected and accelerated cluster-bootstrapped standard errors (1000 replications). Sequence data of honey samples collected in 2017 and 2018 (PRJNA601326) were also included in association analysis. Family-wise Bonferroni correction of the p-value was applied to control for Type I error. The analysis of the environmental correlates of ARGs in honey samples from 26 local beehives that could be geographically localized was performed using spatial autoregressive regression to account for the geographical distribution of the beehives leading to non-independence of observations from nearby locations. Given the low number of observations and outcome events, this analysis was performed at the antimicrobial class level. Statistical analysis and data visualization were carried out in STATA (version 16) and R (version 3.6.3) (https://www.r-project.org/).

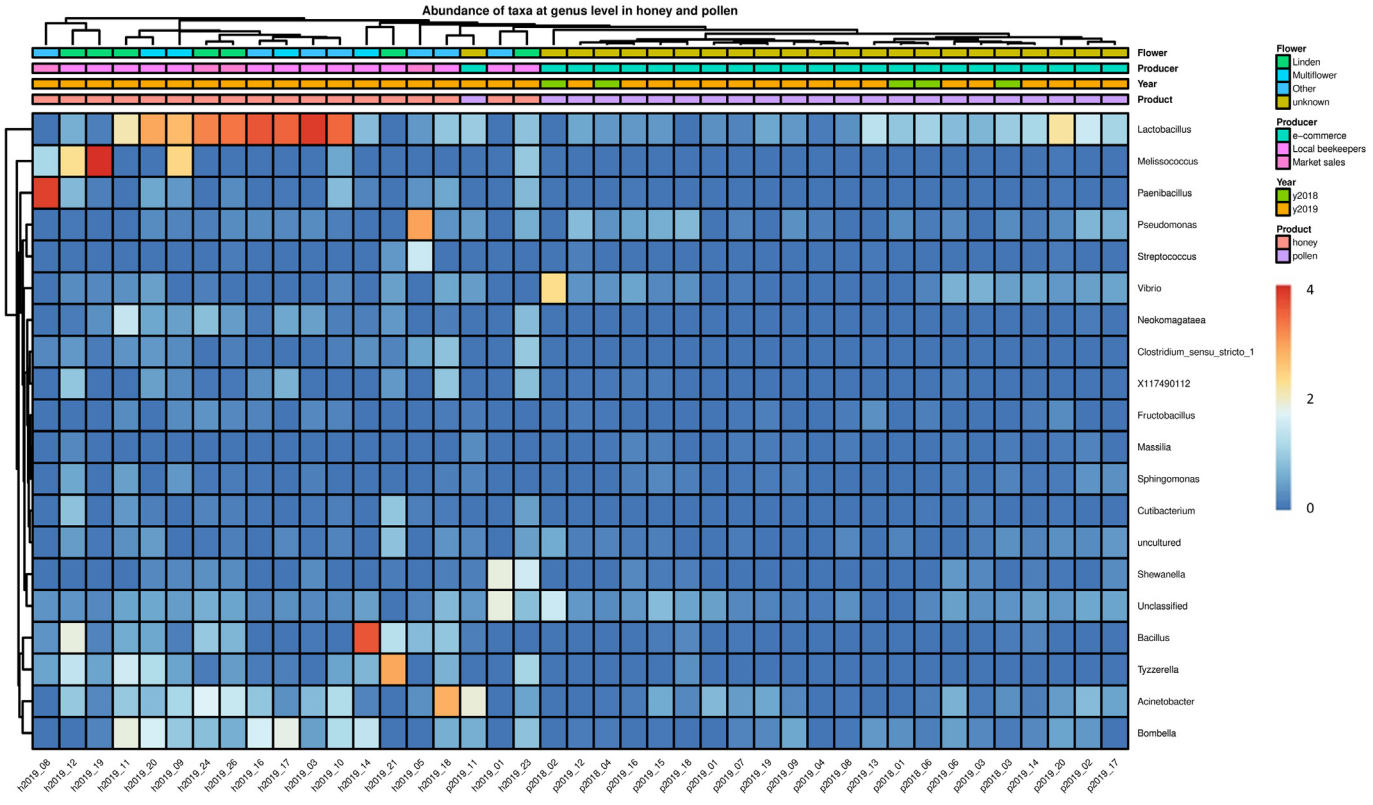

Fig. 1. Heatmap representing the microbial community composition of honey and pollen samples at genus level.

3. Results

3.1. General description of DNA sequences

After the quality-filter step, removal of chimeric fragments and reads merging, a total of 749249 reads was obtained with 1867 different features, with an average of 17424 sequences per individual sample. Filtering by quality, one sample was excluded and the remaining 42 were considered for the characterization of the microbial communities.

3.2. Composition of bacterial communities, α- and β-diversity

Using 16S rRNA gene sequencing, the microbial community structure of honey and pollen samples was characterized. Firmicutes and Proteobacteria dominated honey and pollen microbiota at phylum level, while Bacilli and Alphaproteobacteria were the predominant classes. *Lactobacillus* and *Acinetobacter* were abundant in both beehive products; however, other genera showed a different distribution with *Bacillus*, *Clostridium* and *Bombella* being more abundant in honey ($p < 0.05$) and *Pseudomonas* and *Vibrio* being more abundant in pollen ($p < 0.05$). As a result, the heatmap at genus level (Fig. 1) shows two main clusters, one grouping honey and one pollen samples.

The α- and β-diversity were assessed at the Operational Taxonomic Unit (OTU) level. The α-diversity, expressed using both the Shannon and Simpson indexes, was comparable between the two sample types (Fig. 2). However, PERMANOVA showed that the microbial communities of honey and pollen were significantly ($p = 0.0003$) different from one another. Indeed, the PCoA and NMDS graphs (Fig. 3A, B) show a clear separation between honey and pollen samples. LEfSe analysis (Fig. 3C) identified 21 taxa associated with honey, including the following genera: *Bacillus* (linear discriminant analysis (LDA) = 4.41), *Clostridium* (LDA = 4.04), *Melissococcus* (LDA = 4.38) and *Staphylococcus* (LDA = 3.53). Nine taxa were associated with pollen samples (Fig. 3C), including *Mycoplasma* (LDA = 4.11), *Pseudomonas* (LDA = 4.32) and *Vibrio* (LDA = 4.43) genera.

3.3. Prevalence of ARGs

The prevalence of ARGs in honey ($n = 97$) and pollen ($n = 24$) samples was investigated. Of the 20 ARGs considered, all but *qnrA*, bla_{VIM2}, and *mcr-3* genes were detected in at least one sample, while 91.75% (95% confidence of interval (CI) 85.00–96.64%) of honey and 83.33% (95% CI 67.26–99.41%) of pollen samples resulted positive to at least one ARG. Notably, 63.64% (95% CI 54.94–72.33%) of the samples possessed more than one ARG, 58.68% (95% CI 49.78–67.58%) showed resistance to more than one antimicrobial class, and 14.05% (95% CI 7.76–20.33%) to at least three antimicrobial classes. *ErmB* (52.89%, 95% CI 41.38–61.91%) and bla_{TEM-1} (50.41%, 95% CI 41.38–59.45%) were the most prevalent ARGs, followed by *oqxB* (13.22%, 95% CI 7.1–19.35%), bla_{SHV} and bla_{CMY-2} (12.40%, 95% CI 6.44–18.35%), while for the remaining genes the prevalence ranged from 9.09% (95% CI 3.89–14.29%) of $bla_{CTX\text{-}M\text{-}1LIKE}$ and *mcr-2* to 0.86% (95% CI 0.00–2.46%) of $bla_{OXA\text{-}48}$ (Fig. 4A). Target genes showed a similar distribution in the two sample types, even if some differences were observed; $bla_{OXA\text{-}1}$, $bla_{OXA\text{-}48}$, and *qnrB* were detected in honey, but not in pollen samples (Fig. 4B). Furthermore, a higher prevalence of *ermB* and bla_{TEM-1} was observed in honey (59.79%, 95% CI 49.86–69.73% and 54.64%, 95% CI 44.55–64.73, respectively) than in pollen (25.00%, 95% CI 6.32–43.68% and 33.33%, 95% CI 13.00–53.67%, respectively), and vice versa *mcr-2* was more prevalent in pollen (33.33%, 95% CI 13.00–53.67%) than in honey (3.09%, 95% CI 0.00–6.60%) samples (Fig. 4B). However, there were no significant differences ($p > 0.05$) in the prevalence of ARGs between the two beehive products. Only when considering the antimicrobial class-based multiresistance, a significant ($p = 0.021$) difference was observed between pollen (mean 1.5 resistances per sample, range 0–3) and honey (mean 1.7 resistances per sample, range 1–4). The hierarchical clustering analyses based on the 17 ARGs detected, did not show any clear clustering accordingly to the explanatory variables considered (Supplementary material 2).

The prevalence of *oqxB* was significantly higher ($p = 0.031$) in large-scale market honeys (26.67%, 95% CI 9.87–43.46%) than in those of local beekeepers (8.95.7%, 95% CI 1.93–5.97%) (Fig. 4C), while bla_{TEM-1} was significantly more prevalent ($p = 0.037$) in honey samples of Italian origin (59.09%, 95% CI 48.61–69.57%) than in those of non-Italian origin (27.78%, 95% CI 4.86–50.70%) (Fig. 4D). When considering the other explanatory variables, no other significant differences ($p > 0.05$) in ARGs prevalence were found.

3.4. Associations between microbial communities and ARGs

The ARGs significantly associated with specific taxa (genus level) are reported in Table 1. In total, 7 taxa were found to be associated with at least one ARG. The occurrence of *ermB* and bla_{TEM-1} genes showed the largest number of significant associations with the abundance of specific taxa: *ermB* occurrence was positively associated with *Erwinia*, *Serratia*, *Rosenbergiella* and *Lactobacillus* abundance, while bla_{TEM-1} occurrence was positively associated with *Gilliamella*, *Fructobacillus* and *Lactobacillus* abundance. The occurrence of $bla_{CTX\text{-}M\text{-}1LIKE}$ was positively associated with *Bombella* abundance.

3.5. Environmental correlates of ARGs

Factors significantly associated with the occurrence of resistance to macrolides in the local beekeeper honey samples ($n = 26$) were the

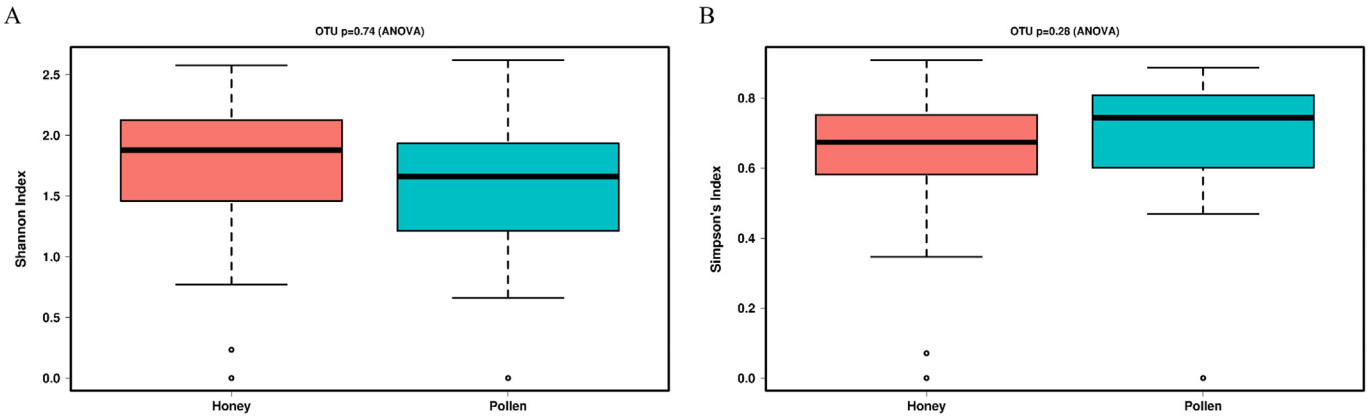

Fig. 2. α-Diversity within honey and pollen samples using Shannon (A) and Simpson (B) indexes. Boxplots represent 25th to 75th percentiles and whiskers showing a maximum of 1.5× the interquartile range (IQR).

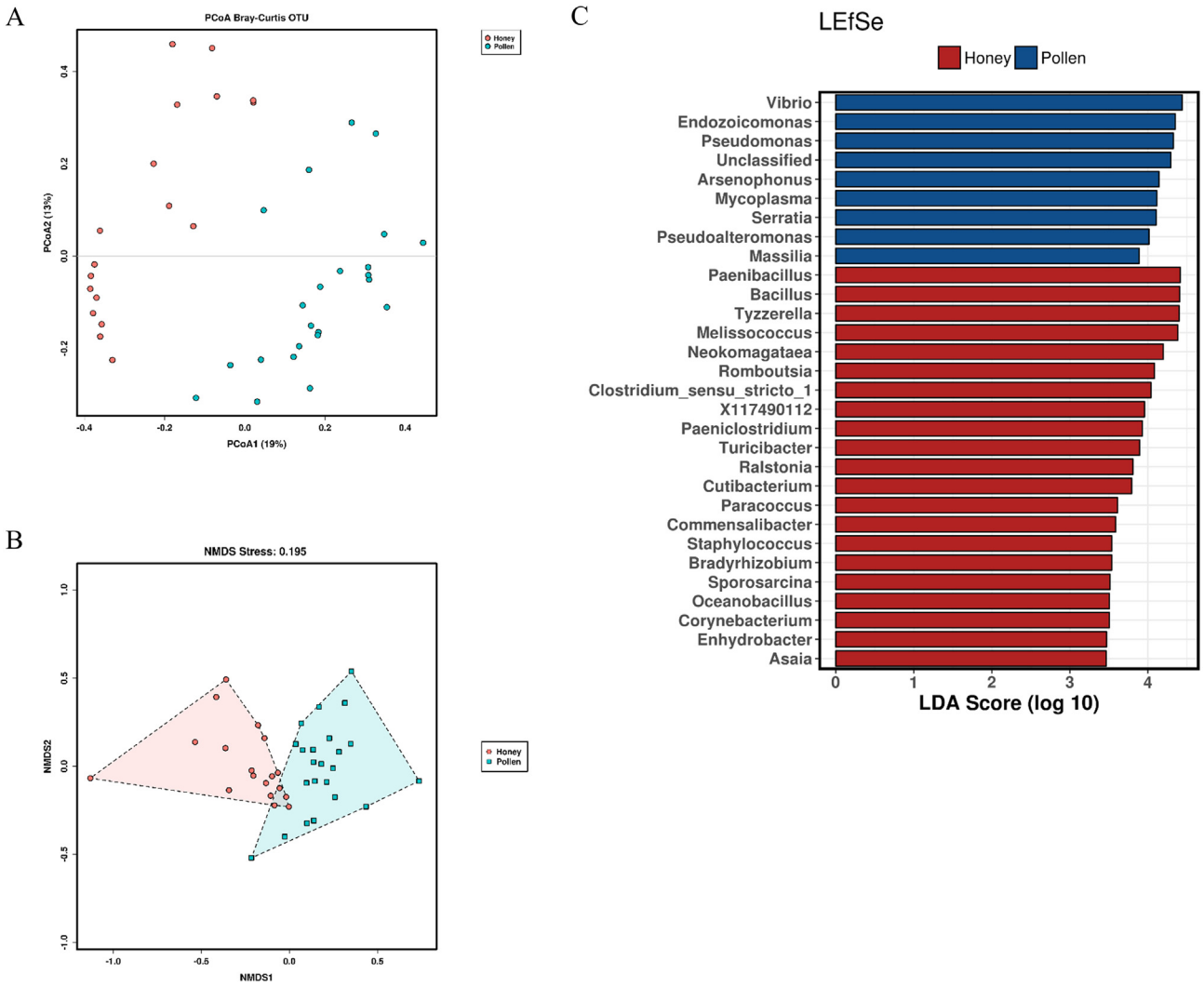

Fig. 3. β-Diversity between honey and pollen samples. In both PCoA (A) and NDMS (B) analysis, samples are clustered according to Bray-Curtis distances. C) LDA scores of LEfSe comparison analysis between honey and pollen samples. The red and blue shading depicts bacterial taxa significantly higher in honey and pollen samples, respectively.

increasing percentage of non-irrigated arable land (β-coefficient = 0.106, p = 0.001) and broad-leaved forest (β-coefficient = 0.021, p = 0.0001) and the decreasing percentage of complex cultivation patterns (β-coefficient = −0.017, p = 0.001) around the beehive. Factors significantly associated with the occurrence of resistance to β-lactams were the increasing percentage of land covered by industrial or commercial units (β-coefficient = 0. 179, p = 0.006) and the decreasing percentage of complex cultivation patterns (β-coefficient = −0.020, p = 0.001) and the decreasing density of poultry farms (β-coefficient = −3.207, p = 0.001). No other significant associations were found.

4. Discussion

4.1. Microbial community composition differs between honey and pollen

The microbial community composition of honey and pollen is in line with previous studies (Ambika Manirajan et al., 2016; Balzan et al., 2020), with the phyla Proteobacteria and Firmicutes being the most abundant in both types of samples. However, β-diversity, as assessed using PERMANOVA analysis, showed a significant difference between honey and pollen microbiome. The microbial communities of pollen could be affected by both the floral nectars microbiota and by the honeybee microbiota. However, in agreement with previous observations, our findings seem to suggest that the former is more influential on pollen microbial community composition (Donkersley et al., 2018). Indeed, among the most abundant genera there were *Pseudomonas*, *Lactobacillus* and *Acinetobacter*, which are commonly found in pollen grains of insect-pollinated plants (Ambika Manirajan et al., 2016; Lenaerts et al., 2016). Honey microbial community composition is the result of more complex interactions between the microbiota resident in honeybee gut and body surfaces, the beehive infrastructures, and the food (i.e. pollen and honey bread) stored in the beehives (Vásquez et al., 2012). Accordingly, together with genera highly abundant in pollen (i.e. *Lactobacillus* and *Acinetobacter*), the most common genera in honey samples included *Bacillus*, *Clostridium*, and *Staphylococcus*, which are also abundant in the honeybee gut (Anderson et al., 2013; Vásquez et al., 2012). Notably, the genus *Melissococcus*, to which belong the species *M. plutonius*, the causative agent of European foulbrood (EFB), a disease which spreads in the hive at the brood level and kills larvae, was more abundant in honey than in pollen. Taken together, our data seem to confirm that pollen microbial communities resemble those of the pollinated flowers, while both pollen and honeybee gut influence honey's microbiota.

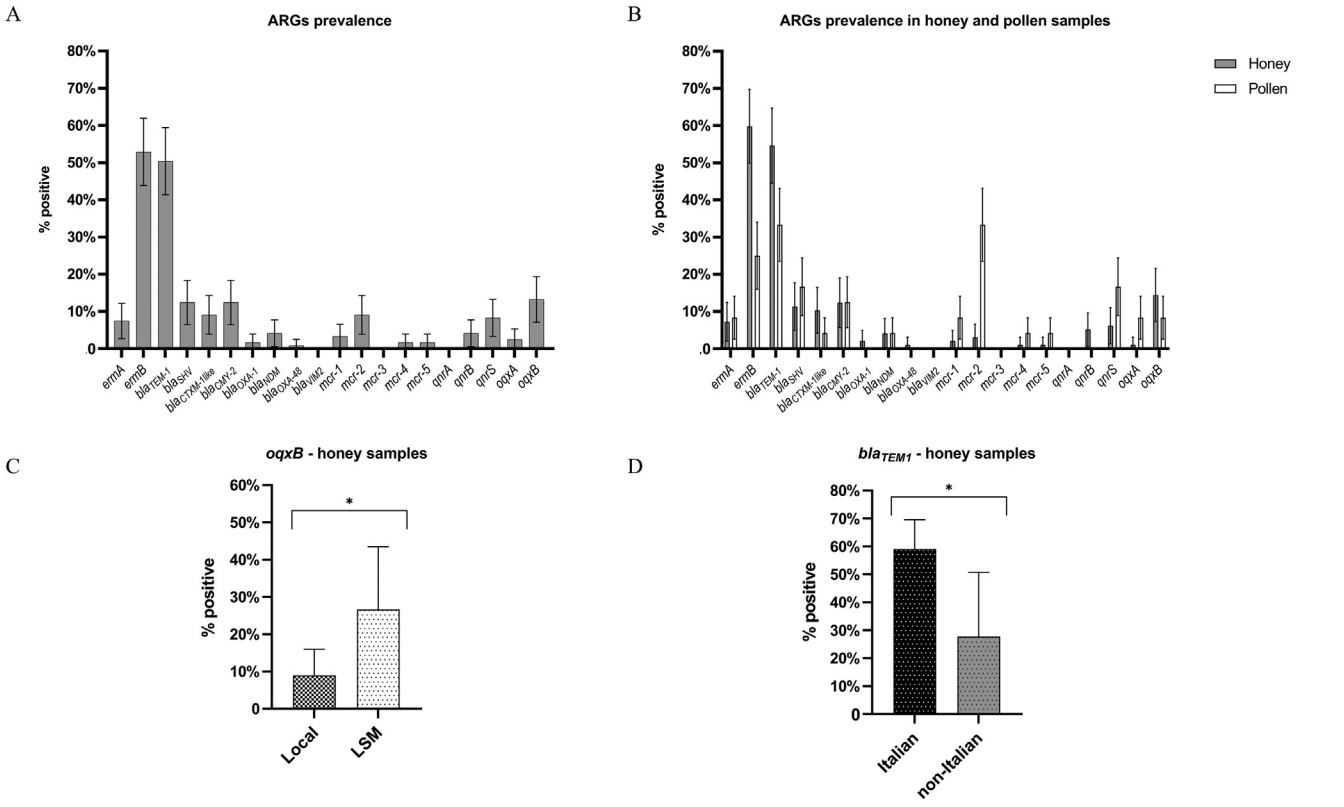

Fig. 4. Prevalence of target genes in honey and pollen samples. A) Overall prevalence of ARGs. B) Prevalence of ARGs in honey (grey) and pollen (white). C) Prevalence of *oqxB* in honey samples obtained from local producers and large-scale market (LSM). D) Prevalence of bla_{TEM-1} in honeys of Italian and non-Italian origin. Bars represent 95% confidence interval (CI). $p < 0.05$ shown as *.

4.2. Honey and pollen show similar ARGs profiles

ARGs were detected in the vast majority of the samples. Remarkably, more than half of the samples showed antimicrobial class multi-resistance, and only three of the targeted genes (i.e. *qnrA*, bla_{VIM2} and *mcr-3*) went undetected. While ARGs against macrolides (i.e. *ermB* and *ermC*) have been previously identified in honey (Okamoto et al., 2021), this study reports for the first time the presence of ARGs conferring resistance to β-lactams, (fluoro)quinolones, and polymyxins, and describes for the first time the detection of ARGs in pollen. The occurrence of ARGs in beehive products might represent a potential risk for human health, as resistance determinants can be transferred among bacteria via mobile genetic elements (MGEs), resulting in the emergence of resistant bacteria, including human pathogens (Martínez et al., 2015). Among the ARGs detected, the carbapenemase genes bla_{OXA-1} and bla_{OXA-48} and the *mcr* genes confer resistance to carbapenems and polymyxins, respectively, which represent last resort antimicrobials against multi-drug resistant Gram-negative bacteria (Poirel et al., 2010; Xia et al., 2019).

Table 1
Significant associations between microbial taxa and antimicrobial resistance genes. β-coefficients and Bonferroni-corrected p-values (within parentheses) are shown.

	$bla_{CTXM-1-LIKE}$	*ermB*	bla_{TEM1}
Gilliamella			0.095 (0.009)
Erwinia		0.139 (0.045)	
Serratia		0.119 (0.036)	
Rosenbergiella		0.169 (0.009)	
Fructobacillus			0.134 (0.036)
Bombella	0.324 (0.027)		
Lactobacillus		0.350 (0.009)	0.390 (0.001)

The presence of ARGs in beehive products might be attributed to the contamination of the foraging environment (e.g. manure amended soil, wastewater, environmental pollution, etc.), the contaminated equipment used during harvesting and processing of beehive products, or the antimicrobials exerting a selective pressure on honeybee gut microbiota. Moreover, large-scale market honeys usually undergo mixing of different batches originating from different geographical areas or even countries. Accordingly, our study reports a higher prevalence of the plasmid-mediated quinolone resistance (PMQR) gene *oqxB* in large-scale markets than in local honeys. Honey and pollen showed comparable ARG profiles, suggesting that most of the ARGs in honey might originate from pollen, and therefore implying a relation between ARGs in beehive products and environmental AMR contamination. According to microbiota dynamics (Daisley et al., 2020; Donkersley et al., 2018), pollen resistome might derive from the environment and influence the resistome in honeybee gut and in honey. Indeed, β-lactamase encoding genes are widespread in the environment (Graham et al., 2016), and β-lactams are not effective against *Paenibacillus larvae* and *M. plutonius*, the causative agents of American and European Foulbrood, respectively (Reybroeck et al., 2012), discarding the hypothesis of being the result of antimicrobial treatments in the beehives and enforcing the hypothesis that the occurrence of these ARGs in honey is due to foraging activities. Indeed, studies investigating β-lactamase encoding genes and β-lactam resistant bacteria in honeybees have previously suggested a possible environmental origin of the observed AMR (Cenci-Goga et al., 2020; Piva et al., 2020). Macrolides are commonly used against diseases of the beehive (i.e. American and European Foulbrood) outside the EU or for illegal treatments (Reybroeck et al., 2012). Hence, their occurrence in honey samples might be also due to the antimicrobial treatments of the beehive, rather than exclusively to environmental contamination. The higher prevalence of *ermB* in honey than in pollen seems to point to a combined effect of AMR environmental contamination and selective pressure on the honeybee gut microbiota.

Even if the contamination during harvesting and processing might play a role in the presence of ARGs in beehive products, and legit (or illegal) treatments cannot be ruled out for some antimicrobial drugs (i.e. macrolides and (fluoro)quinolones), most of the investigated genes seems to be acquired from the environment during foraging activities, suggesting that honey and pollen might represent useful and reliable bioindicators of AMR dissemination. In this regards, future studies should aim at investigating the entire resistome of honey and pollen, by adopting a metagenomics approach.

4.3. Associations between ARGs, microbial taxa and environmental factors

The positive associations between the most prevalent ARGs (i.e. *ermB* and bla_{TEM-1}) and the genera *Lactobacillus* and *Fructobacillus*, which were highly abundant in both beehive products and common in the microbiota of pollinated flowers (Ambika Manirajan et al., 2016; Donkersley et al., 2018), seems to confirm the environmental origin of these resistance determinants. Increased prevalence of $bla_{CTX\text{-}M\text{-}1LIKE}$ extended-spectrum β-lactamases (ESBLs) encoding genes in association with the genus *Bombella*, more abundant in honey than in pollen, might be due to horizontal gene transfer (HGT) events, since this genus dominates brood and honeybee queens gut microbiota, but is seldom detected in their food (i.e. pollen and honey bread) (Smith and Newton, 2020). Significant associations between the land use and resistance to β-lactams and macrolides were also found. Industrial and commercial units were associated with an increased occurrence of ARGs against β-lactams, while non-irrigated arable lands and broad-leave forests were positively associated with resistance to macrolides. The positive association between the occurrence of these ARGs and lands impacted by anthropogenic activities supports the hypothesis of their environmental origin, since they can spread and persist in the environment and both β-lactams and macrolides are used in agriculture, livestock and humans (Graham et al., 2016; Laconi et al., 2021; Lopatto et al., 2019). The positive association between macrolide resistance and semi-natural areas, such as broad-leave forests, might be due to the presence of naturally occurring resistant bacteria in soil, ponds and insect-pollinated flowers present in these environments (Osbiston et al., 2021). Indeed, woodlands represent privileged spots for foraging activities, being associated with increased microbial richness and protein content, which are both beneficial to honeybees (Donkersley et al., 2018). Complex cultivation patterns represent small agricultural activities, e.g. mosaic of parcels of permanent fruit trees, vineyards and hobby gardens, and therefore less likely to involve the use of antimicrobials or the application of antimicrobial-impacted manure, possibly explaining the negative association with the occurrence of both β-lactams and macrolides ARGs. In contrast with previous observations, no positive associations with antimicrobial resistance and livestock productions were found (Cenci-Goga et al., 2020) to the extent that the occurrence of β-lactams was negatively associated with the density of poultry farms within the foraging range, possibly due to the significant reduction of antimicrobial use in the Italian poultry industry (Caucci et al., 2019).

5. Conclusion

In the present study, we analyzed honey and pollen samples of different origin and type over a three-year period. By using gene specific real-time PCR assays, we assessed the prevalence of ARGs in these beehive products and investigated their association with the microbial communities and different environmental factors. The main conclusions of the study are:

- ARGs showed a similar distribution between honey and pollen, suggesting that most of the genes detected in honey might derive from pollen, and therefore might be of environmental origin.
- Resistance to β-lactams and macrolides, which are commonly used in livestock and humans, was positively associated with urbanized and agricultural areas, supporting the hypothesis that the AMR detected in beehive products originated from the foraging environment.
- Honey and pollen may be considered as reliable and non-invasive bioindicators of AMR dissemination in the environment.
- The presence of ARGs against last resort drugs for the treatment of multi-resistant bacteria (i.e. *mcr*, $bla_{OXA\text{-}1}$, and $bla_{OXA\text{-}48}$ genes) in beehive products represents a risk for consumers' health, since resistance genes can be transferred to pathogenic bacteria.

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CRediT authorship contribution statement

Andrea Laconi: Formal analysis, Conceptualization, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Roberta Tolosi:** Investigation, Data curation, Validation. **Lapo Mughini-Gras:** Formal analysis, Writing – original draft, Writing – review & editing. **Matteo Mazzucato:** Formal analysis, Writing – review & editing. **Nicola Ferrè:** Formal analysis, Writing – review & editing. **Lisa Carraro:** Investigation, Validation. **Barbara Cardazzo:** Resources. **Francesca Capolongo:** Writing – review & editing. **Roberta Merlanti:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Alessandra Piccirillo:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – original draft, 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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References

Ambika Manirajan, B., Ratering, S., Rusch, V., Schwiertz, A., Geissler-Plaum, R., Cardinale, M., Schnell, S., 2016. Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity. Environ. Microbiol. 18, 5161–5174. https://doi.org/10.1111/1462-2920.13524.

Anderson, K.E., Sheehan, T.H., Mott, B.M., Maes, P., Snyder, L., Schwan, M.R., Walton, A., Jones, B.M., Corby-Harris, V., 2013. Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (Apis mellifera). PLoS One 8. https://doi.org/10.1371/journal.pone.0083125.

Balzan, S., Carraro, L., Merlanti, R., Lucatello, L., Capolongo, F., Fontana, F., Novelli, E., Larini, I., Vitulo, N., Cardazzo, B., 2020. Microbial metabarcoding highlights different bacterial and fungal populations in honey samples from local beekeepers and market in North-Eastern Italy. Int. J. Food Microbiol. 334, 108806. https://doi.org/10.1016/j.ijfoodmicro.2020.108806.

Bargańska, Ż., Ślebioda, M., Namieśnik, J., 2016. Honey bees and their products: bioindicators of environmental contamination. Crit. Rev. Environ. Sci. Technol. 46, 235–248. https://doi.org/10.1080/10643389.2015.1078220.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439.

Bezirtzoglou, E., 2016. Emerging antibiotic resistance in honey as a Hazard for human health. J. Bacteriol. Mycol. Open Access 2, 6–12. https://doi.org/10.15406/jbmoa.2016.02.00012.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.Bin, Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L.,

Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Bonerba, E., Panseri, S., Arioli, F., Nobile, M., Terio, V., Di Cesare, F., Tantillo, G., Maria Chiesa, L., 2021. Determination of antibiotic residues in honey in relation to different potential sources and relevance for food inspection. Food Chem. 334, 127575. https://doi.org/10.1016/j.foodchem.2020.127575.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Caucci, C., Di Martino, G., Dalla Costa, A., Santagiuliana, M., Lorenzetto, M., Capello, K., Mughini-Gras, L., Gavazzi, L., Bonfanti, L., 2019. Trends and correlates of antimicrobial use in broiler and Turkey farms: a poultry company registry-based study in Italy. J. Antimicrob. Chemother. 74, 2784–2787. https://doi.org/10.1093/jac/dkz212.

Cenci-Goga, B.T., Sechi, P., Karama, M., Ciavarella, R., Pipistrelli, M.V., Goretti, E., Elia, A.C., Gardi, T., Pallottini, M., Rossi, R., Selvaggi, R., Grispoldi, L., 2020. Cross-sectional study to identify risk factors associated with the occurrence of antimicrobial resistance genes in honey bees (Apis mellifera) in Umbria, Central Italy. Environ. Sci. Pollut. Res. 27, 9637–9645. https://doi.org/10.1007/s11356-020-07629-3.

Daisley, B.A., Pitek, A.P., Chmiel, J.A., Gibbon, S., Chernyshova, A.M., Al, K.F., Faragalla, K.M., Burton, J.P., Thompson, G.J., Reid, G., 2020. *Lactobacillus* spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. Commun. Biol. https://doi.org/10.1038/s42003-020-01259-8.

Donkersley, P., Rhodes, G., Pickup, R.W., Jones, K.C., Wilson, K., 2018. Bacterial communities associated with honeybee food stores are correlated with land use. Ecol. Evol. 8, 4743–4756. https://doi.org/10.1002/ece3.3999.

European Parliament and the Council of the European Union, 2010. Commission regulation (EU) No 37/2010. Off. J. Eur. Union L15, 1–72.

Graham, D.W., Knapp, C.W., Christensen, B.T., McCluskey, S., Dolfing, J., 2016. Appearance of β-lactam resistance genes in agricultural soils and clinical isolates over the 20th century. Sci. Rep. 6, 1–8. https://doi.org/10.1038/srep21550.

Hruby, C.E., Soupir, M.L., Moorman, T.B., Shelley, M., Kanwar, R.S., 2016. Effects of tillage and poultry manure application rates on salmonella and fecal indicator bacteria concentrations in tiles draining Des Moines lobe soils. J. Environ. Manag. 171, 60–69. https://doi.org/10.1016/j.jenvman.2016.01.040.

Laconi, A., Mughini-Gras, L., Tolosi, R., Grilli, G., Trocino, A., Carraro, L., Di Cesare, F., Cagnardi, P., Piccirillo, A., 2021. Microbial community composition and antimicrobial resistance in agricultural soils fertilized with livestock manure from conventional farming in northern Italy. Sci. Total Environ. 760, 143404. https://doi.org/10.1016/j.scitotenv.2020.143404.

Lenaerts, M., Pozo, M.I., Wäckers, F., Van den Ende, W., Jacquemyn, H., Lievens, B., 2016. Impact of microbial communities on floral nectar chemistry: potential implications for biological control of pest insects. Basic Appl. Ecol. 17, 189–198. https://doi.org/10.1016/j.baae.2015.10.001.

Lopatto, E., Choi, J., Colina, A., Ma, L., Howe, A., Hinsa-Leasure, S., 2019. Characterizing the soil microbiome and quantifying antibiotic resistance gene dynamics in agricultural soil following swine CAFO manure application. PLoS One 14, e0220770. https://doi.org/10.1371/journal.pone.0220770.

Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Topp, E., 2013. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Appl. Environ. Microbiol. 79, 5701–5709. https://doi.org/10.1128/AEM.01682-13.

Martinez, J.L., 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. Proc. R. Soc. B Biol. Sci. 276, 2521–2530. https://doi.org/10.1098/rspb.2009.0320.

Martínez, J.L., Coque, T.M., Baquero, F., 2015. What is a resistance gene? Ranking risk in resistomes. Nat. Rev. Microbiol. 13, 116–123. https://doi.org/10.1038/nrmicro3399.

Okamoto, M., Kumagai, M., Kanamori, H., Takamatsu, D., 2021. Antimicrobial resistance genes in bacteria isolated from Japanese honey, and their potential for conferring macrolide and lincosamide resistance in the American foulbrood pathogen paenibacillus larvae. Front. Microbiol. 12, 1–16. https://doi.org/10.3389/fmicb.2021.667096.

Osbiston, K., Oxbrough, A., Fernández-Martínez, L.T., 2021. Antibiotic resistance levels in soils from urban and rural land uses in Great Britain. Access Microbiol. 3. https://doi.org/10.1099/acmi.0.000181.

Piva, S., Giacometti, F., Marti, E., Massella, E., Cabbri, R., Galuppi, R., Serraino, A., 2020. Could honey bees signal the spread of antimicrobial resistance in the environment? Lett. Appl. Microbiol. 70, 349–355. https://doi.org/10.1111/lam.13288.

Poirel, L., Naas, T., Nordmann, P., 2010. Diversity, epidemiology, and genetics of class D β-lactamases. Antimicrob. Agents Chemother. 54, 24–38. https://doi.org/10.1128/AAC.01512-08.

Pruden, A., Arabi, M., Storteboom, H.N., 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environ. Sci. Technol. 46, 11541–11549. https://doi.org/10.1021/es302657r.

Reybroeck, W., Daeseleire, E., De Brabander, H.F., Herman, L., 2012. Antimicrobials in beekeeping. Vet. Microbiol. 158, 1–11. https://doi.org/10.1016/j.vetmic.2012.01.012.

Savarino, A.E., Terio, V., Barrasso, R., Ceci, E., Panseri, S., Chiesa, L.M., Bonerba, E., 2020. Occurrence residues in apulian of antibiotichoney: pollution potential riskby anti bioticsof environmental. Ital. J. Food Saf. 9. https://doi.org/10.4081/ijfs.2020.8678.

Smith, E.A., Newton, I.L.G., 2020. Genomic signatures of honey bee association in an acetic acid symbiont. Genome Biol. Evol. 12, 1882–1894. https://doi.org/10.1093/GBE/EVAA183.

Vásquez, A., Forsgren, E., Fries, I., Paxton, R.J., Flaberg, E., Szekely, L., Olofsson, T.C., 2012. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. PLoS One 7. https://doi.org/10.1371/journal.pone.0033188.

Xia, X., Wang, Z., Fu, Y., Du, X.Dang, Gao, B., Zhou, Y., He, J., Wang, Y., Shen, J., Jiang, H., Wu, Y., 2019. Association of colistin residues and manure treatment with the abundance of mcr-1 gene in swine feedlots. Environ. Int. 127, 361–370. https://doi.org/10.1016/j.envint.2019.03.061.

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glöckner, F.O., 2014. The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. Nucleic Acids Res. 42, 643–648. https://doi.org/10.1093/nar/gkt1209.

Zakrzewski, M., Proietti, C., Ellis, J.J., Hasan, S., Brion, M.J., Berger, B., Krause, L., 2017. Calypso: a user-friendly web-server for mining and visualizing microbiome-environment interactions. Bioinformatics 33, 782–783. https://doi.org/10.1093/bioinformatics/btw725.

Zurek, L., Ghosh, A., 2014. Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. Appl. Environ. Microbiol. 80, 3562–3567. https://doi.org/10.1128/AEM.00600-14.