Short communication

Chronic exposure to pesticides disrupts the bacterial and fungal co-existence and the cross-kingdom network characteristics of honey bee gut microbiome

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Abstract

Gut microbiome communities have a significant impact on bee health and disease and have been shown to be shaped by a variety of factors, including exposure to pesticides and inhive chemicals. However, it is unknown whether pesticide exposure affects the coexistence and cross-kingdom network parameters of bee gut microbiome communities because microbes may compete in the gut environment under different stressors. Therefore, we conducted additional analysis of the microbiome data from our previous study in which we discovered that exposure to two novel insecticides flupyradifurone (FPF) and sulfoxaflor (Sulf) or/and a fungicide, azoxystrobin (Azoxy) caused dysbiosis of bee gut microbiota that was associated with an increase in the relative abundance of opportunistic pathogens such as *Serratia marcescens*. We investigated for the first time the potential cross-kingdom fungal-bacterial interactions using co-occurrence pattern correlation and network analysis. We discovered that exposure to FPF or Sulf alone or in combination with Azoxy fungicide influenced the co-existence patterns of fungal and bacterial communities. Significant differences in degree centrality, closeness centrality, and eigenvector centrality distribution indices were also found in single and double-treatment groups compared to controls. The effects of FPF and Sulf alone on cross-kingdom parameters (bacterial to fungal node ratio, degree of centrality, closeness centrality, and eigenvector centrality) were distinct, but this was reversed when they were combined with Azoxy fungicide. The fungal and bacterial hub taxa identified differed, with only a few shared hubs across treatments, suggesting microbial cross-kingdom networks may be disrupted differently under different stressors. Our findings add to our understanding of pesticide effects on the bee gut microbiome and bee health in general, while also emphasizing the importance of cross-kingdom network analysis in future microbiome research.

Keywords: Gut microbiota, microbes' coexistence, pesticides, dysbiosis, interkingdom network analysis, honey bees.

1. Introduction

The western honey bee, *Apis mellifera*, has a gut microbial community that is relatively stable and is thought to improve host health and defend against parasites and pathogens (Dosch et al., 2021; Huang et al., 2023; Kwong and Moran, 2016; Moran et al., 2019; Zheng et al., 2018). Many studies, however, have shown that pesticides and in-hive chemicals used in apiculture can disrupt the honey bee gut microbiome, putting host bee health at risk (Blot et al., 2019; Dai et al., 2018; Hotchkiss et al., 2022; Kakumanu et al., 2016; E. Motta et al., 2018; Motta et al., 2022; Qi et al., 2022). However, the majority of these studies focused on the effects of pesticides on the bacterial communities of the microbiome, despite the fact that the bee microbiome includes members from many more kingdoms (archaea, fungi, protozoa, and viruses) (Engel et al., 2016). For example, until June 2023, only three studies looked at the effects of pesticides on both fungal and bacterial communities, compared to 22 studies that only looked at bacterial communities (personal literature survey, supplementary fig. S1). The relative and absolute abundances of the members of these microbial communities, as well as their interactions, will determine the microbiome's overall contribution to host health.

Previous research found that a decrease in bacterial richness in the gut of solitary bee larvae *Megachile rotundata* after antifungal treatment, implying that changes in the fungal community caused changes in the bacterial community (McFrederick et al., 2014). Moreover, introducing *Wickerhamomyces anomalus* yeast to bees with a well-developed microbiota was immunomodulatory and had an effect on the overall microbial community (Tauber et al., 2019). Therefore, it is crucial to investigate how microbes coexist and interact under different stressors, as well as the effects on the host, including whether they are affected by dysbiosis in other microbiome members. This is because microbes may compete in the gut environment under

different stressors, with some directly or indirectly inhibiting the growth of others (McFrederick et al., 2014).

Cross-kingdom network analysis aided in the identification of hub or keystone non-bacterial species in the microbiome that can influence community stability and connectivity (Kim et al., 2020; Lee et al., 2022; Lemoinne et al., 2020). Although it has many unresolved intrinsic and technical limitations (Faust and Raes, 2012), it is still the only exploratory data analysis technique that allows researchers to infer microbial interactions from sequence data, particularly cross-kingdom interactions. For instance, the network parameters provide key insights into the associations between taxa and the influence of some taxa on particular modules or the whole community (Peschel et al., 2021; Singavarapu et al., 2023). The current state of research assumes a simplified "competition vs. cooperation" framework in the microbiome between the bacterial and fungal kingdoms. As a result, it is critical to uncover potential pesticide effects on cross-kingdom networks to determine how pesticide exposure affects hub or keystone bacterial and non-bacterial species (Lee et al., 2022).

Previously, we discovered that chronic exposure to field realistic concentrations of two new agrochemicals, sulfoxaflor (Sulf) and flupyradifurone (FPF), alone or in combination with azoxystrobin (Azoxy), had a significant impact on the composition of the honey bee microbial community (bacteria and fungi). Fungicide, insecticides, and fungicide-insecticides combinations all had varying effects on the relative abundance of the top ten genera of the bee gut microbiota. Moreover, dysbiosis of the gut microbiota has been linked to an increase in the relative abundance of opportunistic pathogens like *Serratia marcescens* (Al Naggar et al., 2022). However, it is unknown whether exposure to these pesticides affects the coexistence and cross-kingdom network

parameters of bacterial and fungal communities of the bee gut microbiome and its implication to the bee health in bee microbiome.

To fill this knowledge gap, we conducted additional analysis of the microbiome data from our prior study (Al Naggar et al., 2022) to ascertain the impact of these novel insecticides (FPF and Sulf) or/and Azoxy fungicide on the honey bee gut microbial community coexistence and cross kingdom co-occurrence network characteristics. We hypothesized that pesticide exposure, either individually or in combination, would affect the coexistence and interkingdom network parameters of fungal and bacterial communities in the bee microbiome.

2. Materials and methods

2.1 Honey bees

The experimental design was presented in detail in (Al Naggar et al., 2022). Briefly, newly emerged bees were fed the gut homogenate of inhive (nurse) bees diluted 3:8 with sterile 1:1 sucrose-water solution. This mixture was then given to experimental bees housed in metal cages via bulk feeding 24 h as described in detail in our previous work. Then the tube was removed and a new one containing 1:1 sucrose water- solution was provided for 4 days for microbiome establishment, following (Dosch et al., 2021). This method of developing the gut microbiota was chosen because it results in bees with gut communities like those found in natural bee colonies (Kwong et al., 2014; Zheng et al., 2017b).

Sublethal and field-relevant concentrations of FPF (4.30 µg/g) or Sulf (46.97 ng/g) found in nectar were given chronically *ad libitum* to bees via sugar water for 10 days, while 3 % of the LD_{50} of Azoxy (38.18 µg bee^{-1}) was used. At day zero of pesticide exposure, one bee from each of the 18 cages was collected (total 18 bees) and considered a reference sample to document the initial or background gut microbiota. Cages were then assigned to 6 treatments with three cages per

treatment and 30 bees per cage: control, FPF, Sulf, Azoxy, FPF+Azoxy, and Sulf+Azoxy as illustrated in **Fig. 1**. At 5 and 10 days after exposure, subsamples of 3 bees per cage (9 bees per treatment) were collected for microbiome analysis. Then the gut region, including the midgut/ventriculus, the ileum, and the rectum, was dissected out of each bee under sterile conditions and stored at - 80 °C till genomic DNA extraction.

2.2. Sequence data and statistical analysis

The DNA extraction, amplicon library preparation, sequencing and bioinformatic analysis procedures were reported earlier (Al Naggar et al., 2022). We used the same sequence data deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) under BioProject ID PRJNA839609. The respective metadata, ASV matrices, taxonomic tables, representative sequences and phylogenetic trees were imported into R using the phyloseq package (McMurdie and Holmes, 2013). For this study the fungal and bacterial datasets were rarefied to 1042 and 13204 reads per sample, respectively, to get all the treatments and replicates. In total 108 individuals per treatment per time (9 individual x 6 treatments x 2 time points) and 9 time zero controls resulting in 117 individuals were used for analysis.

The data consistency on the fungal and bacterial beta diversity was tested using Permanova and Principal Coordinate Analysis (PCoA) based ordinations using the Bray-Curtis distance with the functions adonis2, ordinate and plot-ordination implemented in phyloseq. The pattern of co-existence of the fungal and bacterial communities in the bee gut across treatments was tested using mantel correlation test between the fungal and bacterial community Bray-Curtis distance matrices using the vegan package (Oksanen et al., 2022). Linear regression model was fitted, and the relationship was statistically tested and plotted using the stat_poly_line and stat_poly_eq functions of the ggpmisc extension of ggplot2 (Wickham, 2016). Pairwise comparison between treatments

was done using pairwise Wilcoxon signed-rank tests followed by BH multiple-testing correction using the rstatix package (Kassambara, 2023).

Bacterial and fungal cross-kingdom co-occurrence networks were constructed for each of the 6 treatments (Control, FPF, Sulf, Azoxy, FPF+Azoxy, Sulf+Azoxy) using the function multi.spiec.easi of SpiecEasi package (Kurtz et al., 2015). SpiecEasi controls the spurious co-occurrences by controlling for the lack of independence in normalized count data, which accounts for the high number of edges in the network-based analysis of amplicon data sets. In addition, it estimates interactions through sparse inverse covariance selection of a transformed compositional data set than correlation. Networks were estimated by the Meinshausen and Bühlmann graph inference method. The nlambda and lambda.min.ratio values were optimized to get a network stability value close to 0.05 and network assessment was done over the nlambda values for every 50 cross-validations. Networks were then plotted using the ggnet2 function of GGally package (Schloerke et al., 2021). Network structural and topological properties, including edges, centrality indices, and modularity were calculated using the igraph package (Csardi, Gabor, 2006). Modularity and modules (elementary components of any biological network, and their identification and characterization give more information about the network's local interaction patterns and their contribution to the overall structure, connectivity, and function of the network) (Layeghifard et al., 2017) that are considered to be subcommunities in each network were determined based on a hierarchical agglomeration algorithm with modularity optimization using the cluster_fast_greedy function. Differences in the distribution of six network centrality measures (Dai et al., 2019; Deng et al., 2012) including node degree (number of links with other taxa also used to identify community hub taxa), betweenness (centrality based on a measure of a taxon's influence in the network), closeness (a measure of a taxon's closeness to all other members), closeness centrality (measures node efficiency in terms of connection to other nodes), eigenvector

centrality (a measure of a taxon's linkage to others accounting for how connected the others are), and transitivity or clustering coefficient (a measure of the tendency of the nodes to cluster together) between treatments microbial networks were tested with pairwise Wilcoxon signed-rank tests followed by BH multiple-testing correction using the rstatix package (Kassambara, 2023). All statistical analyses were performed using R (R Core Team, 2020).

3. Results

3.1 Effects on beta diversity

The effects of pesticides treatment on the microbial community composition of honey bee guts are consistent with our previous study, even though we increased the sample size and decreased the per sample read coverage. The only difference was that no significant interactive effect (treatment x time) on bacterial community composition was found using permutational multivariate analysis of variance (PERMANOVA), which was also marginally significant with $p = 0.045$ in the previous dataset.

3.2 Effects on the coexistence pattern of bee gut microbiota

The co-existence patterns of the fungal and bacterial communities in the honey bee gut were tested across the treatment groups using mantel test which revealed a significant correlation between the two communities only in the control samples ($r = 0.231$, $p = 0.031$). Consistently linear regression model analysis of the two distance matrices also showed a substantial relationship only for the control samples, implying that pesticide exposure disrupts this positive relationship **(Fig. 2)**.

3.2 Effects on cross-kingdom network characteristics

Cross-kingdom bacterial and fungal community network analysis and visualization also revealed a significant impact of pesticides exposure on the co-occurrence network patterns as compared to control communities. The cross-kingdom network of FPF and Sulf, for example, has a higher percentage of modularity (76-79 vs. 69), a 1.7-fold decrease in the ratio of fungal to bacterial nodes (26-27% vs. 46%), a higher percentage of positive edges (91.3 vs. 84.8), and a lower percentage of negative edges (8.7 vs. 15.2) compared to control **(Fig. 3)**. Azoxy's cross-kingdom network was relatively similar to the control. However, the double treatments FPF + Azoxy and Sulf +Azoxy affected these cross-kingdom network relationships differently compared to both control and single treatments **(Fig. 3)**.

In order to evaluate the underlying network community organization as well as the significance of the community members, we tested the distribution of six crucial network centrality indices. We found significant differences ($P < 0.05$) in the distribution's indices of degree, closeness, and eigenvector centrality in single and double treatment groups compared to control **(Table 1)**. The network transitivity was significantly different ($P < 0.05$) in all treated groups compared to controls except in the azoxy alone or combined with FPF treated groups. There were no significant differences in network betweenness centrality ($P > 0.05$) between treated and non-treated (control) groups **(Table 1)**.

When we compared the network centrality indices of double treatments and single treatments, we discovered a significant difference ($p < 0.05$) in both closeness centrality and eigenvector centrality of the double treatment 'FPF plus Azoxy' compared to both FPF insecticide and Azoxy fungicide individually **(Table 1)**. While we found a significant difference ($p < 0.05$) in both closeness and eigenvector centrality of the double treatment 'Sulf plus Azoxy' compared to both Sulf insecticide and Azoxy fungicide individually **(Table 1)**. We discovered that the number

of fungal nodes is greater than the number of bacterial nodes; however, the percentage of bacterial hubs (i..e the most connected OTUs, according to their degree) relative to the number of taxa was greater than the percentage of fungal hubs, and pesticide exposure altered the percentages of hubs in treated groups differently than in control groups **(Fig. 4 a, b)**. Surprisingly, the identified fungal and bacterial hub taxa were distinct, with only a few hubs shared across treatments **(Supplementary file S1)**.

An interesting trend can also be seen in **(Fig. 4 a, c, d, e)**, where we observed a decrease in the ratio of bacterial to fungal nodes, as well as the degree of centrality and closeness centrality while increasing eigenvector centrality in both the FPF and Sulf cross-kingdom networks when compared to the control. However, in cross-kingdom networks of the double treatments, this trend was reversed, indicating the impact of the fungicide Azoxy, as Azoxy alone showed a similar pattern to the control. Furthermore, the cross-kingdom centrality closeness index was significantly lower ($p < 0.05$) in FPF alone or in combination with Azoxy, as well as in Sulf+Azoxy treated groups compared to controls **(Fig. 4 f)**.

4. Discussion

Pesticides can disrupt gut microbes by either directly affecting microbe growth or by causing a decline in host health to the point where the host can no longer properly regulate its gut microbiota (Hotchkiss et al., 2022). It is, however, critical to investigate how microbes coexist and interact in the bee gut under various stressors, as well as the consequences to the host health.

In this study, we investigated for the first time the potential of cross-kingdom fungal-bacterial co-occurrence network analysis to unravel the impact of pesticide exposure on the co-existence of fungi and bacteria in the bee gut, a method first developed for the soil microbiome (Barberán et al., 2012) and later used for the plant-microbiome (Cardinale et al., 2015; Chen et al.,

2018). We found that exposure to two novel insecticides, FPF or Sulf alone or in combination with Azoxy fungicide impacted the co-existence patterns of the fungal and bacterial communities as well as their cross-kingdom network parameters. These results emphasize the detrimental effects of these pesticides and may help to explain the increase in the relative abundance of opportunistic pathogens, such as *Serratia marcescens*, that we previously identified due to dysbiosis of the gut microbiota after exposure to these pesticides (Al Naggar et al., 2022).

High values of degree centrality and betweenness centrality, respectively, may indicate stronger relationships among taxa and a strong influence of some taxa on bridging or communicating between different parts of the network (Ma et al., 2016). In our study, in both the FPF and Sulf cross-kingdom networks, the degree of centrality and closeness centrality decreased while eigenvector centrality increased. This trend, however, was reversed in cross-kingdom networks of the double treatments, indicating the impact of the fungicide Azoxy, as Azoxy alone showed a similar pattern to control. These findings imply that different agrochemicals (insecticides, vs. fungicides) either alone or in combination had different effects on microbial communities cross-kingdom network (Matsuzaki et al., 2023). Previous research also revealed that the effect of pesticides on honey bee gut microbiota is pesticide dependent. For instance, glyphosate herbicide, as well as neurotoxic insecticides such as coumaphos, fipronil, thiamethoxam, and imidacloprid, have been shown to disrupt honey bee gut microbiota (Motta et al., 2018; Motta and Moran, 2020; Rouzé et al., 2019), whereas carbendazim fungicide does not (Wang et al., 2022).

The hub taxa are more ecologically significant than other microbes because their removal would have an impact on the overall community assemblage (Faust and Raes, 2012). The hub microbes can have a significant impact on the microbial community by suppressing or inducing the development of other populations. Even though the number of fungal nodes in the cross-kingdom networks was greater than that of bacteria in the current study, the percentage of bacterial

hubs relative to the number of taxa was greater than that of fungi, and pesticides exposure altered the percentages of hubs in treated groups differently than in control groups. Furthermore, the fungal and bacterial hub taxa identified were distinct, with only a few hub taxa were shared across treatments, suggesting that different agrochemicals had different effects on microbial community's cross-kingdom network connectivity and centrality. These effects may be attributed to the fact that fungi and bacteria coexist and interact both physically and chemically, exhibiting both antagonism and cooperation in a mixed bacterial-fungal complex habitat (Frey-Klett et al., 2011). As a result, under various stressors, the bee gut microbiome cross-kingdom network patterns may be disrupted differently, resulting in a lack of similarity between microbiome hubs (Hernandez et al., 2021).

5. Conclusion

The insecticides FPF or Sulf, alone or in combination with the fungicide Azoxy, disturbed the coexistence patterns of fungal and bacterial communities as well as their cross-kingdom network characteristics, which is consistent with and clarifies our prior findings (Al Naggar et al., 2022). Our research is the first, to our knowledge, to demonstrate how pesticide exposure disrupts cross-kingdom bacterial-fungal relationships in honey bee gut microbiota. In addition, it emphasizes the importance of studying the coexistence of different gut microbiota as well as cross-kingdom network analysis in future bee gut microbiome research to improve our understanding of the effects of pesticide exposure on the reorganization of the bee gut microbiome and its implications for bee health.

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7. Supplementary material

Supplementary file S1: Interkingdom network analysis results for each treatment and summary of hub taxa identified.

Figure S1. Literature review of the number of studies that investigated the effects of pesticides on the gut microbiota of bees between 2003 and 2023.

8. References

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Table 1. Pairwise comparison of fungal and bacterial cross-kingdom network parameters across treatment groups. Analysis output is summarized in three categories of (**A**) control versus all treatments of insecticides (FPF, Sulf), fungicide (Azoxy), and their combinations (FPF+Azoxy and Sulf+Azoxy), (**B**) between insecticides and fungicides, and (**C**) between double treatments and single treatments. In bold are network indices that were significant across treatments with BH adjusted *p* values.

Treatment		***P* value adj.**					
		Degree	**Betweenness centrality**	**Closeness**	**Closeness centrality**	**Eigenvector centrality**	**Transitivity**
A. Control versus all treatments							
Control vs.	FPF	**< 0.001**	1	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	Sulf	**< 0.001**	0.092	0.67	**< 0.001**	**0.012**	**0.005**
	Azoxy	**0.001**	0.21	0.14	**< 0.001**	**< 0.001**	0.62
	FPF_Azoxy	**0.013**	1	**< 0.001**	**< 0.001**	**< 0.001**	0.75
	Sulf_Azoxy	**< 0.001**	0.55	**< 0.001**	**< 0.001**	**< 0.001**	**0.095**
B. Insecticide & fungicide							
FPF vs.	Sulf	0.24	0.1	**0.006**	**0.001**	**< 0.001**	0.41
FPF vs.	Azoxy	**0.01**	0.21	**< 0.001**	**< 0.001**	**< 0.001**	**0.002**
Sulf vs.	Azoxy	**< 0.001**	0.55	0.13	**< 0.001**	**0.002**	**0.04**
C. Interaction between double and single treatments							
FPF_Azoxy vs.	FPF	**0.001**	1	0.3	**0.005**	**< 0.001**	**< 0.001**
	Azoxy	0.44	0.21	**< 0.001**	**< 0.001**	**0.003**	0.53
Sulf_Azoxy vs.	Sulf	**0.011**	0.072	**< 0.001**	0.22	**< 0.001**	0.3
	Azoxy	0.18	0.1	**< 0.001**	**< 0.001**	**< 0.001**	0.3

List of figures

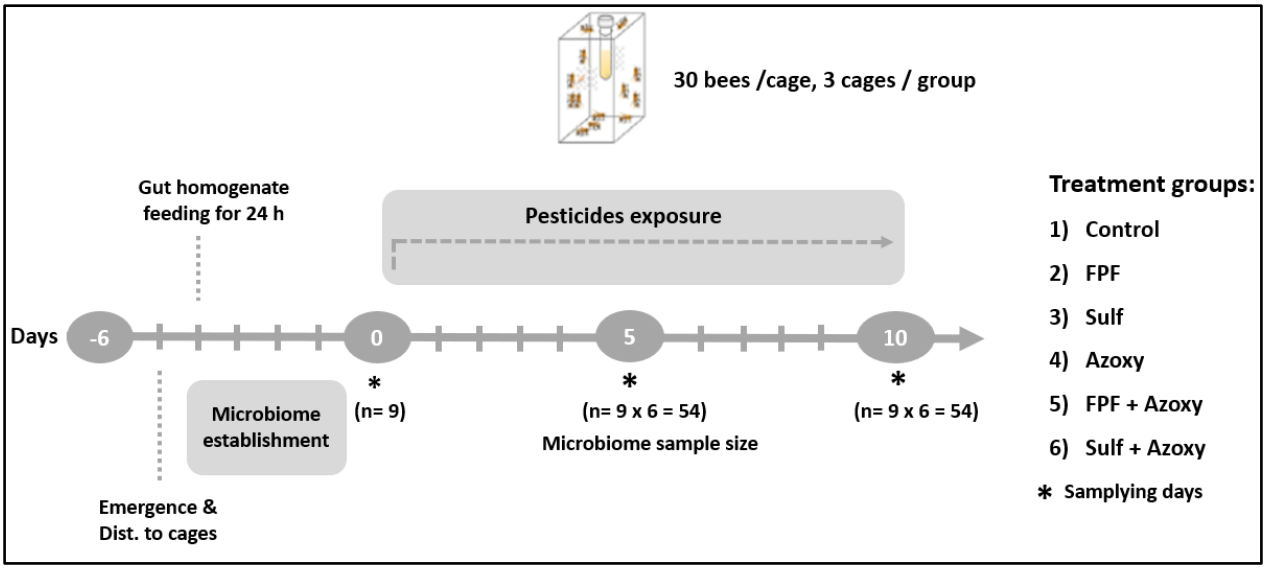

Figure 1. Experimental design showing treatments groups (3 cages per treatment) and timing of microbiome establishment, pesticide treatments and sampling of bees for microbiome analysis (D_0 as control, D_05 and D_10). Dimethoate was used as a reference toxic substance to validate exposure to pesticides (Al Naggar et al. 2022).

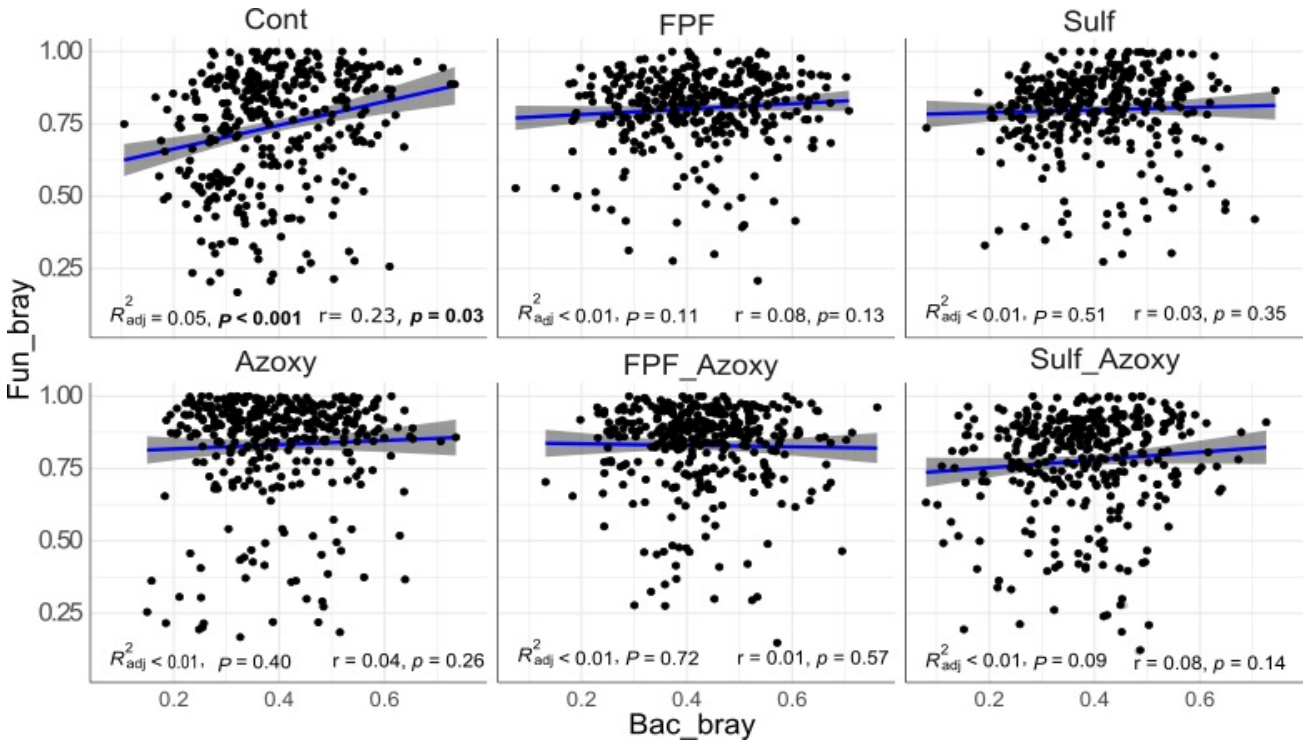

Figure 2. Comparison of the relationship of co-existing fungal and bacterial communities in the honey bee gut microbiome within the treatment groups using mantel test and linear regression model fitting. Each point represents the dissimilarity distance between pairs of 27 samples (9 individuals and 3 time points per treatment since the control time zero is used uniformly as initial community in all treatments) resulting in 351 pairwise comparison based on fungal or bacterial communities. Both mantel test and linear model fitting revealed a significant positive dissimilarity correlation between the two communities only in the control samples (Mantel test: r = 0.23, p = 0.03; linear model fitting: $R^2_{adj} = 0.05$, $P < 0.001$).

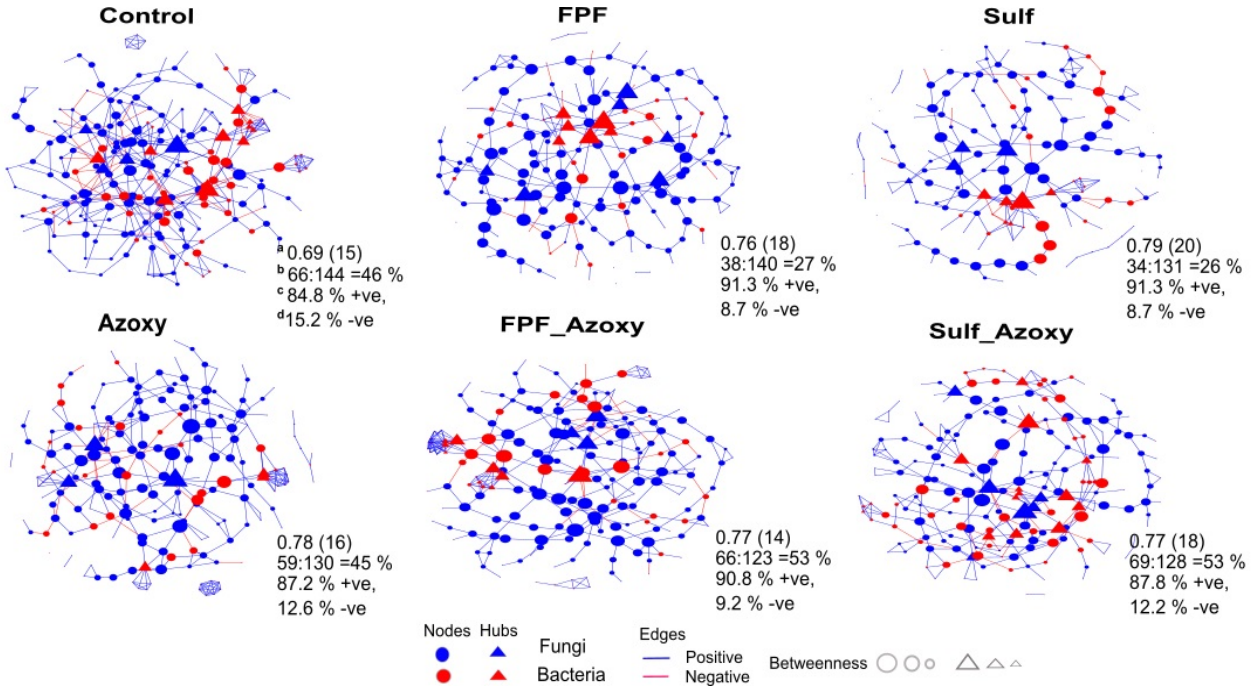

Figure 3. Cross-kingdom co-occurrence network of bacterial and fungal communities in the honey bee gut microbiome across treatments. Node size refers to the betweenness centrality value. The networks are dominated by fungal (blue) members with noticeable bacterial (red) presence. Insecticide and/or fungicide exposure altered cross-kingdom network parameters (**see table 1 for statistical details**). Abbreviations: **[a]** % of modularity, **[b]** ratio of bacterial to fungal nodes, **[c]** % of positive edges, and **[d]** % of negative edges.

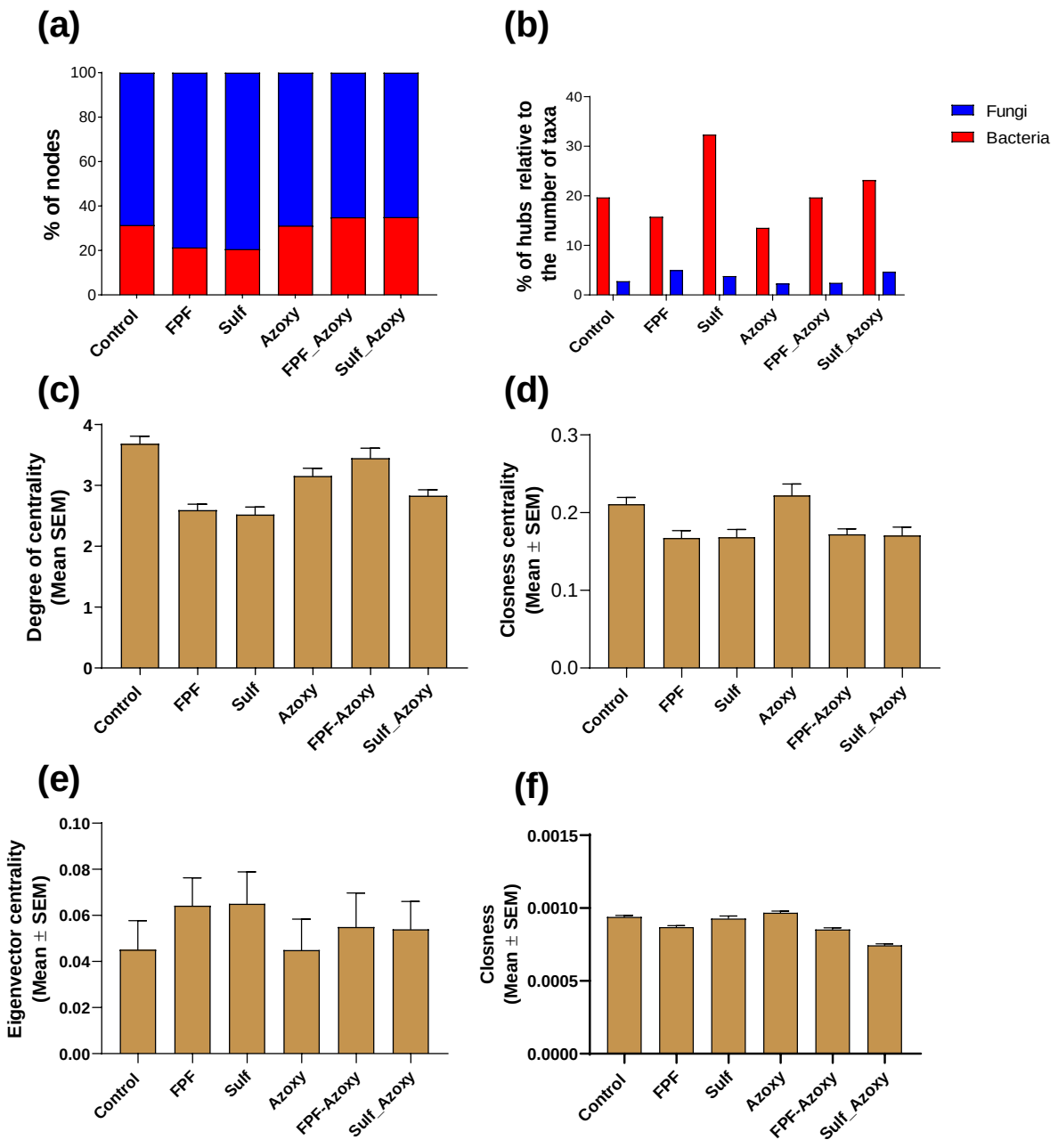

Figure 4. Comparison of cross-kingdom network centrality indices of bacterial and fungal communities in the honey bee gut microbiome across treatments. (**a**) % of nodes, (**b**) % of hubs relative to the number of taxa, (**c**) degree centrality, (**d**) closeness centrality, (**e**) eigenvector centrality, and (**f**) closeness. Pesticide and/or fungicide exposure significantly altered cross-kingdom network characteristics compared to control (**see table 1 for statistical details**).