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Research Article

Fungal Diversity in the Gut Microbiome of Healthy Mice Exposed to Fungicide

Dashne S. Latif, Pshtiwan S. A. Bebane, Rebin M Salih

Department of Medical Laboratory Analysis, College of Science, Charmo University, Kurdistan Region-Iraq

ABSTRACT

Pesticide use is widespread in agricultural systems in Iraq, raising concerns about their potential impact on the gut mycobiome. This study investigated how residues of a fungicide containing metalaxyl-M and azoxystrobin affect the gut fungal microbiome using a mouse model. Thirty 12-week-old male Swiss albino mice (*Mus musculus*) weighing 30–40 g were divided into two groups: A control group (n = 15) fed a regular diet and a treatment group (n = 15) fed the same diet supplemented with 10 ppm of fungicide. Due to high inter-individual variation observed in preliminary analysis and cost constraints, fecal samples from mice within each group were pooled (5 pools/group, 3 mice/pool) before DNA extraction, effectively resulting in n = 5 biological replicates per group for sequencing. Metagenetic libraries of the internal transcribed spacer (ITS) gene were generated and analyzed using QIIME2, DESeq2, DECIPHER, and Phangorn. A total of 191 amplicon sequence variants of fungal taxa were identified across all samples. The bioinformatic analysis revealed that the gut fungal community was significantly altered by fungicide treatment. Some species were enriched, including uncultured *Nakazawaea* and *Neosartorya hiratsukae*, and others were reduced, including *Trichomonascus ciferrii*, *Alternaria conjuncta,* and *Ophiostoma canum*. This study reveals that fungicides used in Iraq have profound effects on gut microbiome communities in mice, and it is recommended to pursue these aspects towards the further development of a research agenda focused on the health implications of dietary fungicide exposure and also on the methods of minimization.

Keywords: Fungicide, mycobiome, gut microbiome, metalaxyl-M, azoxystrobin, internal transcribed spacer sequencing

INTRODUCTION

Synthetic pesticides play a major role in agriculture, particularly in greenhouse systems where fungicides are frequently used to manage fungal diseases.[1] Although pesticides are known to have harmful effects on the liver, neurological system, and endocrine system, recent research indicates that they can also impact the gut microbiome.[2,3] This is highly relevant to human health, as the gut microbiome plays a crucial role in immunity, digestion, and overall well-being.[3]

Besides bacteria, the gut harbors viruses, fungi, and other microbes. Although fungi constitute <1% of the gut microbiota, collectively termed the mycobiome, they have a significant impact on metabolism and immune function.[4] Fungicides are developed with the purpose of removing fungal pathogens, and residues of these substances can negatively affect the mycobiome by altering the diversity and composition of the gut microbiome.[2,5]

Fungicide residues in vegetables, particularly cucumbers, represent a significant source of human exposure. Cucumbers are among the most widely consumed vegetables globally and are frequently treated with fungicides due to their susceptibility to fungal diseases such as downy mildew and powdery mildew.[6,7] Studies have detected metalaxyl and azoxystrobin residues in greenhouse-grown cucumbers at concentrations ranging from 0.05 to 4.69 mg/kg, depending on application rates and harvest intervals.[1,8] While these residues typically remain below maximum residue limits when good agricultural practices are followed, the frequent consumption of cucumber and other fungicide-treated vegetables contributes to chronic, low-level dietary exposure to these compounds.[11]

Open fields and greenhouses are common areas used with pesticide management in Iraq, where agriculture accounts for a large portion of household income. In recent studies on vegetables from Basrah to Erbil, various pesticide

Corresponding Author:
Pshtiwan S. Bebane, Department of Medical Laboratory Analysis, College of Science, Charmo University, Kurdistan Region-Iraq.
E-mail: Pshtiwan.saeed@chu.edu.iq

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residues were observed.[10,11] and farmers had higher levels of pesticide exposure. The active components of commonly used fungicides, including metalaxyl-M and azoxystrobin, active parts in agricultural products,[12,13] are among the common compounds found in them. Azoxystrobin is a strobilurin fungicide that disrupts mitochondrial respiration by obstructing the cytochrome bc1 complex, and metalaxyl-M is a phenylamide fungicide, which inhibits RNA polymerase I in fungi.[9,10] Given their extensive use and frequent detection in vegetables and fruits, such fungicides present a realistic exposure scenario for the Iraqi people.[10,11]

The acceptable daily intake (ADI) for metalaxyl has been established at 0.03 mg/kg body weight per day by the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) meeting on pesticide residues,[16] while azoxystrobin has an ADI of 0.2 mg/kg body weight per day.[17] The ADI is an indication of how much of a substance a person can ingest daily for a lifetime with minimal health risk. For a typical 60 kg adult, the ADI is 1.8 mg/day for metalaxyl and 12 mg/day for azoxystrobin. Long-term exposure to these fungicides at concentrations greater than the ADI in rats was associated with liver weight increases and mild hepatocellular changes, though no carcinogenic effects have been demonstrated.[16,17] The dose used here (10 ppm in diet) was chosen to represent environmentally relevant chronic exposure levels based on estimated dietary intake of contaminated produce consumption.

Fungicides can affect intestinal microflora through several mechanisms. First, direct antimicrobial activity disrupts fungal cell membranes and metabolic processes, potentially affecting commensal fungi in the gut.[18] Metalaxyl-M inhibits fungal RNA synthesis, while azoxystrobin blocks mitochondrial respiration, mechanisms that are not entirely specific to pathogenic fungi and may impact beneficial gut mycobiota.[9,10] Second, fungicide exposure can induce gut dysbiosis by selectively eliminating sensitive fungal species while promoting the growth of resistant opportunistic species.[19] Third, fungicide metabolites and their interaction with the gut epithelium can trigger inflammatory responses and alter the gut barrier function, indirectly affecting microbial community composition.[20] Finally, fungicides may exert selective pressure favoring fungi with alternative respiratory pathways or enhanced efflux pump systems, fundamentally altering the ecological balance of the gut mycobiome.[21,22]

While findings on the influence of pesticides on gut microbiota have been observed around the world, no studies have addressed this matter specifically in Iraqi agriculture. However, the adequacy of risk assessment and health recommendations in Iraq is not fully achieved due to the scarcity of locally relevant data. We conducted a mouse model study to examine the effect of fungicide exposure on gut fungal populations. They aimed to find out if fungicide treatment upsets the natural balance of the gut mycobiome, and which fungal species react most strongly to this exposure. This would serve as a baseline for future biomonitoring studies of pesticide-exposed populations in Iraq, providing some evidence suggesting pesticide safety evaluations should add microbiome impacts assessment along with traditional toxicity testing.

MATERIALS AND METHODS

Pesticide Survey

A survey was conducted to identify the most widely used pesticides in greenhouses in Sulaymaniyah, Iraq, focusing on cucumber cultivation. Between 10 and 15 farmers, agricultural engineers, and pesticide suppliers in the Sulaymaniyah region were interviewed using a structured questionnaire. The questionnaire included questions regarding: (1) Types of fungicides used, (2) frequency of fungicide application, (3) spray intervals between applications and harvest, and (4) timing of produce sales after pesticide application. The survey analysis revealed that fungicides containing metalaxyl-M and azoxystrobin are among the most widely used in the Sulaymaniyah region.

Fungicide Composition

The exposure agent used in this study was a commercial fungicide (Syngenta Crop Protection AG, Basel, Switzerland), a suspo-emulsion formulation containing metalaxyl-M [methyl N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-D-alaninate] at 124 g/L and azoxystrobin [methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate] at 322 g/L. Unless otherwise noted, all additional compounds and reagents were of analytical grade.

Ethics Statement

All animal procedures were carried out in compliance with international standards for the care and use of laboratory animals and were approved by Charmo University's Ethics Committee (Approval No: 9, dated January 22, 2024).

Animal Housing and Experimental Design

Thirty 12-week-old male Swiss albino mice (Mus musculus) weighing 30–40 g were acquired from the experimental animal center of Human Development University in Sulaymaniyah. All animals were maintained in a controlled environment with a 12-h light/dark cycle, stable temperature (25 ± 2°C), and humidity (50 ± 5%). Mice were housed in standard 32 × 20 × 14 cm polypropylene cages with three animals/cage (10 cages total). Following a 2-week acclimatization period, mice were randomly divided into two groups: Control (n = 15) and fungicide treatment (n = 15).

Fungicide Treatment and Sample Collection

The control group received standard laboratory chow[23] and sterile drinking water, while the treatment group received the same diet with a fungicide mixture incorporated into the food pellets at a concentration of 10 ppm. This concentration was selected to represent chronic, low-level dietary exposure based on estimated human consumption patterns and WHO/FAO ADI values.[16,17,24,25] The 10 ppm dose in mouse feed approximates the cumulative exposure humans might experience through regular consumption of fungicide-treated vegetables. After 3 weeks of dietary exposure, fresh fecal samples were collected each morning for three consecutive days. At the end of the exposure period, mice were euthanized, and gut tissue was

aseptically dissected. Both fecal and gut tissue samples were immediately stored at −80°C for subsequent DNA extraction.

DNA Extraction and Internal Transcribed Spacer (ITS) Amplicon Sequencing

Due to high inter-individual variation observed in preliminary analysis and sequencing cost constraints, fecal samples from mice within the same experimental group were pooled (5 pools/group, 3 mice/pool) before DNA extraction to create biological replicates. This pooling strategy effectively resulted in $n = 5$ biological replicates per group for sequencing analysis, which reduced statistical power compared to individual-level sequencing but allowed for comprehensive characterization of overall group-level fungal community differences. Approximately 200 mg of pooled fecal material was used to extract whole genomic DNA using the Neo Biotech Stool Genome DNA Extraction Kit (Catalog No. NB-88-00025-50T, Neo Biotech, Republic of Korea) according to the manufacturer's instructions. DNA concentration and purity were measured using a NanoDrop spectrophotometer. Approximately 200 ng of DNA from each pooled sample was submitted to BGI Tech Solutions Co., Ltd. (Hong Kong) for library preparation and sequencing.

Bioinformatics Analysis

FastQC v0.11.9 was used to evaluate the quality of raw sequencing reads.[26] Quality control, demultiplexing, and taxonomic classification were performed using the QIIME 2 Core v2025.4 pipeline.[14,27]

Differential Abundance Analysis

Total abundance levels were imported to RStudio from feature table biom output. Statistical differences between control and fungicide-treated groups were assessed using the Wilcoxon rank-sum test due to non-normal data distribution. Differentially abundant fungal taxa were identified between control and fungicide groups using the DESeq2 package[15,28] with adjusted $P < 0.05$ and absolute log2 fold change (log2FC) >1 considered significant. Bar plots were generated with the ggplot2 package to visualize log2FC values. Positive and negative log2FC values indicated enrichment in treatment and control groups, respectively. Feature tables were exported from QIIME 2 to RStudio using the qiime tools export command.

Phylogenetic Analysis

Fungal DNA sequences were analyzed using the DECIPHER and phangorn packages.[29,30] Aligning various sequences was accomplished with the AlignSeqs function. Sequences aligned were transformed into a phylogenetic data object, and pairwise distances were calculated with a maximum-likelihood algorithm. A Neighbor-joining tree was created and subsequently optimized via maximum-likelihood estimation in the general time reversible model with invariant sites and gamma rate heterogeneity. Branch support was evaluated via 100 replicates of non-parametric bootstrap analysis and nearest-neighbor interchange optimization. Phylogenetic analysis was performed to assess the evolution of fungal taxa and to place its observed community dynamics within the context of a broader taxonomic perspective. The resulting phylogenetic tree is presented.

RESULTS

Sequencing Quality and Amplicon Sequence Variant (ASV) Generation

High-quality sequencing data were obtained from all samples. Approximately 37.5 ± 0.1 million paired-end reads (300 bp × 2, forward and reverse) were generated per sample. Following quality filtration and trimming, 74.1% of reads passed quality control. Across all samples, 191 ASVs were obtained with a mean sequence length of 303 ± 12 bp. For subsequent analysis, samples were rarefied to a depth of 1,100 reads/sample.

Total abundance levels were observed between the control and fungicide-treated group. The abundance values are shown as a box plot [Figure 1], with each dot representing an individual operational taxonomic unit (OTU) feature. Overall, there was no statistically significant difference between the control and fungicide-treated samples, and the distribution of abundances was comparable between the two groups (Wilcoxon rank-sum test, $P > 0.05$). This suggests that overall abundance levels were not significantly altered by fungicide treatment as compared to the control group.

Fungal Community Composition

Phylogenetic analysis of the ITS region revealed numerous fungal taxa with distinct clustering patterns. Three major lineages were clearly separated: *Filamentous Ascomycota*, *Saccharomycetales*, and *Basidiomycetous yeasts*. The most prevalent *Ascomycota* taxa included genera *Penicillium*, *Cladosporium*, *Alternaria*, *Fusarium*, *Nigrospora*, and *Geotrichum*. These formed well-supported clades, with fungal genera grouped closely with species such as *Penicillium expansum*, *Cladosporium ramotenellum*, *Fusarium graminearum*, and *Geotrichum galactomycetum*.

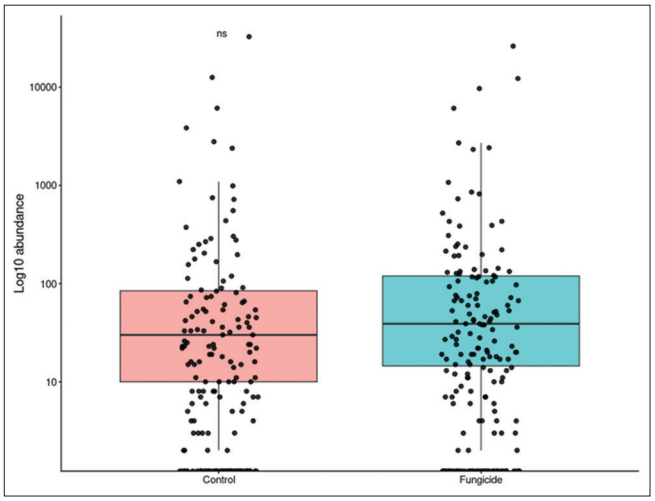

Figure 1: Comparison of OTU abundance between control and fungicide-treated groups. Box plot highlights the median and interquartile range, and each dot indicates an individual OTU. "ns" denotes no significant difference according to the Wilcoxon rank-sum test

Candida tropicalis, *Meyerozyma guilliermondii*, *Wickerhamomyces anomalus*, *Pichia membranifaciens*, and *Starmerella lactis-condensi* were among the yeast species identified. Filamentous ascomycetes and fermentative yeasts were consistently distinguished by the clustering of these yeast species within the *Saccharomycetales* branch. With taxa like *Mrakia*, *Cystofilobasidium*, *Filobasidium*, *Rhodosporidiobolus*, and *Leucosporidium*, *basidiomycetous* yeasts became a separate group. These species group formed a distinct cluster and they are clearly separated the Ascomycota. In addition, data analysis showed that many taxa within the Basidiomycota clade, including *Sporobolomyces salmonicolor* and *Erythrobasidium hasegawianum*. These taxa formed their own cluster and clearly separated from the *Ascomycota*. Notably, several uncultured taxa were identified among diverse clades instead forming a single cluster. The unidentified sequences included uncultured *Ascomycota*, uncultured fungus, and *Basidiomycete* classifications, indicating that the dataset contains multiple environmental or poorly characterized taxa.

Overall, these results demonstrate that the mice gut harbors numerous unidentified species in addition to well-defined fungal genera. The ITS region provided sufficient phylogenetic resolution to distinguish the main lineages observed in the samples, as evidenced by the clear separation of the three main groups.

Differentially Abundant Taxa

Fungicide exposure significantly altered the relative abundance of numerous fungal taxa [Figure 2]. A total of 23 taxa showed significant differential abundance (adjusted $P < 0.05$, $|\log2FC| > 1$).

A group of fungal species exhibited large positive log2FCs, demonstrating that these taxa significantly proliferated in response to fungicide exposure. The most strongly enriched taxa included uncultured *Nakazawaea* (log2FC = +4.2, adjusted $P = 0.003$), *Neosartorya hiratsukae* (log2FC = +3.8, adjusted p = 0.007), uncultured *Ascomycota* sp. (log2FC = +3.5, adjusted $P = 0.009$), and several other fungal taxa showing log2FC values between +2.0 and +3.0. Their persistent up-regulation indicates that the fungicide established a gut environment conducive to their proliferation or persistence.

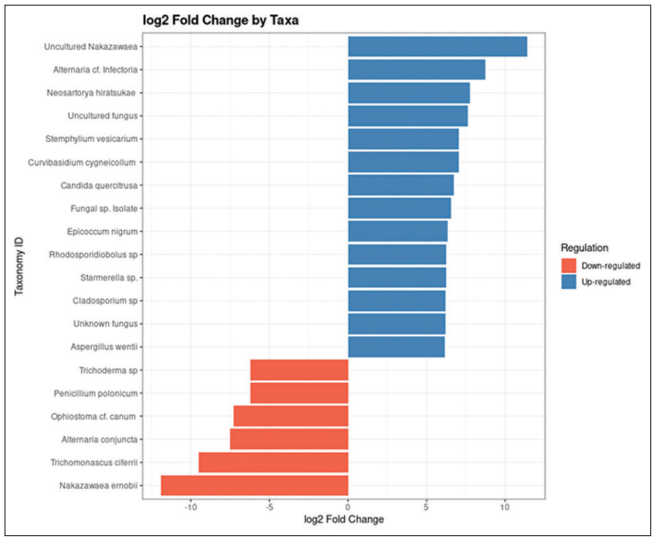

Figure 2: Differential abundance of fungal taxa between control and fungicide-treated groups. Bar plot shows log2 fold change (log2FC) values for significantly altered taxa (adjusted $P < 0.05$, $|\log2FC| > 1$). Taxa suppressed in comparison to the control are shown by negative values (red bars), whereas positive values (blue bars) show taxa enriched in the fungicide group. Standard error is represented by error bars

On the other hand, a smaller set of taxa was strongly reduced following fungicide exposure. The most pronounced decreases were observed for *Trichomonascus ciferrii* (log2FC = −3.6, adjusted $P = 0.004$), *Alternaria conjuncta* (log2FC = −3.2, adjusted $P = 0.006$), and *Ophiostoma canum* (log2FC = −2.8, adjusted $P = 0.012$). Additional suppressed taxa included *Cladosporium* spp., *Fusarium* spp., and several unclassified Basidiomycota. These taxa clustered together on the negative side of the distribution, reflecting a suppressive effect of the fungicide on specific components of the mycobiome.

DISCUSSION

This study demonstrates that a diet rich in fungicide residue greatly changes the composition of gut mycobiome in mice. The observed alterations to the structure of the fungal microbiome may have implications for human populations, such as those in which exposure to the same levels of fungicide and diet intake are significant, but a cautious inferential extrapolation from mouse models to humans would be important.

Mechanisms of Fungicide Effects on Gut Microflora

Gut mycobiome dysbiosis was caused by different interacting mechanisms mediated by the fungicide treatment. Metalaxyl-M and azoxystrobin act as antifungals, working on two important fungal cellular processes – RNA synthesis and mitochondrial respiration, respectively. However, although they were developed to inhibit plant pathogens, they are not a completely selective system and are capable of acting on commensal gut fungi.[8,10,18] The loss of commensal fungal populations establishes ecological niches that can be occupied by opportunistic species, as demonstrated by the enrichment of *Nakazawaea* and *N. hiratsukae* in treated mice.[31,32]

Multiple pathways can route gut microbiota dysbiosis to metabolic disorders in mice. The alteration in composition from alterations of mycobiome composition can weaken gut barrier integrity by increasing intestinal permeability and inducing low-grade inflammation.[33] Fungi may produce metabolites that also can modify host metabolism and modify host metabolism, such as short-chain fatty acid or bile metabolism.[34] Fungi described in this study (*Nakazawaea*, *N. hiratsukae*, *T. ciferrii*, *A. conjuncta*, and *O. canum*) are examples of taxa with different levels of pathogenic activity. Although some are classed as environmental fungi, in gut dysbiosis and immune disturbance conditions, they can also serve as opportunistic pathogens.[35,36]

Alternative Respiratory Enzymes and Efflux Transporters

The enrichment of some types of fungal taxa in fungicide-treated mice could be a consequence of complementary respiratory pathways and enhanced efflux pump systems. Azoxystrobin hits the cytochrome bc1 complex in the electron transport chain, but fungi expressing AOX-mediated (alternative oxidase) pathways can escape this inhibition and have increased resistance to it.[21,37] The enrichment of *Nakazawaea* and *Neosartorya* species found herein would indicate that these taxa may have these compensatory features. Similarly, higher expression of ATP-binding cassette transporters and major facilitator superfamily efflux pumps could lower intracellular fungicide burden, resulting in survival advantages under selective pressure.[22,38] These forms of resistance have great potential for elucidating fungicide effects upon microbial ecology, and perhaps help to mediate the emergence of resistant fungal communities in agricultural and clinical settings.

Study Limitations

A number of limitations of this study need to be acknowledged. Due to the pooling strategy used to reduce costs and inter-individual variation, the effective sample size was decreased ($n = 5$ pools/group, as opposed to $n = 15$ individuals), which limits statistical power and prevents assessment of individual-level responses to fungicide exposure. Future studies with individual-level sequencing would enable more robust statistical inference and interrogation of inter-individual variability in response to fungicide exposure. Furthermore, although mice have been traditionally used as a model for mammalian biology, direct extrapolation to human health requires caution due to species differences in gut microbiome composition, dietary habits, and xenobiotic metabolism. The dose used (10 ppm) was chosen to approximate chronic dietary exposure levels, but the pattern of exposure in humans may differ markedly according to dietary habits, agricultural practices, and food preparation methods. Finally, the present research focused on mycobiome changes without assessing functional consequences or health outcomes and should be studied further in future work.

Implications and Future Directions

Fungicides such as those commonly used in Iraqi agriculture have been reported to dramatically affect gut mycobiome composition. Further studies should explore if these microbial alterations are accompanied by measurable clinical health outcomes, and determine whether the introduction of probiotic supplements or dietary adjustment is able to curtail fungicide-induced dysbiosis. One possible direction could be the potential of beneficial microorganisms like particular probiotic bacteria or yeasts (e.g., *Saccharomyces boulardii*) to promote the degradation of fungicide or to prevent dysbiosis.[39] Moreover, biomonitoring studies of agricultural workers and populations with high consumption of fungicide-treated vegetables would provide valuable data on real-world exposure levels and associated health outcomes. Finally, these data underscore the need to integrate microbiome assessment into pesticide safety assessments alongside traditional toxicity testing.[40]

CONCLUSION

This study shows that exposure to dietary fungicides substantially changes gut mycobiome composition in a mouse model, with certain fungal taxa enriched and others depleted. Results here present preliminary evidence that fungicides commonly used in Iraqi agriculture may impact gut microbiome health and set a basis for further biomonitoring and intervention studies. As the sample pool was limited and an animal model was used, additional investigations are required with greater sizes of the sample and in human individuals to fully understand the health effects of chronic exposure to fungicide through the diet.

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