An integrated host-microbiome response to atrazine exposure mediates toxicity in Drosophila

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The gut microbiome produces vitamins, nutrients, and neurotransmitters, and helps to modulate the host immune system—and also plays a major role in the metabolism of many exogenous compounds, including drugs and chemical toxicants. However, the extent to which specific microbial species or communities modulate hazard upon exposure to chemicals remains largely opaque. Focusing on the effects of collateral dietary exposure to the widely used herbicide atrazine, we applied integrated omics and phenotypic screening to assess the role of the gut microbiome in modulating host resilience in Drosophila melanogaster. Transcriptional and metabolic responses to these compounds are sex-specific and depend strongly on the presence of the commensal microbiome. Sequencing the genomes of all abundant microbes in the fly gut revealed an enzymatic pathway responsible for atrazine detoxification unique to Acetobacter tropicalis. We find that Acetobacter tropicalis alone, in gnotobiotic animals, is sufficient to rescue increased atrazine toxicity to wild-type, conventionally reared levels. This work points toward the derivation of biotic strategies to improve host resilience to environmental chemical exposures, and illustrates the power of integrative omics to identify pathways responsible for adverse health outcomes.
Pesticides include herbicides, fungicides, and insecticides, and are applied in excess of 5.6 billion pounds each year. The resulting ecological distributions of these chemicals give rise to a global health challenge—the World Health Organization estimates that 25 million individuals are hospitalized each year with pesticide-induced maladies. In the US, these illnesses cost over $1.2B dollars in health care alone. Thirty-three million Americans drink atrazine-contaminated water, and more than fifty million experience chronic low-dose exposure to one or more widely used pesticides. During the next 30 years, agricultural productivity will need to increase by over 50% to feed our growing global population, and similarly increasing energy demands met in part by biofuels, will lead to a concomitant scale-up of the environmental distributions of pesticides worldwide.

Remediation strategies for the ecological and human health effects of pesticide usage focus on preventing exposure, but this is challenging given the scale of the problem, and the substantial benefits associated with pesticide use—i.e., the feasibility of supplying fresh produce to our growing population. It is clear that humans exhibit substantial individual variability in responses to pesticide exposure, but the source of variation is poorly understood. Polymorphisms in P450 cytochromes have been associated with increased risk for adverse health outcomes from low dose exposure for particular compounds. Many pesticides, such as the triazine herbicide atrazine, are not effectively transformed by metazoan metabolisms, but are rapidly detoxified by microbes. In some pests, it has been shown that the host gut microbiome plays a role in establishing individual susceptibility to insecticides exposure by modulating effective dosage in the gut compartment. However, few such studies have been conducted, and the capacity of commensal microbial consortia to modulate toxicity through chemical transformation requires additional exploration and detailed case studies. The gut microbiome plays a major role in the metabolism of xenobiotic compounds, including the pharmacokinetics of many drugs. Studies in tractable genetic model systems have revealed complex host-microbiome interactions. Here, we used the genetic model organism, Drosophila melanogaster, to identify microbes that participate in adaptive responses to chronic pesticide exposure in aerobic and anaerobic conditions. We investigated host-microbiome interactions using gnotobiotic studies and multi-omics measurements, including RNA sequencing, 16S profiling, metagenomics, and metabolomics, to identify coregulatory genes, microbes, and molecules associated with adverse host phenotypes. Ultimately, understanding the role of the gut microbiome in adaptation to chronic pesticide exposure may lead to novel therapeutic strategies surrounding the application of pre- and pro-biotics with the potential to improve the health of tens of millions of people worldwide and improve the resilience of keystone pollinators, such as flies and bees, to pesticide-induced population collapse.

Results

Drosophila as a highly tractable exposure model. We designed a multi-omic study to investigate the role of the microbiome on pesticide metabolism using Drosophila melanogaster as a model system (Fig. 1a). We exposed conventionally reared four-day-old flies to atrazine or paratrazine for three consecutive days. To identify, classify and determine the microbial community composition of the fly microbiome, we collected fecal swabs for 16S ribosomal RNA profiling and metabolomics analysis. To monitor the effects of herbicide exposure on the host, we performed RNA-seq analysis of whole flies.

Environmental and food-born microbes rapidly colonize the adult fly gut shortly after eclosion. A number of studies have characterized laboratory and natural populations, and there is substantial variation across strains and environmental conditions. Therefore, prior to starting our exposure studies, we first characterized the gut microbiome of Oregon-R-modENCODE flies in our laboratory environment. Relative to the mouse microbiome we observed approximately 100-fold fewer species in the fly gut, consistent with previous reports (Fig. 1b; Supplementary Data 1 and 2). The fly microbiome was dominated by two phyla: Firmicutes and Proteobacteria; and two genera: Lactobacillus and Acetobacter (Fig. 1c; Supplementary Data 1 and 2). Over time, the relative abundances changed and the microbiome became more complex with an increase in the relative abundance of newly rare species (Fig. 1d) consistent with previous studies.

We exposed adult flies (Oregon-R-modENCODE; BDSC strain 25211) to two commonly used herbicides: paratrazine and atrazine, in chronic, collateral dietary scenarios lasting three to four days (72–96 h, 5% adult lifespan). Each herbicide was added to fly medium at a fixed concentration. First, we determined the LC50 for each herbicide by exposing adult flies to different doses ranging from 5 to 20 mM atrazine and 6 to 40 mM paratrazine. These doses are commensurate with field-proximal exposures expected for pollinators, including Diptera. Atrazine was solubilized in DMSO and paratrazine in water. DMSO and water were used as controls for atrazine and paratrazine exposures, respectively. Interestingly, we observed sex differences in sensitivity to both atrazine and paratrazine (Fig. 2a, b). The LC50, defined as the 50% lethal concentration at 48 h, for atrazine was significantly different between males (5 mM) and females (12 mM) (Fig. 2a). In contrast, the females (LC50 = 20 mM) were more sensitive to paratrazine exposures compared to males (LC50 = 43 mM) (Fig. 2b).

Microbiome remodeling under pesticide exposure. To determine the impact of herbicide exposure on the gut microbiome, fecal swabs were collected for 16S sequencing every 24 h during the 72 h atrazine and paratrazine exposures. Each herbicide resulted in alterations to the microbiome composition (Fig. 2c, d; Supplementary Data 1 and 2)—intriguingly both reduced the relative abundance specifically of Acetobacter relative to Lactobacillus and other genera. Dietary paratrazine exposure resulted in a 33-fold reduction in the relative abundance of all species and strains of Acetobacter by 72 h (Fig. 2d, e). Atrazine also impacted Acetobacter, reducing overall population abundance by two-fold (Mann–Whitney U test; p = 0.036) (Fig. 2d, e). For both atrazine and paratrazine, we observed a robust increased abundance of species belonging to the order Lactobacillales and a decrease in abundance level of the order Rhodospirillales (Fig. 2e). These data show that pesticide exposure impacts specific genera of the gut microbiome. Whether these perturbations are accomplished through the direct action of the herbicide on the microbes or through modulation of host metabolism remains unclear.

Since atrazine is widely used in the US and its effect on terrestrial metazoans is less studied than paratrazine, we focused our studies on the effects of atrazine on the host and microbiome. All data are submitted to public repositories as a resource (Supplementary Data 1).

To investigate the role of the microbiome on the LC50 after atrazine exposure, we conducted a dose-response analysis on germ-free reared (GF) adult flies. Similar to conventionally reared (CR) and exposed flies, male germ-free flies were more sensitive to atrazine compared to female flies (Fig. 2f). Interestingly, significant toxicity was observed for DMSO alone (2.5%) in germ-
free flies, which was not observed in conventional flies (Fig. 2f). Based on this observation we reduced the DMSO concentration 10-fold to 0.25% for subsequent germ-free fly experiments.

Transcriptional responses to pesticide exposure. To investigate the transcriptional response to atrazine we performed whole animal RNA sequencing analysis of conventionally and germ-free reared flies before and after atrazine exposure.

We first studied the differences in gene expression between unexposed CR and GF flies. We found 38 genes in males and 23 genes in females (Supplementary Fig. 1 and Supplementary Data 3 and 4) showing differential gene expression FC ≥ 3, p ≤ 1E
Fig. 1 Baseline microbiome composition in *Drosophila melanogaster*. a Study design. b Rarefaction curves of the observed OTUs to assess species richness in *Drosophila* (orange; obtained from AdMMF at 4–8 days of age) and *Mus musculus* (purple; obtained from C57BL/6J fecal samples at 12 weeks of age). c Metagenomic sequence analysis to determine the bacterial species composition of the *Drosophila* gut microbiome isolated from 20 adult females at 21 days of age. The bar indicates relative abundance level colored at the species level as indicated in the key. d Age-dependent change in the distribution of the *Drosophila* gut microbiome based on 16S sequencing of fecal samples. For each of the 16S experiments, embryos were distributed between 25 bottles containing food. Four days post-eclosion flies were collected and the weight-equivalent of 250 flies were transferred to small cages (100 mm diameter × 150 mm) for the aging study. The food was replaced once daily. Bars indicate relative bacterial abundance colored at the family level as indicated in the key.

Fig. 2 Effect of atrazine and paraquat on survival and gut microbiome in *Drosophila melanogaster*. a Host survival curves in response to 5–20 mM atrazine exposures. L50 at 48 h is 16 mM for females and 7 mM for males. For each dose, we used 30 males and 30 females in triplicate aged 4–5 days post eclosion. Error bars indicate the standard deviation across replicates. b Host survival curves in response to 6–40 mM paraquat exposures. L50 at 48 h is 19 mM for females and 43 mM for males. For each dose, we used 30 males and 30 females in triplicate aged 4–5 days post eclosion. Error bars indicate the standard deviation across replicates. c Principal component analysis of microbial abundance levels in control, atrazine, and paraquat treated flies. The control 0 and 72 h groups are closer to each other than the atrazine and paraquat groups at 72 h. d Bar charts showing the relative abundance of bacterial families in control (*n* = 5), 2 mM atrazine treated (*n* = 3) or 8 mM paraquat treated (*n* = 3) flies at 72 h after treatment. Response to atrazine and paraquat. Both herbicides show a reduction in the amount of the Rhodospirillales grp and an increase in the Lactobacillales group. e Log2 fold change at the genus level for bacteria significantly changed after treatment with atrazine (FDR < 0.06). Bacteria are color-coded at the order level. f Germ-free host survival curves in response to 5 and 7 mM atrazine exposure. For each dose, we used 30 males and 30 females in triplicate aged 4–5 days post eclosion. Error bars indicate the standard deviation across replicates.
—06. The genes that show common males and female differential gene expression include the P450 Cytochromes, Cyp6a16, Cyp30a4a1, and eye transformer (et) that are activated in the absence of a microbiome, and PGRP-SC1a and 1b, pirk and CG9759 that are repressed. Cyp6a16 and Cyp30a4a1 are likely associated with the detoxification of bioactive compounds. The gene et is a negative regulator of JAK/STAT signaling that is induced by and protective against bacterial infection22—why it is induced in the absence of a microbiome is not immediately apparent. Peptidoglycan recognition protein SC1a and b (PGRP-SC1a and b) recognize and degrade bacterial cell wall structures, and pirk is a negative regulator of similar peptidoglycan recognition proteins. The function of gene CG9759 remains unknown and requires further investigation.

It is well known that germ-free animals show altered physiology, an adaptation to the absence of microbes that includes reduced metabolism and changes in immunity. Overall, we see an increased expression in genes encoding proteins with serine-type endopeptidase, oxidoreductase, alkaline phosphatase, ammonium—transmembrane transporter, cytokine receptor, gamma-butyrobetaine dioxygenase, and metalloendopeptidase activity (Fig S1b). We see a decreased expression in genes encoding proteins involved in peptidoglycan (the primary amino acid sugar component of bacterial cell walls) binding and degradation—but consistent with the expression of bacteria-regulating proteins only in the presence of bacteria (Fig S1b).

In males, 72 h after 2.0 mM atrazine exposure, we observed 33 genes at FC ≥ 1.5, adjusted p ≤ 1E−06 and 13 genes at FC ≥ 3, adjusted p ≤ 1E−06 (Fig. 3a; Supplementary Data 5). In females, we observed an increase in the number of deregulated genes compared to males in response to 2.0 mM atrazine exposure (92 genes at FC ≥ 1.5, adjusted p ≤ 1E−06; 47 genes at FC ≥ 3, adjusted p ≤ 1E−06) (Fig. 3a; Supplementary Data 6). Sixteen genes (FC ≥ 1.5, adjusted p ≤ 1E−06) were found deregulated after atrazine exposure in both males and females, which included Cyp6a2, Cyp6a8, and Cyp6d5, involved in response to toxic substances (p < 0.01). In contrast to CR flies, the transcriptional response to atrazine was more pronounced in GF flies. In germ-free treated male flies, we observed a eight-fold greater transcriptional response compared to CR treated male flies as evidenced by 274 genes with altered expression (274 genes at FC ≥ 1.5, adjusted p ≤ 1E−06; 55 genes at FC ≥ 3, adjusted p ≤ 1E−06) (Supplementary Data 7), whereas in treated female flies we observed only a two-fold greater transcriptional response compared to CR treated female flies, corresponding to 207 genes with altered expression (207 genes at FC ≥ 1.5, adjusted p ≤ 1E−06; 86 genes at FC ≥ 3, adjusted p ≤ 1E−06) (Supplementary Data 8). The transcriptional response of the majority of genes found in CR flies also responded in GF flies (32 out of 33 in males and 60 out of 92 in females)—though we observe intriguing differences: in male CR flies, Glutathione S-Transferase D8 is down-regulated ~2-fold, whereas it shows no response in GF animals. More broadly, in comparison to CR flies, GF male and female flies show strong enrichment for genes involved in oxidation-reduction in response to atrazine exposure. In female GF flies, we found strong downregulation of reproductive genes involved in vitelline membrane (including Vm26aa, Vm26ab, Vm32E, and psd) and eggshell (including Cad7a4, Cp16, Cp7fb, Cp7fc, and Mur11Da) formation (p < 0.01) indicating that atrazine exposure affects female fecundity (Fig. 3b). In male GF flies, genes involved in hormone, lipid, and organic acid metabolism were affected by atrazine exposure (p < 0.01) (Fig. 3c).

In addition, similar to female GF flies, genes involved in the male reproductive system including Acp54a1, Acp24aa4 were down-regulated. None of the genes that show differential gene expression in the CR versus GF flies were found in common with the treated samples except for a female repressed gene, Jon66cii, a chymotrypsin-like serine protease (Fig. 3c).

Acetobacter tropicalis rescues atrazine induced toxicity. GF flies showed increased sensitivity to atrazine compared to CR flies. To assess whether specific microbes could rescue atrazine-induced toxicity, we first characterized the microbiome at the species level. We performed metagenomic sequencing and found that the fly gut microbiome contains: Lactobacillus plantarum (34.8%), L. brevis (1.0%) and Acetobacter aceti (45.2%), A. tropicalis (14.6%), and A. pomorum (4.1%) (Fig. 1d). These five species were isolated and sequenced to generate complete genomes (Supplementary Figs. 2—7)23−27. Finally, we cultured GF adult mixed male and female flies (AdMMF) with Acetobacter tropicalis or Lactobacillus brevis—two species observed in the fly gut microbiome. We used AdMMF flies for this experiment to avoid confounding behavioral effects of single-sex housing. Interestingly, we observed that inoculation of DMSO treated GF flies with A. tropicalis reduced survival in comparison to DMSO treated GF flies (Fig. 4a). This finding suggests a potential interaction between A. tropicalis and DMSO that is more toxic though such a mechanism is unknown. Importantly, restoration of the gut microbiome with A. tropicalis reduced atrazine toxicity to the same level as toxicity observed in CR flies (Fig. 4a), whereas L. mesenteroides or L. brevis did not (Supplementary Fig. 8a, b). To identify candidate genes involved in atrazine metabolism that might be present in A. tropicalis, we searched for genes that are similar to genes involved in a well-known atrazine metabolizing pathway28. We found that candidate genes atzA, atzB, and atzC were present in A. tropicalis (Fig. 4b), but not in L. brevis, or any other species sequenced as part of this study (Supplementary Data 2 and Supplementary Figs. 2—7).

Metabolic responses to pesticide exposure. To assess the effects of these exposures on fly metabolism, we studied the fecal metabolome. Feces capture information about both host and microbiome metabolism, of potential importance for understanding toxicity. We used nanoelectrospray ionization (nESI) direct-infusion mass spectrometry (DIMS) to detect metabolites and lipids in each sample (Supplementary Data 9)29. We detected more than 6500 metabolic features occurring in at least 80% of samples. To study metabolic features present only in one condition, e.g., GF versus CR, we lowered our threshold on presence as follows: we retained all metabolic features present in at least 2/3rds (66%) of samples within a condition and then imputed missing values as previously described29. As we observed with transcriptomics, we see an exaggerated metabolic response in GF flies. Differential abundance analysis reveals that after 72 h 2975 metabolic features are modulated under atrazine exposure in CR flies, while 3297 metabolic features change in GF (see “Methods” and Supplementary Data 10).

The effect of exposure to atrazine, and indeed DMSO, dominates the variance we observe in the fecal metabolome (Fig. 4c). In principal component analysis, the first component (53% of variance) is dominated almost entirely by exposure status. Samples segregate by microbiome status in the second principal component (23% of variance). We examined differentially abundant metabolic features that correspond to specific KEGG identifiers (and assigned to pathways) and found that the majority of such metabolites correspond to secondary metabolism, the biodegradation of xenobiotic compounds, and terpenoid and polyketoid metabolism (Fig. 4d and Supplementary Data 9)30. Remarkably, none of the metabolic features observed that change under atrazine exposure are specific to CR animals, indicating that we have identified host, as opposed to microbial metabolites—or at least metabolites that are not unique to microbes.
Fig. 3 Effect of atrazine on host transcription in adult flies. Transcriptional profiling of males and females raised conventionally or germ-free after exposure to atrazine. a Genes with differential expression after 72 h atrazine exposure with fold changes ≥ 3 (adjusted $p \leq 1E^{-06}$) (red). Conventionally reared (CR) germ-Free reared (GF). Genes are ordered on the chromosomes X, 2L, 2R, 3L, and 4. All genes are shown in gray. b Anatomical analysis of the genes identified by DeSeq2 (a) using their organ-specific maximal gene expression to assign each gene to a single organ system. c Gene Ontology analysis using ClueGO in Cytoscape ($p < 0.05$) of genes differentially expressed (FC ≥ 1.5 and adjusted $p \leq 1E^{-06}$) after 72 h in 2 mM atrazine treated CR and GF flies compared to CR and GF untreated control flies, respectively.
We note that nearly all metabolic features that significantly change in abundance upon atrazine exposure to which we were able to assign KEGG identifiers increase, with only a few decreasing. This pattern does not hold for metabolic features in general (those to which we are unable to assign KEGG identifiers), in both GF and CR animals, about half of exposure-modulated metabolic features increase in abundance (and the other half decrease, Supplementary Data 10). The source of this apparent annotation bias is not immediately clear.

Unsurprisingly, glutathione metabolism is significantly represented in both metabolomics and transcriptomics data. However, we note that while glutathione precursors and metabolic byproducts are modulated by atrazine exposure, glutathione itself is not—levels are remarkably steady in both...
Fig. 4 Acetobacter tropicalis partially rescues atrazine toxicity in germ-free flies. a Survival curves of AdMMF GF flies exposed to 2 mM atrazine, 2 mM atrazine supplemented with Atropic. Survival curves of untreated GF, 0.25% DMSO treated GF, and 2 mM atrazine treated CR flies are included as controls. All atrazine treated flies die by 15 days whereas those supplemented with Acetobacter tropicalis survive for at least 23 days following the curve of flies reared conventionally. For each condition, we used 15 males and 15 females in triplicate aged 4–5 days post eclosion. Error bars indicate the standard deviation of the proportion of surviving flies. b Atrazine degradation pathway. Atrazobactericotoxins are green in color with high similarity to those discovered in a Pseudomonas strain ADP, PAPD-1 plasmid (LKAXO1000023), AtzE (ATJ92156.1), Bith (ATJ90877.1), and AtzF (ATJ90895.1). c principal component analysis of metabolic features detected in CR and GF flies at zero or 72 h after atrazine treatment. d Heatmap of putatively annotated metabolites clustered and grouped by KEGG pathway classifications. Note that z-scores correspond to the inverse normal transform of ranks, not a measure of significance—z-scores are used to improve visualization only. e Flyscape visualization of the metabolomics data in the context of metabolic reactions (24, 48, or 72 h) and transcriptional changes in adult female flies (72 h timepoint; adjusted p ≤ 0.01). The network includes only putatively annotated metabolites and genes identified in our analyses.

CR and GF flies. Hence, while the metabolic flux of this key antioxidant in secondary metabolism changes, the active metabolite is effectively maintained at basal levels suggesting effective homeostasis (Fig. 4e).

Discussion

Multi-omic interrogation of atrazine response in Drosophila melanogaster revealed correlated transcriptional, metabolic, and microbial changes over the course of 72 h after exposure. We found that the gut microbiome evolves dynamically during early adult life under conventional rearing conditions, along consistent trajectories. In flies, Lactobacillus and Acetobacter dominate the gut microbiota19,32. We found that dietary exposure to both PAR and GF flies display a lower abundance of Acetobacter relative to other clades.

The host transcriptome also undergoes changes upon atrazine exposure—and gene expression changes are of larger magnitude and more numerous (more genes affected) in gnotobiotic animals as compared to those conventionally reared. Among the genes that change most significantly upon atrazine exposure in gnotobiotic animals are those that are already induced or repressed compared to CR animals in the absence of pesticide exposure. Some genes, e.g., that respond in CR animals are non-responsive in animals that lack a gut microbiome, e.g., Glutathione S-transferase D8 is downregulated ~2-fold in CR versus unchanged in GF flies. However, over-all, genes involved in oxidation-reduction are more strongly induced in GF flies compared to CR in response to atrazine exposure.

There were also intriguing sex-specific effects, with males exhibiting broader stronger transcriptional modulation compared to females, despite the fact that females generally consume more food33,34, and therefore likely more atrazine, than males.

Study of the fecal metabolome revealed that, in contrast to the host transcriptome, where only dozens of genes are significantly modulated in their expression levels, the metabolome undergoes profound quantitative changes, with more than a quarter of detectable mass features changing significantly. Intriguingly, despite the central importance of glutathione in response to oxidative stress, and the modulations of numerous genes and metabolites involved in glutathione cycling, glutathione itself remains in homeostasis upon exposure.

We found that GF flies exhibited more pronounced lethality than CR flies at the same dosage of atrazine. An explanation for this difference is the presence of an atrazine degradation pathway in Acetobacter tropicalis. Indeed, we found that gut colonization of Acetobacter tropicalis in gnotobiotic animals partially rescues lethality due to atrazine exposure. However, we cannot exclude the possibility that some atrazine degradation by A. tropicalis may occur in the fly media. Measurements of atrazine metabolites in bacteria medium in the absence or presence of A. tropicalis showed no significant difference in degradation suggesting that A. tropicalis alone is not sufficient in degrading atrazine (Supplementary Fig. 8c). Although our study used a food dye to exclude starvation as a confounder, we did not quantify food intake and it is possible that A. tropicalis inoculated flies displayed an altered feeding behavior resulting in reduced uptake of atrazine. The relationship between microbiome remodeling and chronic toxicity is therefore intriguing. Given that A. tropicalis can be cultured on atrazine without impacts on growth rates (Supplementary Fig. 8d), the depletion of A. tropicalis in the gut is unlikely to be a direct effect of chemical exposure—rather, an indirect effect of host metabolism on the competitive landscape of the gut—an example of toxicity and resilience that arises through the integration of host-microbiome metabolism. Given that Acetobacter, and not Lactobacillus, is sufficient to reduce atrazine toxicity to wildtype levels in gnotobiotic flies, this appears to be a disadvantageous situation for the host. We posit that increased abundance of Acetobacter is associated with improved outcomes for individuals—future studies will be needed to assess the merit of Acetobacter supplementation to protect against atrazine toxicity.

The fact that we were able to identify genes corresponding to most, but not all of the Atrazine degradation pathway (KEGG Map 00791) is also interesting, and highlights the challenge in the functional annotation of metabolic pathways, particularly in microbes. Whether this is a case of weak homology, or the presence of non-canonical enzymes able to functionally substitute for atzD is unclear. Engineering genetically tractable knockout bacterial strains would be useful to identify which genes enable atrazine detoxification.

Materials and methods

Fly husbandry

Conventional. This study was performed using the sequenced modENCODE D. melanogaster isogenic Oregon R BDSC strain 23211. Flies were reared and maintained at 25 °C on standard Drosophila medium (0.68% (w v−1) agar, 8.5% (w v−1) cornmeal, 0.4% (w v−1) active dry yeast, 0.024% (w v−1) sucrose, 0.028% (w v−1) potassium sodium tartrate, tetrahydrate, 0.004% (w v−1) CaCl2, 8.26% (v v−1) unsulfured dark molasses, 1.12% (w v−1) Tegosept, 0.01% (v v−1) ethanol, 0.017% (v v−1) propionic acid). To collect adults, flies were raised in 250 ml bottles containing 40 ml medium. Experiments including 16S microbiome and metagenomics, metabolomics, LC50 determination, RNA-sequencing, and the resulting experiment were conducted in the absence of Tegosept (see details below).

Age synchronization.

To obtain adults for the treatment protocols we started by synchronizing embryos. Embryos were collected from three large embryo collection cages (3.5 in diameter × 6 in height). Each embryo cage was populated with 1 g of Oregon-R modENCODE flies, fitted with a molasses-agar (3.3% agar, 13% unsulfured molasses, 0.01% ethyl acetate, and 0.15% Tegosept) petri dish smeared with a 1–2 cm diameter circle of yeast paste (Fleischmann’s Baker’s Active Dry Yeast, 67% in water), was populated with 1 g of Oregon-R modENCODE flies and maintained at 25 °C under constant light conditions. Food plates were changed daily for up to one week. On the day of embryo collection, a fresh, yeasted, molasses-agar plate was attached for at least one hour, then replaced with another.
fresh, yeasted, molasses-agar plate for six hours. Embryos were harvested with 10–14 mL of sterile, 1×PBS buffer3, followed by 2–3 washes with 1×PBS. Thirty-two mature embryos were transferred to sterile bottles containing standard fly food, one small Kimwipes, and active dry yeast pellets. The bottles were incubated at 25 °C, with constant light. On Day 9, bottles were cleared of any early-eclosing adults. On Day 10, adult flies (0–24 h post-eclosion) from multiple bottles were combined under brief CO2 anesthesia. Mixed-sex thirty AdMMF bibulous paper-covered, inverted glass petri dish on ice. To avoid confounding and 16S PCR methods.

M. dilata (15 female plus 15 male) were transferred into each sterile, glass shell vial, containing 5 mL Yeast-Glucose (YG) fly food (10% w/v active dry yeast, 10% w/v glucose and 1.2% w/v Bacto-agar;53 a strip of Whatman paper (2” by 12”); 2 mM atrazine in DMSO (0.25% v/v); DMSO only, or YG food only; with and without Acetobacter tropicalis or two members of the family Lactobacillaceae (L. brevis and L. mesenteroides) (100 million CFU in sterile 1×PBS) applied to the food surface. Acetobacter tropicalis was grown in Difco YPD broth (Yeast Extract-Peptone-Dextrose, BD Biosciences) at 30 °C to log phase. OD600 measurements were taken using a Nanodrop ND-1000 spectrophotometer, and the culture was pelleted and resuspended in sterile 1×PBS to an OD600 of 0.5 (107 cfu/mL). This mixture of resuspended culture was applied to the surface of sterile food in a glass shell vial. L. brevis and L. mesenteroides were grown in Difco Lactobacilli MRS (BD Biosciences) at 30 °C to log phase. OD600 measurements were taken using a Nanodrop ND-1000 spectrophotometer, and the culture was pelleted and resuspended in sterile 1×PBS to an OD600 of 0.2 (106 cfu/mL). Ten microliters of resuspended culture was applied to the surface of sterile food in a glass shell vial. Exposed and inoculated flies were transferred to fresh vials every 3–4 days during the 30-day exposure period. All materials were steam-sterilized at 121 °C in an autoclave. Glass shell vials containing YG food were sealed in an autoclave prior to sterilization and were only removed from the bag inside a biosafety cabinet. Light-sealed cultures were closed with light-sealable pupae. Genese Scientific. Experiments were maintained in closed plastic boxes in an incubator at 25 °C with >60% relative humidity, and on a cycle of 12 h light and 12 h dark. All procedures were performed in a Class II biosafety cabinet, except during transport between the incubator and the biosafety cabinet. All surfaces were sterilized with 70% ethanol before entering the biosafety cabinet.

Bacterial genomic DNA isolation. Bacteria were grown at 30–37 °C in an incubator at 150 RPM under aerobic conditions. Cells were transferred to a 50 mL Falcon tube when the OD600 (Beam Instrument DU640 spectrophotometer) was between 0.3 and 1 and pelleted. Genomic DNA was extracted using a modified phenol/chloroform method. Cell pellets were resuspended in 5 mL TE pH8.0; freshly prepared lysozyme (100 mg mL−1) was added to a final concentration of 3–5 mg mL−1 and incubated for 30–60 min at 37 °C. SDS (10% w/v) was added to a final concentration of 0.5% v/v; then 50 µL protease K (20 mg mL−1) was added and incubated at 56 °C for 1–3 h. The lysate was frozen in liquid nitrogen for 1 min then heated in 80 °C water bath for 3 min. DNA was then isolated by phenol/chloroform (12.5 mL NaCl4 (M) and 12 mL CTAB/NaCl solution 10% CTAB w/v in 0.7 M NaCl, heated to 65 °C) were added and incubated for 10 min at 65 °C. The lysate was transferred to an Oak Ridge tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) pH8.0 was added. The solution was mixed by inversion, centrifuged at 10,000 × g for 20 min, and the aqueous layer was transferred to a new Oak Ridge tube; then the phenol/chloroform extraction was repeated once. A final extraction was done with an equal volume of chloroform/isoamyl alcohol (24:1 v/v), mixed by inversion, and centrifuged for 10 min at 23,000 × g. The aqueous layer was transferred to a 50 mL Falcon tube and the nucleic acids precipitated with 0.7 volumes of isopropanol at –20 °C overnight. DNA was pelletified for 20 min at 16,000 × g, washed with 70% ethanol, centrifuged for 10 min at 16,100 × g, air-dried, and resuspended in 200 µL 1× TE (pH8.0). RNA was removed by digestion with 10 µL of RNase I and incubated for 1 h at 37 °C. To remove the enzyme, we performed phenol/chloroform extractions and precipitated the RNA as above. The final pellet was resuspended in 100 µL 1×TE (pH8.0). The concentration was determined by Qubit and Nanodrop.

Microbiome analyses

16S rRNA sequencing. A DNA-free, sterile cotton swab was pre-wetted with HL3.1 buffer40 then used to collect feces from the wall of a large embryo collection cage. The tip was placed into a PureLink Microbiome DNA Purification Kit (Invitrogen) bead tube, and the wooden handle was broken off. The supernatant was recovered and used for genomic DNA extraction. Genomic DNA was extracted from homogenized fly samples collected daily after transfer of flies to fresh vials (Supplementary Figure 5) using the Invitrogen PureLink Microbiome DNA Purification Kit according to the manufacturer’s instructions. PCR amplification of the V4 region of the 16S rRNA gene was performed using the protocol developed by the Earth Microbiome Project and described in15 using updated primers described by81. 16S sequencing was performed in a 20 µL reaction volume using 10X PCR Buffer without MgCl2 (1X); 50 mM MgCl2 (1.5 mM); RTPCR grade water, 10 mM dNTP mix (0.3 mM each); 16S rRNA gene primers (V4 region) 515 F (0.2 µM, 5′GTCGACTCGTGCGCTGTA3′) and 806 R (0.2 µM, 5′GGACTACHVGGGTWTCTAAT3′); Platinum Taq DNA polymerase (1U), fecal DNA prep (<500 ng). PCR was performed on ABI 9600 thermal cyclers using the following conditions: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min; 72 °C for 10 min; 4 °C hold. PCR clean-up was performed using AMPure XP beads. Sequencing libraries were made using the NEBNext Ultra DNA Library Prep Kit for Illumina Rev 3.0 with the following modifications: 5 µL PCR product plus 50.5 µL nuclease-free water were used for the end prep stage; cleanup of adaptator-ligated DNA was performed without size selection; 9 cycles of PCR enrichment were performed using NEBNext Q5 Hot Start HiFi PCR Master Mix Library. Sequences were filtered on an Illumina MiSeq using the 500-cycle (2×250nt) MiSeq Reagent Kit v2 (MS-102-1101, Illumina) according to the manufacturer’s instructions.

Metagenomic analyses. For metagenomic analysis, bacterial DNA was isolated from adult 21-day old female flies. Whole flies were briefly anesthetized with CO2, washed once with 3% sodium hypochlorite and once with 70% ethanol, then dissected in sterile 1×PBS. The dissected guts were homogenized in 100ul. 1×PBS buffer using a motorized disposable, autoclavable pestle. Following centrifugation at 1,780 × g for 3 min, the supernatant was discarded and the pellet was resuspended in 100ul of QuickExtract DNA extraction solution (Q590050, Lucigen) and 1% ReadyLyse Lysozyme Solution (30,000 U/ul, R1804, Lucigen). Fragmentation was performed using NEBNext ddDNA Fragmentase (M0348S, New England Biolabs) and EDTA was removed with 1.5 volumes of AMPure XP (A63882, Beckman-Coulter Life Sciences) beads. Libraries were made using NEBNext Ultra DNA Library Prep Kit for Illumina (E73705, New England Biolabs) and sequenced using a MiSeq v2 Reagent Kit (MS-102-1101, Illumina).
was centrifuged for 8 min at 12,000 RPM in an Eppendorf centrifuge 5415 and RNA was eluted with 35 µl of buffer. Prepared RNA was quantified using the Bioanalyzer chip (RNA nano 6000) and the Qubit. The smaller of the two concentrations were used to calculate the amount of RNA needed for RNA library preparation (500 ng). The NEBNext Ultra Directional RNA Library Preparation Kit (E7420L, Illumina) together with NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490L, Illumina) was used for RNA library preparation with the following modifications: Section 1.2 mRNA Isolation, Step 37, Fragmentation and Priming Total RNA, we decreased the incubation time from 15 to 5 min; Section 1.3 First Strand cDNA Synthesis, Step 2, we increased the incubation time from 15 to 30 min; for the size selection we used an insert size of 300–450 bp and a final library size of 400–550 bp; and finally in Section 1.9A, PCR Library Enrichment Step 2, we used 14 cycles for PCR cycling. RNA was stored at −80 °C prior to RNA sequencing. Strand-specific RNA-seq libraries were prepared from the treated and untreated samples using the NEBNext protocol. Samples were sequenced in biological triplicate. Libraries were sequenced on the Illumina HiSeq4000 platform using single-end 100 bp chemistry.

**Metabolomics**

### Conventionally raised flies

Fecal samples were collected by placing a standard glass microscope slide inside a large embryo collection cage, avoiding contact with the food plate. Fecal deposits were collected for one day, then stored at −80 °C until metabolomics analysis.

**Germ-free raised flies**

Flies were transferred from food vials to empty 2 mL cryovials and placed horizontally into the fly incubator at 25 °C for 1 h then returned to their original vials. Cryovials containing fecal samples were placed into the −80 °C for storage until metabolomics analysis.

**Nano-electro spray ionization (nESI) direct-infusion mass spectrometry (DIMS)-based metabolomics and lipidomics** were performed as previously reported48. Briefly, metabolites and lipids were extracted from fecal samples using a biphasic solvent extraction method. Extracts were analyzed by positive and negative ionization modes using an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a direct-infusion, chip-based nano-electrospray ionization source (Triversa, Advion Biosciences, Ithaca, NY, US).

**Computational analysis**

**16S microbiome**. QIIME 1.9.1 was used to demultiplex, quality filter, and join MiSeq barcodes45,46. VSEARCH 2.4.1 was used to dereplicate, sort by abundance, remove single reads, and then to cluster at 99% similarity. VSEARCH was also used to check clusters for chimeras and construct an abundance table by mapping labeled reads to chimera-checked clusters45,47. Taxonomy was assigned to the centroid of each cluster using the Qime script parallel_assign_taxonomy._ucclust.py and the Greengenes database56. Statistical analysis and visualization were performed in R using the packages Phyloseq, DESeq2, and ggplot249,51. For bar plots, the 50 most abundant OTUs, representing 98.95% of the data, were agglomerated at the family and converted to relative abundances. Rarefaction curves were generated by subsampling reads (uniformly) from our 16S sequencing data with replacement and then computing species-level and agglomerated abundances using QIIME, as above. Operational taxonomic units were agglomerated as above. Ordination was performed and plotted with the Phyloseq package using the Principal Coordinates Analysis method and Bray–Curtis distances. Differential abundance analyses were performed on the OTU level using DESeq2 1.22.1. The effects of atrazine treatment over time were determined using a two-factor design (Treatment + Time + Treatment:Time) and extracting the results for the interaction term. Differentially expressed clades were thresholded at a log fold change of 1.5 and an FDR adjusted p ≤ 0.01.

**Metagenomics**. To identify gut bacterial species, we used bowtie2 to remove reads that aligned to the Drosophila reference genome (Release 6)52, yeast (S288C, E7420L, Illumina) together with NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490L, Illumina) was used for RNA library preparation with the following modifications: Section 1.2 mRNA Isolation, Step 37, Fragmentation and Priming Total RNA, we decreased the incubation time from 15 to 5 min; Section 1.3 First Strand cDNA Synthesis, Step 2, we increased the incubation time from 15 to 30 min; for the size selection we used an insert size of 300–450 bp and a final library size of 400–550 bp; and finally in Section 1.9A, PCR Library Enrichment Step 2, we used 14 cycles for PCR cycling. RNA was stored at −80 °C prior to RNA sequencing. Strand-specific RNA-seq libraries were prepared from the treated and untreated samples using the NEBNext protocol. Samples were sequenced in biological triplicate. Libraries were sequenced on the Illumina HiSeq4000 platform using single-end 100 bp chemistry.

**GO analysis**. Gene Ontology biological annotations (p < 0.05) of differentially expressed genes (fold-change ≥ 1.5 and adjusted p ≤ 0.01) were determined using ClueGO50 and visualized in Cytoscape25.

**Integrative analysis**. FlyScan (version 1.0.1) in Cytoscape (version 3.7.1) was used to visualize the metabolomics data in the context of metabolic reactions and transcriptional changes. Transcriptional changes in atrazine-treated female flies (72 h timepoint; P adjust < 0.01) were combined with the union of all metabolic changes after 24, 48, or 72 h of atrazine treatment and visualized in Flyscape29. The network was reduced to only include metabolites and genes from our input lists. Heatmaps were created using the ggplot v3.0.1 R package.

**Statistics and reproducibility**. R was used for all statistical analysis. Statistical significance was determined using statistical tests as indicated, including Student’s t-test and the Mann–Whitney U test. The number of animals and/or independent experiments are indicated in figure legends and the associated methods sections. Adjusted p-values are indicated as statistically significant where appropriate throughout the manuscript.

**Reporting summary**. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Strains are available from the BDSC public repositories. Complete 16S, RNAseq, and metagenomics datasets and all metadata are available from our BioProject accession number PRJNA718558. Data presented in Figs. 2a, b, and 4a are available in Supplementary Data 11.

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Author contributions
J.-H.M., A.M.S., J.B.B., and S.E.C. conceived the project. K.W., J.B.B., and S.E.C. designed the fly experiments. S.E.C. managed the project. A.H. isolated BDGP fly gut microbes, C.Y. made genomic DNA, K.W., S.P., and S.M. performed fly animal husbandry. R.W. made RNA and built libraries for fly samples. B.W.B. and S.L. analyzed the RNA sequence data. S.M. and K.W. isolated fecal samples and K.W. made 16S RNA libraries. S.P. performed all the gnotobiotic and rescue experiments. K.W. generated microbial genomic DNA for sequencing and S.M. and K.E.C. wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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