Abstract

This study explored the effects of flunitrazepam, a benzodiazepine, on the microbiome diversity of *Calliphora vicina* Robineau-Desvoidy, 1830 (Diptera: Calliphoridae). By examining the microbial shifts throughout developmental stages, the research contributes valuable data to the field of forensic entomotoxicology.

Two colonies of 500 adults were fed minced beef liver, spiked and non-spiked with 25 mg of flunitrazepam, and reared under controlled conditions (24°C, relative humidity of 45%, 12:12 light-dark cycle). Following oviposition, egg clusters were transferred, and the experiment was carried out in triplicate under the same experimental conditions. A total of 54 specimens, including all developmental stages, were collected for microbiome investigation via Illumina MiSeq.

Both colonies had a 19-day development cycle from eggs to teneral. However, flunitrazepam-fed specimens were heavier, particularly during the pupa and teneral stages. Microbiome analysis revealed significant differences in diversity and composition between the colonies and across developmental stages. Pseudomonadota (Proteobacteria) dominated the control adults, while Bacteroidota and Bacillota (Firmicutes) were more prevalent in flunitrazepam-fed adults. Additionally, Enterobacterales, Lactobacillales, and Morganellaceae showed notable variations across different stages.

This study highlights the significant impact of flunitrazepam on the microbiome dynamics of *C. vicina*, revealing notable morphological changes related to the specimens' weight towards the end of the development cycle and alterations in microbiome composition. These findings have important implications for forensic entomotoxicology, particularly in the accurate estimation of the minimum postmortem interval (mPMI).

Keywords: forensic entomology; entomotoxicology; benzodiazepines; blow flies; drug-induced dysbiosis.

Highlights:

- Flunitrazepam alters microbiome diversity in *Calliphora vicina* across all life stages.
- Microbiome shifts include dominance of Bacteroidota and Bacillota in flunitrazepam adults.
- Enterobacterales and Lactobacillales vary significantly with developmental stage and treatment.
- Drug exposure increases pupa and teneral stages weight.
- Findings can help enhance mPMI estimation accuracy in forensic entomotoxicology.

Introduction

Entomotoxicology is a specialized field within forensic entomology that involves the study of various insect species to detect the presence of toxic substances, providing valuable information in cases where traditional toxicological samples (like blood or urine) are unavailable due to advanced decomposition [1]. Since the first study in 1977 [2], over 70 papers focused on entomotoxicology have been published. Although studies have been reported since the 70s, the term forensic entomotoxicology was defined in 1991 by Pounder [3]. In early 2000 [4], entomotoxicology was still considered a relatively new branch of forensic entomology. Nevertheless, insects have proven useful for the detection of drugs in the absence of tissues or fluids [5–7], making entomotoxicology a crucial tool in forensic investigations.

Studies demonstrating the bioaccumulation of various metals, including mercury, in different Diptera species have been reported since 1977 [2]. Other earlier studies [8,9] investigated the insects' presence and dynamics as a response to different poisons and their accumulation. Different drugs have been

successfully identified from the larval tissues or puparia cases, as opposed to some organs [10,11]. Moreover, substances were detected even from insect traces such as frass and puparia cases [12–15].

Building on these foundational studies, it is critical to understand how different substances are influencing the development cycle of the primary necrophagous insect species, to have a better understanding of this effect for the minimum postmortem interval (mPMI) estimations. As previously demonstrated, certain drugs can speed up or slow down the rate of development [16–18]; however, special consideration must be given to the concentration used to test the drug effect [19]. Another earlier report [20] showed that larvae collected from the nasopharyngeal area of an individual deceased from a cocaine overdose were observed to be twice as big in length, in contrast to the larvae collected from other body areas. As demonstrated, the manner of drug ingestion and distribution in different tissues and fluids is important when analyzing the pharmacokinetic processes of these substances in both human and insect samples [1]. Further, the metabolization of different substances depends on their physiochemical properties [21–23]. All these earlier studies revealed that there are certain limitations when studying and analyzing the bioaccumulation of drugs in different insect tissue; thus, a more in-depth investigation on the effect of drugs at a bacterial level could reveal more information that could help understand the microbiome shifts as a response to drugs ingestion by insects.

Studying the effect of benzodiazepines, namely flunitrazepam, on *Calliphora vicina* Robineau-Desvoidy, 1830, is justified by the species' primary forensic role and importance as entomological evidence during death investigations [24]. Necrophagous insect species, particularly Diptera species, represented by Calliphoridae, are the predominant insects collected at a crime scene or from decomposed animal remains during experimental studies [24]. As the developmental stages and insects' succession are used to estimate the mPMI, understanding their response to different drugs and

poisons found in the feeding substrate proves to be crucial during death investigations where insects are used as forensic evidence. Understanding how drugs like flunitrazepam affect the microbial composition can provide insights into the overall health and development of this insect species. The bioaccumulation of flunitrazepam in *C. vicina* larvae and pupae samples has been previously tested to develop a sensitive method for benzodiazepines quantification via liquid chromatography–tandem mass spectrometry (LC-MS-MS) [25]. Flunitrazepam, a psychotropic drug, belongs to the Benzodiazepine group, classified as a Schedule IV controlled substance under the Controlled Substances Act in the USA [26], and has been listed as a prohibited drug since 1992 [27]. While this drug is often prescribed as a treatment for anxiety and insomnia (Mishima, 2024), unfortunately, it has often been involved in rape cases, overdoses, and accidental deaths [28,30]. Moreover, benzodiazepines deaths also involved opioids [31]. The most recent data revealed that the number of people suffering from drug use disorders worldwide has risen to 64 million [32]. As the use of psychoactive substances, including antidepressants such as flunitrazepam, has substantially increased in the last decade [33,34], it is imperative to understand how this drug is affecting the main entomological forensic indicators, such as *C. vicina*.

While several studies [35–37] have investigated the microbiome of different Diptera species, the current research is the first one, to our knowledge, to investigate *C. vicina* microbiome variations as a response to the presence of flunitrazepam in the feeding substrate. Despite advancements in entomotoxicology, there are still limitations in understanding how drugs affect insect microbiomes. This study aims to address these gaps by investigating the effects of flunitrazepam on microbial composition. Specifically, this study seeks to answer two key questions: 1) Are the microbiomes of *C. vicina* flunitrazepam-treated and control colonies different throughout the developmental stages? 2) If

variations are recorded, what implications might these have on the development of this necrophagous insect species?

Materials and Methods

Research Design

To investigate the effect that flunitrazepam has through the development cycle of *C. vicina*, and on microbiome diversity and dynamics, two initial colonies of 500 adult specimens each were used during this research. Larvae specimens were purchased from a local live bait vendor and reared within an insect rearing chamber (Caron, USA) (Figure 1). The experiment started with the emergence of all adult specimens. Both initial larvae received at the laboratory and a few adult specimens were taxonomically identified to species using the taxonomic identification keys provided by the scientific literature [38] via a stereomicroscope Leica S9 D (Leica, USA). The experiment received approval from the Institutional Biosafety Committee (IBC2312-0057).

Both adult colonies were provided with 200 g of minced beef liver each, spiked and non-spiked with flunitrazepam. A solution of 25 mg of flunitrazepam was prepared by dissolving it in 50 ml of ultrapure water. This solution was subsequently homogenized with 200 g of minced beef liver for each rearing container. The concentration selected corresponds to the higher threshold of a lethal dose [39]. Water and honey were provided daily for colony maintenance.

The colonies were maintained at a constant temperature of 24°C and a constant humidity of 45%, under a 12:12 light-dark cycle, within an insect rearing chamber (Caron, USA). For the entire duration of the experiment, the colonies were monitored three times a day, between the hours of 8:00 and 18:00. After the adults fed for four days, oviposition was recorded. The egg clusters were transferred, and the

experiment was carried out in triplicate for each control and flunitrazepam colony under the same experimental conditions. The beef liver was medium minced by using a chopper (Ninja Express Chop, USA) with four pulses for each mincing.

Specimens were randomly collected in triplicate, with each replicate representing a distinct rearing container, including all developmental stages: adult, eggs, first instar larvae, second instar larvae, third instar larvae (mid-stage), pupa (midpoint of emergence), and teneral (fully emerged specimens that had released their meconium). Six adult and teneral specimens respectively, were randomly sampled, representing triplicate of female and male specimens. Developmental stages were determined by daily monitoring and random specimens' life stage identification via a stereomicroscope Leica S9 D (NCI, USA). A total of 54 samples were collected and analyzed, while the development cycle was monitored and compared between the two colonies. The collected specimens were weighed using an analytical balance (Ohaus Pioneer, USA).

Microbiome Investigation

Upon sampling, the specimens were rinsed in distilled water and immediately stored at -20°C (ThermoFisher, USA) without any buffer. Total genomic DNA was isolated from each entire specimen via Qiagen Blood and Tissue (Qiagen, USA) modified protocol that included a disruption step of the tissues via surgical tweezers.

DNA concentration and purity were assessed via a NanoDrop One spectrophotometer (Thermo Scientific, United States). All experimental steps, including DNA isolation and all molecular assays,

were performed under aseptic conditions to avoid contamination, via a purifier-filtered PCR enclosure (Labconco, United States).

The purified DNA samples were sent for sequencing at the McGill Genome Centre, Canada, using the Illumina MiSeq PE300 sequencing platform, and the primer pair 341F/805R64 for the PCR amplification of the 16S rRNA gene fragments (V3–V4 variable region). Three samples, represented by a teneral male specimen from the control colony, one teneral female specimen and one teneral male specimen from the flunitrazepam colony, failed amplification. The remainder of the investigation was conducted with 51 samples. As previously demonstrated [40], certain core bacterial taxa tend to be consistently present across different individuals, even when analyzing a lower number of specimens.

Data Processing and Analysis

Paired-end readings from each sample were sequenced separately for forward and reverse readings and processed using the QIIME2 microbiome bioinformatics platform (Quantitative Insights into Microbial Ecology 2), version 2024.5 [41]. We adopted the newest taxonomic nomenclature available, but we included where needed (in brackets) the older nomenclature to facilitate comparisons with previous works. The 51 samples gave a total of 19,375,754 raw sequences, with 358,810.25 mean reads per sample.

Quality control and denoising, including chimera removal, were conducted with the DADA2 plugin (v.1.26.0) [42], and low-quality sequences were filtered by trimming forward reads at 20 bp, truncating forward reads to 200 bp, and reverse reads to 160 bp.

Taxonomic classification was performed using the Silva v.138.2 database (97% OTUs full-length sequences) [43]. Statistical analysis was carried out in the R version 4.4.1 (2024-06-14) computing

environment [44], and taxon abundances were calculated and visualized using the 'phyloseq' package (v.1.48.0) [45].

Data were converted into relative abundances where needed, to ensure comparability amongst samples. Alpha diversity was used to assess variations within treatments and developmental stages, with significance tested through global Kruskal-Wallis and Wilcoxon signed-rank test pairwise (significance set at $\alpha < 0.05$). Beta diversity, exploring differences between groups, was analyzed using Principal Coordinate Analysis (PCoA). Data analysis focused on linear discriminant analysis (LDA) effect size (LEfSe) method for microbiome biomarker discovery implemented in 'lefer' [46] using Kruskal-Wallis and Wilcoxon signed-rank test pairwise cutoff set at 0.05 and LDA cutoff of 2. Unique ASVs for each developmental stage were identified using an in-house function.

Permutational multivariate analysis of variance (PERMANOVA) was performed using the *vegan* package (version 2.6–10) in R to assess differences between groups.

Supplementary Table 1 includes full taxonomic attributions to the ASVs, and their associated unique identifiers used for simplicity in the manuscript.

Results

The duration of the complete life cycle, from eggs to teneral, was 19 days for both treated and untreated fly colonies. While no temporal differences were observed between the two colonies, the weights of specimens varied during the developmental stages (from larvae to adult) under the two treatment conditions as shown in Figure 2. In the adult stage, there is a significant difference in weight between the treatments, with the flunitrazepam group having a higher median weight than the control (p =

0.0022). For the first and second instar larval stages, there are no significant weight differences between treatments, both showing p-values of 1. This suggests that flunitrazepam does not impact weight during these early larval stages. Similarly, in the third instar larval stage, no significant effect of flunitrazepam on weight is observed, with a p-value of 1. In the pupal stage, the data show a difference in weight, with the flunitrazepam group having a slightly higher median weight, but this difference is not statistically significant. Finally, in the teneral stage (newly emerged adult), a significant weight difference is observed, with the flunitrazepam group exhibiting a higher median weight compared to the control ($p = 0.015$). This pattern indicates that flunitrazepam may affect weight primarily in later developmental stages (pupae and teneral), while having little to no impact in earlier stages.

While no significant differences were observed in the richness and Shannon diversity metrics between the two colonies (Figure 3A), there were notable genus-level differences and slight non-significant variations between the first and second generations (Figure 3B). Statistically significant differences were observed in the richness and Shannon diversity of the microbiome at various developmental stages in both treated and untreated groups (Figure 3C). Specifically, the highest levels of diversity were found within the first and second instar larval stages, and the lowest ones within adult and teneral stages.

The PCoA analysis, grouped by each developmental stage, highlighted some similarities between the microbiome of treated and non-treated specimens at the pupal stage, whereas all other stages showed some differences between treated and non-treated colonies. This approach helped in identifying specific microbial shifts and their potential impacts on development. Interestingly, the egg stage shows quite distinct clusters between the control and the treated specimens, with no outliers identified. On the contrary, other stages such as adult, larvae, and teneral all show some outliers in the plots (Figure 4).

In the control group, Bacillota (Firmicutes) and Pseudomonadota (Proteobacteria) dominate the microbial community across all sample types (Figure 5A). Pseudomonadota constitute approximately 89.10% of the total adult community, demonstrating their significant prevalence at this stage. However, this trend shifts between the egg and pupal stages, where Bacillota accounts for over 50% of the overall community, indicating a marked transition in microbial composition. Bacteroidota are also prominent during these stages but are notably absent in the pupal phase. Finally, in the teneral stage, Pseudomonadota emerges as the dominant phylum, comprising 75% of the microbial community, followed by Bacteroidota, which accounts for the remaining 25%.

Upon exposure to flunitrazepam, significant changes in microbial composition are observed (Figure 5B). In the early sample types, Bacillota maintain their dominance. However, in later stages, there is a pronounced reduction in Bacillota and a corresponding increase in Pseudomonadota, suggesting a shift in the community dynamics. Notably, Actinomycetota (Actinobacteria), which were absent from the control group, appear in detectable percentages during the teneral stage of the flunitrazepam group, accounting for 16.27% of the community. This suggests a potential selective enrichment of Actinomycetota under treatment conditions. Bacteroidota also displays minor fluctuations in abundance, but their overall contribution remains relatively small.

These findings demonstrate that flunitrazepam exposure significantly alters the structure of the microbial community, particularly by increasing the relative abundance of Pseudomonadota and enabling the emergence of Actinomycetota. These shifts suggest that Pseudomonadota and Actinomycetota may be more adaptable or resilient to the treatment conditions (Supplementary Table

1). Further investigation is warranted to understand the mechanisms underlying these changes and their implications for microbial function and host health.

Results of the linear discriminant analysis size effect (Figure 6) highlight significant microbial markers associated with specific developmental stages. For the adult stage, several features belonging to the phylum Pseudomonadota, specifically within the family Yersiniaceae, were highly enriched. The marker genus *Serratia* (LDA = 5.10, padj = 0.001) and its associated taxa were dominant. These taxa underline the predominance of Pseudomonadota in the adult phase, suggesting their functional importance during this stage (Supplementary Figure 1).

During the egg stage, most of the enriched markers were unclassified at higher taxonomic levels, with many identified only as "Unassigned" across multiple taxonomic ranks. These markers demonstrated high LDA scores (ranging from 5.50 to 5.50) and significant adjusted p-values (padj = 0.013), indicating that these uncharacterized taxa may play critical roles during the egg stage. In the first instar larvae, enrichment was observed within the phylum Bacillota, specifically in the order Lactobacillales. Key taxa included Streptococcaceae and *Lactococcus* (LDA = 5.24, padj < 0.001). These results highlight the dominance of Bacillota taxa in early larval development, which may contribute to nutritional or protective functions.

In the second instar larvae, the dominant enriched taxa were again from Bacillota, particularly within the family Lactobacillaceae. *Leuconostoc* and its species (LDA = 4.79, padj = 0.006) were strongly associated with this stage, reinforcing the importance of lactic acid bacteria in mid-larval development. In the third instar larvae, enrichment patterns showed significant contributions from both Bacillota and Pseudomonadota. Lactic acid bacteria, such as *Latilactobacillus* (LDA = 4.98, padj = 0.020), were

prevalent, alongside *Moellerella* from the Morganellaceae family (LDA = 5.06, padj = 0.002). These taxa likely reflect a complex interplay of microbial populations during this developmental peak. The pupa stage showed a more diverse enrichment pattern, with significant taxa belonging to both Bacillota and Pseudomonadota. Notable Bacillota taxa included Planococcaceae and *Savagea* (LDA ~ 4.72, padj = 0.006). Additionally, taxa like *Faecalibacterium* (LDA = 4.74, padj = 0.006) were enriched, suggesting the role of both gut-associated and environmental taxa in this transitional developmental phase. The teneral stage revealed significant enrichment in Pseudomonadota, particularly within the family Morganellaceae. Taxa such as *Providencia* and its species (LDA = 5.39, padj < 0.001) were dominant. These findings suggest that Morganellaceae-related taxa may play a key role in the transition to adult functionality. Supplementary Table 2 and supplementary Figure 2 show the taxonomy of all enriched ASV and associated statistics.

Considering the behavior of the single ASVs across stages and divided between treated versus untreated, Figure 7 shows six selected markers. Specifically, ASV4 seems to have significantly different trends according to the treatment with expression only in egg and teneral stages for the treated portion of the sample while there is presence of this ASV in adult, egg, second instar and teneral phase for the control sample. This ASV with unique identifier fdbfdd695cc322a1337638c0bff5343d was unassigned preventing any biological interpretation. In contrast, ASV17 is from the genus *Serratia* in the family Enterobacteriaceae and showed higher abundances in the control group for the adult stage to then disappear in this group and remain in the flunitrazepam-treated individuals until the second larval stage. Mostly present in the flunitrazepam in second and third instar is ASV27, genus *Moellerella* family Enterobacteriaceae. Very different is the behavior of ASV28 from the genus *Providencia* that belongs to the family Enterobacteriaceae. This ASV appears only from the third instar larval stage to

progressively increase with the development. Both groups seem to have a similar abundance except for the teneral stage, when the control sample shows higher mean abundance. Finally, ASV98 and ASV99 are both from the genus *Lactococcus,* however their behavior differs; ASV98 peaks in both control and treatment in the first instar larvae, whereas ASV99 is higher in the control than in the treatment, specifically in eggs, first and third instar, and higher in the treatment instead in the second instar. Supplementary Table 3 provides the statistical differences between the several developmental stages.

Discussion

The effect of flunitrazepam has been previously tested on *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) specimens [47]. The authors tested four concentrations of 4, 16, 18, and 32 ng/g. It is worth noting that even at these lower concentrations, compared to the current study, where 25 mg of flunitrazepam per 200 g of minced liver was used, the weights of the pupae and adult specimens were higher than in the control colony. In the current study, we observed that the weights of the pupae and adult specimens in the flunitrazepam group were significantly higher than those in the control group ($p = 0.0022$ for adults and $p = 0.015$ for teneral stage). This indicates that flunitrazepam has a pronounced effect on weight during the later developmental stages. However, at the larval level, our study revealed little to no weight differences between the treated and control groups, suggesting that flunitrazepam may have little to no effect on these earlier developmental stages, when tested at these high concentrations.

Another study [48] demonstrated that the 7-aminoflunitrazepam, a metabolite of flunitrazepam, causes the *C. vicina* larvae to grow in length. When testing the effect of other benzodiazepines, like nordiazepam, on *C. vicina*, no morphological changes were reported [49]. In other previous studies on

calliphorids [50], including *C. vicina* [51] it was observed that both diazepam and lorazepam increased the larval weight and length.

Our study did not record a difference in the development time of the overall cycle or individual developmental stages between the treated and control groups, consistent with Baia and colleagues [47]. This suggests that even at higher concentrations, flunitrazepam does not alter the duration of the development cycle.

When testing nordiazepam effects on *C. vicina*, no life cycle modifications were recorded [49], however, other benzodiazepines, such as lorazepam, were observed to delay the development cycle of *C. vicina* [51]. Another study [50] researching the effect of diazepam, another benzodiazepine drug, on the development cycle of *Chrysomya albiceps* (Wiedemann, 1819) and *Chrysomya putoria* (Wiedemann, 1830), revealed that the larvae developed more rapidly, and the adult emergence was delayed. Thus, the presence of the drug influenced the duration of the development cycle.

As previously reported from these studies, different benzodiazepines have diverse effects on the Calliphoridae development cycle. However, an increase in larval and pupal weight and length represents a common finding regardless of the benzodiazepine and blowfly species investigated. These findings suggest that the fundamental reaction to benzodiazepines can be attributed to metabolic and physiological changes as a stress response. More in-depth studies on nutrient absorption, hormone regulation, and stress responses are needed to elucidate the metabolic and physiological mechanisms by which benzodiazepines influence insect growth, with special consideration to Calliphoridae species. Nevertheless, the presence of benzodiazepines must be considered when using different developmental

stages to estimate the mPMI, since as previously mentioned its accuracy will depend on the assumption that insect development proceeds under typical/normal conditions [52].

The microbiome associated with blow flies and house flies has been investigated to demonstrate the potential of flies to be proxies for human pathogens distribution, having public health implications [53,54]. Most of the studies to date [55] used 16S rRNA sequencing for the identification of microbial taxa. As demonstrated [55], necrophagous insect species can acquire part of the microbiome encountered within the feeding substrate, while also being able to transfer their own microbiome onto the feeding and breeding environment [56,57]. A previous study investigating the 16S rRNA profiles of *C. vicina* larvae and adults (males and females) revealed taxonomic diversity similarities between males and females and a clear distinction between larvae and adults [54], findings supported by the current study. *Lactobacillus* dominated the larvae specimens [54], similar to the current study, while Morganellaceae prevailed in the adult specimens of the previous study and in the teneral specimens of the current study.

While certain bacterial species can be insect-specific [58–60], more than 50% of their microbiome is acquired from the environment where they feed and breed [53]. The authors tested *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae) and *C. megacephala* microbiomes via whole-genome shotgun sequencing. Proteobacteria, Bacteroidetes, and Firmicutes representatives were the most abundant for both blow flies and house flies [53,58,61], confirmed by the current study, with a clear distinction in abundances between the control and flunitrazepam specimens, as well as a higher percentage of Actinomycetota in the teneral specimens exposed to flunitrazepam. A previous study [62] investigating the effect of flunitrazepam and its metabolite 7-aminoflunitrazepam revealed that the gut-liver microbiome of zebrafish showed significant differences when exposed to this drug, altering the

abundances of Proteobacteria. Even though this previous study targeted a different organism, such as the zebrafish, it is worth noting that the presence of this drug had the same effect on the blowfly species investigated in the current study. Pseudomonadota had undetectable abundances in the egg, first and second instar larva specimens from the control colony, being noticeably abundant in specimens from the flunitrazepam colony. These results could suggest that flunitrazepam is affecting the microbiome composition by impacting the growth and survival of certain bacteria. This could also be a stress response, similar to the effect on the insects' weight and length, as a result of the drug impact on the immune system or the metabolic processes. As previously demonstrated for vertebrate species [63–65], the γ-aminobutyric acid (GABA), an important inhibitory neurotransmitter, has receptors for benzodiazepines, including flunitrazepam, similar to invertebrates [66]. The stress response affecting both morphology and microbiome can be a repercussion of the neuronal GABA receptors, disrupting normal physiological and microbial processes. Noteworthy, when using the zebrafish as an organism model to study the effect of flunitrazepam and its main metabolite on the brain [67], it was demonstrated that the drug can accumulate in the brain tissue, being able to cause neuronal damage.

It is noteworthy that the microbial shifts observed in the current study may have broader implications in forensic science beyond insect physiology. Taxa such as Pseudomonadota, Bacillota and Actinomycetota are commonly associated with decomposition and have been identified as potential indicators of the postmortem interval estimation in previous studies [68–70]. The current findings suggest that drug exposure can significantly alter the insect microbiome, which may in turn influence the microbial community associated with decomposing remains. As Pechal and collaborators [68] have shown, the presence or absence of insects can impact the postmortem microbiome of the remains over

time. Therefore, drug-induced changes in insect-associated microbiome could potentially affect the interpretation of microbial succession as it relates to the PMI estimation.

When interpreting the results of the effect of flunitrazepam on *C. vicina* or other calliphorid species, special consideration must be given to the moment when the inoculated feeding substrate is made available to the fly colonies (e.g. during the initial feeding and oviposition stage or later in the process). Consideration must also be given when analyzing different drugs, at different concentrations, on different feeding substrates and using different insect species. Noteworthy, the administration of the drugs is critically important, as there is a clear distinction between the feeding substrate represented by a pig carcass deceased of an overdose, a minced beef liver manually homogenized with the drug, and an artificial diet. While the first option might be difficult to implement by many laboratories, using liver or decomposed organic matter as feeding substrate might provide a more realistic model when discussing the importance of feeding substrate in interpreting the results. A standardization of these experimental conditions is needed to have a more reliable and comparable data set.

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Figure legends

Figure 1. Research design: methodology and data collection.

Figure 2. Box plots show the weight (in mg) of the insects at different developmental stages. Statistical differences were evaluated by means of Wilcoxon test reported in the plot.

Figure 3. Observed richness and Shannon diversity metrics across: (A) samples at all stages grouped by treatment type; (B) adult female and male specimens grouped between first and second generation;

(C) developmental stages for both treated and untreated sample. Statistical significance for the Wilcoxon test is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. Principal coordinates analysis (PCoA) on weighted-UniFrac distance for all samples grouped by stage. Treatments are represented with different shapes (circle for controls, triangle for flunitrazepam).

Figure 5. Bar plots show the relative abundance of bacterial phyla across different sample types, comparing the (A) control group to the (B) treatment group exposed to flunitrazepam.

Figure 6. LEfSe analysis identifying differentially abundant features across developmental stages, highlighting those with both statistical and biological significance and ranking them by effect size.

Figure 7. Line plot of mean abundance of selected ASV across different developmental stages under control and flunitrazepam-treated conditions. Error bar shows standard deviation.

Supplementary Figure 1. Bar plots show the relative abundance of microbial class across different developmental stages under (A) control and (B) Flunitrazepam-treated conditions.

Supplementary Figure 2. Line plot of mean abundance of each ASV across different developmental stages under control and flunitrazepam-treated conditions. Error bars show standard deviation.

Supplementary Table 1. Taxonomy table for the Phyloseq object showing both assigned ASV name and unique identifier.

Supplementary Table 2. Significant markers for LEfSe and relevant enriched groups.

Supplementary Table 3. Pairwise comparison between normalized abundance of four selected ASVs across developmental stages using Wilcoxon test with false discovery rate (FDR) p-value adjustment. Sample is divided by treatment. Statistical significance for the Wilcoxon test is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.