



# Heavy metal accumulation and fecal microbiota response in black soldier fly larvae: identification of *Leminorella grumontii* as a potential indicator species

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## Abstract

With growing environmental concerns about marine pollution from heavy metals, black soldier fly larvae (BSFL) are gaining attention for their potential use in sustainable waste-to-feed systems. In this study, BSFL were raised on diets containing cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As) to assess their growth, metal accumulation patterns, and changes in microbiota. While Cd mainly accumulated in the larval body, Pb, Hg, and As were primarily excreted through exuviae and feces. This pattern remained consistent even when larvae were fed naturally contaminated mackerel heads. Microbiome analysis of fecal samples showed that *Leminorella grumontii* had a strong positive correlation with Hg and As concentrations, suggesting its potential as a microbial indicator species for heavy metal exposure. These findings support the use of BSFL in bioconversion systems and suggest *L. grumontii* as a new biomarker for environmental monitoring.

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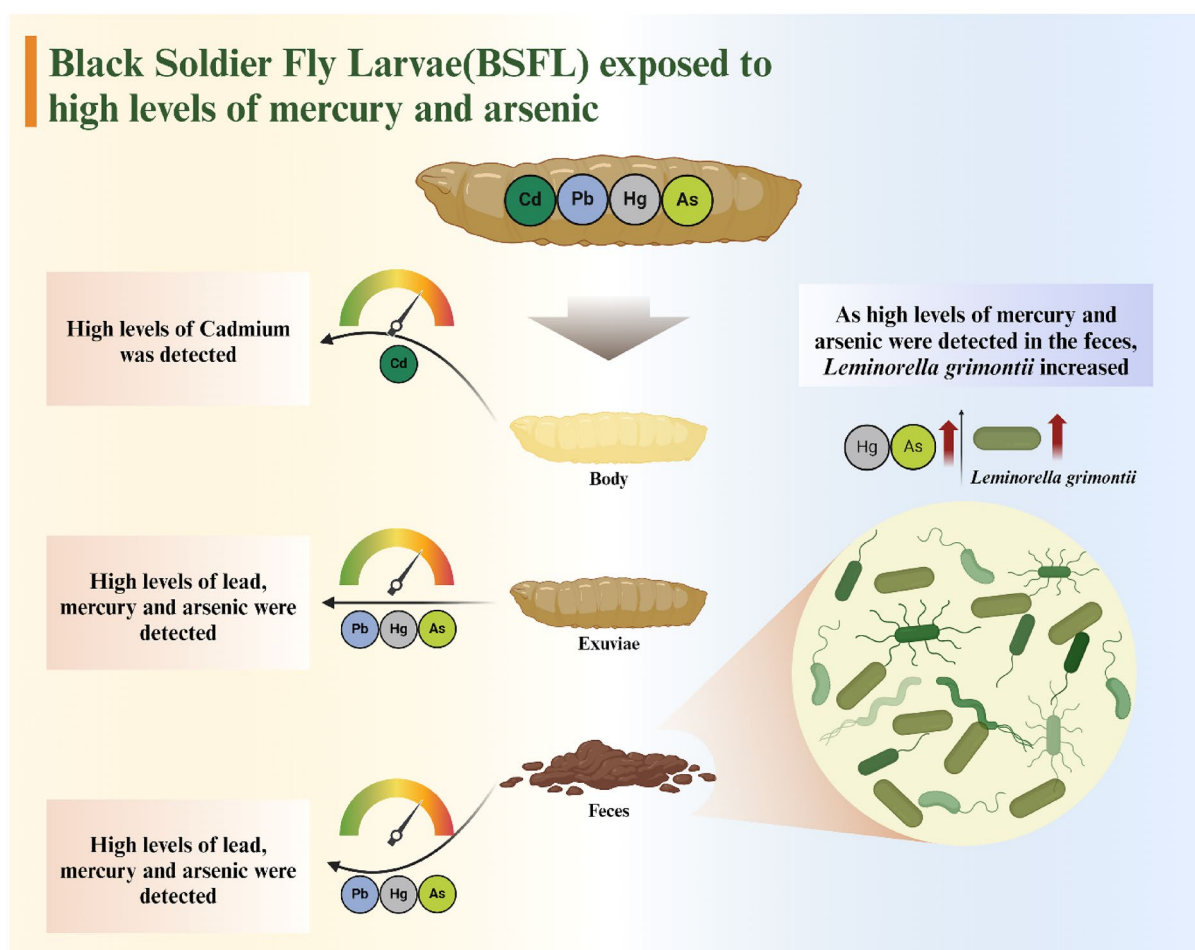
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## Graphical abstract



**Scheme 1** Experimental procedure and fecal microbiome results

**Keywords** Black soldier fly larva (BSFL) · Heavy metal · Mackerel head · *Leminorella grimontii*

## Introduction

The global surge in economic development has led to the overproduction of natural resources and a substantial increase in waste generation, underscoring the urgent need for effective waste management (Raksasat et al., 2020). As the amount of organic waste increases, waste disposal and environmental protection are emerging as important issues (Diener et al., 2015; Nachana'a Timothy, 2019).

Recently, the exploration of insects as a viable solution for various purposes has garnered interest. Among these, there is growing interest in research on Black Soldier Fly Larvae (BSFL). BSFL exhibit remarkable proficiency in degrading organic waste and are characterized by substantial amounts of proteins and lipids, along with a diverse array of nutrients, including essential amino acids and

fatty acids (Elwert et al., 2010; Spranghers et al., 2017). Unlike pests that biodegrade waste, BSFL do not transmit bacteria or diseases and are known to inhibit the activity of *E. coli* and *Salmonella* (Erickson et al., 2004; Higa et al., 2021; Lalander et al., 2013). Black soldier fly adults do not require additional nutrients for survival and accumulate nutrients for adult survival during larval development (Tomberlin et al., 2002). With its diverse range of characteristics, BSFL has been employed in various fields of research.

BSFL is highly valued for its nutritional richness and is used as a feed for a diverse range of organisms. Research has confirmed its use as a feed for various animals, including pigs, chickens, and marine life. Furthermore, the nutritional composition of BSFL varies depending on the diet (Bondari and Sheppard, 1981; Newton et al., 1977). Furthermore, research has shown that when BSFL consume feeds rich

in protein and lipids, they exhibit excellent digestion, indicating that the provision of protein and lipids enhances the performance of BSFL (Barragan-Fonseca et al., 2018; Danielsen et al., 2013; Gold et al., 2020, 2018).

BSFL can be cultivated using a diverse range of organic materials, including household waste and fecal matter. However, household organic waste is often contaminated with various substances, including environmentally problematic heavy metals. Therefore, BSFL are exposed to heavy metals through various routes when reared in organic waste (Gold et al., 2018). To address this issue, in the case of insects, reducing heavy metal concentrations through excretion is considered an effective method to prevent excessive accumulation in the body (Jiang et al., 2018). However, research on the feces and pupal excretions of BSFL exposed to various heavy metals is lacking.

An increase in heavy metal concentration can result in growth inhibition, developmental abnormalities, reduced reproductive performance, decreased hatching rates, and alterations in respiratory and metabolic processes in BSFL. In the case of lead, BSFL accumulate it in their exuviae, and, as with other insects, there is a high likelihood of a decrease in lead content during molting. According to previous studies, cadmium tends to accumulate in larvae, whereas arsenic is detected in larvae, and a significant amount of arsenic discharge has been investigated (van der Fels-Klerx et al., 2016). Furthermore, when consuming waste containing mercury, the mercury content decreases, and it has been observed that mercury biologically accumulates in BSFL, hindering its growth (Attigbe et al., 2019).

Environmental pollutants can alter the gut microbiota of animals, and the gut microbiome can vary significantly across species and types of contaminants. Preliminary studies indicate that accumulation of various heavy metals can affect BSFL development; however, few studies have examined changes in BSFL intestinal microflora resulting from heavy metal accumulation. Therefore, this study aims to investigate the effects of four heavy metals (cadmium, mercury, lead, and arsenic) on BSFL growth, the distribution and accumulation of these metals across the body, exuviae, and feces, and to explore changes in intestinal microflora, particularly focusing on the potential of *Lemnorea grimon-tii* as a microbial indicator of heavy metal exposure.

## Material and methods

### Materials

The BSFL were purchased from a BSFL breeding facility located in Hamyang County, South Korea. Cadmium, arsenic, lead, and mercury used in the experiment were

purchased from Kanto. The chick feed used as BSF food was purchased online.

### Manufacturing process of feed containing heavy metals

The chick feed was ground for 10 min using a grinder and homogenized using a 100-mesh sieve. The homogenized chick feed was prepared by mixing the feed with water at a ratio of 1:2. The concentrations of heavy metals used in the feed (classified as low, medium, and high) were selected based on reported contamination ranges in marine organisms, particularly in fish by-products like mackerel heads, to ensure environmental relevance. This classification reflected levels of contamination reported in previous studies of marine life and fish-derived byproducts (Biancarosa et al., 2018; van der Fels-Klerx et al., 2016). Approximate reference values used were: Cd (0.5–5 mg/kg), Hg (0.4–5 mg/kg), Pb (0.7–10 mg/kg), and As (0.3–9 mg/kg), as reflected in Table 1.

### Rearing environment and methods for BSFL

The larvae were reared in an incubator at 60% humidity and 26 °C for 7 days. To homogenize BSFL, a 100-mesh sieve was used. Each treatment group consisted of three independent rearing containers, each containing approximately 5 g of BSFL. Approximately 15 g of feed was provided every two days, and additional moisture was added to maintain a humidity of 60%. After 7 days, the BSFL was rinsed with distilled water and placed on a 1-day fast. Subsequently, the body, exuviae, and excreta of BSFL were collected for use as experimental samples.

### Treatment of BSFL using the mackerel head

The rearing environment and methods were carried out in the same manner as the Method 2.3. Feed consisted of chick feed and mackerel heads. It was prepared in three concentrations (feed containing 30, 40, and 50% mackerel heads) and fed.

### Heavy metals analysis method

Heavy metal analyses, including sample pretreatment and analysis, were performed by the YS Institute of Environmental Technology in accordance with the Food Code (MFDS Notice No. 2023-28) of Korea. We accurately weighed approximately 0.2 g of the sample, placed it in a microwave container, and added 9 mL of nitric acid. After the container was attached to the microwave, it decomposed until it became colorless or pale yellow, depending on the microwave operating conditions.

**Table 1** Heavy metal contents measured in the body, exuviae, and feces of BSFL

|                   | Body (mg/kg)  | Exuviae (mg/kg) | Feces (mg/kg)  | Total (mg/kg)  |
|-------------------|---------------|-----------------|----------------|----------------|
| Cd (0.5–5 mg/kg)  |               |                 |                |                |
| Low               | 0.806 ± 0.087 | 0.246 ± 0.041   | -0.123 ± 0.078 | 0.929 ± 0.079  |
| Mid               | 4.383 ± 0.334 | 0.842 ± 0.361   | 0.101 ± 0.240  | 5.326 ± 0.858  |
| High              | 5.025 ± 0.396 | 1.002 ± 0.261   | 0.081 ± 0.068  | 6.108 ± 0.633  |
| Pb (0.7–10 mg/kg) |               |                 |                |                |
| Low               | 0.734 ± 0.139 | 5.460 ± 1.623   | 1.613 ± 2.232  | 7.357 ± 3.721  |
| Mid               | 3.382 ± 0.167 | 10.877 ± 1.882  | 4.661 ± 1.256  | 18.921 ± 1.887 |
| High              | 3.892 ± 0.618 | 9.896 ± 0.612   | 7.592 ± 2.263  | 21.380 ± 2.746 |
| Hg (0.4–5 mg/kg)  |               |                 |                |                |
| Low               | 0.057 ± 0.005 | 0.257 ± 0.007   | 0.114 ± 0.012  | 0.428 ± 0.019  |
| Mid               | 0.296 ± 0.052 | 1.432 ± 0.121   | 0.833 ± 0.109  | 2.562 ± 0.229  |
| High              | 0.796 ± 0.081 | 5.015 ± 0.393   | 5.400 ± 0.414  | 11.210 ± 0.750 |
| As (0.3–9 mg/kg)  |               |                 |                |                |
| Low               | 0.311 ± 0.017 | 1.440 ± 0.105   | 0.613 ± 0.084  | 2.364 ± 0.069  |
| Mid               | 2.072 ± 0.090 | 6.250 ± 0.455   | 5.194 ± 0.441  | 13.517 ± 0.894 |
| High              | 1.823 ± 0.202 | 8.309 ± 0.502   | 8.717 ± 0.240  | 18.849 ± 0.794 |

The decomposed sample was cooled to room temperature and then transferred to a 50 mL DigiTUBE (SCP Science). We washed the tertiary-purified water container and lid, added tertiary-purified water to the 50 mL scale, filtered it through a 0.45 µm membrane filter, and used the resulting solution as the sample. Complete pretreatment samples were analyzed by ICP-MS (Agilent 7700x, Agilent Technologies).

### Microbiome analysis

For microbiome analysis, fecal samples were collected from all three replicate containers per treatment group, pooled to obtain sufficient material for DNA extraction, and one composite sample per group was sequenced. Microbiome analysis was performed by Macrogen (Seoul, South Korea). Briefly, DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), quantified using Quant-IT PicoGreen (Invitrogen, Carlsbad, CA), and the V3 and V4 regions were amplified according to the Illumina 16S Metagenomic Sequencing Library protocols. The first PCR included heat activation for 25 cycles, followed by a second PCR for 10 cycles. Sequencing was conducted using a MiSeq platform (Illumina, San Diego, CA, USA) after purification and quantification.

### Diversity analysis

The computation of alpha and beta diversity metrics utilized the q2-diversity module. For alpha diversity, Shannon's diversity index (a qualitative measure of community richness), observed features (a qualitative indicator of community richness), Faith's phylogenetic diversity (a qualitative measure considering the phylogenetic relationships between

features), and Pielou's evenness (a metric for community evenness) were employed. Similarly, beta diversity metrics included Jaccard distance (a qualitative measure of community dissimilarity), Bray–Curtis distance (a quantitative measure of community dissimilarity), unweighted UniFrac distance (a qualitative measure of community dissimilarity incorporating phylogenetic relationships between features), and weighted UniFrac distance (a quantitative measure of community dissimilarity considering phylogenetic relationships between features).

### Linear discriminant analysis effect size analysis

The differential abundance of taxonomic groups was assessed in three comparisons using linear discriminant effect Size analysis. Differential abundance analysis used the Biome file format, focusing on level 7, which represents the species-level collapse table. Following the format adjustment, LEfSe analysis was performed using a cloud-based Galaxy service. Cladograms and bar charts were created to illustrate the LEfSe outcomes for taxonomic groups exhibiting differential abundances.

### Correlation analysis between metal concentration and microbiome

The taxonomic results were collapsed to the species level (7th level) and used to calculate Pearson correlations in R. The correlation between microbiomes and the concentrations of all four studied metals (in food, body, exuviae, and feces) was calculated. The correlation matrix was plotted using the corrplot function in the R software.

## Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and two-way ANOVA using GraphPad Prism 10.2.0 (GraphPad Software, San Diego, CA, USA). Three replicates were performed for each experiment.

## Results and discussion

### Impact of heavy metal (lead, mercury, arsenic, cadmium) concentrations on the growth of BSFL

The growth rate variation of BSFL exposed to various concentrations of four heavy metals (Pb, Hg, As, and Cd) is illustrated in Fig. 1. No significant changes were observed in the growth rate of BSFL fed with feed containing four types of heavy metals (lead, mercury, arsenic, and cadmium) at low, medium, or high concentrations. It was confirmed that the predetermined concentrations of each heavy metal did not inhibit BSFL growth. Overall, BSFL growth across all groups exposed to heavy metals was similar to that in the control group. Studies have reported no significant weight changes or mortality in

BSFL exposed to various concentrations of Cu and Cd during the experimental period (Wu et al., 2020). Similarly, BSFL exhibited high resistance to diets supplemented with a mixture of heavy metals (Cai et al., 2018). These results suggest that BSFL may be resistant to the concentrations of the four heavy metals used in this study over the 7-day exposure period.

### Measurement of heavy metal contents in BSFL body, exuviae, and feces

The content of each heavy metal presented in Fig. 2 and Table 1 represents net accumulation, calculated by subtracting the values measured in the control group. The content of each heavy metal was calculated by subtracting the heavy metal content in the control group. Pb was predominantly detected in the exuviae, with lower concentrations found in the body and feces, suggesting excretion via molting. Mercury levels increased significantly in the body, exuviae, and feces, depending on food concentration, with higher levels in exuviae and feces than in the body. Cd levels increased significantly in the body, exuviae, and feces, depending on the feed concentration, with higher accumulation in the body and minimal detection in the feces. A significant increase was observed in the exuviae and feces with increasing feed concentrations, and higher levels were detected in the exuviae and feces than in the body.

When assessing the accumulation levels of four heavy metals in BSFL fed varying concentrations, it was evident that the concentrations of all heavy metals, excluding As, in larvae significantly increased with elevated metal concentrations, particularly in those fed low concentrations. Due to the incomplete collection of exuviae and feces during the rearing period, some variability in the measured values may have occurred. In particular, the unexpectedly high arsenic concentration observed in the medium exposure group may represent an outlier resulting from this sampling limitation. BSFL exposed to four heavy metals exhibited diverse patterns of biological accumulation. In the body, Cd showed the highest accumulation. This high body Cd accumulation is consistent with previous findings (Biancarosa et al., 2018; Diener et al., 2015; van der Fels-Klerx et al., 2016). In the exuviae, Pb was highly accumulated. Pb was detected at higher levels in the exuviae than in the body, consistent with previous studies indicating that Pb is excreted during the molting process of BSFL. (Diener et al., 2015). As concentrations increased significantly, high levels of As were detected in the exuviae and feces. As was also detected in the body, but at higher levels in the exuviae and feces, consistent with previous studies indicating that As was excreted in the skin and feces (van der Fels-Klerx et al., 2016). Hg tended to accumulate more in the exuviae and feces than in the larvae.

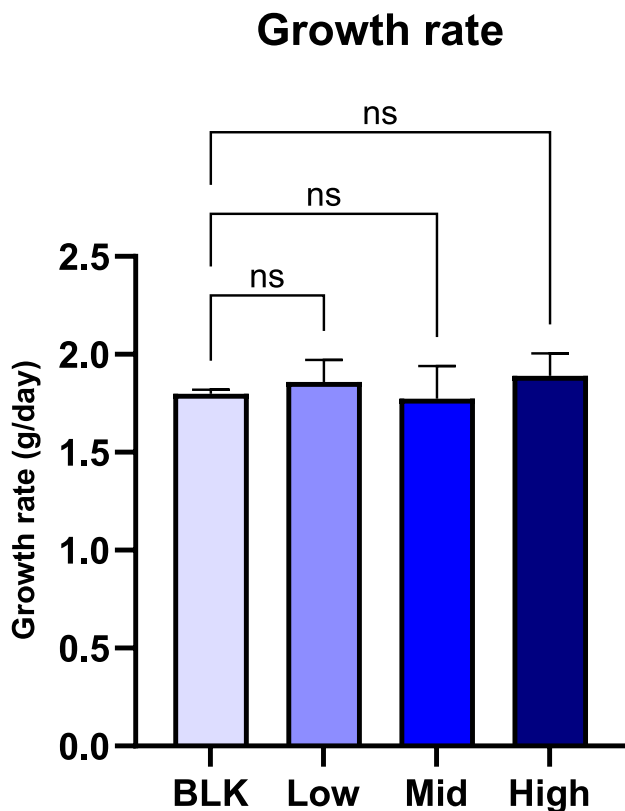


**Fig. 1** Growth rates of BSFL exposed to four heavy metals at different concentrations

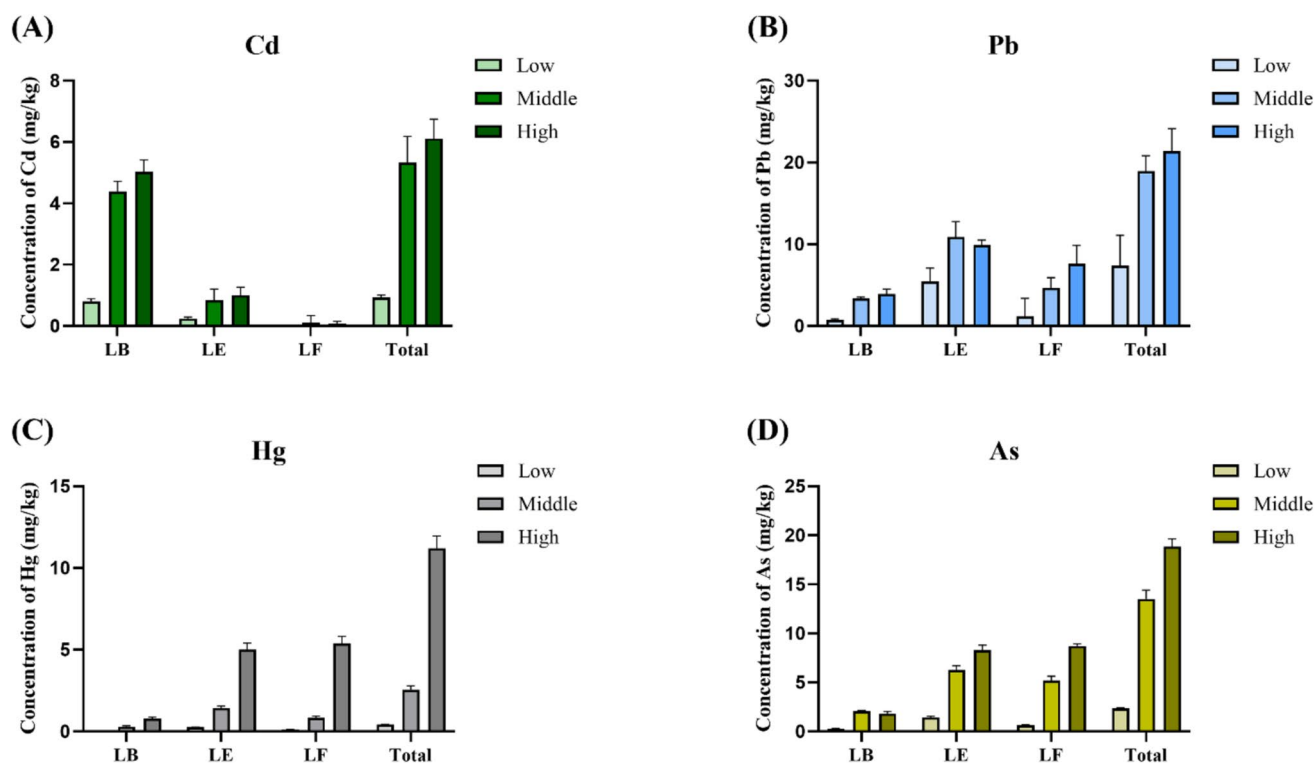


**Fig. 2** Measurement of four heavy metal contents in BSFL, specifically in the body (LB), exuviae (LE), and feces (LF). **A** Cadmium, **B** lead **C** mercury, **D** arsenic. LB, LE, LF denote the larval body, exuviae, and feces, respectively. L, M, and H indicate the low, medium, and high concentrations of each respective heavy metal

These results provide insights into distinct patterns of heavy metal accumulation across different components of BSFL, highlighting concentration-dependent effects.

### Changes in fecal arsenic and mercury content according to changes in mackerel head content

It was confirmed that mackerel heads contained a large amount of mercury and arsenic, as shown in Fig. 3. As the content of mackerel heads increased, the content of mercury and arsenic in the feces increased. The content of BSFL and

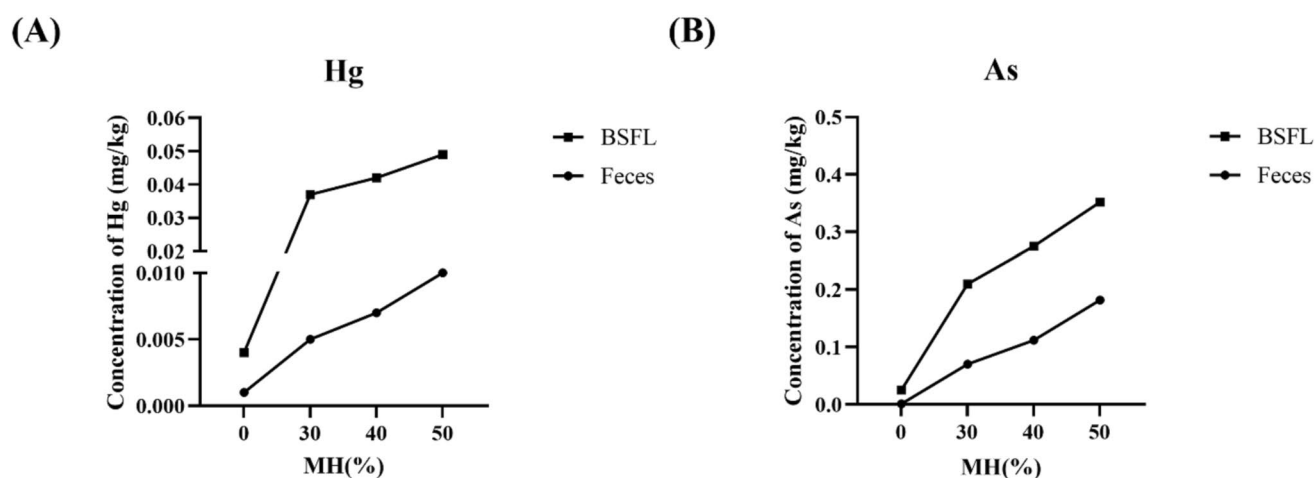


**Fig. 3** Variation in mercury (A) and arsenic (B) contents in the body, exuviae, and feces of BSFL according to mackerel head contents

feces increased according to the content of mackerel heads, which was similar to the overall increase in mercury and arsenic content, as shown in Fig. 2.

Hg and As, known for their toxicity, are significant contaminants among heavy metals that pose a threat to aquatic environments. Hg and As accumulate through the gills and skin after exposure, affecting humans through the food chain (Kim and Kang, 2015; Kumari et al., 2017; Tolkou et al., 2023). Therefore, monitoring was conducted using mackerel heads to investigate the effects of heavy metal content on marine organisms. This study confirmed that the amounts of Hg and As excreted in the feces of BSFL increased as the mackerel head content in the feed increased. As shown in Fig. 2, Hg and As were mainly detected in the exuviae and feces of BSFL, indicating that a significant amount of Hg and As could be detected in the exuviae and feces of BSFL that had ingested feed with high mackerel head content.

It is important to note that this study measured total arsenic content and did not distinguish between inorganic and organic species. As inorganic arsenic is more toxic than its organic counterparts, future studies should consider arsenic speciation. Previous research has reported that a substantial proportion of arsenic accumulated in BSFL exposed to contaminated substrates exists in the inorganic form, although the ratio can vary depending on the source and form of arsenic in the feed (Biancarosa et al., 2018; van der Fels-Klerx et al., 2016).

### Analysis of alpha and beta diversity in the gut microbiome

Alpha diversity was assessed using Faith's Phylogenetic Diversity and Pielou's Evenness. Faith's PD was significantly higher in the low metal concentration group compared to both the control ( $p=0.027$ ) and high concentration groups ( $p=0.041$ ), indicating greater phylogenetic richness at lower exposure. No significant differences in Pielou's Evenness were observed between the groups. The lowest alpha diversity was observed in the control group. Significant differences in alpha diversity were observed only in comparisons between the control and low, control and medium, and high and low groups. There was no significant difference in alpha diversity evenness between the groups in this study.

For beta diversity, samples from different groups were clustered at different locations on the 3D graph (Supplement Fig. 1). The control group was clustered near the central axis, whereas the low-metal concentration group was clustered in the upper-right quadrant. The medium metal concentration group was clustered near axis 1 on the right side of the graph, while the high metal concentration group was clustered on the right side of the medium metal concentration group (Supplement Fig. 1 B). There was a significant difference in the beta diversity between the control and

metal-exposed groups. However, no significant difference was observed between individual group comparisons.

A significant change in alpha diversity was observed between the control and the metal-exposed BSFL, suggesting a significant impact of metal exposure on the gut microbiome of BSFL, as reported in the literature, which indicates that toxic metal exposure may impact the gut microbiome (Assefa and Kohler, 2020). Similarly, a significant change in beta diversity was observed between the control and metal-exposed BSFL. However, changes in beta diversity were not significant between the individual groups, which may be owing to the small number of samples used in the study. It is suggested that more samples from the control and metal-exposed groups be studied in the future.

### Changes in microbiota taxonomic composition according to heavy metal exposure

The classification results involved generating bar plots and Krona plots to compare significant taxa in the gut microbiome of the different experimental groups. Through metagenomic analysis, the differential abundance of the gut microbiome was studied using Linear Discriminant Analysis Effect Size (LEfSe) at the species level, which revealed that *Dysgonomonas gadei*, *Corynebacterium simulans*, *Lactococcus garviae*, and *Milissococcus plutonius* were abundant in the low-concentration metal group, and *Leminorella grimontii* was abundant in the high-concentration metal group (Fig. 4).

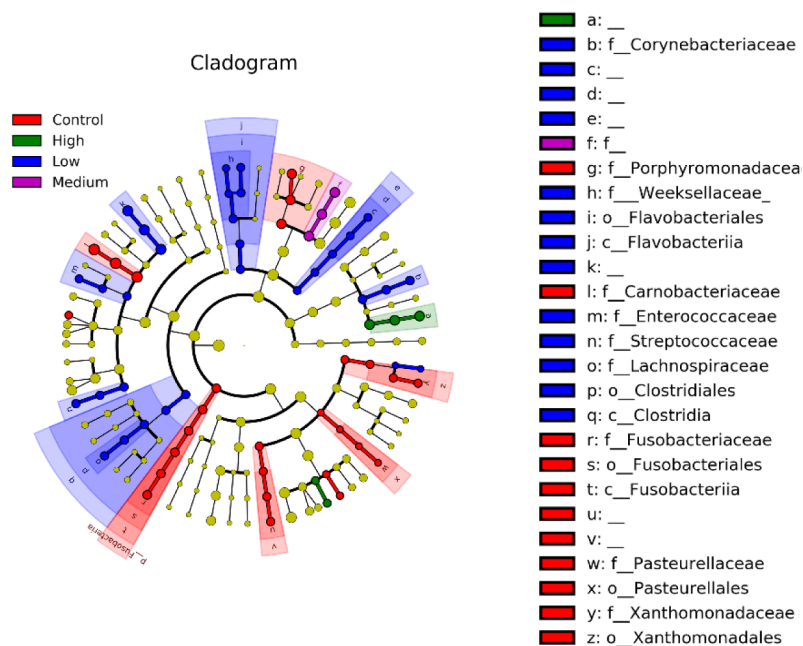
Microbiome analysis of BSFL feces confirmed a strong association between *Leminorella grimontii* and elevated levels of mercury (Hg) and arsenic (As), with the bacterium being more abundant under high metal exposure conditions. *L. grimontii* is known to produce hydrogen sulfide ( $H_2S$ ), a gas with a characteristic odor (Hickman-Brenner et al., 1985), which has been reported to mediate various abiotic stress responses and may act as an antioxidant by reducing reactive oxygen species (ROS) and maintaining low  $H_2O_2$  levels (Chen et al., 2017). These characteristics suggest that *L. grimontii* could be involved in microbial responses to heavy metal stress. However, its precise role in mediating heavy metal tolerance in BSFL remains speculative and requires further functional validation.

In contrast, other bacterial species such as *Dysgonomonas gadei*, *Corynebacterium simulans*, *Lactococcus garviae*, and *Milissococcus plutonius* were enriched in the low-concentration metal groups, suggesting that these taxa may be more sensitive to metal exposure.

### Correlation analysis between heavy metals and microbiome

Correlation analysis revealed that *Leminorella grimontii* abundance was strongly and positively correlated with the

(A)



(B)

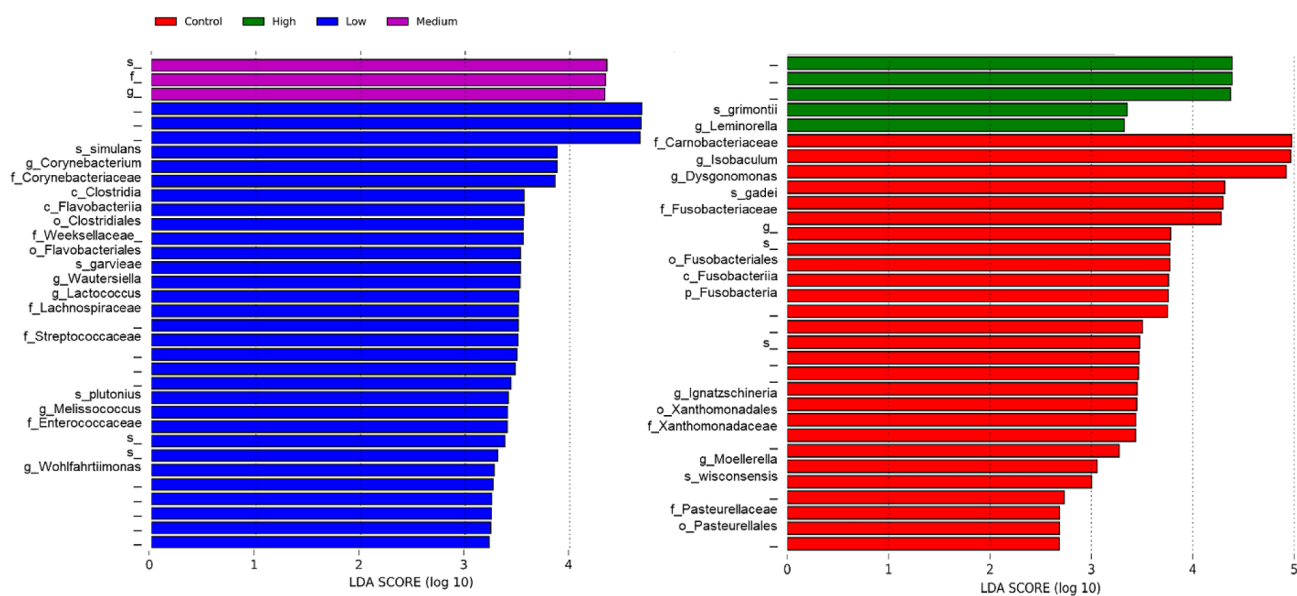
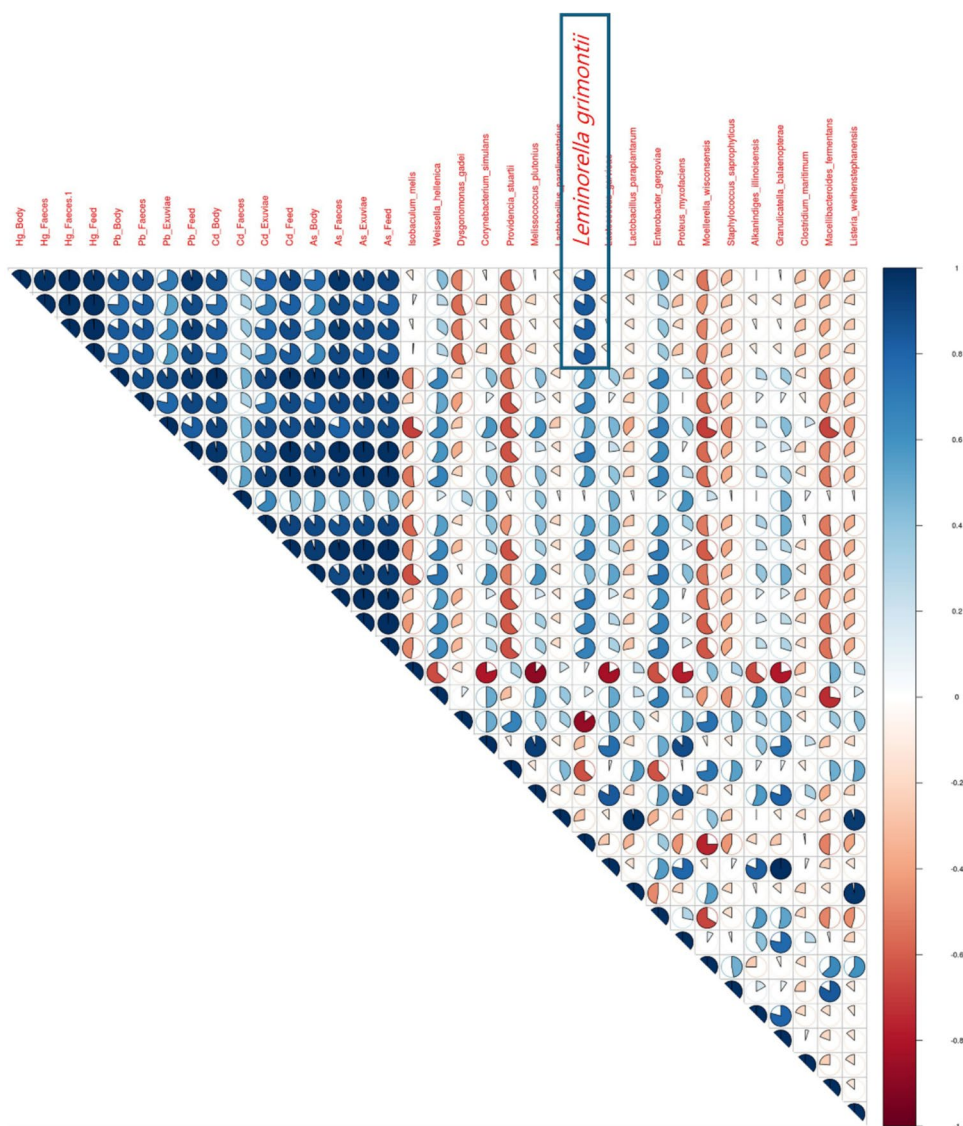


**Fig. 4** Differential abundance of taxonomic groups across levels of heavy metal exposure. **A** Cladogram, **B** bar plot illustrating bacterial taxonomic groups according to LDA. Groups based on Low (PL) and High (PH) heavy metal intake

**Fig. 5** Correlation between metal concentrations and microbiomes



concentrations of all four tested heavy metals, particularly mercury (Hg) and arsenic (As). While these associations suggest a potential involvement of *L. grimontii* in microbial responses to heavy metal exposure, they do not confirm a causal role. Further experimental validation is required to determine whether *L. grimontii* actively contributes to metal tolerance mechanisms in BSFL. In contrast, negative correlations were observed for *Moellerella wisconsensis*, *Providencia stuartii*, and *Dysgonomonas gadei*, whereas *Enterobacter gergoviae* showed positive correlations with all metals (Fig. 5). These species may also be involved in metal sensitivity or tolerance and warrant further investigation.

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## Declarations

**Conflict interest** The authors declare that they have no conflict of interest.

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