Research Paper

Investigating safety aspects of using insect farming to reduce pig and chicken wastes at semi-commercial and lab-scale

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ABSTRACT

Increasing global demand for food is driving the need to reduce wastes produced by agriculture to minimise environmental impacts. Black Soldier Fly, *Hermetia illucens*, can reduce livestock wastes, but research into the safety and scalability of the system is required. Insect bioconversion concerns include potential bioaccumulation of pathogens, antimicrobial resistance genes and heavy metals in larvae and substrates. Here, a semi-commercial-sized insect rearing facility was used to rear larvae on pig slurry, alongside a lab-based experiment using chicken manure. Larval microbiome composition was impacted by substrate, with increased Clostridia in larvae reared on slurry and manure. Pathogens largely decreased in the larvae from starting levels. Both slurry and manure substrates showed time-related changes regardless of insect presence or absence except for *E. coli* in chicken manure which was reduced in substrates with larvae added (−2.840 LFC vs −1.168 LFC; p<0.05), suggesting that time-associated alterations in the substrate could be more significant than larval presence. Antimicrobial resistance gene changes were dependent on the substrate and gene, with increases found for tetM in chicken manure after larval bioconversion (9.000 vs 10.370 LFC; p<0.001), and for sul2 in larvae reared on chicken manure (3.509 vs −0.985 LFC; p=0.001). In pig slurry-reared larvae, tetM decreased (−1.578 LFC; p<0.001) but there was no difference in sul2. Heavy metal contents generally met permissible standards for animal feed and organic fertilizers. However, there was some non-significant evidence for bioaccumulation of cadmium in slurry-reared larvae 0.18 to 0.70 mg/kg) compared to starter larvae (0.25mg/kg) requiring further study.

1. Introduction

The global population is expected to rise to over 9 billion people by 2050 (United Nations). The resultant increase in food production in the agricultural sector invariably will lead to an augmentation in waste production. Approximately 140.1 m tonnes of manure was produced in the UK between 2016 and 2019 (Köninger et al., 2021). Of this, around 6 million tonnes are produced annually from pig farms, and 4.6 million from poultry farms, with the majority coming from cattle farming (Smith and Williams, 2016). Much of the manure produced on UK farms is spread on agricultural land (Hutchison et al., 2005; Köninger et al., 2021), though, it can also be used in anaerobic digestion to produce biogas (Nasir et al., 2012). Agricultural waste can have significant environmental and economic costs (Skinner et al., 1997; Nagendran, 2011), including water pollution from runoff and leaching, as well as contamination of soils (Ogbuewu et al., 2012). These wastes can contain pathogenic bacteria (Hutchison et al., 2005) and heavy metals

(Nicholson et al., 2006), as well as other contaminants such as microplastics (Sheriff et al., 2023). However, these wastes have potential for use in circular agricultural systems (Rehman et al., 2022).

Insect farming is increasingly proposed as a viable prospect for reducing livestock wastes (Pastor et al., 2015; Fu et al., 2025). In particular, the Black Soldier Fly, *Hermetia illucens*, has been identified as a suitable species (Sheppard et al., 1994). A generalist feeder, the larvae (BSFL) can be reared on a variety of wastes, including pig (Parodi et al., 2021) and chicken (Oonincx et al., 2015) manure. Though differences vary between studies (Oonincx et al., 2015), BSFL have been shown to efficiently reduce animal wastes, decreasing pig manure by up to 56% (Newton et al., 2005). Larval growth is also impacted by the type of manure (Oonincx et al., 2015), associated with levels of nutrients and fibre (Gold et al., 2020). Some studies have reported increased yields and conversion performances for larvae reared on pig manure compared to chicken manure (Boafo et al., 2023; Diola et al., 2024), whilst others found an increase on chicken manure (Li et al., 2011; Xiao et al., 2020;

Wu et al., 2024).

Larval frass (composed of larval excretions and remaining substrates) has potential as a fertilizer (Lomonaco et al., 2024). Frass contains minerals and microbes that could increase plant growth (Khayrova et al., 2019; Lopes et al., 2022; Tanga et al., 2022). However, frass composition has been shown to vary depending on substrate (Lopes et al., 2022), and the presence of contaminants could carryover or even bioaccumulate in the frass (Addeo et al., 2024; Kawasaki et al., 2020). As such, further processing may also be required before frass can be used as a fertilizer. BSFL could provide a protein source for livestock, reducing the current reliance on soya products (Van Huis and Gasco, 2023) due to their high protein and amino acid content (Cullere et al., 2016; Tschirner and Simon, 2015). Studies have found partial inclusion of BSFL in diets of different species show similar or improved performance compared to classic feeds, though there are often reductions at higher levels of supplementation (Barragan-Fonseca et al., 2017).

Despite these potential benefits, questions remain over the safety of outputs (Hoek-van den Hil et al., 2023). Animal wastes can contain pathogenic bacteria, such as *E. coli* (Fernández-Labrada et al., 2023), and pathogens such as *Salmonella* spp. have been isolated from larvae reared on manure (Hoek-van den Hil et al., 2023). Accumulation of pathogenic bacteria in the larvae could enter the food chain through introduction into livestock feed or via contaminated frass addition to crop soils. However, pathogen suppression could also occur (Shelomi, 2024), via production of antimicrobial peptides, competition from the larval microbiota (Erickson et al., 2004; Elhag et al., 2022), or passage through the acidic larval midgut (Bruno et al., 2019). Another major concern is antimicrobial resistance. Antibiotics are used to treat and control infections in livestock (Marshall and Levy, 2011), however, high usage selects for antimicrobial resistance (Bava et al., 2024). Pigs have been found to excrete up to 75% of tetracycline antibiotics (Feinman and Matheson, 1978), 96% of sulfadiazine given to pigs can subsequently be found in the manure (Heuer et al., 2008), and chicken manure application has been shown to increase levels of antibiotics in soil (Xu et al., 2022). Larvae could also bioaccumulate antibiotic resistance genes (Bohm et al., 2024). Heavy metals can result in a number of health problems (Sall et al., 2020), and animal waste may contain high levels (Provolo et al., 2018). Studies have reported accumulation of heavy metals in BSFL, particularly cadmium (Diener et al., 2015; Biancarosa et al., 2018; Hoek-van den Hil et al., 2023). Further complications may arise from larval excretion of heavy metals into the frass (Jiang et al., 2022).

Understanding how larval bioconversion of animal wastes influences these hazards is critical before they can be fully integrated into agricultural systems (Lievens et al., 2021; McDowall and McDermott, 2025), and more research is needed on commercial scales (Grassauer et al., 2023; Biteau et al., 2024). In this pilot study, BSFL were used to reduce pig slurry in a semi-commercial-sized insect rearing unit or chicken manure in a lab-scale assay. Microbiome composition, pathogenic bacteria, antimicrobial resistance genes, and heavy metal content of the larvae and the substrates before and after bioconversion were investigated.

2. Material and Methods

2.1. Experiment 1: Pig Slurry

Experiment 1 compared larvae reared on pig slurry with those fed a chicken feed control diet (Chickcrumbs) and was performed at the University of Leeds National Pig Centre (NPC, Leeds, UK) using the bespoke EntoExplore unit designed by Entocycle (London, UK) which allows BSFL to be reared at scale, under semi-commercial conditions.

BSFL were obtained from Beta Bugs Ltd (Midlothian, UK) at 5 days old and shipped overnight to the NPC. Larvae were pooled and briefly sieved to remove excess feedstuff before weighing. Approximately 175 g of larvae were weighed and added to the centre of each tray (40x60x20 cm; ~4,900 larvae). The bioreactor environmental conditions were set to 27°C and 85% humidity and maintained through a heating, ventilation, and air conditioning (HVAC) monitoring system custom built by Entocycle sitting in a separate module ontop of the insect rearing unit. Control was achieved using an automated reverse flow airflow system flowing through the heating and humidifier elements sitting above the bioreactor. The air extraction and ventilation system had a maximum capacity of 1400 m^3h^{-1}, blowing air through the sides and changing direction in forward and reverse mode to help maintain homogeneity. Air was drawn into the HVAC and back into the bioconversion space at either side of the tray racking. A total of 16 sensors monitored temperature, humidity, CO_2, NH_3 and H_2S (WinCC Unified Comfort v18; Siemens, Germany). The set up can be seen in Fig. 1.

The control feed used was chicken feed (Chickcrumbs), a commonly used feedstock for Black Soldier Fly larvae (Hoek-van den Hil et al., 2023; Nguyen et al., 2015; Oonincx et al., 2015). Chickcrumbs were made up by mixing 75 % water, 24 % chickcrumb (Dodson & Horrell Chick Crumbs; nutritional composition: 19 % Protein, 3.3 % Oil, 3.3 % Fibre, 5.1 % Ash (Dodson & Horrell)) and 1 % caster sugar. Pig slurry from the growing herd at the NPC was transferred into the mixing tank (pH 8.19). Substrates were dosed into trays the day before larval addition to allow them to come up to temperature. Trays contained 6kg of substrate (Chickcrumbs or pig slurry) and replicates (n=6) were haphazardly allocated to each position to minimise environmental variation, whilst ensuring each was represented on the different levels (Fig. 1A). Trays were included that received slurry but no larvae ('Slurry Only') as a control to determine changes to the substrate over time. Larvae were left to feed for 20 days. Monitoring during the growth phase was limited due to the nature of the container, i.e., to enter the rearing compartment, doors must be opened to allow air flow to remove any noxious gasses from the digestion of slurry, lowering the temperature and humidity. Larvae were checked in person 2-3 times during each experimental run and temperature and humidity remotely monitored throughout the experimental period. Larvae were undisturbed as much as possible to minimise fluctuations in environmental parameters. Following bioconversion, samples were separated using an automatic separator or by hand. Samples of larvae and the substrate (or frass) were collected at the start and at harvesting for DNA extractions and frozen at −80 °C or pooled in 1 litre bottles for heavy metal analysis (~400 g, -20 °C). The experiment was independently replicated 3 times.

2.2. Experiment 2: Chicken Manure

Experiment 2 compared larvae reared on chicken manure with those fed a Chickcrumbs control diet. This experiment was performed at laboratory scale. Chicken manure could not be used within the insect bioreactor due to biosecurity restrictions at the NPC.

BSFL larvae were handled as for Experiment 1. Larvae (10 g) were weighed and added to each tub (16x14x10 cm). The lid of the tub was modified to include mesh allowing air flow but preventing escape. The incubator (Sanyo, MIR-553) was set to 27 °C. Chickcrumbs were mixed as previously described. Chicken manure was sourced fresh the day of larval arrival from a local chicken farm (pH 8.87). Due to the dryness of the substrate, 750ml water was mixed into 340 g of the manure. Experimental tubs of each type (n=6) were set-up per run, including chicken manure without larvae ('Manure Only'). Each shelf contained 2 of each sample type, with the location of each haphazardly changed between replicates. Samples were collected as above. Larvae were frequently monitored, and Chickcrumbs-fed larvae were harvested at day 13, when larvae were beginning to pupate. At this stage, larvae stop crawling and turn dark in colour. Chicken manure larvae were harvested at day 19 when larvae had also reached the pre-pupal stage. Unlike with the insect rearing unit, monitoring was much easier in the laboratory, allowing for more specific timing of harvesting. As this assay was also carried out after the pig slurry assay, this provided a timescale foundation that could be used in future experiments. A total of 3 independent
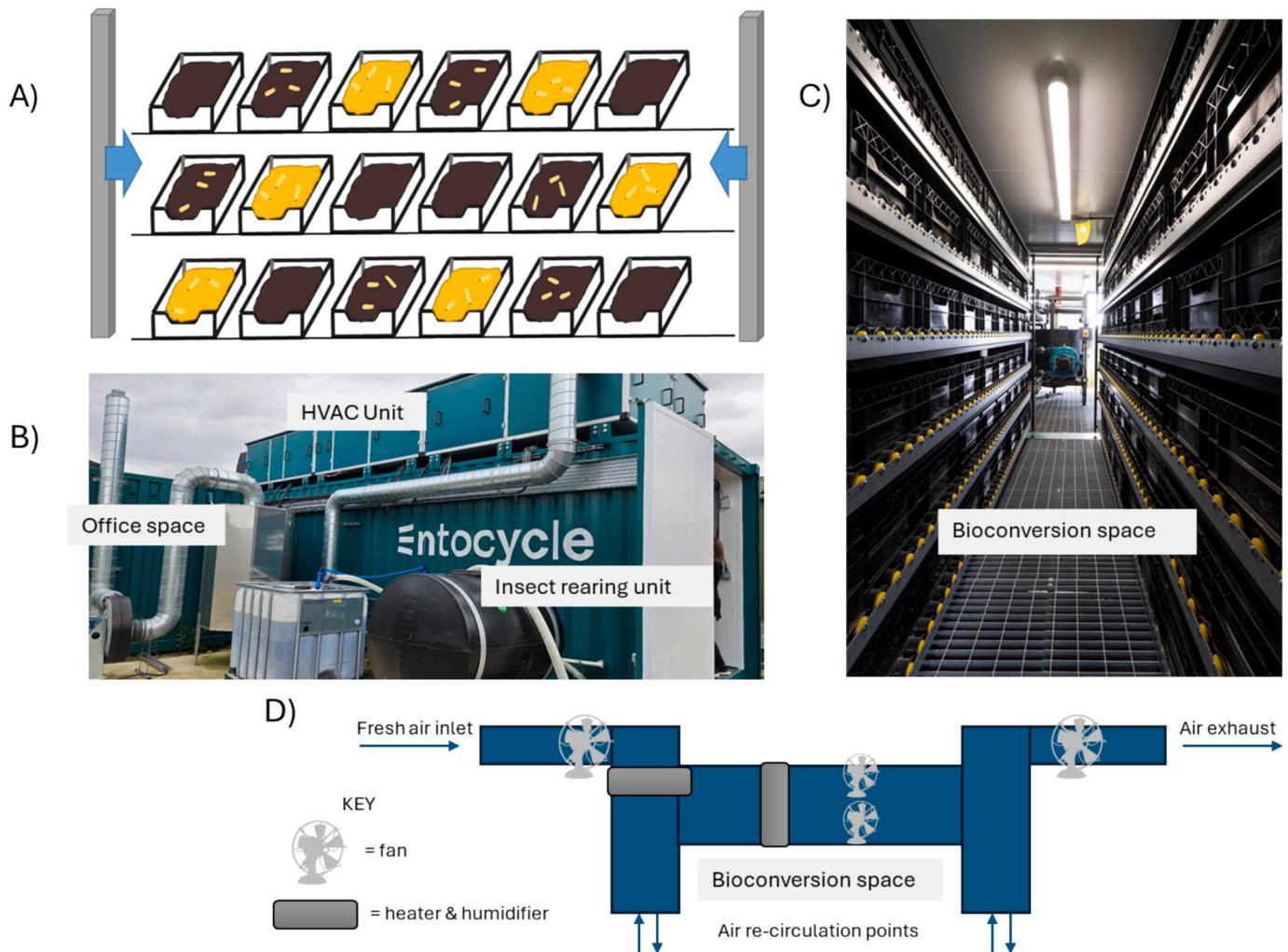
Fig. 1. Set-up of the Insect Rearing Unit A) showing an example of the placement of substrate types across feeding trays. Yellow = chickcrumb, dark brown = pig slurry. Trays are shown either with or without black solider fly larvae (not to scale). Trays without larvae were included as substrate only controls. N=6 per substrate type. Blue arrows indicate the movement of air across the trays either in forward or reverse mode (maximum capacity of 1400 m3 h-1). B) Photo of the semi-commercial EntoExplore unit showing the insect rearing unit (containing a pre-processing area for dosing trays, a bioconversion space for insect rearing and a post-processing space for harvesting of insects) and the heating, ventilation and air conditioning module (HVAC unit) a separate container with office space is also visible. C) Photo of the bioconversion space showing the tray racking which contains insect larvae and slurry. D) A simplified schematic (not to scale) of the HVAC air conditioning process. The HVAC contains a series of 16 sensors to monitor temperature, humidity and gasses including CO_2, NH_3 and H_2S. Air is drawn in and out of the HVAC at either side of the tray racking and is directed across the rearing trays. Direction of airflow switched between a forward and reverse mode to help maintain homogeneity.

runs were carried out.

2.3. General Analysis Methods

DNA extractions were performed using the DNeasy PowerSoil Pro Kits (Qiagen, Germany) according to the manufacturer's instructions. For DNA extraction of frass, 300 mg was weighed into a 2 ml Eppendorf tube. Due to the dryness of the samples and the volume of particles, all frass samples, slurry only or manure only samples had 500 µl of sterile, RNAse/DNase free water added. From this and for all other samples, 270mg of sample was added to a Powerbead Tube and extractions were carried out following the manufacturer's protocol, including 10 minutes of homogenising (TissueLyser LT, Qiagen) at maximum speed. Yields were quantified using a Nanodrop 2000. For larval samples, 10 larvae were added to a 5ml Eppendorf tube. Larvae were then rinsed with 70 % ethanol followed by a further 2 rinses with distilled water to sterilise their outer surface (Vitenberg and Opatovsky, 2022; De Filippis et al., 2023) and homogenised manually with a metal spatula to break the cuticle, followed by automatic homogenisation (Art-Miccra D-8; 23,500

min^{-1}). Equipment was cleaned with 70 % ethanol and distilled water between samples. Homogenised larvae (300 mg) were then transferred into the Powerbead tubes and DNA extracted following the manufacturer's protocol.

For microbiome analyses, DNA was sent to Novogene for sequencing of the V4 region using the Illumina NovaSeq platform and paired end 250bp strategy. 3 samples per replicate run were submitted for sequencing (n=9). Forward and reverse sequences were aligned with the SILVA SEED v138.2 database using mothur and following the mothur SOP (https://mothur.org/wiki/miseq_sop/). Analysis was then carried out in R using phyloseq v1.42.0, vegan v2.6-4, ggplot2 v3.5.1, ANCOMBC v2.0.3 and FSA v0.9.5 packages. Alpha Diversity was measured using the Chao1 index and beta diversity with PERMANOVA. ANCOMBC analysis was used to compare log fold changes to a reference group. For larvae, starter larvae were used as the reference group, and for substrates, this was set as the starting Chickcrumbs.

Antimicrobial resistance genes tetM and sul2 were chosen as they confer resistance to tetracyclines and sulphonamides respectively, both used at the NPC (Aminov et al., 2001; Wang et al., 2014). Common

human food pathogens were also selected, taking into consideration the possibility of using the insects in animal feed (and therefore entering the food chain) or using the frass as a fertilizer. These included spore-forming bacteria such as *Bacillus cereus* and *Clostridium perfringens,* that produce toxins and are difficult to eliminate (Carroll et al., 2019), as well as common pathogens found in livestock, such as *Escherichia coli* (Begum et al., 2014). Primers (Supplementary Table 1) were tested via PCR using GoTaq Green Master Mix (10 μM). Annealing temperatures were checked on a 16 °C gradient (Techne TC-512), with a middle temperature of 58 °C. PCR conditions were 3 mins at 94°C (30s at 94°C, 30s at annealing, 30 s at 72 °C) x30, 7 min at 72 °C, and hold at 4 °C. Samples were then checked on a 1 % agarose gel (80 V, 40 minutes). Primers were tested for qPCR efficiency (BioRad CFX96 Connect qPCR machine; 300 nM using 5-6 10x serial dilutions), with a starting concentration of 2 ng/μl, SYBR Green and the selected annealing temperature from the gradient PCR. Plates were briefly spun down in a plate centrifuge followed by shaking in a plate shaker at 450 rpm for 45 seconds. The conditions for qPCR were 95°C for 3 mins, followed by 95 °C for 10 seconds and the appropriate annealing temperature for 30 seconds for 40 cycles, then a ramp from 65°C to 95°C in 0.5 °C increments. Primers were accepted with an efficiency of 90-100% and $R^2 \geq 0.98$, except for *Salmonella enterica* primers (efficiency = 86.3 %) and *E. coli* (85.6 %), as these were the best of the multiple primer pairs tested. Substrate and larvae samples were run on separate plates. Each sample was run in triplicate and accepted if the Cq values of at least two of these were within 0.5. qPCR data was analysed via the Pfaffl method (Pfaffl, 2001), with the average of the Chickcrumbs samples as the reference for the substrates and starter larvae as the reference for the larvae plates, to give relative quantities (RQ). Values were normalised to the RQ values from the 16S primers and logged to give the relative log fold change. The data was tested for normality and analysed via T-tests or ANOVA (for normally distributed data) or Mann Whitney U or Kruskal-Wallis tests (for non-normal data). *Post hoc* Dunn's Tests with Benjamini-Hochberg corrections were carried out where appropriate.

Samples for heavy metals were pooled from all trays of the same sample type per replicate, and stored at −20 °C. Larvae samples were briefly rinsed with water and sieved, with a final rinse with ddH₂O to remove excess substrate, then patted dry before returning to the freezer. Samples were shipped to NRM (Cawood Scientific) for analyses. The data was analysed using Kruskal-Wallis tests, and Dunn's tests with Benjamini-Hochberg corrections for *post-hoc* analyses.

3. Results

3.1. Experiment 1: microbiome composition in pig slurry assay

For pig slurry, there was an overall significant difference in Chao1 alpha diversity measures across all sample types (KW=65.137; df=7; p<0.001) (Fig. 2a). *Post-hoc* analyses find a number of significant differences (Supplementary Table 2), including an increase for Chickcrumbs Frass compared to Chickcrumbs (p=0.019) and an increase in Slurry Larvae compared to Chickcrumbs Larvae (p=0.049). Slurry was not significantly different from Slurry Frass (p=0.276) or Slurry Only (p=0.239). There was a significant difference in beta diversity (PERMANOVA: F=20.781; df=7,71; p<0.001) (Fig. 2b), with larvae reared on slurry more similar to the slurry substrates than to the larvae reared on Chickcrumbs. Relative abundances also differed between sample types (Fig.2c). ANCOMBC analysis revealed there were more significant differences between the starter larvae and the Slurry Larvae than with Chickcrumbs Larvae (Fig.2d). There was a greater abundance of Clostridia in both Slurry and Chickcrumbs Larvae compared to the five-day old starter larvae (p<0.001), and decreased Alphaproteobacteria in Chickcrumbs Larvae (p=0.002), with decreased Bacilli and Gammaproteobacteria in Slurry Larvae (p<0.001)

There were a number of significant differences between the three slurry groups (slurry at the start 'Slurry', slurry at the end of the experiment 'Slurry Frass' and samples from trays at the end that contained only slurry and no insects 'Slurry Only') and Chickcrumbs (Fig. 2e). Bacilli were reduced in all slurry samples compared to Chickcrumbs (p<0.001). Increases in Clostridia were found in all groups, though only significantly so in the slurry samples (p<0.001). Gammaproteobacteria was significantly reduced in the slurry groups (p<0.05). Actinobacteria was increased in all groups compared to Chickcrumbs, including Chickcrumbs Frass (p<0.001), and Alphaproteobacteria was significantly increased in the Slurry Frass (p<0.05) and Slurry Only (p<0.001) samples, but not in Slurry (p=0.992).

3.2. qPCR of antimicrobial resistance genes in pig slurry assay

There was a significant difference in the Log Fold Change (LFC) of tetM in Chickcrumbs Larvae compared to Slurry Larvae (MWU=288; N=36; p<0.001), with a reduction observed for those reared on slurry (-1.578 vs 0.173 LFC) (Fig.3a). There was also a significant difference in the LFC of sul2 (MWU=77; n=18; p=0.006), however, in this case, Chickcrumbs Larvae had lower relative LFC than the Slurry Larvae (-2.103 vs -0.007 LFC) (Fig. 3b). For substrates, there was a significant overall difference in LFC values for tetM (KW=12.471; df=3; p=0.006), however, *post-hoc* analyses show the only significant difference occurred between Chickcrumbs Frass and Slurry (p=0.003) (Fig. 3c), though there was also a large amount of variation. There was no difference in the level of sul2 between substrates (KW=4.371; df=3; p=0.224) (Fig. 3d). Both tetM and sul2 levels were generally higher relative to the Chickcrumbs for all substrates. Overall, for slurry groups, larval bioconversion did not appear to have an effect on the levels of these AMR genes.

3.3. qPCR of Pathogenic Bacteria in Pig Slurry Assay

For pathogenic bacteria, *Salmonella enterica* and *Campylobacter jejuni* were not detectable. Both types of larvae (pig slurry and Chickcrumbs) had lower levels of *Bacillus cereus* and *E.coli* relative to the five day old starter larvae. There was no significant difference found between larvae types for *Bacillus cereus* at the end of the assay (MWU=185; N=36; p=0.481) (Fig.4a). Chickcrumbs larvae had greater median levels of *Clostridium perfringens* than Slurry Larvae (2.101 vs -0.271 LFC; MWU=249; N=36; p=0.001) (Fig. 4b). *E. coli* levels were significantly lower for Slurry Larvae compared to Chickcrumbs Larvae (−2.428 vs −1.518 LFC; t=2.573; df=33.682; p=0.015) (Fig. 4c). For substrates, samples generally had decreased levels for *Bacillus cereus* and *E.coli*, but increased *Clostridium perfringens* relative to the Chickcrumbs at the start. There was a significant difference in log fold change for *B. cereus* across samples (F=19.41; df=3,64; p<0.001) (Fig. 4d). Pairwise comparisons demonstrated significantly reduced LFC for Chickcrumbs Frass compared to all other groups (p<0.001), and a significant reduction in Slurry Frass compared to Slurry (−6.023 vs −2.232 LFC; p=0.002), but not for Slurry and Slurry Only (p=1.000) or Slurry Frass and Slurry Only (p=0.077). LFC was significantly different for *C. perfringens* across substrates (KW=48.703; df=3; p<0.001), with *post-hoc* analysis showing differences across all groups (p<0.05), except for Slurry and Slurry Only (p=0.262), and Slurry Frass and Slurry Only samples (p=0.175) (Fig. 4e). For *E. coli*, there was a significant overall difference (KW=9.878; df=3; p=0.020), however *post-hoc* analysis showed the only significant differences occurred between Chickcrumbs Frass and Slurry (p=0.038) and Chickcrumbs Frass and Slurry Only samples (p=0.023) (Fig. 4f).

3.4. Heavy Metal and Nutrient Content in Pig Slurry Assay

Heavy metals and nutrients were measured in pooled samples (Table 1). There were no significant differences for larvae in any of the constituents where values were determined (KW p>0.05) (Supplementary Table 3). There were significant differences between
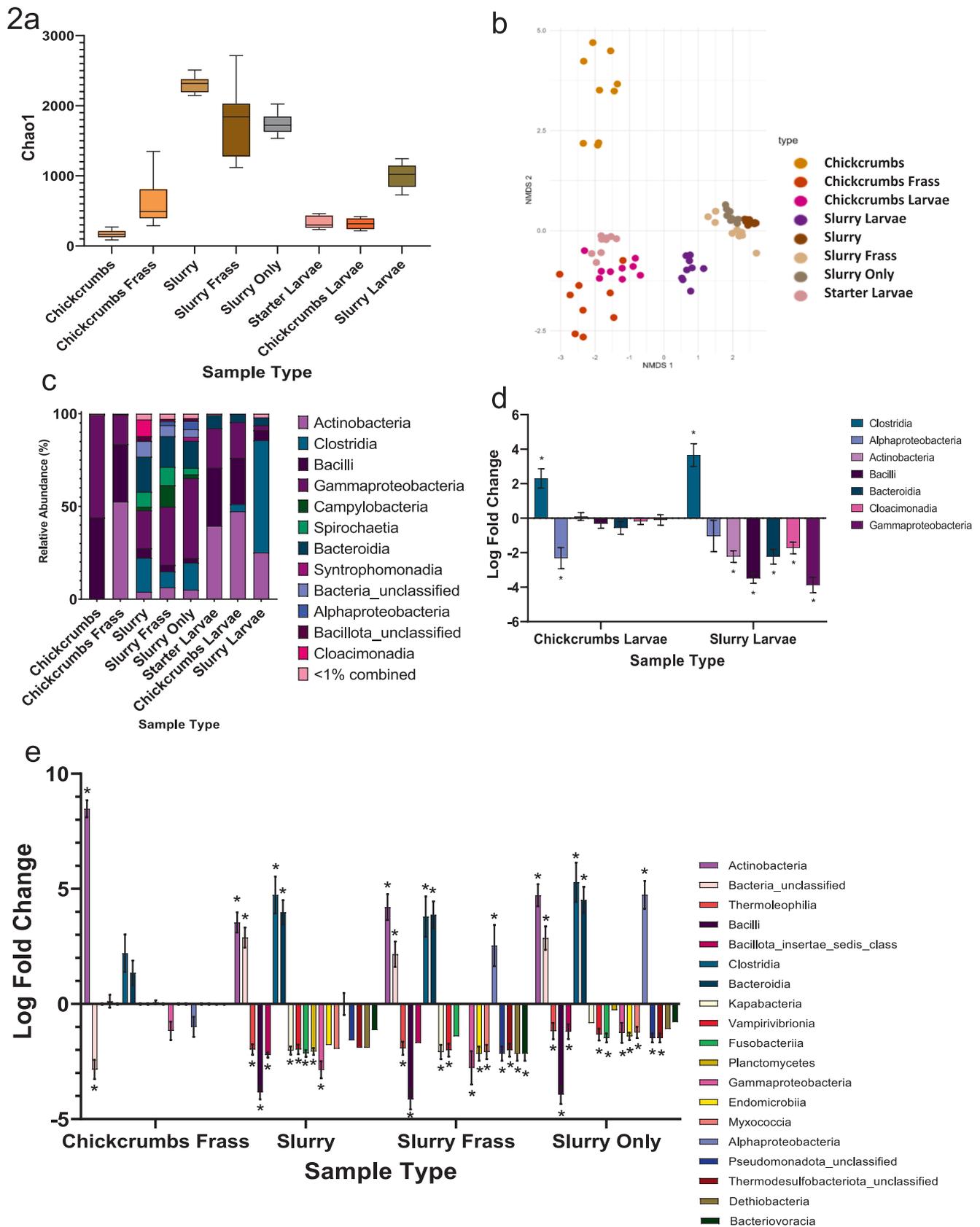
Fig. 2. Alpha and Beta Diversity, Relative Bacterial Class Abundance and Log Fold Changes for larvae and substrates. Alpha Diversity using Chao1 index (a) and Beta Diversity NMDS plot (b). Relative abundance of bacterial classes, combining all classes with <1% abundance (c). ANCOMBC Log Fold Change for larvae reared on Chickcrumbs and pig slurry relative to the 5 day old starter larvae ± S.E.M. (d). ANCOMBC Log Fold Change for substrates relative to starting Chickcrumbs ± S.E.M. (e). Only classes that were significant in at least one sample type are shown. * p<0.05.
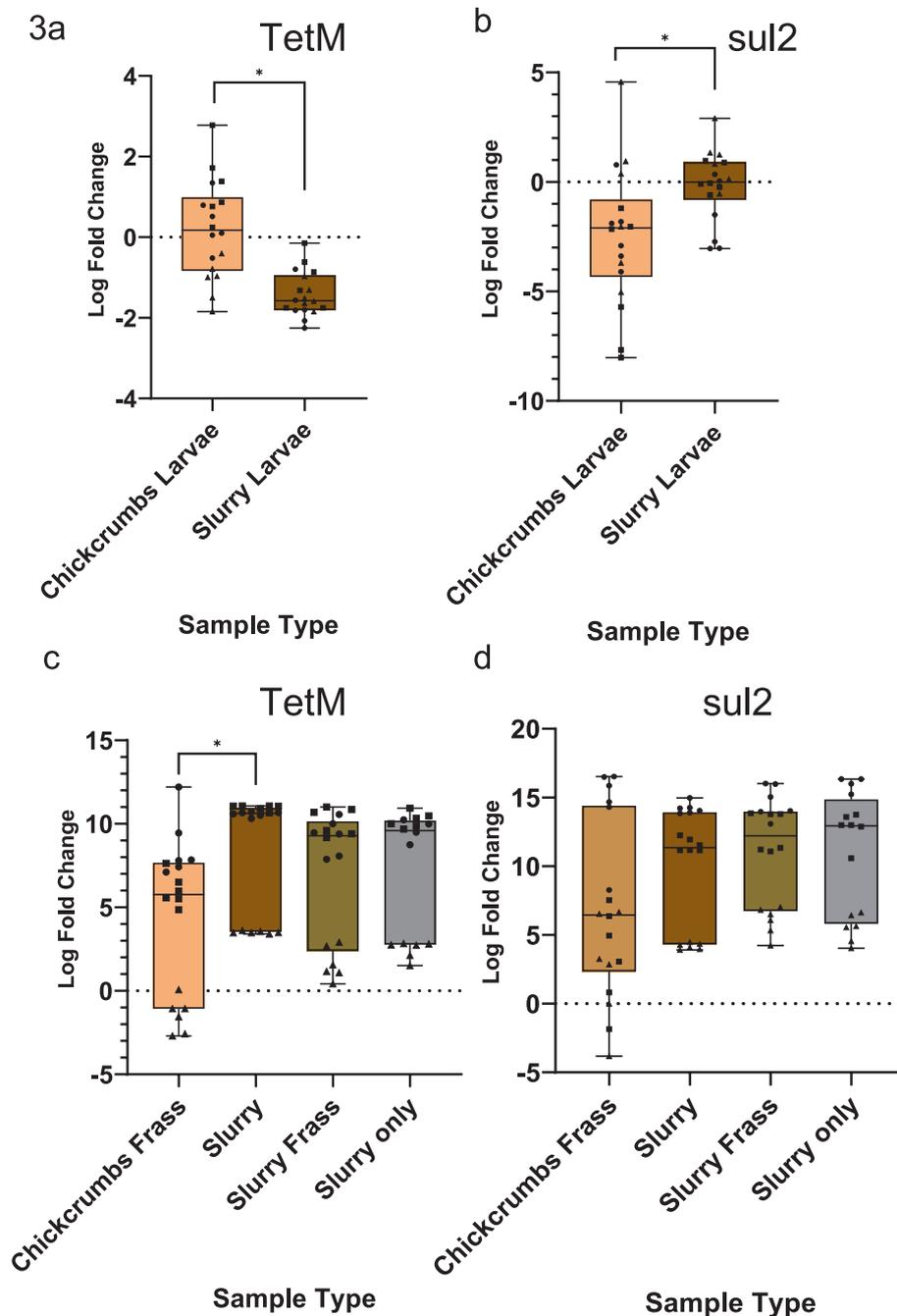
Fig. 3. Log Fold Change (LFC) for Antimicrobial Resistance Genes in Larvae and Substrates. LFC for tetM (a) and sul2 (b) in larvae reared on Chickcrumbs or pig slurry relative to starter larvae (five days old) and normalised to 16S gene (n=18). LFC for tetM (c) and sul2 (d) in substrates relative to Chickcrumbs and normalised to 16S gene. * p<0.05.

substrates, however, this was mostly between Chickcrumbs and Slurry types. Chickcrumbs Frass did have a significantly higher ammonium nitrogen than Chickcrumbs (8348.00 vs 166.30 mg/kg; p=0.014), and Slurry had significantly less sodium than Slurry Frass (452.33 vs 9080.67 mg/kg; p=0.046) and Slurry Only samples (452.33 vs 8787.00 mg/kg; p=0.044).

3.5. Experiment 2: Microbiome Composition in Chicken Manure Assay

Alpha Diversity Chao1 values were significantly different across sample types (KW=59.107; df=7; p<0.001) (Fig.5a). Manure-type substrates had higher species richness than their Chickcrumbs counterparts (p<0.05). There was also a significant increase for Manure Larvae

compared to both starter larvae (p=0.029) and Chickcrumbs Larvae (p<0.001) (Supplementary Table 2). Beta Diversity differed between samples (PERMANOVA F=11.543; df=7,71; p=0.001) (Fig.5b). Generally, the microbiome of chicken manure larvae was more similar to the manure substrates than the Chickcrumbs Larvae. As such, there were differences observed in relative abundances between groups, particularly between Chickcrumbs and manure types (Fig.5c). ANCOMBC analysis was used to investigate these differences in more detail.

Relative to starter larvae, both Chickcrumbs and Manure Larvae had decreased Actinobacteria and increased Clostridia. Bacilli increased in Chickcrumbs Larvae, but decreased in Manure Larvae, whilst Gammaproteobacteria significantly decreased in Manure Larvae (all p<0.001) (Fig.5d). For substrates, relative to Chickcrumbs, there was an
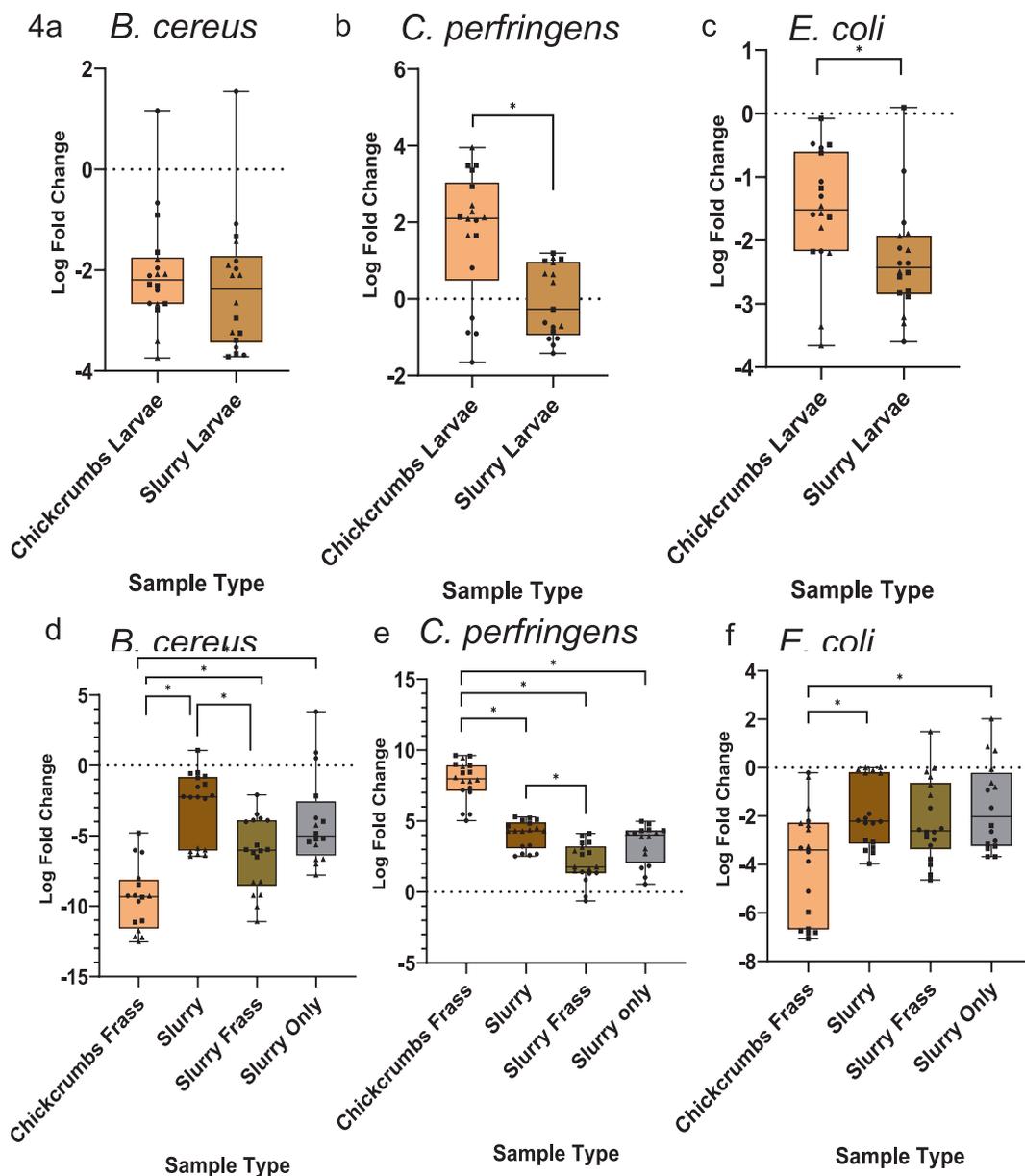
Fig. 4. Log Fold Change (LFC) for Pathogenic Bacteria in Larvae and Substrates. LFC for *Bacillus cereus* (a), *Clostridium perfringens* (b) and *Escherichia coli* (c) in larvae reared on Chickcrumbs or pig slurry relative to five day old starter larvae and normalised to 16S gene (n=18). LFC for *Bacillus cereus* (d), *Clostridium perfringens* (e) and *Escherichia coli* (f) in substrates relative to Chickcrumbs and normalised to 16S gene. * p<0.05.

increase observed in both Clostridia and Actinobacteria (p<0.001). Bacilli was increased in all groups (p<0.05), except for Manure Only samples (p=0.668). Chickcrumbs Frass had decreased Bacteroidea, however, this was increased in Manure, Manure Frass and Manure Only samples (p<0.001). There were a number of significant differences observed for Manure relative to the Chickcrumbs, including decreased Alphaproteobacteria (p<0.001) and Gammaproteobacteria (p=0.001; Fig.5e).

3.6. qPCR of Antimicrobial Resistance Genes in Chicken Manure Assay

There was no significant difference in the Log Fold Change (LFC) of tetM in Chickcrumbs Larvae compared to Manure Larvae (MWU= 114; df=1; p=0.134), with both showing an increase relative to starting larvae (Fig.6a). There was a significant difference in LFC of sul2 between larval types compared to starter larvae, with an increase in Manure Larvae, but not Chickcrumbs (3.509 vs -0.985 LFC; MWU=53; df=1;

p=0.001) (Fig. 6b). For substrates, there was a significant overall difference in LFC values for tetM (F=10.13; df=3, 68; p<0.001) (Fig. 6c), with *post-hoc* analyses showing significant differences between Chickcrumbs Frass and Manure Frass (p<0.001), Manure and Manure Frass (9.000 vs 10.370 LFC; p<0.001) and Manure Frass and Manure Only (10.370 vs 9.317 LFC; p=0.007). There was no difference for other comparisons. Overall, levels were increased relative to the starting Chickcrumbs, with a greater increase in the Manure Frass compared to the other substrates. Additionally, there was a significant effect of substrate type on sul2 levels (KW=36.338; df=3; p<0.001) (Fig.6d). *Post-hoc* analyses show significant differences between Chickcrumbs Frass and Manure Frass (4.673 vs 12.080 LFC); Manure and Manure Frass (9.426 vs 12.080 LFC); Chickcrumbs Frass and Manure Only (4.673 vs 11.220 LFC); Manure and Manure Only (9.426 vs 11.220 LFC); all p<0.001. There was no difference in sul2 LFC between Chickrumbs Frass and Manure (p=0.356) or Manure Frass and Manure Only (p=0.880).

Table 1

Heavy Metal and Nutrient Composition of Larvae Reared on Chickcrumbs or Pig Slurry and Substrates before and after insect bioconversion (mg/kg). 6 trays of each sample type were pooled per replicate (n=3). Values are means across replicates or ranges where exact values could not be measured.

	Starter larvae	Chickcrumbs larvae	Slurry larvae	Chickcrumbs	Chickcrumbs frass	Slurry	Slurry frass	Slurry only
Oven Dry Solids	34.80	32.37	24.33	21.15	80.83	3.10	28.27	39.77
Conductivity	2882.00	719.67	488.00	1870.00	8905.67	2906.33	4018.33	6606.33
Total Kjeldahl Nitrogen	6.11	6.72	7.02	1.49	3.98	0.26	3.19	2.95
Nitrate Nitrogen	<10 to 21.3	<10 to 15.3	<10 to 13.5	<10 to 11.1	<10	<10	<10	<10 to 12.8
Ammonium Nitrogen	3143.33	409.00	400.00	166.30	8348.00	1370.67	791.00	889.00
Total Phosphorus	14373.67	9229.33	14319.67	3632.33	20423.33	729.00	13788.00	17509.00
Total Potassium	21100.00	10973.67	15528.67	4329.00	23324.67	2141.67	43698.33	41627.00
Total Magnesium	5308.67	3914.67	6145.00	848.67	3843.33	472.00	8854.33	11418.33
Total Copper	50.40	48.70	104.93	6.37	45.57	7.42	158.67	176.67
Total Zinc	254.33	400.67	965.33	50.13	282.67	38.87	847.67	1006.67
Total Sulphur	5237.33	3422.33	4720.33	1362.00	6868.00	313.67	7347.00	7368.67
Total Calcium	20086.67	37009.67	45459.33	4521.33	11154.00	912.00	20323.67	26866.33
Total Lead	<1	<1	<1	<0.5 to <1.0	1 to <1	<0.5	1.36	1.49
Total Cadmium	0.25	0.27	0.46	<0.1 to 0.01	<0.1	0.02	0.37	0.44
Total Mercury	<0.1	<0.1	<0.1	<0.1 to <0.05	<0.1	<0.05	<0.1	<0.1 to 0.21
Total Nickel	2.74	<1 to 3.71	<1 to 9.99	0.74	4.55	0.64	11.22	11.67
Total Chromium	4.34	<2 to 3.04	<2 to 4.08	<0.2 to <2	2.58	0.33	3.22	3.81
Total Sodium	3347.67	1171.33	1943.33	693.33	4671.00	452.33	9080.67	8787.00
pH	7.56	7.21	7.29	5.34	6.78	8.19	8.68	8.62
Total Arsenic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Total Selenium	0.78	0.55	1.07	0.14	0.79	0.07	1.63	1.69

3.7. qPCR of Pathogenic Bacteria in Chicken Manure Assay

There was no difference in *Bacillus cereus* for either larvae type, with a reduction compared to starter larvae (MWU=103; df=1; p=0.064) (Fig.7a). There was a significant effect of larval rearing substrate for *Clostridium perfringens* levels compared to the starting larvae (MWU=269; n=18; p<0.001) (Fig.7b), with a reduction observed for larvae reared on chicken manure (-1.113 vs 0.255 LFC). There was a reduction in *Escherichia coli* levels for both larvae types, with a greater reduction for larvae reared on Chickcrumbs (-2.431 vs -1.558 LFC; MWU=84; n=18; p=0.013) (Fig.7c).

For substrates, there was a significant difference for *B. cereus* (KW=50.125; df=3; p<0.001), with *post-hoc* analyses showing differences between all groups (p<0.05) except for Manure Frass and Manure Only (-6.695 and -5.921 LFC; p=0.069). All substrates were reduced compared to starting Chickcrumbs (Fig.7d). There was a significant difference for *C. perfringens* levels compared to starting Chickcrumbs (KW=41.976; df=3; p<0.001) (Fig.7e). *Post-hoc* analyses show significant differences for Chickcrumbs Frass compared to Manure (8.122 vs 6.602 LFC), Chickcrumbs Frass compared to Manure Frass (8.122 vs 5.096 LFC), Chickcrumbs Frass compared to Manure Only (8.122 vs 4.855 LFC) and Manure compared to Manure Only (6.602 vs 4.855 LFC) (all p<0.05). There was no significant difference between Manure and Manure Frass (p=0.191) or Manure Frass and Manure Only (p=0.498). All substrates had increased levels relative to starter Chickcrumbs, with the highest observed in Chickcrumbs Frass. There was also a significant difference for *E. coli* (KW=43.623; df=3; p<0.001) (Fig. 7f), with *post-hoc* analyses showing significant differences between all groups (p<0.05), except Manure and Manure Only (-1.021 and -1.168 LFC; p=0.886). Substrates generally showed reduced *E. coli* relative to starting Chickcrumbs, with the lowest levels observed in Chickcrumbs Frass (-6.141 LFC).

3.8. Larvae and substrates heavy metal and nutrient content in chicken manure assay

Heavy metals and nutrients were measured in pooled samples (Table 2). Zinc was significantly reduced in Chickcrumbs Larvae compared to starter larvae (65.43 vs 287.67 mg/kg; p=0.034), and Manure Larvae had increased calcium compared to starter larvae (62282 vs 20519; p=0.022) (Supplementary Table 4). For substrates with exact measurements, calcium was significantly different overall (p=0.025),

however, *post-hoc* analysis found no pairwise differences.

4. Discussion

In this study, BSFL were reared on pig slurry in a bespoke, semi-commercial-sized insect rearing unit or on chicken manure in a lab-scale study. Substrates and experimental scale affected microbiome composition, levels of the pathogenic bacteria and antimicrobial resistance genes in the larvae.

Microbiome analysis of pig slurry found a greater species richness in slurry samples compared to Chickcrumbs. Larvae reared on slurry had a greater species richness than Chickcrumbs-fed larvae and a microbiome composition more similar to the slurry substrates. Increases in Clostridia, and decreases in Bacilli and Gammaproteobacteria were observed in the slurry-reared larvae, and reductions in Bacilli and Gammaproteobacteria were also found in the slurry samples compared to the Chickcrumbs. The similarity in larvae compared to substrates may simply represent the simple processes of intake and excretion, but there could also be alterations related to selection for particular microorganisms by the larvae that perform important functions (Schreven et al., 2022). Increases in spore-forming bacteria, such as *Clostridium* spp. are a particular concern as they are difficult to eliminate and can produce neurotoxins (Romero-Rodríguez et al., 2023). For example, Grenda et al., (2021) found higher *Clostridium* spp. levels and botulinum toxin-producing genes in insect protein-containing poultry feed. However, antimicrobial peptide activity from the larvae could also inhibit certain pathogens, such as *C. perfringens*, resulting in significant shifts in the diversity of larval microbial communities, with potential effects for animals that have consumed insect extracts (Dong et al., 2021).

Similarly, Ao et al., 2021 found a reduction of *Providencia* (Class: Gammaproteobacteria) and *Enterococcus* (Bacilli) in larvae reared on pig manure, with *Enterococcus* increasing in larvae reared on chicken manure. These changes were associated with differences in the presence of elements such as organic matter, total nitrogen and potassium (Ao et al., 2021). However, Bacilli was decreased in the chicken manure larvae in this study. Moisture content of pig manure has also been shown to affect larval microbiome composition (Wang et al., 2024). Changes in the microbiome composition observed in this study may be related to alterations in the substrate. In this study, pH of slurry samples did not vary, though there were increases in nutrients such as potassium, phosphorus, magnesium and sodium in the frass. More research should explore how specific changes in the substrate influence microbiota
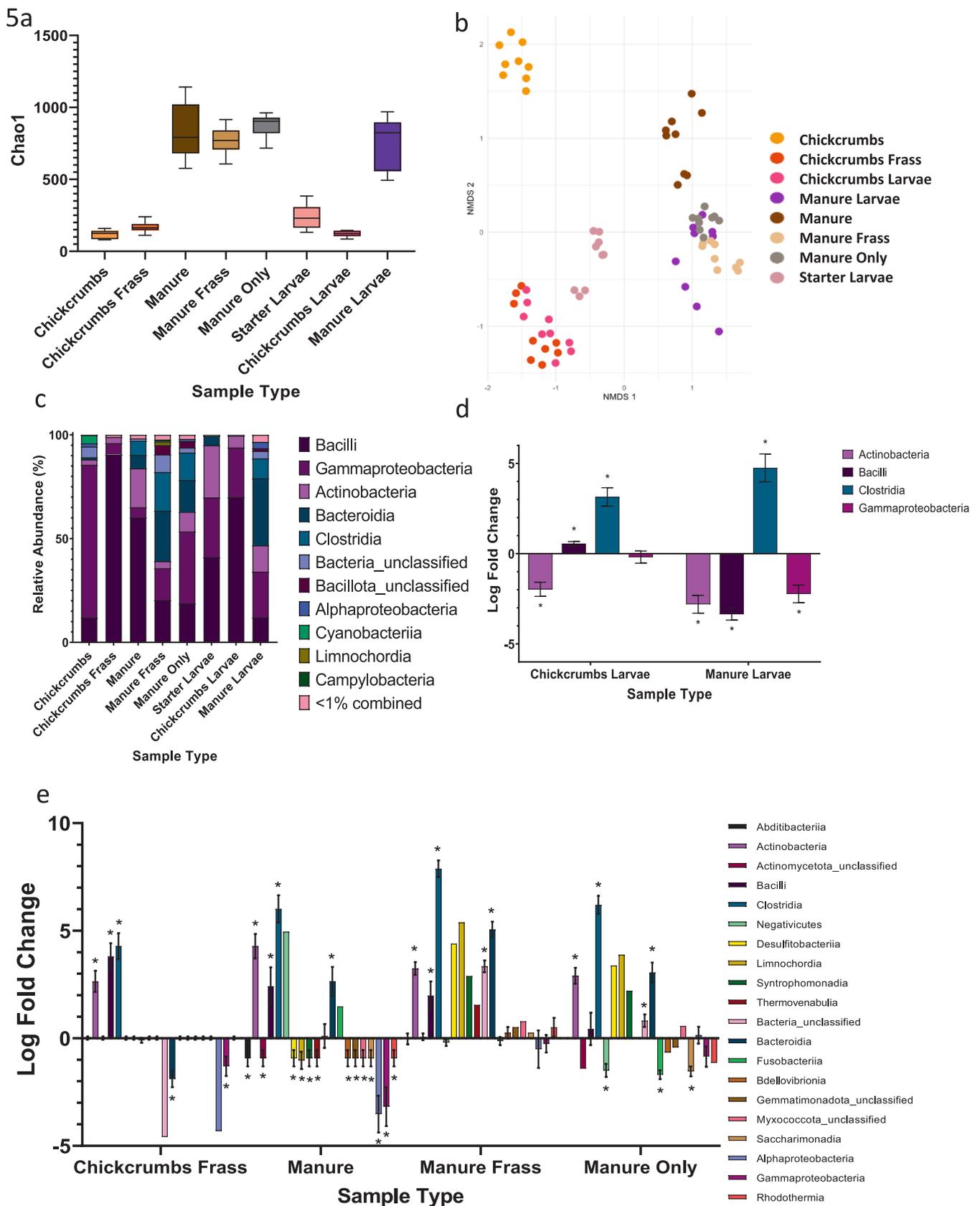
Fig. 5. Alpha and Beta Diversity, and Relative Bacterial Class Abundance and Log Fold Changes (LFC) for larvae and substrates in the chicken manure assay. Alpha Diversity using Chao1 index (a) and Beta Diversity NMDS plot (b). Relative abundance of bacterial classes for all sample types, with all classes with <1% abundance combined (c). ANCOMBC LFC for larvae reared on Chickcrumbs and chicken manure relative to the five day old starter larvae ± S.E.M. (d). ANCOMBC LFC for substrates relative to Chickcrumbs ± S.E.M. (e). Only classes that were significant in at least one sample type are included. * p<0.05
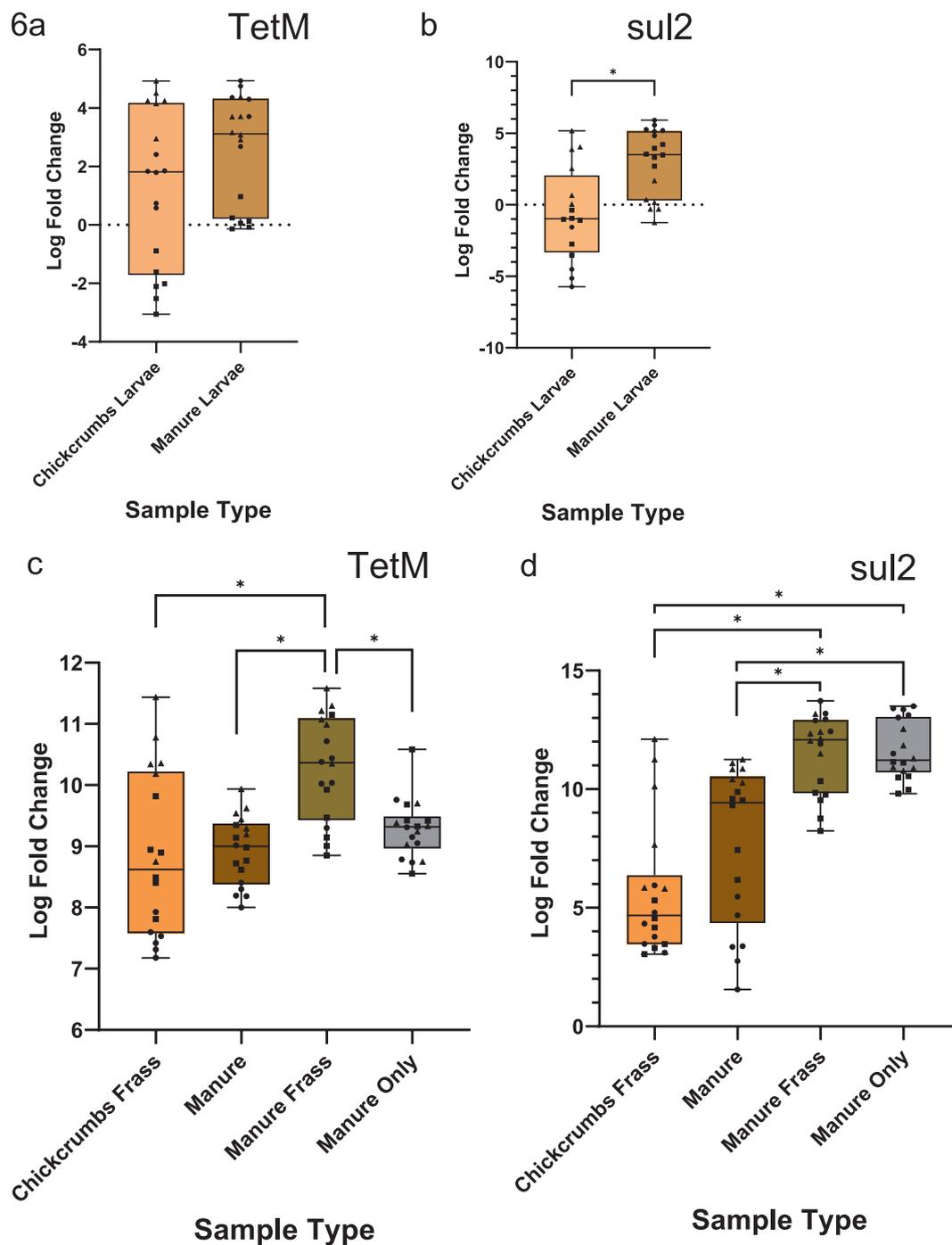
Fig. 6. Log Fold Change (LFC) for Antimicrobial Resistance Genes in Larvae and Substrates. LFC for tetM (a) and sul2 (b) in larvae reared on Chickcrumbs or chicken manure relative to five day old starter larvae and normalised to 16S gene (n=18). LFC for tetM (c) and sul2 (d) in substrates relative to Chickcrumbs and normalised to 16S gene. * $p < 0.05$

composition, and the possibility of larval selection for microbes with beneficial functions. Further, these assays used surface-sterilised whole larvae, and it would be useful for future research to examine changes occurring specifically in the gut microbiome.

Due to biosecurity controls in place at the NPC, the chicken manure assay was carried out in a laboratory setting. As such, results with the pig slurry assay are not directly comparable, and it would be advantageous for future research to carry out a test with chicken manure on a larger-scale. In this assay, there was a greater species richness for chicken manure samples compared to Chickcrumbs, with larvae reared on chicken manure having a higher species richness. Though the two assays

are not directly comparable, there were similarities. For example, Bacilli and Gammaproteobacteria decreased in manure larvae relative to starting larvae, and Clostridia increased. However, whilst Actinobacteria decreased in both slurry and manure larvae in their respective experiments, there was only a reduction in Chickcrumbs larvae from the chicken manure assay. Density differences between the two assays could impact on the microbial communities, for example, through changes in the temperature (Klammsteiner et al., 2025), pH and moisture content of the substrate (Schreven et al., 2022). For example, increasing larval density has been shown to alter abundance of bacteria such as *Lactobacillus* and *Corynebacterium* in chicken manure (Schreven et al., 2022).
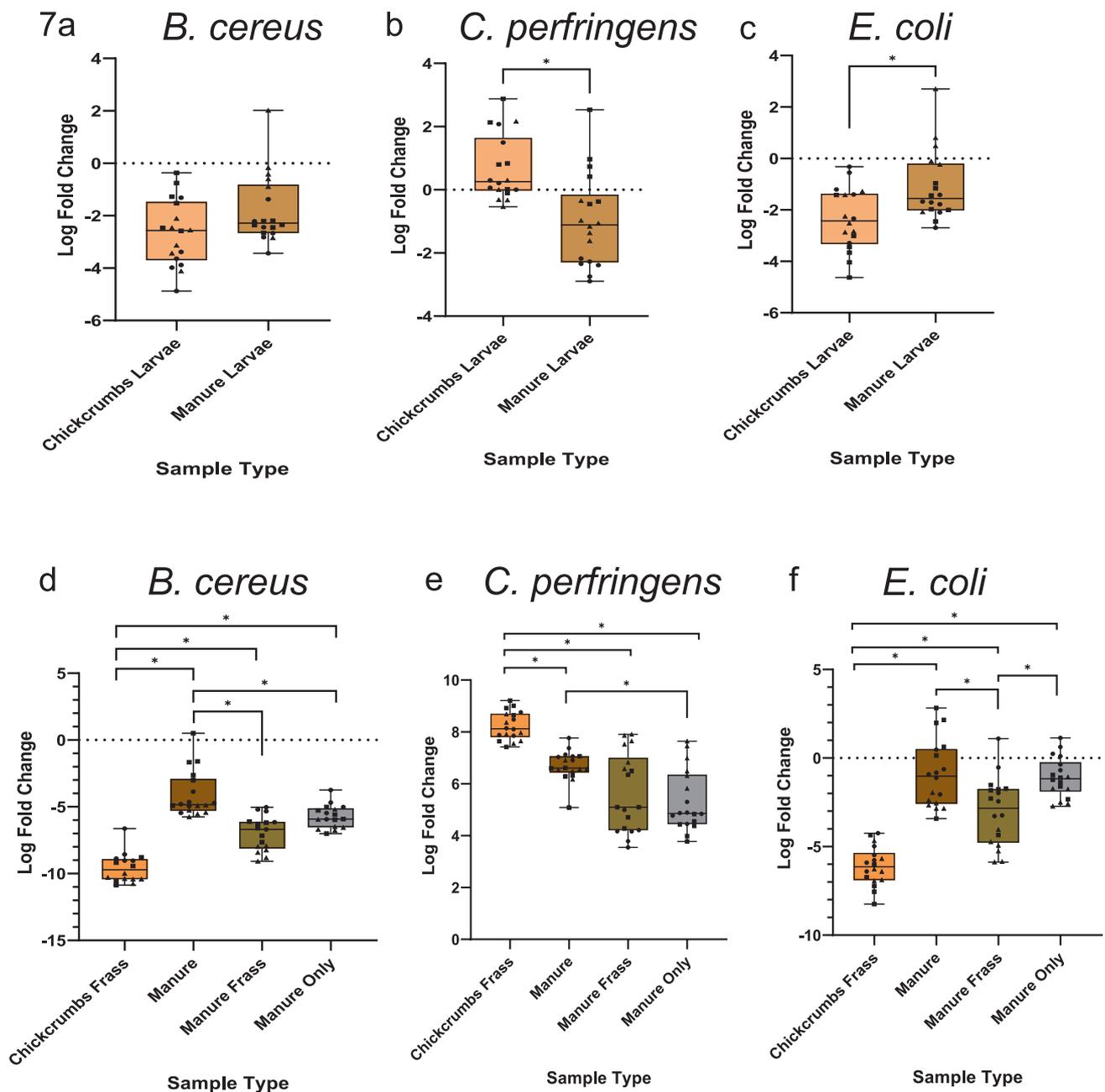
Fig. 7. Log Fold Change (LFC) for Pathogenic Bacteria in Larvae and Substrates. LFC for *Bacillus cereus* (a), *Clostridium perfringens* (b) and *Escherichia coli* (c) in larvae reared on Chickcrumbs or chicken manure relative to five day old starter larvae and normalised to 16S gene (n=18). LFC for *Bacillus cereus* (d), *Clostridium perfringens* (e) and *Escherichia coli* (f) in substrates relative to Chickcrumbs and normalised to 16S gene. * p<0.05

Table 2
Heavy Metal and Nutrient Composition of Content of Larvae Reared on Chickcrumbs or Chicken Manure and Substrates (mg/kg). 6 trays of each sample type were pooled per replicate (n=3). Values are means across replicates or ranges where exact values could not be measured.

	Starter larvae	Chickcrumbs larvae	Manure larvae	Chickcrumbs	Chickcrumbs frass	Manure	Manure frass	Manure only
Oven Dry Solids (%)	29.87	41.67	35.43	34.83	46.33	20.27	23.13	24.9
Total Copper	35.3	8.61	23.07	5.83	16.27	41.15	73.1	62.33
Total Zinc	287.67	65.43	150.47	41.90	120.33	229.13	403.97	353.4
Total Calcium	20519	28990.33	62282.33	4340.67	7338	31680.33	46125.33	47065.33
Total Lead	<1	<1	<1 to 1.04	<0.5 to <1	<1	<0.5 to 1.28	0.57 to 2.56	0.52 to 2.3
Total Cadmium	0.34	0.19	<0.1 to 0.54	0.01 to <0.1	<0.1	0.28	0.51	0.44
Total Mercury	<0.1	<0.1	<0.1	<0.05 to <0.1	<0.1	<0.05 to <0.1	<0.05 to <0.1	<0.05 to <0.1
Total Nickel	1.85	<1 to 1.08	7.95	0.78	11.06	7.07	20.54	5.67
Total Chromium	3.68	<2 to 3.38	4.69	0.28 to <2	2.98	10.08	2.92	2.06
Total Arsenic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Total Selenium	0.76	0.36	1.1	0.21	0.48	0.60	0.95	0.87

Higher larval densities can result in lower larval weight, and influence the gut microbiome (Yang et al., 2021). As some trays in the pig slurry assay had used much of the feedstock by the end of the bioconversion period, densities were reduced for the lab-scale assay.

For pathogenic bacteria, BSFL reared on pig slurry had reduced *B. cereus* and *E. coli* relative to the starter larvae, though there was no change in the levels of *C. perfringens* in Slurry Larvae. Slurry frass had lower *B. cereus* and *C. perfringens* compared to slurry. There was no difference with the slurry only samples that did not have larvae present, suggesting these changes are not related to the presence of the insects. For the chicken manure assay, only *E.coli* demonstrated an impact of insect presence on manure. Pathogenic bacteria have previously been found to decrease in frass from larvae reared on pig manure (Wu et al., 2021) and chicken manure (Zhao et al., 2023). BSFL produce antimicrobial peptides (Xia et al., 2021) and could suppress pathogenic bacteria via their production or competition with their microbiome (Shi et al., 2024). For example, *Salmonella* spp. were reduced in human faeces treated with larvae (Lalander et al., 2013), and Zhao et al., 2023 found a reduction in pathogens including *E. coli* and *Staphylococcus aureus*, in chicken manure following larval addition. The substrate pH could have an effect (Shi et al., 2024). Differences may also vary with larval life stage (Alagappan et al., 2025), that may be connected to changes in larval lauric acid concentration (Alagappan et al., 2025), which has antimicrobial properties (Hurtado-Ribeira et al., 2023). Furthermore, as development to the pre-pupal stage took longer in the pig slurry larvae, there could be changes in the substrate with this extra time which impacts on the microbiota or differences in metabolic factors in the slower developing larvae that could affect their microbiome or levels of pathogens (Barragan-Fonseca et al., 2018). Pathogenic bacteria have been found to increase in chicken manure substrates with increasing densities of larvae (Schreven et al., 2022). It is worth noting that use of qPCR to examine the levels of specific bacteria in these samples does not allow us to distinguish between viable bacteria (Wolffs et al., 2005) and does not consider differences in 16S copy number (Klappenbach et al., 2000). Issues also arise with efficiency of the DNA extraction from different samples (Bonk et al., 2018). Nonetheless, this does allow for an initial indication of differences between the groups, and further studies should look to address absolute levels.

Antimicrobial resistance is a significant global health concern (Bava et al., 2024). Commonly used antibiotics in agriculture include tetracyclines and sulfonamides (OIE, 2018), and both types are used on the farm that provided the pig slurry for this study. As such, two antimicrobial resistance genes (ARG), tetM and sul2, were selected. tetM prevents tetracycline inhibition of protein synthesis (Burdett, 1996), and sul2 encodes a variant of the sulphonamide target enzyme that is insensitive to inhibition (Sköld, 2000). tetM has been identified in a large number of genera, including *Enterobacter* and *Escherichia* (Roberts, 2005), and in slurry-treated soils, sul2 has been found in genera such as *Acinetobacter* (Byrne-Bailey et al., 2009). In this study, slurry samples had higher starting levels of these ARGs than Chickcrumbs. Bioconversion did not affect levels of either in slurry substrates. However, there was variation between replicates, perhaps because of variations in substrates or in environmental settings in the insect rearing unit. Future experiments would benefit from increased replicates, and improvements to homogeneity of environmental conditions. Indeed, work is ongoing to improve the consistency of conditions across the internal environment. For the larvae, tetM decreased for those reared on slurry compared to the starter larvae, whilst there was no detectable change for sul2, which may be connected to the microbial community composition (Bohm et al., 2024).

Conversely, in the chicken manure assay, both tetM and sul2 ARGs were increased in larvae reared on chicken manure. Both genes were increased in frass, and there was a significant increase in tetM in manure frass compared to the starting manure and the manure without larvae, suggesting the presence of the larvae impacted levels. There was no difference found for sul2 between manure frass and manure only,

suggesting this increase is due to factors other than larval presence. These results suggest that larval bioconversion can, under certain circumstances, including rearing substrate, affect levels of ARGs. Antibiotic concentration in substrates was not determined and may represent the most likely reason for the difference observed between substrate types. Other studies have found decreases in levels of ARGs in chicken manure (Cai et al., 2018; Zhao et al., 2023). Chen et al., 2022 found a decrease in tetracycline ARGs in chicken manure frass, though there was an increase in tetW at low density, suggesting differences between genes with an increase observed in pakchoi plants on soil treated with high larval density frass. Differences in ARGs in this study may represent variations in composition of the substrates, levels of antibiotics or differences in the experimental design (discussed below). Horizontal Gene Transfer, the process of transferring of genetic material between unrelated organisms (Villa et al., 2019), could be a significant driver for the spread of ARGs in this system. The diversity of the microbial community of livestock manures, combined with antibiotic presence, and the co-occurrence with heavy metals could provide suitable conditions for this process (Lima et al., 2020). Thus, differences between these factors could influence the levels of ARGs in the substrates and larvae. Further, the distinct effects in different ARGs warrants greater investigation, including of a wider range of antimicrobial resistance genes, as well as how factors may interconnect to influence ARG levels. This is particularly pertinent where larvae may be added back into the system as a livestock feed, for example, ARGs can be spread to zebrafish fed on larvae (Milanović et al., 2021), or where ARG-containing frass is added to agricultural soils (Chen et al., 2022). Long-term consumption or exposure could have significant consequences and needs to be more thoroughly understood.

It is important to note that whilst differences were observed between the substrate types i.e. pig slurry and chicken manure, the experimental design was different between the two assays and therefore direct comparisons between the two feedstocks should be analysed with caution. The two assays varied in rearing scale, larval density, and had differences in environmental control which constitute a major limitation of this study. Whilst differences observed between the assays may have been due to the feedstock, it is important that future work compares these under identical rearing conditions to confirm the findings observed here. Yakti et al., (2022) showed significant differences in larval growth depending on the scale of the rearing container used (whilst maintaining density and feed availability). They attributed this to the higher temperatures achieved at a larger scale with a difference of more than 5°C between the smallest and largest containers. They also showed that density affected the concentration of minerals e.g. S, Mg, K, P, Fe, Zn and amino acids in the larvae themselves. Nayak and Klüber (2025) also explored scale using different substrates. They showed that highest individual larvae weight and highest substrate reduction were achieved at different scales and concluded that density and scale need to be adjusted to maximise nutrient conversion efficiency when looking at different insect feedstocks.

A further consideration when comparing between the results from the two feedstocks used in this study is the difference in time taken to reach the pre-pupae stage. BSFL reared on pig slurry took longer than those reared on chicken manure and were therefore harvested later. The difference in physiological age between the larvae in the two studies, may have been a confounding factor for outcomes such as pathogen reduction and the accumulation of ARGs. Physiology of the larvae is developmentally regulated, and as such impacts the gut microbiome and metabolic activity. For example, the gut microbiome of BSFL has been shown to vary with larval age and this interacted with diet composition and genetic factors (Silvaraju et al., 2024). It has also been found that 6th instar larvae (prepupae) have lower microbial counts that those at the 5th instar (Alagappan et al., 2025). The authors attributed this to the difference in some of the fatty acids present in the larvae and the antimicrobial effect that they confer. As the gut microbiota of BSFL can inhibit the proliferation of potentially pathogenic microorganisms (Shi et al., 2024), any changes to the community composition due to age at

harvesting may have affected the results presented here.

Feeding on contaminated substrates can result in bioaccumulation of heavy metals in insects (Malematja et al., 2023) which can negatively impact larval growth (Purschke et al., 2017). There may also be changes in frass through concentration or excretion from the larvae (Addeo et al., 2024), though levels depend on substrate type (Alagappan et al., 2024). Frass from pig manure-fed larvae containing different levels of cadmium has been shown to increase quantities in the soil and maize plants (Wang et al., 2020). BSFL have been shown to accumulate heavy metals such as lead (Purschke et al., 2017), mercury and arsenic (Biancarosa et al., 2018). High levels of cadmium and copper have been shown to lower the larval gut microbiome diversity (Wu et al., 2020). High levels of accumulation in larvae is often observed for cadmium (Biancarosa et al., 2018; Wang et al., 2021; Wang et al., 2022). In this study, larvae reared on slurry had Cd concentrations that ranged from 0.18 to 0.70 mg/kg, within previously reported ranges (Wang et al., 2021; Wang et al., 2022; Lin et al., 2023; Hoffmans et al., 2024). Whilst not statistically significant, due to the low sample size (n=3), the mean cadmium concentration in slurry-reared larvae exceeded that of slurry (0.02 mg/kg), and starting larvae (0.25mg/kg), this suggests there has been some bioaccumulation in the larvae. This trend should be investigated further with greater replication.

However, this is lower than the permissible limit for the animal feed in the EU (2mg/kg) (Regulation (EU) No 32/2002). Slurry frass showed a non-significant increase in cadmium compared to the starting slurry, but within the permissible EU limit for organic fertilisers (1.5mg/kg; Regulation (EU) No 1009/2019). There was no difference in lead, arsenic or mercury in larvae reared on slurry, demonstrating that these did not bioaccumulate in our system, despite small, non-significant increases in lead and mercury in the slurry substrates. Larvae met the EU permissible limits for Animal Feed for lead, arsenic and mercury for both the slurry and chicken manure assays (Regulation (EU) No 32/2002), and both slurry frass and chicken manure frass met the permissible limit for organic fertilizers for these heavy metals (Regulation (EU) No 1009/2019). Whilst calcium increased in larvae reared on chicken manure, there was no significant bioaccumulation of cadmium observed in the larvae of this assay, however, a trend for higher accumulation in the manure-reared larvae warrants further investigation with more replicates. Understanding how heavy metals change in this system is important (Malematja et al., 2023), including how co-selection may impact the spread of antimicrobial resistance genes (Wales and Davies, 2015). For example, tetM abundance in soils correlates with nickel, chromium, iron and lead levels (Knapp et al., 2011).

Further research must continue to address these hazards. Questions also remain over other hazards such as prions (Benestad et al., 2024), and though evidence suggests polychlorinated biphenyls and pharmaceuticals do not generally accumulate (Siddiqui et al., 2023), confirmation is required. Further research should also be undertaken to observe long-term effects of hazard exposure via insect farming to both animals and humans (Mufungwe et al., 2025). Regulation for the use of insects in animal feed are often limited to general requirements for animal feed and fertilizer, and would benefit from more specific limits, based on factors including substrates and welfare (Meijer et al., 2025).

In conclusion, whilst pathogenic bacteria levels varied, these largely decreased in larvae reared on slurry or manure compared to starter larvae in their respective assays. In substrates, levels were generally related to changes in the substrates themselves, rather than insect presence. Thus, the impact of BSFL bioconversion was often minimal compared to the maturation of substrates. This is an important consideration for future research and may indicate that the larvae may not have as significant an impact on pathogen loads as previously considered. The bioconversion process did not significantly pose a heavy metal risk, with contents generally meeting EU permissible feed or fertilizer limits, though there were small trends of bioaccumulation in some instances. Critically, there was a substrate-dependent effect on ARGs, including increases in the chicken manure assay. Therefore, the safety of BSFL waste valorization is highly dependent on the waste stream used and/or the experimental scale examined, and rigorous, substrate-specific risk assessments must be carried out before large-scale implementation.

CRediT authorship contribution statement

L.S. McDowall: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **A. Collado:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **R. Clarkson:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Investigation, Conceptualization. **G. Reiss:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **K. McDermott:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2025.115278.

Data availability

Data has been made available at the University of Leeds Data Repository under this manuscript title.

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