Investigating Safety Aspects of Using Insect Farming to Reduce Pig and Chicken Wastes at Semi-Commercial and Lab-Scale

L.S.McDowall[a], A.Collado[b], R.Clarkson[a], G.Reiss[a] and K.McDermott[a]

[a] *School of Biology, Faculty of Biological Sciences, University of Leeds, Woodhouse Lane, Leeds, UK, LS29JT*

[b]*Entocycle, 29 Shand Street, London, UK, SE1 2ES*

Corresponding author: Katie McDermott, k.mcdermott@leeds.ac.uk

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.

1.1 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 140.1m 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 (Tschirner and Simon, 2015; Cullere et al., 2016). 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

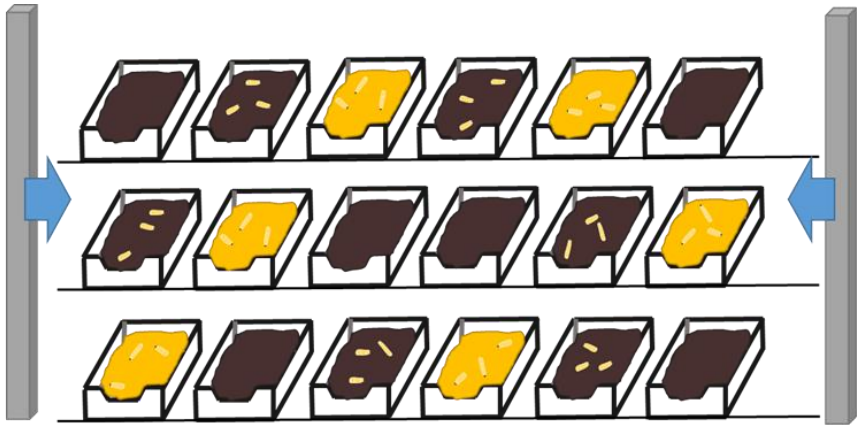
2.1 Experiment 1: Pig Slurry

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 175g 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 $m^3 h^{-1}$, 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 Figure 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 (~400g, -20°C). The experiment was independently replicated 3 times.

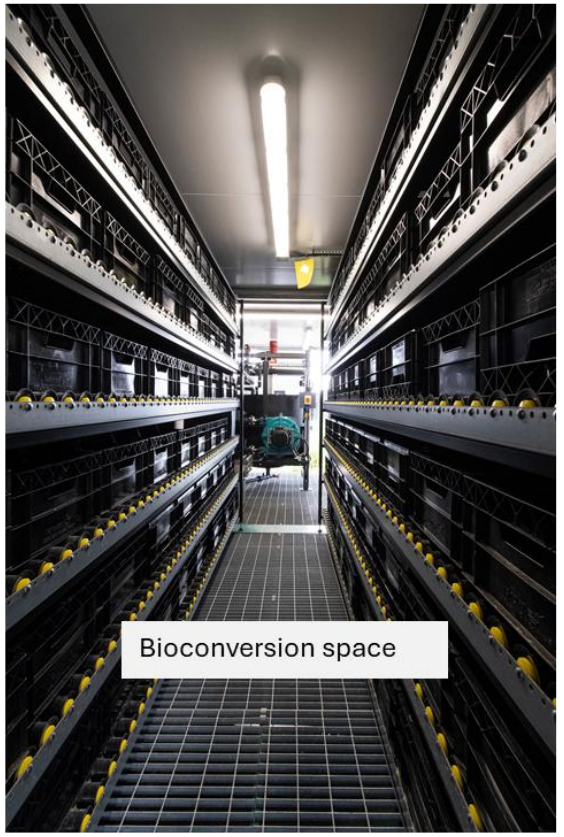
A)



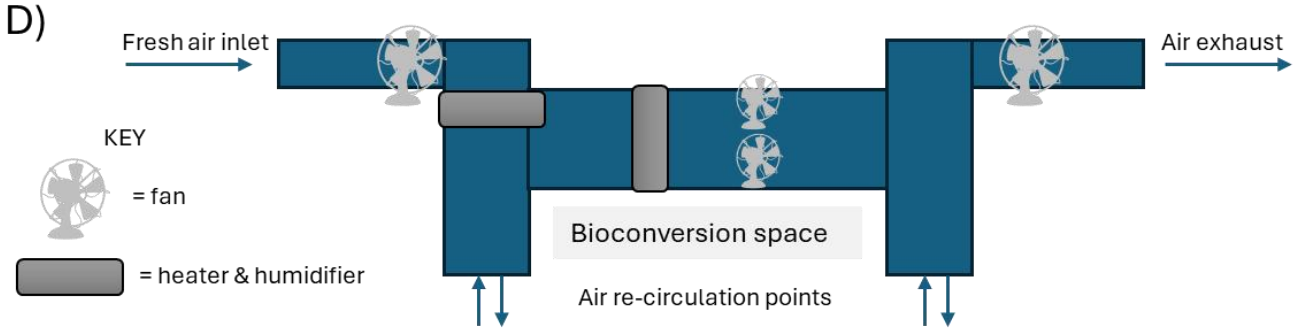
B)


HVAC Unit
Office space
Entocycle
Insect rearing unit

C)


Bioconversion space

D)


Fresh air inlet
Air exhaust
KEY
= fan
= heater & humidifier
Bioconversion space
Air re-circulation points

137

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.

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 (10g) 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 340g 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 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, 300mg was weighed into a 2ml Eppendorf tube. Due to the dryness of the samples and the volume of

particles, all frass samples, slurry only or manure only samples had 500µl 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 (300mg) 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.

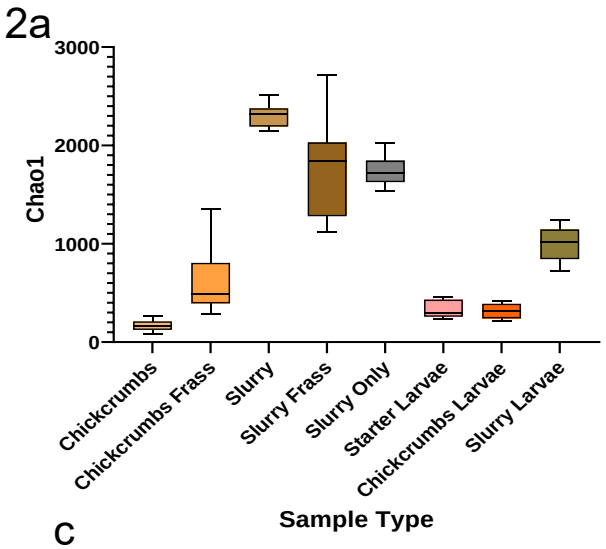
193 Antimicrobial resistance genes tetM and sul2 were chosen as they confer resistance to
194 tetracyclines and sulphonamides respectively, both used at the NPC (Aminov et al., 2001;
195 Wang et al., 2014). Common human food pathogens were also selected, taking into
196 consideration the possibility of using the insects in animal feed (and therefore entering the
197 food chain) or using the frass as a fertilizer. These included spore-forming bacteria such as
198 *Bacillus cereu*s and *Clostridium perfringens,* that produce toxins and are difficult to eliminate
199 (Carroll et al., 2019), as well as common pathogens found in livestock, such as *Escherichia*
200 *coli* (Begum et al., 2014). Primers (Supplementary Table 1) were tested via PCR using GoTaq
201 Green Master Mix (10µM). Annealing temperatures were checked on a 16°C gradient
202 (Techne TC-512), with a middle temperature of 58°C. PCR conditions were 3 mins at 94°C
203 (30s at 94°C, 30s at annealing, 30s at 72°C) x30, 7 min at 72°C, and hold at 4°C. Samples
204 were then checked on a 1% agarose gel (80V, 40 minutes). Primers were tested for qPCR
205 efficiency (BioRad CFX96 Connect qPCR machine; 300nM using 5-6 10x serial dilutions), with
206 a starting concentration of 2ng/µl, SYBR Green and the selected annealing temperature
207 from the gradient PCR. Plates were briefly spun down in a plate centrifuge followed by
208 shaking in a plate shaker at 450 rpm for 45 seconds. The conditions for qPCR were 95°C for
209 3 mins, followed by 95°C for 10 seconds and the appropriate annealing temperature for 30
210 seconds for 40 cycles, then a ramp from 65°C to 95°C in 0.5°C increments. Primers were
211 accepted with an efficiency of 90-100% and $R^2 \geq 0.98$, except for *Salmonella enterica* primers
212 (efficiency = 86.3%) and *E. coli* (85.6%), as these were the best of the multiple primer pairs
213 tested. Substrate and larvae samples were run on separate plates. Each sample was run in
214 triplicate and accepted if the Cq values of least two of these were within 0.5. qPCR data was
215 analysed via the Pfaffl method (Pfaffl, 2001), with the average of the Chickcrumbs samples
216 as the reference for the substrates and starter larvae as the reference for the larvae plates,

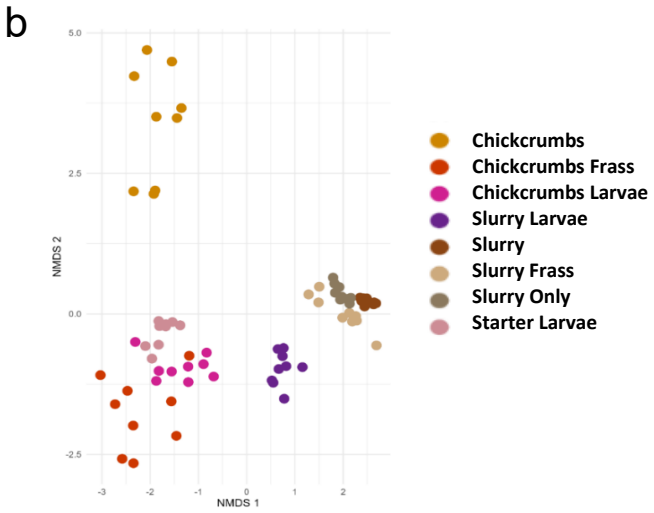
217 to give relative quantities (RQ). Values were normalised to the RQ values from the 16S
218 primers and logged to give the relative log fold change. The data was tested for normality
219 and analysed via T-tests or ANOVA (for normally distributed data) or Mann Whitney U or
220 Kruskal-Wallis tests (for non-normal data). *Post hoc* Dunn's Tests with Benjamini-Hochberg
221 corrections were carried out where appropriate.

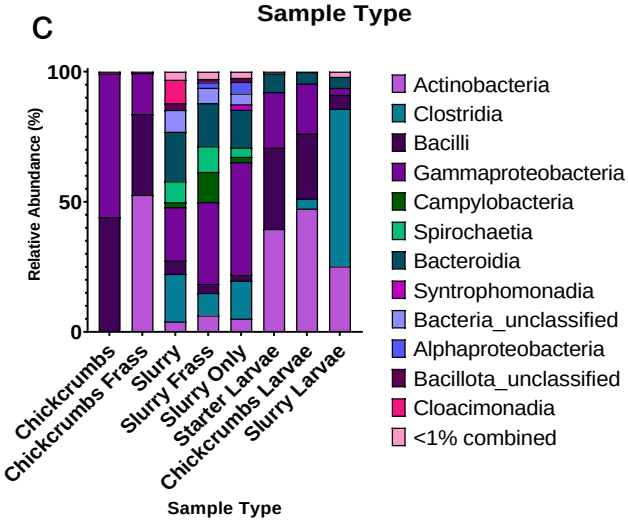
222 Samples for heavy metals were pooled from all trays of the same sample type per replicate,
223 and stored at -20°C. Larvae samples were briefly rinsed with water and sieved, with a final
224 rinse with ddH_2O to remove excess substrate, then patted dry before returning to the
225 freezer. Samples were shipped to NRM (Cawood Scientific) for analyses. The data was
226 analysed using Kruskal-Wallis tests, and Dunn's tests with Benjamini-Hochberg corrections
227 for *post-hoc* analyses.

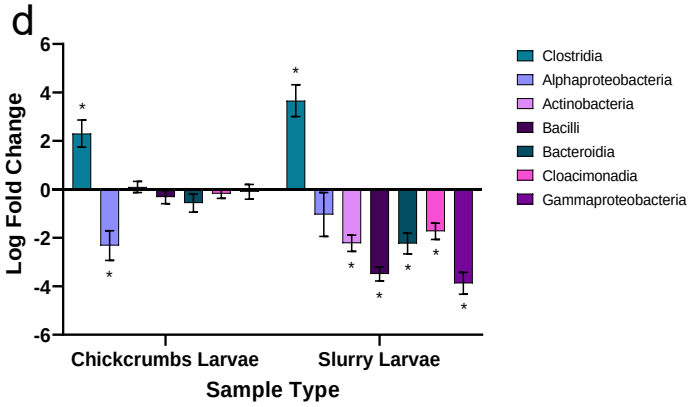
228 3. Results

3.1.1 Experiment 1: Microbiome Composition in Pig Slurry Assay









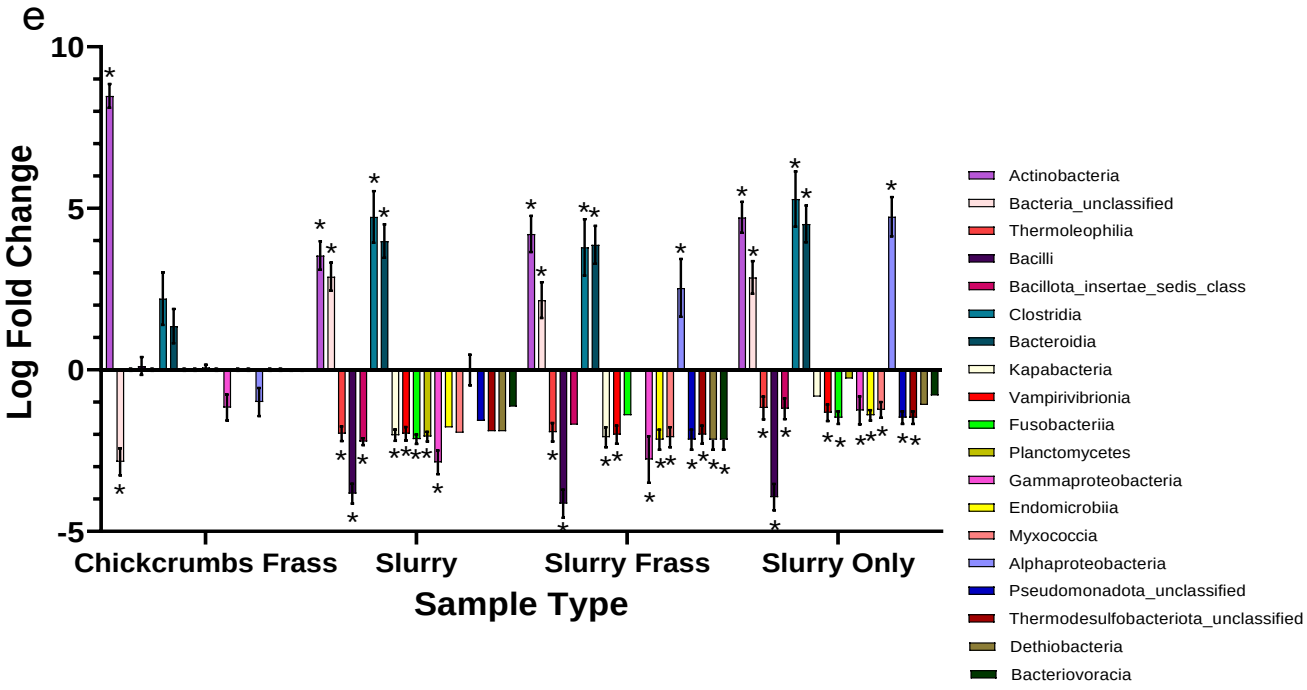


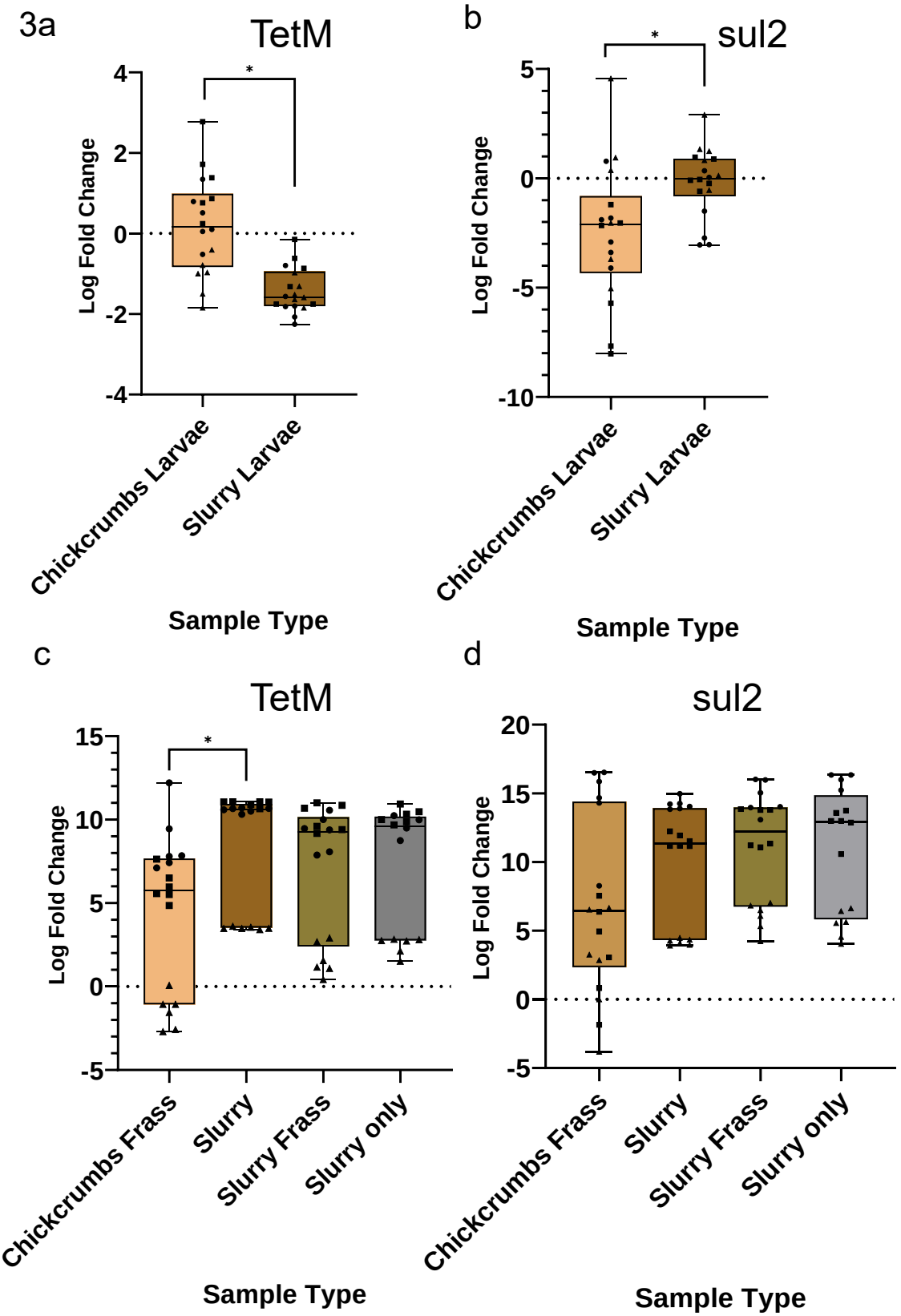
Fig. 2 Alpha and Beta Diversity, Relative Bacterial Class Abundance and Log Fold Changes for larvae and substrates. Alpha Diversity using Chao1 index (a) and Beta Diversity NMDS plot (b). Relative abundance of bacterial classes, combining all classes with <1% abundance (c). ANCOMBC Log Fold Change for 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. * $p<0.05$.

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 ($p=0.992$).

3.1.2 qPCR of Antimicrobial Resistance Genes in Pig Slurry Assay



262

263 **Fig.3 Log Fold Change (LFC) for Antimicrobial Resistance Genes in Larvae and Substrates.**

264 LFC for tetM (a) and sul2 (b) in larvae reared on Chickcrumbs or pig slurry relative to starter

265 larvae (five days old) and normalised to 16S gene (n=18). LFC for tetM (c) and sul2 (d) in
266 substrates relative to Chickcrumbs and normalised to 16S gene. * $p<0.05$.

267 There was a significant difference in the Log Fold Change (LFC) of tetM in Chickcrumbs
268 Larvae compared to Slurry Larvae (MWU=288; N=36; $p<0.001$), with a reduction observed
269 for those reared on slurry (-1.578 vs 0.173 LFC) (Fig.3a). There was also a significant
270 difference in the LFC of sul2 (MWU=77; n=18; p=0.006), however, in this case, Chickcrumbs
271 Larvae had lower relative LFC than the Slurry Larvae (-2.103 vs -0.007 LFC) (Fig. 3b). For
272 substrates, there was a significant overall difference in LFC values for tetM (KW=12.471;
273 df=3; p=0.006), however, *post-hoc* analyses show the only significant difference occurred
274 between Chickcrumbs Frass and Slurry (p=0.003) (Fig. 3c), though there was also a large
275 amount of variation. There was no difference in the level of sul2c between substrates
276 (KW=4.371; df=3; p=0.224) (Fig. 3d). Both tetM and sul2 levels were generally higher
277 relative to the Chickcrumbs for all substrates. Overall, for slurry groups, larval bioconversion
278 did not appear to have an effect on the levels of these AMR genes.

3.1.3 qPCR of Pathogenic Bacteria in Pig Slurry Assay

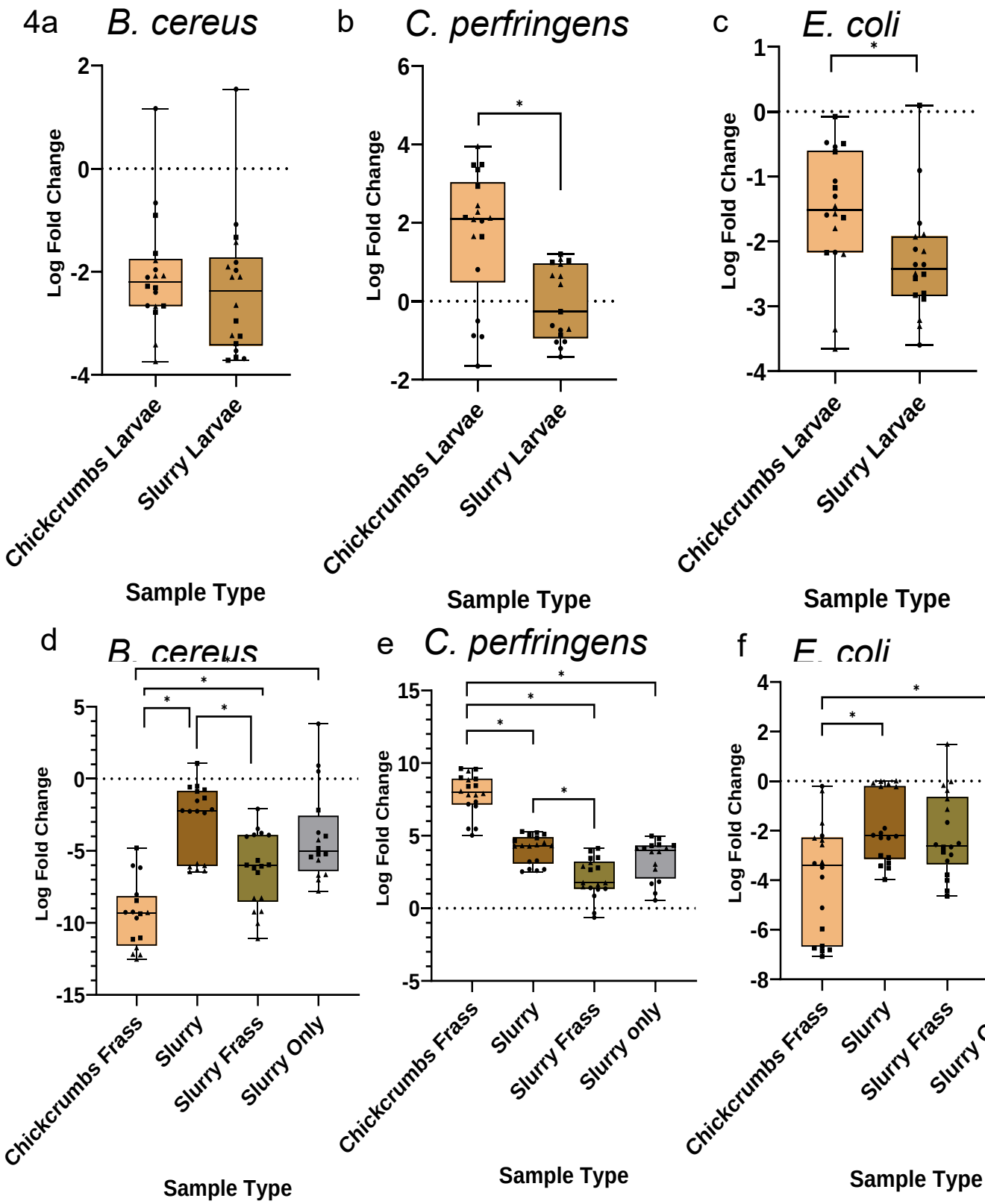


Fig.4 Log Fold Change (LFC) for Pathogenic Bacteria in Larvae and Substrates. LFC for *Bacillus cereus* (a), *Clostridium perfringens* (b) and *Escherichia coli* (c) in larvae reared on Chickcrumbs or pig slurry relative to five day old starter larvae and normalised to 16S gene (n=18). LFC for *Bacillus cereus* (d), *Clostridium perfringens* (e) and *Escherichia coli* (f) in substrates relative to Chickcrumbs and normalised to 16S gene. * $p<0.05$.

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).

3.1.4 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 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; $p=0.044$).

315 **Table 1: Heavy Metal and Nutrient Composition of Larvae Reared on Chickcrumbs or Pig Slurry and Substrates before and after insect**

316 **bioconversion (mg/kg).** 6 trays of each sample type were pooled per replicate (n=3). Values are means across replicates or ranges where

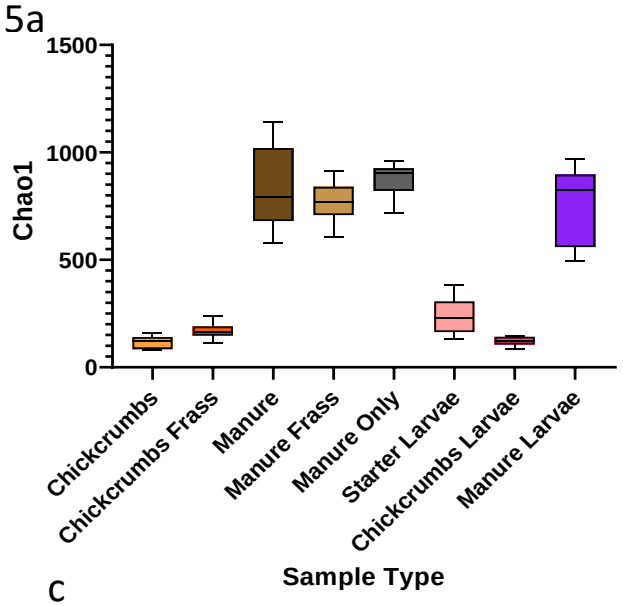
317 exact values could not be measured.

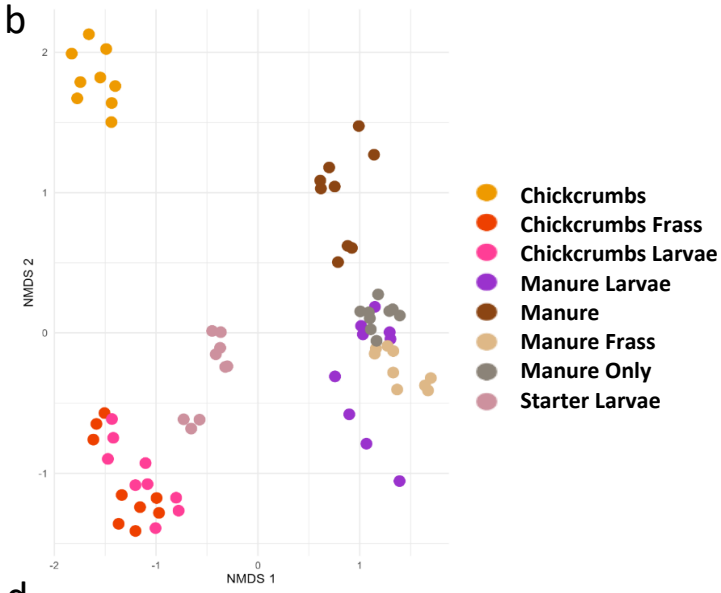
	Starter Larvae	Chickcrumbs Larvae	Slurry Larvae	Chickcrumbs	Chickcrumbs Frass	Slurry	Slurry Frass	Slurry Only
Oven Dry Solids	34.80	32.37	24.33	21.15	80.83	3.10	28.27	39.77
Conductivity	2882.00	719.67	488.00	1870.00	8905.67	2906.33	4018.33	6606.33
Total Kjeldahl Nitrogen	6.11	6.72	7.02	1.49	3.98	0.26	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

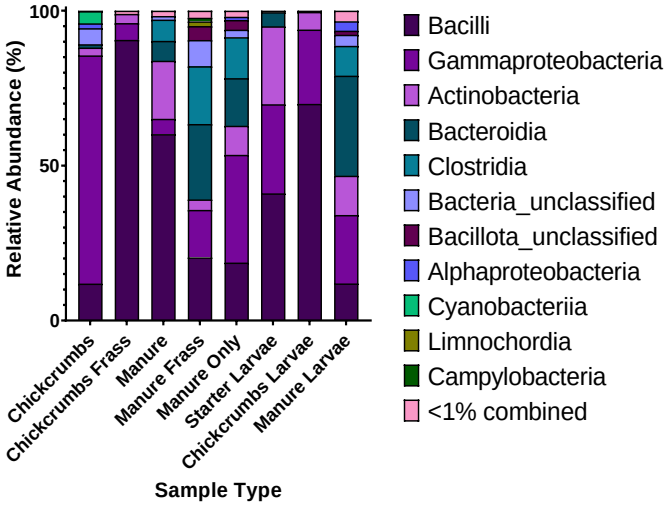
Total Zinc	254.33	400.67	965.33	50.13	282.67	38.87	847.67	1006.67
Total Sulphur	5237.33	3422.33	4720.33	1362.00	6868.00	313.67	7347.00	7368.67
Total Calcium	20086.67	37009.67	45459.33	4521.33	11154.00	912.00	20323.67	26866.33
Total Lead	<1	<1	<1	<0.5 to <1.0	1 to <1	<0.5	1.36	1.49
Total Cadmium	0.25	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

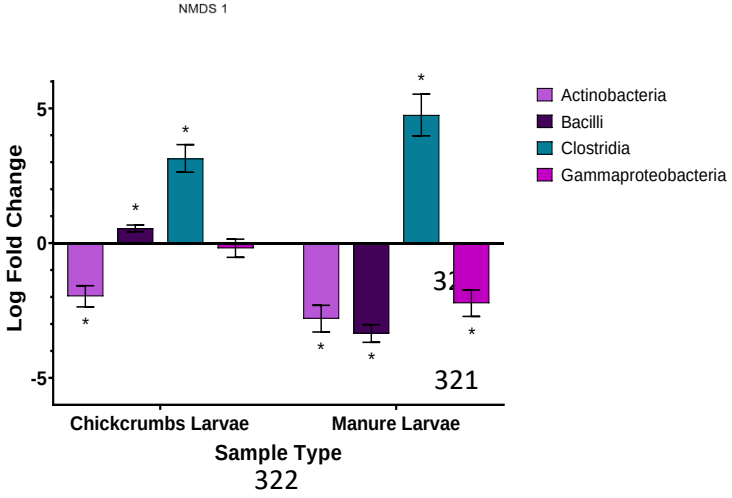
318

3.2.1 Experiment 2: Microbiome Composition in Chicken Manure Assay



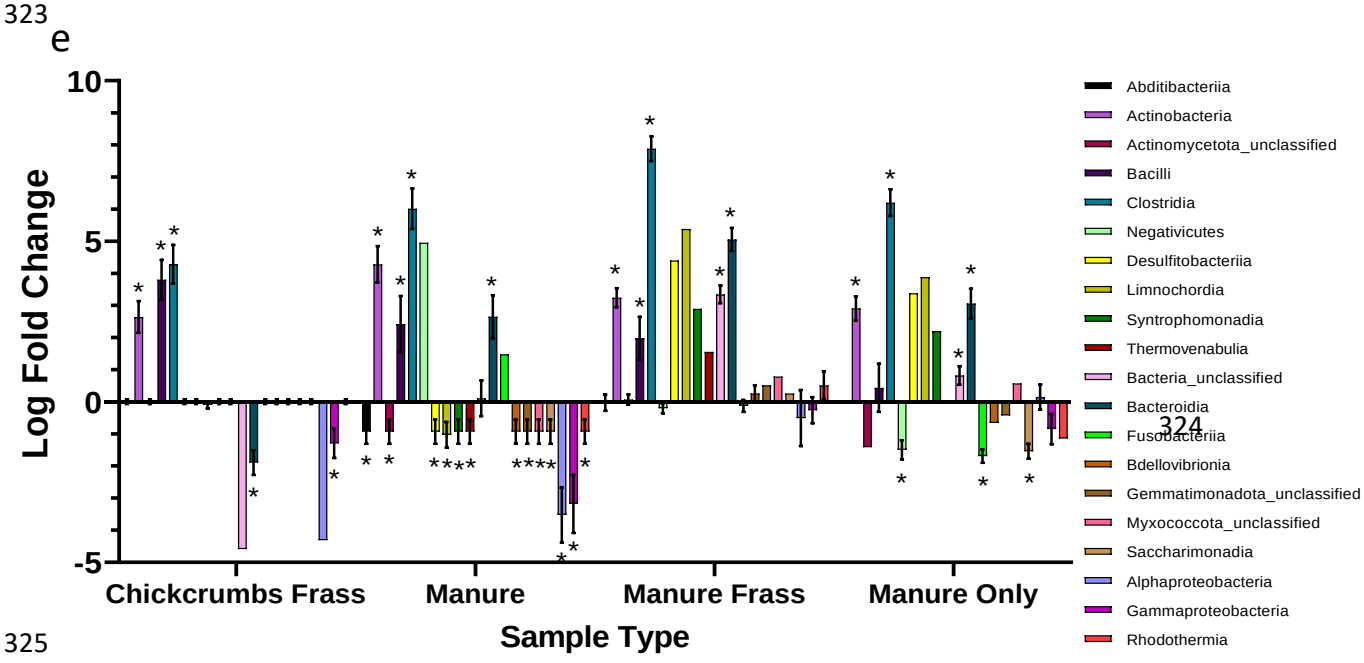






322

323



324

325

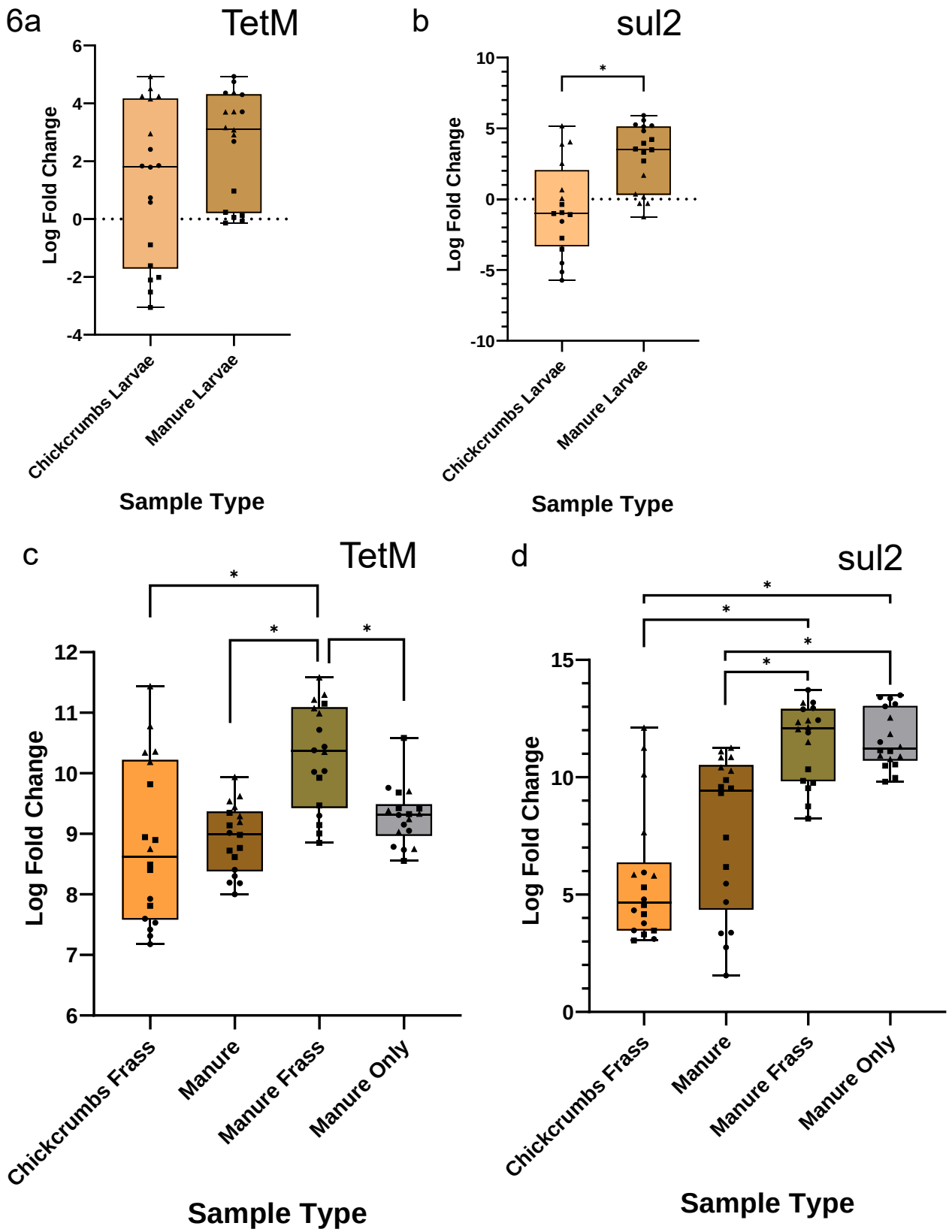
Fig. 5 Alpha and Beta Diversity, and Relative Bacterial Class Abundance and Log Fold Changes (LFC) for larvae and substrates in the chicken manure assay. Alpha Diversity using Chao1 index (a) and Beta Diversity NMDS plot (b). Relative abundance of bacterial classes for all sample types, with all classes 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. * $p<0.05$

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 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).

355 3.2.2 qPCR of Antimicrobial Resistance Genes in Chicken Manure Assay

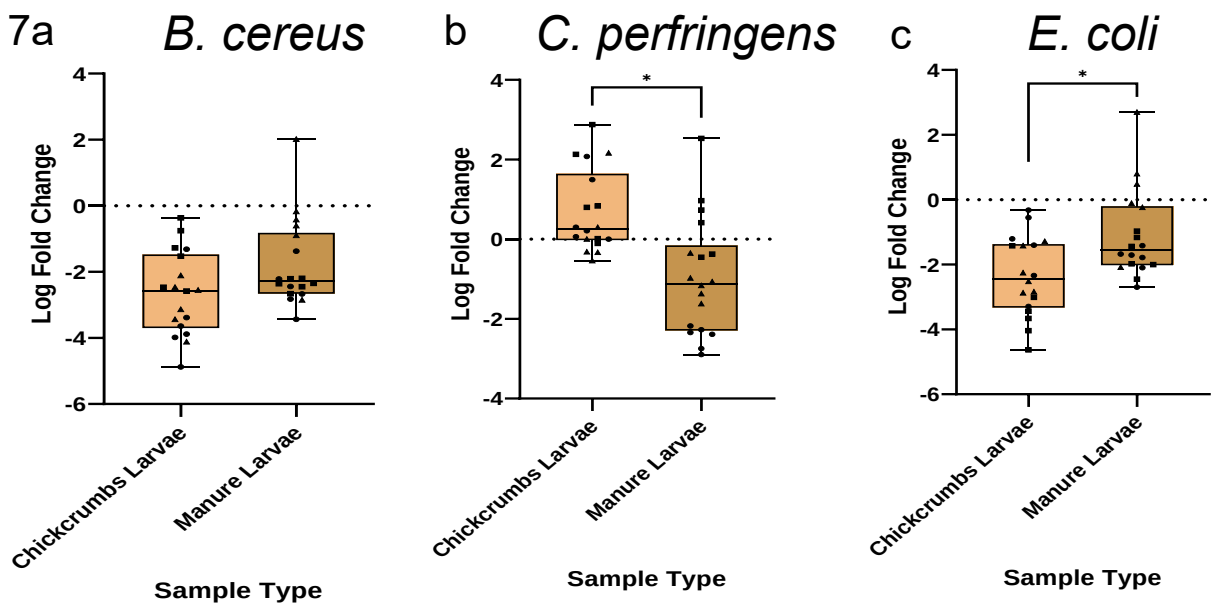


356

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. * $p<0.05$

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).

3.2.3 qPCR of Pathogenic Bacteria in Chicken Manure Assay



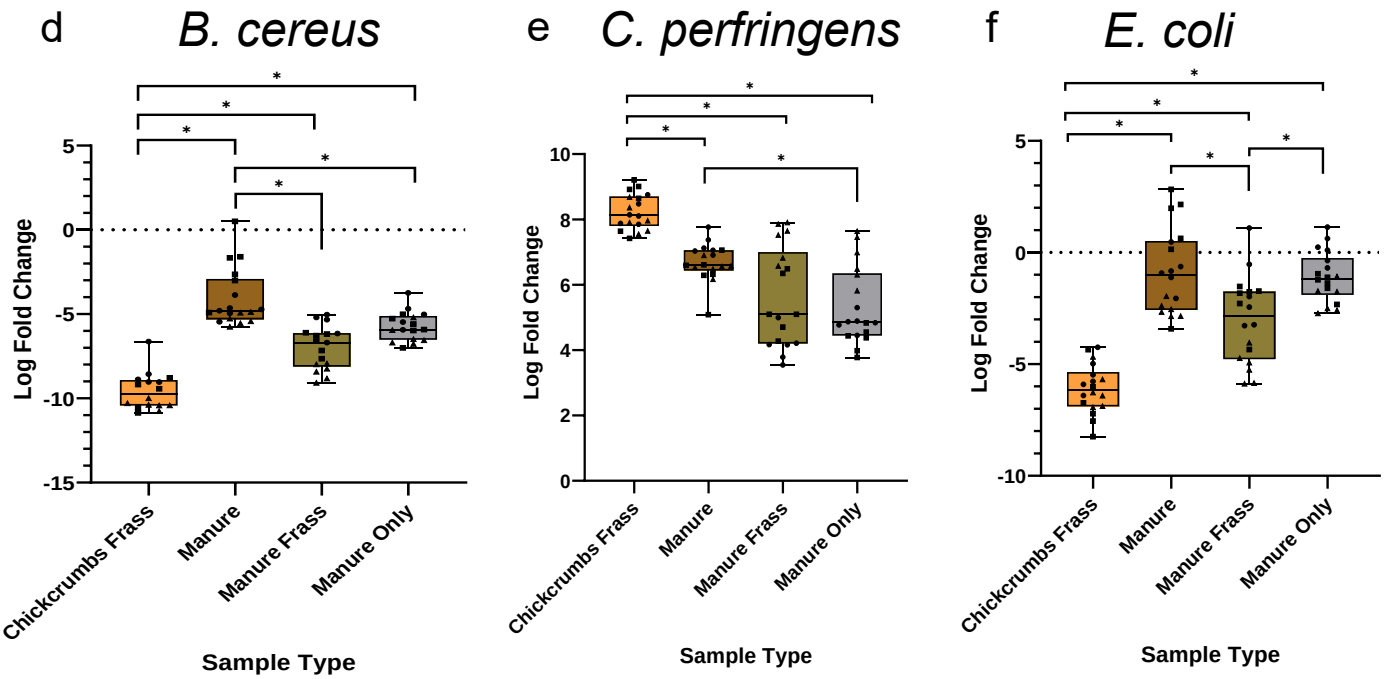


Fig.7 Log Fold Change (LFC) for Pathogenic Bacteria in Larvae and Substrates. LFC for *Bacillus cereus* (a), *Clostridium perfringens* (b) and *Escherichia coli* (c) in larvae reared on Chickcrumbs or chicken manure relative to five day old starter larvae and normalised to 16S gene (n=18). LFC for *Bacillus cereus* (d), *Clostridium perfringens* (e) and *Escherichia coli* (f) in substrates relative to Chickcrumbs and normalised to 16S gene. * $p<0.05$

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

406 Chickcrumbs Frass. There was also a significant difference for *E. coli* (KW=43.623; df=3;
407 p<0.001) (Fig. 7f), with *post-hoc* analyses showing significant differences between all groups
408 (p<0.05), except Manure and Manure Only (-1.021 and -1.168 LFC; p=0.886). Substrates
409 generally showed reduced *E. coli* relative to starting Chickcrumbs, with the lowest levels
410 observed in Chickcrumbs Frass (-6.141 LFC).

411 3.2.4 Larvae and Substrates Heavy Metal and Nutrient Content in Chicken Manure Assay

412 Heavy metals and nutrients were measured in pooled samples (Table 2). Zinc was
413 significantly reduced in Chickcrumbs Larvae compared to starter larvae (65.43 vs 287.67
414 mg/kg; p=0.034), and Manure Larvae had increased calcium compared to starter larvae
415 (62282 vs 20519; p=0.022) (Supplementary Table 4). For substrates with exact
416 measurements, calcium was significantly different overall (p=0.025), however, *post-hoc*
417 analysis found no pairwise differences.

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

422

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 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). 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

517 to the starter larvae, whilst there was no detectable change for sul2, which may be
518 connected to the microbial community composition (Bohm et al., 2024).

519 Conversely, in the chicken manure assay, both tetM and sul2 ARGs were increased in larvae
520 reared on chicken manure. Both genes were increased in frass, and there was a significant
521 increase in tetM in manure frass compared to the starting manure and the manure without
522 larvae, suggesting the presence of the larvae impacted levels. There was no difference
523 found for sul2 between manure frass and manure only, suggesting this increase is due to
524 factors other than larval presence. These results suggest that larval bioconversion can,
525 under certain circumstances, including rearing substrate, affect levels of ARGs. Antibiotic
526 concentration in substrates was not determined and may represent the most likely reason
527 for the difference observed between substrate types. Other studies have found decreases
528 in levels of ARGs in chicken manure (Cai et al., 2018; Zhao et al., 2023). Chen et al., 2022
529 found a decrease in tetracycline ARGs in chicken manure frass, though there was an
530 increase in tetW at low density, suggesting differences between genes with an increase
531 observed in pakchoi plants on soil treated with high larval density frass. Differences in ARGs
532 in this study may represent variations in composition of the substrates, levels of antibiotics
533 or differences in the experimental design (discussed below). Horizontal Gene Transfer, the
534 process of transferring of genetic material between unrelated organisms (Villa et al., 2019),
535 could be a significant driver for the spread of ARGs in this system. The diversity of the
536 microbial community of livestock manures, combined with antibiotic presence, and the co-
537 occurrence with heavy metals could provide suitable conditions for this process (Lima et al.,
538 2020). Thus, differences between these factors could influence the levels of ARGs in the
539 substrates and larvae. Further, the distinct effects in different ARGs warrants greater
540 investigation, including of a wider range of antimicrobial resistance genes, as well as how

541 factors may interconnect to influence ARG levels. This is particularly pertinent where larvae
542 may be added back into the system as a livestock feed, for example, ARGs can be spread to
543 zebrafish fed on larvae (Milanović et al., 2021), or where ARG-containing frass is added to
544 agricultural soils (Chen et al., 2022). Long-term consumption or exposure could have
545 significant consequences and needs to be more thoroughly understood.

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

562 A further consideration when comparing between the results from the two feedstocks used
563 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) (EU Animal Feed Directive 2022/32/EC). 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; EU Organic Fertilizer 2019/1009/EC). 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 (EU Animal Feed Directive 2022/32/EC), and both slurry frass and chicken manure frass met the permissible limit for organic fertilizers for these heavy metals (EU Organic Fertilizer 2019/1009/EC). 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

Conceptualization: all authors; Data curation: LSM, KM; Formal Analysis: LSM, KM, Funding Acquisition: KM, GR, AC; Investigation: KM, LSM, RC; Methodology: KM, LSM, RC; Project administration: KM, RC, LSM, AC; Resources: KM, RC, AC; Software: LSM, KM;

Supervision:KM; Validation: LSM, KM, RC; Visualization: KM, LSM; Roles/Writing - original draft: LSM, KM; and Writing - review & editing: all authors

Declaration of competing interest

None

Acknowledgements

We thank Lauren Radtke and Joshua Barnett for their assistance in setting up the insect rearing unit, Jack Stephenson at Harper Farm for supplying the chicken manure and Beta Bugs for supplying the larvae.

Funding

This work was supported by an Innovate UK grant, jointly funded by BBSRC (InSAFE: Securing the Future of the UK's Insect Farming Industry; grant number: 10075954).

Data Accessibility

Data associated with this paper is available from the University of Leeds Data Repository.

References

Addeo, N.F., Marcello, S., Simone, V., Fulvia, B., A., A.K., Shatha, A., Antonio, C.N., and Severino, L., 2024. Mineral profile and heavy metals bioaccumulation in black soldier fly (*Hermetia illucens*, L.) larvae and frass across diverse organic substrates. *Italian Journal of Animal Science*, 23, 179-188. doi: https://doi.org/10.1080/1828051X.2024.2302845.

Alagappan, S., Dong, A., Hoffman, L., Cozzolino, D., Mantilla, S.O., James, P., Yarger, O., Mikkelsen, D., 2025. Microbial safety of black soldier fly larvae (*Hermetia illucens*) reared on

food waste streams. *Waste Management*, 194, 221-227,doi:https://doi.org/10.1016/j.wasman.2025.01.019.

Alagappan, S., Mallard, S., Cozzolino, D., Mikkelsen, D., James, P., Mantilla, S.O., Yarger, O., Hoffman, L., 2024. Effect of larval instar and post-harvest treatments on heavy metals in BSFL and frass reared on commercial food waste streams. *International Journal of Food Science and Technology*, 59, 8214-8223. doi: https://doi:10.1111/ijfs.17511.

Aminov, R.I., Garrigues-Jeanjean, N., Mackie, R.I., 2001. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Applied Environmental Microbiology*, 67, 22-32. doi: https://doi:10.1128/aem.67.1.22-32.2001.

Ao, Y., Yang, C., Wang, S., Hu, Q., Yi, L., Zhang, J., Yu, Z., Cai, M., Yu, C., 2021. Characteristics and nutrient function of intestinal bacterial communities in black soldier fly (*Hermetia illucens* L.) larvae in livestock manure conversion. *Microbial biotechnology*, 14, 886-896. doi:https://doi.org/10.1111/1751-7915.13595.

Bacchetti De Gregoris, T., Aldred, N., Clare, A.S., Burgess, J.G., 2011. Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. *Journal of Microbiological Methods*, 86, 351-356. doi:https://doi.org/10.1016/j.mimet.2011.06.010.

Barragan-Fonseca, K.B., Dicke, M. and van Loon, J.J., 2017. Nutritional value of the black soldier fly (Hermetia illucens L.) and its suitability as animal feed–a review. *Journal of Insects as Food and Feed*, *3,* 105-120, doi: https://doi.org/10.3920/JIFF2016.0055.

Barragan-Fonseca, K.B., Dicke, M. and van Loon, J.J., 2018. Influence of larval density and dietary nutrient concentration on performance, body protein, and fat contents of black soldier fly larvae (Hermetia illucens). *Entomologia experimentalis et applicata*, *166,* 761-770, doi: https://doi.org/10.1111/eea.12716.

678 Bava, R., Castagna, F., Lupia, C., Poerio, G., Liguori, G., Lombardi, R., Naturale, M.D., Mercuri,
679 C., Bulotta, R.M., Britti, D., Palma, E., 2024. Antimicrobial Resistance in Livestock: A Serious
680 Threat to Public Health. *Antibiotics*, 13, 551, doi:
681 https://doi.org/10.3390/antibiotics13060551.
682 Begum, S., Hazarika, G.C. and Rajkhowa, S., 2014. Prevalence of Escherichia coli from pigs
683 and cattle. *Journal of Animal Health and Production*, *2,* 38-39, doi:
684 http://dx.doi.org/10.14737/journal.jahp/2014/2.3.38.39.
685 Benestad, S.L., Tran, L., Malzahn, A.M., Liland, N.S., Belghit, I., Hagemann, A., 2024.
686 Retention of prions in the polychaete *Hediste diversicolor* and black soldier fly, *Hermetia*
687 *illucens*, larvae after short-term experimental immersion and feeding with brain
688 homogenate from scrapie infected sheep. *Heliyon, 10,* doi:
689 https://doi.org/10.1016/j.heliyon.2024.e34848.
690 Beyers, M., Coudron, C., Ravi, R., Meers, E., Bruun, S., 2023. Black soldier fly larvae as an
691 alternative feed source and agro-waste disposal route – A life cycle perspective. *Resources,*
692 *Conservation and Recycling*, 192, 106917, doi:
693 https://doi.org/10.1016/j.resconrec.2023.106917.
694 Biancarosa, I., Liland, N.S., Biemans, D., Araujo, P., Bruckner, C.G., Waagbø, R., Torstensen,
695 B.E., Lock, E.J., Amlund, H., 2018. Uptake of heavy metals and arsenic in black soldier fly
696 (Hermetia illucens) larvae grown on seaweed-enriched media. *Journal of the Science of Food*
697 *and Agriculture,* 98, 2176-2183, doi: https://doi.org/10.1002/jsfa.8702.
698 Biteau, C., Bry-Chevalier, T., Crummett, D., Loewy, K., Ryba, R., Jules, M.S., 2024. Have the
699 environmental benefits of insect farming been overstated? A critical review (preprint).
700 *EcoEvoRxiv*, doi: https://doi.org/10.32942/X2W60R

Boafo, H.A., Gbemavo, D.S.J.C., Timpong-Jones, E.C., Eziah, V., Billah, M., Chia, S.Y., Aidoo, O.F., Clottey, V.A., Kenis, M., 2023. Substrates most preferred for black soldier fly *Hermetia illucens* (L.) oviposition are not the most suitable for their larval development. *Journal of Insects as Food and Feed,* 9, 183-192, doi:https://doi.org/10.3920/JIFF2022.0034.

Bohm, K., Taylor, W., Gyawali, P., Pattis, I., Gutiérrez Ginés, M.J., 2024. Black soldier fly-based bioconversion of biosolids: Microbial community dynamics and fate of antibiotic resistance genes. *Science of The Total Environment,* 930, 172823, doi:https://doi.org/10.1016/j.scitotenv.2024.172823.

Bonk, F., Popp, D., Harms, H. and Centler, F., 2018. PCR-based quantification of taxa-specific abundances in microbial communities: Quantifying and avoiding common pitfalls. *Journal of microbiological methods*, *153*, 139-147, doi: https://doi.org/10.1016/j.mimet.2018.09.015

Bruno, D., Bonelli, M., Filippis, F.D., Lelio, I.D., Tettamanti, G., Casartelli, M., Ercolini, D., Caccia, S., 2019. The Intestinal Microbiota of *Hermetia illucens* Larvae Is Affected by Diet and Shows a Diverse Composition in the Different Midgut Regions. *Applied and Environmental Microbiology,* 85, doi: https://doi.org/10.1128/AEM.01864-18.

Burdett, V., 1996. Tet(M)-promoted release of tetracycline from ribosomes is GTP dependent. J*ournal Of Bacteriology* 178, 3246-3251, doi: https://doi.org/10.1128/jb.178.11.3246-3251.1996.

Byrne-Bailey, K.G., Gaze, W.H., Kay, P., Boxall, A.B.A., Hawkey, P.M., Wellington, E.M.H., 2009. Prevalence of Sulfonamide Resistance Genes in Bacterial Isolates from Manured Agricultural Soils and Pig Slurry in the United Kingdom. *Antimicrobial Agents and Chemotherapy,* 53, 696-702. doi: https://doi.org/10.1128/aac.00652-07.

Cai, M., Ma, S., Hu, R., Tomberlin, J.K., Thomashow, L.S., Zheng, L., Li, W., Yu, Z., Zhang, J., 2018. Rapidly mitigating antibiotic resistant risks in chicken manure by *Hermetia illucens*

725 bioconversion with intestinal microflora. *Environmental Microbiology*, 20, 4051-4062.
726 doi:https://doi.org/10.1111/1462-2920.14450.
727 Carroll K.C., & Hobden J.A., & Miller S, & Morse S.A., & Mietzner T.A., & Detrick B, & Mitchell
728 T.G., & McKerrow J.H., & Sakanari J.A., 2019 Spore-forming gram-positive bacilli: bacillus
729 and clostridium species., *Jawetz, Melnick, & Adelberg's Medical Microbiology, 27,*
730 https://accessmedicine.mhmedical.com/content.aspx?bookid=1551&sectionid=94106389
731 Chen, J., Cai, Y., Deng, W., Xing, S., Liao, X., 2022. Transmission of tetracycline resistance
732 genes and microbiomes from manure-borne black soldier fly larvae frass to rhizosphere soil
733 and pakchoi endophytes. *Frontiers in Microbiology,* 13,
734 doi:https://doi.org/10.3389/fmicb.2022.1014910.
735 Chiu, C.H., Ou, J.T., 1996. Rapid identification of Salmonella serovars in feces by specific
736 detection of virulence genes, invA and spvC, by an enrichment broth culture-multiplex PCR
737 combination assay. *Journal of Clinical Microbiology*, 34, 2619-2622, doi:
738 https://doi.org/10.1128/jcm.34.10.2619-2622.1996.
739 Csordas, A.T., Barak, J.D., Delwiche, M.J., 2004. Comparison of primers for the detection of
740 *Salmonella enterica* serovars using real-time PCR. *Letters in Applied Microbiology,* 39, 187-
741 193. doi: https://doi.org/10.1111/j.1472-765X.2004.01559.x.
742 De Filippis, F., Bonelli, M., Bruno, D., Sequino, G., Montali, A., Reguzzoni, M., Pasolli, E., Savy,
743 D., Cangemi, S., Cozzolino, V., Tettamanti, G., Ercolini, D., Casartelli, M., Caccia, S., 2023.
744 Plastics shape the black soldier fly larvae gut microbiome and select for biodegrading
745 functions. *Microbiome*, 11, 205. doi: https://doi.org/10.1186/s40168-023-01649-0.
746 Diener, S., Zurbrügg, C., Tockner, K., 2015. Bioaccumulation of heavy metals in the black
747 soldier fly, Hermetia illucens and effects on its life cycle. *Journal of Insects as Food and*
748 *Feed,*1, 261-270. doi:https://doi.org/10.3920/JIFF2015.0030.

Diola, C.S., Nacilla, E.J., Pardillo, C.A., Alosbaños, R.S., Evacitas, F., Maglangit, F.J.P.J.o.S., 2024. Waste Reduction and Bioconversion of Quail, Chicken, and Pig Manure by Black Soldier Fly (*Hermetia illucens* L.). *Philippine Journal of Science*, 153, doi: https://doi.org/10.56899/153.02.11.

Dong, L., Ariëns, R.M., America, A.H., Paul, A., Veldkamp, T., Mes, J.J., Wichers, H.J. and Govers, C., 2021. *Clostridium perfringens* suppressing activity in black soldier fly protein preparations. *LWT – Food Science and Technology*, 149, 111806, doi: https://doi.org/10.1016/j.lwt.2021.111806.

Elhag, O., Zhang, Y., Xiao, X., Cai, M., Zheng, L., Jordan, H.R., Tomberlin, J.K., Huang, F., Yu, Z., Zhang, J., 2022. Inhibition of Zoonotic Pathogens Naturally Found in Pig Manure by Black Soldier Fly Larvae and Their Intestine Bacteria. *Insects*, 13, 66, doi: https://doi.org/10.3390/insects13010066.

Erickson, M.C., Islam, M., Sheppard, C., Liao, J., Doyle, M.P., 2004. Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Enteritidis in Chicken Manure by Larvae of the Black Soldier Fly. *Journal of Food Protection*, 67, 685-690, doi:https://doi.org/10.4315/0362-028X-67.4.685.

Feinman, S.E., Matheson, J.C., 1978. Draft environmental impact statement: subtherapeutic antibacterial agents in animal feeds. Department of Health, Education, and Welfare, Public Health Service, Food and Drug Administration, Bureau of Veterinary Medicine.

Fernández-Labrada, M., López-Mosquera, M.E., García, L., Barrio, J.C., López-Fabal, A., 2023. Hazards of swine slurry: Heavy metals, bacteriology, and overdosing—Physicochemical models to predict the nutrient value. *Animal Science Journal,* 94, e13849. doi:https://doi.org/10.1111/asj.13849.

772 Fu, C., Cheema, W.A., Mobashar, M., Shah, A.A., Alqahtani, M.M., 2025. Insects as
773 Sustainable Feed: Enhancing Animal Nutrition and Reducing Livestock Environmental
774 Impression. *Journal of Animal Physiology and Animal Nutrition*, 109, 280-290.
775 doi:https://doi.org/10.1111/jpn.14055.
776 Gold, M., Cassar, C.M., Zurbrügg, C., Kreuzer, M., Boulos, S., Diener, S., Mathys, A., 2020.
777 Biowaste treatment with black soldier fly larvae: Increasing performance through the
778 formulation of biowastes based on protein and carbohydrates. *Waste Management*, 102,
779 319-329, doi:https://doi.org/10.1016/j.wasman.2019.10.036.
780 Grassauer, F., Ferdous, J., Pelletier, N., 2023. Manure Valorization Using Black Soldier Fly
781 Larvae: A Review of Current Systems, Production Characteristics, Utilized Feed Substrates,
782 and Bioconversion and Nitrogen Conversion Efficiencies. *Sustainability*, 15, 12177, doi:
783 https://doi.org/10.3390/su151612177.
784 Grenda T, Kwiatek K, Goldsztejn M, Sapała M, Kozieł N, Domaradzki P., 2021. Clostridia in
785 Insect Processed Animal Proteins—Is an Epidemiological Problem Possible? *Agriculture,* 11,
786 270, doi: https://doi.org/10.3390/agriculture11030270
787 Haenelt, S., Wang, G., Kasmanas, J.C., Musat, F., Richnow, H.H., da Rocha, U.N., Müller, J.A.,
788 Musat, N., 2023. The fate of sulfonamide resistance genes and anthropogenic pollution
789 marker intI1 after discharge of wastewater into a pristine river stream. *Frontiers in*
790 *microbiology,* 14, doi: https://doi.org/10.3389/fmicb.2023.1058350.
791 Heuer, H., Focks, A., Lamshöft, M., Smalla, K., Matthies, M., Spiteller, M., 2008. Fate of
792 sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial
793 resistance genes in manure and manured soil. *Soil Biology and Biochemistry*, 40, 1892-1900,
794 doi:https://doi.org/10.1016/j.soilbio.2008.03.014.

795 Hoek-van den Hil, E.F., Meijer, N.P., Van Rozen, K., Elissen, H., van Wikselaar, P.G., Brust, H.,
796 Te Loeke, N.A.J.M., de Rijk, T., Tienstra, M., van de Schans, M.G.M., Wanrooij, J.,
797 Van der Weide, R., Veldkamp, T., van der Fels-Klerx, H.J., 2023. Safety of black soldier fly
798 (*Hermetia illucens*) larvae reared on waste streams of animal and vegetal origin and manure.
799 *Journal of Insects as Food and Feed*, 10, 771-783, doi:https://doi.org/10.1163/23524588-
800 20230080.
801 Hoffmans, Y., Veldkamp, T., Meijer, N.P., Brust, G.M.H., van der Schans, M.G.M., Prins, T.W.,
802 van Rozen, K., Elissen, H., van Wikselaar, P., van der Weide, R., van der Fels-Klerx, H.J., Hoek-
803 van den Hil, E.F., 2024. Can black soldier fly larvae (*Hermetia illucens*) be reared on waste
804 streams for food and feed? – A safety perspective. *Journal of Insects as Food and Feed*, 10,
805 1211-1221, doi:https://doi.org/10.1163/23524588-20230169.
806 Hurtado-Ribeira, R., Silvan, J.M., Fornari, T., Vázquez, L., Martinez-Rodriguez, A.J., Martin,
807 D., 2023. Modulation of the lipolysis and subsequent antibacterial activity of the fat from
808 black soldier fly (*Hermetia illucens*) by the combined selection of slaughtering, drying and
809 defatting methods of the larvae. *Innovative Food Science & Emerging Technologies,* 90,
810 103510, doi:https://doi.org/10.1016/j.ifset.2023.103510.
811 Hutchison, M.L., Walters, L.D., Avery, S.M., Munro, F., Moore, A., 2005. Analyses of
812 Livestock Production, Waste Storage, and Pathogen Levels and Prevalences in Farm
813 Manures. *Applied and Environmental Microbiology*, 71, 1231-1236, doi:
814 https://doi.org/10.1128/AEM.71.3.1231-1236.2005.
815 Inglis, G.D., Kalischuk, L.D., 2004. Direct Quantification of *Campylobacter jejuni* and
816 *Campylobacter lanienae* in Feces of Cattle by Real-Time Quantitative PCR. *Applied and*
817 *Environmental Microbiology*, 70, 2296-2306, doi: https://doi.org/10.1128/AEM.70.4.2296-
818 2306.2004.

819 Jiang, D., Jiang, K., Li, R., Zhao, L., Liu, Z., Xiong, B., Jin, D., Hao, X., Zhu, L., Kang, B., Bai, L.,
820 2022. Influence of different inoculation densities of black soldier fly larvae (*Hermetia*
821 *illucens*) on heavy metal immobilization in swine manure. *Environmental Science and*
822 *Pollution Research International*, 29, 54378-54390, doi: https://doi.org/10.1007/s11356-
823 022-19623-y.
824 Kawasaki, K., Kawasaki, T., Hirayasu, H., Matsumoto, Y., Fujitani, Y., 2020. Evaluation of
825 Fertilizer Value of Residues Obtained after Processing Household Organic Waste with Black
826 Soldier Fly Larvae (*Hermetia illucens*). *Sustainability*, 12, 4920, doi:
827 https://doi.org/10.3390/su12124920.
828 Khayrova, A., Lopatin, S., Varlamov, V.J.I.J.S., 2019. Black soldier fly *Hermetia illucens* as a
829 novel source of chitin and chitosan. *International Journal of Sciences*, 8, 81-86, doi:
830 http://dx.doi.org/10.18483/ijSci.2015
831 Klammsteiner, T., Heussler, C.D., Insam, H., Schlick-Steiner, B.C. and Steiner, F.M., 2025.
832 Larval density drives thermogenesis and affects microbiota and substrate properties in black
833 soldier fly trials. *iScience*, 28, doi: https://doi.org/10.1016/j.isci.2025.112794.
834 Klappenbach, J.A., Dunbar, J.M. and Schmidt, T.M., 2000. rRNA operon copy number reflects
835 ecological strategies of bacteria. *Applied and Environmental Microbiology*, 66, 1328-1333,
836 doi: https://doi.org/10.1128/AEM.66.4.1328-1333.2000
837 Knapp, C.W., McCluskey, S.M., Singh, B.K., Campbell, C.D., Hudson, G. and Graham, D.W.,
838 2011. Antibiotic resistance gene abundances correlate with metal and geochemical
839 conditions in archived Scottish soils. *PloS one*, *6, doi:*
840 *https://doi.org/10.1371/journal.pone.0027300*

Köninger, J., Lugato, E., Panagos, P., Kochupillai, M., Orgiazzi, A., & Briones, M. J. (2021). Manure management and soil biodiversity: Towards more sustainable food systems in the EU. *Agricultural Systems*, 194, 103251, doi: https://doi.org/10.1016/j.agsy.2021.103251

Lalander, C., Diener, S., Magri, M.E., Zurbrügg, C., Lindström, A., Vinnerås, B., 2013. Faecal sludge management with the larvae of the black soldier fly (*Hermetia illucens*) — From a hygiene aspect. *Science of The Total Environment*, 458-460, 312-318, doi: https://doi.org/10.1016/j.scitotenv.2013.04.033.

Li, Q., Zheng, L., Cai, H., Garza, E., Yu, Z., Zhou, S., 2011. From organic waste to biodiesel: Black soldier fly, *Hermetia illucens*, makes it feasible. *Fuel,* 90, 1545-1548, doi:https://doi.org/10.1016/j.fuel.2010.11.016.

Lievens, S., Poma, G., De Smet, J., Van Campenhout, L., Covaci, A., Van Der Borght, M., 2021. Chemical safety of black soldier fly larvae (*Hermetia illucens*), knowledge gaps and recommendations for future research: a critical review. *Journal of Insects as Food and Feed,* 7, 383-396, doi:https://doi.org/10.3920/JIFF2020.0081.

Lima, T., Domingues, S. and Da Silva, G.J., 2020. Manure as a potential hotspot for antibiotic resistance dissemination by horizontal gene transfer events. *Veterinary sciences*, *7*, 110, doi: https://doi.org/10.3390/vetsci7030110.

Lin, C., Xia, X., Li, Y., Ma, R., Zhu, L., Li, X., Tang, Y., Wang, C., 2023. Heavy metals transport patterns and risk evaluation in the pig manure- black soldier fly-tilapia food chain. *Environmental Pollution*, 337, 122565, doi:https://doi.org/10.1016/j.envpol.2023.122565.

Lomonaco, G., Franco, A., De Smet, J., Scieuzo, C., Salvia, R., Falabella, P., 2024. Larval Frass of *Hermetia illucens* as Organic Fertilizer: Composition and Beneficial Effects on Different Crops. *Insects*, 15, doi:10.3390/insects15040293.

Lopes, I.G., Yong, J.W.H., Lalander, C., 2022. Frass derived from black soldier fly larvae treatment of biodegradable wastes. A critical review and future perspectives. *Waste Management*, 142, 65-76, doi:https://doi.org/10.1016/j.wasman.2022.02.007.

Malematja, E., Manyelo, T.G., Sebola, N.A., Kolobe, S.D., Mabelebele, M., 2023. The accumulation of heavy metals in feeder insects and their impact on animal production. *Science of The Total Environment*, 885, 163716, doi:https://doi.org/10.1016/j.scitotenv.2023.163716.

Marshall, B.M., Levy, S.B. 2011. Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews Journal*, 24, 718-733, doi: https://doi.org/10.1128/cmr.00002-11.

McDowall, L.S., McDermott, K., 2025. Opportunities and challenges for the pork industry from circular livestock waste. *animal*, doi: https://doi.org/10.1016/j.animal.2025.101547.

Meijer, N., Safitri, R.A., Tao, W. and Hoek-Van den Hil, E.F., 2025. European Union legislation and regulatory framework for edible insect production–Safety issues. *animal*, https://doi.org/10.1016/j.animal.2025.101468.

Milanović, V., Cardinali, F., Aquilanti, L., Maoloni, A., Garofalo, C., Zarantoniello, M., Olivotto, I., Riolo, P., Ruschioni, S., Isidoro, N. and Cattalani, M., 2021. Quantitative assessment of transferable antibiotic resistance genes in zebrafish (*Danio rerio*) fed Hermetia illucens-based feed. *Animal Feed Science and Technology*, *277*, https://doi.org/10.1016/j.anifeedsci.2021.114978.

Molina, F., López-Acedo, E., Tabla, R., Roa, I., Gómez, A., Rebollo, J.E., 2015. Improved detection of *Escherichia coli* and coliform bacteria by multiplex PCR. *BMC Biotechnology*, 15, 48, doi: https://doi.org/10.1186/s12896-015-0168-2.

887 Mufungwe, J., Namukonde, N., Mwaanga, P., 2025 Critical safety concerns in the production
888 of black soldier Fly (*Hermetia illucens*) larvae in Africa. *Discovery Food* **5,**
889 https://doi.org/10.1007/s44187-025-00355-0
890 Nagendran, R., 2011. Agricultural waste and pollution. In *Waste* (341-355). Academic Press,
891 doi: https://doi.org/10.1016/B978-0-12-381475-3.10024-5
892 Nasir, I.M., Mohd Ghazi, T.I., Omar, R., 2012. Anaerobic digestion technology in livestock
893 manure treatment for biogas production: A review. *Engineering in Life Sciences*, 12, 258-
894 269, doi:https://doi.org/10.1002/elsc.201100150.
895 Nayak, A. and Klüber, P. 2025. The hidden drivers: Unravelling the impact of density,
896 moisture, and scale on Hermetia illucens rearing. *PLOS ONE*. 20(1), doi:
897 https://doi.org/10.1371/journal.pone.0317049
898 Newton, L., Sheppard, C., Watson, W.D., Burtle, G., Dove, R., 2005. Using the black soldier
899 fly, *Hermetia illucens*, as a value-added tool for the management of swine manure. *Animal*
900 *Poultry Waste Management Center*, North Carolina State University, Raleigh, NC 17, 18.
901 Nguyen, T.T., Tomberlin, J.K. and Vanlaerhoven, S., 2015. Ability of black soldier fly (Diptera:
902 Stratiomyidae) larvae to recycle food waste. *Environmental Entomology*, *44,* 406-410, doi:
903 https://doi.org/10.1093/ee/nvv002.Nicholson, F.A., Smith, S.R., Alloway, B.J., Carlton-Smith,
904 C., Chambers, B.J., 2006. Quantifying heavy metal inputs to agricultural soils in England and
905 Wales. *Water and Environment Journal*, 20, 87-95, doi:https://doi.org/10.1111/j.1747-
906 6593.2006.00026.x.
907 Ogbuewu, I., Odoemenam, V., Omede, A., Durunna, C., Emenalom, O., Uchegbu, M., Okoli,
908 I., Iloeje, M.J.S.J.o.r., 2012. Livestock waste and its impact on the environment. *Scientific*
909 *Journal of Review*, 1, 17-32.

910 OIE, 2018. Annual report on antimicrobial agents intended for use in animals. In World
911 Organization for Animal Health Paris, France, Better Understanding of the Global Situation.
912 Oonincx, D.G.A.B., van Huis, A., van Loon, J.J.A., 2015. Nutrient utilisation by black soldier
913 flies fed with chicken, pig, or cow manure. *Journal of Insects as Food and Feed,* 1, 131-140,
914 doi:https://doi.org/10.3920/JIFF2014.0023.
915 Parodi, A., Gerrits, W.J.J., Van Loon, J.J.A., De Boer, I.J.M., Aarnink, A.J.A., Van Zanten,
916 H.H.E., 2021. Black soldier fly reared on pig manure: Bioconversion efficiencies, nutrients in
917 the residual material, greenhouse gas and ammonia emissions. *Waste Management*, 126,
918 674-683, doi:https://doi.org/10.1016/j.wasman.2021.04.001.
919 Pastor, B., Velasquez, Y., Gobbi, P., Rojo, S.J.J.o.I.a.F., Feed, 2015. Conversion of organic
920 wastes into fly larval biomass: bottlenecks and challenges. *Journal of Insects as Food and*
921 *Feed*, 1, 179-194, doi: https://doi.org/10.3920/JIFF2014.0024.
922 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-
923 PCR. *Nucleic Acids Research*, 29, e45. doi:https://doi.org/10.1093/nar/29.9.e45 .
924 Provolo, G., Manuli, G., Finzi, A., Lucchini, G., Riva, E., Sacchi, G.A., 2018. Effect of Pig and
925 Cattle Slurry Application on Heavy Metal Composition of Maize Grown on Different Soils.
926 *Sustainability*, 10, 2684, doi: https://doi.org/10.3390/su10082684
927 Purschke, B., Scheibelberger, R., Axmann, S., Adler, A., Jäger, H., 2017. Impact of substrate
928 contamination with mycotoxins, heavy metals and pesticides on the growth performance
929 and composition of black soldier fly larvae (*Hermetia illucens*) for use in the feed and food
930 value chain. *Food Additives & Contaminants*, 34, 1410-1420, doi:
931 https://doi.org/10.1080/19440049.2017.1299946.

Rai, P.K., Lee, S.S., Zhang, M., Tsang, Y.F., Kim, K.-H., 2019. Heavy metals in food crops: Health risks, fate, mechanisms, and management. *Environment International*, 125, 365-385, doi:https://doi.org/10.1016/j.envint.2019.01.067.

Rehman, K.u., Hollah, C., Wiesotzki, K., Rehman, R.u., Rehman, A.U., Zhang, J., Zheng, L., Nienaber, T., Heinz, V., Aganovic, K., 2022. Black soldier fly, *Hermetia illucens* as a potential innovative and environmentally friendly tool for organic waste management: A mini-review. *Waste Management & Research*, 41, 81-97,doi: https://doi.org/10.1177/0734242X221105441.

Roberts, M.C., 2005. Update on acquired tetracycline resistance genes. *FEMS Microbiology Letters*, 245, 195-203, doi: https://doi.org/10.1016/j.femsle.2005.02.034.

Romero-Rodríguez A, Ruiz-Villafán B, Martínez-de la Peña CF, Sánchez S., 2023. Targeting the Impossible: A Review of New Strategies against Endospores. *Antibiotics*, 12, 248, doi: https://doi.org/10.3390/antibiotics12020248

Sall, M.L., Diaw, A.K.D., Gningue-Sall, D., Efremova Aaron, S., Aaron, J.J., 2020. Toxic heavy metals: impact on the environment and human health, and treatment with conducting organic polymers, a review. *Environmental Science and Pollution Research*, 27, doi: https://doi.org/10.1007/s11356-020-09354-3.

Schreven, S.J.J., de Vries, H., Hermes, G.D.A, Smidt, H., Dicke, M. and van Loon, J.J.A., 2021. Relative contributions of egg-associated and substrate-associated microorganisms to black soldier fly larval performance and microbiota. *FEMS Microbiology Ecology*, 97, doi: https://doi.org/10.1093/femsec/fiab054

Schreven, S.J.J., de Vries, H.., Hermes, G.D.A., Zeni, G., Smidt, H., Dicke, M., Loon, J.J.A.v., 2022. Black Soldier Fly Larvae Influence Internal and Substrate Bacterial Community

955 Composition Depending on Substrate Type and Larval Density. *Applied and Environmental*
956 *Microbiology*, 88, doi: https://doi.org/10.1128/aem.00084-22.
957 Shelomi, M., 2024. Mitigation Strategies against Food Safety Contaminant Transmission
958 from Black Soldier Fly Larva Bioconversion. *Animals*, 14, 1590, doi:
959 https://doi.org/10.3390/ani14111590.
960 Sheppard, C.D., Newton, L.G., Thompson, S.A., Savage, S., 1994. A value added manure
961 management system using the black soldier fly. *Bioresource Technology*, 50, 275-279.
962 doi:https://doi.org/10.1016/0960-8524(94)90102-3.
963 Sheriff, I., Yusoff, M.S., Manan, T.S.B.A., Koroma, M., 2023. Microplastics in manure:
964 Sources, analytical methods, toxicodynamic, and toxicokinetic endpoints in livestock and
965 poultry. *Environmental Advances*, 12, 100372,
966 doi:https://doi.org/10.1016/j.envadv.2023.100372.
967 Shi, C., Xie, P., Ding, Z., Niu, G., Wen, T., Gu, W., Lu, Y., Wang, F., Li, W., Zeng, J., Shen, Q.,
968 Yuan, J., 2024. Inhibition of pathogenic microorganisms in solid organic waste via black
969 soldier fly larvae-mediated management. *Science of The Total Environment,* 913, 169767,
970 doi:https://doi.org/10.1016/j.scitotenv.2023.169767.
971 Siddiqui, S.A., Fernando, I., Nisa, K., Shah, M.A., Rahayu, T., Rasool, A., Aidoo, O.F., 2023.
972 Effects of undesired substances and their bioaccumulation on the black soldier fly larvae,
973 *Hermetia illucens* (Diptera: Stratiomyidae)-a literature review. *Environmental Monitoring*
974 *and Assessment*, 195, 823. doi: https://doi.org/10.1007/s10661-023-11186-w.
975 Silvaraju, S., Zhang, Q.-h., Kittelmann, S. and Puniamoorthy, N. 2024. Genetics, age, and diet
976 influence gut bacterial communities and performance of black soldier fly larvae (Hermetia
977 illucens). *Animal Microbiome.* 6(1). doi: https://doi.org/10.1186/s42523-024-00340-5

978 Skinner, J.A., Lewis, K.A., Bardon, K.S., Tucker, P., Catt, J.A., Chambers, B.J., 1997. An
979 Overview of the Environmental Impact of Agriculture in the U.K. J*ournal of Environmental*
980 *Management,* 50, 111-128, doi:https://doi.org/10.1006/jema.1996.0103.
981 Sköld, O., 2000. Sulfonamide resistance: mechanisms and trends. *Drug Resistance Updates* ,
982 3, 155-160, doi:https://doi.org/10.1054/drup.2000.0146.
983 Smith, K.A., Williams, A.G., 2016. Production and management of cattle manure in the UK
984 and implications for land application practice. *Soil Use Management*, 32, 73-82,
985 doi:https://doi.org/10.1111/sum.12247.
986 Tanga, C.M., Beesigamukama, D., Kassie, M., Egonyu, P.J., Ghemoh, C.J., Nkoba, K.,
987 Subramanian, S., Anyega, A.O., Ekesi, S., 2022. Performance of black soldier fly frass fertilizer
988 on maize (*Zea mays* L.) growth, yield, nutritional quality, and economic returns. *Journal of*
989 *Insects as Food and Feed* 8, 185-196, doi:https://doi.org/10.3920/JIFF2021.0012.
990 United Nations, Global Issues: Population. URL:https://www.un.org/en/global-
991 issues/population
992 Villa, T.G., Feijoo-Siota, L., Sánchez-Pérez, A., Rama, J.R. and Sieiro, C., 2019. Horizontal gene
993 transfer in bacteria, an overview of the mechanisms involved. *Horizontal gene transfer:*
994 *Breaking borders between living kingdoms*, 3-76, doi: https://doi.org/10.1007/978-3-030-
995 21862-1_1
996 Vitenberg, T., Opatovsky, I., 2022. Assessing Fungal Diversity and Abundance in the Black
997 Soldier Fly and its Environment. *Journal of Insect Science*, 22, doi:
998 https://doi.org/10.1093/jisesa/ieac066.
999 Wales, A.D., Davies, R.H., 2015. Co-Selection of Resistance to Antibiotics, Biocides and Heavy
1000 Metals, and Its Relevance to Foodborne Pathogens. *Antibiotics*,4, 567-604, doi:
1001 https://doi.org/10.3390/antibiotics4040567.

Wang, L., Wang, S., Yang, R., Zhang, B., Xu, L., Hu, Q., Zhao, Z., Cao, Z., 2024. Effect of moisture content on larval gut microbiome and the conversion of pig manure by black soldier fly. *Science of The Total Environment*, 912, 169579, doi:https://doi.org/10.1016/j.scitotenv.2023.169579.

Wang, N., Yang, X., Jiao, S., Zhang, J., Ye, B., Gao, S., 2014. Sulfonamide-Resistant Bacteria and Their Resistance Genes in Soils Fertilized with Manures from Jiangsu Province, Southeastern China. *PLoS One*, 9, doi: https://doi.org/10.1371/journal.pone.0112626.

Wang, R.-F., Cao, W.-W., Cerniglia, C.E., 1997. A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. *Journal of Applied Microbiology*, 83, 727-736, doi:https://doi.org/10.1046/j.1365-2672.1997.00300.x.

Wang, X.-b., Wu, N., Cai, R.-j., Geng, W.-n., Xu, X.-y., 2021. Changes in speciation, mobility and bioavailability of Cd, Cr and As during the transformation process of pig manure by black soldier fly larvae (*Hermetia illucens*). *Journal of Integrative Agriculture*, 20, 1157-1166, doi:https://doi.org/10.1016/S2095-3119(20)63333-0.

Wang, X., Cai, R., Geng, W., Mao, Z., Zhao, Y., Jiang, H., Xu, X., 2020. Migration and changes of heavy metals during biotransformation of pig manure by black soldier fly. *Transactions of the Chinese Society of Agricultural Engineering*, 36, 263-268, doi:https://doi.org/10.11975/j.issn.1002-6819.2020.20.031.

Wang, X., Wu, N., Ma, Y., Wang, Z., Cai, R., Xu, X., 2022. Migration and Transformation of Cd in Pig Manure-Insect Frass (*Hermetia illucens*)-Soil-Maize System. *International Journal of Environmental Research and Public Health*, 20, doi: https://doi.org/10.3390/ijerph20010060.

Williams, M.L., Ghanem, M., 2022. Evaluation of candidate reference genes stability for gene expression analysis by reverse transcription qPCR in *Clostridium perfringens*. *Scientific Reports*, 12, 19434, doi: https://doi.org/10.1038/s41598-022-23804-7.

Wolffs, P., Norling, B. and Rådström, P., 2005. Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. *Journal of Microbiological Methods*, *60*, 315-323, doi: https://doi.org/10.1016/j.mimet.2004.10.003

Wu, N., Ma, Y., Yu, X., Wang, X., Wang, Q., Liu, X., Xu, X., 2024. Black soldier fly larvae bioconversion and subsequent composting promote larval frass quality during pig and chicken manure transformation process. *Bioresource Technology,* 402, 130777, doi:https://doi.org/10.1016/j.biortech.2024.130777.

Wu, N., Wang, X., Xu, X., Cai, R., Xie, S., 2020. Effects of heavy metals on the bioaccumulation, excretion and gut microbiome of black soldier fly larvae (*Hermetia illucens*). *Ecotoxicology and Environmental Safety*, 192, 110323, doi:https://doi.org/10.1016/j.ecoenv.2020.110323.

Wu, N., Wang, X., Yan, Z., Xu, X., Xie, S., Liang, J., 2021. Transformation of pig manure by passage through the gut of black soldier fly larvae (*Hermetia illucens*): Metal speciation, potential pathogens and metal-related functional profiling. *Ecotoxicology and Environmental Safety*, 211, 111925, doi:https://doi.org/10.1016/j.ecoenv.2021.111925.

Xia, J., Ge, C., Yao, H., 2021. Antimicrobial Peptides from Black Soldier Fly (*Hermetia illucens*) as Potential Antimicrobial Factors Representing an Alternative to Antibiotics in Livestock Farming. *Animals*, 11, doi: https://doi.org/10.3390/ani11071937.

Xiao, Y., Geng, W., Yang, Y., Wang, X., Xu, X., 2020. Study on the Difference of Transformation of Livestock and Poultry Feces by Black Soldier Fly. *IOP Conference Series:*

Earth and Environmental Science, 450, 012122, doi: https://doi.org/10.1088/1755-1315/450/1/012122.

Xu, L., Wang, W., Xu, W., 2022. Effects of tetracycline antibiotics in chicken manure on soil microbes and antibiotic resistance genes (ARGs). *Environmental Geochemistry and Health*, 44, 273-284, doi: https://doi.org/10.1007/s10653-021-01004-y.

Yakti, W., Schulz, S., Marten, V., Mewis, I., Padmanabha, M., Hempel, A.-J., Kobelski, A., Streif, S. and Ulrichs, C.J.S., 2022. The effect of rearing scale and density on the growth and nutrient composition of *Hermetia illucens* (L.)(Diptera: Stratiomyidae) larvae. *Sustainability* 14(3), doi: https://doi.org/10.3390/su14031772

Yang, F., Tomberlin, J.K. and Jordan, H.R., 2021. Starvation alters gut microbiome in black soldier fly (Diptera: Stratiomyidae) larvae. *Frontiers in Microbiology*, *12*, 601253, doi: https://doi.org/10.3389/fmicb.2021.601253.

Zhao, Z., Yang, C., Gao, B., Wu, Y., Ao, Y., Ma, S., Jiménez, N., Zheng, L., Huang, F., Tomberlin, J.K., Ren, Z., Yu, Z., Yu, C., Zhang, J., Cai, M., 2023. Insights into the reduction of antibiotic-resistant bacteria and mobile antibiotic resistance genes by black soldier fly larvae in chicken manure. *Ecotoxicology and Environmental Safety*, 266, 115551, doi:https://doi.org/10.1016/j.ecoenv.2023.115551.