Sci

MDPI

Article

Diet-Driven Modulation of Antibiotic Resistance Genes and Microbial Risk During the Bioconversion of Agro-Industrial Residues by *Hermetia illucens*

Vesna Milanović, Andrea Marcelli, Alessio Ilari, Giorgia Rampanti *, Kofi Armah Boakye-Yiadom, Federica Cardinali, Andrea Osimani, Cristiana Garofalo, Ester Foppa Pedretti and Lucia Aquilanti

Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy; v.milanovic@staff.univpm.it (V.M.); a.marcelli@staff.univpm.it (A.M.); a.ilari@staff.univpm.it (A.I.); k.a.boakye@staff.univpm.it (K.A.B.-Y.); f.cardinali@staff.univpm.it (F.C.); a.osimani@staff.univpm.it (A.O.); c.garofalo@staff.univpm.it (C.G.); e.foppa@staff.univpm.it (E.F.P.); l.aquilanti@staff.univpm.it (L.A.)

* Correspondence: g.rampanti@staff.univpm.it

Abstract

Background: *Hermetia illucens* larvae provide a sustainable bioconversion pathway that transforms agro-industrial residues into protein- and nutrient-dense biomass and frass, suitable for animal feed and soil amendment, respectively. Nevertheless, the potential spread of antibiotic resistance (AR) genes and pathogenic microorganisms poses biosafety concerns. This study examined the impact of four residue-based diet formulations; peas and chickpea (D1), peas and wheat (D2), onion and wheat (D3), and wheat with digestate (D4), on microbial safety during the bioconversion process. **Methods**: *Enterococcus* spp. (viable counts), *Salmonella* spp. (presence/absence), and 13 AR genes associated with resistance to tetracyclines, macrolide-lincosamide-streptogramin B, β-lactams, vancomycin, and aminoglycosides were quantified in single substrates, diets, larvae, and frass using qPCR. **Results**: Principal component analysis revealed diet-driven AR gene profiles. D1 lowered the levels of the greatest number of tested AR genes, particularly *erm*(B), tetracycline, and β-lactam genes in frass, as well as *tet*(O) and *vanB* in mature larvae. In contrast, D2 increased the AR gene levels in frass. All diets except D4 eliminated *Salmonella* spp. *Enterococcus* spp. loads varied by diet and larval stage, with D2 reducing counts in frass. **Conclusions**: Diet composition directly shapes microbial dynamics and AR gene dissemination, indicating that legume-based substrates may enhance biosafety in bioconversion systems.

Keywords: agro-industrial residues; *Hermetia illucens*; antibiotic resistance genes; microbial safety; waste valorization; circular bioeconomy; *Salmonella* spp.; *Enterococcus* spp.

Academic Editor: Jose Navarro Pedreño

Received: 6 November 2025
Revised: 12 December 2025
Accepted: 31 December 2025
Published: 8 January 2026

1. Introduction

The rapid expansion of the global population, coupled with increasing urbanization and rising food insecurity, has intensified challenges associated with the management of agri-food and industrial waste. Organic residues generated by agriculture and food processing now represent a significant environmental issue that requires sustainable and innovative solutions [1]. In line with circular economy principles, strategies that emphasize reduction, reuse, and recycling have gained prominence as effective means to reduce the environmental footprint of organic waste [2]. Among these strategies, insect-based bioconversion has gained attention as a practical approach that transforms low-value

residues into high-value products. This approach supports waste reduction and contributes to sustainable protein production [3].

The black soldier fly (*Hermetia illucens*) efficiently converts diverse organic materials into nutrient-rich larval biomass characterized by high protein and lipid content, as demonstrated by previous studies [4,5]. This bioconversion process reduces the environmental burden of agri-food residues while generating valuable secondary products of agronomic interest. Among these products, frass, which consists of larval excreta, shed cuticles, and unconsumed substrate, has attracted growing attention as an organic fertilizer that can improve soil fertility and stimulate plant growth [6,7]. At the same time, mature larvae can be processed into feed ingredients for livestock, aquaculture, and companion animals. This use provides a sustainable alternative to conventional protein sources such as fishmeal and soybean meal, thereby alleviating pressure on overexploited marine and agricultural systems [8,9]. This circular strategy converts organic waste streams into high-value nutrient resources. It also strengthens environmental and economic sustainability objectives [10,11]. In addition to plant-derived residues, researchers are increasingly exploring organic waste substrates with complex microbial compositions, including digestate from anaerobic digestion, for *H. illucens* rearing. Although nutrient-rich digestate can improve soil fertility, its recalcitrant organic fractions degrade slowly and may limit direct application [12]. Using digestate as a larval substrate offers a promising strategy to recycle nutrients and increase protein yields [12,13].

Despite these benefits, the microbiological safety of *H. illucens* products remains a crucial issue, particularly regarding the potential spread of antibiotic resistance (AR) genes. The microbial composition of both larvae and their rearing substrates directly influences the hygienic quality of the resulting frass and biomass [14]. Studies indicate that rearing environments can harbor pathogens and AR determinants, and these elements may persist during the bioconversion process [14–17]. However, *H. illucens* larvae do not function only as passive carriers of microorganisms. They use immune responses, antimicrobial peptides, and gut microbial competition to counter microbial threats. These mechanisms suppress potential pathogens, such as *Salmonella* spp., and lower AR gene abundance in organic substrates [14].

Building on this background, the present study investigated the microbiological safety of *H. illucens*-mediated bioconversion of agri-food residues and anaerobic digestate. Specifically, it analyzes how four diet formulations affect microbial loads and AR gene profiles in larvae and frass and determines whether larval activity can reduce associated risks. Larvae were reared on four experimental diets prepared by combining agro-industrial residues: peas with chickpea (D1), peas with wheat (D2), onion with wheat (D3), and wheat with digestate (D4). The diet formulations included cereals, legumes, vegetables, and digestate to supply sufficient protein and carbohydrates for *H. illucens* larvae. This design minimized nutritional limitations that might impair larval growth and development. Microbiological analyses quantified presumptive *Enterococcus* spp. and detected *Salmonella* spp. in individual substrates, formulated diets, young larvae collected before bioconversion, mature larvae collected after bioconversion, and the resulting frass. Quantitative PCR (qPCR) assessed 13 AR genes that confer resistance to major antibiotic classes. These included tetracyclines [*tet*(M), *tet*(S), *tet*(W), *tet*(K), *tet*(O)], macrolide-lincosamide-streptogramin B (MLS_B) [*erm*(A), *erm*(B), *erm*(C)], β-lactams (*mecA*, *blaZ*), vancomycin (*vanA*, *vanB*), and aminoglycosides [*aac(6′)-Ie aph(2″)-Ia*]. The selected genes serve as representative indicators of clinically and environmentally relevant resistance mechanisms across multiple antibiotic classes. These genes are commonly detected in agricultural residues, animal-derived wastes, and insect-associated microbial communities [16,17]. This study offers new insights into how diet composition influences microbial safety and AR gene dynamics during *H. illucens*

bioconversion. The findings support the safe incorporation of insect-derived products into sustainable waste management, agriculture, and feed production systems.

2. Materials and Methods

2.1. Experimental Setup and Sampling

Rearing trials of *H. illucens* larvae were conducted under controlled conditions (30 °C, 80% relative humidity) in a specialized bioconversion facility, as described in detail by Marcelli et al. [18]. Four agro-industrial residue–based diets were tested: D1 (peas and chickpeas), D2 (peas and wheat), D3 (onions and wheat), and D4 (wheat and digestate). The individual substrates were weighed, ground twice to an average particle size of approximately 4 mm and manually mixed to produce homogeneous formulations. The fresh-to-dry ratios were 3.5:1 for D1 and D2, 3:1 for D3, and 2:1 for D4.

Each 12 kg batch of the resulting diet formulations was placed into polypropylene trays measuring 60 × 40 × 23 cm. About 150,000 *H. illucens* larvae, aged four to six days, were sourced from SmartBugs s.s. (Villorba, TV, Italy) and evenly distributed in the trays to reach a density of 4.88 larvae/cm^2. All treatments were maintained under uniform feeding conditions, with substrate moisture adjusted to 70% and each larva receiving 100 mg of feed per day. Each diet was tested in 12 replicate trays, except D4, which was tested in 6 replicates due to limited material availability.

Larvae were reared for 10 days, followed by a 2-day drying phase to facilitate larval and frass separation. Single substrates, formulated diets, young larvae (before the bioconversion process), as well as frass and mature larvae (after the bioconversion process), were aseptically collected and pooled to obtain three biological replicates per sample type for subsequent microbiological and molecular analyses.

2.2. Viable Counts

Prior to microbiological analyses, surface disinfection of *H. illucens* larvae was performed, and samples were homogenized for viable counts following the protocol described by Marcelli et al. [18]. Ten grams of each sample (substrates, diet formulations, larvae, and frass) were homogenized in sterile peptone water using a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy). The homogenates were serially diluted tenfold and plated on Enterococcus Selective Agar (Merck KGaA, Darmstadt, Germany) to enumerate presumptive *Enterococcus* spp. Plates were incubated at 37 °C for 48 h. Results from three biological and two technical replicates were expressed as the logarithm of colony-forming units per gram of sample (log CFU/g) and reported as mean ± standard deviation.

For the detection of *Salmonella* spp., 25 g of each sample were submitted to the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati". The analysis applied the enzyme-linked fluorescent assay (ELFA) method according to the AFNOR BIO 12/16-09/05 standard [19]. Results were reported as the presence or absence of *Salmonella* spp. in 25 g of sample.

2.3. DNA Extraction and qPCR Quantification of AR Genes

DNA was extracted from 1 mL aliquots of each homogenate (1:10 dilution) used for viable counts, following the protocol of Marcelli et al. [18]. Extraction blanks (negative controls without sample material) were included in each batch to monitor potential contamination. DNA quality and quantity were assessed spectrophotometrically. Successful extraction was confirmed by PCR using universal bacterial primers 27f–1495r [20].

qPCR was employed to detect and quantify genes associated with resistance to clinically relevant antibiotics, including MLS_B [*erm*(A), *erm*(B), *erm*(C)], vancomycin

(*vanA*, *vanB*), tetracyclines [*tet*(M), *tet*(O), *tet*(S), *tet*(K), *tet*(W)], β-lactams (*mecA*, *blaZ*), and aminoglycosides (*aac(6′)-Ie aph(2″)-Ia,* abbreviated as *aac-aph*), as described by Milanović et al. [16,17]. Calibration curves were generated by preparing tenfold serial dilutions of genomic DNA from 13 reference bacterial strains, each carrying one of the AR genes analyzed in this study. Amplifications were conducted on a CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA). Each qPCR reaction had a total volume of 10 µL, consisting of 4 µL of DNA, 5 µL of Type-it 2X HRM PCR Master Mix (Qiagen, Hilden, Germany), the appropriate forward and reverse primers for each AR gene (Table S1), and nuclease-free water. The thermal cycling program started with an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s. The instrument automatically calculated the amplification efficiencies (E) and correlation coefficients (R^2) from the slopes of the standard curves. The curves covered a range of <1 to 7 log gene copies per reaction. The specificity of the amplified products was verified through melt-curve analysis, in which the temperature increased gradually from 65 °C to 95 °C at a rate of 0.2 °C/s. Negative controls were included in all qPCR runs to confirm the absence of contaminating DNA in the reagents.

Gene copy numbers were quantified by comparing the amplification signals of the samples with those of the calibration standards. Results were expressed as the logarithm of the average gene copy number per gram of sample (log gene copies/g), calculated from three biological and three technical replicates, and reported as mean ± standard deviation.

2.4. Statistical Analysis

A one-way analysis of variance (ANOVA) assessed statistical differences among samples. Tukey–Kramer's Honest Significant Difference (HSD) post hoc test identified pairwise differences at a significance level of $p < 0.05$. Statistical analyses were performed using JMP software (version 11.0.0; SAS Institute Inc., Cary, NC, USA).

To explore overall patterns and relationships among AR gene profiles across sample types, principal component analysis (PCA) was conducted on quantitative AR gene copy numbers for all genes detected above the limit of quantification in at least one sample. Variables were mean-centered and scaled to unit variance to ensure that all genes contributed equally to the multivariate analysis. PCA was performed and visualized in R (version 4.4.3) using the *factoextra* and *ggplot2* packages.

3. Results and Discussion

3.1. Rearing Performance

Diets D1, D3, and D4 supported robust larval growth, producing comparable biomass (~1.6 kg of mature larvae). In contrast, D2 yielded substantially lower biomass. This reduced performance may result from high substrate fermentability, which increases temperatures during bioconversion and adversely affects larval activity and survival. As a result, frass yield was higher for D2 and lower for the other diets.

3.2. Viable Counts

The results of the viable counts of presumptive *Enterococcus* spp. in the samples are reported in Table 1.

For samples related to D1 (peas and chickpeas), the highest *Enterococcus* spp. count occurred in *H. illucens* larvae sampled after the bioconversion process (8.50 ± 0.46 log CFU/g). Higher counts in mature larvae compared to younger ones likely result from physiological and microbial dynamics. As larvae mature, changes in gut morphology, pH, and retention time create a more favorable environment for bacterial growth [21]. No substantial

differences were observed in this microbial group among the D1 formulation, its individual substrate components, and the frass produced after rearing.

Table 1. Viable counts (log CFU/g) of *Enterococcus* spp. and detection of *Salmonella* spp. in samples associated with *Hermetia illucens* larvae fed four agro-industrial residue-based diets (D1–D4), before (single substrates, diet formulations, young larvae) and after (frass, mature larvae) the bioconversion process.

Diet	Sample	*Enterococcus* spp. *	*Salmonella* spp. **
D1	Peas	7.25 ± 0.09^{b}	-
	Chickpea	6.40 ± 0.55^{b}	-
	Diet 1 (peas + chickpea)	6.96 ± 0.32^{b}	-
	Young *H. illucens* larvae	6.95 ± 0.04^{b}	+
	Frass	6.44 ± 0.34^{b}	-
	Mature *H. illucens* larvae	8.50 ± 0.46^{a}	-
D2	Peas	8.17 ± 0.13^{a}	-
	Wheat	$<1^{c}$	-
	Diet 2 (peas + wheat)	7.81 ± 0.08^{a}	-
	Young *H. illucens* larvae	6.97 ± 0.02^{b}	+
	Frass	6.86 ± 0.29^{b}	-
	Mature *H. illucens* larvae	8.03 ± 0.01^{a}	-
D3	Onion	3.26 ± 0.11^{c}	-
	Wheat	4.56 ± 0.35^{b}	-
	Diet 3 (onion + wheat)	3.54 ± 0.11^{c}	-
	Young *H. illucens* larvae	7.21 ± 0.03^{a}	+
	Frass	7.05 ± 0.38^{a}	-
	Mature *H. illucens* larvae	7.52 ± 0.22^{a}	-
D4	Digestate	3.61 ± 0.03^{c}	-
	Wheat	3.58 ± 0.35^{c}	-
	Diet 4 (digestate + wheat)	4.67 ± 0.06^{b}	-
	Young *H. illucens* larvae	7.14 ± 0.12^{a}	+
	Frass	5.07 ± 0.12^{b}	+
	Mature *H. illucens* larvae	7.33 ± 0.25^{a}	+

* Values are expressed as means of log CFU/g from three biological and two technical replicates ± standard deviation. For each sample related to the same diet, values with different superscript letters are significantly different ($p < 0.05$) according to the Tukey–Kramer (HSD) test; ** Values are expressed as the presence (+) or absence (-) of *Salmonella* spp. in 25 g of the sample.

Members of the *Enterococcus* genus are widely recognized as indicators of fecal contamination in food and environmental matrices because of their resilience under diverse conditions [22]. Moreover, *Enterococcus* spp. consistently appear in the gut microbiota of *H. illucens* larvae, with some strains potentially modulating host appetite and stimulating increased feeding activity, independent of diet composition [23]. Previous studies have shown a positive association between the presence of *Enterococcus* spp. and *H. illucens* larvae grown on fermented substrates. Certain species in this genus carry genes encoding enzymes such as endoglucanases and β-glucosidases, which aid in cellulose breakdown. These bacteria can therefore facilitate decomposition of plant material within the substrate [24]. Additionally, specific *Enterococcus* species, such as *Enterococcus mundtii*, produce antimicrobial peptides [25].

For D2 (peas and wheat), *Enterococcus* spp. were not detected in the wheat waste substrate, indicating that their presence in the formulated diet likely originated from the pea component. High counts in peas (8.17 ± 0.13 log CFU/g) were similar to those observed in peas used for D1, suggesting that peas provide a favorable environment for bacterial growth by offering nutrients, moisture, and other supporting factors. A lower bacterial load appeared in the frass compared to the D2 formulation, possibly due to the

physical and chemical composition of the frass, including variations in moisture, organic matter, and microbial competition. Additionally, antimicrobial substances produced by the larvae may further limit bacterial growth [26,27]. A similar trend occurred for D1, where *Enterococcus* spp. counts were lower in the frass, although the difference was not statistically significant. These observations suggest that while peas support higher bacterial loads, they may also indirectly influence the microbial community in the frass, altering microbial dynamics and interactions. Overall, these findings highlight the importance of substrate composition in shaping bacterial populations and microbial interactions, which ultimately affect bacterial load and survival in rearing environments. As observed for D1, adult *H. illucens* larvae carried a higher bacterial load than young larvae.

Regarding D3 (onion and wheat waste), the loads of *Enterococcus* spp. were the lowest in onion (3.26 ± 0.11 log CFU/g), which matches previous findings reporting levels between 2.0 and 5.0 log CFU/g [28]. This trend may be attributed to the antimicrobial properties of onion, which contains bioactive compounds such as flavonoids and sulfur-containing molecules that inhibit bacterial growth [29]. In contrast, the highest microbial loads were observed in *H. illucens* larvae both after (7.52 ± 0.22 log CFU/g) and before (7.21 ± 0.03 log CFU/g) the bioconversion. The frass collected after rearing showed significantly higher counts (7.05 ± 0.38 log CFU/g) than the initial D3 formulation (3.54 ± 0.11 log CFU/g), indicating substantial bacterial proliferation during the bioconversion process.

Finally, for the D4 formulation (wheat waste and digestate), the highest *Enterococcus* spp. counts were recorded in mature *H. illucens* larvae (7.33 ± 0.25 log CFU/g), with no significant difference compared to young larvae. A similar pattern was observed between the initial D4 formulation and the frass collected after bioconversion, showing stable *Enterococcus* levels throughout the rearing process.

Regarding the presence of *Salmonella* spp., the analysis revealed its occurrence only in young *H. illucens* larvae and in two samples associated with D4: the frass obtained after the bioconversion process and the mature larvae fed with D4. These findings suggest that the bioconversion of the other three diets (D1, D2, and D3) effectively eliminated *Salmonella* spp. present in young larvae. Furthermore, *Salmonella* spp. was absent in the frass associated with these diets. These results support earlier research showing that *H. illucens* larvae can reduce *Salmonella* spp. in organic waste, including animal manure and sewage sludge. These studies indicate that both the rearing substrate and ambient temperature strongly influence pathogen reduction [14,30–33]. When used as animal feed, *H. illucens* larvae typically undergo additional processing, such as heat treatment, to further reduce bacterial load, in accordance with EU Regulation (EC) No. 142/2011 [34] for processed animal proteins. Nonetheless, it remains crucial to verify that the final product is completely free of *Salmonella* spp., as this regulation mandates the absence of *Salmonella* spp. in 25 g samples of feed ingredients.

3.3. qPCR Quantification of AR Genes

Assessing AR genes in *H. illucens* larvae destined for animal feed and in frass intended as a soil amendment is crucial to prevent the spread of resistance through the food chain and the environment. In larvae, AR genes could be transferred to farmed animals and, subsequently, to humans, while frass can act as a vector for AR genes in soil, potentially affecting crops and surrounding ecosystems. This concern is addressed by EU legislation, such as Commission Regulation (EU) 2017/893 [35] on the use of insects as feed, and Regulation (EU) 2019/1009 [36] concerning fertilizer products, alongside European Food Safety Authority (EFSA) recommendations [37] that emphasize a One Health approach to managing antibiotic resistance. Accordingly, the present study investigated how different diet formulations influence AR gene profiles in both the larvae and the resulting frass, and whether larval activity can reduce these microbial risks.

Standard curves for each of the 13 AR genes demonstrated satisfactory amplification efficiencies, ranging from 90% to 110%, with R^2 values exceeding 0.99 for all reactions. The detection limit for all genes was below 1 log gene copy per reaction. Quantitative results for the AR genes detected in the samples are summarized in Table 2.

In the analyzed samples, AR genes conferring resistance to tetracyclines and MLS_B were the most prevalent and abundant. This finding agrees with previous studies, as such genes are frequently reported in industrially reared insects, including *H. illucens* [16,17,38–41]. Among the tetracycline resistance determinants, *tet*(M) and *tet*(S) were the most abundant, exceeding 10 log gene copies per gram in mature *H. illucens* larvae reared on D4 (digestate and wheat waste). The high prevalence of *tet*(M) was expected because of its broad host range, facilitated by its association with mobile genetic elements that promote gene transfer among bacteria. Similarly, *tet*(S), which shares 79% amino acid identity with *tet*(M), occurs in Firmicutes and Gammaproteobacteria across diverse ecological niches [42].

The frequent detection of *erm*(B) results from its integration into mobile genetic elements, including transposons (Tn917, Tn1545) and plasmids, which promote horizontal gene transfer. This gene is particularly prevalent among Firmicutes, especially in *Enterococcus* and *Streptococcus* genera, and its widespread distribution is driven further by the extensive use of macrolides in agriculture and veterinary medicine [43].

Conversely, the *vanA* gene, which confers resistance to vancomycin, and the *aac-aph* gene, which confers resistance to aminoglycosides, were absent in all tested samples. This result confirms previous studies reporting the absence or very low abundance of these genes in samples associated with *H. illucens* larvae [16,17].

For samples related to D1 (peas and chickpea residues), among the genes conferring resistance to MLS_B, *erm*(A) and *erm*(C) were not detected in peas or in the young *H. illucens* larvae sampled before the bioconversion process, indicating chickpea residues as their source. The highest copy number of the *erm*(A) gene was found in the frass (7.26 ± 0.01 log gene copies/g), which was significantly higher than the D1 formulation (6.20 ± 0.02 log gene copies/g), showing accumulation during bioconversion. The same trend appeared for the *erm*(C) gene. Conversely, for the *erm*(B) gene, frass showed a significant reduction (5.75 ± 0.02 log gene copies/g) compared to the starting D1 formulation (6.14 ± 0.01 log gene copies/g). The highest quantity of this gene was found in the mature *H. illucens* larvae (6.84 ± 0.01 log gene copies/g), indicating accumulation during rearing. Regarding tetracycline resistance genes, the peas used as a single substrate in the D1 formulation were identified as the main source of the *tet*(S), *tet*(W), and *tet*(O) genes. This finding aligns with previous studies showing the presence of AR genes in various agricultural products, including vegetables, likely reflecting environmental contamination from agricultural practices, such as the application of manure or wastewater containing antibiotics [44]. This underscores the widespread dissemination of AR genes in agricultural ecosystems and their potential incorporation into food chains. Interestingly, the bioconversion process by *H. illucens* larvae significantly reduced all tetracycline genes except *tet*(O) in the frass compared to the initial D1 formulation. Mature larvae sampled at the end of bioconversion, however, accumulated the *tet*(M), *tet*(W), and *tet*(K) genes. Only the quantity of the *tet*(O) gene decreased in mature larvae compared to young larvae. Regarding vancomycin resistance genes, the *vanA* gene was never detected, while the *vanB* gene was detected only in young larvae samples. The *mecA* and *blaZ* genes, conferring resistance to β-lactams, were not detected in either young or mature larvae. The highest quantities of these genes were found in the chickpeas used for the D1 formulation, identifying them as the main source. However, bioconversion reduced these genes, with significantly lower quantities in the resulting frass compared to the initial D1 formulation.

Table 2. Log copy number of antibiotic resistance (AR) genes per gram of samples related to *Hermetia illucens* larvae fed four agro-industrial residue-based diets: peas and chickpea (D1), peas and wheat (D2), onion and wheat (D3), and wheat with digestate (D4), before (single substrates, diet formulations, young larvae) and after (frass, mature larvae) the bioconversion process.

Diet	Sample	Antibiotic Resistant Genes (Log Gene Copies/g ± Standard Deviation)												
		MLS_B			Tetracyclines					Vancomycin		β-Lactams		Aminoglycosides
		erm(A)	*erm*(B)	*erm*(C)	*tet*(M)	*tet*(S)	*tet*(W)	*tet*(K)	*tet*(O)	*van*A	*van*B	*mecA*	*blaZ*	*aac-aph*
D1	Peas	n.d.	5.30 ± 0.02^{f}	n.d.	8.60 ± 0.00^{d}	9.38 ± 0.01^{a}	7.32 ± 0.03^{a}	4.62 ± 0.09^{f}	7.74 ± 0.02^{a}	n.d.	n.d.	4.30 ± 0.01^{d}	n.d.	n.d.
	Chickpea	6.50 ± 0.01^{b}	5.37 ± 0.03^{e}	6.04 ± 0.01^{b}	8.33 ± 0.01^{e}	8.43 ± 0.01^{c}	5.48 ± 0.02^{e}	9.32 ± 0.03^{a}	5.52 ± 0.13^{d}	n.d.	n.d.	7.49 ± 0.01^{a}	5.37 ± 0.03^{a}	n.d.
	Diet 1 (peas + chickpea)	6.20 ± 0.02^{c}	6.14 ± 0.01^{b}	6.05 ± 0.01^{b}	9.09 ± 0.01^{a}	8.89 ± 0.00^{b}	5.93 ± 0.02^{c}	8.41 ± 0.04^{b}	6.48 ± 0.11^{c}	n.d.	n.d.	6.64 ± 0.01^{b}	5.44 ± 0.06^{a}	n.d.
	Young *H. illucens* larvae	n.d.	5.51 ± 0.02^{d}	n.d.	8.01 ± 0.00^{f}	7.54 ± 0.06^{d}	5.50 ± 0.04^{e}	4.93 ± 0.01^{e}	7.81 ± 0.02^{a}	n.d.	4.75 ± 0.02	n.d.	n.d.	n.d.
	Frass	7.26 ± 0.01^{a}	5.75 ± 0.02^{c}	6.52 ± 0.01^{a}	8.98 ± 0.01^{b}	7.25 ± 0.01^{e}	5.75 ± 0.07^{d}	6.85 ± 0.08^{c}	6.60 ± 0.02^{c}	n.d.	n.d.	5.84 ± 0.01^{c}	4.99 ± 0.04^{b}	n.d.
	Mature *H. illucens* larvae	5.59 ± 0.04^{d}	6.84 ± 0.01^{a}	4.33 ± 0.01^{c}	8.90 ± 0.01^{c}	7.57 ± 0.04^{d}	6.81 ± 0.00^{b}	5.28 ± 0.02^{d}	7.22 ± 0.01^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
D2	Peas	5.64 ± 0.02^{c}	6.72 ± 0.01^{a}	3.58 ± 0.03^{d}	9.18 ± 0.02^{b}	8.87 ± 0.04^{a}	7.32 ± 0.03^{b}	7.56 ± 0.00^{a}	7.74 ± 0.02^{a}	n.d.	n.d.	6.26 ± 0.01^{a}	5.71 ± 0.01^{a}	n.d.
	Wheat	n.d.	4.09 ± 0.04^{f}	4.13 ± 0.11^{c}	6.11 ± 0.06^{e}	5.78 ± 0.13^{e}	5.61 ± 0.06^{e}	4.99 ± 0.10^{d}	5.14 ± 0.14^{d}	n.d.	n.d.	n.d.	n.d.	n.d.
	Diet 2 (peas + wheat)	4.34 ± 0.07^{d}	5.75 ± 0.03^{b}	3.14 ± 0.01^{e}	8.37 ± 0.02^{d}	8.34 ± 0.03^{b}	6.57 ± 0.01^{d}	6.22 ± 0.01^{c}	7.00 ± 0.02^{b}	n.d.	n.d.	4.90 ± 0.06^{c}	5.35 ± 021^{b}	n.d.
	Young *H. illucens* larvae	3.76 ± 0.05^{e}	5.36 ± 0.04^{c}	n.d.	8.35 ± 0.02^{d}	7.81 ± 0.01^{c}	5.51 ± 0.01^{f}	4.85 ± 0.04^{e}	7.87 ± 0.01^{a}	n.d.	4.54 ± 0.02	n.d.	n.d.	n.d.
	Frass	7.08 ± 0.04^{a}	5.22 ± 0.01^{d}	6.58 ± 0.02^{a}	9.51 ± 0.02^{a}	8.92 ± 0.03^{a}	7.04 ± 0.03^{c}	7.00 ± 0.00^{b}	6.93 ± 0.02^{b}	n.d.	n.d.	5.28 ± 0.02^{b}	5.08 ± 0.03^{c}	n.d.
	Mature *H. illucens* larvae	6.49 ± 0.03^{b}	4.96 ± 0.04^{e}	5.26 ± 0.01^{b}	8.75 ± 0.04^{c}	7.36 ± 0.04^{d}	7.76 ± 0.02^{a}	5.02 ± 0.03^{d}	6.15 ± 0.06^{c}	n.d.	n.d.	n.d.	n.d.	n.d.
D3	Onion	5.45 ± 0.04^{b}	5.97 ± 0.01^{c}	4.14 ± 0.06^{d}	8.09 ± 0.01^{c}	7.31 ± 0.08^{d}	6.82 ± 0.06^{b}	5.75 ± 0.06^{d}	6.02 ± 0.03^{d}	n.d.	n.d.	3.88 ± 0.07^{b}	n.d.	n.d.
	Wheat	4.37 ± 0.08^{c}	4.96 ± 0.02^{f}	4.01 ± 0.11^{d}	6.55 ± 0.05^{d}	6.60 ± 0.11^{e}	6.29 ± 0.08^{c}	6.59 ± 0.09^{c}	6.62 ± 0.09^{bc}	n.d.	n.d.	5.04 ± 0.11^{a}	5.56 ± 0.11^{a}	n.d.
	Diet 3 (onion + wheat)	5.45 ± 0.03^{b}	6.23 ± 0.02^{b}	4.35 ± 0.01^{c}	8.73 ± 0.02^{a}	8.13 ± 0.02^{b}	6.98 ± 0.03^{a}	6.76 ± 0.02^{b}	6.75 ± 0.01^{b}	n.d.	n.d.	4.96 ± 0.06^{a}	5.58 ± 0.04^{a}	n.d.
	Young *H. illucens* larvae	3.60 ± 0.14^{d}	5.42 ± 0.05^{e}	n.d.	8.78 ± 0.00^{a}	7.95 ± 0.03^{c}	5.53 ± 0.02^{e}	5.35 ± 0.04^{e}	7.96 ± 0.02^{a}	n.d.	4.55 ± 0.00^{a}	n.d.	n.d.	n.d.
	Frass	6.61 ± 0.01^{a}	7.05 ± 0.01^{a}	6.28 ± 0.03^{a}	8.42 ± 0.01^{b}	9.81 ± 0.02^{a}	6.30 ± 0.05^{c}	7.79 ± 0.01^{a}	6.47 ± 0.02^{c}	n.d.	n.d.	5.05 ± 0.04^{a}	5.57 ± 0.05^{a}	n.d.
	Mature *H. illucens* larvae	5.37 ± 0.02^{b}	5.52 ± 0.05^{d}	5.14 ± 0.03^{b}	8.77 ± 0.01^{a}	8.03 ± 0.05^{bc}	6.15 ± 0.05^{d}	5.27 ± 0.06^{e}	5.77 ± 0.21^{d}	n.d.	4.54 ± 0.00^{a}	n.d.	n.d.	n.d.
D4	Digestate	6.97 ± 0.04^{b}	8.21 ± 0.02^{a}	6.44 ± 0.01^{b}	9.65 ± 0.01^{b}	7.95 ± 0.07^{b}	9.73 ± 0.01^{a}	7.37 ± 0.01^{c}	9.11 ± 0.01^{a}	n.d.	n.d.	n.d.	6.09 ± 0.05^{c}	n.d.
	Wheat	7.08 ± 0.03^{b}	7.66 ± 0.02^{b}	5.23 ± 0.01^{e}	9.17 ± 0.01^{c}	8.10 ± 0.04^{b}	6.32 ± 0.01^{d}	7.66 ± 0.01^{b}	8.76 ± 0.02^{b}	n.d.	n.d.	5.02 ± 0.12^{a}	6.48 ± 0.05^{a}	n.d.
	Diet 4 (digestate + wheat)	6.33 ± 0.22^{c}	5.07 ± 0.26^{d}	5.43 ± 0.11^{d}	7.22 ± 0.13^{e}	7.52 ± 0.22^{c}	7.83 ± 0.15^{b}	7.05 ± 0.03^{d}	7.50 ± 0.11^{e}	n.d.	n.d.	4.94 ± 0.14^{a}	6.30 ± 0.04^{b}	n.d.
	Young *H. illucens* larvae	3.88 ± 0.08^{d}	5.46 ± 0.03^{c}	n.d.	8.65 ± 0.10^{d}	7.80 ± 0.01^{bc}	5.56 ± 0.04^{e}	5.02 ± 0.03^{f}	7.87 ± 0.01^{d}	n.d.	4.54 ± 0.01	n.d.	n.d.	n.d.
	Frass	6.74 ± 0.21^{b}	5.12 ± 0.09^{d}	6.03 ± 0.03^{c}	8.56 ± 0.03^{d}	7.91 ± 0.14^{b}	6.60 ± 0.04^{c}	5.83 ± 0.02^{e}	7.44 ± 0.01^{e}	n.d.	n.d.	n.d.	5.60 ± 0.02^{e}	n.d.
	Mature *H. illucens* larvae	8.21 ± 0.01^{a}	7.42 ± 0.01^{b}	7.94 ± 0.02^{a}	10.03 ± 0.01^{a}	10.29 ± 0.01^{a}	7.94 ± 0.02^{b}	8.51 ± 0.01^{a}	8.30 ± 0.03^{c}	n.d.	n.d.	4.87 ± 0.03^{a}	5.87 ± 0.09^{d}	n.d.

MLS_B, macrolide-lincosamide-streptogramin B; n.d., not detected. For each parameter, values with different superscript letters are significantly different ($p < 0.05$) according to the Tukey–Kramer's (HSD) test.

For samples related to D2 (peas and wheat residues), among the genes conferring resistance to MLS_B, *erm*(A) was not detected in the wheat substrate, whereas *erm*(C) was not detected in the young *H. illucens* larvae samples. As observed for D1, the highest quantity of *erm*(A) was found in the frass (7.08 ± 0.04 log gene copies/g), which was significantly higher than in the D2 formulation (4.34 ± 0.07 log gene copies/g), indicating substantial accumulation during bioconversion. A similar trend was observed for *erm*(C), with a log copy number per gram increasing from 3.14 ± 0.01 in the D2 formulation to 6.58 ± 0.02 in the resulting frass. Additionally, *erm*(A) and *erm*(C) accumulated in mature larvae compared to young larvae. The highest quantity of *erm*(B) was found in the pea-based substrate (6.72 ± 0.01 log gene copies/g). Unlike the other MLS_B genes, *erm*(B) significantly decreased in the frass (5.22 ± 0.01 log gene copies/g) compared to the starting D2 formulation (5.75 ± 0.03 log gene copies/g). In contrast to D1, mature larvae reared on D2 carried lower *erm*(B) quantities than young larvae. Genes conferring resistance to tetracyclines were detected in all analyzed samples. As for D1, the highest *tet*(O) copy number was found in the pea substrate, which also served as the main source of *tet*(K). Frass samples contained the highest quantities of *tet*(M) (9.51 ± 0.02 log gene copies/g) and *tet*(S) (8.92 ± 0.03 log gene copies/g), indicating accumulation during bioconversion. A similar trend was observed for *tet*(W) and *tet*(K), whereas *tet*(O) showed no significant difference between the initial D2 formulation and the resulting frass. Regarding tetracycline genes in mature larvae, *tet*(M), *tet*(W), and *tet*(K) accumulated, while *tet*(S) and *tet*(O) decreased. Concerning vancomycin resistance genes, as for D1, *vanB* was detected only in young larvae (4.54 ± 0.02 log gene copies/g). The *mecA* and *blaZ* genes, conferring resistance to β-lactams, were not detected in either young or mature larvae. The highest quantities of these genes were found in the pea-based substrate used for D2, identifying it as the main source. However, bioconversion led to accumulation of *mecA* in the frass, while *blaZ* decreased significantly in the resulting frass compared to the D2 formulation.

Regarding the samples related to D3 (onion and wheat residues), the frass obtained after the bioconversion process contained the highest log copy number per gram of all tested MLS_B genes, ranging from 6.28 ± 0.03 [*erm*(C)] to 7.05 ± 0.01 [*erm*(B)], indicating accumulation compared to the initial D3 formulation. As with the previous diets, *erm*(C) was not detected in young *H. illucens* larvae. However, the quantity of MLS_B genes increased significantly in mature larvae, particularly for *erm*(A) and *erm*(C). Regarding tetracycline resistance genes, the highest quantity of *tet*(M) was found in the D3 formulation as well as in larvae before and after bioconversion, with no significant differences among them. In contrast, bioconversion reduced *tet*(M) in the resulting frass. *tet*(S) accumulated in the frass after bioconversion, with no significant differences between young and mature larvae. A similar trend occurred for *tet*(K). The highest log gene copy number per gram for *tet*(W) was detected in the initial D3 formulation and decreased significantly in the frass, whereas it increased significantly in mature larvae. Finally, *tet*(O) decreased significantly in both the frass and mature larvae after bioconversion. Regarding vancomycin resistance genes, *vanB* was detected only in young and mature larvae, with no significant differences between them. The β-lactam resistance genes *mecA* and *blaZ* were not detected in larvae. Moreover, *blaZ* was absent in the onion substrate, indicating wheat waste as its only source. No significant differences were observed between the D3 formulation and the corresponding frass, indicating that bioconversion did not affect the quantity of β-lactam resistance genes.

The qPCR screening revealed that among samples associated with D4 (wheat residues and digestate), the highest quantities of the MLS_B resistance genes *erm*(A) and *erm*(C) were found in mature *H. illucens* larvae, indicating accumulation during rearing. A similar trend occurred for *erm*(B). Moreover, *erm*(A) and *erm*(C) accumulated in the frass after bioconversion, whereas *erm*(B) showed no significant difference between the D4 formulation

and the corresponding frass. The highest quantities of *tet*(M), *tet*(S), and *tet*(K), which confer resistance to tetracyclines, were detected in mature larvae, whereas the highest load of *tet*(W) and *tet*(O) was found in the digestate used for the D4 formulation. Bioconversion increased all tested tetracycline genes in mature larvae, while *tet*(W) and *tet*(K) decreased significantly in the resulting frass compared to the initial D4 formulation. For *tet*(O), no significant differences were observed between the initial diet and the frass. As with D1 and D2, *vanB* was detected exclusively in young larvae. The *mecA* gene, which confers resistance to β-lactams, was not detected in the digestate, young larvae, or the D4 formulation, and no significant differences were observed in log gene copies per gram among the remaining samples. The *blaZ* gene was most abundant in the wheat substrate but decreased notably in the frass obtained after bioconversion. This gene was absent in young larvae but present in mature larvae, showing significant accumulation during rearing.

Generally, considering genes conferring resistance to MLS_B, the highest quantities of *erm*(A) and *erm*(C) were found in frass samples related to diets D1, D2, and D3, whereas for D4, the highest quantities appeared in mature *H. illucens* larvae. *erm*(A) and *erm*(C) typically occur in Gram-positive bacteria, particularly *Staphylococcus* and *Streptococcus* species, where they confer resistance through 23S rRNA methylation [45]. The bioconversion of all diets led to the accumulation of these genes in both frass and mature larvae compared to the initial diet formulations and young larvae, respectively. This accumulation was most evident for *erm*(C), which was never detected in young larvae. Similarly, Ravoitytė et al. [41] reported *erm*(A) in all frass samples obtained after rearing *H. illucens* on a diet of expired pasta, fruits, vegetables, and barley malt. Regarding larvae, Milanovic et al. [17] reported the highest copy number of this gene in *H. illucens* used as zebrafish feed. Another study [16] found *erm*(A) exclusively in larvae reared on substrates containing 20% *Isochrysis galbana*. The same study detected *erm*(C) exclusively in frass obtained after larval rearing on substrates supplemented with the same alga. In the present study, bioconversion of D1 and D2 formulations, both containing green peas, reduced *erm*(B) in the frass. However, a similar trend occurred only in mature larvae reared on D2. Bohm et al. [46] reported that *H. illucens*-based bioconversion of biosolid substrates significantly reduced the relative abundance of *erm*(B), likely due to a decline of AR gene carrying bacteria in the substrates. In contrast, bioconversion of wheat bran was associated with an increase in *erm*(B) abundance.

Regarding genes conferring resistance to tetracyclines, their abundance ranged from approximately 4 to 10 log gene copies per gram, largely matching values reported by Cifuentes et al. [39] for larval and pupal guts of *H. illucens* analyzed via qPCR. Bioconversion of the D1 formulation reduced the highest number of tetracycline genes tested [all except *tet*(O)] in the resulting frass compared to the initial diet. Legume-based substrates provide distinctive amino acids, dietary fibers, and bioactive phytochemicals, including phenolic compounds and saponins, which can modulate microbial community structure and reduce the persistence of specific AR determinants [47]. Conversely, the frass derived from bioconversion of D2 showed a significant increase in all tetracycline genes except *tet*(O). Additionally, the abundance of *tet*(W) in frass was significantly lower than in the initial formulations of diets D3 and D4. This finding agrees with Böhm et al. [46], who reported a significant decrease in *tet*(W) abundance during the bioconversion of biosolid substrates by *H. illucens* larvae. In mature larvae, tetracycline genes accumulated across all diets. Nevertheless, some exceptions occurred, such as a reduction in *tet*(O) in larvae fed D1, D2, and D3, and a decrease in *tet*(S) in adult larvae fed D2. Similarly, Böhm et al. [46] reported a significant increase in *tet*(W) in mature larvae reared on biosolid-based diets. The dynamics of *tet* genes during bioconversion may reflect their distinct resistance mechanisms. *tet*(M), *tet*(O), and *tet*(S) encode ribosomal protection proteins that prevent tetracycline binding to the ribosome and are often carried on mobile genetic elements, promoting

horizontal transfer [48]. In contrast, *tet*(K) encodes an efflux pump commonly found in Gram-positive bacteria and frequently detected in food and environmental samples [49]. *tet*(W), widely distributed in animal and human gut microbiota and associated with anaerobes from *Clostridium* and *Bacteroides* genera, may serve as a marker for tracking tetracycline resistance in microbiota shaped by organic substrates [50].

Among the vancomycin resistance genes, only *vanB*, commonly associated with enterococci and frequently detected in microbial communities linked to waste [51], was found in young *H. illucens* larvae, suggesting initial environmental contamination likely originating from the rearing substrate. Additionally, *vanB* was detected exclusively in mature larvae fed D3 (onion and wheat residues), indicating that this substrate may promote the persistence of bacteria carrying this gene. This pattern may result from diet-driven microbial selection, as sulfur-containing compounds and flavonoids in onion reduce microbial diversity, favoring intrinsically resistant or stress-tolerant taxa such as *Enterococcus* spp., while wheat-derived carbohydrates provide fermentable substrates that support their persistence [52].

Among the β-lactam resistance genes, *blaZ*, which encodes a β-lactamase capable of hydrolyzing penicillin, and *mecA*, which encodes the alternative penicillin-binding protein PBP2a responsible for methicillin and other β-lactam resistance in staphylococci [53], showed significant reductions in frass derived from D1 and D4. This reduction likely results from larval activity or shifts in microbial communities during bioconversion. In contrast, D2 caused a decrease in *blaZ* but an unexpected increase in *mecA*, suggesting that substrate composition may differently influence the persistence or proliferation of specific resistance genes. Previously, Milanović et al. [16] detected *mecA* exclusively in frass obtained after larval rearing on substrates supplemented with *I. galbana*, highlighting that certain feed components may enrich specific AR genes. The absence of these genes in young larvae indicates that they were not present in the rearing environment before diet exposure. Interestingly, only mature larvae fed D4 (wheat residues and digestate) carried β-lactam resistance genes, possibly reflecting exposure to resistant microbes in the digestate or selective enrichment during larval development.

PCA (Figure 1) was performed to explore overall patterns and relationships among AR gene profiles across the tested samples. The two principal components (PC1 and PC2) accounted for approximately 46.4% and 20.7% of the total variance, respectively. The ordination revealed distinct clustering influenced by both diet and sample type. The primary separation along PC1 clearly distinguished the final larval product from most feed inputs and waste materials. Specifically, feed substrates and frass samples, including chickpea, D1, D2, and D3 mixtures, clustered predominantly on the negative side of PC1 (left side). This cluster was driven by high loadings of *tet*(K), *erm*(C), and *mecA*, reflecting relatively lower contributions of these genes to the positive PC1 direction. In contrast, mature larvae from all diets except D4 positioned distinctly on the positive side of PC1 (right side). Young larvae formed a well-defined cluster in the upper right quadrant (positive PC1 and PC2), driven primarily by the strong positive loading of *vanB*, which was detectable only at this stage. Digestate, wheat, and mature larvae from D4 showed a distinct separation from other diets, clustering in the upper left quadrant, corresponding to positive loadings of *erm*(B), *tet*(M), and *tet*(S).

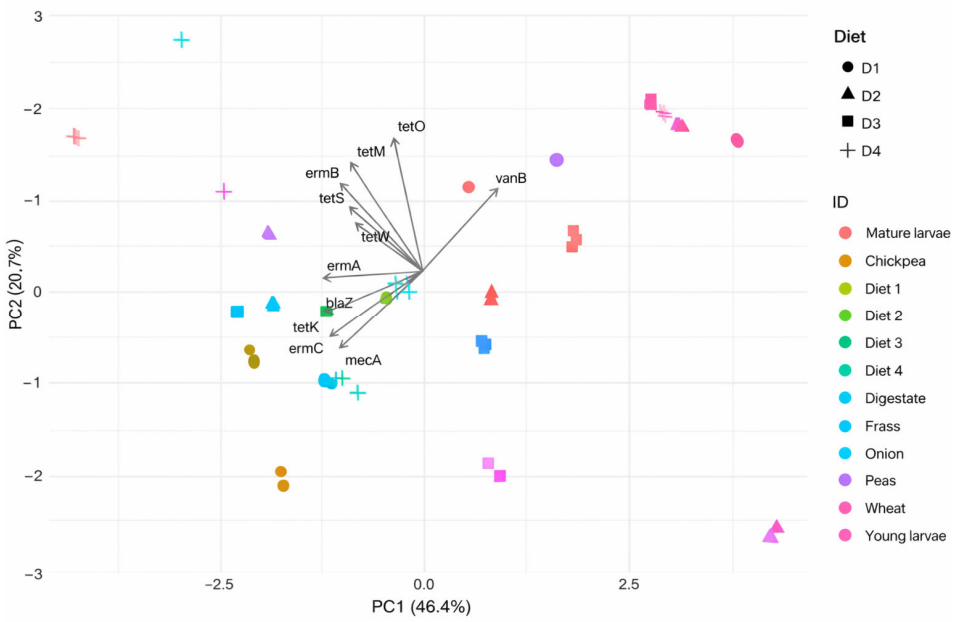

Figure 1. Principal component analysis (PCA) illustrating the overall patterns and relationships among antibiotic resistance gene profiles across the samples. Samples related to the same diet are distinguished by geometric symbols within the plot: circles for D1 (peas and chickpeas), triangles for D2 (peas and wheat), squares for D3 (onion and wheat), and plus signs for D4 (digestate and wheat). Within each diet, the individual substrates, diet formulations, *Hermetia illucens* larvae (young and mature), and frass are differentiated by color.

Overall, considering the efficacy of *H. illucens*-led bioconversion of agro-industrial residues, bioconversion of peas and chickpea residues (D1) reduced the greatest number of tested AR genes in both frass and mature larvae. Specifically, frass contained significantly lower quantities of *erm*(B), *tet*(M), *tet*(S), *tet*(W), *tet*(K), *mecA*, and *blaZ* compared to the initial D1 formulation, whereas mature larvae showed significantly lower quantities of *tet*(O) and *vanB*. The second most effective diet in terms of AR gene reduction in frass was D4 (wheat residues and digestate), showing lower quantities of tetracycline genes *tet*(W) and *tet*(K) and both tested β-lactam genes. Frass obtained from D3 contained lower quantities of *tet*(M), *tet*(W), and *tet*(O). In contrast, bioconversion of D2 (peas and wheat residues) was less efficient in reducing AR genes in frass. This diet reduced *erm*(B), *tet*(S), and *tet*(O) in mature larvae compared to young larvae. Additionally, larvae reared on D3 showed a reduction only in *tet*(O), while D4 did not reduce any of the tested AR genes. On the other hand, the D2 formulation led to accumulation of the highest number of tested AR genes in frass, including *erm*(A), *erm*(C), *tet*(M), *tet*(S), *tet*(W), *tet*(K), and *mecA*. Similarly, mature larvae reared on D4 exhibited significant accumulation of all tested MLS_B, tetracycline, and β-lactam genes.

The observed diet-driven differences in AR gene profiles likely result from multiple interacting mechanisms. Horizontal gene transfer mediated by mobile genetic elements such as plasmids and transposons drives AR gene dissemination in complex microbial communities, including those associated with insect guts and organic substrates [43,54]. Genes such as *tet*(M), *tet*(O), and *erm*(B) are frequently linked to conjugative transposons, facilitating transfer among Firmicutes and other taxa during bioconversion [55]. Furthermore, selective pressures from residual antibiotics or plant-derived compounds in substrates can favor specific microbial strains and shift overall microbial diversity, potentially enriching stress-tolerant taxa [52,56]. Finally, microbial shifts caused by larval digestion and substrate fermentation can alter community composition, creating ecological niches that favor bacteria carrying specific AR determinants. Previous studies showed that gut restructuring

during larval development and the production of antimicrobial peptides can suppress some taxa while enabling others to proliferate [21,40]. These mechanisms collectively explain the accumulation or reduction in AR genes observed across diets and sample types in this study.

4. Conclusions

This study demonstrates that diet composition strongly shapes microbial communities and AR gene profiles during *H. illucens* larval bioconversion. Among the diets tested, D1 (peas and chickpea residues) was most effective at reducing a broad spectrum of AR genes in both frass, a potential soil amendment, and mature larvae, a potential animal feed. In contrast, D2 (peas and wheat residues) led to the accumulation of the highest number of tested AR genes in frass, underscoring the critical role of substrate choice in limiting AR spread during *H. illucens*-mediated waste valorization. PCA analysis further confirmed the strong influence of diet on AR gene distribution, with samples clustering primarily according to dietary treatment. Observed shifts in enterococci abundance across substrates and larval stages highlight dynamic microbial changes driven by diet and larval development. Moreover, bioconversion effectively eliminated *Salmonella* spp. in most diets, emphasizing the importance of substrate selection and the need for additional processing to ensure feed safety. Overall, these findings underscore the potential of *H. illucens*-based bioconversion not only for organic waste management but also as a strategy to reduce environmental spread of antimicrobial resistance and foodborne pathogens. Adjusting substrate composition is key to maximizing the benefits of this approach. Future investigations should integrate metagenomic and functional analyses to systematically identify microorganisms carrying AR genes and clarify metabolic interactions governing their persistence. Such targeted investigations will deepen the mechanistic understanding of AR gene dynamics and provide a scientific basis for developing safer, more efficient bioconversion systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/sci8010011/s1, Table S1: Primers used in qPCR assays for 13 antibiotic resistance (AR) genes, with details on amplicon size (bp) and primer concentration (nM).

Author Contributions: Conceptualization, L.A. and E.F.P.; Methodology, A.M., A.I. and V.M.; Formal analysis, V.M. and G.R.; Investigation, V.M., A.M. and A.I.; Resources, L.A., E.F.P., A.O. and C.G.; Data curation, V.M., A.M. and G.R.; Writing—original draft preparation, V.M.; Writing—review and editing, A.M., A.I., K.A.B.-Y., L.A. and G.R.; Visualization, V.M.; Supervision, L.A., E.F.P. and A.O.; Project administration, F.C. and E.F.P.; Funding acquisition, E.F.P. and L.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Marche Region through the Rural Development Program (PSR) 2014–2020-Call: MEASURE 16 "COOPERAZIONE", Sottomisura 16.1 "Sostegno per la costituzione e la gestione dei gruppi operativi del PEI in materia di produttività e sostenibilità dell'agricoltura"—Azione 2 "Fase di gestione del G.O. e realizzazione del Piano di Attività", Project: "Allevamento e produzione sostenibile di insetti. Processi di bio-conversione di residui della filiera agroindustriale tramite *H. illucens*", acronym "BSFlyGreen" (ID 42668). Additionally, this research was conducted within the Agritech National Research Center and received funding from the European Union under the NextGenerationEU initiative (National Recovery and Resilience Plan—PNRR, Mission 4, Component 2, Investment 1.4—Ministerial Decree No. 1032 dated 17 June 2022, Project Code: CN00000022).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AR	Antibiotic Resistance
MLS_B	Macrolide-lincosamide-streptogramin B
qPCR	Quantitative Polymerase Chain Reaction
EU	European Union
EFSA	European Food Safety Authority

References

1. Fróna, D.; Szenderák, J.; Harangi-Rákos, M. The Challenge of Feeding the World. *Sustainability* **2019**, *11*, 5816. [CrossRef]
2. Urugo, M.M.; Teka, T.A.; Gemede, H.F.; Mersha, S.; Tessema, A.; Woldemariam, H.W.; Admassu, H. A Comprehensive Review of Current Approaches on Food Waste Reduction Strategies. *Compr. Rev. Food Sci. Food Saf.* **2024**, *23*, e70011. [CrossRef]
3. Ojha, S.; Bußler, S.; Schlüter, O.K. Food Waste Valorisation and Circular Economy Concepts in Insect Production and Processing. *Waste Manag.* **2020**, *118*, 600–609. [CrossRef]
4. Naser El Deen, S.; van Rozen, K.; Elissen, H.; van Wikselaar, P.; Fodor, I.; van der Weide, R.; Hoek-van den Hil, E.F.; Rezaei Far, A.; Veldkamp, T. Bioconversion of Different Waste Streams of Animal and Vegetal Origin and Manure by Black Soldier Fly Larvae *Hermetia illucens* L. (Diptera: Stratiomyidae). *Insects* **2023**, *14*, 204. [CrossRef]
5. Siddiqui, S.A.; Ristow, B.; Rahayu, T.; Putra, N.S.; Yuwono, N.W.; Nisa', K.; Mategeko, B.; Smetana, S.; Saki, M.; Nawaz, A.; et al. Black Soldier Fly Larvae (BSFL) and Their Affinity for Organic Waste Processing. *Waste Manag.* **2022**, *140*, 1–13. [CrossRef] [PubMed]
6. Lomonaco, G.; Franco, A.; De Smet, J.; Scieuzo, C.; Salvia, R.; Falabella, P. Larval Frass of *Hermetia illucens* as Organic Fertilizer: Composition and Beneficial Effects on Different Crops. *Insects* **2024**, *15*, 293. [CrossRef] [PubMed]
7. Poveda, J. Insect Frass in the Development of Sustainable Agriculture: A Review. *Agron. Sustain. Dev.* **2021**, *41*, 56. [CrossRef]
8. Barragan-Fonseca, K.B.; Dicke, M.; van Loon, J.J.A. Nutritional Value of the Black Soldier Fly (*Hermetia illucens* L.) and Its Suitability as Animal Feed—A Review. *J. Insects Food Feed* **2017**, *3*, 105–120. [CrossRef]
9. Govorushko, S. Global Status of Insects as Food and Feed Source: A Review. *Trends Food Sci. Technol.* **2019**, *91*, 436–445. [CrossRef]
10. da Silva, W.C.; Silva, É.B.R.; Silva, J.A.R.; Martorano, L.G.; Belo, T.S.; Sousa, C.E.L.; Camargo-Júnior, R.N.C.; Andrade, R.L.; Santos, A.G.S.; Carvalho, K.C.; et al. Nutritional Value of the Larvae of the Black Soldier Fly (*Hermetia illucens*) and the House Fly (*Musca domestica*) as a Food Alternative for Farm Animals—A Systematic Review. *Insects* **2024**, *15*, 619. [CrossRef]
11. Osuch, B.; Barszcz, M.; Tomaszewska-Zaremba, D. The Potential of Black Soldier Fly (*Hermetia illucens* L.) Larvae in Chicken and Swine Nutrition—A Review. *J. Anim. Feed Sci.* **2024**, *33*, 454–468. [CrossRef]
12. Mohan, K.; Sathishkumar, P.; Rajan, D.K.; Rajarajeswaran, J.; Ganesan, A.R. Black Soldier Fly (*Hermetia illucens*) Larvae as Potential Feedstock for Biodiesel Production: Recent Advances and Challenges. *Sci. Total Environ.* **2023**, *860*, 160235. [CrossRef] [PubMed]
13. Spranghers, T.; Ottoboni, M.; De Clercq, P.; De Smet, S. Nutritional Composition of Black Soldier Fly (*Hermetia illucens*) Prepupae Reared on Different Organic Waste Substrates. *J. Sci. Food Agric.* **2017**, *97*, 2594–2600. [CrossRef] [PubMed]
14. Brulé, L.; Misery, B.; Baudouin, G.; Yan, X.; Guidou, C.; Trespeuch, C.; Foltyn, C.; Anthoine, V.; Moriceau, N.; Federighi, M.; et al. Evaluation of the Microbial Quality of *Hermetia illucens* Larvae for Animal Feed and Human Consumption: Study of Different Types of Rearing Substrates. *Foods* **2024**, *13*, 1587. [CrossRef]
15. European Food Safety Authority Scientific Committee (EFSA). Risk Profile Related to Production and Consumption of Insects as Food and Feed. *EFSA J.* **2015**, *13*, 4257. [CrossRef]
16. Milanović, V.; Roncolini, A.; Cardinali, F.; Garofalo, C.; Aquilanti, L.; Riolo, P.; Ruschioni, S.; Corsi, L.; Isidoro, N.; Zarantoniello, M.; et al. Occurrence of Antibiotic Resistance Genes in *Hermetia illucens* Larvae Fed Coffee Silverskin Enriched with *Schizochytrium limacinum* or *Isochrysis galbana* Microalgae. *Genes* **2021**, *12*, 213. [CrossRef]
17. Milanović, V.; Cardinali, F.; Aquilanti, L.; Maoloni, A.; Garofalo, C.; Zarantoniello, M.; Olivotto, I.; Riolo, P.; Ruschioni, S.; Isidoro, N.; et al. Quantitative Assessment of Transferable Antibiotic Resistance Genes in Zebrafish (*Danio rerio*) Fed *Hermetia illucens*-Based Feed. *Anim. Feed Sci. Technol.* **2021**, *277*, 114978. [CrossRef]
18. Marcelli, A.; Ilari, A.; Milanović, V.; Foppa Pedretti, E.; Boakye-Yiadom, K.A.; Cardinali, F.; Rampanti, G.; Osimani, A.; Garofalo, C.; Aquilanti, L. Impact of Dietary Inputs on Carbapenem Resistance Gene Dynamics and Microbial Safety During Bioconversion of Agri-Food Waste and Anaerobic Digestate by *Hermetia illucens* Larvae. *Genes* **2025**, *16*, 907. [CrossRef]
19. *AFNOR BIO 12/16-09/05*; Validation of Alternative Analysis Methods—VIDAS® Easy *Salmonella* (Enzyme-Linked Fluorescent Assay for Detection of *Salmonella* spp.); AFNOR Certification: La Plaine Saint-Denis, France. 2025. Available online: https://nf-validation.afnor.org/en/wp-content/uploads/sites/2/2025/07/BIO-12-16-09-05_en.docx_.pdf (accessed on 30 December 2025).

20. Weisburg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J. 16S Ribosomal DNA Amplification for Phylogenetic Study. *J. Bacteriol.* **1991**, *173*, 697–703. [CrossRef]
21. Klammsteiner, T.; Walter, A.; Bogataj, T.; Heussler, C.D.; Stres, B.; Steiner, F.M.; Schlick-Steiner, B.C.; Arthofer, W.; Insam, H. The Core Gut Microbiome of Black Soldier Fly (*Hermetia illucens*) Larvae Raised on Low-Bioburden Diets. *Front. Microbiol.* **2020**, *11*, 993. [CrossRef]
22. Boehm, A.B.; Sassoubre, L.M. Enterococci as Indicators of Environmental Fecal Contamination. In *Enterococci: From Commensals to Leading Causes of Drug-Resistant Infection*; Massachusetts Eye and Ear Infirmary: Boston, MA, USA, 2014; pp. 99–122.
23. Yang, F.; Tomberlin, J.K.; Jordan, H.R. Starvation Alters Gut Microbiome in Black Soldier Fly (Diptera: Stratiomyidae) Larvae. *Front. Microbiol.* **2021**, *12*, 601253. [CrossRef]
24. Klüber, P.; Tegtmeier, D.; Hurka, S.; Pfeiffer, J.; Vilcinskas, A.; Rühl, M. Diet Fermentation Leads to Microbial Adaptation in Black Soldier Fly (*Hermetia illucens*; Linnaeus, 1758) Larvae Reared on Palm Oil Side Streams. *Sustainability* **2022**, *14*, 5626. [CrossRef]
25. Van Looveren, N.; IJdema, F.; van der Heijden, N.; Van Der Borght, M.; Vandeweyer, D. Microbial Dynamics and Vertical Transmission of *Escherichia coli* Across Consecutive Life Stages of the Black Soldier Fly (*Hermetia illucens*). *Anim. Microbiome* **2024**, *6*, 29. [CrossRef]
26. Franco, A.; Scieuzo, C.; Salvia, R.; Pucciarelli, V.; Borrelli, L.; Addeo, N.F.; Bovera, F.; Laginestra, A.; Schmitt, E.; Falabella, P. Antimicrobial Activity of Lipids Extracted from *Hermetia illucens* Reared on Different Substrates. *Appl. Microbiol. Biotechnol.* **2024**, *108*, 167. [CrossRef] [PubMed]
27. Gorrens, E.; Van Looveren, N.; Van Moll, L.; Vandeweyer, D.; Lachi, D.; De Smet, J. *Staphylococcus aureus* in Substrates for Black Soldier Fly Larvae (*Hermetia illucens*) and Its Dynamics During Rearing. *Microbiol. Spectr.* **2021**, *9*, e0218321. [CrossRef]
28. Kanarek, P.; Breza-Boruta, B.; Bogiel, T. In the Depths of Wash Water: Isolation of Opportunistic Bacteria from Fresh-Cut Processing Plants. *Pathogens* **2024**, *13*, 768. [CrossRef]
29. Puišo, J.; Žvirgždas, J.; Paškevičius, A.; Arslonova, S.; Adlienė, D. Antimicrobial Properties of Newly Developed Silver-Enriched Red Onion–Polymer Composites. *Antibiotics* **2024**, *13*, 441. [CrossRef] [PubMed]
30. Alagappan, S.; Dong, A.; Hoffman, L.; Cozzolino, D.; Olarte Mantilla, S.; James, P.; Yarger, O.; Mikkelsen, D. Microbial Safety of Black Soldier Fly Larvae (*Hermetia illucens*) Reared on Food Waste Streams. *Waste Manag.* **2025**, *194*, 221–227. [CrossRef]
31. Amrul, N.F.; Kabir Ahmad, I.; Ahmad Basri, N.E.; Suja, F.; Abdul Jalil, N.A.; Azman, N.A. A Review of Organic Waste Treatment Using Black Soldier Fly (*Hermetia illucens*). *Sustainability* **2022**, *14*, 4565. [CrossRef]
32. Erickson, M.C.; Islam, M.; Sheppard, C.; Liao, J.; Doyle, M.P. Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Enteritidis in Chicken Manure by Larvae of the Black Soldier Fly. *J. Food Prot.* **2004**, *67*, 685–690. [CrossRef]
33. Lalander, C.H.; Diener, S.; Magri, M.E.; Zurbrügg, C.; Eriksson, S. Bioconversion of Organic Matter in Municipal Bio-Waste, Sewage Sludge, and Agro-Industrial Waste through Black Soldier Fly Larvae—From the Feed Production Perspective. *J. Clean. Prod.* **2015**, *97*, 219–229.
34. European Union. Commission Regulation (EU) No 142/2011 of 25 February 2011. *Off. J. Eur. Union* **2011**, *L 54*, 1–254.
35. European Union. Regulation (EU) 2017/893 of 24 May 2017 Amending Annexes I and IV to Regulation (EC) No 999/2001 and Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011 as Regards the Provisions on Processed Animal Protein. *Off. J. Eur. Union* **2017**, *L 138*, 92–116.
36. European Union. Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 on Making Fertilising Products Available on the Market. *Off. J. Eur. Union* **2019**, *L 190*, 1–83.
37. European Food Safety Authority (EFSA). The European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food in 2017. *EFSA J.* **2019**, *17*, e05709. [CrossRef]
38. Cai, M.; Ma, S.; Hu, R.; Tomberlin, J.K.; Thomashow, L.S.; Zheng, L.; Li, W.; Yu, Z.; Zhang, J. Rapidly Mitigating Antibiotic Resistant Risks in Chicken Manure by *Hermetia illucens* Bioconversion with Intestinal Microflora. *Environ. Microbiol.* **2018**, *20*, 4051–4062. [CrossRef]
39. Cifuentes, Y.; Glaeser, S.P.; Mvie, J.; Bartz, J.-O.; Müller, A.; Gutzeit, H.O.; Vilcinskas, A.; Kämpfer, P. The Gut and Feed Residue Microbiota Changing During the Rearing of *Hermetia illucens* Larvae. *Antonie Van Leeuwenhoek* **2020**, *113*, 1323–1344. [CrossRef] [PubMed]
40. Liu, C.; Yao, H.; Chapman, S.J.; Su, J.; Wang, C. Changes in Gut Bacterial Communities and the Incidence of Antibiotic Resistance Genes During Degradation of Antibiotics by Black Soldier Fly Larvae. *Environ. Int.* **2020**, *142*, 105834. [CrossRef]
41. Ravoitytė, B.; Varnelytė, G.; Lukša-Žebelovič, J.; Trakevičius, S.; Burokas, A.; Baltriukienė, D.; Servienė, E. Microbial Safety of Industrially Reared *Hermetia illucens* Larvae and Frass: Bacterial Dynamics and Prevalence of Antibiotic Resistance Genes. *J. Insects Food Feed* **2025**, *11*, 1739–1755. [CrossRef]
42. Novais, C.; Freitas, A.R.; Silveira, E.; Baquero, F.; Peixe, L.; Roberts, A.P.; Coque, T.M. Different Genetic Supports for the *tet*(S) Gene in Enterococci. *Antimicrob. Agents Chemother.* **2012**, *56*, 6014–6018. [CrossRef]
43. Roberts, M.C. Update on Macrolide-Lincosamide-Streptogramin, Ketolide, and Oxazolidinone Resistance Genes. *FEMS Microbiol. Lett.* **2008**, *282*, 147–159. [CrossRef]

44. Ali, I.; Naz, B.; Liu, Z.; Chen, J.; Yang, Z.; Attia, K.; Ayub, N.; Ali, I.; Mohammed, A.A.; Faisal, S.; et al. Interplay among Manures, Vegetable Types, and Tetracycline Resistance Genes in Rhizosphere Microbiome. *Front. Microbiol.* **2024**, *15*, 1392789. [CrossRef]
45. Roberts, M.C.; Sutcliffe, J.; Courvalin, P.; Jensen, L.B.; Rood, J.; Seppälä, H. Nomenclature for Macrolide and Macrolide-Lincosamide-Streptogramin B Resistance Determinants. *Antimicrob. Agents Chemother.* **1999**, *43*, 2823–2830. [CrossRef]
46. Bohm, K.; Taylor, W.; Gyawali, P.; Pattis, I.; Ginés, M.J.G. Black Soldier Fly-Based Bioconversion of Biosolids: Microbial Community Dynamics and Fate of Antibiotic Resistance Genes. *Sci. Total Environ.* **2024**, *930*, 172823. [CrossRef]
47. Duarte, R.D.C.; Iannetta, P.P.M.; Gomes, A.M.; Vasconcelos, M.W. More than a Meat- or Synthetic Nitrogen Fertiliser-Substitute: A Review of Legume Phytochemicals as Drivers of 'One Health' via Their Influence on the Functional Diversity of Soil- and Gut-Microbes. *Front. Plant Sci.* **2024**, *15*, 1337653. [CrossRef]
48. Chopra, I.; Roberts, M. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 232–260. [CrossRef] [PubMed]
49. Yamaguchi, A.; Shiina, Y.; Fujihira, E.; Sawai, T.; Noguchi, N.; Sasatsu, M. The Tetracycline Efflux Protein Encoded by the *tet*(K) Gene from *Staphylococcus aureus* Is a Metal-Tetracycline/H+ Antiporter. *FEBS Lett.* **1995**, *365*, 193–197. [CrossRef]
50. Scott, K.P.; Melville, C.M.; Barbosa, T.M.; Flint, H.J. Occurrence of the New Tetracycline Resistance Gene *tet*(W) in Bacteria from the Human Gut. *Antimicrob. Agents Chemother.* **2000**, *44*, 775–777. [CrossRef] [PubMed]
51. Young, S.; Nayak, B.; Sun, S.; Badgley, B.D.; Rohr, J.R.; Harwood, V.J. Vancomycin-Resistant Enterococci and Bacterial Community Structure Following a Sewage Spill into an Aquatic Environment. *Appl. Environ. Microbiol.* **2016**, *82*, 5653–5660. [CrossRef] [PubMed]
52. Barker, G.C.; Pistritto, G.; Muñoz, A.; Blanco, J.E.; Marín, M.; de la Cruz, F. Antimicrobial Plant Compounds and Their Influence on Bacterial Stress Tolerance and Resistance Selection. *Front. Microbiol.* **2017**, *8*, 2265.
53. Carretto, E.; Visiello, R.; Nardini, P. Methicillin Resistance in *Staphylococcus aureus*. In *Pet-to-Man Travelling Staphylococci: A World in Progress*; Savini, V., Ed.; Macmillan Publishers: New York, NY, USA, 2018; pp. 225–235.
54. Haudiquet, M.; de Sousa, J.M.; Touchon, M.; Rocha, E.P.C. Selfish, Promiscuous and Sometimes Useful: How Mobile Genetic Elements Drive Horizontal Gene Transfer in Microbial Populations. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2022**, *377*, 20210234. [CrossRef] [PubMed]
55. Lunde, T.M.; Hjerde, E.; Al-Haroni, M. Prevalence, Diversity and Transferability of the Tn916-Tn1545 Family ICE in Oral Streptococci. *J. Oral Microbiol.* **2022**, *13*, 1896874. [CrossRef] [PubMed]
56. Tello, A.; Austin, B.; Telfer, T.C. Selective Pressure of Antibiotic Pollution on Bacteria of Importance to Public Health. *Environ. Health Perspect.* **2012**, *120*, 1100–1106. [CrossRef] [PubMed]