

Changes in Potential Pathogens and Antibiotic Resistance-Related Functional Profiles during the Bioconversion of Chicken Manure by Black Soldier Fly Larvae

Xueyi Ren, Shuai Han, Xinyu Geng, Baolu Wang, Nan Wu *

College of Engineering and Technology, Tianjin Agricultural University, Tianjin, 300392, China

* Corresponding author: Nan Wu

Abstract

Black soldier fly larvae (BSFL) bioconversion is considered a promising approach for the harmless treatment and resource utilization of livestock manure. In this study, chicken manure and its residues after BSFL bioconversion and secondary composting were investigated to evaluate changes in potential pathogens and antibiotic resistance-related functions using high-throughput sequencing-based analysis. A total of 12 potential pathogenic species were detected throughout the treatment process. Chicken manure (CM) showed the highest abundance of potential pathogens, with *Escherichia coli* being dominant. Its relative abundance decreased from 0.2166 in CM to 0.0001 after secondary composting, and the other potential pathogens also exhibited an overall declining trend. Analysis of antibiotic resistance-related functional profiles showed differential responses among functional categories. The relative abundance of vancomycin resistance-related functions decreased steadily from approximately 0.280% to 0.220%, whereas β -lactam resistance-related functions changed little after BSFL bioconversion and increased to approximately 0.450% after secondary composting. These results indicate that BSFL bioconversion combined with secondary composting can effectively reduce some potential pathogens and certain antibiotic resistance-related functions in chicken manure, but the responses of different resistance types vary. Further attention should therefore be paid to the persistence and potential dissemination risk of specific resistance traits during manure resource utilization.

Keywords

Black Soldier Fly; Antibiotic Resistance; Pathogenic Bacteria; Secondary Composting; High-Throughput Sequencing.

1. Introduction

With the rapid expansion of intensive poultry farming, the generation of chicken manure has increased substantially, making it a major source of organic solid waste. Chicken manure is characterized by a high moisture content and rapid biodegradability, and its improper disposal or uncontrolled accumulation can cause odor emissions, soil and water contamination, and potential biosafety risks [1]. Moreover, the prophylactic and therapeutic use of antibiotics in intensive poultry production may contribute to the enrichment of pathogenic bacteria and antibiotic resistance genes (ARGs) in chicken manure. Pathogens such as *Salmonella* spp. and *Escherichia coli* can persist in manure for extended periods, while ARGs associated with resistance to antibiotics such as tetracyclines and sulfonamides can disseminate through soil, water, and airborne particles, posing potential risks to environmental and public health through food chains and human-animal contact [2]. Conventional treatment methods, including composting and air drying, may have limited effectiveness in eliminating ARGs and therefore may not completely prevent their environmental dissemination [3].

The black soldier fly (*Hermetia illucens*) is an efficient bioconversion insect capable of rapidly degrading organic matter in chicken manure and reducing the abundance of pathogenic microorganisms, demonstrating considerable potential for the treatment and valorization of organic wastes [4]. However, previous studies have primarily focused on waste reduction and odor mitigation during a single-stage BSFL bioconversion process. Systematic investigations into the subsequent composting of BSFL residues for further reducing potential pathogenic bacteria and ARGs remain relatively limited [5].

Therefore, this study investigated the effects of BSFL bioconversion followed by secondary composting on the dynamics and reduction of potential pathogenic bacteria and ARGs in chicken manure. The objectives were to elucidate the potential mechanisms underlying the enhanced sanitization achieved by this combined treatment and to provide a theoretical basis for the environmentally sustainable treatment and resource recovery of livestock and poultry wastes.

2. Materials and Methods

2.1. BSFL Bioconversion Experiment

BSFL eggs were obtained from the Research and Development Center for Organic Waste Resource Utilization Technology, Tianjin Agricultural University, China. The eggs were incubated for 48 h at 30 °C and 80% relative humidity. After hatching, the larvae were transferred to wheat bran with a moisture content of 80% and reared for 8 days before being used in the manure bioconversion experiment. Chicken manure was collected from a poultry farm in Tianjin, China.

Four treatments were established: untreated raw chicken manure (CM), frass obtained after larval bioconversion of chicken manure (CBF), composted chicken-manure-derived BSFL frass (CBV), and conventionally composted chicken manure (CV). Each treatment was performed in triplicate.

The BSFL were allowed to convert the chicken manure for 8 days. Subsequently, feeding was discontinued for 24 h to allow the larvae to evacuate the residual contents of their digestive tracts. The larvae were then separated from the resulting frass, which was collected for subsequent treatment and analysis.

2.2. Identification of Potential Pathogens

High-throughput sequencing of 16S rRNA gene was performed on all samples, and the resulting operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) were compared against the NCBI database. Potential pathogenic bacteria were identified through taxonomic annotation, and their relative abundances were calculated [6].

2.3. Prediction of Antibiotic Resistance-Related Functions

Functional prediction of microbial communities was conducted based on 16S rRNA gene sequences with reference to the KEGG database using PICRUSt2. After normalization, OTU abundance tables were subjected to functional prediction analysis to obtain functional gene abundance matrices for each sample. β -lactam and vancomycin resistance-related functional entries were extracted and quantified.

3. Results and Discussion

3.1. Composition of Potential Pathogenic Bacteria in Samples

The composition of potential pathogenic bacteria in different samples is shown in Fig. 1. A total of 12 potential pathogenic bacterial species were identified. Among them, *Escherichia coli* and

Clostridium perfringens accounted for the highest proportions, both at 11.15%, followed by *Pseudomonas fluorescens* (9.13%) and *Serratia marcescens* (8.11%). *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Bacillus cereus* each accounted for 7.10%, while *Stenotrophomonas maltophilia* accounted for 6.80%. In contrast, *Vibrio vulnificus* and *Vibrio aestuarianus* exhibited the lowest proportions, both at 1.10%. Overall, a diverse range of potential pathogenic bacteria was identified in chicken manure, with *E. coli*, *C. perfringens*, and *P. fluorescens* representing the predominant species. These results indicated that untreated chicken manure may harbor a variety of potentially pathogenic microorganisms, suggesting potential biosafety risks associated with its direct disposal or environmental application without appropriate treatment.

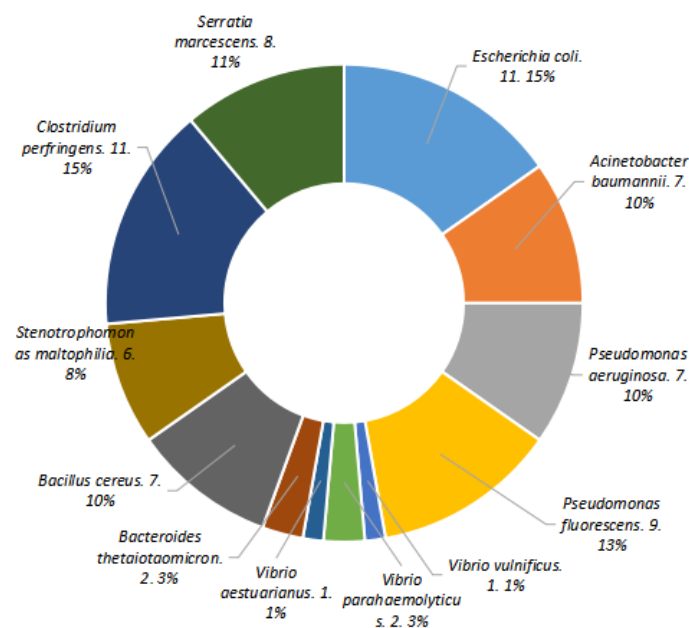


Fig 1. Relative proportions of the identified potential pathogenic bacteria.

3.2. Changes in Potential Pathogenic Bacterial Communities across Different Treatment Stages

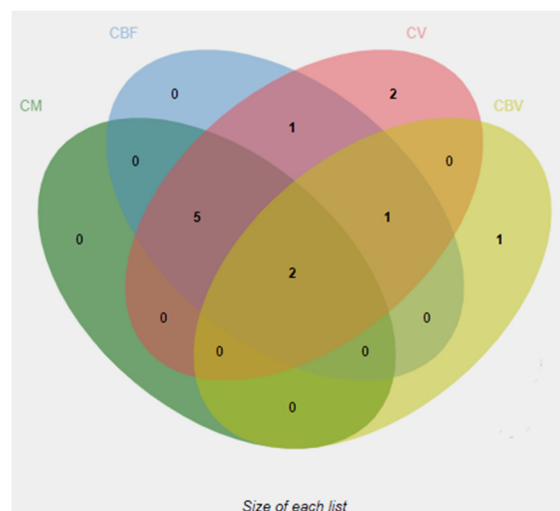


Fig 2. Venn diagram of potential pathogenic bacteria among different treatment groups.

As shown in the Venn diagram (Fig. 2), two potential pathogenic bacterial species were shared among the CM, CBF, CV, and CBV groups, indicating that some potential pathogenic bacteria

persisted throughout the different treatment stages. No unique potential pathogenic bacterial species were detected in either CM or CBF, whereas two and one unique species were identified in CV and CBV, respectively. Overall, differences in the composition of potential pathogenic bacterial communities were observed among the different treatment stages. The results indicated that the community composition of potential pathogenic bacteria underwent dynamic changes during BSFL bioconversion, conventional composting, and subsequent composting of BSFL frass.

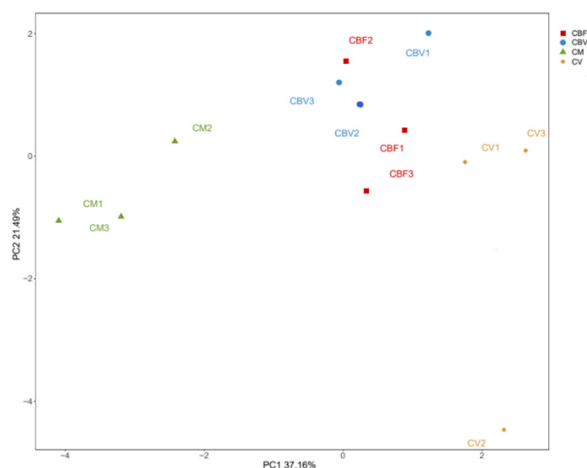


Fig 3. Principal component analysis (PCA) of potential pathogenic bacterial communities.

The principal component analysis (PCA) of potential pathogenic bacterial communities in different samples is shown in Fig. 3. PC1 and PC2 explained 37.16% and 21.49% of the total variation in community composition, respectively, accounting for a cumulative 58.65% of the variation. The different treatment groups exhibited a certain degree of separation in the ordination space. In particular, CM samples were mainly distributed along the negative axis of PC1 and were relatively distant from the other treatment groups, indicating differences in the composition of potential pathogenic bacterial communities between untreated chicken manure and the treated samples.

The CBF and CBV samples were positioned relatively close to each other, with samples within each group showing relatively tight clustering. This pattern suggested that the composition of potential pathogenic bacterial communities in BSFL frass remained relatively similar after subsequent composting.

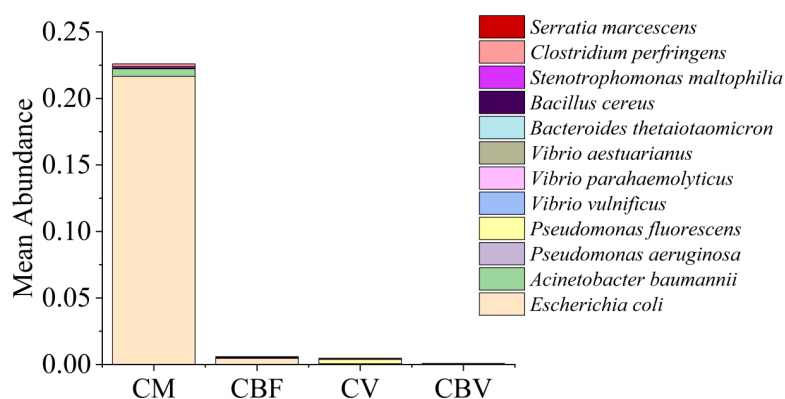


Fig 4. Changes in the mean relative abundance of potential pathogenic bacteria among different samples.

As shown in Fig. 4, the overall relative abundance of potential pathogenic bacteria was relatively high in untreated chicken manure (CM). Following BSFL bioconversion, the relative abundances of various pathogenic bacteria decreased to different extents in the resulting frass (CBF), which is consistent with previous findings showing that BSFL treatment can reduce the abundance of pathogenic bacteria in manure [7]. After subsequent composting, the overall abundance of potential pathogenic bacteria further decreased in CBV.

Among the identified potential pathogenic bacteria, *Escherichia coli* exhibited the most pronounced decrease. Its mean relative abundance was 0.2166 in CM and decreased to 0.0046 following BSFL bioconversion (CBF). After subsequent composting, its mean relative abundance further decreased to 0.0001 in CBV. These results indicate that BSFL bioconversion substantially reduced the abundance of *E. coli* in chicken manure, while subsequent composting further enhanced its reduction.

3.3. Correlation Analysis between Environmental Factors and Potential Pathogenic Bacteria

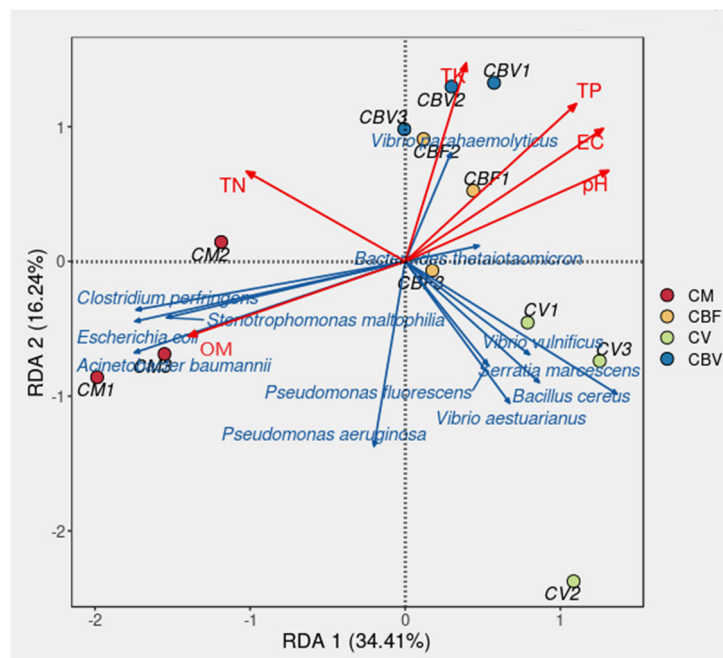


Fig 5. Redundancy analysis (RDA) of the relationships between potential pathogenic bacterial abundance and environmental factors.

RDA1 and RDA2 explained 34.41% and 16.24% of the variation in potential pathogenic bacterial communities, respectively (Fig. 5), accounting for a cumulative 50.65% of the total variation. Regarding the ordination of environmental factors, EC and pH were primarily oriented along the positive direction of RDA1, whereas OM and TN were mainly oriented along the negative direction of RDA1. In contrast, TK and TP were primarily oriented along the positive direction of RDA2, indicating that different environmental factors exhibited distinct patterns of association with the distribution of potential pathogenic bacterial communities.

Specifically, *Vibrio parahaemolyticus* was positively correlated with TK, TP, EC, and pH, with a relatively strong correlation observed with TK. *Acinetobacter baumannii* and *Clostridium perfringens* were positively correlated with OM but negatively correlated with EC and pH. Overall, these results suggest that changes in environmental factors during the treatment processes may be closely associated with shifts in the community structure of pathogenic bacteria.

3.4. Prediction of Antibiotic Resistance-Related Functions

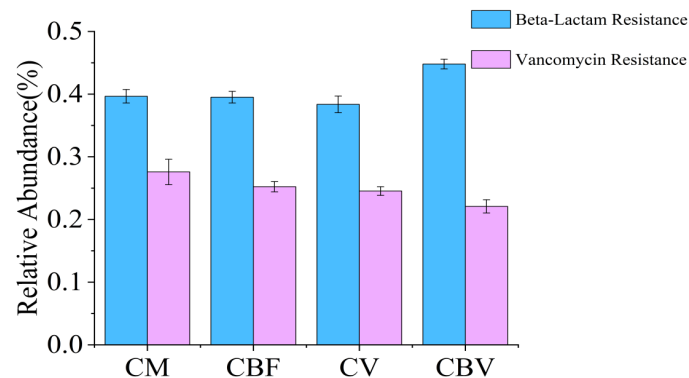


Fig 6. Changes in the relative abundance of antibiotic resistance-related pathways among different treatment groups.

As shown in Fig. 6, the relative abundance of β -lactam resistance-related functions was consistently higher than that of vancomycin resistance-related functions across all treatment groups. The relative abundance of β -lactam resistance-related functions ranged from 0.38% to 0.45%, with similar levels observed in the CM, CBF, and CV groups, whereas an increase to approximately 0.45% was observed in the CBV group. The increased relative abundance of β -lactam resistance-related functions in CBV may be associated with microbial community restructuring and changes in environmental conditions during the treatment process; however, the underlying mechanisms require further investigation.

In contrast, vancomycin resistance-related functions exhibited an overall decreasing trend. Their relative abundance decreased from approximately 0.28% in CM to approximately 0.25% in CBF and further declined to 0.22% in CBV following subsequent composting. A decrease was also observed in CV compared with CM, with the relative abundance declining to approximately 0.24%. Overall, these results indicate that different antibiotic resistance-related functions exhibited distinct responses to BSFL bioconversion and subsequent composting.

Previous studies have demonstrated that BSFL bioconversion can alter both the microbial community structure and resistome composition of livestock and poultry manure, although different ARG types may respond differently to the treatment process. Cai et al. reported that BSFL bioconversion of chicken manure significantly reduced the abundance of multiple ARGs and integron-associated genes, accompanied by changes in potential bacterial hosts of ARGs[8]. Similarly, Zhao et al. found that BSFL treatment reduced the relative abundance of ARGs in chicken manure, although the extent of reduction varied among different ARG types[9]. Therefore, the contrasting trends observed for β -lactam and vancomycin resistance-related functions in the present study may be associated with changes in microbial community composition and environmental conditions during BSFL bioconversion and subsequent composting.

As shown in Fig. 7, Pearson correlation analysis revealed that β -lactam resistance-related functions were highly significantly and positively correlated with pH, total phosphorus (TP), electrical conductivity (EC), and total potassium (TK) ($P < 0.01$), but significantly and negatively correlated with organic matter (OM) ($P < 0.05$). Vancomycin resistance-related functions were also positively correlated with pH, TP, EC, and TK; however, these correlations were not statistically significant ($P > 0.05$).

Overall, β -lactam resistance-related functions exhibited stronger correlations with the physicochemical properties of the composting system than vancomycin resistance-related functions. These findings suggest that variations in the relative abundance of β -lactam resistance-related functions may be closely associated with changes in physicochemical factors.

In contrast, vancomycin resistance-related functions showed relatively weak correlations with the measured environmental factors, suggesting that their variation may also be associated with microbial community composition and other environmental factors [10].

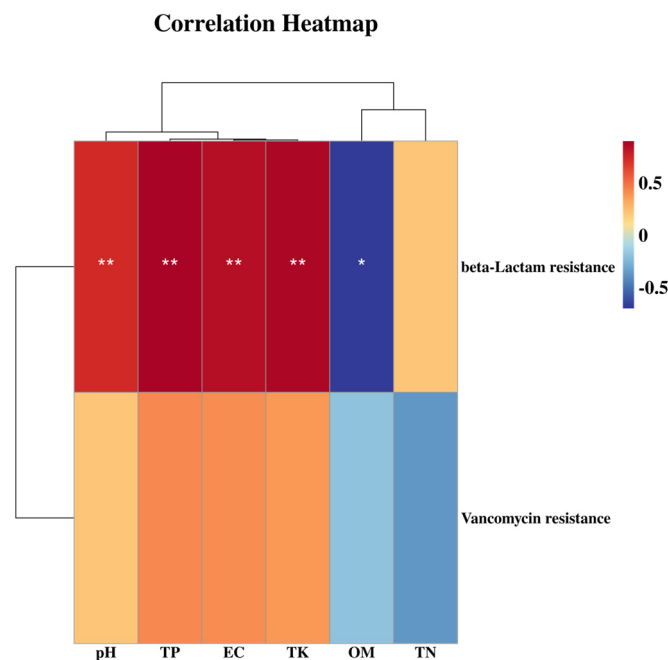


Fig 7. Pearson correlation heatmap between antibiotic resistance-related functions and environmental factors. Note: * indicates a significant correlation at $P < 0.05$, and ** indicates a highly significant correlation at $P < 0.01$.

4. Conclusion

This study investigated changes in potential pathogenic bacteria and antibiotic resistance-related functions during BSFL bioconversion of chicken manure and subsequent composting. The results showed that BSFL bioconversion effectively reduced the relative abundance of potential pathogenic bacteria in chicken manure, with *Escherichia coli* exhibiting the most pronounced reduction. Subsequent composting further enhanced the reduction of *E. coli*. The composition of pathogenic bacterial communities varied across different treatment stages and showed different degrees of correlation with environmental factors. Different antibiotic resistance-related functions exhibited distinct responses to the treatment processes. The relative abundance of vancomycin resistance-related functions showed an overall decreasing trend, whereas β -lactam resistance-related functions slightly increased after subsequent composting of BSFL frass.

Overall, the combination of BSFL bioconversion and subsequent composting contributed to reducing the potential risks associated with pathogenic bacteria in chicken manure. However, its effects on different antibiotic resistance-related functions varied, indicating that potential antibiotic resistance risks should still be carefully considered during the resource utilization of chicken manure.

Acknowledgments

This work was supported by Scientific Research Project of Tianjin Education Commission (2023KJ003).

References

- [1] Tang, W. X., Sun, X. B., Gao, H. Z., et al. (2020). Isolation and identification of multidrug-resistant bacteria in feces from large-scale livestock and poultry farms and their antibiotic-resistance characteristics. *Journal of Agro-Environment Science*, 39(1), 207–216. (in Chinese)
- [2] Zhang, W. Y., Cao, Z. W., Zhi, J. Q., et al. (2023). Contents of various components in manure at intensive livestock farms in Beijing. *Animal Husbandry & Veterinary Medicine*, 55(4), 41–49. (in Chinese)
- [3] Li, Y., Shen, L., Gao, H. Z., et al. (2025). Occurrence characteristics of conjugative antibiotic resistance plasmids and antibiotic resistance genes in manure from large-scale livestock and poultry farms. *Environmental Science*, 46(2), 1245–1254. (in Chinese)
- [4] Jiang, X. R., Li, T., Sun, X. B., et al. (2022). Dissipation kinetics of multidrug-resistant bacteria, antibiotic resistance genes and integrase genes during simulated composting of chicken manure. *Research of Environmental Sciences*, 35(4), 1045–1055. (in Chinese)
- [5] Lu, L. Z., Deng, D., Ma, P., et al. (2020). Research progress in composting with black soldier fly to promote the decomposition of livestock and poultry manure. *Guangdong Agricultural Sciences*, 47(8), 110–117. (in Chinese)
- [6] Fang, H., Wang, H. F., Cai, L., et al. (2015). Prevalence of antibiotic resistance genes and bacterial pathogens in long-term manured greenhouse soils as revealed by metagenomic survey. *Environmental Science & Technology*, 49(2), 1095–1104.
- [7] Awasthi, M. K., Liu, T., Awasthi, S. K., et al. (2020). Manure pretreatments with black soldier fly *Hermetia illucens* L. (Diptera: Stratiomyidae): A study to reduce pathogen content. *Science of the Total Environment*, 737, 139842.
- [8] Cai, M. M., Ma, S. T., Hu, R. Q., et al. (2018). Rapidly mitigating antibiotic resistant risks in chicken manure by *Hermetia illucens* bioconversion with intestinal microflora. *Environmental Microbiology*, 20(11), 4051–4062.
- [9] Zhao, Z. Z., Yang, C. R., Gao, B. Q., et al. (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.
- [10] Peng, J., Wang, K., Gu, Y., et al. (2019). Effects of environmental factors on tetracycline and macrolides resistance genes in cattle manure composting systems. *Environmental Science*, 40(3), 1439–1445. (in Chinese)