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RESEARCH ARTICLE

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Hermetia illucens L. larvae–associated intestinal microbes reduce the transmission risk of zoonotic pathogens in pig manure

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Abstract

Black soldier fly (BSF) larvae are considered a promising biological reactor to convert organic waste and reduce the impact of zoonotic pathogens on the environment. We analysed the effects of BSF larvae on *Staphylococcus aureus* and *Salmonella* spp. populations in pig manure (PM), which showed that BSF larvae can significantly reduce the counts of the associated *S. aureus* and *Salmonella* spp. Then, using a sterile BSF larval system, we validated the function of BSF larval intestinal microbiota in vivo to suppress pathogens, and lastly, we isolated eight bacterial strains from the BSF larval gut that inhibit *S. aureus*. Results indicated that functional microbes are essential for BSF larvae to antagonise *S. aureus*. Moreover, the analysis results of the relationship between the intestinal microbiota and *S. aureus* and *Salmonella* spp. showed that *Myroides*, *Tissierella*, *Oblitimonas*, *Paenalcalignes*, *Terrisporobacter*, *Clostridium*, *Fastidiosipila*, *Pseudomonas*, *Ignatzschineria*, *Savagea*, *Moheibacter* and *Sphingobacterium* were negatively correlated with *S. aureus* and *Salmonella*. Overall, these results suggested that the potential ability of BSF larvae to inhibit *S. aureus* and *Salmonella* spp. present in PM is accomplished primarily by gut-associated microorganisms.

Yuanpu Zhang and Xiaopeng Xiao contributed equally to this work.

INTRODUCTION

With rapid economic growth, the standard of living in many locations has gradually improved resulting in increasing demand for high-quality protein sources from livestock production. However, the risk for environmental pollution from the resulting manures also increases (Zhang et al., 2021). Not only the quantity of manure is a problem but also its quality due to antibiotics is often added as additives to animal feed (Hashemi, 2018). Many countries have banned the use of antibiotics as growth promoter since 2006 (Ben et al., 2017; Carlota et al., 2021; Xue et al., 2021). Manure is also regarded as a significant reservoir of several pathogens such as *Staphylococcus*, *Salmonella*, *Listeria*, *Escherichia coli*, *Streptococcus*, *Clostridium*, *Campylobacter*, *Corynebacterium* and *Mycobacterium* (Chen & Jiang, 2014; Chung et al., 2021; Shepherd et al., 2010). Therefore, the manure disposition needs much consideration to prevent spreading of these pathogens, because the direct release of manure from farms into environment before treatment could not only harm humans but also even overall ecosystems (Giwa et al., 2020). Currently, composting and anaerobic digestion are the main manure treatment methods (Awasthi et al., 2019). However, concerns have been raised about their efficient ability to eliminate pathogenic bacteria, and researchers have found that vegetables treated with composted manure as a fertiliser can also be contaminated (Ingham et al., 2004). Therefore, new and environmentally friendly manure treatment methods are urgently needed to decrease the environmental pollution with zoonotic pathogens in manure. Using insects to convert poultry manure into insect biomass can treat manure in an environmentally friendly way and also produce proteins for further use (Mazza et al., 2020). Manure is the main food source for many insects, which can efficiently convert manure into biomass whilst keeping a low environmental cost and recycling waste (Lalander et al., 2019). One of the most environmentally friendly and most economically viable insect species is the black soldier fly (BSF, *Hermetia illucens*; Diptera: Stratiomyidae) (Abdel-Latif et al., 2021; Abdel-Tawwab et al., 2020; Mazza et al., 2020).

Black soldier fly is widely distributed throughout temperate and tropical regions and is often associated with livestock manure (Li et al., 2011). BSF larvae can feed on decaying organic wastes, such as animal manure and plant material, and exhibit activity against zoonotic pathogens in organic wastes (Elhag et al., 2017; Lalander et al., 2015). This disinfection progress is mainly associated with BSF larva gut microorganisms. The microbiome associated with BSF larvae is relatively abundant. A large number of microorganisms are distributed in the egg surface and mid gut. They can promote the digestion of organic matter, growth and reproduction of BSF. The abundance and diversity of intestinal microflora of BSF larvae differed significantly under different feeding conditions and rearing substrates, and the dominant intestinal flora in the mid gut included Firmicutes, Bacteroidetes and Proteobacteria (Zhan et al., 2020). Some studies have demonstrated that BSF larva gut microbiota might be shaped by different substrates (Bruno et al., 2019; Jeon et al., 2011). However, the correlation between BSF larva gut microbes and zoonotic pathogens and the change of microbial structure in pig manure during the transformation of BSF larvae remain poorly understood.

Data indicated that symbiotic microbes are important partners with their hosts (Colman et al., 2012; Rehman et al., 2017). Insects use several mechanisms to suppress pathogens (Wei et al., 2016), such as membrane disruption, targeting cytoplasmic components and inhibiting their metabolism such as insect employs miRNAs to induce cross-kingdom silencing of pathogen virulence-related genes, conferring resistance to infection (Wu et al., 2018; Wang et al., 2021). Several antimicrobial substances are produced by the insect's digestive tract to prevent microbial infection (Choi et al., 2012). Previous research indicated that insect symbiotic microbes can suppress the colonisation of several invasive microbes (Cirimotich et al., 2011; Dong et al., 2009). Moreover, gut bacteria can also suppress human pathogen invasion through the gastrointestinal system (Krismer et al., 2017; Piewngam et al., 2018). Some gut bacteria have developed various adaptive strategies, including the production of antimicrobial substances, such as bacteriocins and microcins, against Gram-negative bacteria. Some of these substances are highly stable in the gastrointestinal tract, which then target pathogens with minimal impacts on the already present beneficial gut microbes (Telhig et al., 2020).

In a previous study, we isolated a gut bacterium, *Bacillus subtilis*, from BSF larvae that showed strong inhibitory effects against *Xanthomonas oryzae* PXo99 and *Rhizoctonia solani* AG-8. We have speculated that the active substances involved in this antagonism may be the lipopeptides iturin and surfactin (Zhou et al., 2012). However, more studies are still required to explore the antagonistic activities of insect gut-associated microorganisms and to identify a novel approach for intestinal bacterial application.

Intestinal microbes of BSF larvae play an important role. More specifically, we isolated intestinal microbes antagonistic to *S. aureus* from the gut of BSF larvae and found that the antagonistic ability of sterile BSF larvae against *S. aureus* could be improved after being inoculated these microbes. These functional microorganisms may directly produce antagonistic substances against *S. aureus* in the intestinal

of the BSF larvae, reducing the number of *S. aureus*. Gut microbes are critical to the growth and development of their hosts, and it is possible that these microorganisms may enhance the ability of the BSF larvae to produce antimicrobial peptides, thereby reducing the number of *S. aureus* and *Salmonella* spp. In addition, intestinal microorganisms can promote the transformation of the BSF larvae, and reduce the nutrients and water content in the material, making *S. aureus* and *Salmonella* spp. not suitable for the growth in this circumstance. In this concern, Lalander et al. (2015) found that BSF larvae had the ability to reduce zoonotic pathogen bacteria especially *Salmonella* spp., when used in the treatment of organic wastes. Furthermore, compared with other Diptera, the BSF genome encodes more peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) (Zhan et al., 2020). Further studies showed that the immune genes *BsfDuox* and *BsfTLR3* can regulate the homoeostasis of intestinal bacteria *Providencia* and *Dysgonomonas* to inhibit zoonotic pathogens (Huang et al., 2020).

Several studies have preliminarily investigated the intestinal microbiota of BSF larvae under limited conditions (Bruno et al., 2019; Erickson et al., 2004; Jeon et al., 2011; Mazza et al., 2020; Wynants et al., 2019; Zheng et al., 2013). However, few studies have analysed the complete distribution of intestinal microbiota of BSF larvae under the dynamics of common manure types and feeding times. In this study, we aimed to establish the relationship between BSF larvae intestinal microorganisms and zoonotic pathogens (*Staphylococcus aureus*, *Salmonella* spp.) in manure and the changes of manure microbial community structure during the transformation of BSF larvae. We used BSF larvae to convert fresh pig manure (PM). Meanwhile, we screened out purely culturable larva gut microorganisms antagonising *S. aureus* for possible potential industrial applications. As it was known, obtaining pure bacteria by microbial isolation and identification is necessary for research purposes and industrial applications (Prakash et al., 2013). We examined the changing trend of *S. aureus* and *Salmonella* spp. in PM during the transformation of BSF larvae and then analysed the changes of larva gut microflora and PM microflora during the transformation of BSF larvae. Pure strains with antagonism against *S. aureus* were isolated from the larva gut. Because BSF larvae are often used in the industrial conversion of organic waste, they can be used as animal feed, and the converted residue can be used as organic fertiliser (Barrag'an-Fonseca et al., 2017). Purifying the antagonistic microorganisms against *S. aureus* and understanding the relationships of these larva gut microbes with zoonotic pathogens may help improve the microbiological safety of insects as livestock feed and converted residue as fertiliser (Bosch et al., 2019).

EXPERIMENTAL PROCEDURES

Insects

Black soldier fly adults were maintained in a colony located in a greenhouse. Eggs were collected in corrugated cardboard strips on a moist substrate of bran and microbial fermentation broth from the BSF colony of the State Key Laboratory of Agricultural Microbiology of Huazhong Agricultural University, Wuhan, China. The colony was maintained in a greenhouse at 27 ± 1°C with 60%–70% relative humidity and 12-h light/12-h dark photoperiod. For the first 5 days, larvae were fed with a diet based on bran with 65% water content. Feeding was stopped on the sixth day, and larvae were separated from the feed.

PM

Fresh PM was collected from the National Engineering and Technology Research Center for Livestock of Huazhong Agricultural University, Wuhan, China.

PM conversion systems and sampling

Approximately 7500 6-day-old BSF larvae with similar weight were added to 5 kg fresh PM with 70% water content in a plastic container (length: 25 cm, width: 20 cm, height: 12 cm) and labelled as 'PM inoculated larva group' (PM + BSFL). The control PM did not contain larvae. Each treatment was set up in three replicates and kept in a greenhouse for 8 days at 27 ± 1°C and 65% ± 5% relative humidity.

The dynamic changes of *S. aureus* and *Salmonella* spp. were detected during the transformation of PM by BSF larvae. The methods were described as previously described (Huang et al., 2020). In brief, about 5 g of faecal residue was sampled at 0, 2, 4, 6 and 8 days of each replicate. Samples were placed in an individual 50-ml sterile plastic tube (Biosharp) and added into 45-ml sterile water. After vortex shock for 5 min, the samples were kept at room temperature for 5 min, and the supernatant was pipetted for 10-fold gradient dilution. About 100 μl of each gradient dilution was pipetted and coated with selective medium (Qingdao Hope Bio-technology Co., Ltd) for *S. aureus* and *Salmonella* spp counting respectively. Each gradient dilution was set up in three replicates on each selective medium (Qingdao Hope Bio-technology Co., Ltd) and cultured in a 37°C constant temperature incubator for more than 36 h. The bacterial numbers of the two zoonotic pathogens

were recorded. The number of the colony on the plate around 30–300 CFU was used to take count of the total bacteria.

PM samples (10 g) were randomly collected from each replicate every 2 days and placed in an individual 50-ml sterile plastic tube (Biosharp). Additionally, 10 larvae were sampled from each replicate, placed in a 15-ml sterile plastic bottle (Biosharp) and starved for 24 h. All samples (Table 1) were frozen in liquid nitrogen for 1 h and preserved at −80°C for subsequent microbiome analysis.

Verification of intestinal microorganism function

Sterile BSF larva preparation was described as previously described (Cai et al., 2018). The intestinal extracting solution was prepared by transforming 8-day-old larvae from PM. The larvae were starved for 1 day, disinfected with 75% alcohol for 3 min and rinsed with sterile water three times. Intestinal tracts were dissected, and five intestinal tracts were placed into one group. The intestinal extract was prepared after homogenisation. Another batch of PM was collected from the National Engineering and Technology Research Center for Livestock of Huazhong Agricultural University, Wuhan, China, before being flushed using water. The water content of PM was adjusted to 70%. Thereafter, the treatment experiments were as follows: (1) 300 sterile BSF larvae and 200 g fresh PM (BSF-S, n = 3); 300 conventional BSF larvae and 200 g fresh PM (BSF-N, n = 3); 300 sterile BSF larvae with intestinal extracting solution and 200 g fresh PM (BSF-I, n = 3); 200 g fresh PM and no BSF larvae (CK, n = 3). Manure samples were collected at 0, 4, 6, 8 and 12 days after conversion, and the changes in bacterial counts of *Salmonella* spp. and *S. aureus* in manure were detected by using selective medium (Qingdao Hope Bio-technology Co., Ltd).

Isolation of strains from BSF larva gut and against *S. aureus* in vitro

Luria–Bertani (LB) medium (tryptone 10.0 g/L, yeast extract 5.0 g/L, NaCl 10.0 g/L, agar 20.0 g/L, distilled water 1 L, pH 7.0 ± 0.2), Gause I Medium (soluble starch 20 g/L, KNO_3 1 g/L, K_2HPO_4 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, NaCl 0.5 g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L, agar 20.0 g/L, distilled water 1 L, pH 7.2–7.4), potato dextrose agar medium (potato 200 g/L, glucose 20 g/L, agar 20.0 g/L, distilled water 1 L), inorganic agar medium (K_2HPO_4 1.0 g/L, KH_2PO_4 0.3 g/L, $MgSO_4{\cdot}7H_2O$ 0.1 g/L, NaCl 1.0 g/L, NH_4NO_3 1.0 g/L, agar 15 g/L, distilled water 1 L, pH 7.0), MRS medium (beef extract 10 g/L, glucose 20 g/L, peptone 10 g/L, K_2HPO_4 2 g/L, yeast extract 5 g/L, ammonium citrate dibasic 2 g/L, sodium acetate 5 g/L, $MnSO_4{\cdot}4H_2O$ 0.25 g/L, $MgSO_4{\cdot}7H_2O$ 0.58 g/L, Tween-80 1 g/L, distilled water 1 L, pH 6.2 ± 0.2) were used to isolate and purify the intestinal culturable microorganisms of BSF larva transformed PM for 0, 2, 4, 6 and 8 days. The BSF larva gut isolate strains were tested for antagonistic effect on Petri dishes by the dual culture method (Zivkovic et al., 2010) against *S. aureus*. *S. aureus* and all isolates were grown to the logarithmic phase at 37°C in the corresponding liquid medium. Two hundred microlitres of *S. aureus* fermentation liquid were added into 100 ml of cooled but molten LB agar medium and then poured into plates. Two hundred microlitres of fermentation broth were added to the Oxford cup on the indicator plate. The fermentation broth was also centrifuged at 8000 r/min for 2 min, and the supernatant was filtered through a 0.22-μm filter membrane. About 200 μl of the filtrate of each strain was added to the Oxford cup on the indicator plate. Plates were incubated at 30°C for 24 h. All experiments were performed in triplicate.

Determination of intestinal microbe antagonism against *S. aureus* in vivo

The preliminarily screened intestinal microorganisms were shaken in LB liquid medium at 28°C for 36 h and diluted to 10^9 CFU/ml with sterile water. After incubation, sterile BSF larvae were inoculated with 5% (v/m) intestinal bacterial suspensions and incubated at 28°C until the eighth day. Eight-day-old BSF larvae were isolated from the raw material and starved for 1 day. The body surface was disinfected with 75% alcohol for 3 min. The larvae were washed with sterile water three times and inoculated into sterile wheat bran with

TABLE 1 Samples used for bacterial diversity analysis

Time	Pig manure inoculated larvae	Pig manure	BSF larva gut
0 day	IPM0001–IPM0003	IPM0001–IPM0003	IM0001–IM0003
2 days	PMIL0201–PMIL0203	PM0201–PM0203	IM0201–IM0203
4 days	PMIL0401–PMIL0403	PM0401–PM0403	IM0401–IM0403
6 days	PMIL0601–PMIL0603	PM0601–PM0603	IM0601–IM0603
8 days	PMIL0801–PMIL0803	PM0801–PM0803	IM0801–IM0803

Abbreviations: IM, intestinal microorganism; IPM, initial pig manure; PM: pig manure; PMIL: pig manure inoculated larvae.

80 larvae per bottle. BSF larvae without intestinal bacteria were used as the control group. Each bottle was inoculated with 10^9 CFU/ml 1% *S. aureus* and kept at a constant temperature of 28°C. *S. aureus* selective medium (Qingdao Hope Bio-technology Co., Ltd) was used to detect the number of *S. aureus* bacteria in the feed after 10 days of transformation.

DNA extraction and analysis

The sampled BSF larvae were starved for 24 h to reduce the effect of feed and faeces with non-symbiotic bacteria. Following starvation, BSF larvae were washed with 75% alcohol for 3 min and then rinsed three times with sterile water (Crippen & Sheffield, 2006). BSF larvae were then dissected, and the gut was removed and placed in a 1.5-ml sterile plastic tube. The FastDNA SPIN extraction kit (MP Biomedicals) was used for DNA extraction of BSF larva gut and PM samples. The quantity and quality of extracted DNAs were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis respectively. PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5′–ACTCCTACGGGAGGCAGCA–3′) and the reverse primer 806R (5′–GGACTACHVGGGTWTCTAAT–3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μl of Q5 reaction buffer (5×), 5 μl of Q5 High-Fidelity GC buffer (5×), 0.25 μl of Q5 High-Fidelity DNA Polymerase (5 U/μl), 2 μl (2.5 mM) of dNTPs, 1 μl (10 uM) of each Forward and Reverse primer, 2 μl of DNA Template and 8.75 μl of ddH_2O. Thermal cycling consisted of initial denaturation at 98°C for 2 min, followed by 25 cycles consisting of denaturation at 98°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, with a final extension of 5 min at 72°C. PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2×300 bp sequencing was performed using the Illlumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Latd.

Microbiome bioinformatics were performed with QIIME 22019.4 (Bolyen et al., 2019) with slight modification according to the official tutorials (https://docs.qiime2.org/2019.4/tutorials/). Briefly, raw sequence data were demultiplexed using the demux plugin following by primers cutting with cutadapt plugin (Martin, 2011). Sequences were then quality filtered, denoised, merged and chimera removed using the DADA2 plugin (Callahan et al., 2016). Non-singleton amplicon sequence variants (ASVs) were aligned with mafft (Katoh et al., 2002) and used to construct a phylogeny with fasttree2 (Price et al., 2010). Alpha diversity metrics (observed features and Faith's Phylogenetic Diversity [Faith, 1992]), beta diversity metrics (weighted UniFrac (Lozupone et al., 2007), unweighted UniFrac (Lozupone & Knight, 2005), Jaccard distance and Bray–Curtis dissimilarity were estimated using the diversity plugin with samples were rarefied to 900 sequences per sample. Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in feature-classifier plugin (Bokulich et al., 2018) against the Greengenes 13_8 99% OTUs reference sequences (McDonald et al., 2012).

Statistical analyses

Statistical analyses were performed by SPSS 26.0 (SPSS). Transformation-based redundancy analysis (tb-RDA) was performed with CANOCO 5.0. Prior to analysis, data were checked for normal distribution with one-sample Kolmogorov–Smirnov test and for homogeneity of variances with Levene's test. Bacterial populations were converted to $\log_{10}$ CFU/g before statistical analysis. All mean values with standard deviation were calculated for each bioindicator using Microsoft Excel. One-way ANOVA was used to determine the significant difference between groups, and Duncan's multiple range tests were employed to perform multiple comparisons between means. Statistical significance was considered at $p < 0.05$. Unpaired *t*-test was used to measure statistical difference at $p < 0.05$ (*), $p < 0.01$(**) and $p < 0.001$ (***) using the software GRAPHPAD PRISM 8.0 (GRAPHPAD Software).

RESULTS

Evaluation of BSF larvae efficiency in reducing *S. aureus* and *Salmonella* spp. populations in PM

Black soldier fly larvae can significantly reduce *S. aureus* populations in PM ($p < 0.05$) compared with the control group. A reduction in *S. aureus* population was observed from day 2 up to day 8 (Figure 1A). The effect of BSF larvae on *Salmonella* spp. viability in manure was shown in Figure 1B. BSF larvae significantly reduced *Salmonella* spp. concentration in PM ($p < 0.05$) compared with the control group. This effect was observed on day 2, and a gradual decline in *Salmonella* spp. count was measured 6 days after inoculating manure with the larvae. Few *Salmonella* spp. were observed at the end of the experiment.

At the eighth day of conversion, *S. aureus* and *Salmonella* spp. populations in the PM were significantly reduced by BSF larvae. However, *S. aureus* and

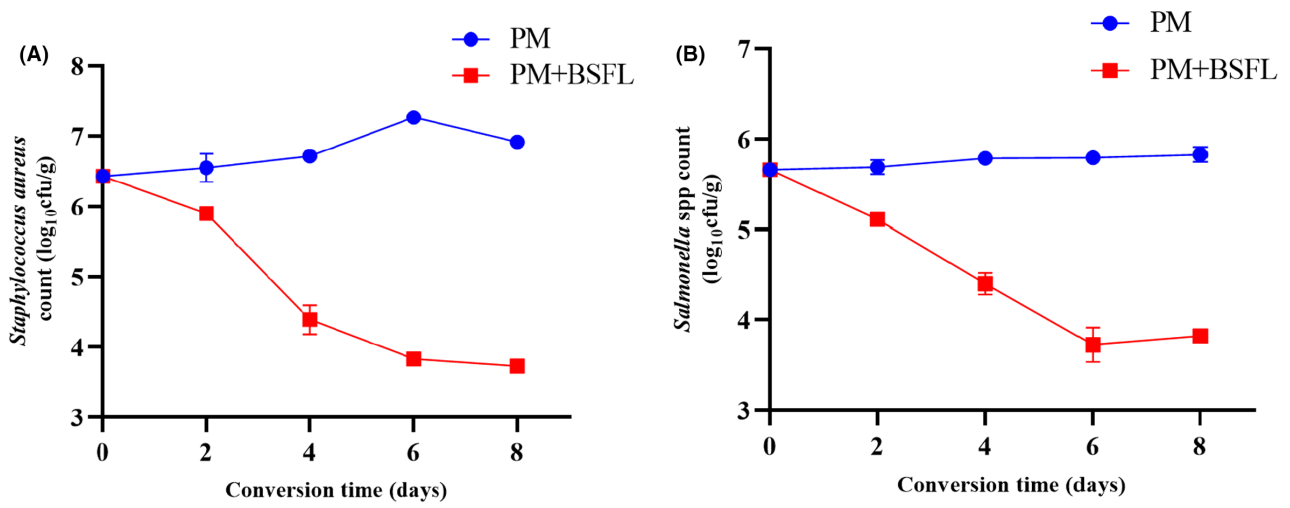

FIGURE 1 Effects of BSF larvae on pathogen populations in PM. Impacts of BSF larvae on *S. aureus* (A) and *Salmonella* spp. (B) in PM. PM as a control. Each point represents the mean of three independent trials ($n = 3$). ***Results***:Evaluation of BSF larvae efficiency in reducing natural pathogen populations in PM.

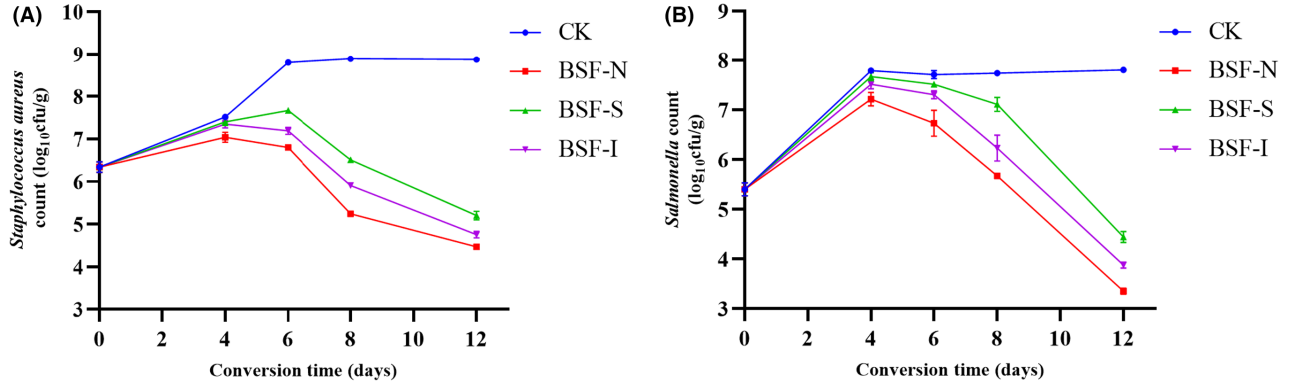

FIGURE 2 Effects of intestinal microbes on pathogen populations in PM. Effect of BSF larvae on *S. aureus* (A) and *Salmonella* spp. (B) by inoculating with conventional BSF larvae (BSF-N), sterile BSF larvae (BSF-S) and sterile BSF larvae with intestinal extracting solution (BSF-I) in PM. PM as a control (CK). Each point represents the mean of three independent trials ($n = 3$). ***Results***:Intestinal microbes enhance the ability of BSF larvae to antagonise pathogens.

Salmonella spp. still showed minimal counts in the PM. Compared with the control group, *S. aureus* was reduced by 8.31×10^6 CFU/g, whilst *Salmonella* spp. were reduced by 6.69×10^5 CFU/g. Taken together, these results suggest that BSF larvae can significantly reduce *S. aureus* and *Salmonella* spp. populations in PM, but there are still a certain number of *S. aureus* and *Salmonella* spp. in PM.

Intestinal microbes enhance the ability of BSF larvae to antagonise *S. aureus* and *Salmonella* spp.

We hypothesised that intestinal microbes could increase the capacity of BSF larvae to antagonise *S. aureus and Salmonella* spp. We compared the effects of the addition of nonsterile BSF larvae, sterile BSF larvae and sterile BSF larvae with intestinal extracting solution on *S. aureus* (Figure 2A) and *Salmonella* spp. (Figure 2B) in PM. We found that sterile BSF larvae were antagonistic against *S. aureus* and *Salmonella* spp. occurring in PM, and the ability to antagonise *Salmonella* spp. and *S. aureus* was enhanced after the intestinal extracting solution was added. Nonsterile BSF larvae were most effective in inhibiting *S. aureus* and *Salmonella* spp. in PM. Taken together, these results suggested that intestinal microbes can enhance the ability of BSF larvae to antagonise *S. aureus and Salmonella* spp.

Intestinal microorganisms of BSF larvae antagonise *S. aureus* in vitro

One hundred and eighty-five strains were isolated from the gut of BSF larvae. Using the plate confrontation method, we found that eight bacterial strains isolated

from the larval gut were able to antagonise *S. aureus* in vitro. The fermentation broth and supernatant of the bacterial broth antagonised *S. aureus*, with similar bacteriostatic zones. Here, clear inhibition zones were observed when eight bacterial strains isolated from BSF larvae were used against *S. aureus*. We speculated that these bacteria can produce certain metabolites, such as volatile or non-volatile metabolites including short-chain fatty acids and lipopeptides, to antagonise *S. aureus* (Figure 3). Then, these strains were identified by 16S rRNA phylogenetic analysis as *Bacillus* spp. (Table 2).

Intestinal microorganisms of BSF larvae antagonise *S. aureus* in vivo

We inoculated these strains into sterile BSF larvae to verify their ability to antagonise *S. aureus* in vivo. As shown in Figure 4, the sterile BSF larvae did not have the ability to antagonise *S. aureus*, but the intestinal microorganisms (BSF-CL, D2406, PD0810, MR0402, M2405, LB0415, PD0603, M2602) could inhibit *S. aureus* in vitro and enhance the inhibition of *S. aureus* by the larvae in vivo. Moreover, the concentration of *S. aureus* was two log cycles lower than the initial state.

Analysis of intestinal microbial community of BSF larvae

The intestinal microorganisms of BSF larvae were mainly composed of Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, accounting for more than 99% of the levels of all phyla (Figure 5A). Actinobacteria (6.82%), Proteobacteria (47.06%) and Bacteroidetes (19.61%) initially decreased, reaching the lowest values on day 4; then increased; and finally reached the initial state. Spirochetes (0.00%) and Firmicutes (26.51%) first increased and then decreased, and the maximum values were observed on the second and fourth day, respectively, finally reaching their original concentrations.

The genus level of BSF larvae during conversion of PM is complex (Figure 5B). In the process of PM transformation, the dominant genera of BSF larva intestinal microbes dramatically changed, and their relative abundances varied with conversion times. The relative abundance of *Enterococcus*, *Providencia*, *Dysgonomonas* and *Koukoulia* accounted for 70.65% of the BSF larvae at IM00. After 8 days of conversion time, the relative abundance of *Enterococcus* (24.81%) and *Koukoulia* (13.43%) decreased to 0.19% and 0.02% respectively. *Providencia* (18.28%) and *Dysgonomonas* (14.13%) were cleared from the intestine at IM20 and

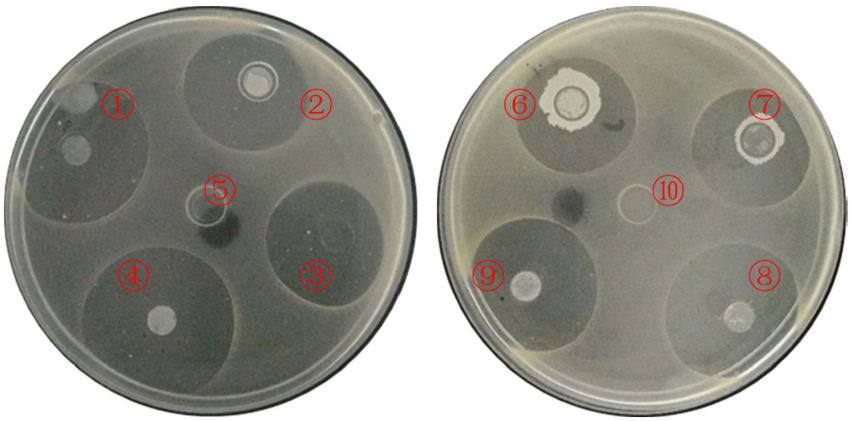

FIGURE 3 Inhibition of *S. aureus* by the intestinal microorganisms of BSF larvae in vitro. *Note*: ①②: *B. velezensis* D2406 fermentation; ③④: *B. velezensis* D2406 fermentation supernatant; ⑥⑦: *B. subtilis* BSF-CL fermentation; ⑧⑨: *B. subtilis* BSF-CL fermentation supernatant; ⑤⑩: LB medium. ***Results***:Intestinal microorganisms of BSF larvae antagonise pathogens in vitro.

TABLE 2 Intestinal microorganisms of BSF larvae against *S. aureus*

Strain	Closest NCBI library strain (accession no.)	Similarity (%)	References
D2406	*Bacillus velezensis* MK310268.1	100.00	Wang et al. (2020)
LB0415	*B. amyloliquefaciens* KC790325.1	100.00	Li et al. (2014)
M2405	*B. subtilis* JQ308580.1	99.93	Wang et al. (2013)
M2602	*B. amyloliquefaciens* MW559345.1	99.86	Dikit et al. (2019)
MR0402	*B. subtilis* KX791428.1	99.93	Huang et al. (2017)
PD0810	*B. amyloliquefaciens* MT613661.1	99.93	Zheng et al. (2021)
PD0603	*B. amyloliquefaciens* KC790325.1	100.00	Li et al. (2014)
BSF-CL	*B. subtilis* KU551205.1	99.93	Li et al. (2016)

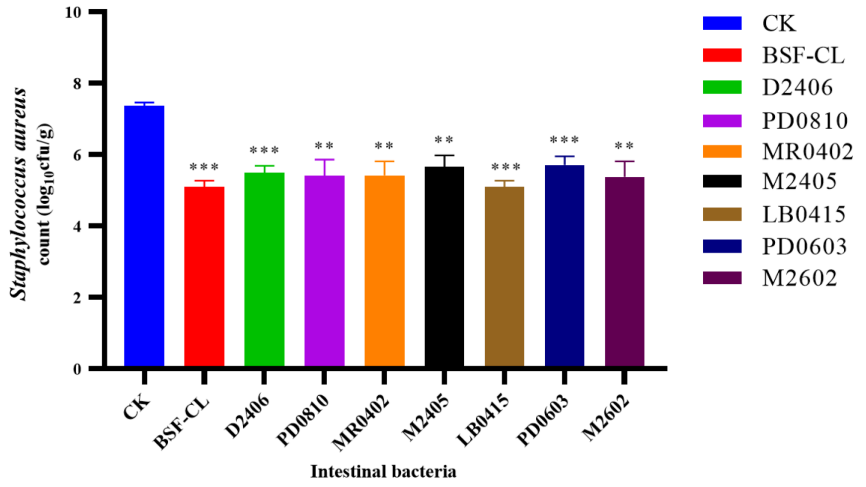

FIGURE 4 Effect of intestinal bacterial inhibition of *S. aureus* in vivo. CK: non-intestinal bacteria group. Statistical analyses were performed using unpaired *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared with CK. ***Results***:Intestinal microorganisms of BSF larvae antagonise pathogens in vivo.

IM06 respectively. After conversion, *Pseudomonas*, *Sphingobacterium* and Clostridiaceae became the dominant genera of BSF larvae. The proportion of *Pseudomonas* (2.63%) increased to 31.48%, and that of *Sphingobacterium* (0.12%) increased to 8.05%. Clostridiaceae was 0.02% in the intestine at IM00, but it had a proportion of 8.52% at IM08. The abundance of *Psychrobacter*, *Tissierella*, *Ignatzschineria*, *Bacteroides* and *Fastidiosipila* increased first and then decreased, and finally reached the initial concentration. These modulations in relative abundance may be associated with stress responses related to environmental changes and adverse factors in PM, such as pathogenic bacteria, antibiotics and heavy metals. It was found that the abundance of other genera, including *Enterococcus*, *Providencia*, *Dysgonomonas*, *Koukoulia*, *Actinomyces* and *Myroides*, has been significantly decreased. This finding suggests that these genera were gradually eliminated from the intestine during manure conversion by BSF larvae, which may be related to the microbial population with a continuous increasing trend in abundance or may be because the entomopathogens in PM stimulated the immune system of BSF larvae. Meanwhile, the Shannon indices of the intestinal microbes in gut samples at days 4, 6 and 8 were significantly higher than those at day 0 (Figure 5C). These results indicated that the diversity of microorganisms in the intestine was significantly increased after the transformation of PM by the BSF larvae.

We found 20 bacterial communities with high abundance in the intestines of BSF larvae. To determine whether microflora correlated with population responses of two zoonotic pathogens (*S. aureus* and *Salmonella* spp.), we carried out a transformation-based redundancy analysis (tb-RDA) based on Hellinger transformed distances (Figure 5D). Twelve bacterial communities, including *Myroides*, *Tissierella*, *Oblitimonas*, *Paenalcaligenes*, *Terrisporobacter*, *Clostridium*, *Fastidiosipila*, *Pseudomonas*, *Ignatzschineria*, *Savagea*, *Moheibacter* and *Sphingobacterium*, were negatively correlated with *S. aureus* and *Salmonella* spp. These bacterial genera may play an active role in inhibiting pathogenic bacteria in BSF larvae and assist the BSF larvae in inhibiting the *S. aureus* and *Salmonella* spp. in PM. The remaining genera including *Atopostipes*, *Bacteroides*, *Psychrobacter*, *Actinomyces*, *Dysgonomonas*, *Providencia*, *Koukoulia* and *Enterococcus* showed a positive correlation with the *S. aureus* and *Salmonella* spp. These bacterial genera might play a positive role in the colonisation of pathogens in manure through some currently unidentified mechanisms.

Analysis of PM microbial community

Taxonomic composition analysis of initial PM suggested that Firmicutes and Bacteroidetes accounted for 91.93% of the microbial community. Firmicutes (60.27%) remained at high levels throughout the conversion period, and the relative abundances were 48.28% and 49.69% at PMIL08 (Figure 6A) and PM08 (Figure 6B) respectively. Conversely, Bacteroidetes (31.66%) decreased to 13.44% at PMIL08 and 8.48% at PM08. The proportion of Proteobacteria constantly increased in the control group and BSF larva–treated group. Spirochaetes, Kiritimatiellaeota and Patescibacteria were cleared in PM after conversion by BSF larvae for 2 days.

After inoculation with BSF larvae, we found that *Prevotellaceae* (4.07%), *Prevotella* (3.68%), *Lachnospiraceae* (3.55%) and *Treponema* (5.42%) were cleared at day 2. Meanwhile, *Lactobacillus* (15.19%) and *Streptococcus* (6.42%) were cleared at day 4. *Muribaculaceae* (15.48%) and *Ruminococcaceae* (5.22%) were cleared at day 6 (Figure 6C). However, in the control group, *Streptococcus*, *Prevotella*, *Treponema*, *Lactobacillus*, *Lachnospiraceae*, *Ruminococcaceae* and *Muribaculaceae* constantly existed in the PM over time (Figure 6D).

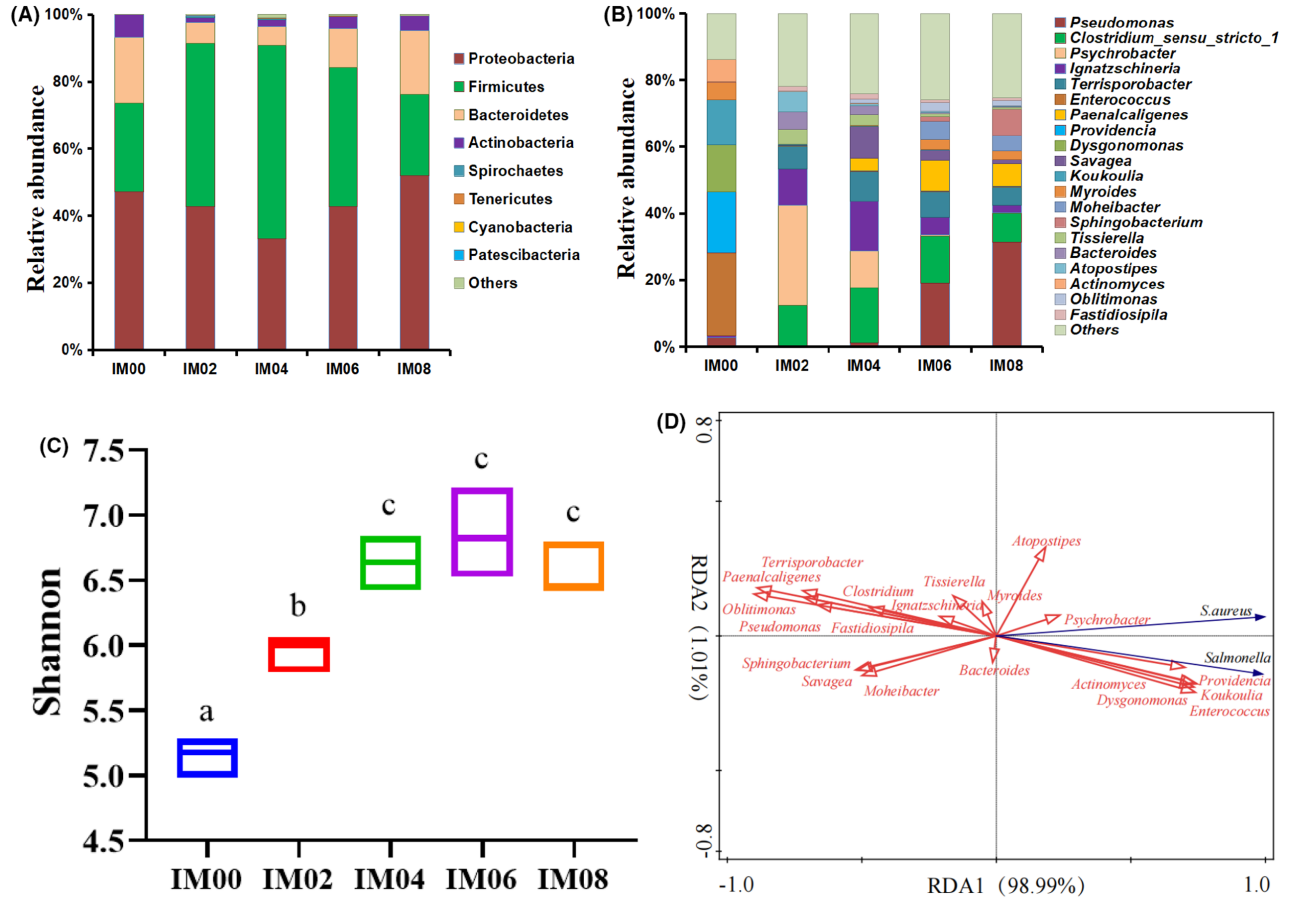

FIGURE 5 BSF larval intestinal bacterial communities in manure conversion systems. Abundance profiles of BSF larva intestinal bacteria at phylum (A) and genus levels (B). (C) Shannon indices for each group sample ($n = 3$), different letters indicate significant differences ($p < 0.05$); (D) tb-RDA of pathogens (*S. aureus* and *Salmonella* spp.) and intestinal genus communities in BSF larva converting PM system. *Note*: IM00: intestinal microbes of initial BSF larvae. IM02, IM04, IM06 and IM08: intestinal microbes of BSF larvae at the second, fourth, sixth and eighth day after the conversion of PM respectively.

DISCUSSION

In this study, we examined the capability of BSF larvae to eliminate *S. aureus* and *Salmonella* spp. in PM and deciphered the role of the associated intestinal microbes with this process. Different pathogen strains, including *S. aureus* and *Salmonella* spp., were analysed in PM after inoculating the BSF larvae. We determined that BSF larvae can reduce *S. aureus* and *Salmonella* spp. populations in PM and intestinal microbes can enhance the ability of BSF larvae to antagonise *S. aureus and Salmonella* spp. We also analysed the taxonomic composition of the intestinal microorganism of BSF larvae and isolated eight intestinal microorganisms that could inhibit *S. aureus* in vitro. These microorganisms could enhance the inhibitory ability of BSF larvae against *S. aureus* after being inoculated to sterile BSF larvae in vivo. Meanwhile, the taxonomic composition analysis of PM showed that BSF larvae can effectively clear *Streptococcus*, *Prevotella*, *Treponema*, *Lactobacillus*, *Lachnospiraceae*, *Ruminococcaceae* and *Muribaculaceae* in PM.

House flies (Diptera: Muscidae), blow flies (Diptera: Calliphoridae), flesh fly maggots (Diptera: Sarcophagidae) and BSF are used to process manure. Their offspring can reduce bacterial numbers in manure either by competing with bacteria for nutrients or eliminating them (Čičková et al., 2015). Moreover, the disinfection action of the larvae may be achieved by ingesting and digesting microorganisms, as well as through releasing different types of antimicrobial peptides (Chiam et al., 2021). The resulting digested waste could be used as a safe soil amendment, which is highly valued given issues with food-borne pathogens associated with compost as fertiliser (Devarajan et al., 2021).

Food-borne diseases are endemic in many parts of the world, with at least one in 10 people becoming sick by eating contaminated food and 420,000 people dying each year according to the World Health Organisation (2015). The US Center for Disease Control estimates that in the United States alone, 48 million people, or one in six Americans, suffer from food-borne illness each year (Painter et al., 2013). In many parts of the world, organic systems use more manure than conventional

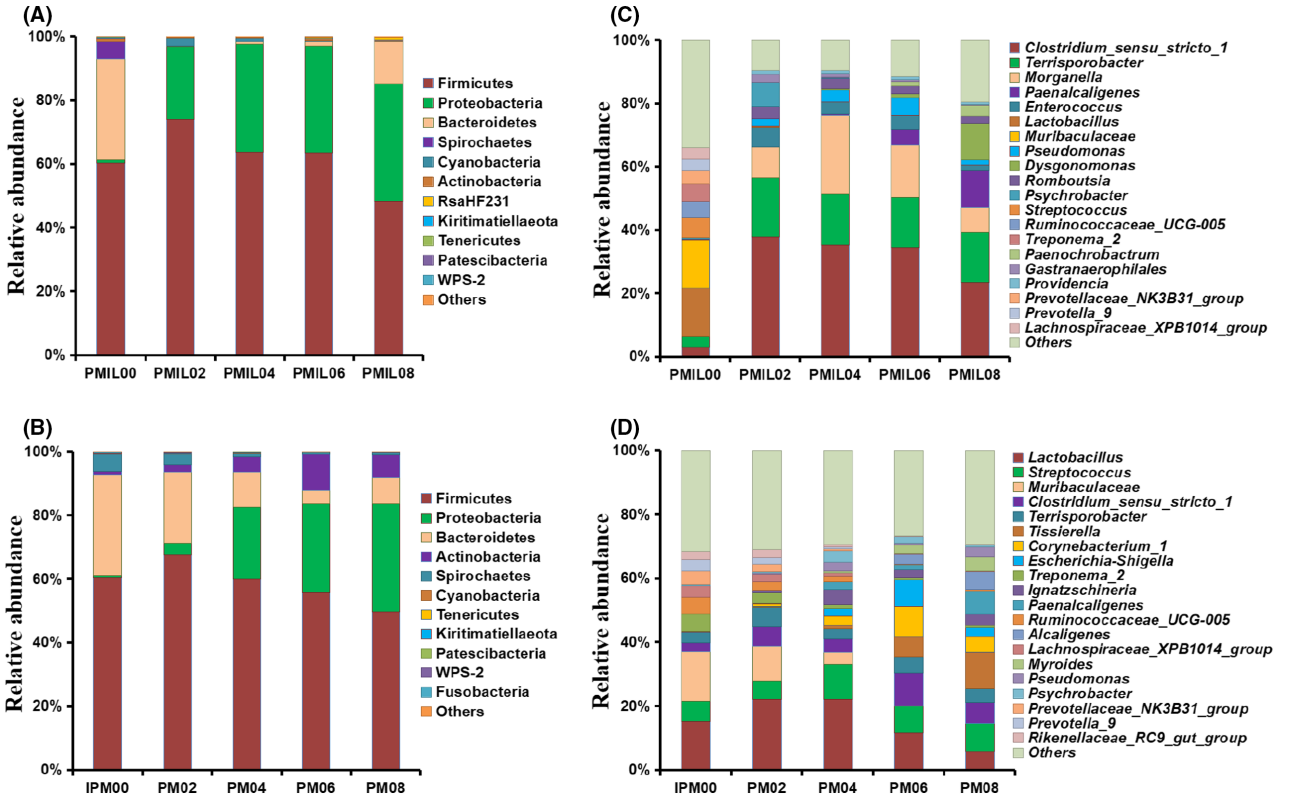

FIGURE 6 Abundance profiles of PM bacteria at phylum (A, B) and genus levels (C, D). *Note*: IPM00: fresh PM; PMIL02, PMIL04, PMIL06 and PMIL08: samples of PM converted by BSF larvae at 2, 4, 6 and 8 days respectively. PM02, PM04, PM06 and PM08: samples of PM not inoculated with BSF larvae at 2, 4, 6 and 8 days. **Results:** Analysis of PM microbial community.

systems, and chemical treatments against pathogens are prohibited in organic farming. Raw manure is widely used as soil amendments as manure is a cost-effective source of nutrition for agricultural plants (Goss et al., 2013; Manyi-Loh et al., 2016). Under these circumstances, pathogens may be spread through the direct interaction of vegetable surfaces with manure or resulting compost. In the last decades, a food-borne illness associated with fresh produce has been correlated with the increased use of animal manure as fertiliser (Alegbeleye et al., 2018).

Because the production system of BSF larvae is simple and economical and relies primarily on recycling organic waste into nutritious biomass, the use of BSF larvae as animal feed for poultry, fish and pigs has been considered feasible (Shumo et al., 2019). In a previous study, the effects of BSF larvae on reduction of *S. enteritidis* populations in chicken manure have been reported (Erickson et al., 2004). In that study, methanol extracts of BSF larvae showed antibacterial activities against Gram-negative bacterial strains, including *Shigella sonnei*, *Neisseria gonorrhoeae* and *Klebsiella pneumonia*. BSF larvae have been demonstrated to rapidly inhibit the proliferation of the pathogen and cause a significant reduction of *E. coli* count in chicken (Erickson et al., 2004) and also in cow manure (Liu et al., 2008). Regarding their potential dangers, *S. aureus* is the main causative agent of skin infections and respiratory diseases, and *Salmonella enteritidis*, a zoonotic enteric pathogen, is a commonly isolated serotype of infected domestic animals and may be excreted in their faeces. In this study, we investigated the effectiveness of BSF larvae in reducing the *S. aureus* and *Salmonella* spp. populations in PM. BSF larvae can significantly reduce *S. aureus* and *Salmonella* spp. populations in PM. *S. aureus* and *Salmonella* spp. populations in PM were assessed, and a gradual decrease was observed during the study period.

We found that during the whole conversion process, Firmicutes, Proteobacteria and Bacteroidetes were the main bacterial communities of BSF larvae, accounting for more than 90% of the microbial community structure, which was consistent with the results of previous studies (Ao et al., 2021; Zhan et al., 2020; Zheng et al., 2013). Firmicutes play a pivotal role in the digestion of animal faeces because these bacteria secrete various proteases and pectinases and are also involved in the degradation of indigestible carbohydrates in straw-associated compost (Sun et al., 2015; Zhang et al., 2018). Bacterial genes encode enzymes, such as proteases, cellulases and lipases, in the gut of BSF larvae, facilitating the decomposition and recycling of biological waste and other accumulated nutrients by hydrolysing cellulose, proteins and lipids in the BSF larvae

(Jeon et al., 2011; Lee et al., 2014). Feeding material is a pivotal factor for the change of intestinal microbiota in BSF larvae (Bruno et al., 2019). After 8 days of conversion, the dominant bacterial community in the gut of BSF larvae changed from *Enterococcus*, *Providencia*, *Dysgonomonas* and *Koukoulia* to *Pseudomonas*, *Sphingobacterium* and *Clostridium*. These microorganisms may promote the adaptation of BSF larvae to the PM environment and then transform the PM.

Microorganisms play significant roles in insect digestion. They may offer essential nutrients to insects that are not found in their food substrates (Douglas, 2014). For many insects, microorganisms also enable complex polysaccharides found in their feed to be hydrolysed (Muhammad et al., 2017). They also help insects reduce toxic substances such as the secondary metabolites produced by plants and pathogenic agents (Meylaers et al., 2003). In this study, 185 bacterial strains isolated from the gut of BSF larvae were tested in vitro for preliminary screening in order to discover their potential antagonistic effects against the PM-associated zoonotic pathogenic bacteria. However, among the 185 isolated strains from the gut of BSF larvae, we did not screen out the strains that can antagonise *Salmonella* spp. in vitro. This may be because our separation method is not comprehensive as there are still a large number of anaerobic microorganisms and unculturable microorganisms in the gut of BSF larvae. De et al. pointed out that the antagonism of BSF larvae against *Salmonella* was limited when BSF larvae were reared on chicken feed that was inoculated with different levels of *Salmonella* (De Smet et al., 2021). In our study, we found that after conversion, BSF larvae could significantly reduce the *S. aureus* and *Salmonella* spp. populations, but there are still a certain number of *S. aureus* and *Salmonella* spp. remaining in PM. Therefore, the PM after transformation by BSF larvae may need further treatment to improve its safety before use as organic fertiliser. Culturable and pure cultures are still necessary for research purposes and industrial applications (Prakash et al., 2013). Due to the above concerns, we isolated culturable functional gut microorganisms from BSF larva gut. When using BSF larvae to convert organic waste, these functional bacteria can be inoculated to enhance the inhibition of pathogens by BSF larvae.

These results indicate that BSF larvae are capable of inhibiting *S. aureus* and *Salmonella* spp. in PM through the mechanism of gut-associated microorganisms, and the strains isolated from the larva gut may have the potential for further industrial applications to improve the safety of using BSF larvae as organic waste converter. It is also a limitation that the study only isolated eight strains from the larval gut that can antagonise *S. aureus* in vitro and in vivo without screening out the strains that can antagonise *Salmonella* spp. in vitro. Another limitation is that this study only focused on the decreasing number of pathogenic bacteria in manure in a short time period. Further research is needed to make sure if the pathogenic bacteria will grow and contaminate frass or after being applied in the field. In summary, our results showed that *S. aureus* and *Salmonella* spp. populations in PM were significantly decreased after treatment with BSF larvae compared with the control group. We also determined that intestinal microbes associated with BSF larvae play a key role in this process and we screened out eight purely culturable bacterial strains from the larval gut that can antagonise *S. aureus* in vitro and in vivo.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author.

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