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Black Soldier Fly Gut Microbiota Resists Invasion by *Bacillus subtilis* 168 and *Pseudomonas putida* KT2440

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Abstract

Due to its high bioconversion efficiency and nutritional value, the black soldier fly (*Hermetia illucens* L. 1758) is a promising insect species for sustainable animal feed production. However, concerns remain regarding microbial safety when larvae are reared on substrates contaminated by pathogenic or spoilage bacteria. This study investigated the effects of substrate inoculation with *Bacillus subtilis* 168 or *Pseudomonas putida* KT2440 on larval performance and gut microbiota composition. Larvae reared on contaminated diets showed no significant differences in survival or development time compared to controls. However, a short-term reduction in growth was observed in the *Bacillus*-exposed group. qPCR analyses confirmed the temporary presence of *Bacillus* taxa in larval guts, while *Pseudomonas* taxa were effectively excluded. Amplicon sequencing of the 16S rRNA gene revealed that the contamination did not affect gut bacterial microbiota richness and composition. Instead, the bacterial communities evolved naturally with *Lactobacillales*-related bacteria dominating early stages and *Morganellaceae* taxa becoming more abundant in prepupae. Our findings demonstrate the stability and resilience of *H. illucens* gut bacterial microbiota, reinforcing the safety and suitability of *H. illucens* as a feed ingredient, even when reared under challenging microbial conditions.

Keywords: black soldier fly; intestinal microbiota; *Bacillus*; *Pseudomonas*; *Lactobacillus*; probiotics

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1. Introduction

The black soldier fly (BSF, *Hermetia illucens* L. 1758; Diptera Stratiomyidae) is found throughout the world's tropical, subtropical, and warm-temperate regions [1]. The life cycle of BSF consists of four stages: egg, larva, pupa, and adult. According to its saprophytic dietary habits, BSF larvae can feed on organic matter from plant and animal origins [2,3]. At the end of the larval stage, which lasts approximately 11–14 days, the body weight can reach about 220 mg [4]. Importantly, BSF adults will not actively enter human habitats or be attracted to food, nor can they act as vectors of human diseases, unlike the house fly, *Musca domestica* L. 1758 [5]. Due to these excellent characteristics, the bioconversion of organic waste by BSF larvae has attracted significant attention in recent decades.

BSF has gained attention as an efficient bioconverter of various organic wastes, including food scraps, manure, and other agricultural by-products [4,6]. The success of BSF larvae in waste processing is not only due to their biological traits, such as low substrate requirements and adaptability, but also strongly influenced by their gut microbiota. This

gut microbial community plays a key role in nutrient digestion, immune function, and overall larval development, making it a crucial factor in the bioconversion process. Recent studies have identified a core gut microbiome in BSF larvae that is relatively stable across different rearing conditions and substrates. This core microbiome mainly comprises genera such as *Enterococcus*, *Providencia*, *Morganella*, and *Lactobacillus*, which are believed to support essential physiological functions and contribute to the resilience of the larval gut environment [7].

Studies have also shown that the composition of the rearing substrate significantly affects the growth and nutrient profile of BSF larvae [7–10]. For example, the conversion efficiency of digested food in the larval stage ranged from 21.5% to 49.8%, depending on the composition of the feeding substrate [7]. The substrates harbor microbial communities that likely contribute to the bioconversion processes involving BSF larvae by potentially predigesting the substrate and influencing the larval gut microbiome [11]. Like all organisms, insects live in close association with multiple microorganisms, including bacteria, archaea, fungi, protists, and viruses, either permanently or transiently. As a result, the gut microbiota is often regarded as an independent "organ" of insects [12–14]. The insect gut microbiota functions as an open system, permitting colonization and interaction between external microbes and resident gut microorganisms. Consequently, dietary changes can modify the composition and structure of these microbial communities [15,16]. The gut microbiota of BSF larvae reared on various organic waste streams at different facilities is highly variable during the rearing phase [17] and exhibits a diverse bacterial composition across the different midgut regions [16]. The versatility of the gut microbiota is an opportunity to use specific microorganisms to enhance the bioconversion process. For example, Deng et al. (2025) demonstrated that combining bacterial inoculation with BSF larvae synergistically enhances kitchen waste detoxification [18].

However, if the substrate used to feed the insects is contaminated with foodborne pathogens, the larvae's gut may become a conducive environment for these microorganisms to survive and multiply, potentially creating biosafety concerns. This is particularly relevant because insects are typically consumed whole, including their gut [19]. For example, Fernandez-Cassi et al. (2018) classified a high microbial load in *Acheta domesticus* (L. 1758) as a moderate hazard [19]. Similarly, foodborne pathogens such as *Salmonella* spp. have been detected in *Tenebrio molitor* (L. 1758) larvae fed with contaminated wheat bran [17]. Given the feeding habits of black soldier fly larvae, the risk of contamination is notably increased [20]. Moreover, as both black soldier fly larvae and mealworms are widely used as protein sources in aquaculture and poultry feeds, contamination of their rearing substrates can facilitate the transfer of pathogenic bacteria like *Salmonella* and *Listeria monocytogenes* to the larvae, which may subsequently enter the animal feed chain [21,22]. These findings emphasize the critical need for rigorous hygiene controls in substrate management to minimize biosafety risks in insect-based feed production.

Pseudomonas Migula 1894 and *Bacillus* Cohn 1872 genera comprise taxa widely distributed in natural environments [23,24]. *Bacillus* species are Gram-positive, spore-forming bacteria that are commonly distributed in the environment, although their primary habitat is soil and rizhosphere. These bacterial microorganisms are usually found in decaying organic matter, dust, vegetables, water, and some species are part of the normal gut microbiota. Although some *Bacillus* species are pathogenic, *Bacillus subtilis* 168 is considered "Generally Recognized As Safe" (GRAS). This laboratory strain, originally derived from a soil isolate, has been extensively studied for decades and has served as a central model organism in illuminating bacterial development, sporulation, and cell biology due to its high genetic tractability and the early availability of a fully sequenced and heavily annotated genome [25]. The 2023 update of its genome annotation further refined insights

into its metabolism, biofilm formation capabilities, and gene regulation dynamics, reinforcing its value in both environmental and industrial contexts [25]. Beyond its model status, *B. subtilis* is also considered as a beneficial bacterium applied as a probiotic for plant [24] and animals [26]. The absence of virulence factors and antibiotic production in strain 168 further supports its safety and suitability for experimental and industrial applications. *Pseudomonas* species are Gram-negative bacteria that are very common in fresh foods due to their association with water, soil, and vegetation. Among them, *Pseudomonas putida* KT2440 has become a cornerstone in synthetic biology and metabolic engineering. De Lorenzo et al. [27] trace its evolution from a soil-dwelling biodegrader to a robust "synthetic biology chassis," a status earned thanks to its non-pathogenic nature, versatile metabolism, resilience under stress conditions, and extensive molecular toolset, including a fully sequenced and well-annotated genome. This strain is particularly valued for its ability to metabolize aromatic compounds and other pollutants, making it an attractive host for bioremediation as well as biosynthetic applications. Naturally, these two genera were found in some BSF larval and/or residual samples [17,28]. A few species of these bacteria are either spoilage, opportunistic pathogens, or potential foodborne pathogens. Some pathogenic bacteria, such as *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus*, are negatively affected by BSF larvae [29,30]. However, other pathogens can survive in the BSF gut. For example, *Bacillus cereus* contaminated substrate has been shown to produce contaminated BSF larvae by vegetative cells or endospores [31,32]. Consequently, since BSF larvae can be used as feed, it is crucial to identify which bacteria can coexist with BSF microbiota to prevent contamination of the food chain and to adjust sanitization processes accordingly.

In this study, we hypothesized that the core gut bacteria of BSF larvae can influence the colonization of specific bacterial microorganisms in the rearing substrate and limit the transmission of external microbes to the larval gut. We established a bacterial challenge evaluation model in the BSF feeding phase to test this hypothesis. The feeding substrate (CF) was inoculated with the *Pseudomonas putida* KT2440 (CFP) or *Bacillus subtilis* 268 (CFB) culture as bacterial models to obtain a high contamination level. Then, the survival of specific bacterial microorganisms in the CF and their transmission to and colonization of the BSF were determined. Most importantly, BSF's biomass gain, developmental progress, and gut microbial community structure (i.e., richness and composition of bacterial communities) were assessed. With these findings, our study highlighted the necessity of having future independent research on the local and core bacterial communities in the BSF larval gut.

2. Materials and Methods

2.1. Acquisition of 6-Day-Old Larvae

The *H. illucens* larvae used in this study come from a stock colony established at the Functional and Evolutionary Entomology laboratory of Gembloux Agro-Bio Tech (University of Liège, Belgium). The whole life cycle of BSF, including artificial reproduction, egg incubation, pre-fattening larvae, fattening larvae, and prepupae collection, was completed in a shipping container as described by Hoc et al. (2017) in the following abiotic conditions: 27 ± 2 °C and 60 ± 5% RH [33]. Two grams of egg were collected and incubated in 30 × 15 × 5 cm non-transparent plastic boxes, covered with nets for aeration. The BSF larval population under 6 days old was maintained on a diet based on a 2:2:1 mixture of brewers' grains, carrot puree, and water, and was checked every 12 h until hatch. Newly emerged larvae were allowed to feed for six days in the environmental chamber before use in the experiment.

2.2. Substrate Preparation

Chicken feed and flax cake were ground to a particle size of 0.750 mm for diet formulation. Based on these two components, three different diets were produced. The control diet (i.e., CF) consisted of 40% chicken feed and 60% flax cake, supplemented with 40% saline water. Regarding the contaminated feed, the dried compounds of the control feed were rehydrated with 40% saline water containing 2.5×10^7 cells/mL of bacteria. Two bacterial species were used as model contaminants: *Bacillus subtilis* 168 (CFB) and *Pseudomonas putida* KT2440 (CFP).

2.3. Bioassay

Experiments were conducted in a controlled, dark rearing room ($2.45 \times 2.06 \times 2.72$ m) with temperature and relative humidity maintained at 27 ± 1 °C and 60 ± 5%, respectively. Six batches of 100 7-day-old larvae were manually collected and inoculated into each container containing 150 g of feed ($108 \times 82 \times 50$ mm), covered with a transparent plastic lid and a square mosquito net (12×12 mm) in the center for ventilation. All replicates, both with and without larvae, were randomly arranged at the same height in the rearing room for 18 days, and no additional food or water was added till prepupae collection. Twenty larvae were collected on days 0 and 3, ten larvae were collected on days 7 and 11, and 10 prepupae were collected on day 18 from each container, constituting a sample, respectively. The samples of day 0 were derived from the collective initial population before the separation of larvae and the introduction of new diets. When approximately half the population had reached the prepupal stage, the batches of larvae were washed with distilled water, dried with a paper towel, fasted for 24 h, and then frozen at −20 °C until analysis.

2.4. Intrinsic Parameters

For each treatment, larval growth was determined by measuring the combined weight of 30 randomly selected larvae at each sampling day. At the end of the experiment, the number of surviving larvae and prepupae was counted. For each treatment, the biomass growth of larvae was determined by measuring the total weight of 30 randomly selected larvae on each sampling day. At the end of the experiment, the survival rate was assessed by counting the number of larvae and pupae in each repetition. Data were analyzed using R Studio (version 2023.12.1+402, Boston, MA, USA). Firstly, normality was assessed using the Shapiro–Wilk test, and homoscedasticity was checked using the Bartlett test ("bartlett.test" command, R-package "stats", [34]). Then, one-way analysis of variance (ANOVA) was performed ("aov" command, R-package "stats", [34]) and followed by a post hoc Tukey test ("TukeyHSD" command, R-package "stats", [34]).

2.5. Gut Removal and DNA Extraction

Before removing the gut, we keep the larvae at −20 °C for 30 min to inactivate the larvae. After disinfecting the larval surfaces with 70% ethanol, a few millimeters of the anterior part were cut off with a sterile scalpel. The 200 mg of gut tissues were pulled out using sterile forceps and then transferred into a sterile microcentrifuge tube. Following the manufacturer's protocol, the DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (QIAGEN, Hilden, Germany) and stored at −20 °C until analysis.

2.6. q-PCR

qPCR was performed with primers specific for *Bacillus subtilis*, *Pseudomonas putida*, and total bacteria in the insect using the Bio-Rad Real-Time PCR System in a 20 µL reaction volume consisting of the following reagents: DNA template, 300 nM target primer

for total bacteria or 100 nM target primer for *Bacillus subtilis* and *Pseudomonas putida* (see Supplementary Table S1), and 1× SYBR Green Mix. The amplification protocol was 95 °C for 20 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. All reactions were performed in triplicate; each run contained one negative and one positive control. The efficiency of all primers was tested using the standard curve method, as shown in Supplementary Figure S1. The CFX Maestro Software analyzed the data to obtain the crossing threshold (Ct) values.

2.7. High-Throughput Sequencing (HTS) on Bacterial Community

Sample purity and concentration of all DNA extracts were controlled using a Nanodrop spectrophotometer ND1000 V3.8.1 (Nanodrop, Wilmington, DE, USA) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), respectively. We followed the Illumina MiSeq System protocol for the library preparation of the 16S ribosomal RNA gene (Illumina, San Diego, CA, USA, 2024). DNA concentrations were normalized at 5 ng/µL and stored at −20 °C. We amplified the V3-V4 region (464 bp) of the 16S rRNA gene using the primer pair S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (see Supplementary Table S1). All PCR reactions were performed in a volume of 25 µL containing 2.5 µL of normalized sample DNA (at 5 ng/µL), 5 µL of each primer at 1 µM, and 12.5 µL of 2x KAPA Hifi HotStart Ready Mix (KAPA Biosystems, Wilmington, MA, USA). The PCR profile was set up with an initial DNA denaturation at 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and finished with an extension part at 72 °C for 5 min. If necessary, we stored DNA amplicons at 4 °C. For quality control, 5 µL of PCR products were loaded into a 1% electrophoresis gel and visualized under UV light to verify the presence of the expected 464 bp band. The PCR products were then sent to the Institute of Biotechnology at Cornell University (Ithaca, NY, USA) for completion of the 16S library preparation and Illumina sequencing. Briefly, PCR products were purified using a PCR clean-up protocol that included the AMpure XP beads system (Beckman Coulter, Brea, CA, USA). Nextera XT index Kit (Illumina, San Diego, CA, USA) was used to attach dual indices and Illumina sequencing adapters for all samples. A second PCR was performed on sample mixtures containing 5 µL of each sample amplicon (at 3 to 5 ng/µL following manufacturer instructions), 25 µL of 2x KAPA HiFi HotStart ReadyMix, 5 µL of each Nextera XT index primer, and 10 µL of PCR-grade water, for a total volume of 50 µL. The PCR profile was set up with an initial DNA denaturation at 95 °C for 3 min, followed by eight cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and finished with an extension part at 72 °C for 5 min, followed by storage at 4 °C. A second PCR clean-up was performed using the same AMpure XP beads system as the index PCR, followed by library quantification. DNA samples were normalized to a concentration of 4 nM and were then prepared for sequencing according to the Denature and Dilute Libraries Guide of the Illumina system. Paired-end sequencing (2 × 250 nt) was processed on the MiSeq Illumina system.

2.8. Bioinformatic Analysis

In total, 72 samples were sequenced (i.e., N = 24/diet treatment or N = 18/experimental day of sampling). Raw Illumina sequencing data were deposited on the GENBANK platform with the PRJNA1295584 accession number. Paired-end demultiplexed read files were imported on QIIME2 Core 2020.11 distribution [35]. In the QIIME2 environment, DADA2 was used to trim the primers from the reads, 17 bp for the forward and 21 bp for the reverse, to filter and remove chimeric and low-quality sequences [36]. We produced amplicon sequence variants (ASVs) taxonomy and the ASVs community matrix of our samples by querying our sequences against the reference database (i.e., known taxonomic composition): SILVA release 138 at 99% similarity [37,38]. We used the naïve-Bayes classifier

implemented in QIIME2 to compare our queried sequences with the SILVA database [39]. Finally, we removed singletons, chloroplast sequences, and mitochondrial contaminants from the ASVs community matrix. In our ASVs community matrix, we removed unassigned taxa and suppressed contaminant ASVs detected in the bacterial identifications of our blank sample [40]. In the R environment, we created a *phyloseq* object that combined the ASV taxonomy, the ASV community matrix, and the metadata [41]. The *phyloseq* object was then converted to a *MicrobiotaProcess* object for further analysis [42]. Rarefaction curves and the alpha diversity metrics (i.e., bacterial taxa richness or ASVs richness, Chao1's estimator, and Shannon index) were estimated for diet treatments and larval development by a split number of 100 chunks using the *mp_cal_rarecurve* function [42]. Principal Coordinates Analysis (PCoA) using a Bray–Curtis dissimilarity matrix corrected by Hellinger's transformation was then performed to visualize the results obtained from the multivariate analysis. Differences in microbial community composition according to the larval treatments and the larval development were tested using permutational multivariate ANOVA (PERMANOVA) with 9999 permutations under a reduced model, based on Bray–Curtis dissimilarities [42].

3. Results

3.1. Growth, Development Time, and Survival of Larvae

The development time required for *H. illucens* reared on different treatments, from 6-day-old larvae to the prepupal stage, ranges from 17.6 to 18.0 days (Table 1). The first prepupae were collected after 15, 15.5, and 15.5 growing days in *B. subtilis* 168-contaminated feed (CFB), the control (CF), and *P. putida* KT2440-contaminated feed (CFP), respectively. Bacterial contamination while rearing *H. illucens* was not found to significantly affect the development time required for larval development ($F_{2,6}$ = 3.10; p = 0.69). Similarly, the survival rate between CFB, CFP, and CF was not significant ($F_{2,6}$ = 0.33; p = 0.73). Overall survival rate of larvae exceeded 91% in these experiments (Table 1).

Table 1. Development time and survival rate of *Hermetia illucens* larvae fed control chicken feed (CF), *Bacillus subtilis* 168 contaminated chicken feed (CFB), and *Pseudomonas putida* KT2440 contaminated chicken feed (CFP).

Treatments	Development Time (days ± SD)	Survival Rate (% ± SD)
CF	17.8 ± 1.11 a	93.00 ± 2.67 a
CFB	18.3 ± 0.78 a	91.33 ± 1.77 a
CFP	17.8 ± 0.89 a	91.67 ± 1.11 a

[a] Means within the same column with different letters differ significantly.

Larval development occurred over 11 days, with individuals from the CFB treatment being significantly lighter on days 7 and 11 compared to those reared on the CF and CFP diets (Figure 1). In contrast, the growth curves of larvae fed feed contaminated with *P. putida* KT2440 were more like those of larvae fed on CF, except for day 7, when the mean weight per larva was found to be heavier. Between days 11 and 18, larvae evolved into prepupae. *B. subtilis* 168 and *P. putida* KT2440 produce prepupal weights identical to those of CF (Figure 1).

3.2. Bacillus spp. and Pseudomonas spp. Abundance

Total bacterial expression was stable across modalities at day 7 (Ct = 10.44 ± 0.33). As shown in Figure 2, larvae fed CFB exhibited a significantly higher abundance of *Bacillus* spp. in their gut at day 7 compared to those fed control feed ($F_{1,10}$ = 104.74; $p < 0.001$). In contrast, larvae fed CFP showed a significantly lower abundance of *Pseudomonas* spp. than the control group ($F_{1,10}$ = 392.14; $p < 0.001$).

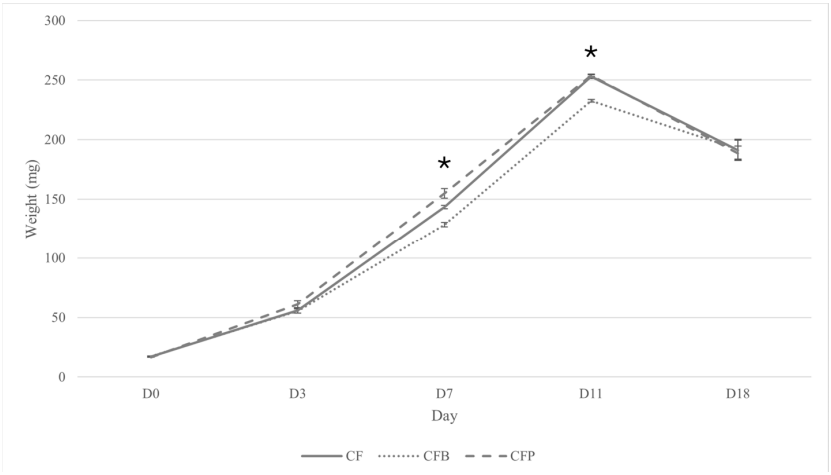
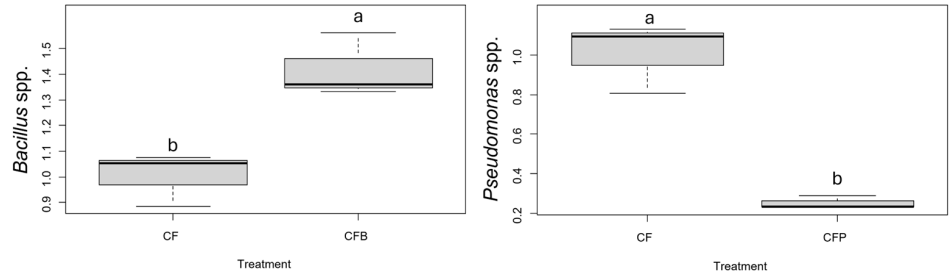


Figure 1. Growth of *Hermetia illucens* larvae fed control chicken feed (CF), *Bacillus subtilis* 168-contaminated chicken feed (CFB), and *Pseudomonas putida* KT2440-contaminated chicken feed (CFP). Each point on the curves represents the mean individual weight (mg) calculated from weighing 30 larvae randomly selected from a population of 100 larvae (n = 3). Error bars indicate SEM, and asterisks indicate significant differences across modalities, as determined by ANOVA followed by Tukey's post hoc test. On day 7, all modalities differ significantly.



(**A**) (**B**)

Figure 2. Bacterial abundance in the gut of *Hermetia illucens* larvae fed contaminated chicken feed with model contaminant strains *Bacillus subtilis* 168 and *Pseudomonas putida* KT2440 at day 7. (**A**) *Bacillus* spp. abundance. (**B**) *Pseudomonas* spp. abundance. Mean value (n = 3) ± SEM per bacterial group. Data are expressed as the normalized fold expression to control diet (i.e., CF), which is 1.000. Different letters above columns indicate significant differences ($p < 0.05$). Total bacteria were selected as the reference gene and were stable across treatments.

3.3. Gut Bacterial Microbiome Diversity and Community Structure

The effective bacterial read numbers of the homogenized larval gut samples ranged from 3697 to 50,809 reads, with a mean of 24,602 ± 1207 reads per sample after filtering out low-quality reads and trimming adapters. A total of 41 distinct bacterial genera were identified within the gut microbiome of the BSF.

Rarefaction curve analysis demonstrated adequate sequencing depth across all samples, with curves approaching saturation for most treatments and developmental stages (Figure 3A,B). No significant variations in bacterial diversity were observed in BSF gut-fed control, *Bacillus subtilis* 168-contaminated, or *Pseudomonas putida* KT2440-contaminated treatments (Figure 3C). Alpha diversity metrics, including bacterial taxa richness, Chao1 estimator, and Shannon index, remained consistent across treatments, indicating that the specific bacterial contamination in the substrate had minimal impact on overall gut microbial diversity. Principal Coordinates Analysis (PCoA) using Bray–Curtis dissimilarity matrix revealed no significant effect of treatments on community composition ($F = 1.48$, $R^2 = 0.04$, $p = 0.14$; Figure 3E), confirming that exogenous bacterial contamination did

not substantially alter the overall structure of the BSF gut bacterial community. However, significant differences were observed in intestinal microbial diversity between larvae and prepupae, with the microbial composition during the larval fattening stage (days 0–11) evolving progressively as feeding time increased (Figure 3B,D). A second PERMANOVA analysis revealed a highly significant effect of larval development on community composition ($F = 36.09$, $R^2 = 0.61$, $p < 0.001$; Figure 3F), demonstrating that developmental stage represents the primary driver of gut microbiota structure.

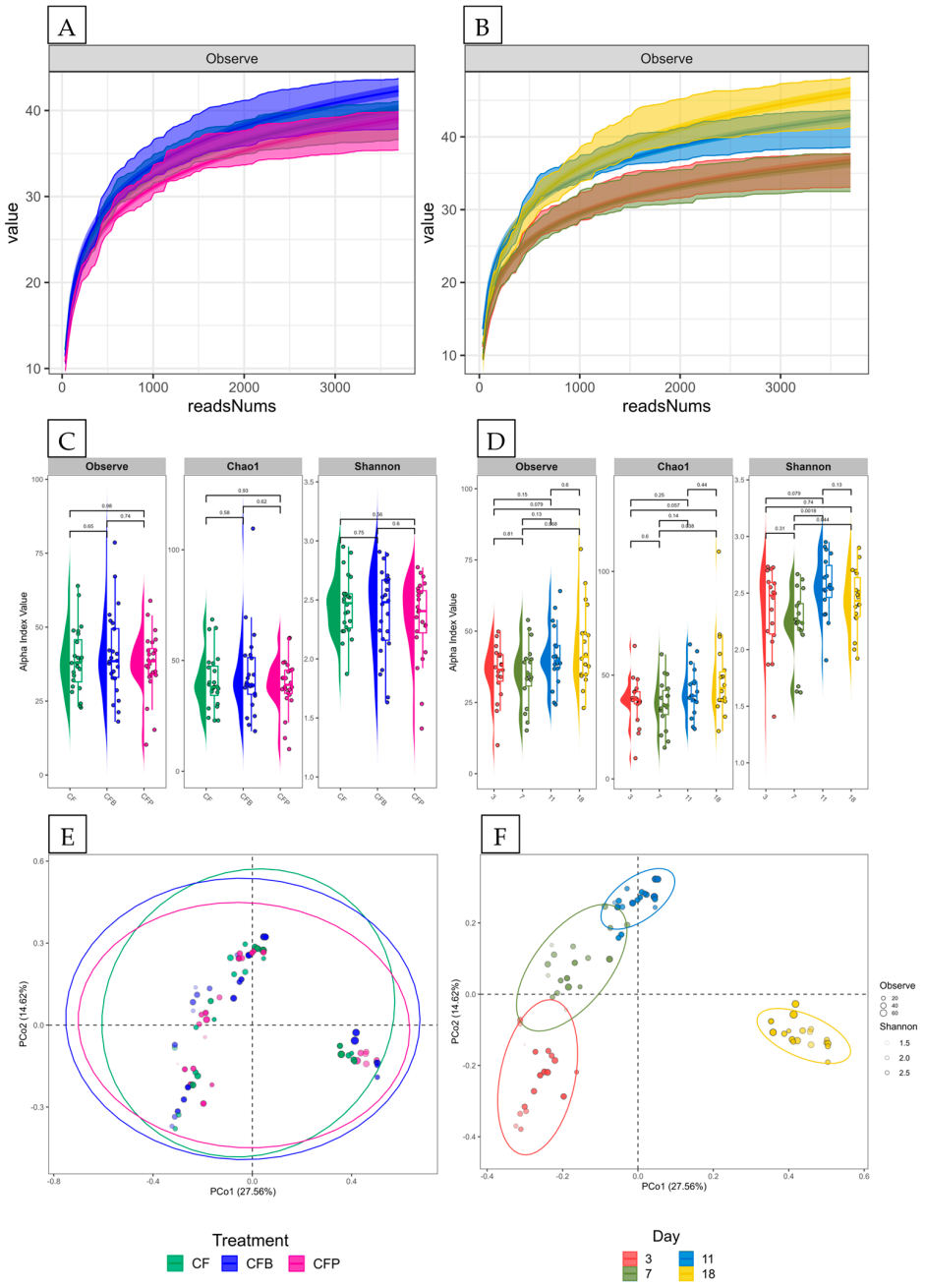


Figure 3. (**A**,**B**) Rarefaction curves for diet treatment (**A**) and *Hermetia illucens* larval development (**B**). Shaded areas of both curves correspond to the 95% confidence interval. (**C**,**D**) Alpha diversity metrics comparing diet treatment (**C**) and BSF larval development (**D**) for bacterial taxa richness in ASVs, Chao1 richness estimator, and Shannon's index. The *p*-value of the Kruskal–Wallis test is indicated on every boxplot. (**E**,**F**) Principal component analysis (PCoA) comparing bacterial communities of diet treatment (**E**) and BSF larval development (**F**). The dot size is scaled according to the ASVs' richness, and the opacity is scaled according to Shannon's index.

3.4. Dominant Bacterial Genera Composition

Six core bacterial genera were consistently identified across the three diets, with *Lactobacillus* spp. representing the dominant genus in the larval gut (Figure 4). The relative abundance of dominant genera showed treatment-specific patterns while maintaining overall community stability. *Lactobacillus* species comprised 35.61 ± 4.66% of the gut microbiota in control larvae, 30.43 ± 5.44% in *Bacillus subtilis* 168-contaminated treatment, and 35.18 ± 4.51% in *Pseudomonas putida* KT2440-contaminated treatment. *Lactobacillus* spp. showed a peak at day 7 throughout the progression of BSF larval development, while genera from *Morganellaceae* bacteria (i.e., *Morganella* spp. and *Providencia* spp.) dominated the bacterial proportions for the BSF prepupal stage (Figure 4B).

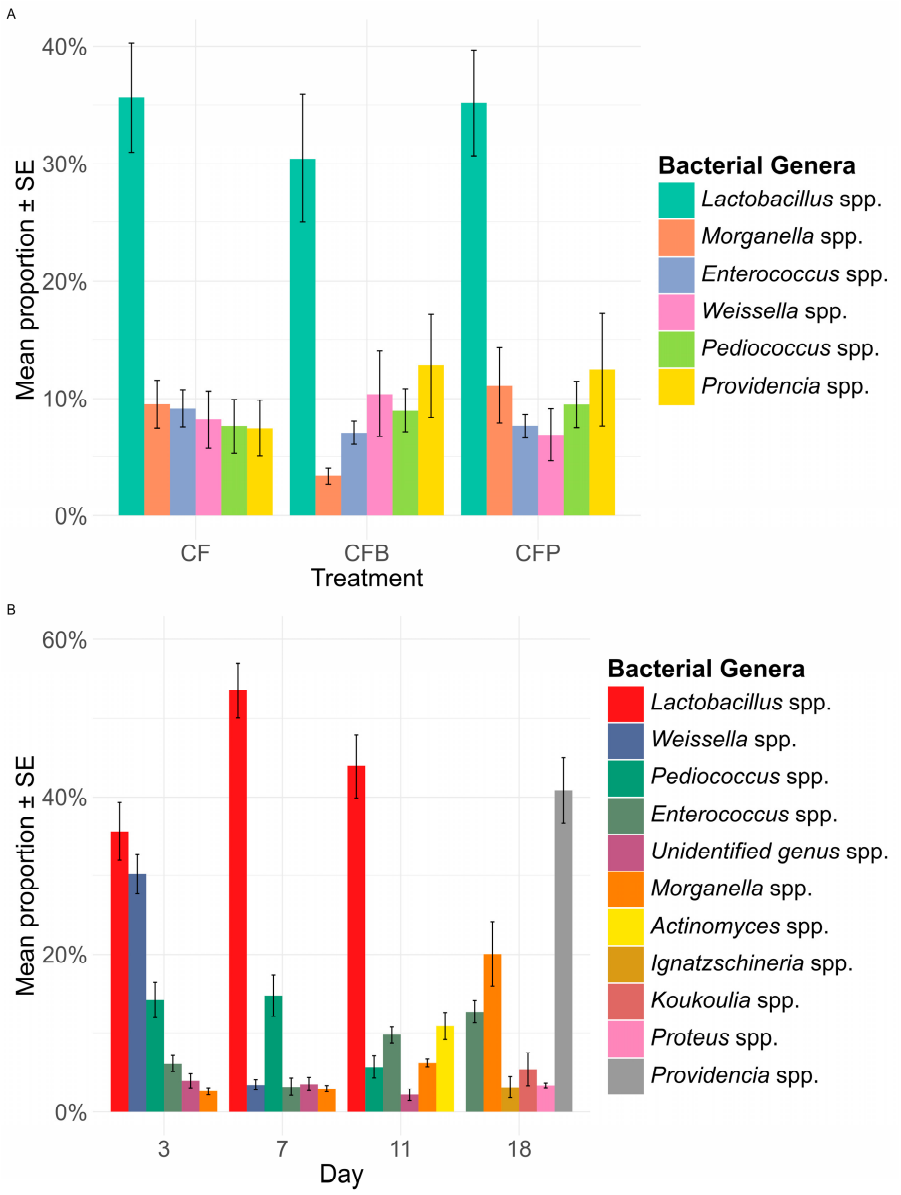


Figure 4. (**A**,**B**) Composition of bacterial genera in *Hermetia illucens* gut microbiota across feed treatments (**A**) and throughout larval development (**B**). (**A**) Relative abundance of dominant bacterial genera in the gut microbiota of *Hermetia illucens* larvae reared on three feed treatments: Control, *Bacillus subtilis* 168-contaminated feed, and *Pseudomonas putida* KT2440-contaminated feed. (**B**) Temporal changes in the relative abundance of dominant bacterial genera within the *Hermetia illucens* gut microbiota at days 3, 7, 11, and 18 of development.

4. Discussion

This study aimed to investigate the effect of contamination by model contaminant strains *Pseudomonas putida* KT2440 and *Bacillus subtilis* 168 on the development of BSF larvae and to evaluate the temporal changes in their gut bacterial community structures. Six-day-old BSF larvae were fed chicken feed contaminated by either *P. putida* KT2440 (CFP) or *B. subtilis* 168 (CFB). Over 18 days, observations showed that a high contamination level of exogenous bacteria in the substrate had only a slight effect on BSF larvae growth. Specifically, at day 7, larvae fed contaminated feed were lighter than those fed the control diet. In the CFB group, this initial slowdown in growth correlates with a significantly higher abundance of *Bacillus* spp. in their gut compared to controls. Despite this early growth delay, larvae showed tolerance to high *B. subtilis* 168 abundance, demonstrated by comparable survival rates and final weights to controls. This tolerance may reflect not only the robustness of BSF larvae but also potential beneficial interactions. *B. subtilis* is known to produce a range of extracellular enzymes—including proteases, amylases, and lipases—that facilitate nutrient breakdown in organic substrates, potentially improving nutrient assimilation by the larvae despite initial microbial competition [43]. Such enzymatic activity may support substrate degradation and enhance feed conversion efficiency over time, contributing to the observed recovery in larval growth. Xiao et al. [44] demonstrated that inoculating BSF larvae with *B. subtilis* at a large scale enhanced the reduction of organic matter. Zhao et al. [45] isolated 13 strains of *B. subtilis* from the surface and the gut of BSF larvae and showed that these strains improved the valorization of bird manure. Similarly, Qiu et al. [46] reported that a strain of *B. cereus*, isolated from the BSF gut and identified as a beneficial microorganism, improved larval performance when cultivated and inoculated into the feed. Concerning the larvae fed CFP, the abundance of *Pseudomonas* spp. was lower after 7 days than their abundance in the gut of control larvae. This result suggests that inoculated *P. putida* KT2440 did not find favorable conditions for development in the decaying substrate or the larval gut. Secondly, the abundance of *P. putida* KT2440 at the start of the experiment may have overstimulated an immune response in BSF larvae against this microorganism, leading to a lower abundance level by day 7.

According to Vogel et al. [47], the expression of antimicrobial peptides (AMPs) was remarkably induced and expanded in *H. illucens* larvae by feeding diets containing high bacterial loads. Previously, these AMPs expressed in a diet-dependent mode endow larvae with inhibitory activities against *Pseudomonas* spp. [47]. Moreover, another key immune factor, the BsfDuox-TLR3 signaling axis, has shown targeted suppression of Pseudomonaceae in BSF larvae [48], reinforcing the hypothesis that host immune activity contributed to the decline in *P. putida* KT2440.

Previous studies have shown that exogenous bacteria can considerably shape the insect gut, and it has even been claimed that caterpillar intestines are settled (or invaded) by the food microbiome, without any resident gut microbiota [49]. The general view is that the composition of the intestinal microbiota of BSF depends on the source of nutrition; for example, the intestinal bacterial structure varies depending on diets [11,15,16,50–52]. Despite these genus-specific differences revealed by qPCR, high-throughput sequencing showed that *Bacillus* and *Pseudomonas* were not dominant members of the gut microbial community. This interpretation is supported by the PCoA analysis (Figure 3E), which shows no clear clustering by treatment, and by the similar alpha diversity indices across treatments (Figure 3C). Together, these results indicate that the presence of exogenous bacteria in the substrate did not significantly alter the overall structure of the BSF gut bacterial community.

However, several limitations should be considered. This study focused on genus-level bacterial profiles, which may mask strain-level interactions that could influence larval

performance. While both qPCR and high-throughput sequencing were employed, we did not evaluate the actual viability or persistence of the inoculated strains in the substrate before ingestion. Furthermore, the experimental conditions—controlled laboratory settings and the use of a uniform chicken feed substrate—may not fully represent the complexity of waste-based or industrial rearing environments. Additionally, the limited invasion observed with the two model species could be attributed to strain-specific traits, such as the lack of virulence factors and/or antibacterials, which we did not assess. Lastly, while host immune responses were proposed as a mechanism shaping gut colonization, these responses were not directly measured. Future studies incorporating immune gene expression and functional microbial assays would help validate these hypotheses.

In this study, only the abundance of *Bacillus* in the larval gut showed a clear correlation with the initial inoculum, whereas the *Pseudomonas* community neither transferred effectively nor accumulated within the BSF intestines. Two hypotheses are given here to explain this difference. First, *H. illucens* appears to exert selective and interactive control over colonization by exogenous microorganisms. Multiple studies confirm that the insect gut environment generally restricts the migration and colonization of exogenous bacteria, and BSF feeding behavior can even reduce the proliferation of bacteria in the feeding substrate, such as Enterobacteriaceae [29,30,47,53]. On the other hand, there is substantial evidence that part of the microbiota in the BSF intestinal tract has been transferred from the diet. In this study, we hypothesize that *Bacillus* spp. did not highly proliferate in the intestines of *H. illucens* due to suboptimal abiotic and biotic conditions for their development. It is known that *B. subtilis* prefers alkaline to neutral environments, with growth occurring over a pH range of approximately 4.8 to 9.2 and an optimum near pH 6.8, although these values and optima are strain-specific [54,55]. Moreover, *B. subtilis* grows across a wide temperature range from about 5.5 °C to 55.7 °C, with an optimal growth temperature near 47 °C, but again, these thermal preferences can vary between strains [56]. Notably, the pH within the *H. illucens* larval gut varies substantially, from acidic conditions in the middle midgut to alkaline conditions in the posterior midgut, with the anterior midgut estimated to be near neutral pH, potentially suitable for *B. subtilis* growth [16]. *B. subtilis* is also considered a facultative aerobe, and its growth slows down in an anaerobic environment, such as the larval gut lumen. Finally, *B. subtilis*, as a Gram-positive bacterium, masters sporulation under unfavorable conditions, unlike *P. putida* [55]. It can be assumed that the abundance of *Bacillus* spp. in the larval gut is distributed between active cells and endospores.

The analysis of biotic interactions revealed that the inoculated bacteria primarily competed with *Lactobacillales* and Enterobacterales, indicating that the abiotic conditions of the luminal environment were more favorable to these orders. The *Lactobacillales* abundances were mainly represented across all treatments by the following genera: *Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp., and *Weissella* spp. The Enterobacterales were represented by *Morganella* spp. and *Providencia* spp. The relative homogeneity of the bacterial communities across treatments confirms that the diet composition is a primary modulator, as previously described by several studies [7,50,57]. The genera listed above were previously found in the gut of *H. illucens* larvae [57]. The persistence and high abundance of *Enterococcus* spp. throughout the experiment were expected, as this genus has been identified as a prominent member of the core gut microbiota in *H. illucens* larvae [7,57]. *Morganella* spp. was also considered a core member by Wynants et al. [17]. Although this was debated by Klammsteiner et al. [7], our results support the core member status of *Morganella* spp., especially given its high abundance at the prepupal stage, as observed by Wynants et al. [17] and Wang et al. [58]. This genus appears to be less stable than *Enterococcus* spp. and may be more influenced by biotic factors, such as the presence of exogenous bacteria. *Providencia* spp. also reached an abundance level above 40% at the end of larval

development, despite the use of a 100% plant-based feed. Comparable levels have only been reported in BSF fed with animal protein by Vecherskii et al. [57], suggesting that other nutrients could also shape this microbial profile. The high abundance of *Lactobacillus* spp. during larval growth was unexpected, as this genus typically accounts for only 0.05% to 5.00% of the gut microbiota [7,16,17,57]. Previous studies have shown that when BSF larvae process fruits and vegetables, the substrate may display elevated levels of *Lactobacillus* spp.; however, this increase does not necessarily translate to higher abundance within the larval gut [16]. *Lactobacillus* spp. are known to confer health benefits [59]. Therefore, some studies have tested the inoculation of *Lactobacillus* sp. into the feeding substrate of *H. illucens*, reporting beneficial effects such as improved conversion rates and enhanced nutritional content in the larvae [60]. A positive correlation was observed between *Lactobacillus* spp. abundance and trypsin and peptidase activities in the gut of BSF larvae, suggesting that these bacteria may support host protein digestion [61]. However, the initial inoculation of *Lactobacillus* spp. did not increase its abundance level as observed in our experiment. Interestingly, Wang et al. [58] observed a variable abundance of *Lactobacillus* spp. and *Enterococcus* spp. in BSF-fed food waste related to substrate particle size. *Lactobacillus* spp. reached a similar abundance when the particle size distribution ranged mainly from 0 mm to 2 mm. This distribution corresponds to the particle size distribution observed for our substrate (0.750 mm milled chicken feed). Particle size is a crucial factor for the digestion of *H. illucens* and microorganisms. Indeed, reducing particle size increases nutrient solubilization and the surface area available for microbial community colonization. Thus, particle size indirectly modifies the feed composition. It would be interesting to investigate further this parameter on different substrates, especially the control substrate used in studies on insect gut microbiota.

Finally, in addition to the abiotic and biotic conditions imposed by the digestive tract of *H. illucens*, the stable colonization of exogenous bacteria in BSF is constrained by its developmental mode. From the perspective of microbial colonization of bacteria, the insect gut as a microbial habitat is often unstable [11,13,14,16]. During larval development, the insect molts and the midgut continuously produce and shed the peritrophic matrix. This dynamic process disrupts or removes most of the associated bacterial populations. Only a small fraction of bacteria cross into the region adjacent to the midgut epithelium, which offers a more stable surface for colonization and supports microbial persistence [13]. In short, numerous challenges must be overcome before exogenous microbes colonize insect intestines and even achieve intergenerational vertical transmission, especially in holometabolous insects, which have distinct larval, pupal, and adult stages. The gut and other organs undergo a radical remodeling during metamorphosis, eliminating the entire larval gut and its contents. In *H. illucens*, this remodeling begins with the prepupal stage, and this phenomenon is perfectly highlighted in this study by the sudden abundance of the *Morganellaceae* genera at day 18. To conclude, the establishment of exogenous microorganisms in the *H. illucens* gut is not an overnight process; they must evolve to form a stable and specific association with this niche, rather than being acquired from the environment by each generation. In mass-reared insects, good rearing practices must be implemented to promote the establishment of insect lines associated with a desired exogenous microorganism selected for its beneficial traits.

5. Conclusions

Our results show that inoculating exogenous *B. subtilis* 168 and *P. putida* KT2440 into the substrate of early-stage *H. illucens* larvae does not disrupt the pre-established bacterial community in the larval gut. Moreover, this study highlights that *Lactobacillales*-related bacteria are naturally capable of colonizing the digestive system of *H. illucens* larvae.

Among these bacteria, the genus *Lactobacillus* spp. is well known for its beneficial effects on health. Its abundance in fully developed larvae further enhances the potential of using these larvae as feed.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/applmicrobiol5030082/s1: Figure S1: The standard curves of specific primers for detection of bacteria; Table S1: Primer pairs used during the experiment and their application [62–64].

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Abbreviations

The following abbreviations are used in this manuscript:

BSF	Black Soldier Fly
CF	Larvae Fed Chicken Feed
CFB	Larvae Fed Chicken Feed + *Bacillus subtilis*
CFP	Larvae Fed Chicken Feed + *Pseudomonas putida*

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