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Gut bacterial communities in black soldier fly larvae *Hermetia illucens* from different rearing locations using metagenomic and culture-dependent approaches

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Abstract. *Phuengmun P, Suwanchaisri K, Roddee J, Wangkeeree J. 2026. Gut bacterial communities in black soldier fly larvae* Hermetia illucens *from different rearing locations using metagenomic and culture-dependent approaches. Biodiversitas 27 (1): d270123. https://doi.org/10.13057/biodiv/d270123.* The black soldier fly larva (BSFL; *Hermetia illucens*) has gained increasing attention as an alternative protein source for animal feed. Knowledge of its gut bacterial communities is critical for ensuring microbial safety and utilization in rearing systems. In this study, BSFL samples were collected from six different rearing locations, and their gut microbiota were characterized using both 16S rRNA amplicon sequencing (V3-V4 region) and culture-dependent techniques. Twelve amplicon libraries were constructed from pooled samples corresponding to different rearing sites. After quality filtering, the number of clean reads ranged from 73,108 to 75,871 sequences, and clustering of these reads yielded between 98 and 717 operational taxonomic units (OTUs). Alpha diversity indices, including Shannon (3.51-6.07), Simpson (0.82-0.97), and Chao1 (98.04-717.00), indicated that the BSFL gut harbored a diverse and dynamic bacterial community, with variation in microbial richness and evenness. The most abundant phyla were Proteobacteria, Firmicutes, and Bacteroidota. At the genus level, dominant taxa commonly detected included *Dysgonomonas*, *Proteus*, *Enterococcus*, *Klebsiella*, and *Providencia*. Among the detected taxa were potentially pathogenic bacteria, such as *Campylobacter*. Through culture-dependent analysis, 57 bacterial isolates belonging to Proteobacteria and Firmicutes were identified by 16S rRNA gene sequencing. The most frequently cultured genera included the foodborne pathogen *Bacillus cereus*, along with other species such as *Bacillus amyloliquefaciens* and *Priestia megaterium*. This study represents the first investigation of BSFL gut bacterial communities from multiple locations in Thailand integrating metagenomic and culture-dependent approaches. The findings expand current knowledge of BSFL gut bacterial communities and highlight important biosafety considerations. In addition, identifying gut-derived bacterial isolates represent promising candidates for further evaluation of their beneficial roles in improving BSFL rearing efficiency.

Keywords: Black soldier fly larvae, culture-dependent isolation, gut microbiota, metagenomic analysis

INTRODUCTION

The black soldier fly larva (BSFL; *Hermetia illucens* (L.) Diptera; Stratiomyidae) has gained widespread attention as a sustainable alternative protein source for animal feed (Wang and Shelomi 2017). BSFL possesses a high-quality nutritional composition, containing approximately 40% protein, essential amino acids, and about 30% fat, which includes beneficial fatty acids (Smets et al. 2020). Their protein content can partially replace fishmeal or soybean meal in feed formulations for aquaculture, livestock, poultry, and domestic animals (Su et al. 2025). Beyond their nutritional value, BSFL possess several advantageous attributes; they are neither pest nor a disease vector, and most notably, they contribute to environmental sustainability (Rehman et al. 2023). The larvae are capable of bioconversion, decomposing a wide range of low-value residues, including food waste, vegetable and fruit residues, animal manure, and agri-food industry waste products, then convert these substrates into nutrient-rich biomass (Raksasat et al. 2020; Surendra et al. 2020). In addition, BSFL-derived product such as frass, can be used as organic fertilizers (Siddiqui et al. 2024). Recently, BSFL have demonstrated for large-scale industrial farming, providing dual benefits; through the recycling of organic waste and the producing of high-quality biomass (Tomberlin and van Huis 2020).

Owing to their saprophagous feeding behavior and wide dietary intake, BSFL harbor diverse microbial taxa within their gut (Vandeweyer et al. 2023). Previous studies have identified a set of core bacterial genera consistently present in BSFL guts, such as *Dysgonomonas*, *Enterococcus*, *Klebsiella*, *Morganella*, *Proteus*, and *Providencia* (IJdema et al. 2022). These gut-associated bacteria contribute to nutrient utilization, and in some cases, exhibit potential probiotic functions that may enhance BSFL growth performance (Li et al. 2023; Luo et al. 2023; Yu et al. 2023). However, gut-associated bacteria may pose potential pathogenic risks with adverse effects on human and animal health, such as *Escherichia coli*, *Bacillus cereus*, *Salmonella*, *Campylobacter*, and *Staphylococcus* sp. (Wynants et al. 2019; Khamis et al. 2020; Shumo et al. 2021; Tanga et al. 2021). In addition to safety considerations, there is increasing interest in the practical applications of gut-associated bacteria in BSFL rearing. Several bacterial strains, including *Bacillus licheniformis*, *Bacillus subtilis*, *Lysinibacillus sphaericus*, *Bacillus velezensis*, *Citrobacter* sp., *Klebsiella* sp., and *Stenotrophomonas maltophilia*, have been isolated from BSFL guts and reported as probiotics to improve growth performance and overall productivity (Gorrens et al. 2023). Understanding the gut bacterial community of BSFL is important for two main reasons: (i) ensuring microbial safety in BSFL-derived food and feed, and (ii) identifying beneficial microbes that may improve rearing efficiency.

The bacterial communities in the BSFL gut are influenced by several factors, with diet being the most important. Different nutrient profiles and substrate compositions introduce distinct initial microbes and create selective pressures that shape the overall gut bacterial community (Schreven et al. 2022). Besides diet, the developmental stage presents distinct physiological and metabolic conditions that favor specific bacterial groups. Genetic variation among BSFL populations may contribute to differences in gut bacterial composition (Silvaraju et al. 2024). Additionally, geographical location introduces further variation through differences in local environmental microbiota and substrate-associated microbes (Wynants et al. 2019). Given these influencing factors, comprehensive analyses of BSFL gut bacteria across different locations are needed to understand variation in microbial diversity and composition. This is particularly relevant as BSFL farming has expanded rapidly among smallholder farmers and at industrial scales across Southeast Asia (Barragán-Fonseca et al. 2024). Despite growing interest, knowledge of gut bacterial communities in BSFL from different rearing locations remains limited, particularly in Thailand, where such investigations have not previously been conducted.

To address this knowledge gap, the present study characterized the gut bacterial communities of BSFL collected from six different rearing locations using high-throughput V3-V4 16S rRNA amplicon sequencing combined with culture-dependent isolation techniques. By integrating these approaches, this study provides information on BSFL gut bacterial profiles and contributes to an improved understanding of BSFL-associated bacterial communities. Amplicon sequencing provides an overview of bacterial diversity and structure, whereas culture-dependent methods enable the isolation of viable strains for future functional evaluation and potential applications.

MATERIALS AND METHODS

Sampling location of BSFL

Based on BSF rearing locations, larvae were collected from six provinces that represent geographically distinct regions across Thailand, including Nakhon Pathom, Ratchaburi, Roi Et, Surin, Chiang Mai, and Chumphon Provinces (Table 1). At each site, BSFL were reared on locally available feeding substrates specific to each facility throughout the rearing cycle. Approximately 100 of late-instar larvae (200±25 mg/individual) were randomly selected. Sampling bias was minimized by collecting larvae during the daytime using a consistent method across all locations. The specimens were immediately preserved in absolute ethanol, transported to the laboratory in sterile containers under refrigerated conditions, and stored at -20°C until further use. For amplicon sequencing, larvae collected from two collection cycles were used, whereas specimens from the first rearing cycle were reserved for bacterial isolation. The sampling dates and designated sample codes for each location are summarized in Table 1.

BSFL gut dissection

Approximately forty BSFL from each location were surface-sterilized to remove external contaminants. The larvae were first immersed in 70% ethanol for 3 min, followed by soaking in 6% (v/v) sodium hypochlorite for 1 min. They were then washed twice for 1 min with sterile distilled water, rinsed three times with sterile phosphate-buffered saline (PBS; 130 mM NaCl, 7 mM $Na_2HPO_4{\cdot}2H_2O$, 3 mM $NaH_2PO_4{\cdot}2H_2O$, pH 7.0), and dried on sterile filter paper. To ensure data reliability, microbial contamination was strictly controlled throughout the study. All instruments, forceps, and containers were autoclaved before use. The entire gut was dissected under a sterile laminar flow hood using a stereomicroscope and sterile gloves, and tools were used for each sample set to avoid cross-contamination. Dissected guts were pooled according to location and immediately stored at -20°C until further processing.

Gut DNA extraction

For each location and collection cycle, ten dissected guts were pooled into a single sample for DNA extraction. Genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality and quantity of the extracted DNA were evaluated using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). All DNA samples were stored at -80°C until further analysis.

High-throughput sequencing and bioinformatic analysis

Genomic DNA from the pooled gut samples (one pooled sample per location per collection cycle, for a total of 12 samples) was used to prepare the amplicon libraries targeting the V3-V4 hypervariable regions of the bacterial 16S rRNA gene. Sample pooling was employed as an exploratory strategy to provide a preliminary overview of BSFL gut bacterial profiles across different rearing locations. Amplicon sequencing was performed on the Illumina MiSeq platform using a paired-end strategy (2 × 250 bp) by Biomarker Technologies (BMKGENE, Beijing, China).

Table 1. Sampling sites of rearing locations for BSFL collection, collection date, and assigned sample codes

Geographic region	Location district, Province	Latitude and longitude	Feeding substrates	Collection date	Sample codes
Central	Kamphaeng Saen, Nakhon Pathom	13.98472, 99.99396	Food waste, vegetable and fruit waste	7 Sep 2024	NPT1
			Food waste, vegetable and fruit waste	24 Jan 2025	NPT2
Central	Ban Pong, Ratchaburi	13.81488, 99.87158	Soybean residue, Supermarket waste	4 Jun 2024	RBR1
			Soybean residue, Supermarket waste	1 Sep 2024	RBR2
North-eastern	Mueang, Roi Et	16.05387, 103.65461	Food waste, bread waste	21 Mar 2025	RET1
			Food waste, bread waste	1 Oct 2024	RET2
North-eastern	Mueang, Surin	14.88327, 103.49244	Food waste, Vegetable waste	9 Nov 2024	SRN1
			Food waste, Vegetable waste	1 Feb 2025	SRN2
Northern	Doi Saket, Chiang Mai	18.87172, 99.13661	Rice bran, food waste, Fruit waste	15 Oct 2024	CMI1
			Rice bran, food waste, Fruit waste	21 Feb 2025	CMI2
Southern	Tha Sae, Chumphon	10.66568, 99.17285	Palm cake, food waste, Bread waste	8 Nov 2024	CPN1
			Palm cake, food waste, Bread waste	21 Feb 2025	CPN2

All downstream bioinformatic analyses were conducted using the Quantitative Insights Into Microbial Ecology (QIIME2) platform (version 2021.8) (Bolyen et al. 2019). Primer sequences were trimmed from the reads using the Cutadapt plugin (Martin 2011), followed by quality filtering to remove low-quality reads and ambiguous bases. Paired-end reads were merged using the VSEARCH plugin (Rognes et al. 2016), and chimeric sequences were identified and removed using the UCHIME algorithm. The resulting high-quality reads were clustered into operational taxonomic units (OTUs) at 97% similarity using the VSEARCH plugin. Representative sequences of each OTU were then aligned and taxonomically classified by comparison against the SILVA v.138 database (Quast et al. 2013), applying a minimum bootstrap confidence threshold of 80% for taxonomic assignment. OTU feature tables and representative sequences were generated and used to calculate alpha diversity indices (Chao1, Shannon, and Simpson) for each sample to assess bacterial richness and evenness. These diversity metrics were derived from single-pooled samples per location and collection cycle. Relative abundance plots were created at the phylum and genus levels to identify dominant bacterial taxa and illustrate community shifts across different rearing locations and collection cycles. Beta diversity analyses were not conducted because sequencing data were generated from single-pooled samples per location and collection cycle, limiting statistical comparison among rearing locations.

Isolation of cultivable bacteria

For each location, three dissected guts were pooled into a single sample for homogenization, and three such pooled samples per location were prepared and plated independently on each type of culture medium. All procedures were conducted aseptically under a sterile laminar flow hood. To remove external contaminants, the gut tissues were surface-sterilized following the procedure described above and subsequently homogenized in 300 µL of sterile phosphate-buffered saline (PBS) using sterile glass pestles. The homogenates were serially diluted tenfold (10^{-5} to 10^{-9}) in PBS, and 100 µL of each dilution was spread onto two general-purpose media: Nutrient Agar (NA) and Brain Heart Infusion Agar (BHI). Inoculated plates were incubated aerobically at 30°C for 24-48 h in darkness. After incubation, colony-forming units (CFU) were enumerated on plates containing 30-300 colonies. Bacterial load was calculated and expressed as CFU per milliliter (CFU/mL) using the following formula:

CFU/mL = (number of colonies × dilution factor) / volume plated (mL)

The final PBS wash was plated as a sterility control; if bacterial growth was observed, the corresponding sample was discarded.

For bacterial classification, morphologically distinct colonies were selected from each medium. Eight to ten well-isolated colonies per medium were randomly chosen and purified through repeated streaking onto fresh agar plates, followed by incubation at 30°C for 24-48 h. Purified single colonies were then picked and preserved in 75% glycerol stocks at -80°C until further molecular identification. These isolates were later subjected to molecular identification based on 16S rRNA gene sequencing.

DNA extraction of bacterial isolates

A total of 60 selected bacterial isolates were transferred to liquid media according to their original isolation (Nutrient Broth (NB) and Brain Heart Infusion (BHI) broth), and cultured overnight at 30°C with shaking at 150 rpm.

Bacterial cells were then collected by centrifugation, and genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol for Gram-positive bacterial cell lysates. The quality and concentration of the extracted DNA were assessed using a NanoDrop spectrophotometer (NanoDrop Lite; Thermo Fisher Scientific, DE, USA). Purified DNA samples were stored at -80°C until further molecular analyses.

Molecular identification of bacterial isolates

For species identification, the 16S rRNA region of bacterial gene was amplified by PCR using the universal primer pair 27F and 1492R (Shumo et al. 2021). Each PCR reaction was performed in a 25 μL volume containing 10× PCR buffer, 0.25 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.3 μM of each primer, and 2 μL of template DNA (~100 ng). The cycling conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min. PCR products (~1,500 bp) were analyzed by electrophoresis on 1% agarose gels stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and visualized under UV illumination. The amplicons were purified using the PureLink Quick PCR Purification Kit (Life Technologies, Carlsbad, CA, USA) and subjected to bidirectional sequencing with both primers at Macrogen, Inc. (Seoul, Korea). Forward and reverse reads were trimmed for low-quality bases, assembled into consensus sequences, and subsequently analyzed using the NCBI BLASTN algorithm (http://www.ncbi.nlm.nih.gov/blast). A ≥97% sequence identity threshold was applied for species-level identification.

Statistical analysis

CFU/mL values obtained from different rearing locations were calculated and assessed for normality using the Kolmogorov-Smirnov test. As the data were normally distributed, no data transformation was applied prior to analysis. Differences in bacterial counts among locations within the same media type were determined using one-way analysis of variance (ANOVA), and the comparisons of the means were performed using Tukey's Honest Significant Difference (HSD) test. Statistical significance was considered at $p<0.05$. All analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA), and results are presented as mean ± standard error (SE) from three biological replicates.

Data availability

The raw 16S rRNA amplicon sequencing data from the metagenomic analysis have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1346758. In addition, the nearly full-length 16S rRNA gene sequences of the cultured bacterial isolates have been deposited in the NCBI GenBank database under accession numbers PX363095 to PX363151.

RESULTS AND DISCUSSION

Gut bacterial community composition in BSFL from different rearing locations

To explore the composition and diversity of bacterial communities in the gut of BSFL, DNA was extracted from pooled gut samples from two collection cycles at six locations (Table 1) and subjected to Illumina sequencing targeting the V3-V4 region of the 16S rRNA gene. A total of 12 sequencing amplicons generated 79,707-80,221 raw reads and 73,108-75,871 high-quality clean reads. Subsequent clustering of these sequences yielded between 98 and 717 OTUs. Alpha diversity indices are presented in Table 2, and it should be noted that these metrics were derived from single pooled samples for each location and collection cycle. The Shannon index ranged from 3.51 to 6.07, the Simpson index from 0.82 to 0.97, and the Chao1 index from 98.04 to 717.00. These results indicate variation in gut microbial richness and evenness in the BSFL gut samples.

Table 2. Diversity indices of bacterial communities in BSFL gut samples collected from six locations

Samples[1/]	Raw reads	Clean reads	Number of OTUs	Shannon	Simpson	Chao1
NPT1	80,032	73,922	400	5.82	0.96	400.05
NPT2	79,939	73,929	486	4.55	0.85	486.15
RBR1	79,966	73,108	329	5.89	0.97	329.04
RBR2	79,789	74,603	717	6.07	0.97	717.00
RET1	79,831	73,600	228	4.94	0.94	228.00
RET2	79,909	75,613	379	5.42	0.95	379.00
SRN1	79,929	74,996	339	4.52	0.93	339.16
SRN2	79,997	74,987	378	4.64	0.93	378.00
CMI1	79,707	75,871	98	3.51	0.86	98.04
CMI2	79,827	75,329	174	3.55	0.86	174.05
CPN1	80,221	74,117	220	4.77	0.93	220.25
CPN2	79,917	75,100	318	3.93	0.82	318.00

Note: [1/] Representative of the BSFL gut samples collected from six different rearing sites located in the following provinces: NPT: Nakhon Pathom, RBR: Ratchaburi, RET: Roi Et, SRN: Surin, CMI: Chiang Mai, and CPN: Chumphon

Taxonomic composition of gut bacteria in BSFL from different rearing locations

The OTUs were taxonomically classified by comparing representative sequences against the SILVA database. Analysis revealed that the identified sequences belonged to 7 bacterial phyla. Among these, members of Proteobacteria, Firmicutes, and Bacteroidota were dominant phyla that were found across gut samples, although their relative abundance varied among locations and collection cycles (Figure 1). Specifically, the dominant phyla in each sample were as follows: NPT1: Firmicutes (37.49%) and Bacteroidota (31.13%), NPT2: Proteobacteria (77.57%), RBR1: Firmicutes (39.75%) and Bacteroidota (33.72%), RBR2: Bacteroidota (34.00%) and Firmicutes (27.58%), RET1: Bacteroidota (32.17%) and Proteobacteria (27.40%), RET2: Bacteroidota (35.21%) and Proteobacteria (21.10%), SRN1: Proteobacteria (77.79%), SRN2: Proteobacteria (78.68%), CMI1: Proteobacteria (59.16%) and Campylobacterota (19.54%), CMI2: Proteobacteria (56.51%) and Campylobacterota (19.68%), CPN1: Proteobacteria (41.94%) and Firmicutes (37.34%), and CPN2: Proteobacteria (60.90%) and Firmicutes (18.18%). Other phyla with relative abundances below 20% included Actinobacteriota (2.09-14.87%), Campylobacterota (0.02-19.54%), Desulfobacterota (0.01-9.42%), and Fusobacteriota (0.01-1.00%).

At the genus level, considerable variation in relative abundances was exhibited across different rearing locations and gut collection cycles. The average relative abundances of the 25 most dominant bacterial genera that exceeded 1% are presented in Figure 2. Specifically, the dominant genera in each sample were as follows: NPT1: *Lachnoclostridium* (14.43%) and *Campylobacter* (10.19%), NPT2: *Providencia* (48.06%), RBR1: *Dysgonomonas* (12.05%) and *Sedimentibacter* (10.23%), RBR2: unclassified Dysgonomonadaceae (15.00%), *Morganella* (12.95%), and *Oligella* (10.48%), RET1: unclassified Burkholderiaceae (15.26%), unclassified Dysgonomonadaceae (11.68%), unclassified Tannerellaceae (11.39%), RET2: unclassified Tannerellaceae (23.20%), *Lachnoclostridium* (14.20%), and *Campylobacter* (11.20%), SRN1: *Proteus* (23.66%), *Providencia* (17.31%), and *Enterococcus* (13.33%), SRN2: *Proteus* (21.51%), *Providencia* (17.94%), and *Pseudomonas* (11.31%), CMI1: *Providencia* (44.71%), *Campylobacter* (19.54%), and *Enterococcus* (17.56%), CMI2: *Providencia* (41.99%), *Campylobacter* (19.68%), and *Enterococcus* (18.49%). CPN1: *Klebsiella* (31.56%), *Enterococcus* (19.37%), and *Paraclostridium* (14.11%), CPN2: *Morganella* (55.17%) and unclassified Dysgonomonadaceae (12.21%). In addition to these dominant taxa, several genera were detected with varying relative abundances, including *Actinomyces* (0.14-9.70%), *Breznakia* (4.29%), *Desulfovibrio* (0.01-9.42%), *Erysipelothrix* (0.01-2.50%), *Ignatzschineria* (0.01-6.13%), *Leminorella* (0.10-3.65%), *Myroides* (5.20-7.08%), *Paenalcaligenes* (9.27%), *Proteiniphilum* (1.16-8.20%), unclassified Beutenbergiaceae (0.03- 6.45%) and unclassified Orbaceae (10.87-12.16%). These results suggest that BSFL gut microbiota is composed of a combination of dominant and low-abundance genera, with significant variability across locations and collection cycles, likely influenced by diet, local environment, and larval origin.

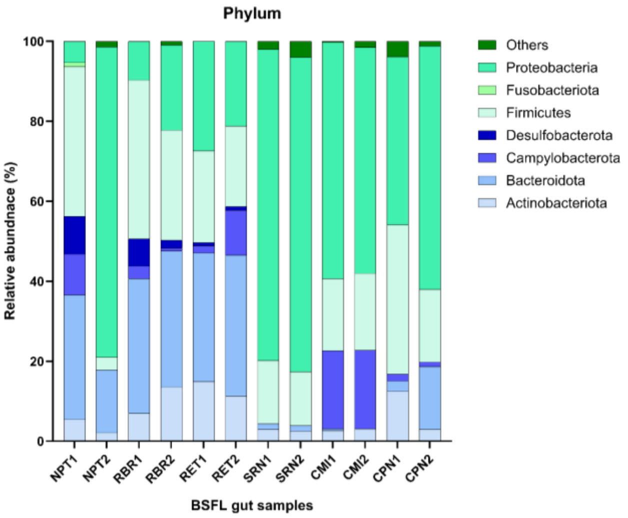

Figure 1. Relative abundance (%) of bacterial communities at the phylum level present in the BSFL gut samples. Twelve amplicon libraries correspond to samples collected from rearing sites located in the following provinces: NPT: Nakhon Pathom, RBR: Ratchaburi, RET: Roi Et, SRN: Surin, CMI: Chiang Mai, and CPN: Chumphon. The taxa with relative abundance lower than 0.1% and unclassified are indicated as 'Others'

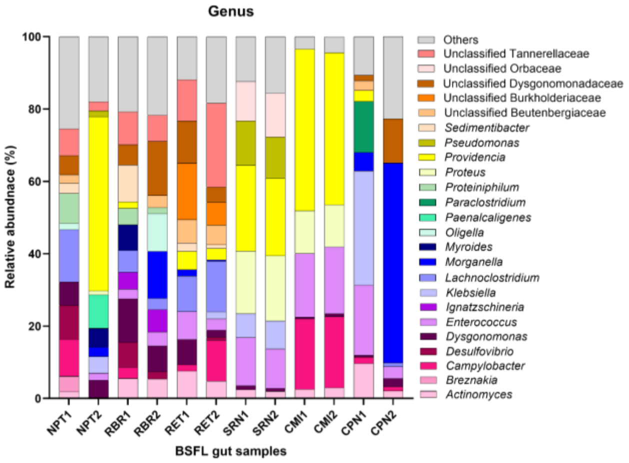

Figure 2. Relative abundance (%) of bacterial communities at the genus level present in the BSFL gut samples. Twelve amplicon libraries correspond to samples collected from rearing sites located in the following provinces: NPT: Nakhon Pathom, RBR: Ratchaburi, RET: Roi Et, SRN: Surin, CMI: Chiang Mai, and CPN: Chumphon; only genera with relative abundances ≥1% are shown individually, while taxa below this threshold are grouped as 'Others'

These findings align with previously published studies. Actinobacteroidota Proteobacteria, Bacteroidota, and Firmicutes were reported as the dominant phyla in the BSFL gut sample. However, their presence and relative abundances varied across studies. For instance, Silvaraju et al. (2024) reported Firmicutes and Proteobacteria as the most abundant phyla, while Gorrens et al. (2021) similarly identified these two phyla as predominant. In contrast, Klammsteiner et al. (2020) found Actinobacteriota and Bacteroidota to be the major phyla. Moreover, previous studies have shown that certain bacterial genera are consistently detected across multiple studies, geographic locations, and diverse dietary substrates. Examples of these shared taxa include *Actinomyces*, *Enterobacter*, *Klebsiella*, *Dysgonomonas*, *Enterococcus*, *Escherichia*, *Morganella*, *Providencia,* and *Proteus* (Lin and Shelomi 2024). Nevertheless, the presence and relative abundances of bacterial communities in the BSFL gut often vary both among and within studies (Cai et al. 2018; Klammsteiner et al. 2020; Galassi et al. 2021). The results of the present study are consistent with these previous findings, as the dominant genera identified here included *Dysgonomonas, Enterococcus, Klebsiella, Proteus,* and *Providencia*.

Several factors contribute to fluctuations in the presence and relative abundances of bacterial communities, including abiotic factors such as temperature, feeding substrate, and pH, as well as biotic factors like the initial microbial communities and potentially the BSFL genotype (Wynants et al. 2019). Among these, the type of feed substrate appears to be the primary driver shaping the bacterial communities, as different substrates harbor distinct initial microbiota and strongly influence the gut microbiota profile (Schreven et al. 2022). Moreover, properties of the feed substrate, such as macronutrient composition (protein, carbohydrates, and overall nutrient content), can induce significant shifts in the composition and relative abundances of gut bacterial communities. Such shifts are associated with the potential function, which facilitates the BSFL's ability to efficiently digest and metabolize the provided substrates (Klammsteiner et al. 2021; Chen et al. 2023).

These results suggest a potential association in which the type of feeding substrate may partially influence the enrichment of specific bacterial taxa within the BSFL gut. Certain predominant genera were shared among rearing locations that provided similar feeding substrates. For example, *Providencia* dominated in gut samples from NPT2, SRN1, SRN2, CMI1, and CMI2, where BSFL were primarily reared on food waste and fruit-vegetable residues. Previous studies have linked *Providencia* to substrate utilization, reporting positive correlations with total nitrogen and carbohydrate content, suggesting that this genus plays a key role in protein and lipid conversion within the BSFL gut (Ao et al. 2021). *Klebsiella* was most abundant in samples from CPN1, which used palm cake, food waste, and bread residues as feeding substrates. According to Gorrens et al. (2021), *Klebsiella* is often associated with cellulose- and hemicellulose-rich substrates, where it contributes to the degradation of plant-derived polysaccharides. Similarly, *Dysgonomonas* predominated in RBR1 and RBR2, where larvae were reared on soybean residue and supermarket waste. This genus has been reported to play a key role in the digestion of complex polysaccharides and lignocellulosic materials (Bruno et al. 2019). Nevertheless, no consistent relationship was observed between specific feeding substrates and dominant bacterial genera across all rearing sites and collection cycles. Interestingly, even locations using similar substrate types exhibited different dominant genera, and conversely, the same genus was occasionally dominant in locations with different substrate compositions. This suggests that complex interactions between feeding substrate and gut microbial communities may occur, potentially influenced by additional local environmental and management factors. Notably, most previous studies reporting strong substrate-microbiota correlations were conducted under controlled laboratory conditions, whereas the present study reflects natural variation in rearing environments. However, the bacterial diversity and composition of the feeding substrates were not examined in this study. Characterizing the substrate-associated microbiota would provide important complementary information for understanding the origins and transmission pathways of BSFL gut bacteria.

While the presence of certain bacterial taxa in the BSFL gut reflects their functional potential, it also has important implications for biosafety. Some of these taxa, including opportunistic pathogens, may pose a risk of contamination or microbiological hazards when BSFL are reared and subsequently utilized as an animal feed ingredient (Alagappan et al. 2025). We will now discuss the most frequently detected bacterial genera in the BSFL gut across rearing locations, with particular consideration of biosafety risks. In the present study, *Providencia* was the most consistently detected genus, being present in nine gut samples with relative abundances ranging from 1.71 to 48.06%. Previous studies have highlighted its significance in BSF biology, including stimulating oviposition (De Smet et al. 2018). However, members of this genus have also been identified as opportunistic pathogens associated with humans, causing infections such as diarrhea (Yuan et al. 2020). *Enterococcus* was also a frequently detected genus, being present in 11 gut samples, with relative abundances ranging from 1.92 to 19.37%. This genus has traditionally been considered a normal component of the gut microbiota across diverse species. Nevertheless, certain species, such as *Enterococcus faecalis*, have recently been recognized as opportunistic pathogens capable of causing nosocomial infections in humans (Maleb et al. 2020). *Klebsiella* was detected in five gut samples, with relative abundances ranging from 1.86 to 31.56%. Various *Klebsiella* strains are known opportunistic pathogens, posing significant public health risks and causing a range of human infections worldwide (Dong et al. 2022). *Proteus* was detected in five gut samples, with relative abundances ranging from 1.13 to 17.94%. This genus plays important functional roles in BSFL, such as supporting protein and carbohydrate digestion, thereby enhancing biomass production (Mazza et al. 2020). Nevertheless, certain *Proteus* species are opportunistic human pathogens capable of causing infections, including urinary tract infections (Drzewiecka 2016). In addition to the presence of these genera, *Campylobacter* was commonly detected in this study, which was identified in eight gut samples and had relative abundances ranging from 1.08 to 19.68%. Tanga et al. (2021) reported that the dominance of *Campylobacter* in BSFL guts likely originated from their feed, such as animal manure. However, *Campylobacter* is considered an opportunistic gastrointestinal pathogen capable of causing diarrhea in humans and is commonly found as a contaminant in various animal-derived food products (Sakran et al. 2020).

Despite providing information on the potential presence of pathogenic taxa, microbiological risk assessment based solely on metagenomic data has limitations. Metagenomic approaches detect microbial DNA, which may originate from either viable or non-viable cells. Consequently, sequencing data do not confirm the presence of live or infectious pathogens, nor do they indicate toxin production (Vandeweyer et al. 2021). Therefore, species- or strain-specific pathogen detection assays are required for accurate microbial risk assessment.

Enumeration, isolation, and identification of cultivable gut bacteria

Despite the advances in metagenomic analysis, culture-dependent methods remain important for characterizing the gut bacteria, as this technique allows isolation of pure cultures and accurate taxonomic classification via full-length 16S rRNA gene sequencing. Cultivable isolates can also be further studied for physiological and functional properties, as well as potential applications. From this perspective, and with a view toward the further application of bacterial isolates in BSFL mass rearing, cultivation techniques were employed in the present study.

The results of gut bacterial isolation and enumeration from the gut of BSFL samples collected from different locations are presented in Table 3. Significant difference in CFU/mL among samples was found ($p<0.001$). Using NA medium, the highest count was observed in the CPN sample, followed by NPT and RET. A consistent result was obtained from the BHI medium; the highest count was also observed in the CPN, followed by RET and CHB. A great variability in the quantity and types of microbes in the gut of BSFL larvae that were reared at different facilities was also reported by Wynants et al. (2019). These authors suggest that the rearing environment, especially the feeding substrate, can lead to significant shifts in the BSFL gut microbiota. Moreover, the cultivation techniques and type of media are important factors that affect the diversity of isolated bacteria. In this study, the two media differed in chemical composition and content, which are suitable for the growth of different bacterial groups.

Based on the morphological characteristics, a total of 60 bacterial isolates were identified, which were subsequently identified through 16S rRNA gene sequencing. Pairwise comparison of sequence similarities revealed that 57 sequences exhibited ≥97% identity with sequences available in the NCBI nucleotide collection database. Three isolates were discarded due to <85% similarity with their closest relatives, possibly indicating inaccurate or misleading sequence data.

The 57 bacterial isolates were classified into two phyla, Firmicutes (47 isolates) and Proteobacteria (10 isolates). These were further assigned to 2 classes, 4 orders, 6 families, 11 genera, and 22 species. Within the Firmicutes phylum, the isolates belonged to seven genera: *Bacillus*, *Priestia*, *Cytobacillus*, *Niallia*, *Kurthia*, *Enterococcus*, and *Lysinibacillus*. The Proteobacteria phylum comprises four genera: *Klebsiella*, *Providencia*, *Proteus*, and *Pseudomonas*. Their prevalence in BSFL gut samples according to sampling site is summarized in Figure 3.

The result of the species-level identification revealed that 22 species were found in six BSFL gut samples collected from different locations (Figure 4). Among these, the most frequently detected species were *B. cereus* (12 isolates), followed by *B. amyloliquefaciens* (9 isolates), and *Priestia megaterium* (5 isolates). *B. amyloliquefaciens* and *B. cereus* were common across multiple gut samples. Other species, including *B. altitudinis, B. velezensis, Klebsiella aerogenes,* and *K. pneumoniae,* were detected in three to five isolates each. Remaining species were less abundant, with one or two isolates per species, including *Kurthia gibsonii*, *Lysinibacillus fusiformis*, *Lysinibacillus macroides*, *Lysinibacillus* sp., *Lysinibacillus mangiferihumi*, *Niallia circulans*, *E. faecalis*, *E. termitis*, *Cytobacillus kochii*, *Providencia rettgeri*, and *Pseudomonas plecoglossicida.*

Comparison of metagenomic and culture-dependent data showed partial overlap in detected genera, with *Proteus*, *Enterococcus*, *Klebsiella*, and *Providencia* identified by both approaches. However, the dominant taxa differed between methods; for example, *Bacillus* was frequently recovered by cultivation but was not detected in sequencing data. These differences are expected, as amplicon sequencing provides a broad overview of gut bacterial diversity including uncultivable taxa. Whereas, culture-based methods enabled the isolation of viable bacteria relevant for risk assessment and further functional studies. In addition, some abundant taxa detected by sequencing are difficult to culture using standard media, while certain cultured taxa may occur at low relative abundance in sequencing data.

Table 3. Total colony-forming units (CFU/ mL ± SE) of bacteria isolated from the BSFL gut samples collected from six locations

BSFL gut sample [1/]	**NA (CFU/ mL ± SE×10^{5})**	**BHI (CFU/ mL ± SE×10^{4})**
NPT	13.10±1.61^{b} [2/]	2.78±0.05^{c}
RBR	0.26±0.02^{d}	1.83±0.14^{cd}
RET	12.80±1.12^{b}	15.51±2.33^{b}
SRN	0.63±0.08^{cd}	1.27±0.06^{d}
CMI	1.04±0.12^{c}	14.92±1.87^{b}
CPN	89.50±10.93^{a}	48.00±4.25^{a}
F [3/]	103.473	126.762
p-value	<0.001	<0.001

Note: [1/] BSFL gut samples collected from rearing sites located in the following provinces: NPT: Nakhon Pathom, RBR: Ratchaburi, RET: Roi Et, SRN: Surin, CMI: Chiang Mai, CPN: Chumphon. [2/] Values represent the mean ± standard error; within columns followed by different letters are significantly difference at $p<0.05$ (Tukey's HSD test). [3/] Mean (±SE) differ significantly at $p<0.05$ (ANOVA)

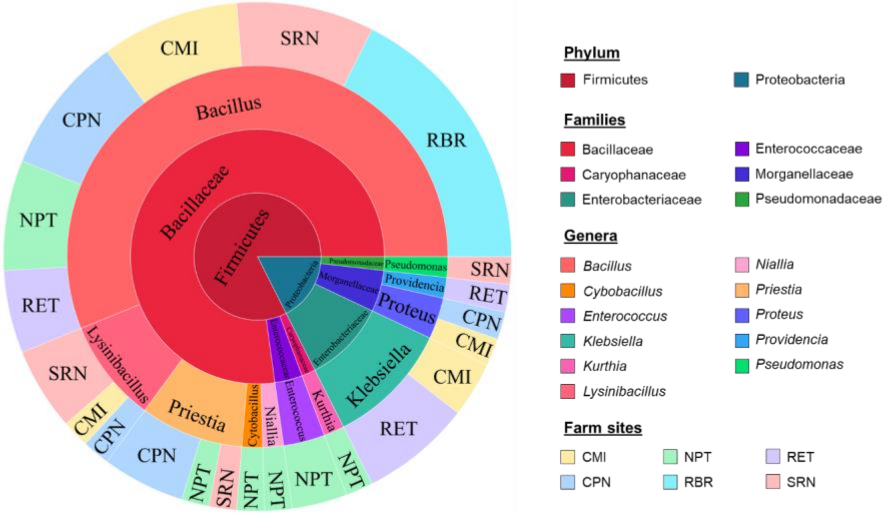

Figure 3. Phyla (inner circle), families, and genera (middle circle) of culturable bacteria isolated from BSFL gut samples collected from six different rearing sites (outer circle) located in the following provinces: NPT: Nakhon Pathom, RBR: Ratchaburi, RET: Roi Et, SRN: Surin, CMI: Chiang Mai, and CPN: Chumphon

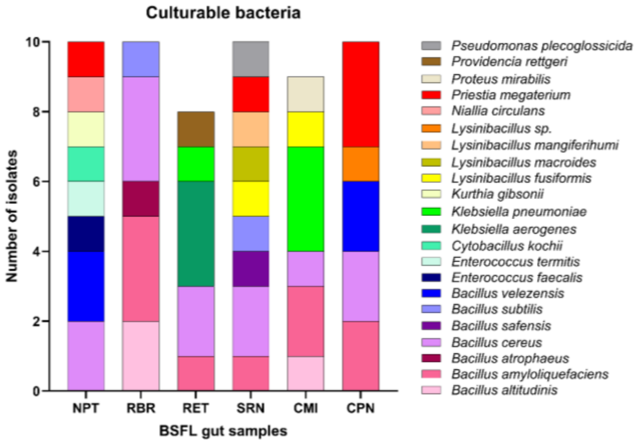

Figure 4. Culturable bacteria isolated from BSFL gut samples collected from rearing sites located in the following provinces: NPT: Nakhon Pathom, RBR: Ratchaburi, RET: Roi Et, SRN: Surin, CMI: Chiang Mai, and CPN: Chumphon

The cultivable isolates identified in this study were consistent with earlier findings. Tegtmeier et al. (2021) reported *Bacillus*, *Enterococcus*, *Klebsiella*, *Lysinibacillus*, *Providencia*, and *Proteus* as cultivable members of the BSFL gut microbiota. Similarly, Gorrens et al. (2021) isolated *Bacillus*, *Enterococcus*, *Lysinibacillus*, *Klebsiella*, and *Providencia*. Cifuentes et al. (2022) identified *Bacillus*, *Enterococcus*, *Morganella*, *Providencia*, and *Proteus* in BSFL guts. However, variations among studies have been observed; for example, Shumo et al. (2021) reported *Brevibacterium*, *Bordetella*, *Morganella*, and *Staphylococcus*. These differences likely reflect experimental factors such as feeding substrates, larval age, genetic background, and environmental conditions, consistent with the patterns observed in metagenomic analyses.

Among the cultivable isolates, it should be emphasized that the foodborne bacterium *B. cereus* was detected in this study. *B. cereus* is a known cause of foodborne illness in humans, commonly associated with gastrointestinal symptoms such as diarrhea and abdominal pain (Wynants et al. 2019). The detection of pathogenic taxa provides important guidance and underscores the need to develop practical management measures in BSFL rearing systems. To further minimize potential risks and enhance the safety of BSFL when used as a feed ingredient, recommended farm management practices include applying appropriate heat or microbial reduction treatments to harvested larvae, maintaining strict hygiene and sanitation within rearing units, and regularly testing larvae and feeding substrates for major foodborne pathogens to enable early detection and risk mitigation.

Of note, several major foodborne pathogens, including *Clostridium perfringens*, *Cronobacter* spp., *E. coli*, *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*, have been reported in BSFL samples in previous studies (Hoek-van den Hil et al. 2023; Brulé et al. 2024). However, none of these pathogens were detected in this study by either metagenomic analysis or the culture-dependent approach. This is an important observation, indicating that the rearing systems examined may exhibit a low or no prevalence of these pathogens under the conditions assessed. However, to ensure comprehensive microbial safety, further investigations using targeted, species- or strain-specific detection methods are recommended to confirm the presence of pathogenic contaminants.

From an application perspective, bacteria isolated from the BSFL gut have been utilized as feed supplements to improve larval growth performance, particularly when larvae are reared on nutritionally poor diets. For example, Xiao et al. (2018) report that gut bacterium *B. subtilis* could enhance BSFL weight and manure conversion efficiency. Similarly, Callegari et al. (2020) identified *B. licheniformis*

HI169 and *S. maltophilia* HI121 from the BSFL gut, and these bacteria significantly increased larval final weight and growth rate. Li et al. (2023) demonstrated that gut bacterial isolates such as *Citrobacter*, *Dysgonomonas*, *Klebsiella*, *Ochrobactrum*, and *Providencia* promoted BSFL development by increasing larval weight gain and shortening the life cycle. The underlying mechanism of these growth-promoting effects is comparable across studies: these bacteria exhibit high enzymatic activities that degrade complex substrate components, thereby supporting BSFL reared on difficult-to-digest substrates by enhancing substrate decomposition and nutrient availability. Moreover, Pei et al. (2022) identified that *B. velezensis* 10B enhances BSFL survival by supplying riboflavin and increasing larval protein content through modulating amino acid synthesis and related metabolic pathways. Therefore, the gut-derived bacteria identified in this study (i.e., *B. subtilis B. amyloliquefaciens*, *B. velezensis*, and *Lysinibacillus*) represent promising candidates for application in local BSFL rearing facilities. However, these candidate strains require further evaluation to determine their beneficial properties and practical efficacy.

Despite providing important data, the current study has a few limitations. First, sequencing analyses were based on single pooled gut samples per location and collection cycle, which may limit the assessment of within-location variability. Increasing the number of biological replicates per location would improve statistical robustness. In addition, DNA extraction blanks and PCR-negative controls were not included, which may limit the ability to assess potential background contamination. Second, the presence of uncharacterized taxa in some samples indicates incomplete taxonomic resolution, which may partially affect the interpretation of the bacterial community composition. This limitation may be attributed to the use of the 16S rRNA V3-V4 region, as these shorter variable regions often provide insufficient resolution for species-level identification (Jeong et al. 2021). Another possible explanation is that these unclassified taxa may represent novel microorganisms that are not yet included in current reference databases. To address this limitation, future studies should integrate whole-metagenome shotgun sequencing or long-read sequencing technologies to achieve species- and strain-level identification. Additionally, combining metatranscriptomic and metabolomic analyses could achieve higher taxonomic resolution and explore microbial functionality in greater depth. Third, only general-purpose culture media were used for bacterial isolation, which likely restricted the recovery of anaerobic or fastidious species. More comprehensive results could be achieved by applying specific culture conditions and selective media optimized for diverse bacterial groups. Such approaches could facilitate the isolation of a broader range of taxa, including novel microbes with potentially important roles in host-microbe interactions.

In conclusion, this study represents the first comprehensive investigation of the gut bacterial communities of BSFL across different geographical rearing locations in Thailand. High-throughput amplicon sequencing revealed that several dominant genera were consistently detected across locations, including *Dysgonomonas*, *Enterococcus*, *Klebsiella*, *Proteus*, and *Providencia*. In addition, potential opportunistic taxa, such as *Campylobacter*, were also detected. The presence and relative abundances of these taxa varied across different rearing locations and collection cycles. Culture-dependent analysis revealed a bacterial community dominated the foodborne bacterium *B. cereus*. In addition, gut-derived bacterial isolates, including *B. amyloliquefaciens*, *B. subtilis*, *B. velezensis*, and *Lysinibacillus*, were identified and represent promising candidates for further evaluation of their beneficial properties. Overall, these findings contribute to a broader understanding of BSFL-associated bacterial communities across different rearing locations. The detection of potentially pathogenic bacteria highlights the importance of continued microbial monitoring and supports the development of microbial safety guidelines for local BSFL rearing systems. Future research should employ multi-omics approaches to elucidate the functional roles of BSFL gut microbiota. In addition, the beneficial properties of cultivable bacterial strains identified in this study should be further assessed, particularly through in vivo feeding trials to evaluate their effects on larval growth performance and waste reduction efficiency.

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