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Research Article

Gut Microbiota of 3 Beetle Larvae and Their Potential for Humic Acid Transformation

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Abstract

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With the rise in global temperature, the removal and long-term sequestration of CO_2 from the atmosphere have become a shared global goal. Agricultural waste can be transformed by microorganisms into humic substances (HS) that are less prone to decomposition in soil, thereby increasing soil carbon sequestration. This study analyzed the gut microbiota of larvae from 3 different beetle species with varying diets. The gut microbiota of larvae from *Trypoxylus dichotomus*, which feed on decaying wood, were found to be richer and harbored a greater diversity of bacterial species compared to those of *Alphitobius diaperinus* and *Araecerus fasciculatus*. Further analysis of the gut microbiota isolated from 3 beetle larvae species examined the activity of enzymes involved in humic acid biosynthesis, including cellulase, ligninase, laccase, and tyrosinase. Among them, *Bacillus megaterium* BM01 and *B. aryabhattai* BA01 exhibited activities for all 4 enzymes, while *B. subtilis* BS01 showed activities for the first 3 enzymes. To further examine the ability of strains to convert rice straw into humic acid, *B. megaterium* BM01, *B. aryabhattai* BA01, and *B. subtilis* BS01 exhibited an increased humic acid conversion efficiency of 2.4%, 2.3%, and 2.1%, respectively, compared to the control group without inoculation. These findings suggest potential applications in on-site conversion and decomposition of residual rice straw post-harvest to humic acid, thereby enhancing soil carbon sequestration.

Key words: Beetle, Gut microbiota, Humic acid, Soil carbon sequestration.

INTRODUCTION

Human industrial activities have transitioned significantly towards the consumption of fossil fuels since the Industrial Revolution in the 18th century, leading to a steady increase in greenhouse gas (GHG) concentrations such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) in the atmosphere. According to the Sixth Assessment Report (AR6) released by the Intergovernmental Panel on Climate Change (IPCC) in 2023, the atmospheric CO_2 concentration has reached its highest point in 2 million years. Between 2011 and 2020, the average global temperature increased by 1.09℃ compared to the period from 1850 to 1900. Among the contributors to this temperature rise, CO_2 accounted for the highest warming

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effect at 0.79℃, followed by CH_4 at 0.5℃. The assessment report also clearly indicates a nearly linear relationship between cumulative anthropogenic CO_2 emissions and global temperature rise (Lee *et al*. 2023). As global temperature rises, the frequency and intensity of extreme weather events are becoming more severe. Removing CO_2 from the atmosphere and storing it long-term have become a common goal for humanity. In agricultural environments, increasing soil carbon sink capacity stabilizes carbon in the soil, mitigates climate change, offers various benefits such as soil quality restoration, ecosystem functioning, and enhances water and nutrient retention, sustainable agricultural practices, and food security (Lal 2008; Lal *et al*. 2015).

Humic substances (HS) in soil exhibit properties such as recalcitrance, enhancing soil aggregation, and prolonging soil carbon retention (Hassett *et al*. 1987; Fortun *et al*. 1990; Spaccini *et al*. 2002). Agricultural organic waste can be transformed into HS by microbial conversion. This conversion process involves the degradation of organic matter followed by polymerization into HS, with humic acid (HA) and fulvic acid (FA) being the main components (Guo *et al*. 2019). Taiwan generates approximately 5 million tons of agricultural residues annually, with rice straw accounting for 1.5 million tons. After harvest, rice straw is often left in fields requiring labor-intensive and time-consuming management. Utilizing these plant residues on-site to convert them into HS capable of enhancing soil carbon sequestration could contribute significantly to soil carbon storage (Martens 2000).

The diverse species, large populations, and various diets of insects contribute to the rich symbiotic bacterial ecology within their intestines. Intestinal symbiotic bacteria assist hosts in food digestion, detoxification, resistance against various insect pathogens, and maintenance of basic insect survival functions (Jang & Kikuchi 2020). It is known that plant substrates, after being consumed and digested by insects, can be converted to feces rich in HA. For instance, larvae of the white-spotted flower chafer (*Protaetia brevitarsis* Lewis) efficiently convert consumed plant residues into feces with high levels of HA (Li *et al*. 2019). While the exact mechanism of formation remains unclear, studies have found that dominant bacterial strains in insect guts play a role in decomposition and transformation. These strains possess the ability to degrade hemicellulose and cellulose and aid in the decomposition of plant debris (Lou *et al*. 2022). The formation of HS involves the degradation of lignin, followed by the action of tyrosinase and laccase, which polymerize small phenolic compounds, amino acids, and aromatic compounds to form stable molecular structures (Gerke 2018; Piccolo *et al*. 2018). Furthermore, gut bacteria in the alkaline and anaerobic environments of insect intestines can transform ingested organic matter, suggesting a link between HA biosynthesis and gut bacteria (Huang *et al*. 2010; Hobbie *et al*. 2012). Many studies have utilized microorganisms to promote humic substance biosynthesis, such as the use of the white rot fungus *Phanerochaete chrysosporium* and the actinomycete *Streptomyces badius* to degrade lignin in rice straw and convert it into HA and FA (Huang *et al*. 2008). The addition of *Bacillus aryabhattai* to discarded coconut fiber substrates enhances humic substance biosynthesis through the enzymatic activities of tyrosinase and laccase, consequently reducing ethylene levels in plants and promoting plant growth (Ngangom *et al*. 2019; Muniraj *et al*. 2021a, 2021b). Following the planting of winter wheat and spring barley, the addition of *B. megaterium*, *Trichoderma reesei*, and *Acinetobacter calcoaceticus* to the soil led to increased total soil organic carbon content, soil carbon-to-nitrogen ratio, and HA content, and also enhanced microbial diversity (Jurys & Feizienė 2021).

Additionally, the supplementation of *B. subtilis* in composts composed of cow dung and rice straw boosts the levels of total organic carbon and HS, facilitates the humification of dissolved organic matter (DOM) in rice straw

composts, and enriches microbial community diversity within the compost (Duan *et al*. 2020; Qu *et al*. 2024).

This study aims to investigate the gut microbiota of beetles and identify strains capable of converting plant substrates into HA, thereby facilitating the decomposition of agricultural waste. By locally decomposing agricultural residues such as straw into HS, soil fertility can be improved, chemical fertilizer application reduced, soil carbon sequestration enhanced, and agricultural carbon emissions mitigated.

MATERIALS AND METHODS

Collection and taxonomic identification of beetle larvae

Three species of beetle larvae were collected from discarded wood piles in Wufeng District, Taichung City, grain warehouse in Toucheng Township, Yilan County, and decaying jackfruit fruits in Citong Township, Yunlin County. Due to the difficulty of identifying insect larvae based on external morphology, the mitochondrial cytochrome oxidase subunit 1 (COI) gene was amplified using polymerase chain reaction (PCR) and sequenced for species identification using DNA barcoding. First, larval epidermal tissue was placed in a 1.5 mL microcentrifuge tube, and 300 μL of TNES buffer containing 0.1 μg of Proteinase K (50 mM Tris, pH 7.5, 400 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS)) was added. The reaction was carried out at 37℃ for 3−18 h. Subsequently, 85 μL of 5 M NaCl was added, mixed with a Vortex (Vortex-Genie® 2; Scientific Industries, NY, USA) for 15 s, and centrifuged at 14,000 g for 5 min. The supernatant was collected and mixed with an equal volume of 99% ethanol, followed by centrifugation to precipitate DNA. Finally, the DNA pellet was washed with 70% ethanol, centrifuged again, and resuspended in 20 μL of ddH_2O (Sunnucks & Hales 1996). PCR was conducted using the Takara Taq™ kit (R001B, Takara Bio USA Inc., San Jose, CA, USA) in a reaction volume of 50 μL. Each reaction included 5 μL of the aforementioned DNA solution and 0.5 μL of a primer mix consisting of 20 mM each of the following primers: LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*. 1994), along with 0.5 μL of Taq polymerase, 5 μL of 10× buffer, 4 μL of dNTPs, and 34.5 μL of ddH_2O. Amplification was carried out using a thermal cycler (GeneAmp® PCR System 2400, PerkinElmerInc., Shelton, CT, USA). The PCR conditions consisted of an initial denaturation step at 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 50℃ for 1 min, 72℃ for 1 min, and a final extension at 72℃ for 7 min. The PCR products were sequenced by Genomics Inc. (New Taipei, Taiwan) using an ABI 3730XL DNA Analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The obtained DNA sequences were compared to the COI gene sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information (NCBI), Bethesda, MD, USA) to identify the beetle species.

Gut microbiota analysis of 3 beetle larvae

Three larvae from each of the three beetle species were dissected to isolate their gut tissues. The gut tissues were then sent to Genomics Inc. (New Taipei, Taiwan) for metagenomic analysis. PCR amplification of the V3−V4 region of the 16S rRNA gene was performed using the 341F and 805R primers. The PCR products were purified using the AMPure XP system (Beckman Coulter, Brea, CA, USA) and used to construct libraries with the Nextera XT Index Kit v2 Set (Illumina Inc., San Diego, CA, USA). Library quality was monitored using the Qubit 2.0 and Qsep400 System (Bioptic Inc., New Taipei, Taiwan). Finally, sequencing of 300 bp paired-end reads was conducted using the Illumina MiSeq system (Illumina Inc., San Diego, CA, USA).

Isolation and identification of gut bacteria in beetle larvae

The larvae of the 3 beetle species were surface-sterilized with 70% ethanol. Subsequently, their gut tissues were dissected using scalpels and forceps and placed in lysogeny broth (LB). The LB was then serially diluted tenfold and spread onto nutrient agar (NA) and LB agar plates. After incubation at 30℃ for 72 h, individual bacterial colonies were transferred into 1.5 mL microcentrifuge tubes containing 10 μL of 1 mM Tris-hydrochloric acid (HCl) buffer. The tubes were subjected to a heat-cold treatment: 5 min in ice, followed by 1 min at 95℃, 30 s in ice, repeated for a total of 4 cycles, and a final 5-min ice bath to lyse the bacterial cells and release DNA into the Tris-HCl buffer.

PCR was performed using the Takara Taq™ kit (R001B, Takara Bio USA Inc., San Jose, CA, USA) with a reaction volume of 50 μL. Each reaction contained 1 μL of the aforementioned DNA solution, 0.5 μL of a primer mix consisting of 20 mM each of the following primer pairs: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3'), or 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Frank *et al*. 2008; Armingohar *et al*. 2014), along with 0.5 μL of Taq polymerase, 5 μL of 10× Buffer, 4 μL of dNTPs, and 34.5 μL of ddH_2O. PCR amplification was carried out using a thermal cycler (GeneAmp® PCR System 2400, PerkinElmer, Inc., Shelton, CT, USA). The PCR conditions consisted of an initial denaturation step at 94℃ for 10 min, followed by 35 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 1 min, with a final extension step at 72℃ for 7 min to amplify the 16S rRNA gene sequence. The PCR products were sequenced by Genomics Inc. (New Taipei, Taiwan) using an ABI 3730XL DNA Analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The obtained DNA sequences were compared to the 16S rRNA gene sequences in the GenBank database using the BLAST (NCBI, Bethesda, MD, USA) to confirm gut bacteria.

Bacteria selection for HA synthesis

Gut bacteria isolated from the 3 beetle larvae were tested for cellulase, ligninase, laccase, and tyrosinase activities. Cellulase activity was assessed using 0.1% (w/v) carboxymethyl cellulose (CMC)-LB agar plates, followed by Congo red staining (Gohel *et al*. 2014). Ligninase activity was measured in a medium containing 2.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, 1.0 g K_2HPO_4, 0.5 g NaCl, and 5.0 g alkaline lignin, supplemented with 1% (w/v) aniline blue (Xiong *et al*. 2020). Laccase activity was determined using a medium containing 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Ahmed *et al*. 2018), while tyrosinase activity was screened in a medium supplemented with 0.1% L-tyrosine (Valipour & Arikan 2015).

Evaluation of bacterial conversion of rice straw into HA

Detection of strains with HA biosynthetic enzymes for their HA conversion efficiency on rice straw was measured at 14, 28, and 42 d. Three Erlenmeyer flasks were prepared with 10 g of ground dry rice straw each, sterilized at 120℃ for 20 min. Each flask was inoculated with 1 mL of bacterial culture and incubated at 35℃ while shaken at 200 rpm (LM-570R, Yih Der, Taipei, Taiwan) for 2 d, followed by the addition of sterile water to achieve 65−70% moisture content. Humification was carried out at 30℃, while the control group received only 1 mL of LB. HA content was measured every 14 d from one flask for each treatment, with 3 replicates per treatment. The HA detection method followed the HA inspection procedure outlined in the Taiwan Agricultural Fertilizer Management Standard (AFS2102-1, announced under agricultural food supply letter No. 1091068958A on April 24, 2020).

Rice straw treated with the bacteria was dried at 105℃ for 24 h, and 0.5 g of the dried rice straw powder was placed into each of 8 centrifuge tubes. To each tube, 25 mL of 0.05% sodium lauryl sulfate solution containing 4% HCl was added, shaken at 120 rpm for 1 h,

centrifuged at 2,990 g for 20 min, and the supernatant was removed. This process was repeated with 25 mL of 0.0005% sodium lauryl sulfate solution containing 0.04% HCl, shaken vigorously for 1 min, centrifuged at 2,990 g for 20 min, and repeated once. Subsequently, 25 mL of 0.25 N sodium hydroxide (NaOH) solution was added to each tube, shaken at 120 rpm for 1 h, centrifuged at 7,656 g for 20 min, and the supernatant was transferred to a 100 mL beaker. This step was repeated twice. The combined supernatant was adjusted to a pH ≤ 1 with 6 N HCl, allowed to stand for 10 min, centrifuged at 7,656 g for 20 min, and the supernatant was removed. Subsequently, 25 mL of distilled water was added, shaken vigorously for 1 min, centrifuged at 2,990 g for 20 min, and the supernatant was removed, repeated twice. The precipitate from the 8 tubes was combined, washed into a crucible with distilled water, dried at 105℃ for 24 h, cooled to room temperature, and weighed (W1). The crucible with the precipitate was then placed in a high-temperature furnace ash, heated gradually to 600℃ , maintained at this temperature for 4 h, cooled to 100℃, cooled to room temperature in a desiccator, and weighed again (W2).

The conversion efficiency of the bacteria in converting rice straw to HA was calculated using the formula: conversion efficiency (%) = (W1 – W2) / W × 100.

RESULTS

Identification of beetle larvae

Three beetle larvae used in this study were collected from discarded wood piles in Wufeng District, Taichung City, grain warehouse in Toucheng Township, Yilan County, and decaying jackfruit fruit in Citong Township, Yunlin County. Mitochondrial COI gene fragments of 600, 632, and 643 bp were amplified and sequenced using LCO1490/HCO2198 primers (Appendix). The sequences showed 99.3%, 100.0%, and 98.9% similarity to that of *Trypoxylus dichotomus* (GenBank LC074686), *Alphitobius diaperinus* (GenBank MT610905), and *Araecerus fasciculatus* (GenBank KM446808) in the GenBank database, respectively. Therefore, these 3 beetle species were identified as *Tr. dichotomus*, *Al. diaperinus*, and *Ar. fasciculatus*.

Gut microbiota analysis of 3 beetle larvae

The gut microbiota of 3 beetle larvae were analyzed using the V3−V4 region of the 16S rRNA gene. Significant differences were observed in their gut microbiota, with only 1 bacterium, *Corynebacterium* sp., found across all species. *Tr. dichotomus* had a greater diversity and abundance of bacteria compared to the other 2 beetles (see Figs. 1–4). The dominant bacterial families differed among the 3 species: *Tr. dichotomus* had a balanced presence of Ruminococcaceae and Lachnospiraceae, each comprising about 20% of its gut microbiota; *Al. diaperinus* larvae were dominated by Streptococcaceae (around 59%); while *Ar. fasciculatus* larvae from grain warehouse and decayed Polomiti fruits had a simpler gut microbiota, dominated by Lactobacillaceae (approximately 58%).

Isolation and identification of gut bacteria in beetle larvae

We isolated and identified 118 strains of symbiotic bacteria from the intestines of larvae of 3 beetle species: *Tr. dichotomus*, *Al. diaperinus*, and *Ar. fasciculatus*, with 72, 27, and 19 strains isolated, respectively (Table 1). Among the 118 strains of bacteria, the most abundant genus was *Bacillus*, with 34 strains, of which 31 were isolated from the gut of *Tr. dichotomus* larvae. This was followed by *Staphylococcus* with 29 strains, *Citrobacter* with 20 strains, and *Acinetobacter* with 12 strains; all other genera had fewer than 10 strains.

Bacteria selection for HA synthesis

We used selective culture media to assess the cellulase, ligninase, laccase, and tyrosinase activities of 118 bacterial strains isolated from

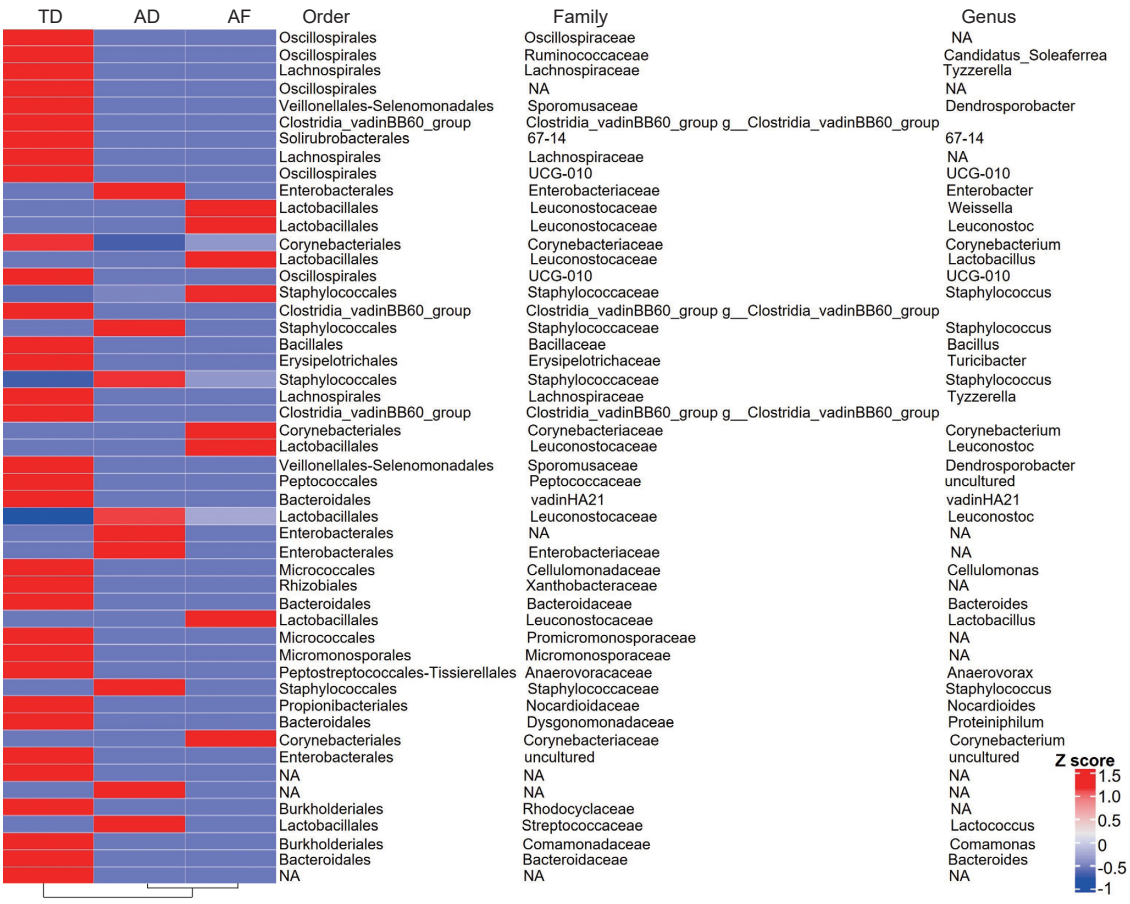

Fig. 1. Heat map of the top 50 abundance microbes in the gut of 3 beetle larvae *Trypoxylus dichotomus* (TD), *Alphitobius diaperinus* (AD), and *Araecerus fasciculatus* (AF).

larvae of 3 beetle species. Among these, 12 strains from *Tr. dichotomus* larvae displayed these enzyme activities. Specifically, *B. aryabhattai* BA01 and *B. megaterium* BM01 exhibited all 4 enzyme activities; *B. subtilis* BS01 showed the first three, while *B. cereus* BC01 and *Neobacillus ginsengisoli* N01 had 2 enzyme activities (Table 2). In contrast, bacteria from *Al. diaperinus* and *Ar. fasciculatus* larvae only showed cellulase activity (Tables 3–4).

Evaluation of bacterial conversion of rice straw into HA

We selected 5 bacterial strains known for their synthesis of HA-related enzymes: *B. subtilis* BS01, *B. megaterium* BM01, *B. aryabhattai* BA01, *B. cereus* BC01, and *N. ginsengisoli* N01, to assess their ability to convert rice straw into HA. After treating the rice straw for 14, 28, and 42 d, all 5 strains demonstrated higher HA conversion efficiencies compared to the control group without bacteria. At 14 d, *B. megaterium* BM01 showed the highest conversion efficiency, increasing by 2.2% compared to the non-inoculated control, followed by *B. subtilis* BS01 at 2.1%, with the remaining 3 strains below 2.0%. At 28 d, *B. subtilis* BS01 led with a 2.3% increase, followed by *B. megaterium* BM01 and *B. aryabhattai* BA01 at 2.2% and 2.1%, respectively, with the other 2 strains below 2.0%. By 42 d, *B. subtilis* BS01 again showed the highest effi-

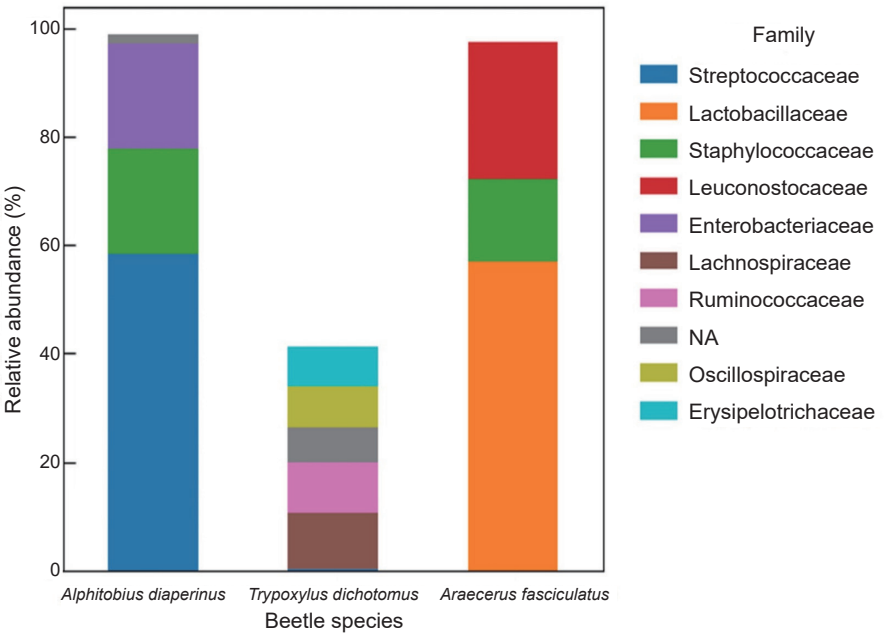

Fig. 2. Relative abundances of bacterial phyla at the family level (top 10) present in the gut of 3 beetle larvae.

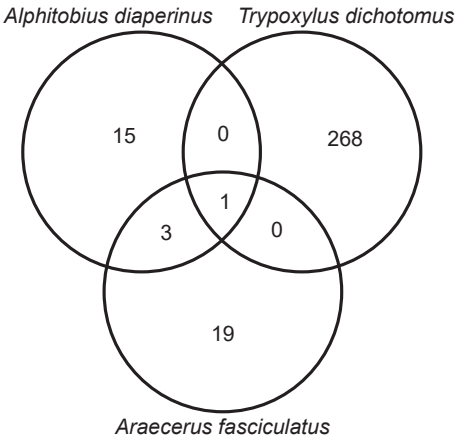

Fig. 3. Venn diagram shows the intersection of the species level in the gut of 3 beetle larvae.

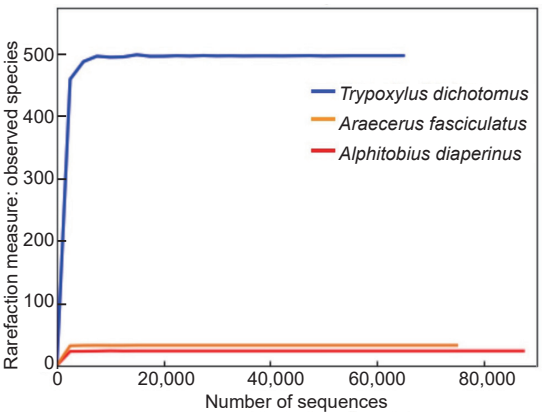

Fig. 4. Rarefaction curves illustrating the species richness of gut microbiota in 3 beetle larvae across different sequencing depths of the 16S rRNA gene.

ciency with a 2.4% increase, followed by *B. aryabhattai* BA01 and *B. megaterium* BM01 at 2.3% and 2.2%, respectively, with the other 2 strains below 2.0%. This indicates that *B. subtilis* BS01, *B. aryabhattai* BA01, and *B. megaterium* BM01 have the highest potential for accelerating the humification of rice straw (Fig. 5).

DISCUSSION

This study examined the gut microbiota of larvae from 3 beetle species with distinct diets. The gut microbiota of *Tr. dichotomus* larvae were found to be richer compared to those of *Al. diaperinus* and *Ar. fasciculatus* larvae. This difference may be due to *Tr. dichotomus* larvae

Table 1. Symbiotic bacteria and their quantities extracted from the guts of 3 beetle species.

Beetle	Species	Numbers	Total numbers
Trypoxylus dichotomus	*Acinetobacter* sp.	12	72
	Bacillus sp.	31	
	Chryseobacterium sp.	1	
	Citrobacter sp.	20	
	Lysinibacillus sp.	2	
	Kluyvera sp.	1	
	Neobacillus sp.	1	
	Streptomyces sp.	1	
	Staphylococcus sp.	3	
Alphitobius diaperinus	*Enterobacter* sp.	8	27
	Pseudomonas sp.	7	
	Sta. sp.	12	
Araecerus fasciculatus	*Bacillus* sp.	3	19
	Paenisporosarcina sp.	1	
	Sta. sp.	14	
	Sporosarcina sp.	1	

Table 2. The enzymatic activity of gut symbiotic bacteria from *Trypoxylus dichotomus*.

Species	Cellulase	Ligninase	Laccase	Tyrosinase
Acinetobacter baumannii A01	+[z]	+	-[y]	-
Bacillus sp.	+	-	-	-
B. subtilis BS01	+	+	+	-
B. megaterium BM01	+	+	+	+
B. aryabhattai BA01	+	+	+	+
B. cereus BC01	+	-	+	-
Chryseobacterium sp.	+	-	-	-
Citrobacter koseri	+	-	-	-
Lysinibacillus xylanilyticus	+	-	-	-
Kluyvera sp.	+	-	-	-
Neobacillus ginsengisoli N01	+	-	-	+
Streptomyces sp.	+	-	-	-

[z] +: Enzyme activity was detected.
[y] -: No enzyme activity was detected.

living in decaying woodpiles in natural soil habitats, which harbor a richer microbial community. When feeding on the decaying substrates within these woodpiles, various microbes are ingested and retained in the gut. Subsequently, we screened the gut microbiota of *Tr. dichotomus* larvae and identified 3 microbial strains, *B. megaterium* BM01, *B. aryabhattai* BA01, and *B. subtilis* BS01, which exhibited enzymes related to HA synthesis. The 3 strains showed the highest potential for accelerating the humification of rice straw. In the future, these strains could assist in on-site decomposition of leftover rice straw after harvest, converting it into HA. This could

Table 3. The enzymatic activity of gut symbiotic bacteria from *Alphitobius diaperinus*.

Species	Cellulase	Ligninase	Laccase	Tyrosinase
Enterobacter sp.	+[z]	-[y]	-	-
E. cloacae Bc01	+	-	-	-
Pseudomonas taiwanensis Bt01	+	-	-	-
Staphylococcus kloosii Bk01	+	-	-	-
Sta. edaphicus Be01	+	-	-	-

[z] +: Enzyme activity was detected.
[y] -: No enzyme activity was detected.

Table 4. The enzymatic activity of gut symbiotic bacteria from *Araecerus fasciculatus*.

Species	Cellulase	Ligninase	Laccase	Tyrosinase
Bacillus anthracis Ba01	+[z]	-[y]	-	-
B. licheniformis Bl01	+	-	-	-
Paenisporosarcina sp.	+	-	-	-
Staphylococcus sp.	+	-	-	-
Sporosarcina sp.	+	-	-	-

[z] +: Enzyme activity was detected.
[y] -: No enzyme activity was detected.

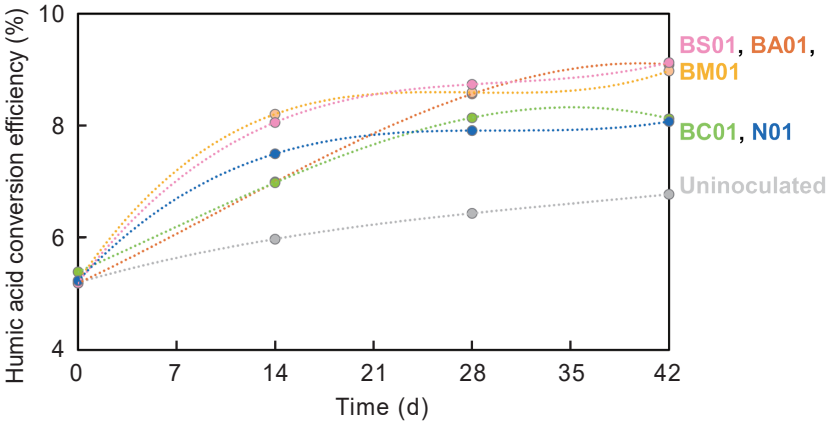

Fig. 5. The convert efficacy of humic acid from rice straw by *Bacillus subtilis* BS01, *B. megaterium* BM01, *B. aryabhattai* BA01, *B. cereus* BC01, and *Neobacillus ginsengisoli* N01.

enhance soil fertility and promote soil carbon sequestration.

The gut microbiota of insects are closely related to their dietary habits. In this study, we examined the gut microbiota of *Al. diaperinus* larvae obtained from grain warehouse and *Ar. fasciculatus* larvae found in decaying jackfruit fruit. Due to their simpler diets, these larvae exhibited less diverse gut microbiota. In contrast, *Tr. dichotomus* larvae, which inhabit environments with more complex food sources, showed a more diverse gut microbiota. The dominant bacterial families identified were Ruminococcaceae and Lachnospiraceae of the phylum Bacillota (formerly Firmicutes). This finding aligns with the research by Huang *et al.* (2022) on scarab larvae, where Bacillota was the most abundant phylum in *Tr. dichotomus*

larvae guts, associated with polysaccharide fermentation.

Additionally, the differences in insect gut microbiota are closely associated with the composition and sources of their diets (Huang *et al*. 2022; Mannaa *et al*. 2023). The richer gut microbiota observed in *Tr*. *dichotomus* compared to *Al*. *diaperinus* and *Ar*. *fasciculatus* larvae may stem from the decaying substrates or surrounding soil in their habitats. Previous research has shown that the cetoniid beetle larvae *Pachnoda ephippiata* selectively utilize HA peptides and polysaccharides from soil substrates and produce higher levels of HA in their feces, suggesting a connection with their gut microbiota (Li & Brune 2005).

The gut microbiota of *Oryctes rhinoceros* larvae, a species belonging to the Scarabaeidae family, have been found to contain bacteria capable of degrading hemicellulose and cellulose, predominantly belonging to the *Bacillus* genus (Sari *et al*. 2016). Similarly, our study identified 12 bacterial strains with cellulose-degrading capabilities in the gut of *Tr*. *dichotomus* larvae, a species also belonging to the Scarabaeidae family. This suggests that Scarabaeidae beetles have the potential to degrade plant residues through their gut microbiota.

Microbial redox enzymes such as tyrosinase and laccase play a role in organic matter degradation and humification (Binner *et al*. 2011). These enzymes are crucial in the synthesis of HS as they can degrade lignin-like polymers in organic matter and catalyze the cross-linking of amino acids, promoting the formation of humic polymer skeletal structures and ultimately aiding in carbon sequestration (Zavarzina *et al*. 2011).

Several microorganisms, including *Str.* sp., *Azospirillum lipoferum*, *B*. *aryabhattai*, *B*. *megaterium*, *Rhizobium* sp., *Thermomicrobium roseum*, and *Vibrio tyrosinaticus*, have been reported to exhibit tyrosinase activity (Pomerantz & Murthy 1974; Kong *et al*. 2000; Claus & Decker 2006; Piñero *et al*. 2007; Fairhead & Thöny-Meyer 2012; Kanteev *et al*. 2013; Guo *et al*. 2015). In our study, both *B*. *aryabhattai* BA01 and *B*. *megaterium* BM01 showed tyrosinase activity, and the tyrosinase activity of *N*. *ginsengisoli* N01 was reported for the first time.

Bacillus species are known for their ability to degrade lignocellulose and are often used in straw composting studies. For instance, inoculating straw with *B*. *siamensis* (H1), *B*. *halophilus* (H2), and *B*. *parahemolyticus* (S1) has been shown to increase the HA content (Zhao *et al*. 2024). In our study, we isolated 3 *Bacillus* strains with the highest HA conversion capabilities from the gut of *Tr*. *dichotomus* larvae: *B*. *megaterium* BM01, *B*. *aryabhattai* BA01, and *B*. *subtilis* BS01. Muniraj *et al*. (2021a, 2021b) found that *B*. *aryabhattai* TG5 exhibits laccase and tyrosinase activities. Treatment with its tyrosinase resulted in the formation of HS from coir pith within 3 d, confirming its role in HS formation. Moreover, *B*. *megaterium* AS019 has been shown to convert vinasse (sugarcane mill wastewater) into HA, helping to reduce pollution from vinasse disposal (Li *et al*. 2018).

Laccase enzymes are typically found in higher plants and fungi but have recently been discovered in some bacteria as well (Shraddha *et al*. 2011) such as *Str. cyaneus* (Arias *et al*. 2003), *Monocillium indicum* (Thakker *et al*. 1992), and *Marinomonas mediterranea* (Jimenez-Juarez *et al*. 2005). In this study, laccase activity was also observed in *B*. *subtilis* BS01, *B*. *megaterium* BM01, *B*. *aryabhattai* BA01, and *B*. *cereus* BC01.

Moreover, these 3 bacterial strains have been the focus of various agricultural studies. *B*. *subtilis* can act as a plant growth-promoting agent, aiding in soil phosphorus solubilization, enhancing nitrogen fixation, and producing siderophores to facilitate plant root growth (Hashem *et al*. 2019). Recent findings have shown that *B*. *subtilis* exhibits antagonistic effects against various pathogens, such as *Phytophthora capsici*, *Blumeria graminis* f. sp. *tritici*, and *Fusarium oxysporum* f. sp. *cucumerinum*, making it one of the most promising microbes for sustainable development (Lin *et al*. 2010; Cao *et al*. 2011; Xie *et al*. 2021).

B. *megaterium* has been found to suppress

the damage caused by rice root-knot nematode (*Meloidogyne graminicola*) and rice sheath blight (*Rhizoctonia solani*). Even after being formulated into granular preparations and stored for 2 years, it still demonstrated effective control against rice sheath blight (Padgham & Sikora 2007; Chumthong *et al*. 2008). In terms of promoting plant growth, *B*. *megaterium* possesses the ability to synthesize auxins and cytokinins and easily aggregates in the rhizosphere and soil, promoting plant growth under stress conditions (Nascimento *et al*. 2020).

B. *aryabhattai* is a phosphate-solubilizing bacterium that converts phosphate into a plant-available form, enhances nitrogen uptake, promotes plant growth, and increases crop yields (Ramesh *et al*. 2014; Wu *et al*. 2019). These findings broaden the potential applications for the 3 selected strains in this study, *B*. *megaterium* BM01, *B*. *aryabhattai* BA01, and *B*. *subtilis* BS01, particularly in enhancing carbon sequestration capabilities.

CONCLUSION

We explored the gut microbiota of 3 beetle species with different diets, confirming that diet influences the gut microbiota composition. With the aim of recycling agricultural waste and enhancing soil carbon sequestration, we screened for bacterial strains capable of transforming HA and verified their ability to convert straw into HA. Among them, *B*. *subtilis* BS01, *B*. *aryabhattai* BA01, and *B*. *megaterium* BM01 exhibited the highest potential for straw humification. These strains can accelerate the decomposition of agricultural waste in the field, facilitating the return of HS to the soil, increasing the residence time and stability of organic carbon in the soil, and enhancing soil carbon sequestration to promote carbon-negative farming practices. Furthermore, both *B*. *subtilis* and *B*. *megaterium* have been reported to suppress plant diseases, while *B*. *aryabhattai* has been found to promote crop growth; therefore, further expansion of the potential applications of these 3 bacterial strains is warranted in the future.

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Appendix. Partial mitochondrial cytochrome oxidase subunit 1 (COI) gene sequences of the three beetle larvae.

Trypoxylus dichotomus

CAGAATTAGGGAATCCAGGCTCTCTGATTGGAGATGATCAAATTTATAATGTAATTGTAACTGCCCATGCTTTTAT-
TATAATTTTCTTTATAGTAATACCTATTATAATTGGAGGATTTGGTAATTGACTTGTGCCTTTAATACTTGGGGCTC-
CAGATATAGCATTTCCCCGAATAAATAATATAAGATTTTGACTTTTACCCCCTTCTTTAACTCTTTTATTAG-
CAAGAAGAATAGTAGAAAATGGGGCTGGAACAGGATGAACAGTTTACCCTCCACTATCTGCTAATATTGCTCATAGTG-
GAGCATCTGTTGATTTAGCTATTTTTAGTCTTCATCTAGCAGGAATTTCATCTATTTTAGGGGCTGTAAATTTTATTACAA-
CAGTAATTAATATACGATCAACAGGCATAACTTTTGACCGAATACCTCTATTTGTATGATCAGTTATACTAACAGCCATCT-
TACTCCTTCTTTCATTGCCTGTATTAGCAGGGGCTATTACTATACTTCTTACTGACCGAAATATTAATACTTCATTTTTT-
GACCCTGCAGGTGGAGGAGACCCAATTCTATACCAACATCTCTTTTGATTTTTTG

Alphitobius diaperinus

TTGGTACTTCTCTAAGACTACTTATTCGAGCTGAACTTGGAAATCCTGGATCACTAATTGGGGATGACCAAATTTATA-
ACGTTATTGTTACAGCTCACGCATTCATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGGTTTGGAAACT-
GACTTGTTCCCCTTATACTTGGTGCCCCAGACATAGCATTCCCTCGAATAAACAATATAAGATTCTGACTTCTC-
CCACCCTCTTTAACCCTTCTTCTAATAAGAAGAATTGTAGAAAGAGGAGCAGGAACTGGATGAACAGTGTAC-
CCCCCACTCTCTTCCAATATTGCACACGGAGGGTCTTCTGTAGACCTAGCCATTTTCAGACTCCATTTAGCTGG-
TATTTCATCAATCCTCGGAGCTGTAAATTTTATCACAACAGTAATTAATATACGACCACAAGGAATAACTTTTGATC-
GAATACCTTTATTTGTATGAGCTGTCGTAATTACAGCAGTTCTTCTTCTTCTTTCACTCCCTGTTCTAGCAGGAGCAAT-
TACAATATTACTTACAGACCGAAATATTAATACCTCATTCTTTGACCCAGCTGGGGGAGGAGACCCAATTTTATATCAA-
CACTTATTTTGATTTTTTTGG

Araecerus fasciculatus

TTGAGCAGGAATAATAGGAACATCCTTAAGAATCTTAATTCGAACAGAATTAGGGAATCCCGGGTCTCTAATTGGAGAT-
GACCAAATCTATAACGTAATCGTAACTGCCCACGCATTCGTTATAATCTTTTTTATAGTAATGCCGACTATAATTGGAG-
GATTTGGGAATTGATTAGTTCCGTTAATACTTGGTGCTCCTGATATAGCATTCCCTCGACTTAACAATATGAGATTTT-
GATTGCTGCCCCCTTCCTTGTCTCTATTGACCTTAAGAAGAATCGTTGAAAGAGGTGCGGGGACTGGTTGAA-
CAGTTTACCCCCCACTATCAGCAAATATTGCCCATAATGGATCTTCTGTTGACTTGGCTATCTTTAGTCTTCATTTAGCAG-
GGGTATCTTCTATTTTAGGAGCAGTTAACTTTATTACTACGATTATCAACATGCGTCCGAGAGGTATATCCCCTGATC-
GTTTACCCCTCTTTGTCTGATCTGTCGGAATCACCGCCCTTCTTCTTTTATTATCTTTACCTGTACTTGCTGGAGCAAT-
TACGATACTCTTAACTGATCGAAATCTAAACACTTCTTTTTTTGACCCCGCAGGAGGAGGTGATCCAATTCTCTACCAA-
CATTTATTTTGATTTTTT

三種甲蟲幼蟲腸道菌相與菌種轉化腐植酸潛力

王泰權[1] 葉千榕[2] 林祐丞[2] 林柏文[3] 姚美吉[4] 張淑貞[4,*]

摘要

王泰權、葉千榕、林祐丞、林柏文、姚美吉、張淑貞。2024。三種甲蟲幼蟲腸道菌相與菌種轉化腐植酸潛力。台灣農業研究 73(3):181–196。

隨著全球氣溫攀升，移除大氣中的 CO_2 並長期封存已是全球的共同目標。農業廢棄物則可藉由微生物轉化為在土壤中不易分解的腐植質 (humic substances; HS)，以達到增加土壤碳匯的目標。本研究分析 3 種不同食性的甲蟲幼蟲腸道微生物相，其中以取食腐朽木材的獨角仙 (*Trypoxylus dichotomus*) 幼蟲腸道菌相較外米擬步行蟲 (*Alphitobius diaperinus*) 及長角象鼻蟲 (*Araecerus fasciculatus*) 的幼蟲腸道菌相較豐富、菌種數量亦較多。進一步分析 3 種甲蟲幼蟲腸道菌的腐植酸生合成相關酵素的活性，包括纖維素酶、木質素酶、漆酶及酪胺酸酶，其中 *Bacillus megaterium* BM01 與 *B. aryabhattai* BA01 可見上述 4 種酵素活性，*B. subtilis* BS01 則有前 3 種酵素活性。進一步檢驗菌株將稻草轉化為腐植酸的能力，其中 *B. megaterium* BM01、*B. aryabhattai* BA01 及 *B. subtilis* BS01，腐植酸轉換效率較未接菌種的對照組，各增加 2.4%、2.3% 及 2.1%。期待未來可應用於稻穀收穫後，田間殘存稻草的現地轉化分解成腐植酸，以達到增加土壤碳匯的目標。

關鍵詞：甲蟲、腸道菌、腐植酸、土壤碳匯。

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