



Nitrogen Fixation and Diazotrophic Community in Plastic-Eating Mealworms *Tenebrio molitor* L

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Abstract

Mealworms, the larvae of a coleopteran insect *Tenebrio molitor* L., are capable of eating, living on, and degrading non-hydrolyzable vinyl plastics as sole diet. However, vinyl plastics are carbon-rich but nitrogen-deficient. It remains puzzling how plastic-eating mealworms overcome the nutritional obstacle of nitrogen limitation. Here, we provide the evidence for nitrogen fixation activity within plastic-eating mealworms. Acetylene reduction assays illustrate that the nitrogen-fixing activity ranges from 12.3 ± 0.7 to 32.9 ± 9.3 nmol ethylene·h⁻¹·gut⁻¹ and the corresponding fixed nitrogen equivalents of protein are estimated as 8.6 to 23.0 µg per day per mealworm. Nature nitrogen isotopic analyses of plastic-eating mealworms provide further evidence for the assimilation of fixed nitrogen as a new nitrogen source. Eliminating the gut microbial microbiota with antibiotics impairs the mealworm's ability to fix nitrogen from the atmosphere, indicating the contribution of gut microbiota to nitrogen fixation. By using the traditional culture-dependent technique, PCR and RT-PCR of *nifH* gene, nitrogen-fixing bacteria diversity within the gut was detected, and the genus *Klebsiella* was demonstrated to be an important nitrogen-fixing symbiont. These findings first build the relationship between plastic degradation (carbon metabolism) and nitrogen fixation (nitrogen metabolism) within mealworms. Combined with previously reported plastic-degrading capability and nitrogen-fixing activity, mealworms may be potential candidates for up-recycling of plastic waste to produce protein sources.

Keywords Nitrogen fixation · Plastic waste · Biodegradation · Mealworm · *Klebsiella*

Introduction

An increasing accumulation of non-hydrolyzable vinyl plastic wastes, such as polystyrene (PS), polyethylene (PE), polypropylene (PP), and polyvinyl chloride (PVC), has become a severe environmental and social concern [43, 56]. In 2015, mealworm, the larva of a coleopteran insect *Tenebrio molitor* L., was first reported to be able to eat PS foam (Styrofoam) as sole diet and lived as well as those fed with a normal food (wheat bran) over a period of 1 month

[57, 58]. Ingested Styrofoam was demonstrated to be efficiently degraded after passage through the larval gut within a retention time of less than 24 h and further mineralized to CO₂ and incorporated into lipids [57]. Subsequently, the ability of mealworm to live on and degrade Styrofoam was ubiquitously confirmed by expanding assessments from 22 countries [55]. Additionally, multiple recent studies showed that mealworm was also capable of surviving by eating other synthetic vinyl plastics, such as PE, PVC, PP, and styrene-butadiene rubber (SBR), as sole diet, and rapidly degrading these ingested vinyl plastics within the larval gut [1, 7, 37, 54, 56]. These findings have provided implications for future applications of mealworms in plastic waste management. However, there is a controversy that vinyl plastics of PS, PE, PVC, PP, and SBR are carbon-rich but nitrogen-deficient diet, which could not meet the nitrogen requirements for the biosynthesis of proteins, nucleic acids, and other metabolites within mealworms. Therefore, it is intriguing to know how mealworms overcome the nutritional obstacle of nitrogen limitation when they are fed with vinyl plastic as sole diet.

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Many insects, like termite, shipworm, stag beetle, and red turpentine beetle, intrinsically feed on nitrogen-deficient wood diet [4]. These xylophagous insects have been extensively demonstrated to be capable of supplementing their diets by fixing nitrogen from atmosphere based on the acetylene reduction assays or nitrogen isotopic tracers [3, 5, 6, 8, 11, 23, 27, 36, 40, 44, 47, 53]. The nitrogen-fixing activity of xylophagous insects could be abolished by feeding with antibiotics, indicating that gut bacteria may contribute to nitrogen fixation [6, 8, 11]. These findings promote a search for insect gut bacteria that are involved in nitrogen fixation [22, 30, 35]. By applying the traditional culture-dependent technique of growing the bacteria on a nitrogen-free medium, a few cultures of nitrogen-fixing bacteria, identified as the species from the genera of *Azotobacter*, *Spirochaeta*, *Citrobacter*, *Enterobacter*, *Treponema*, *Klebsiella*, *Beijerinckia*, *Clostridium*, and *Raoultella*, have been isolated from the guts of various nitrogen-fixing insects [5, 9, 11, 15, 18, 20, 26, 28, 39, 42, 50]. Nevertheless, the culture-dependent method may detect only a limited number of diazotrophic species because of the inability to culture most of environmental isolates. As the gene *nifH* encoding dinitrogenase reductase is genetically conserved [59], the culture-independent molecular techniques based on the amplification of *nifH* gene has been used to detect an unexpected diversity of nitrogen-fixing bacteria within guts of these nitrogen-fixing insects [3, 12, 14, 16, 33, 34, 52]. However, the successful amplifications of *nifH* gene do not always indicate that the encoding dinitrogenase reductase is actually expressed by the corresponding bacteria, because the expressions of *nifH* genes are regulated at the transcriptional and post-transcriptional level [59]. Thus, detection of mRNA by a reverse transcription polymerase chain reaction (RT-PCR) is usually used to further support the expression of *nifH* genes and determine the actual activity of specific gut microbes in nitrogen fixation [3, 12, 14, 31, 32].

Like xylophagous insects, mealworm's gut also provides an ecological niche for the resident of a complex mixed microbial community [51, 58]. The composition of gut bacterial community within mealworms from different sources has been investigated using both culture-dependent and culture-independent methods [21, 25, 41, 45, 48, 49, 51, 58]. The culture-independent pyrosequencing-based analyses show that *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Tenericutes* are the dominant phylum [21, 25, 41, 45, 48, 49]. The detected species belong to phylum *Proteobacteria* or *Firmicutes*, mainly *Citrobacter* spp., *Klebsiella* spp., *Enterobacter* spp., *Pantoea* spp., *Pseudomonas* spp., or *Clostridium* spp., are known potential nitrogen-fixing bacteria [4]. These available data provide circumstantial evidence that nitrogen fixation may occur in mealworms.

However, the direct evidence for the nitrogen-fixing activity of vinyl plastic-eating mealworm and the involvement of gut nitrogen-fixing bacteria is still lacking.

Therefore, the specific aims of this study are to (i) determine whether the gut bacteria-mediated nitrogen fixation takes place within mealworms when they were fed with Styrofoam as sole diet by using the acetylene reduction assays and stable nitrogen isotope composition analyses, (ii) to uncover the diazotrophic diversity of the gut microbial symbiont by culture-dependent technique and culture-independent amplification of *nifH* genes, and (iii) to illustrate whether the *nifH* genes encoding dinitrogenase reductase are actually expressed by gut nitrogen-fixing bacteria. The overall aim is to extend our present understanding that plastic-eating mealworms can adapt the nitrogen-deficient plastic diet by employing the nitrogen-fixing capacity of gut microbiota.

Materials and Methods

Collection and Culture of Mealworms

Mealworms (growth age at approximately 3–4 instars) were purchased from six different insect-breeding plants in northern (Beijing), eastern (Shanghai and Ningbo), western (Chengdu), southern (Guangzhou), and central (Luoyang) region of China, respectively. The distributions of body weight and size of the sampled mealworms are shown in SI Tables S1. The conventional diet for these sampled mealworms was bran.

Sampled mealworms from each source were divided into two groups. One group (denoted as normal group, $n=3$) were reared with Styrofoam blocks as a sole diet for 28 days. Other group (labeled as Antibiotic group, $n=3$) were first fed with the antibiotic diet (60 mg gentamicin per 1 g bran) until the number of active gut bacteria became approximately zero. The number of active gut bacteria was detected by the series dilution method of plate counting on solid nitrogen-free plates (1.5% agar) [10]. These antibiotic-treated mealworms were subsequently fed with Styrofoam for 28 days. Both of "normal" or "antibiotic" groups were used for further investigations.

Acetylene Reduction Assays

Ten mealworms were randomly selected from each group and were quickly surface sterilized by immersion in 75% ethanol for 1 min and then rinsed 2 times with sterile phosphate saline buffer (PBS) prior to gut excision. Their guts were sterilely drawn out and pooled into a Hungate tube containing 5 mL of sterile PBS buffer. Then, Hungate tubes were stoppered with rubber plugs, and the inside atmosphere was exchanged with argon gas (Ar). Acetylene was

injected into the Hungate tubes to a final concentration of 20% (v/v) by replacement of an identical volume of argon gas. The Hungate tubes without guts but with acetylene gas were used as blank controls. The tubes for each group of mealworms and controls were prepared in triplicate. After 5 h of incubation at 30 °C, to determine the reduction of acetylene to ethylene, 0.5 mL of gas sampled from the top of the tubes was analyzed by gas chromatography (GC, PANNA 91A, Changzhou, China) equipped with a flame ionization detector (FID) and HP-PLOT Al₂O₃ S column (25 m × 0.32 mm × 8 µm, Agilent, USA). The operating temperature for inlet, column oven, and FID was 150 °C, 100 °C and 280 °C, respectively. The gas flow rate for N₂, H₂, and air was set as 20 mL·min⁻¹, 40 mL·min⁻¹, and 450 mL·min⁻¹, respectively. Purified ethylene was used as the standard for the quantitative analysis. To estimate the amount of fixed nitrogen, the molar ratio of ethylene to nitrogen was defined as 4:1, and the average working time for nitrogen fixation by bacteria was assumed to be 16 h per day [5]. The fixed nitrogen equivalent of protein was calculated based on a value of 16% nitrogen in protein [5].

Stable Nitrogen Isotope Composition Measurements

Ten mealworms were randomly selected from each group and lyophilized, ground to a homogeneous powder. The powder (about 1.0 mg) was packed in tin capsules and combusted to CO₂ and N₂ at 1000 °C in an on-line combustor. Subsequently, N₂ was separated in a He carrier stream on a Carbosieve G column and further introduced to an isotope ratio mass spectrometer (Finnigan MAT 253, Thermo Electron, Waltham, MA). Nitrogen isotope compositions were reported as δ¹⁵N values relative to atmospheric nitrogen (a pure N₂ gas reference, 0.00‰). The system was calibrated using multiple runs of a powdered working standard (ammonium sulfate and sucrose mixture with δ¹⁵N versus air of 1.33‰), and the detection method was periodically calibrated against international isotope standards (IAEA N1 and N3).

Isolation and Characterization of Nitrogen-Fixing Bacteria

Thirty mealworms from each group were randomly collected and quickly surface sterilized by immersion in 75% ethanol for 1 min and then rinsed twice with sterile PBS. Their guts were drawn out and pooled in a 10-mL centrifuge tube containing 5 mL sterile PBS. The solution was roiled on a vortex mixer for 5 min in order to adequately release the microbial contents from the guts. After that, the clearing gut tissues were carefully picked out with a pipette, while the solution containing gut contents was used as gut microbial inoculum, which was tenfold

diluted and inoculated on the plates containing semi-solid nitrogen-free media (0.5% agar) [10]. These inoculated plates were incubated for 5 days at 30 °C. Individual colonies were randomly picked and streaked repeatedly on Luria–Bertani (LB) agar to obtain pure cultures. Genomic DNA extraction, PCR amplification, sequencing, and phylogenetic analysis of the 16S rRNA gene from these pure cultures were carried out according to the previously described method [51].

For acetylene reduction assays, pure cultures were inoculated in LB media and incubated in agitation at 30 °C for 24 h, with a well-known nitrogen-fixing bacterium *Azospirillum brasilense* was used as positive control [38]. The LB cultures were centrifuged at 4 °C, 12,000 rpm for 5 min, and then the bacterial pellets were collected and washed 3 times and re-suspended with sterile PBS. The OD₆₀₀ values of all bacterial suspensions were determined and adjusted to the same value (OD₆₀₀ = 1.0, 10× dilution). Then, 100 µL of each suspension was inoculated in 25 mL test tubes containing 15 mL of semi-solid nitrogen-free media. The test tubes stoppered with cotton plugs were incubated for 72 h at 30 °C. Subsequently, the cotton plugs of test tubes were replaced with rubber plugs, and 1 mL of acetylene was injected into the tubes. Ethylene production was measured periodically by GC as described above.

PCR amplifications of the nitrogenase (*nifH*) gene (about 360 bp) from the genomic DNA of the nitrogen-fixing isolates were performed using a two-stage nest PCR procedure with a pair of universal primers (SI Table S2). Genomic DNA extraction of the pure cultures were carried out according to the previously described method [51]. In the first round, the genomic DNA extract (1 µL, 300 ng·µL⁻¹), 1 µL of forward *nifFo* and 1 µL of reverse *nifRe* primer, was added to 11 µL of the PCR mixtures (2×M5 Taq HiFi PCR mix, Mei5 Biotechnology, Beijing). The final volume of PCR mixtures was 20 µL by adding double-distilled H₂O (ddH₂O). PCR was carried out with one denaturation step of 5 min at 95 °C, 40 cycles of 94 °C for 11 s, 92 °C for 15 s, 54 °C for 8 s, 56 °C for 30 s, 74 °C for 10 s, and 72 °C for 10 s and a final extension step of 4 min at 72 °C. In the second round, the amplification products of the first round (1 µL, 300 ng·µL⁻¹), 1 µL of forward *polF* and 1 µL of reverse *polR* primer, were added to 11 µL of the PCR mixtures (2×M5 Taq HiFi PCR mix, Mei5 Biotechnology, Beijing). The final volume of PCR mixtures was 20 µL by adding double-distilled H₂O (ddH₂O). PCR was carried out with one denaturation step of 5 min at 95 °C, 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 30 s and a final extension step of 4 min at 72 °C. The final amplification products were analyzed by electrophoresis in 1.5% (w/v) agarose gels in 1×TAE buffer. PCR products of the expected size were gel-purified using a GeneJET Gel Extraction Kit (Thermo Scientific, USA). Nucleotide sequencing was done

using ABI 3730XL genetic analyzer (Applied Biosystems, CA, USA).

DNA Extraction, PCR Amplification, Cloning, and Sequencing of *nifH* Genes

Fifty active mealworms were randomly picked out from each group and were surface sterilized with the same method described above. Their guts were drawn out and pooled into a 50 mL centrifuge tube containing 25 mL sterile PBS on ice, respectively. After being roiled on a vortex mixer for 5 min, the microbial contents were released from the guts, and the empty guts were carefully removed from the tube by using a pipette. The solution containing gut microbial contents was further centrifuged at 4 °C, 12,000 rpm for 5 min. The supernatants were discarded, and the precipitates were used for the DNA extraction by using the TIANamp Stool DNA Kit (DP 328, TIANGEN, Beijing, China) according to the manufacturer's instructions. The average concentration of yielded genomic DNA was determined by NanoDrop One Microvolume UV–Vis Spectrophotometer (Thermo Scientific, USA).

An internal fragment of the *nifH* gene (about 360 bp) was amplified with using each set of previously reported primer pairs (Table S2). The genomic DNA extract (1 µL, 300 ng·µL⁻¹), 1 µL of forward and 1 µL of reverse primer, was added to 11 µL of the PCR mixtures (2×M5 Taq HiFi PCR mix, Mei5 Biotechnology, Beijing). The final volume of PCR mixtures was 20 µL by adding double-distilled H₂O (ddH₂O). Negative controls without DNA were also included to test for contamination. PCR was carried out with one denaturation step of 8 min at 95 °C, 35 cycles of 94 °C for 0.5 min, 47 °C for 0.5 min, and 72 °C for 1 min and a final extension step of 7 min at 72 °C. Amplification products were analyzed by electrophoresis in 1.5% (w/v) agarose gels in 1×TAE buffer. PCR products of the expected size were gel-purified using a GeneJET Gel Extraction Kit (Thermo Scientific, USA). PCR products were ligated into pJET1.2/blunt cloning vector and transformed into *Escherichia coli* XL-10-Gold cells using the CloneJET PCR Cloning Kit (Thermo Scientific, USA). Plasmid DNA was isolated from individual clones and purified using TIANprep Mini Plasmid Kit (DP103, Tiangen, Beijing, China). Nucleotide sequencing was done using ABI 3730XL genetic analyzer (Applied Biosystems, CA, USA).

RNA Extraction and RT-PCR

Gut microbial contents were prepared with the same method as described above for DNA extraction. Total RNA was extracted from gut contents by using TRIzol™ Reagent (Invitrogen, USA). RNA samples were purified using RapidOut DNA Removal Kit (Thermo Scientific, USA) to

remove DNA. Reverse transcription reactions were performed using GoScript™ Reverse Transcription System (Promega, USA). After reverse transcription, *nifH* genes were amplified in cDNA samples using PCR experimental conditions as described in the previous section. Each set of previously reported primer pairs was tested in the PCR reaction (Table S2). RNA samples without reverse transcription step were used as controls to check for residual DNA in the RNA preparations. RT-PCR and PCR negative controls were also included to test for reagent contamination. The generation of clone libraries and sequencing of RT-PCR amplified *nifH* products were performed by the same method described in the above section.

Phylogenetic Analysis of *nifH* Clones

All detected *nifH* gene nucleotide sequences were translated into amino acid sequences by using ExPASy bioinformatics resource tool [19]. Amino acid sequence with at least one amino acid change was considered as a unique operational taxonomic units (OTUs). The closest phylogenetic neighbors were obtained by aligning the representative amino acid sequence of each OTU against NCBI database using protein Basic Local Assignment Search Tool (BLASTp) [2]. Sequence alignment of all amino acid sequences was performed by using clustal×2.0 software [24]. Aligned sequences were used to reconstruct the maximum-likelihood (ML) phylogenetic trees using the program MEGA 6.0 [17, 46], with bootstrap values based on 1000 replications.

Re-infection Treatment

For the re-infection treatment, the nitrogen-fixing isolate was grown in liquid nutrient broth (NB) medium for 12 h, respectively. The cells were collected via centrifugation (10,000 rpm) and rinsed with sterile salt water to remove the residual medium. Next, all collected cells were re-suspended in sterile salt water to obtain a mixed cell suspension of approximately 10⁸ cells per mL. New sterile bran diet was prepared and supplemented with bacterial cell suspension with a ratio of 1:1 (w/v) and then was dried at 35 °C for 1 h. Antibiotic-treated mealworms were fed with the bacteria-inoculated diet for 10 days. Then, the re-infected mealworms as well as normal and antibiotic-treated mealworms (300 as a group, *n* = 3) were fed with Styrofoam blocks as sole diet for 16 days. The survival rate (SR) and the average dry body weight (ADBW) of each group were determined.

Statistical Analysis

Statistical analyses were performed in Excel (version 2016). To assess differences in acetylene-reducing activity and δ¹⁵N values between three groups (normal, antibiotic,

and bran-feeding mealworms) and six different populations (Beijing, Shanghai, Ningbo, Chengdu, Guangzhou, and Luoyang), a two-way ANOVA was performed. The *t* test with Tukey's correction was used to assess differences in acetylene-reducing activity between nitrogen-fixing bacteria and differences in SR and ADBW between three groups (normal, antibiotic, and re-infected mealworms).

Results

Nitrogen-Fixing Activity of Styrofoam-Eating Mealworm

We used acetylene reduction assay to examine the nitrogen-fixing activity of mealworm, because both acetylene and nitrogen are unsaturated compounds with covalent triple bonds and the conversion of acetylene to ethylene has been widely used as an indicator of nitrogen-fixing activity for both terrestrial and aquatic systems [29]. As shown in Fig. 1, ethylene production from acetylene was detected by Styrofoam-eating mealworms (normal group) from six different sources, with the rate from 12.3 ± 0.7 to 32.9 ± 9.3 nmol ethylene·h⁻¹·gut⁻¹. The acetylene-reducing (nitrogen-fixing) activity of Styrofoam-eating mealworm was similar to that of individual field-collected Mediterranean fruit fly (18.0 ± 1.5 nmol ethylene·h⁻¹·gut⁻¹) [5] but was one or two orders of magnitude higher than that of termite (1.25 ± 0.37 nmol acetylene·h⁻¹·gut⁻¹) and stag beetle (0.03 nmol acetylene·h⁻¹·gut⁻¹) [8, 23]. When mealworms were fed with antibiotic diet (60 mg gentamicin per g bran), all detectable acetylene-reducing (nitrogen-fixing)

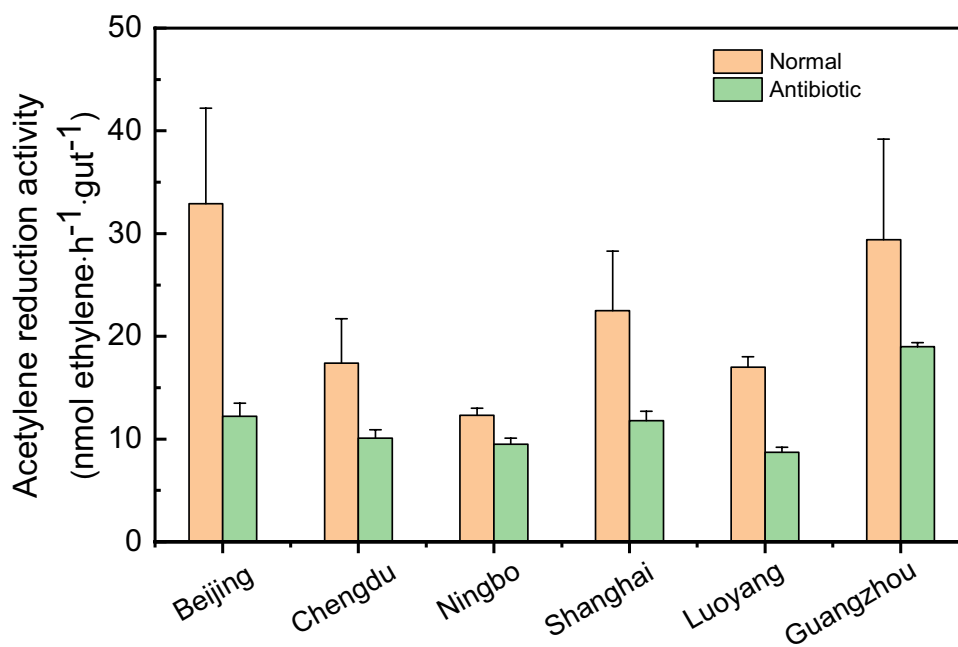
activity decreased to the range of 8.7 ± 0.5 to 19.0 ± 0.4 nmol ethylene·h⁻¹·gut⁻¹ (Fig. 1), correlating with the suppression of gut bacterial numbers detected by the serial dilution method of plate counting (SI Fig. S1). Statistical analyses with a two-way ANOVA (SI Table S3–S4) showed that the effect of antibiotic treatment was significant ($F = 17.05 > F_{crit}$, $p = 0.009 < 0.05$), while the effect of tested mealworms population was non-significant ($F = 3.33 < F_{crit}$, $p = 0.11 > 0.05$) on the acetylene-reducing (nitrogen-fixing) activity. These results indicated that nitrogen fixation really occurred in Styrofoam-eating mealworms and nitrogen-fixing bacteria were present within their guts.

Moreover, we also determined the acetylene-reducing (nitrogen-fixing) activity of mealworms fed with the conventional diet of bran (rich in carbohydrates and proteins). The bran-feeding mealworms had an acetylene-reducing (nitrogen-fixing) activity of 8.1 ± 2.1 to 12.2 ± 0.3 nmol ethylene·h⁻¹·gut⁻¹, which was significantly lower than that of Styrofoam-eating mealworms (two-way ANOVA, $F = 13.76 > F_{crit}$, $p = 0.014 < 0.05$) (SI Table S5–S6), agreeing with the previously known phenomenon that the available nitrogen in the diet would repress the nitrogenase synthesis of nitrogen-fixing systems [8].

Assimilation of Fixed Nitrogen into Styrofoam-Eating Mealworm's Tissues

According to the acetylene-reducing rate, the fixed nitrogen equivalents of protein were estimated as 8.6 to 23.0 µg per day per Styrofoam-eating mealworm using the previously reported method [5]. The investigation of natural stable nitrogen isotope compositions in organisms also can provide

Fig. 1 Nitrogen fixation activity measured by acetylene reduction assays for normal Styrofoam-eating mealworms or antibiotic-treated mealworms from six different sources. All results are shown as means \pm standard deviation (SD) ($n = 3$ samples, 10 worms as a sample). Statistical analyses using a two-way ANOVA were performed to evaluate the effects of the tested mealworm population and antibiotic treatment on the acetylene-reducing activities of mealworms (Table S3–S4). The results showed a significant main effect for antibiotic treatment ($F = 17.05 > F_{crit}$, $p = 0.009 < 0.05$) but not for the tested mealworm population ($F = 3.33 < F_{crit}$, $p = 0.11 > 0.05$)



further evidence for the incorporation of fixed nitrogen as a source of new nitrogen, because the $\delta^{15}\text{N}$ value of atmospheric N_2 is 0‰ and nitrogen-fixing organisms often have $\delta^{15}\text{N}$ close to 0‰ [47]. Thus, we analyzed the $\delta^{15}\text{N}$ values of Styrofoam-eating mealworms, antibiotic-treated mealworms, and bran-feeding mealworms that were sampled from six different source.

As seen in Fig. 2, all Styrofoam-eating mealworms had $\delta^{15}\text{N}$ values between 1.7 and 1.9‰, which were significantly lower than that of antibiotic-treated mealworms (2.3–2.4‰) (two-way ANOVA, $F = 160.0 > F_{\text{crit}}$, $p = 0.00005 < 0.05$) (SI Table S7–S8). Moreover, the $\delta^{15}\text{N}$ values of bran-feeding mealworms were also analyzed and found to be in the range of 1.9 to 2.1‰, which were significantly higher than that of Styrofoam-eating mealworm (two-way ANOVA, $F = 19.62 > F_{\text{crit}}$, $p = 0.0068 < 0.05$) (SI Table S9–S10). These results were consistent with the decreased acetylene-reducing (nitrogen-fixing) activities within antibiotic-treated and bran-feeding mealworms in comparison to Styrofoam-eating mealworms. The low $\delta^{15}\text{N}$ values of Styrofoam-eating mealworms could be attributed to the presence of gut nitrogen-fixing bacteria that have fix more nitrogen from atmosphere nitrogen and provided a substantial new nitrogen source for the mealworm's metabolism.

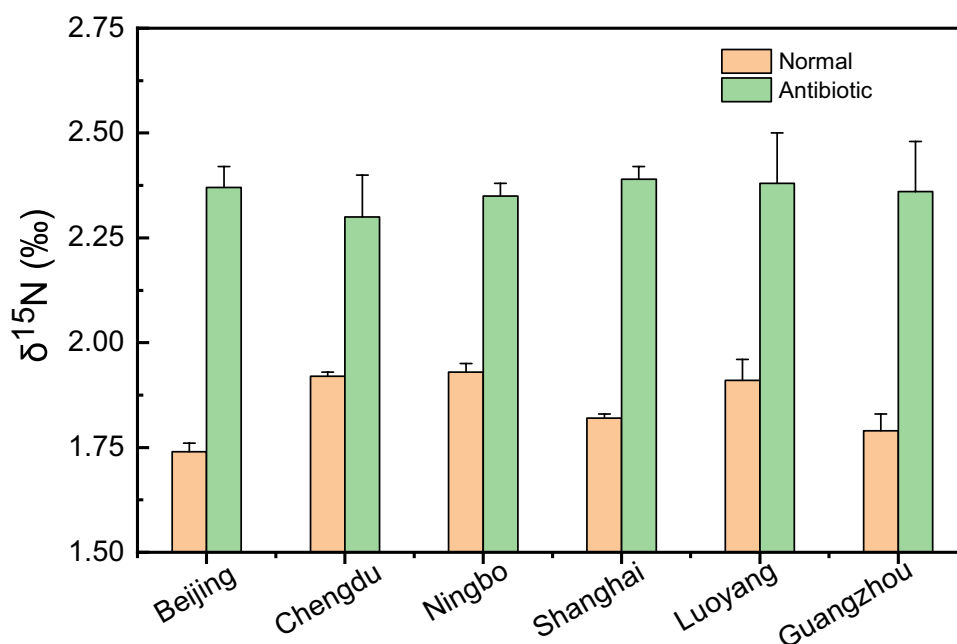
Nitrogen-Fixing Bacterial Cultures Isolated from Mealworm's Gut

To assess the level of potential nitrogen-fixing bacteria in gut microbiota of Styrofoam-eating mealworms, gut contents from Styrofoam-eating mealworms were serially diluted and

inoculated in a semi-solid nitrogen-free enrichment medium for the growth of nitrogen-fixing bacteria. The typical colonies of nitrogen-fixing bacteria came out on all plates inoculated with gut contents of Styrofoam-eating mealworms from six different sources (SI Fig. S1). The number of potential nitrogen-fixing bacteria was estimated at 5.5×10^4 CFU per gut. In contrast, the size of the total cultivable fraction on the a general LB medium, inoculated with the same dilution series, was estimated to be 5.0×10^6 CFU per gut [58]. Therefore, the level of functional nitrogen fixers was estimated at about 1.1% of the total bacterial population within the gut of Styrofoam-eating mealworm.

To identify the specific gut microbes responsible to nitrogen fixation, we conducted extensive streaking isolations using nitrogen-free medium. Colonies isolated from the nitrogen-free plates were identified by amplification and sequencing of the almost full-length 16S rRNA gene. About 110 pure colonies were obtained, but some were duplicates based on their similar colony morphologies and identical 16S rRNA sequences. After removing duplicates, there were only 24 distinct isolates, and their acetylene-reducing abilities were determined. As shown in Fig. 3a, four isolates, stains BIT-1, BIT-56, BIT-96, and BIT-105, were able to reduce acetylene into ethylene. In comparison to a positive control of a known free-living diazotrophs *Azospirillum brasilense* ATCC 29,145, stain BIT-96 and BIT-56 showed a comparable nitrogen-fixing rate (t test, $p > 0.05$), while stains BIT-1 and BIT-105 had a relative lower rate (t test, $p < 0.05$). To further ascertain the nitrogen-fixing ability of these isolates, PCR amplifications of *nifH* gene from the genomic DNA of these isolates were performed using a

Fig. 2 $\delta^{15}\text{N}$ values [N isotopic ratio, per mil (‰)] of normal Styrofoam-eating mealworms and antibiotic-treated mealworms from six different sources. All results are shown as mean \pm standard deviation (SD) ($n = 3$ samples, 10 worms as a sample). Statistical analyses using a two-way ANOVA were performed to evaluate the effects of the tested mealworm population and antibiotic treatment on the $\delta^{15}\text{N}$ values of mealworms (Table S7–S8). The results showed a significant main effect for antibiotic treatment ($F = 160.0 > F_{\text{crit}}$, $p = 0.00005 < 0.05$) but not for the tested mealworm population ($F = 0.52 < F_{\text{crit}}$, $p = 0.76 > 0.05$)



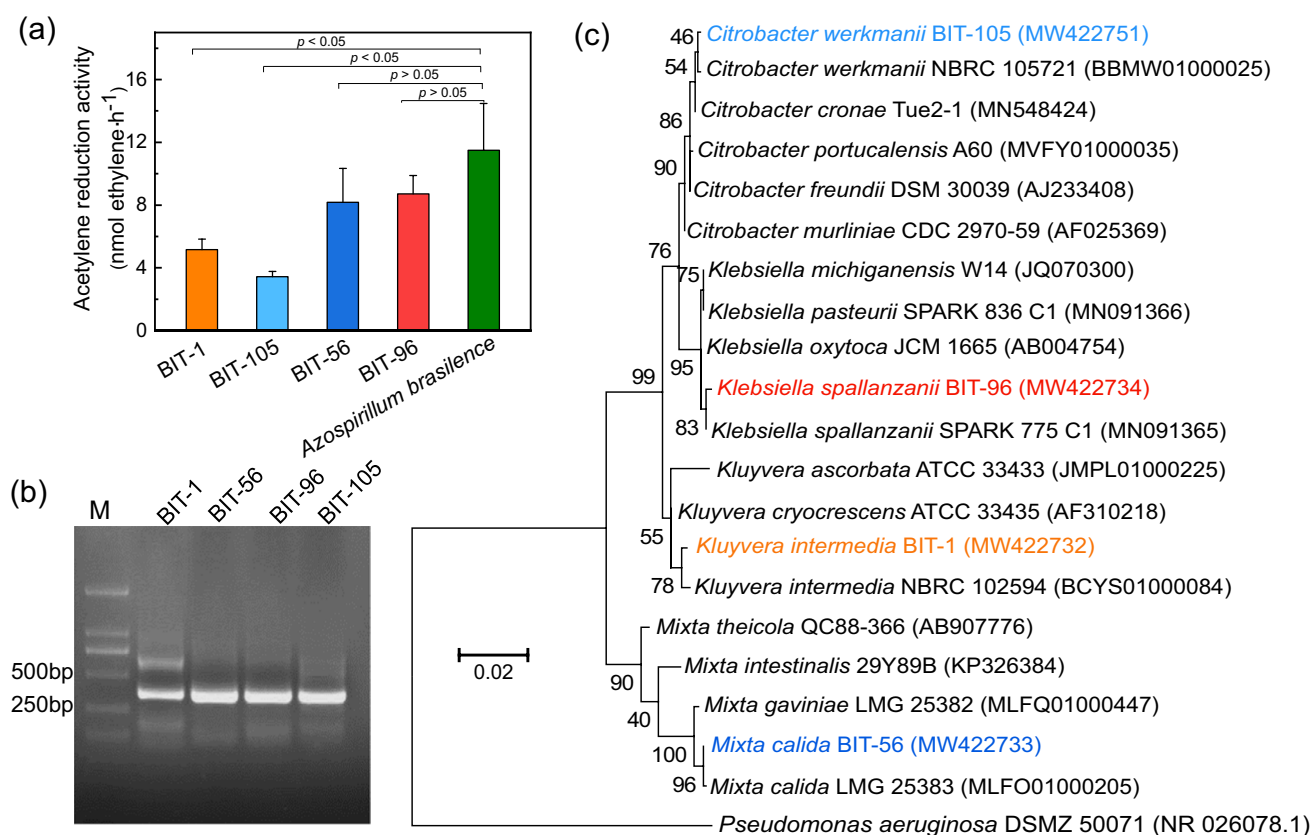


Fig. 3 Nitrogen-fixing bacteria isolated from the mealworm's gut. **a** Nitrogen-fixing activity measured by acetylene reduction assays for bacterial isolates obtained from the gut of mealworms. *Azospirillum brasilense* ATCC 29,145, a known free-living nitrogen-fixing bacterium, was used as a positive control. Results are shown as mean \pm standard deviation (SD) ($n=3$). The *t* test with Tukey's correction was used to assess differences in acetylene-reducing activity

between nitrogen-fixing bacteria. **b** PCR products of *nifH* gene from the genomic DNA of potential nitrogen-fixing gut isolates. **c** The 16S rRNA gene-based maximum-likelihood (ML) phylogenetic tree of nitrogen-fixing bacteria isolated from the gut of plastic-eating mealworms. The 16S rRNA sequence of *Pseudomonas aeruginosa* DSMZ 50,071 was used as outgroup. Scale bar indicates 2% estimated sequence divergence

two-stage nest PCR procedure. The results revealed that the presence of *nifH* amplicon of 360 bp nucleotides was found in all four isolates, strongly suggesting that these four gut isolates are true nitrogen fixers (Fig. 3b).

Phylogenetic analyses based on 16S rRNA (Fig. 3c) showed that these four isolates belonged to the genera of *Klebsiella*, *Mixta*, *Kluyvera*, and *Citrobacter* within the order of *Enterobacteriales*, with the highest similarities to *Kluyvera intermedia* NBRC 102,594 (99.52%), *Mixta calida* LMG 25,383 (99.93%), *Klebsiella spallanzanii* SPARK 775 C1 (99.36%), and *Citrobacter werkmanii* NBRC 105,721 (99.78%), respectively (SI Table S11). Since their highest 16S rRNA similarities were lower than the generally accepted species-level boundary of 98.7% [13], these four isolates could be identified as *K. intermedia* BIT-1, *M. calida* BIT-56, *K. spallanzanii* BIT-96, and *C. werkmanii* BIT-105, respectively. Given the noticeable nitrogen fixation rates measured for these four isolates and their prevalence in the guts of Styrofoam-eating mealworms from six different

sources, we can therefore deduce that the gut species within the order of *Enterobacteriales* are the important nitrogen-fixing symbiont of Styrofoam-eating mealworms.

***nifH* Genes Diversity in Styrofoam-Eating Mealworm's Gut**

To further determine whether more uncultivable nitrogen-fixing bacteria resided in the Styrofoam-eating mealworm's gut, *nifH* genes were amplified from the DNA extracted from gut contents. We have tested four previously reported degenerate primer pairs (SI Table S2) and found that only the primer pairs PolF-PolR could successfully produce the PCR products (about 360 bp) from all DNA samples of gut contents (Fig. 4a). Nevertheless, these results revealed the presence of nitrogen-fixing bacteria within the gut microbiota of Styrofoam-eating mealworms. These PCR products were then cloned and sequenced to analyze their *nifH* identity. In total, more

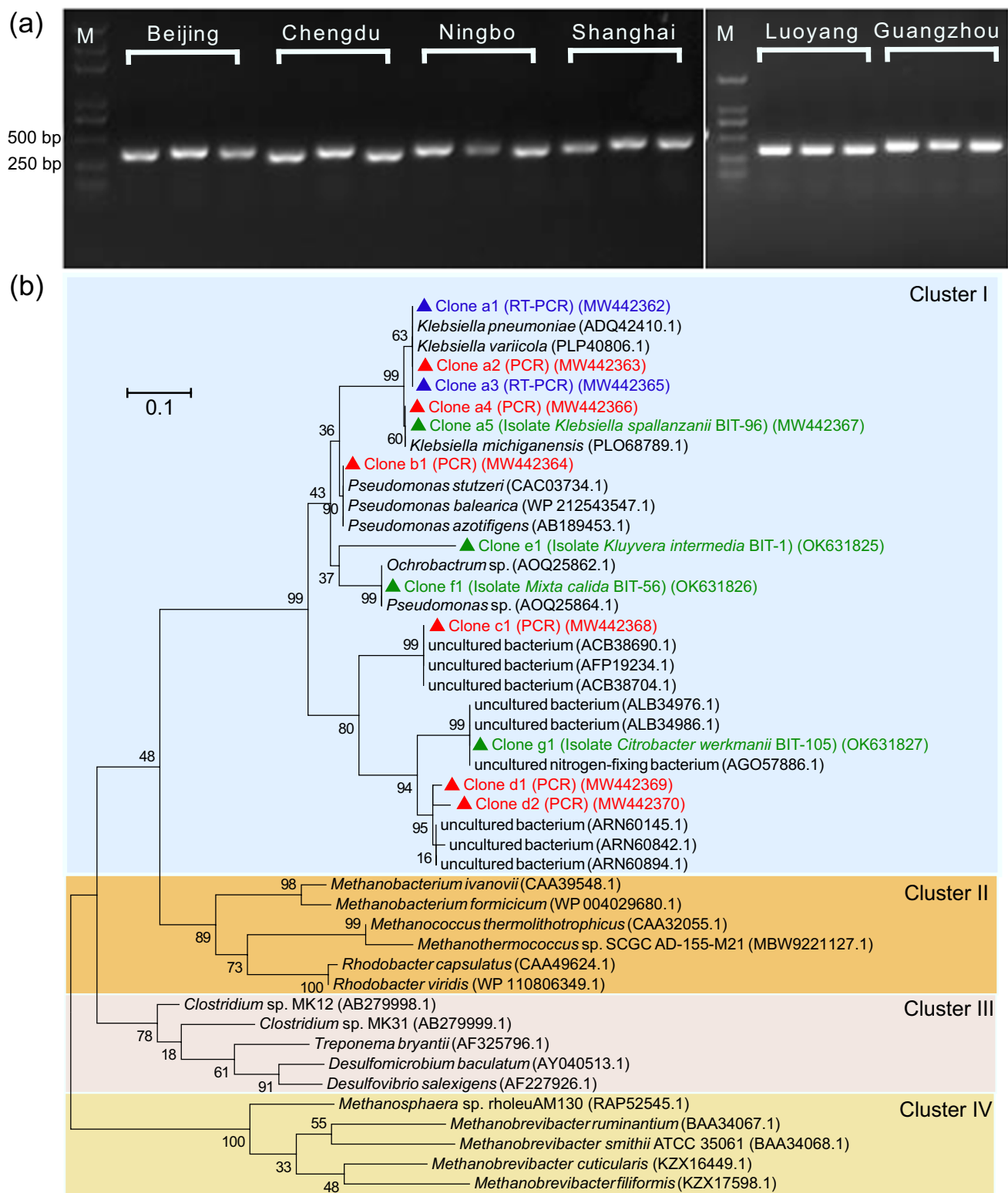


Fig. 4 PCR amplification of *nifH* DNA from the gut microbiota of plastic-eating mealworms. **a** PCR products of *nifH* gene from the genomic DNA of potential nitrogen-fixing gut isolates. **b** *nifH* phylogenetic tree. Phylogenies were reconstructed from *nifH* amino acid sequences recovered from DNA (PCR), RNA (RT-PCR), and isolated

cultures from the gut microbiota of plastic-eating mealworms. Isolate, PCR-based, and RT-PCR derived phylotypes are indicated in green, red, and blue color. The tree was constructed as described in the Materials and Methods section

than 100 clones from clone libraries of PCR products were picked and sequenced. However, several clones had similar DNA sequences and identical deduced amino acid sequences. The clones with identical amino acids sequence were classified into one OTU, and only one sequence was taken as representative for each OTU. Finally, six clones, representing different *nifH* amino acid sequences, were chosen for further study (SI Table S12).

Phylogenetic analysis of the obtained *nifH* amino acid sequences with their closest neighbors was shown in Fig. 4b. According to Zehr et al. [59], *nifH* protein sequences can be classified into four major clusters (clusters I–IV) in phylogeny. We found that all *nifH* clones from mealworm's gut fell into the previously described cluster I, which consists of *nifH* genes from conventional eubacterial Mo-Fe operons and some *vnfH* [59]. The sequence of clone a2, clone a4, and clone b1 was 99.2%, 99.5%, and 100% identical to the *nifH* gene of *K. pneumonia*, *K. michiganensis*, and *Pseudomonas stutzeri*, respectively. Three other sequences of clone c1, clone d1, and clone d2 shared a highest similarity (98.3–99.2%) with the *nifH* gene from uncultured nitrogen-fixing bacteria in soil (Table S12). These results indicate that Styrofoam-eating mealworms have a unique *nifH* gene diversity, which is different from of the *nifH* genes within termite gut that generally belong to cluster II or III which compose sequences from a diverse group of strict anaerobic microorganisms such as clostridia, sulfate reducers, spirochetes, and methanogens [16, 52].

Expression of *nifH* Genes in Styrofoam-Eating Mealworm's Gut

To demonstrate whether the *nifH* genes encoding dinitrogenase reductase were actually expressed by gut nitrogen-fixing bacteria within Styrofoam-eating mealworms, transcribed *nifH* mRNA in the gut microbial communities was analyzed using RT-PCR. Amplifications were detected in the cDNA samples of gut contents from six different Styrofoam-eating mealworms but not in the control RNA sample of gut contents without the RT reaction step (Fig. 5), indicating that the RNA preparation did not have any residual DNA after the DNase treatment and *nifH* transcripts virtually presented in the total RNA pool extracted from Styrofoam-eating mealworms' guts. In comparison, no amplifications were observed in the cDNA samples of gut contents from antibiotic-treated and bran-feeding mealworms (SI Fig. S2), in line with their relative weaker acetylene-reducing (nitrogen-fixing) activities than Styrofoam-eating mealworms.

Clone libraries were constructed from *nifH* cDNA amplified by RT-PCR from total RNA. We recovered two unique representatives from the RT-PCR cDNA clone libraries. Both of them clustered within group 1 of cluster I and were exclusively *Klebsiella* phylotypes (Fig. 4b and SI Table S12). Given the high nitrogen fixation rates measured for the *Klebsiella* isolates BIT-96 (Fig. 3a) and the apparent consistence of *Klebsiella nifH* genes in PCR and RT-PCR clone libraries (Figs. 4 and 5), it is likely that the genus *Klebsiella*, belonging to the order of *Enterobacteriales*, is an important nitrogen-fixing symbiont of Styrofoam-eating mealworms.

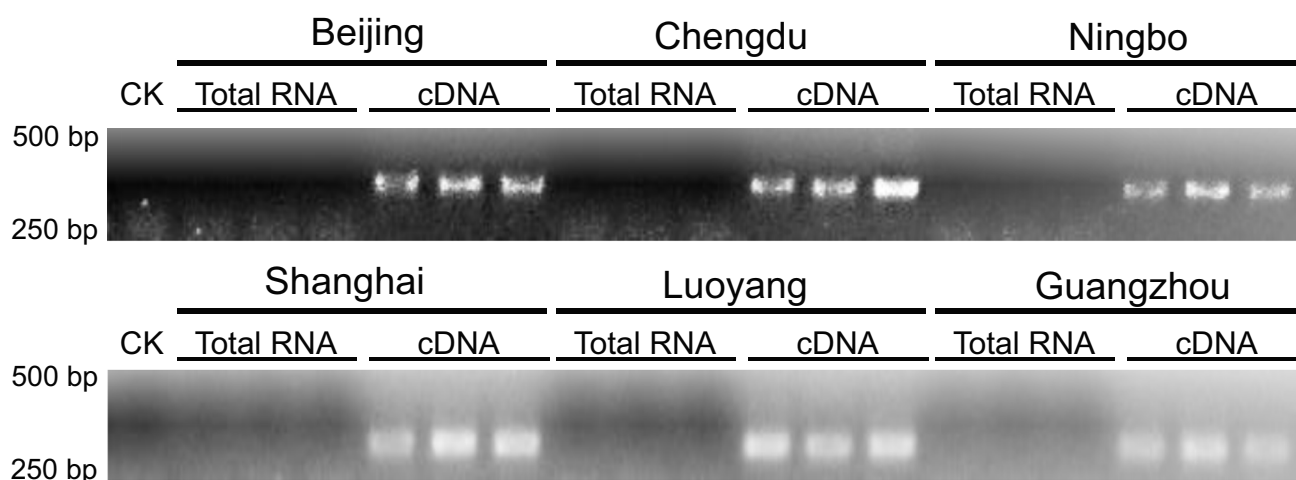


Fig. 5 RT-PCR amplification of *nifH* gene mRNA from the gut microbiota of plastic-eating mealworms. The *nifH*-specific primer PolF-PolR was used in the PCR reaction. RNA preparations treated

with DNase in the absence of RT did not show the presence of PCR products, indicating there are no DNA contaminant in the RNA extracts

Impact of Nitrogen-Fixing Gut Bacteria on Styrofoam-Eating Mealworm's Fitness

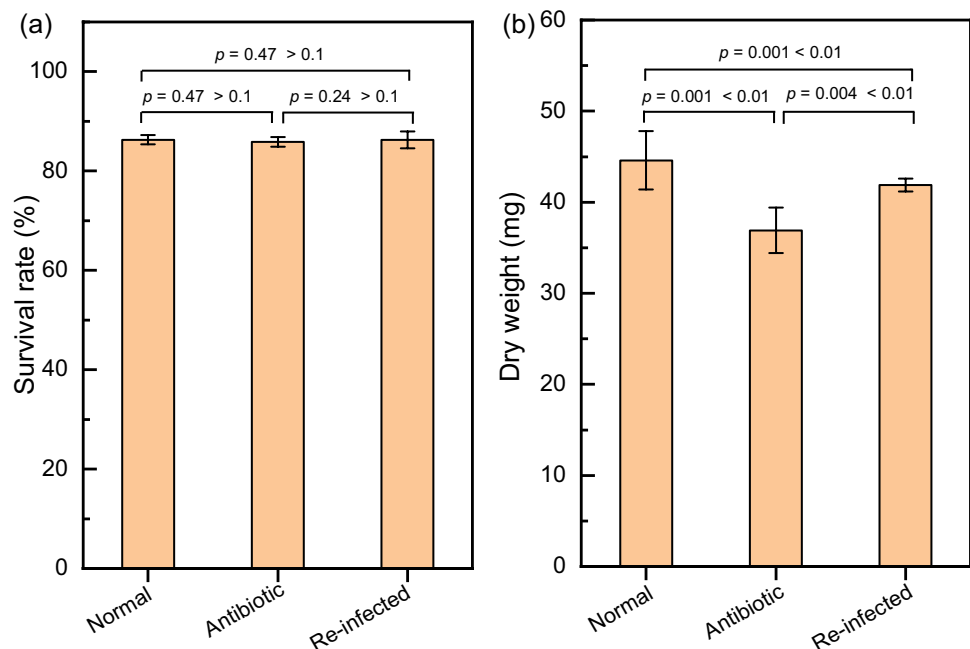
To further examine the influence of nitrogen-fixing bacteria on mealworms' fitness, we re-infected the antibiotic-treated mealworms with nitrogen-fixing gut isolate *K. spallanzanii* BIT-96 and subsequently compared their survival rates (SR) and the average dry body weights (ADBW) with that of Styrofoam-eating mealworms (normal group) and antibiotic-treated mealworms. As shown in Fig. 6a, the SRs of all three groups were not significantly different during a 16-day incubation with Styrofoam as sole diet (one-way ANOVA, $p = 0.89 > 0.1$). By contrast, the ADBW of antibiotic-treated groups was significantly lighter than that of re-infected groups (t test, $p < 0.01$), even though the ADBW of re-infected groups was significantly lighter than that of normal groups (t test, $p < 0.01$) (Fig. 6b). As antibiotic treatment may eliminate both of nitrogen-fixing gut bacteria and other functional bacteria, the highest ADBW loss of antibiotic-treated mealworms may be resulted from the damage of nitrogen-fixing capability and other functions. Re-infection with nitrogen-fixing gut isolate *K. spallanzanii* BIT-96 may help relieve the partial weight loss of antibiotic-treated mealworms by the restoration of the nitrogen-fixing capability. Hence, we could infer that the presence of nitrogen-fixing gut bacteria was important to the biological performance of Styrofoam-eating mealworms.

Discussion

Since mealworm was first introduced as a plastic eater and digester in 2015, this coleopteran insect larva has been proven to survive by eating a series of non-hydrolyzable vinyl plastics, such as PS, PE, PP, and PVC, as sole diet and effectively degrade them [1, 7, 37, 54, 56, 57]. In the perspective of nutrient element content, the petroleum-derived vinyl plastics were carbon-rich but nitrogen-deficient diet. However, from a biology viewpoint, nitrogen was an indispensable element for the biosynthesis of proteins, nucleic acids, and other metabolites in insect [4]. Hence, there was a pending question how mealworms can survive on nitrogen-deficient vinyl plastic as sole diet as well as that feed on normal nutritionally adequate diet (such as bran). Inspired by that the xylophagous insects were capable of supplementing their diets by fixing nitrogen from atmosphere, we attempted to reveal whether mealworms could also fix atmosphere nitrogen.

In the present study, we have provided comprehensive evidence for the occurrence of nitrogen fixation in Styrofoam-eating mealworms from three aspects proposed by Bar-Shmuel et al. [4]. Firstly, our results explicitly demonstrated that Styrofoam-eating mealworm has nitrogen-fixing activity by using the acetylene reduction assays. Secondly, the analyses of natural stable nitrogen isotope compositions in Styrofoam-eating mealworm has provided evidence for the assimilation of atmosphere nitrogen into the biomass. The fixed nitrogen equivalents of protein were estimated as 8.6 to 23.0 μg per day per mealworm based on the

Fig. 6 Influence of nitrogen-fixing ability on the biological performance of mealworms. **a** Survival rate (SR), **b** the average dry body weight (ADBW). The one-way ANOVA and t test with Tukey's correction was used to assess differences in SR and ADBW between the three groups, respectively



acetylene-reducing rate. At last, the antibiotic suppression tests showed that the elimination of gut microbial microbiota would impair the mealworm's nitrogen-fixing ability, indicating the contribution of gut microbiota to nitrogen fixation. By applying the traditional culture-dependent technique, PCR and RT-PCR of *nifH* gene, the presence of nitrogen-fixing bacteria within the gut was demonstrated. These findings advance understandings of the role of gut microbiota in mealworms and suggest that fixing atmosphere nitrogen is a way whereby plastic-eating mealworms overcome the nutritional obstacle of nitrogen limitation.

The four identified nitrogen-fixing gut bacteria isolates from mealworm were found from the genera of *Klebsiella*, *Mixta*, *Kluyvera*, and *Citrobacter* within the order of *Enterobacteriales*. The detected eight distinct *nifH* gene clones from DNA or cDNA of gut microbiota had the greatest sequence similarity to the genera *Klebsiella* and *Pseudomonas* and the *nifH* gene from uncultured nitrogen-fixing bacteria in soil. These nitrogen-fixing bacterial species found in the gut of mealworms were also retrieved from the guts of other nitrogen-fixing insects, such as wood-eating termites [15, 18, 39], the fruit fly *Ceratitis capitata* [5], and the red turpentine beetle *Dendroctonus valens* LeConte [28]. All this suggests that many nitrogen-fixing insects may share the similar gut nitrogen-fixing bacteria regardless of the specific host.

Given that the gut microbiota of mealworms have been demonstrated to be responsible for the plastic degradation in our previous studies [58] and nitrogen fixation within mealworm in the present study, the bacteria from the gut of mealworms possess two properties, plastic degradation and nitrogen fixation. Hence, engineering a synthetic microbial consortium or a combined cell factory, which can degrade plastic waste and fix atmosphere nitrogen at the same time, would be a possible way for up-recycling plastic waste to cell proteins without the additional feed of nitrogen nutrient.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00248-021-01930-5>.

Author Contribution HL, LX, and WJ conducted acetylene reduction assays, isolation of gut nitrogen-fixing bacteria, and PCR and RT-PCR experiments. JG conducted bulk stable isotope measurements on mealworms. YY and HL analyzed data and wrote the manuscript. YY conceived and designed the research.

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Availability of Data and Material The GenBank accession numbers for 16S rRNA of strains *Kluyvera* sp. BIT-1, *Mixta* sp. BIT-56, *Klebsiella* sp. BIT-96, and *Citrobacter* sp. BIT-105 were MW422732,

MW422733, MW422734, and MW422751, respectively. All detected *nifH* gene nucleotide sequences were deposited in GenBank with the accession numbers of MW442362–MW442370 and OK631825–OK631827.

Declarations

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors. All authors read and approved the manuscript.

Competing Interests The authors declare no competing interests.

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