Metagenomic Analysis of Bacterial Community in the Gut of Blister Beetle *Mylabris pustulata* Thunberg

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Abstract. Blister beetles are an important object of study in the field of agriculture and health. Despite being known to be important for the host animal, the gut bacteria of blister beetles have not been investigated deeply. This study aimed to provide the first initial description of the gut bacterial community of *Mylabris pustulata* as one species of blister beetles, based on a culture-independent technique. Adult blister beetles of the same colony were sampled and confirmed as *Mylabris pustulata* Thunberg. The gut content was used in high throughput sequencing, targeting the V3-V4 regions of 16S rRNA gene, and in the cultivation of resident bacteria. The results showed that higher bacterial richness was present in the gut communities when compared to the grown bacterial culture. Proteobacteria was confirmed as the most abundant phylum in the gut of *M. pustulata*, whereby most reads belonged to the class of Gammaproteobacteria. The dominant bacterial genera were determined as *Enterobacter, Acinetobacter, Enterococcus, Klebsiella,* and *Pseudomonas*. In addition, our cultivation attempts led to successful isolation of members of *Klebsiella* and *Enterococcus*. The subject of this study could be explored further to find the potential roles of these microbiota in the gut of the specific beetles and their bioprospects.

Keywords: *16S rRNA; blister beetle; gut bacteria; Mylabris pustulata; metagenomic; next generation sequencing.*

1 Introduction

The blister beetle, classified as a member of the Meloidae family, is a cosmopolite insect that has been intensively studied due to its impact in the agricultural and health sectors. About 120 genera and 3,000 species have been reported in this family [1]. The blister beetle has been reported as a pest that causes economic loss for several crops [1] and may be poisonous for animals and humans in some cases [2]. Despite these negative factors, several benefits for human health have also been reported. In the past, the administration of the blister beetle *Mylabris* spp. was common practice in Chinese traditional medicinal therapies. This included treatment against some toxins and rabies, and miscarriage induction [3].

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The interaction between insects and their microbial community is crucial for the survivability of the host. Gut microbiota play important roles that benefit the host's health and well-being, facilitating essential nutrient provisioning and detoxifying harmful compounds inside the gut, which is important for the host's development [4-6]. Interestingly, the host's physiology can also be influenced by the composition of the bacteria residing in the host's gut [7]. In addition, bacteria associated with beetles have been reported to have the capability of controlling their host [8] and protecting the host against pathogens [9]. Considering the natural and beneficial properties, the gut microbiome that consists of microorganisms and their genomes could be explored for many actions, such as complex carbohydrate hydrolysis, vitamin production, nitrogen, and phenolic compound metabolism [10].

Although known to possess beneficial roles to the host, many of the associated microbes still resist living under given laboratory conditions. The knowledge available between culturable and unculturable microbial taxa, achieved by combining metagenomic and culture-dependent techniques, mostly comes from clinical pathology. For insects, the associated microbes have been known to colonize the exoskeleton, hemocoel, and intestinal gut [11]. A study on the microbiota associated with medicinal maggots *Lucilia sericata*, employing both culture-dependent and culture-independent techniques, showed that only a small fraction of bacteria could be cultivated [12]. Thus, improvement of cultivating techniques is required for achieving a deeper understanding of the influence of these microorganisms on the host animal.

At least 270 insect species of 22 different taxonomic orders have been reported for the microbial community based on culture-independent techniques [5,13]. To our knowledge, no report is currently available on the profiles of the gut bacteria of *Mylabris pustulata*. The bacterial diversity of some other related blister beetles has been investigated, including *Megetra cancellata* and *Epicauta longicollis* [13]. The present study aimed to investigate the gut microbiome of blister beetle *M. pustulata* Thunberg based on a culture-independent technique. In addition, high-throughput sequencing was also employed to detect gut bacteria that are able to grow under specific conditions in the laboratory, to get a distinctive picture of the culturable and non-culturable bacterial taxa in the gut of blister beetles. Although there was a limitation in the number of samples in our study, it is the first study to provide initial information on the bacterial community of the *M. pustulata* blister beetle. Subsequently, the information may be useful to improve the culturomics of the relevant host in the future and there may be further benefits for the agriculture and health sectors as well.

2 Materials and Methods

2.1 Sample Collection

A colony of adult blister beetles was collected in April 2018 from the vegetation of *Hibiscus rosa-sinensis* L. (China rose) nearby Cibinong Ecology Park and Botanic Garden, West Java, Indonesia (6°29'43.2"S 106°51'06.3"E) by dragging a 1-m^2 white blanket in the area. A total of seven male beetles from the same colony were divided into two groups: five for taxonomic identification of the insects and as collection specimens at the Museum Zoologicum Bogoriense (MZB), Indonesian Institute of Sciences (currently called the National Research and Innovation Agency, BRIN); and two for microbiological study. The identification of the insects was conducted using standard taxonomic keys. The collection specimens have the accession numbers MZB COLE 173081, MZB COLE 173082. MZB COLE 173083, MZB COLE 173084, and MZB COLE 173085. For the microbiological study, individual beetles (approx. 0.8 g in weight) were anesthetized using chloroform, then washed in 70% (v/v) ethanol and sterilized deionized water to remove microbial contaminants from the surface of the insects. The gut content was collected aseptically and stored prior to high-throughput sequencing and bacterial cultivation.

2.2 Cultivation of Bacteria from the Gut of Blister Beetles

In order to obtain the natural conditions in the gut, which are likely to be anaerobic, the total culturable bacteria inside the guts of two beetles (guts of individuals A and B) were estimated in nutrient agar (Merck), employing incubation at 37 °C for 48 h under anaerobic conditions in an anaerobic jar (Merck) supplemented with Anaerocult (Merck). The grown colonies were counted for the determination of the number of living-culturable cells (cfu/g). The most predominant colonies were subsequently purified in fresh nutrient agar (Merck) as a single bacterial isolate and then identified based on the 16S rRNA gene sequences obtained through Sanger sequencing. In parallel, 50 µg of gut aliquot of individual B was inoculated into 50 ml of overnight, pre-reduced nutrient broth (NB) (Merck) and incubated anaerobically. After 48 h of incubation at 37 °C, this culture (referred as Culture B) was subjected to genomic DNA extraction and high-throughput sequencing.

2.3 Preparation of Genomic DNA and High-Throughput Sequencing

Genomic DNA of the gut of individuals A and B of the blister beetles (Gut A and Gut B) and the bacterial culture of Gut B (Culture B) were extracted using a ZymoBIOMICS DNA MiniPrep kit (Zymo Research), following the instructions of the manufacturer. For high-throughput sequencing, the V3-V4 hypervariable

regions of the 16S rRNA gene were amplified using the primer pair F336 (5′-GTACTCCTACGGGAGGCAGCA-3′) and R806 (5′--GTGGACTACHVGGGTWTCTAAT-3′) according to the method of Liu *et al.* [14]. The quality of the amplicons was checked with gel electrophoresis. The high-throughput sequencing was performed at Novogene Bioinformatics Technology Co., Ltd. with an Ion S5™ XL platform (Thermofisher). Single-end reads were assigned to a specific sample based on the incorporated unique barcode. The barcodes and primers were truncated from the sequences prior to the community analysis.

2.4 Data Analysis

After quality filtering in Qiime, the OTU table, taxonomy table, and phylogenetic tree were imported to R using the phyloseq package [15]. The calculation of alpha diversity matrices (richness, Shannon, and Simpson) was performed using the same package while sample coverage estimates were determined by iNEXT [16,17]. The PCoA plot was constructed based on weighted UniFrac distances, which were calculated with phyloseq. Unless mentioned otherwise, all plots were visualized using the R ggplot2 package [18].

2.5 Identification of Cultivated Bacterial Isolates by Sanger Sequencing

To obtain single isolates, streaking the Culture B on fresh agar medium was performed. Colony PCR amplification of the 16S rRNA gene was applied with universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The PCR mixture contained 1 μL 10 pmoles of Primer 27F, 1 μL 10 pmol of primer 1492R, and 10 ng of DNA template, 25 μL GoTaq Green Master Mix (Promega) brought up to a final volume of 50 μL with ultra-pure water. The reactions were performed on a Takara Thermal Cycler Dice (Takara Co. Inc.) under the following conditions: 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, and a final extension step at 72 °C for 5 min [19]. Aliquots of 5 μL of each reaction were analyzed on 1% (w/v) agarose gel with GelRed Nucleic Acid Gel Stain (Biotium) in TBE buffer. The sequencing was performed using the Sanger method at the 1st BASE Laboratories. Sequences were aligned to the EzBioCloud database (https://www.ezbiocloud.net/) to find the closest relatives [20]. The neighbor-joining phylogenetic tree was constructed using Clustal X version 2.0.11.13. In addition, the sequences were also aligned against sequences available in the community dataset using ViroBlast [21] to identify their closest operational taxonomic unit (OTU) relatives.

2.6 Microscopic Observation by Scanning Electron Microscopy (SEM)

Microbial cells inside the insect gut (Gut A and Gut B) and Culture B were observed using a scanning electron microscope (SEM) available at ELSA BRIN (https://elsa.brin.go.id/). Microbial suspensions of the gut aliquot were made by centrifugation at 9.000 x g for 1 min, followed by fixation with 2.5% glutaraldehyde. The cells were dehydrated by sequential washing treatments using 50%, 70%, 85%, 95%, and absolute ethanol. The dehydrated cells on the slide were sputter-coated with gold and observed under a JSM-IT200 SEM (JEOL Ltd.) with an accelerating voltage of 3.0 kV.

3 Results

The blister beetles were identified as *Mylabris pustulata* Thunberg (Coleoptera: Meloidae). Adult male blister beetles (2.5 to 2.7 cm in length) were identified based on their morphology, which is characterized by four red and black alternating bands on the elytra. The beetles released yellowish liquids when they were anesthetized, which are most likely a protectant against specific environmental stimuli or external threats. The gut contents of the two chosen adults (Gut A and Gut B) had distinct appearances even though they were taken from the same colony, where Gut A was observed to have a dark-yellow color and Gut B had a red color (Figure 1).

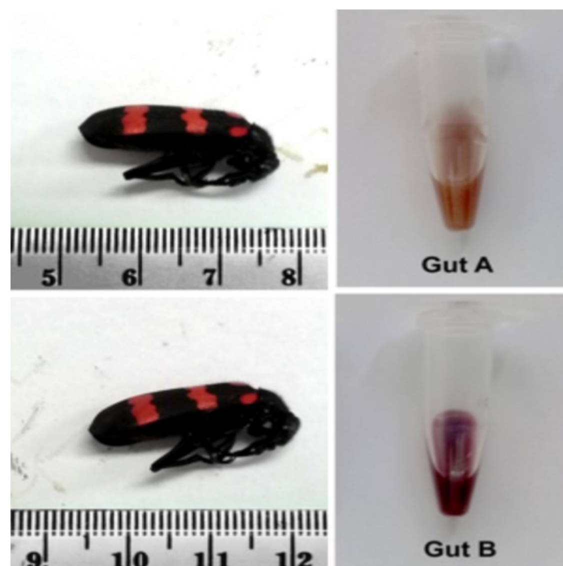

Figure 1 Morphological appearances of two blister beetle specimens of *M. pustulata* Thunberg and their gut contents (Gut A and Gut B).

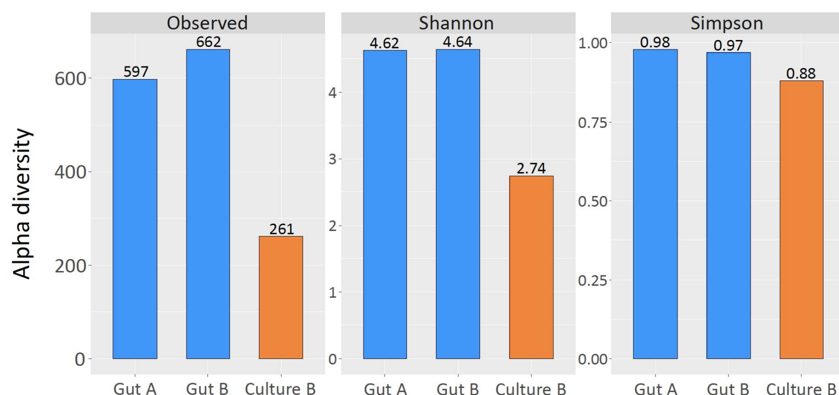

Figure 2 Alpha diversity metrics (richness, Shannon diversity, and Simpson) at OTU level for gut samples of individuals A and B (Gut A and Gut B), and bacterial culture originated from the gut of individual B of blister beetle *M. pustulata* Thunberg (Culture B).

In total, 452,250 reads, which belonged to 16,693 OTUs, were retrieved after processing with Qiime. The rarefaction curves (not shown) imply that our sequence inventory covered most of the taxa that were present in all three samples (Figure 2), including the guts of individuals A and B (Gut A and Gut B), along with Culture B, where the content of gut B was used as inoculant for the cultivation. Samples coverage for all samples was above 98%. All sequences that were associated with either chloroplast or mitochondria were removed beforehand, and the data was further rarified using a sample size of 126,414 to allow fair comparison between the three samples. The calculation of alpha diversity metrics was performed at OTU level. Gut A and Gut B harbored a similar number of OTUs, each with 597 and 662 OTUs, respectively (Figure 2). As expected, the bacterial culture originating from the gut of individual B (261 OTUs) contained lower diversity when compared to that found in the guts of individuals A and B (Figure 2), for both the Shannon and Simpson indices.

The PCoA plot based on weighted UniFrac distance confirmed higher similarity between the Gut A and Gut B bacterial communities compared to when each sample was paired with Culture B, as both were positioned closer together on the ordination plot (Figure 3A). Culture B on the other hand, was positioned further and was separated from the two gut communities along the x axis, which explains as much as 97% of variance across the data. Proteobacteria was the most abundant phylum in the gut of blister beetles, accounting for 75.7% and 81.2% of the reads in Gut A and Gut B, respectively, distributed to five distinct bacterial classes, i.e., *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria,* and *Epsilonproteobacteria* (Figure 3B). The employed

cultivation conditions select the phylum as well, as almost 95% of the reads of Culture B were identified as Proteobacteria. However, it should be noted that the gut contents included some less-explored bacterial phyla such as Saccharibacteria and Tenericutes. Almost no information is available regarding the ecological function of these taxa, especially within the gut environment. Thus, future cultivation efforts should target these taxa as well.

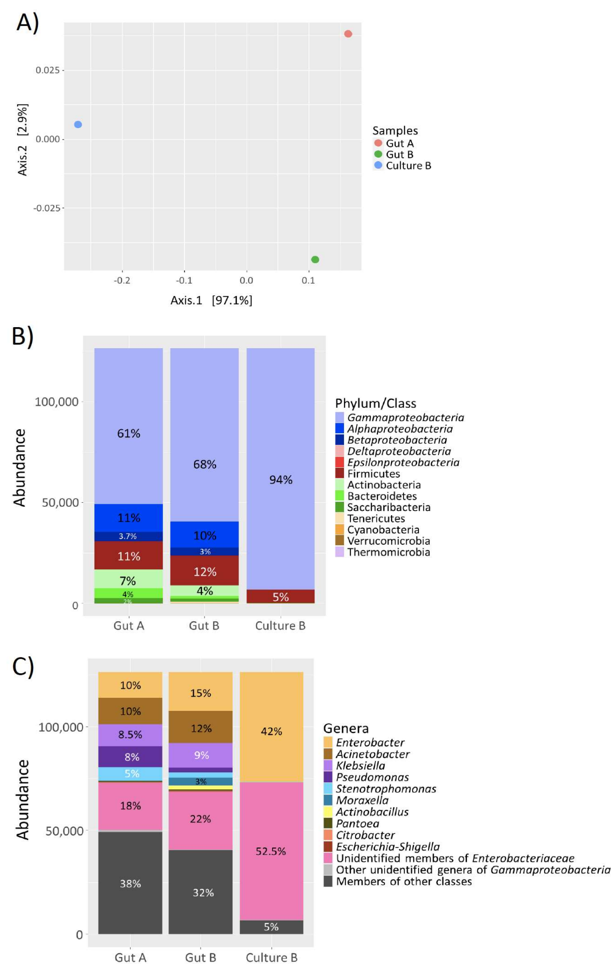

Figure 3 PCoA plot based on weighted UniFrac distances for communities of Gut A, Gut B, and Culture B of blister beetle *M. pustulata* Thunberg, calculated at OTU level (A). Figure B depicts the taxonomy composition of the three samples at the phylum/class level, while C reflects the taxonomic composition at the genus level, with special focus on the class Gammaproteobacteria. Only values of at least 3% were put as labels in the bar plot.

Table 1 a Top ten most abundant genera found in the gut of blister beetle *M. pustulata* Thunberg (Gut A & B) and bacterial culture inoculated with gut of individual B (Culture B).

Genera	Class	Relative abundance (%)		
		Gut A	**Gut B**	**Culture B**
Enterobacter	Gammaproteobacteria	10.0	15.1	41.9
Acinetobacter	Gammaproteobacteria	10.0	12.1	0.0
Enterococcus	Bacilli	8.9	7.5	5.1
Klebsiella	Gammaproteobacteria	8.5	9.4	0.1
Pseudomonas	Gammaproteobacteria	7.8	1.8	0.0
Stenotrophomonas	Gammaproteobacteria	5.3	2.0	0.0
Microbacterium	Actinobacteria	4.6	2,4	0.0
Ochrobactrum	Alphaproteobacteria	2.1	3.0	0.0
Rhizobium	Alphaproteobacteria	2.5	2.5	0.0
Lactococcus	Bacilli	1.8	2.2	0.0

More than 50% of the Proteobacteria reads belonged to *Gammaproteobacteria* in Gut A and Gut B, and almost 100% in Culture B. Of these, 10% and 15% could be identified as *Enterobacter,* 10% and 12% as *Acinetobacter,* 8.5% and 9% as *Klebsiella,* and 7.8% and 1.6% as *Pseudomonas* in the Gut A and B, respectively (Figure 3C). In addition, *Moraxella, Actinobacillus,* and *Pantoea* reads were higher in Gut B when compared to Gut A, with each contributing 3.1%, 1.3%, and 0.8% of the total reads, respectively. Interestingly, many of these reads belonged to as-yet-uncultured members of *Enterobacteriaceae*, contributing 18%, 22%, and 52.5% of the reads within Gut A, Gut B, and Culture B, respectively.

Table 2 Total counts of culturable bacteria in the gut of blister beetle *M. pustulata* Thunberg under anaerobic cultivation.

	Total cells (cfu/g) ± SD
Gut A	$5.44 \pm 0.25 \times 10^6$
Gut B	$4.40 \pm 0.18 \times 10^6$

SD, standard deviation (n=2)

In general, *Enterobacter, Acinetobacter, Klebsiella, Pseudomonas,* and *Stenotrophomonas* were also included in the top ten most abundant genera with the abundance of *Enterobacter* and *Acinetobacter* being the highest, with both contributing 10% of the reads in Gut A, whereas the abundance in Gut B was 15.1% and 12.1%, respectively (Table 1). In addition, members of the class *Bacilli, Alphaproteobacteria,* and *Actinobacteria* were included in the list as well, i.e., *Enterococcus, Lactococcus, Ochrobactrum, Rhizobium,* and *Microbacterium.*

Table 3 Bacterial isolates and the closest relatives based on the Eztaxon database and the closest OTUs along with abundance information.

Isolates	Closest relatives (Eztaxon database)	Similarity (%)	Closest OTU	Similarity (%)	OTUs abundance (%)		
					Gut A	Gut B	Culture B
ADML 141	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044^T	99.7	OTU 12780	99.0	1.3	1.4	3.2
ADML 142	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044^T	99.5	OTU 12780	99.0			
ADML 211	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044^T	99.7	OTU 12780	99.0			
ADML 242	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044^T	99.5	OTU 12780	99.0			
AMML 12	*Enterococcus casseliflavus* MMUTK 20^T	99.8	OTU 2	100.0	1.3	1.1	0.8
AMML 13	*Enterococcus casseliflavus* MMUTK 20^T	100.0	OTU 2	100.0			
ADML 241	*Klebsiella aerogenes* KCTC 2190^T	99.9	OTU 10710	99.0	ND	ND	ND
ADML 221	*Klebsiella aerogenes* KCTC 2190^T	99.7	OTU 6062	99.0	1.0	0.0	0.0

ND = not detected

The data was investigated further to check if there were any OTUs that were unique for specific communities, emphasizing host-specificity even within the same species of the host animal. The result suggests that the most abundant OTUs were shared across samples, contributing 74.7%, 74.5%, and 99.5% of the OTU reads in Gut A, Gut B, and Culture B, respectively (Figure 4). There were 33 OTUs detected to be unique for individual A and 79 OTUs for individual B. However, this OTUs abundance within the gut of both individual A and individual B was less than 3%. It should be noted, however, that amplicon reads do not correlate to the degree of activity of the detected OTUs. Therefore, despite the low abundance, there is still a possibility for these OTUs to be actively metabolizing inside the gut of the blister beetles, which could be observed given appropriate detection methods such as metatranscriptomic, metaproteomic and RNA-based stable isotope probing (RNA-SIP) [22]. Of these unique OTUs, the members of *Moraxella, Actinobacillus, Streptococcus,* and *Klebsiella* were specific for individual B, whereas the members of *Pseudomonas* were specific for individual A. Some unique OTUs found in Gut A and Gut B were identified as members of the same genus, implying that the host-specificity goes up to the OTU level and thus bacterial community analysis should be performed at the deepest taxonomic level possible. Interestingly, there were 3 OTUs that were

detected to be unique for Culture B, despite the fact that it was inoculated with the gut of individual B. This may be explained by the abundance of those 3 OTUs within Gut B being below the detection limit of the sequencing platform and the fact that the coverage of samples with high diversity (here represented by Gut B) may be lower when compared to samples with less diversity (Culture B).

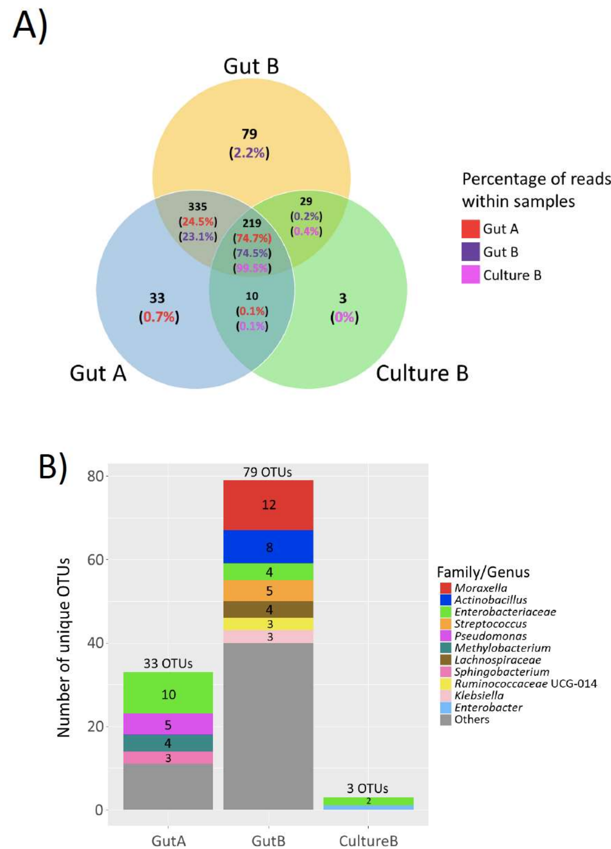

Figure 4 Unique and shared OTUs between Gut A, Gut B, and Culture B (A) of blister beetle *M. pustulata* Thunberg. Figure B depicts the taxonomy of the unique OTUs.

For cultivation, approximately 10^6 cells/g were observed to grow in the cultivation media after inoculation with gut content and subsequent anaerobic incubation (Table 2). After purification, a total of 8 bacterial isolates were retrieved from the gut of individual B (Table 3). Four of the isolates were identified as members of *Klebsiella quasipneumoniae,* while two isolates each were identified as members of *Enterococcus casseliflavus* and *Klebsiella*

aerogenes. The identification based on EzTaxon was also supported by the phylogenetic tree, in which two distinct clades were observed belonging to the genera *Klebsiella* and *Enterococcus* (Figure 5).

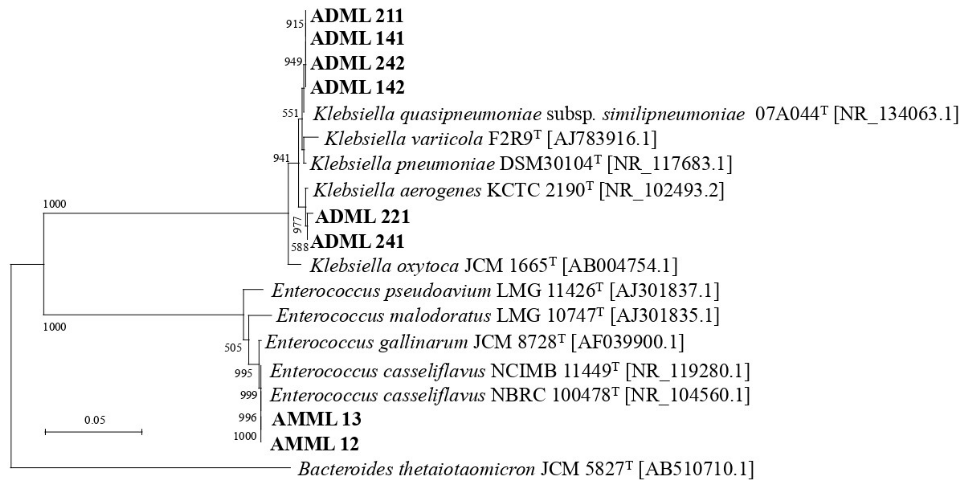

Figure 5 The neighbor-joining (NJ) tree of bacteria isolates from the gut culture of blister beetle *M. pustulata* Thunberg based on the 16S rRNA gene sequences. *Bacteroides thetaiotaomicron* JCM 5827^{T} was used as out-group.

These 8 isolates could be traced back to 4 distinct OTUs, i.e., OTU 12780, OTU 2, OTU 10710, and OTU 6062 (Table 3). Of these, OTU 12780 and OTU 2 may be interesting to be explored further due to the fact that their abundance in both Gut A and Gut B were higher than 1% and thus might be key players in the gut of the corresponding host animal. Of all detected OTUs, only 19 and 22 OTUs were detected with an abundance more than 1% in Gut A and Gut B, respectively (out of 597 and 662 OTUs). These two abundant OTUs could be explored further in the laboratory for deeper analysis concerning functional roles that are relevant in the gut of blister beetles. Surprisingly, one isolate was not detected in both individual gut samples and could not even be found in the Culture B community. After investigation of the raw dataset before rarefaction took place, the single OTU that represented the isolate was found in Culture B but it only corresponded to 2 reads and thus was dismissed during rarefaction. Again, the fact that this OTU was missing from the gut communities might be linked to the detection limit of the sequencing platform.

Observation with scanning electron microscopy (SEM) revealed that Culture B showed a distinct microbial population when compared to the gut contents of individuals A and B. The differences between the gut samples and the bacterial culture could be clearly seen, as only the cells from the gut samples (gut A and

B) were distributed in some type of matrix (Figure 6). In addition, the cells from the bacterial culture appeared to be homogenous and smaller compared to the cells from the gut environment.

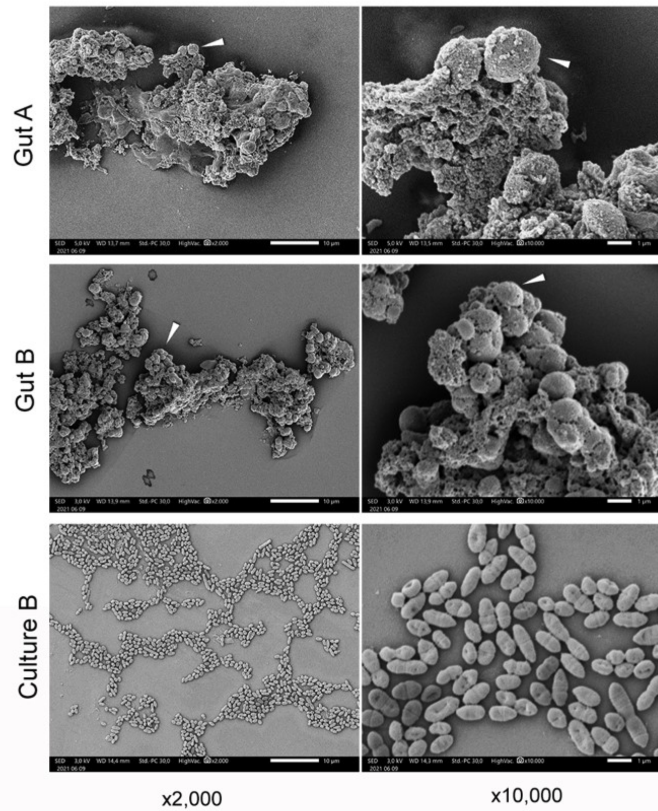

Figure 6 Scanning electron microscopy images of the gut contents of blister *beetle M. pustulata* Thunberg (Gut A and Gut B) and the bacterial culture originated from Gut B (Culture B). The arrow mark highlights the cells observed at magnification of 2,000x (left) and 10,000x (right).

4 Discussion

In this study, gut samples were collected from two individual blister beetles (*Mylabris pustulata*) as a first step in understanding the as-yet-unknown potential of the gut microbiota of the corresponding host animal. In parallel, one gut sample was further used as inoculant to target key players inside the gut of blister beetles. In this study, *M. pustulata* individuals were collected from the clustered flower of *Hibiscus rosa-sinensis*. This species of blister beetle has previously been reported as having a wide variety of flowers as diet source, including *Hibiscus rosa-sinensis* (Malvaceae), *Acacia caesia* (Mimosaceae), *Cassia occidentalis*

(Caesalpiniaceae), *Cleome viscosa* (Capparaceae), *Helicteres isora* (Sterculiaceae), *Mangifera indica* (Anacardiaceae), *Murraya koenigii* (Rutaceae), *Pavonia zeylanica* (Malvaceae), and *Tridax procumbens* (Asteraceae) [23].

Our results suggest that the bacterial richness inside the gut of blister beetle *M. pustulata* reached 600 taxa. This is in agreement with what has been observed for some insects, where the number of OTUs found in the gut of the sand fly (*Lutzomyia evansi*) and the Eri silk moth (*Samia ricini*) was around 500 [24,25]. However, many reports have suggested that the diversity within insect guts is slightly lower, with approximate values between 100 and 300 OTUs, as previously shown for *Thitarodes* larvae and dragonflies [26,27]. Previous reports on gut bacteria from two other blister beetles showed lower OTUs, as demonstrated by *Epicauta longicollis* (177 OTUs) and *Megetra cancellata* (140 OTUs) [13]. Many factors could contribute to these variations in the number of detected OTUs, including distinct environmental factors, diet, and phylogeny of the host animal. Even differences in the developmental stage of the same species may lead to differences in the diversity of the associated microbiomes [5].

It is most likely that a considerable amount of these OTUs is still unculturable. It has been widely accepted that the majority of bacteria at the moment still resist cultivation, with the fraction of the culturable ones, in soil for example, at only less than 1% [28-30]. Unknown growth factors, inappropriate environmental conditions, substrate preferences, short-incubation period, and several other factors are believed to be limiting factors in cultivation efforts [31,32]. The lower diversity in the bacterial culture (Culture B) most likely represents our inability, as of now, to recreate the appropriate growth conditions for the target bacteria. Isolation of bacteria from the gut of *M. pustulata* under anaerobic condition was successfully conducted. Only three species were obtained from the same sample analyzed by high-throughput sequencing (Culture B), which belonged to *Klebsiella quasipneumoniae, Klebsiella aerogenes,* and *Enterococcus casseliflavus* (Table 3). The species of *Enterobacter* was not obtained in the isolation, even though its relative abundance reached 41.9% (Table 1). This study confirmed that general cultivation techniques tend to provide opportunities for certain bacteria that are actually few in nature but are abundant when cultivated with standard cultivation methods in the laboratory.

Techniques such as employment of oligotrophic media and small inoculum size, the utilization of helper bacteria, long incubation period, and targeted sequencing may help to improve cultivation success in the future [33]. In addition, many members of the unculturable fraction of the family *Enterobacteriaceae* were detected in high numbers in our samples. The abundance of the family *Enterobacteriaceae* in insect guts has been linked to improvement of animal host

development, increase in carbon and nitrogen metabolism, better copulatory success, and stronger inhibition of pathogens [34].

An earlier metagenomic study, involving 218 insect species of 21 different taxonomic orders, showed dominance of Proteobacteria (62.1%) and Firmicutes (20.7%) [5]. This has been confirmed by our study, where Proteobacteria was found to be the most dominant phylum in the gut of the blister beetles. Many of the Proteobacteria reads in this study belonged to the *Gammaproteobacteria* class, which is in agreement with what has been observed earlier [5,26]. Bacterial genera that were detected to be abundant in the gut of blister beetle *M. pustulata* as demonstrated in this study, including *Acinetobacter, Enterobacter, Klebsiella, Microbacterium,* and *Pseudomonas*, have also been commonly found in the gut of the oriental fruit fly (*Bactrocera dorsalis*), the pumpkin fly (*B. tau*), the peach fruit fly (*B. zonata*), and the wild tobacco fruit fly (*B. cacuminata*) [35-40]. Another study has reported the enrichment of *Rhodococcus, Enterobacter,* and *Achromobacter* in the hindgut of sawyer beetles (*Monochamus alternatus*), although the composition varied across distinct diet types and insect development stages [41]. Moreover, *Enterobacter, Rosenbergiella, Erwinia, Pseudomonas,* and *Lactococcus* were found to be dominant in the gut of leaf beetles (*Gastrolina depressa*) [42]. The data showed that different insect taxa shared similarity in gut composition, for example, the enrichment of *Enterobacter* and *Pseudomonas*, which may be important for the host animal species.

The enriched bacterial genera in the gut of blister beetles may play important ecological roles inside the gut of the blister beetle, as has been shown by members of *Enterobacter* and *Klebsiella* that help their animal host *Rhagoletis pomonella* in the acquisition of nitrogen through degradation of purines and its derivatives [43]. An earlier study on *M. pustulata* demonstrated that a digestive cellulase system (endo-β–1,4-D-glucanase) is present in the homogenized foregut parts [4]. Although no direct evidence will be presented here, several groups of bacteria have been reported to play a role in the cellulose degradation in the gastrointestinal tract of some insects. In spite of the possibility to be an opportunistic pathogen, *Pseudomonas* has been reported to harbor cellulolytic activities along with amylolytic, xylanolytic, lipolytic, and esterase activities in the gut of *B. mori*, traits that are shared with members of *Acinetobacter*, and therefore could be beneficial for the digestion system of the animal host [6,44].

Moreover, *Pseudomonas* and *Enterococcus* has been reported to degrade the toxic latex and alkaloids included in the basal diet of the host animal [45], whereas *Microbacterium* produces *N*-acyl amino acid hydrolase, which may be helpful for food digestion [46]. Genera such as *Stenotrophomonas* show catalytic activities and perform nitrogen fixation in the gut of the bark beetle (*Dendroctonus rhizophagus*) [47], while a specific strain of *Ochrabactrum* is a

known pesticide degrader [48]. Furthermore, members of *Lactococcus* are actively involved in the degradation of plant polymers and therefore important for improvement of nutrient acquisition of the host animal [7], while *Rhizobium* degrades toxic alkaloids associated with the *Solanaceous* plant [49] and can improve nitrogen uptake [50]. Altogether, these abundant taxa may perform complicated functional systems that benefit the blister beetle.

Insects rely on their gut microbes for the protection against some pathogens [51]. This may include synthesis of specific toxins or modification of the insect immune system [11], which has been well demonstrated in rove beetles (*Paederus* sp.), who produce a potent vesicant agent, namely paederin. Investigation on the *ped* gene cluster of the insect suggests that the production of paederine may be linked to some bacterial symbionts [52], such as the unculturable taxa that are closely related to *Pseudomonas aeruginosa* [53].

Although known as opportunistic pathogens for humans, the most dominant genera in this study, namely, *Enterobacter, Acinetobacter, Klebsiella*, and *Pseudomonas*, are known to possess benefits for humans as well. *Enterobacter* has been reported to produce enterocins and a wide range of antimicrobial lipopeptides [54,55]. *Acinetobacter* induces anti-inflammatory responses and provides protection against allergic sensitization [56]. *Klebsiella pneumoniae* RYC492 produces microcin with potential antitumor activities [57] and *Pseudomonas aeruginosa* produces phenazine with activity against methicillin-resistant *Staphylococcus aureus* [58]. Moreover, bacterial pathogenicity depends on many factors, such as host immunity, number of bacterial cells, and virulence factors [59], and thus the presence of this bacteria does not necessarily reflect occurrence of disease.

A few numbers of insect samples from the same colony being insufficient to yield comprehensive knowledge on *M. pustulata* gut microbiome is recognized as a limitation of this study. However, this study presents an initial dataset that can be built upon in future studies, including on host-microbiota associations and bioprospects interests. The results warrant further study to determine the specific functional roles that are performed by resident bacterial taxa, which may be employed in the near future by applying techniques such as RNA-based community analysis combined with fluorescent labeling.

5 Conclusion

Higher bacterial richness in the gut of blister beetle *M. pustulata* was determined, where Proteobacteria was observed as the most abundant phylum based on metagenomic analysis. The dominant bacterial genera were *Enterobacter, Acinetobacter, Enterococcus, Klebsiella,* and *Pseudomonas*. Our cultivation

effort led to successful isolation of bacterial taxa, i.e., *Klebsiella* and *Enterococcus*, that were detected to be abundant. Altogether, the basic information derived from the culture-dependent and culture-independent techniques can be explored further in the laboratory to determine potential roles of these microbiota in the gut of specific beetles, and their bioprospects.

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