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Bacterial Gut Symbionts of *Antheraea mylitta* (Lepidoptera: Saturniidae)[1]

Resma Rajan, Shampa Deb Chanda, Alekhya Rani, Renuka Gattu[2], Shamitha Vodithala[2], and Anitha Mamillapalli[3]

Department of Biotechnology, Institute of Science, Gandhi Institute of Technology and Management, Visakhapatnam 530 045, India

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The tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae), accounts for 11% of total silk production (Ojha et al. 2009, Acad. J. Entomol. 2:80–84; Reddy et al. 2010, J. Appl. Sci. 10:1902–1909). To our knowledge, there are no reports on the symbiotic microflora associations in wild populations of *A. mylitta* larvae. We, therefore, undertook this study using culture-dependent and metagenomic sequencing of 16S ribosomal RNA (rRNA) to identify bacterial microflora in *A. mylitta* guts with an ultimate objective of improving the nutritional standards for propagating *A. mylitta* in rearing facilities leading to increased yields of silk production.

Fifth-instar *A. mylitta* larvae ($n = 10$), collected 5 d after molting, were dissected and their guts were aseptically extracted and homogenized. The homogenates were serially diluted (10^3–10^7) and drop plated on nutrient agar for initial screening for microbes. This process was repeated five times, with three replicates for each. Each gut sample yielded 30 pure cultures that were subcultured and subsequently subjected to cultural, morphological, and biochemical analyses (Krieg and Holt [eds.], 1984, Bergey's Manual of Systematic Bacteriology, 1st edition, vol. 1, Williams and Wilkins, Baltimore, MD; Sneath et al. [eds.], 1986, Bergey's Manual of Systemic Bacteriology, 1st edition, vol. 2, Williams and Wilkins, Baltimore, MD).

From culture-dependent analyses, we identified *Pseudomonas*, *Erwinia*, *Enterococcus*, *Staphylococcus*, *Bacillus cerus*, *Lactobacillus*, and *Micrococcus* from the cultures isolated from the gut samples. The predominant genera belonged to the *Proteobacteria* community that reportedly aid in the digestion of consumed plant biomass (Suen et al. 2010, PLoS Gen. 6: 1001129).

[1]Received 27 April 2019; accepted for publication 14 May 2019.
[2]Department of Zoology, Kakatiya University, Warangal-506002 Telangana, India.
[3]Corresponding author (email: anitha.mamillapalli@gitam.edu).

Table 1. Richness and diversity index of *Antheraea mylitta* (Am 1 and Am 2). Chao1 was used to estimate OTUs richness. Bacterial diversity index was assessed using Shannon and Simpson indices.

Sample	Shannon	Simpson	Chao1
Am 1	1.193	0.324	541.697
Am 2	0.213	0.043	149.8125

A more in-depth analysis involved genetic sequencing using the Illumina platform with Genotypic Technology Pvt. Ltd. (Bangalore, India) according to standard protocol. The V3 region of the 16S rRNA was amplified using high-quality primer bases (341F, 5′-CCTACGGGAGGCAGCAG-3′ and 518R, 5′-AT TACCGCGGCTGC TGG-3′ with 50 ng of metagenomic DNA). The gut microbial diversity of all the samples was assessed by alpha diversity analysis (Chao1, Shannon, and Simpson indices) and the rarefaction curve (Table 1). Valid reads of 213052 and 21869 were obtained after removing the low-quality reads (Table 2).

The Quantitative Insights into Microbial Ecology methods were used to detect 16S rRNA; clustering and operational taxonomic units (OTUs) were determined by Biom file generation and statistical analysis. Relative abundance values for the samples were used to plot stacked bar diagrams for the top 15 unique organisms identified at the taxonomic levels of phylum and genus (Caporaso et al. 2010, Nat. Methods 7: 335–336; DeSantis et al. 2006, Appl. Environ. Microbiol. 72(7): 5069–5072; Edgar 2010, Bioinformatics 26: 2460–2461; Erik 2013, Open Bioinform. J. 7: 1–8; Qiong et al. 2007, Appl. Environ. Microbiol. 73: 5261–5267; Renaud and Cathal 2010, BMC Bioinformatics 11: 367).

At the phylum level, *Firmicutes* dominated the bacterial flora within the gut of *A. mylitta* (Fig. 1A). At genus level, *Turicibacter* was the most dominant, with *Ruminococcus*, *Rhodococcus*, *Prevotella*, *Delftia*, *Acinetobacter*, *Desulfomicrobium*, *Sphingomonas*, *Faecalibacterium*, *Staphylococcus*, *Ralstonia*, *Bacillus*, *Azospirillum*, *Candidatus*, and *Kocuria* (Fig. 1B). This is the first report of the composition of the bacterial microbiome of the *A. mylitta* gut.

Culture-dependent analysis identified the presence of both *Proteobacteria* and *Firmicutes*, whereas culture-independent analysis showed that >92% of the gut bacterial microbiome is composed of *Firmicutes*. According to previous work (Fonknechten et al. 2010, BMC Genomics 11: 555; Peter et al. 2006, Nature 444:

Table 2. Read count statistics for *Antheraea* mylitta (Am 1 and Am 2) of V3 Region of 16S rRNA genes.

Sample	Total Paired End Reads	Processed Reads	Total Identified rRNA Sequences
Am 1	264524	213052	140897
Am 2	227388	218693	205156

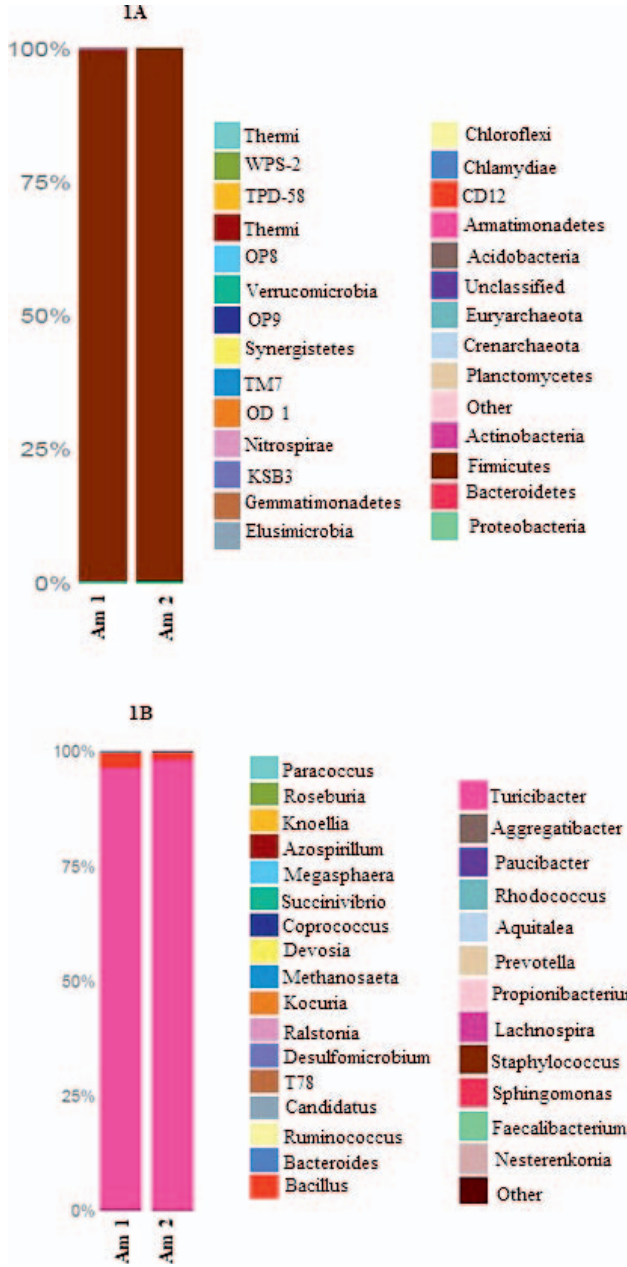

Fig. 1. Stacked bar plots depicting the relative abundance and distribution of bacterial taxa between *Antheraea mylitta* (Am 1 and Am 2) at the phylum (A) and genus (B) levels. Sequences that could not be classified into any group were assigned as unclassified. The *y*-axis represents relative read percentage of different bacterial genome.

1027–1031), *Firmicutes* help host insects withstand extreme conditions and increase their ability to harvest energy from diet. Accordingly, abundant *Firmicutes* microflora in the guts of *A. mylitta* in wild populations is beneficial for survival. Moreover, absence of other microbiota in the *A. mylitta* gut could be due to production of enzyme inhibitors and antibiotics by *Firmicutes* (Pandey et al. 2013, Microb Cell Fact. 12: 35). Apparently, the observed bacterial diversity of *A. mylitta* demonstrates dominance of the lactate-producing *Turicibacter* sp. The abundance of the genus *Turicibacter* sp. has already been reported in the guts of rodents and scarab beetles (Licht et al. 2007, FEMS Microbiol. Lett. 277: 205–209). There are thus far few studies evaluating the interaction of *Turicibacter* with hosts (Egert et al. 2003, Appl. Environ. Microbiol. 69: 6659–6668; Falk et al. 2007, Scand. J. Gastroenterol. 42: 973–985). Furthermore, there is a need for additional studies on the potential interaction of *Turicibacter* sp. and its silkworm hosts to elucidate the influence of diet and habitat on the composition of the gut microbiome and thus its impact on silk production, as shown in previous work with other insect–symbiont relationships (Engel and Moran 2013, FEMS Microbiol. Rev. 37: 699–735; Ley et al. 2008, Science 320: 1647–1651).

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