

Notes and Comments

First report on the characterization of the gut microbiota in *Heliconius erato phyllis* caterpillars (Lepidoptera-Nymphalidae) from southern Brazil

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Microorganisms in the guts of insects play crucial roles in improving nutrition, synthesizing vitamins, establishing metabolic pathways, participating in xenobiotic degradation, and defending against pathogens. Despite its significance, the gut microbiota of *Heliconius* butterflies have been explored in only a few studies to date (Hammer et al., 2014, 2020; van Schooten et al., 2018).

Heliconius (Lepidoptera, Nymphalidae) (Fabricius, 1775) have been well-established model organisms in genetic and evolutionary biology for over 150 years. These neotropical passion-vine butterflies, with around 44 species and over 300 subspecies, inhabit diverse environments from forest fragments to urban areas. Lepidoptera insects play an important role in ecosystems, participating in the pollination and genetic variability of plant species, and shaping the trophic chain of environments. Additionally, several species are also considered agricultural pests and/or of economic interest (Soares et al., 2021). The *Heliconius erato phyllis* subspecies, spanning northeastern Brazil to northern Argentina, exclusively feed on passion vines, which produce cyanogenic glycosides, making the larvae unpalatable to predators (Hammer et al., 2014).

Given the scientific importance of Heliconiines and the roles of microbes in animal biology, understanding the composition and functions of heliconiine-associated microbiomes becomes essential. Until now, there has been no reported information on microbial functions in *H. erato phyllis*. This study aimed to characterize the bacterial community of 5th instar caterpillars, and investigate their interactions with the environment. Specifically, we explored their ability to metabolize toxic components from passion-flower vines, provide protection against pathogens and respond to environmental stressors.

Six fecal samples from 5th instar caterpillars (HEAB2-6, HEAB2-7, HES2-17, HEV2-18, HEV2-26, and HEV2-29) of *H. erato phyllis*, originating from three populations in southern Brazil's forest fragments and urban areas, were analyzed (Table 1) (Huff et al., 2020). DNA extraction, PCR

amplification of the bacterial 16S rRNA gene, sequencing, bacterial community analysis, and functional prediction followed the methodology outlined by Mann et al. (2021). Sequences were deposited in the EMBL database under accession number PRJEB35161. Despite the short read lengths (~290 bp), this targeted gene region is expected to provide sufficient resolution.

A total of 42,212 sequence reads were obtained from preprocessing fecal samples of 5th instar caterpillars of *H. erato phyllis*, yielding 282 operational taxonomic units. Sequence analysis categorized the reads into four phyla, six classes, eight orders, and eight families. Firmicutes (59%) and Proteobacteria (37.42%) were the predominant phyla in all samples. *Lactobacillales* (55.64%) was the most abundant order, followed by *Betaproteobacteriales* (23.64%). At the family level, *Enterococcaceae* (54.38%) was found to be more abundant compared to other families (Figure 1A). Differences in the relative abundance of bacterial families were observed between samples collected from fragment forest and urban areas. However, no significant difference was detected in the diversity metrics used ($p = 0.01$). This study is the first to assess gut bacterial communities in 5th instar caterpillars of *H. erato phyllis* in Brazil. Our findings align with previous research by Hammer et al. (2014, 2020) and van Schooten et al. (2018) on *Heliconius* subspecies from Panama.

Metagenomic functional analysis at the level 2 KEGG pathway predicted that gut microbes possess pathways associated with membrane transport, amino acid metabolism, carbohydrate metabolism, xenobiotic degradation, and more. Upon a more detailed analysis at the level 3 KEGG, the data revealed 19 pathways involved in xenobiotic biodegradation, with benzoate, aminobenzoate, and naphthalene being the most prevalent (Figure 1B). The presence of a bacterial microbiome exhibiting pathways linked to pesticides and aromatic phenolic compounds may indicate environmental stress arising from human-induced pollution within the butterfly

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Table 1. Details of 5th instar caterpillars of *Heliconius erato phyllis* analyzed in this study.

Sample ID	Site	Coordinate
HEAB2-6	Forest fragment	Estação Agronômica de Águas Belas, RS, Brazil
HEAB2-7		
HES2-17		São Francisco de Paula, RS, Brazil
HEV2-18	Urban area	Viamão, RS, Brazil
HEV2-26		
HEV2-29		

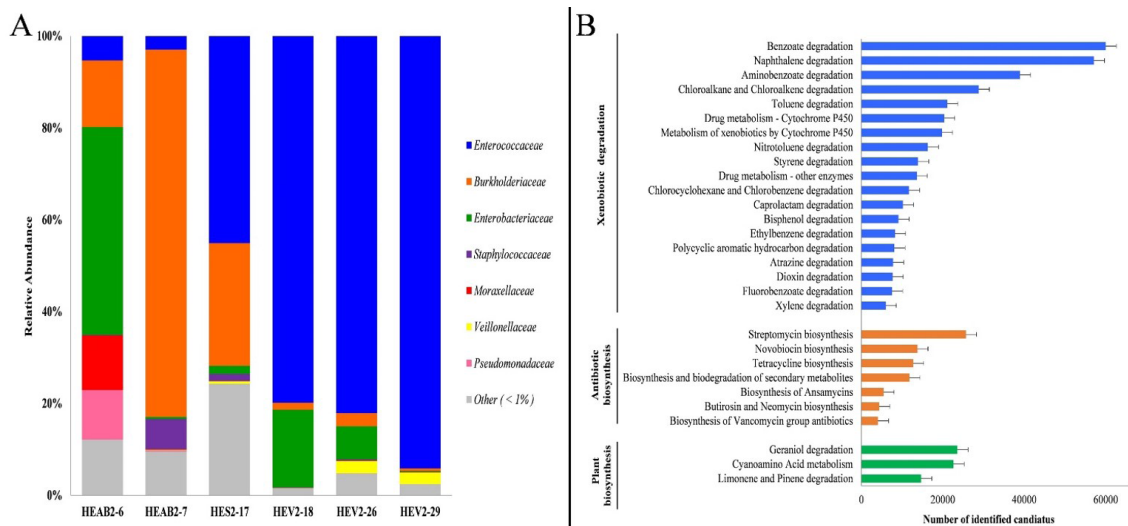


Figure 1. Analysis of gut bacterial communities in 5th instar larvae of *H. erato phyllis* (HEAB2-6, HEAB2-7, HES2-17, HEV2-18, HEV2-26, and HEV2-29). (A) Taxonomic composition at the family level; (B) Average representation of level-3 terms in the KEGG pathway, focusing on xenobiotic degradation, plant degradation components, and antibiotics biosynthesis.

habitat. Additionally, our study observed three pathways associated with the metabolism or degradation of plant components found in passion vines (Figure 1B). *Passiflora* leaves contain cyanogenic glycosides that, upon tissue disruption by herbivore feeding, release hydrogen cyanide (HCN). The occurrence of cyanoamino acid metabolism in the gut microbiota may suggest that these communities assist immatures in metabolizing HCN. The involvement of gut microbiota in assisting insects with digestion of toxic plant compounds has already been reported (Berasategui et al., 2017; Wei et al., 2020). Our study also observed seven antibiotics biosynthesis pathways, with the most abundant being novobiocin biosynthesis, streptomycin biosynthesis, and tetracycline biosynthesis. These antibiotics, biosynthesized by *Streptomyces* spp., show broad-spectrum activity against both Gram-positive and Gram-negative bacteria (Procópio et al., 2012). These results suggest that the gut microbiota potentially benefits *Heliconius* health by protecting against infection through direct antimicrobial antagonism.

In conclusion, this study contributes to our currently limited understanding of the gut microbiota of *Heliconius* species, highlighting potential roles in digesting toxic plant compounds, preventing pathogen invasion, and reflecting

environmental stress induced by anthropogenic pollution in their habitat. Further studies are needed to expand our knowledge of the interactions between gut microbiota and butterfly health.

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