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Do methane and non-methane releasing millipedes depend on their gut microbiome to digest leaf litter?

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Millipedes, one of the most important detritivores in nature, host a community of microorganisms in their guts that may contribute to their nutrition and overall fitness. However, it remains unclear to what extent do millipedes depend on their microbiome. We evaluated the degree of dependence of methane and non-methane releasing millipedes on their gut microbiome using an experimental approach combining chemical inhibitors, microscopy, stable-isotope probing and meta-omics. First, we used either antibiotics or the methanogenesis inhibitor 2-bromoethanesulphate (BES) on juvenile *Epibolus pulchripes* (topical; methane releasing) and *Glomeris connexa* (European; non-methane releasing) to suppress microbial activity. Antibiotics had a large and significant effect on the number of faecal pellets and bacterial plate counts but did not achieve sterilization. It also reduced the weight and CH_4 output in *E. pulchripes* but did not stop it. BES completely inhibited CH_4 production, but recovery was observed after 14-days of feeding on untreated leaves. BES also reduced the abundance of the functional gene for methanogenesis in the faeces—mcrA—but did not affect their weight or faecal pellet production. While the hindguts of antibiotic-treated *E. pulcripes* and *G. connexa* were dominated by Bacteroidota and Proteobacteria, the faeces were dominated by Proteobacteria according to the 16S rRNA amplicon sequencing analysis. Light microscopy and catalysed reporter deposition fluorescence in situ hybridization (CARD-FISH) showed that *E. pulchripes* harbour a multitude of ciliates with ecto- and endo-symbiotic methanogens belonging to Methanobacteriales and Methanomassilicoccales. Surprisingly, these methanogens were still detectable at similar numbers even when methanogenesis was entirely suppressed. We also used RNA stable isotope probing (RNA-SIP) in conjunction with metagenomics to identify key microbial players in the hindguts and distinguish the microbial metabolic potentials of the two millipede species. RNA-SIP results indicated slow labelling of the bacteria over several weeks, with only a few phyla labelled during the first week of feeding on ^{13}C-labelled poplar leaves. We recovered 305 high-quality MAGs (*E. pulchripes* - 282 and *G. connexa* - 33) with ≥ 50% completeness using metagenomics, comprising 18 prokaryotic phyla (*E. pulchripes* - 18 and *G. connexa* - 5). In addition, the MAGs contained some novel bacteria along with some known members of the termite gut microbiota. The results from reconstructed metabolic pathways indicate that the potential role of hindgut bacteria is carbohydrate metabolism, followed by energy metabolism, lipid metabolism, nucleotide metabolism and amino acid metabolism. Analysis of the metatranscriptome is currently ongoing.

Overall, we conclude that while the microbiome is beneficial for the millipede and its composition reflects the prevailing conditions in the gut, it is not essential. Instead, it seems that unlike other methane-releasing animals like termites or ruminants, the millipedes are not dependent on the fermentation products of microorganisms for their nutrition. Together, these results contribute to our understanding of the millipede microbiota and represent the largest genomic resource available to date.