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NOTES AND SHORT COMMUNICATIONS

The Intestinal Microbiota of Tadpoles Differs from Those of Syntopic Aquatic Invertebrates

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Abstract

Bacterial communities associated to eukaryotes play important roles in the physiology, development, and health of their hosts. Here, we examine the intestinal microbiota in tadpoles and aquatic invertebrates (insects and gastropods) to better understand the degree of specialization in the tadpole microbiotas. Samples were collected at the same time in one pond, and the V4 region of the bacterial 16S rRNA gene was sequenced with Illumina amplicon sequencing. We found that bacterial richness and diversity were highest in two studied snail individuals, intermediate in tadpoles, and lowest in the four groups of aquatic insects. All groups had substantial numbers of exclusive bacterial operational taxonomic units (OTUs) in their guts, but also shared a high proportion of OTUs, probably corresponding to transient environmental bacteria. Significant differences were found for all pairwise comparisons of tadpoles and snails with the major groups of insects, but not among insect groups or between snails and tadpoles. The similarity between tadpoles and snails may be related to similar feeding mode as both snails and tadpoles scratch biofilms and algae from surfaces; however, this requires confirmation due to low sample sizes. Overall, the gut microbiota differences found among syntopic aquatic animals are likely shaped by both food preferences and host identity.

Keywords Bacterial community · Gut · Tadpoles · Aquatic invertebrates · 16S rRNA · Illumina sequencing

Associations with bacteria are crucial for the biology of animals [1] and play pivotal roles in the physiology and health of their hosts, which has been intensively studied for the human intestinal microbiota [2, 3]. Animal guts may contain host-adapted core microbiota as well as a flexible, environmentally modulated microbial pool, plus transient environmental bacteria [4], and the proportion of these components may differ among major host groups [4, 5].

Amphibians stand out among vertebrates for their biphasic life history, and in particular, the anuran tadpole stands out as a highly distinct larval form. Their long intestine and filter-feeding apparatus, observed in the majority of species, characterize tadpoles as microphagous animals with a predominantly herbivorous diet, although their trophic status is still understudied [6]. The intestinal microbiotas of tadpoles differ from that of adult frogs in composition and by having a higher diversity [7, 8]. Furthermore, in tadpoles, core community members are shared across continents and appear to be absent in adults [8]. Although tadpole intestinal microbiotas differ among host species [8], they are also influenced by ecological factors, such as aquatic environment and temperature [8, 9].

Here, we examine the intestinal bacterial community of tadpoles in direct comparison to intestinal microbiotas of strictly syntopic aquatic invertebrates (insects and gastropods), to better understand the degree of specialization in the composition of tadpole microbiotas.

Fieldwork was carried out in Itapé, Rio Claro municipality, São Paulo state, Brazil (22.32618 S, 47.712576 W). Specimens were collected opportunistically by dipnetting on 12 and 23 April 2016 in a single small pond (6 m in diameter), taken to the lab in water from the original water body, and processed within 12 h of capture. Invertebrates were sacrificed by freezing and tadpoles by lidocaine overdose. Intestinal tract samples were dissected with sterilized scissors and tweezers,

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stored in separate sterile microcentrifuge vials and immediately frozen. For improved identification of invertebrates, DNA was extracted from small tissue samples of selected specimens and a segment of the mitochondrial COI gene was amplified and sequenced. Our data set contained one species of anuran tadpole (Anura; *Scinax fuscovarius*), one species of snail (Gastropoda; Physidae; *Aplexa* cf. *marmorata*), two species of water beetles (Coleoptera; Hydrophilidae and Dytiscidae), larvae of two species of dragonfly (Odonata; *Micrathyria ocellata* and *Remartinia* sp.), and two species of water bugs (Hemiptera; *Ranatra* sp. and *Lethocerus* sp.). COI sequences of each species were submitted to GenBank (accession numbers KY981498-KY981518).

Genomic DNA was extracted using the MoBio PowerSoil-htp 96-well kit (MoBio, Carlsbad, CA, USA) as in Bletz et al. [10]. We PCR-amplified in duplicate [10] the V4 region of the bacterial 16S rRNA gene using a dual-index approach with barcoded primers 515F and 806R [11]. Pooled samples were purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced with paired-end 2 × 250 v2 chemistry on an Illumina MiSeq sequencer. Raw sequence reads were deposited NCBI short read database (SRA) (bioproject accession PRJNA368741).

We processed sequences using the QIIME pipeline v1.9.1 [12] on Mac OS X. The forward read sequences were quality filtered, demultiplexed, and trimmed to 150 nt. We used the Deblur workflow (*a sub-operational taxonomic unit approach*; [13]) to cluster reads, and taxonomy was assign with the Ribosomal Database Project (RDP) Classifier [14] using the Greengenes 13.8 reference database (May 2013 release; http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). Operational taxonomic units (OTUs) comprising less than 0.001% of the total reads were filtered out and a phylogenetic tree was built with FastTree2 [15]. We rarefied samples to 1000 reads and calculated the number of observed OTUs, Chao1, and Shannon and Faith's phylogenetic diversity as proxies for bacterial richness and diversity. We quantified the similarities between bacterial communities (beta diversity) among groups of individuals using both unweighted and weighted UniFrac metrics with QIIME [16] and used the distance matrices to generate principal-coordinate ordination plots. We calculated core microbiomes as OTUs that were present on 50% of individuals per sample group, discarding OTUs with abundances < 3 reads per group. We calculated shared OTUs among group after removing OTUs with < 5 reads across all samples.

Sequencing of the V4 region of the 16S rRNA gene for the 44 samples analyzed (Supplementary material) yielded 564,825 high-quality reads binned into 3688 sub-operational taxonomic units (sOTUs), with an average of 10,657 reads per sample. Bacteria that typically inhabit insects as endosymbionts (*Wolbachia*, *Rickettsia*, *Arsenophonus*, and *Cardinium*) were exceedingly rare, with only 4 reads of *Wolbachia* in a dragonfly larva and a beetle and 5 reads of *Cardinium* in two dragonfly larvae and a snail. These bacterial taxa are reproductive parasites [17], which might explain why they were uncommon in the intestinal tract of the analyzed species.

Phylogenetic diversity (PD) and number of OTUs were highest in the snail samples, intermediate in tadpoles, and lowest in the four groups of aquatic insects (Fig. 1). Tukey-Kramer pairwise tests revealed significant differences for both metrics ($P < 0.05$) for all pairwise comparisons of tadpoles and snails with major groups of insects, but not among insect groups or between snails and tadpoles.

Microbial composition and structure differed distinctly among groups (PERMANOVA, weighted Unifrac, Pseudo-F = 4.8279, $p = 0.001$; unweighted Unifrac, Pseudo-F = 4.5562, p = 0.001). Pairwise comparisons revealed that the tadpole microbiota differed significantly ($P < 0.01$) from all insect microbiotas, but not from that of snails for both metrics ($P > 0.05$); however, given a sample size of $N = 2$ for snails, this lack of significance is to be taken with caution. In the PCoA, hemipterans were most clearly separated along the first principal coordinate, while tadpoles were rather similar to snails and dragonfly larvae. The second principal coordinate separated tadpoles and the snail from dragonflies. This pattern was also maintained when adding data of other tadpole, fish, and invertebrates from a previous study [8] (Supplementary material).

At the level of bacterial classes, tadpoles differed from invertebrates by (a) an overall more even community, with several classes represented in similar proportions, (b) a substantially greater proportion of Flavobacteria, and (c) along with snails, a relatively high proportion of Bacilli (Supplementary material). All sample groups shared a high number of OTUs, suggesting that a significant proportion of the OTUs found might be transient environmental bacteria. On the other hand, all sample groups also had a substantial number of exclusive bacterial OTUs in their guts (Fig. 1e, f). Of a total of 374 OTUs found in tadpoles, 65 (17%) were not found in any of the invertebrate samples. However, of 47 core-50 OTUs of the tadpoles, 43 (91%) were also found in the core community of invertebrate groups; 42 of these were found in the snails.

The majority of the aquatic insects included in this study are carnivorous while plant matter and algae make up an important part of the diet of tadpoles. Tadpoles also ingest variable proportions of animal matter, and hydrophilid beetles are to a large extent herbivorous; however, the tadpole intestinal microbiotas were more diverse than those of all the insects included in the study. This complies with a general pattern of insects harboring a lower microbial diversity than vertebrates [5, 18]. The snail included in the study can be expected to be herbivorous and had a diverse microbiota as has been seen in other freshwater snails (e.g., [19]). In fact the snail intestinal microbiota observed was diverse with many core taxa shared with the tadpoles, possibly due to feeding

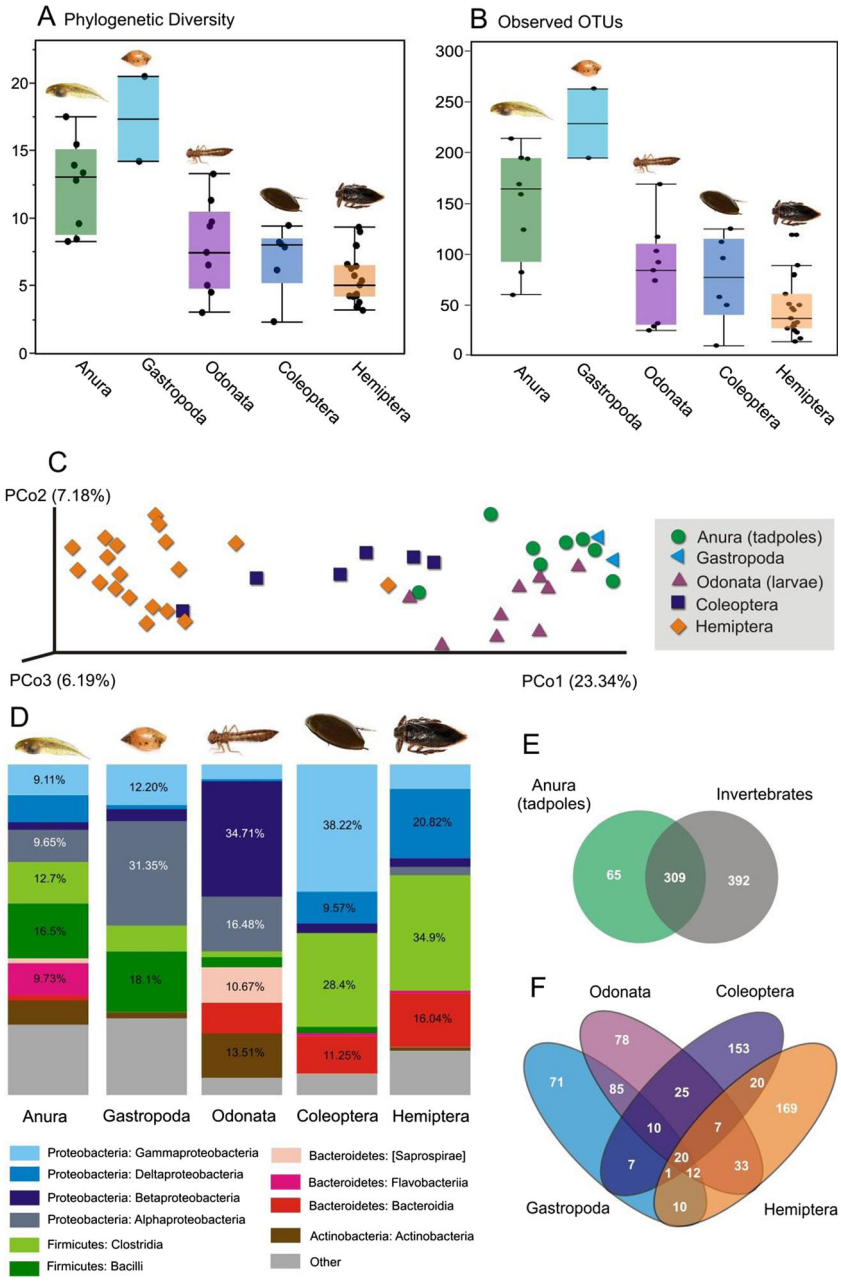

Fig. 1 Variation of the intestinal microbiota of tadpoles (*Scinax fuscovarius*) in comparison to that of syntopic invertebrates in a pond in Itapé, Rio Claro, São Paulo, Brazil. **a** Phylogenetic diversity and **b** total number of bacterial OTUs per individual in gut samples of tadpoles and comparative invertebrates. **c** Principal coordinate plot based on community structure of intestinal microbiota, with percentages indicating the amount of variation explained by each PCo. **d** Composition of intestinal microbiota at the class level; for a complete plot including the other classes (here in gray), see Supplementary materials. **e** Venn diagram showing the number of bacterial OTUs exclusive to and shared by the intestinal microbiota of tadpoles vs. all invertebrates studied and **f** among the four main groups of invertebrates

similarities in scratching biofilms and algae from surfaces. However, due to the very small sample size of only two snail individuals, these preliminary conclusions require confirmation and might not apply beyond the system studied here. Although we did not measure body size of all studied individuals, the invertebrates included in this study had very different body sizes, from 20 to 30 mm for the snails and some beetles to over 80 mm in the giant water bugs of the genus *Lethocerus*; however, there was no obvious correlation of host body size with diversity of their gut microbiota as has previously been detected in vertebrates [20]. In fact, the *Lethocerus* microbiota, similar to that of the other bugs studied, had among the lowest bacterial diversity in our data set.

To summarize, the organisms studied herein were exposed to the same environmental bacterial reservoir, reflected by a high proportion of OTUs shared among the different groups. The differences found among groups in their gut microbiota appear to be predominantly shaped by diet and feeding mode, with smaller but important host-specific effects. Tadpole intestinal microbiotas are overall different from those of aquatic invertebrates, although many of their core microbiota components might be shared with herbivorous invertebrates such as snails.

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Compliance with ethical standards Samples were collected under collection permit SISBIO #52865 and the study has been approved by the ethics committee at the Universidade Estadual Paulista (UNESP; CEUA N36/2015).

References

1. McFall-Ngai M, Hadfield MG, Bosch TC, Carey HV, Domazet-Lošo T, Douglas AE et al (2013) Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. U. S. A. 110:3229–3236
2. Clemente JC, Ursell LK, Parfrey LW, Knight R (2012) The impact of the gut microbiota on human health: an integrative view. Cell 148(6):1258–1270
3. Tuddenham S, Sears CL (2015) The intestinal microbiome and health. Curr. Opin. Infect. Dis. 28(5):464–470
4. Shapira M (2016) Gut microbiotas and host evolution: scaling up symbiosis. Trends Ecol. Evol. 31:539–549
5. Engel P, Moran NA (2013) The gut microbiota of insects—diversity in structure and function. FEMS Microbiol. Rev. 37:699–735
6. Altig R, Whiles MR, Taylor CL (2007) What do tadpoles really eat? Assessing the trophic status of an understudied and imperiled group of consumers in freshwater habitats. Freshw. Biol. 52:386–395
7. Kohl KD, Cary TL, Karasov WH, Dearing MD (2013) Restructuring of the amphibian gut microbiota through metamorphosis. Environ. Microbiol. Rep. 5:899–903
8. Vences M, Lyra ML, Kueneman JG, Bletz MC, Archer HM, Canitz J, Handreck S, Randrianiaina RD, Struck U, Bhuju S, Jarek M, Geffers R, McKenzie VJ, Tebbe CC, Haddad CFB, Glos J (2016) Gut bacterial communities across tadpole ecomorphs in two diverse tropical anuran faunas. Sci Nat 103:25
9. Kohl KD, Yahn J (2016) Effects of environmental temperature on the gut microbial communities of tadpoles. Environ. Microbiol. 18: 1561–1565
10. Bletz MC, Goedbloed DJ, Sanchez E, Reinhardt T, Tebbe CC, Bhuju S, Geffers R, Jarek M, Vences M, Steinfartz S (2016) Amphibian gut microbiota shifts differentially in community structure but converges on habitat-specific predicted functions. Nat. Commun. 7:13699
11. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79:5112–5120
12. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK et al (2010) QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335–336
13. Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Zech Xu Z, Kightley EP, Thompson LR, Hyde ER, Gonzalez A, Knight R (2017) Deblur rapidly resolves single-nucleotide community sequence patterns. mSystems 2:e00191-16
14. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73:5261–5267
15. Price MN, Dehal PS, Arkin AP (2010) FastTree 2—approximately maximum-likelihood trees for large alignments. PLoS One 5:e9490
16. Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71:8228–8235
17. Duron O, Bouchon D, Boutin S, Bellamy L, Zhou L, Engelstädter J, Hurst GD (2008) The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol. 6:27
18. Pernice M, Simpson SJ, Ponton F (2014) Towards an integrated understanding of gut microbiota using insects as model systems. J. Insect Physiol. 69:12–18
19. Van Horn DJ, Garcia JR, Loker ES, Mitchell KR, Mkoji GM, Adema CM, Takacs-Vesbach CD (2012) Complex intestinal bacterial communities in three species of planorbid snails. J Molluscan Studies 78:74–80
20. Godon JJ, Arulazhagan P, Steyer JP, Hamelin J (2016) Vertebrate bacterial gut diversity: size also matters. BMC Ecol. 16:12