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Research Article

A Preliminary Study on Desert Invertebrate Gut - A Metagenomic Evaluation of Bacteria Community

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Abstract

The microbiota of the digestive system have recently been at the heart of many studies. Most of these studies dealt with the human digestive tract, e.g. the effect of different types of microbiota on physical and mental health. The objective of this study was to identify the bacterial and fungal populations in the digestive system of common desert invertebrates of different trophic levels. As a preliminary study, we collected four invertebrates that share the same habitat in a desert system in the northern Negev, Israel, over a period of one year. The organisms in which gastrointestinal microbiota were determined included *Scorpio palmatus*, *Adesmia dilatata*, *Sphincherochila zonata* and *Hemilepistus reaumuri.* Microbial diversity was determined by sequencing DNA harvested from their digestive system. Of a total of 32 orders of bacteria, 26 were present in *S. maurus*, 22 in *S. zonata*, 14 in *A. dilitata* and 8 in *H. reaumuri*. A significant difference in bacterial diversity was found between *S. zonata* and *A. dilatata*. The present study elucidates the importance of the feeding habitat and trophic structure to the microbiotic diversity of the digestive system.

Keywords: Microbiota; Digestive System; Unpredictable Food Source; Extreme Habitat; Diversity

Introduction

The relationships between the host and its gut microbiota range across the entire spectrum of interactions, i.e. from pathogenic to obligate mutualism [1]. Growing evidence indicates that the capacity of organisms to adapt to new habitat conditions basically depends on their ecophysiological plasticity attributes, where their gut commensal microbiota might be an essential impact factor [2]. The gut environment is considered to be an unstable system, due to its multifunctional goals and its response to changes in its surrounding niches, such as food source composition and availability, which affect secretion of digestive enzymes, abiotic physical disturbance and other physiochemical conditions that are typically unfavorable for colonization [3,4]. The gut functions are fulfilled by the microorganisms inhabiting it, and these are influenced by the host's diet, food source availability, developmental stage [5] and geographical location [6]. Despite growing awareness of the importance of bacterial communities in the digestive tract, their presence, diversity and role in the digestive system of desert invertebrates are not well-understood.

Insects are among the most abundant invertebrates in the world, and can be found in all ecosystems. Macrofauna that include invertebrates larger than 2 mm, on average, have a limited proper ability to digest complex organic substrates in the soil. In order to symbiotically exploit soil and organic resources, they developed interactions with microflora. Such an interaction yields micro-food webs that link between the organisms and their predators. Invertebrates inhabiting extreme desert environments are exposed to countless and unpredictable environmental glitches that determine food availability, behavioral activity and functionality [7]. One of the main factors determining gut microbiota in desert environments is the unpredictability of food variety and availability, which

may yield host species that are more phylogenetically and trophically related. Most studies on gut microbial diversity in insects were conducted on termites [8,9], ants [10,11] and beetles [12,13].

The objective of the present study was to identify the bacterial community in the digestive system of common desert invertebrates that share the same habitat. We collected the four most common invertebrates that carry out their entire biological functions in the desert system of the northern Negev, Israel. The organisms selected for determining gastrointestinal bacteria were *Scorpio palmatus*, *Adesmia dilatata*, *Hemilepistus reaumuri* and *Sphincherochila zonata*. Each of these invertebrates was assumed to present potentially distinctive gut microbial communities, since they belong to different trophic groups. *S. palmatus* is a burrowing scorpion, a predator with a foraging strategy of sitting and waiting near the burrow entrance [14]. *A. dilatata*, one of the dominant beetles at the study site [15], is known for its consumption of organic matter, mostly plant-dried substances. However, it also consumes animal-derived food such as insect carcasses. The desert isopod *H. reaumuri* feeds on soil crust and dry plant material and is therefore classified as a saprovore, herbivore and microbivore [16]. The desert snail *S. zonata* spends approximately 95% of the year in the aestivation stage. In the remaining 5%, it fulfills its biological functions, which include feeding on soil crust that contains soil algae, lichens, bacteria and fungi only after rains [17]. Nutrition, i.e. food consumption of an unpredictable variety and source, especially in xeric environments, is one of the main factors that affect colonization of gut symbionts. Each of the four representatives of the desert invertebrates' gut in this study is inhabited by a range of microbial populations that facilitate colonization of the gut environment due to long-term coevolution [18].

The goal of the present study was to elucidate the importance of the feeding habitat to the bacterial diversity of the digestive system and trophic structure. We applied the DNA molecular technique according to Günther., *et al*. [19] and Eitzinger., *et al*. [20] in order to test their gut microbial community that stems from the intake of different foods.

Materials and Methods

Study site

The field study was conducted near Sde Boker (latitude: 30.86144, longitude: 34.779011), a loess plain site in the vicinity of the Desert Research Institute, Sede-Boker Campus, in the Negev Desert Highlands, Israel. Elevation at the site is about 600 m above sea level. The area has a temperate desert climate, with hot summers (mean maximum 32ºC, mean minimum 17.7ºC, in June) and cool winters (mean maximum 14.8ºC, mean minimum 5.4ºC, in January). The soils are brown, shallow, rocky, desert soils (brown lithosols), loessial and grey desert soils (loessian serozems). Vegetation at the research site is a mixture of perennial shrub communities (about 10% of the area), mainly *Hammada scoparia*, *Zygophyllum dumosum*, *Artemisia sieberi*, and a variety of annual plants (differing significantly according to rain) and geophytes [21].

Four different species of organisms were collected at the study site: *S. palmatus*, *A. dilatata*, *S. zonata* and *H. reaumuri* (18 individuals of each organism). The samples of each species were randomly split into two groups in the Terrestrial Ecology Lab, Bar-Ilan University: 9 individuals were transferred to refrigerated storage at 5°C, and 9 to -20°C for digestive system excision and DNA extraction.

Laboratory tests

Prior to DNA extraction from the digestive system, the organisms were washed for a few seconds with 70% ethanol, and each specimen was weighed. Digestive system excision was performed in a sterile environment using sterile tools, e.g. scissors and tweezers, and the extracted gut weight was determined (n = 9 for each species). According to our preliminary study, the amount of extracted gut per specimen was adequate for DNA microbiota analysis.

Molecular analysis

DNA was extracted from the entire digestive tract of each invertebrate (n = 3) using an Invitrogen PureLink® Genomic DNA Mini Kit. DNA concentration was measured using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer.

Polymerase chain reaction and gel electrophoresis

Amplification of the 16S rRNA gene of bacterial sequences was performed using PCR (Applied PCR), Veriti 96-well thermal cycler primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1100R (5'-GGGTTNCGNTCGTTG-3') for the bacterial 16S rRNA. Two PCRs were carried out for each sample, where each contained 25 µL of reaction mixture consisting of: 17.25 µL ultrapure water, 5 µL 5×Phusion® HF reaction buffer, 0.5 µL dNTP mix, 0.5 µL forward primer (with barcode in the second PCR), 0.5 µL reverse primers,

and 0.25 µL Phusion® HF DNA polymerase. In the first PCR, 1 µL of DNA was added to each well, while in the second PCR, 1 µL from the first PCR was added. All PCR products were run on agarose gel with a negative control. In order to prepare the gel, 70 mL TBE were mixed with 1.2 g agarose, and the mix was placed in a microwave oven for 1 min – until the agarose was completely dissolved – and then 1 µL of GelStar™ Nucleic Acid Gel Stain was added. The sample was loaded with buffer and a 1 kb DNA ladder (Thermo Scientific).

PCR purification

The PCR products were purified using the Agencourt AMPure XP PCR purification systems that use Agencourt's solid-phase paramagnetic bead technology for high-throughput purification of PCR amplicons. Each PCR sample was filled with ultrapure water up to 100 µL, followed by an additional 180 µL of AMPure XP. The PCR products were bound to paramagnetic beads, washed with ethanol 70%, and eluted with water. All PCR products obtained from the DNA sample products underwent Ion Torrent sequencing [22].

Bioinformatics analysis

Reads were trimmed by quality values using the fastq filter command from Usearch v9.0 (Edgar 2010). Sequences were filtered by overall read quality using fastq_maxee and a MAX_EE value of 1. The Usearch cluster_otus discarded likely chimeras and clustered OTUs at 97% identity. Determination of OTU taxonomic assignment was performed using QIIME, version 1.7.0 [23], with the greengenes_13_8 reference database. Representative sequences were picked for each OTU, and taxonomic information was annotated for each representative sequence by the RDP classifier in QIIME. Alpha and beta analyses were carried out using QIIME. Heat map and other figures were created using gplot in R (R Core Team 2019, 2019).

Statistical analysis

A two-way ANOVA was carried out using the SAS program (SAS Inst., 1988) with a significance threshold of $p < 0.05$. Duncan and Tukey's mean multiplication test was used to determine variance between the averages. Statistical analyses conducted in R (R Core Team 2019, 2019) were conducted by using the vegan [24] and Ampvis2 [25] R packages.

Results

Bacteria community

A total of 117,029 high-quality sequence reads were identified as belonging to the bacteria domain Each invertebrate sample had average reads as follows: (1) *S. palmatus* (1976); (2) *A. dilitata* (2060); (3) *S. zonata* (2880); and (4) *H. Reaumuri* (4355). The total number of OTUs was 643.

The relative abundance of the 31 bacterial orders belonging to four phyla are presented in figure 1. Of the 31 orders, only three, namely *Acidimicrobiales, Rhodobacterales* (Table 1), were present in all four invertebrates, where *Acidimicrobiales* were represented by 38, 28.4, 17.4 and 10.9% in *H. reaumuri*, *S. maurus*, *A. dilitata* and *S. zonata*, respectively. No unique bacteria were found in *A. dilitata*. However, *Pseudomonadales, Gammaprodeobacteria* and Enterobacteria were present at high rates of 15, 16 and 33%, respectively. These orders were present in *S. maurus* and *S. zonata* at very low rates. Five unique bacteria representatives, *Oscillatoriales, Thermales, Roseiflexales, Legionellales and Caulobacterales* were represented in the guts of *S. maurus* at a relatively low rate. Two unique bacteria were found in the gut of *S. zonata*: *Turicibacterales* represented by 23.9% of the total bacterial community, and Clostridiales with less than 1% (Table 1 and figure 1). Four bacterial orders exhibited presence greater than 20% of the total community in the gut of *H. reaumuri* and *S. palmatus*: *Acidimicrobiale* with 38% and 28.4%, respectively; *Enterobacteriales* with 33.3% in the gut of *A. dilitata*; Turicibacterales with 23.9% in the gut of *S. zonata*; and *Cytophagales* with 34.4% in the gut of *H. reaumuri* (Figure 2).

It can be seen that all 31 bacterial orders are present in all four organisms (Table 1). Each organism has several different OTUs: 13 for *A. dilitata*; 7 for *H. reaumuri*; 25 for *S. maurus*; and 21 for *Z. zonata*.

In order to visualize the distribution of the bacterial community, we applied the phylum level in each of the invertebrates, and a heatmap was generated (Figure 3). A dendrogram was prepared using the Bay-Curtis index to compare similarities between the bacterial communities of the four invertebrates. Each column represents an individual specimen. The columns were clustered according to the similarity of the bacterial abundance profile at the phylum level.

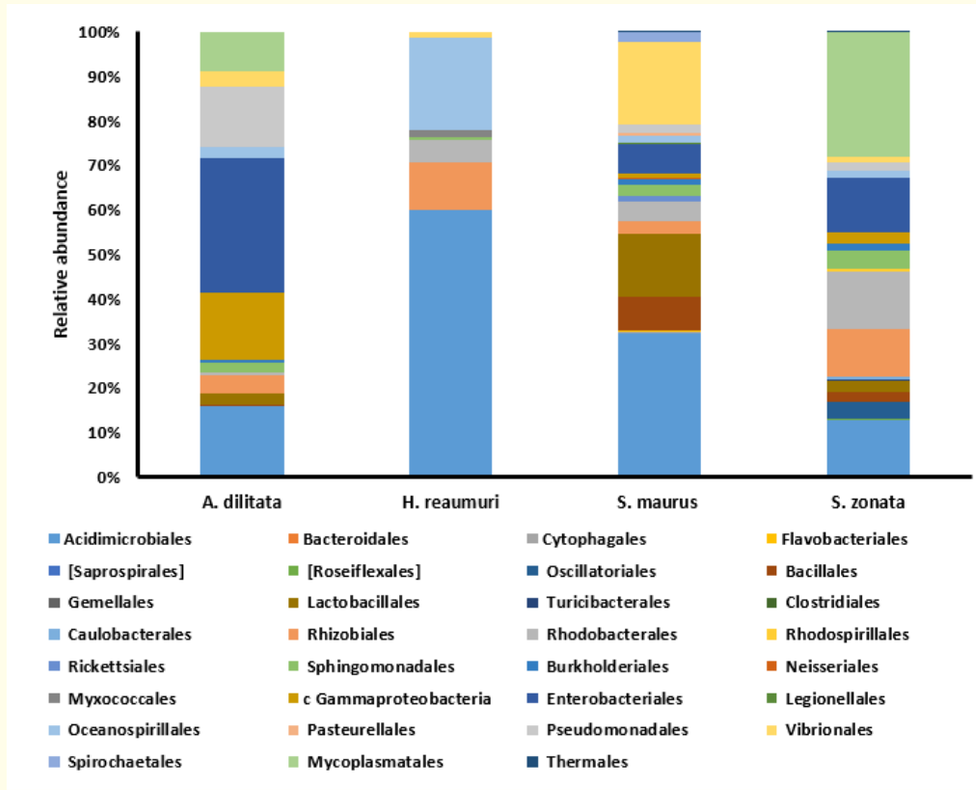

Figure 1: Relative abundance (percentage) of bacterial orders detected in the guts of the four studied invertebrate communities, DNA based on sequencing platform and primer set used.

Total bacterial (order %) present in the gut of the different four invertebrate (* - less than 1% present)					
		A. dilitata	***H. reaumuri***	***S. maurus***	***S. zonata***
	Order			**palmatus**	
	Acidimicrobiales	17.4	38.0	28.4	10.9
	Rhodobacterales	*	*	*	*
	Pasteurellales		*	02.0	03.7
	Cytophagales		34.4	04.3	05.4
	Myxococcales		06.6	02.4	09.1
	Neisseriales		03.3	03.9	11.1
	Saprospirales		13.2	01.2	01.3
	Bacteroidales			*	*
	Flavobacteriales			06.5	01.8
	Gemellales			12.4	02.4
	Rhodospirillales			*	02.0
	Rickettsiales			06.0	10.6
	Spirochaetales			01.8	01.7
	Vibrionales	03.9		*	*
	Pseudomonadales	15.0		01.3	01.2
	Bacillales	*		*	
	Burkholderiales	*		*	
	Gammaproteobacteria	16.3		*	
	Rhizobiales	04.7		*	
	Sphingomonadales	02.3		*	
	Lactobacillales	02.6			*
	Mycoplasmatales	09.6			*
	Oceanospirillales	02.5			*
	Enterobacteriales	33.3			03.2
	Roseiflexales			*	
	Oscillatoriales			01.2	
	Legionellales			*	
	Caulobacterales			*	
	Thermales			01.8	
	Clostridiales				*
	Turicibacterales				23.9
Total	31	13	7	25	21

Table 1

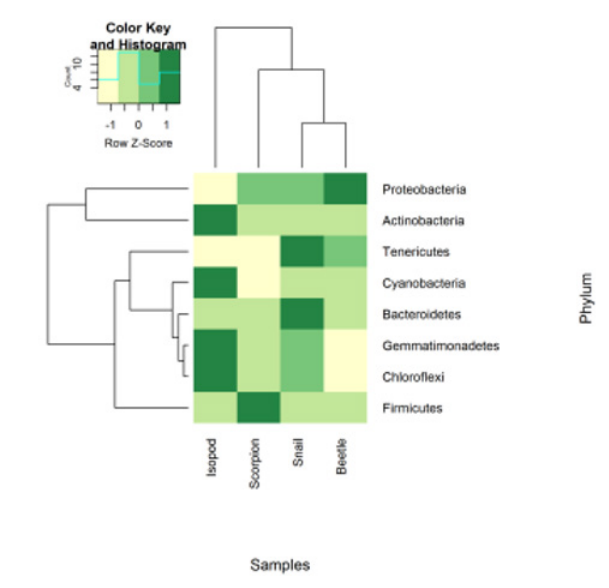

Figure 2: Heat map of order level abundance. Six bacterial community phyla were found in the gut of the four invertebrates. Hierarchical clustering was performed using hclusing default parameters (complete linkage). The green colors represent a Z score used to illustrate the expression of genes for each invertebrate expressed by bacterial community phyla.

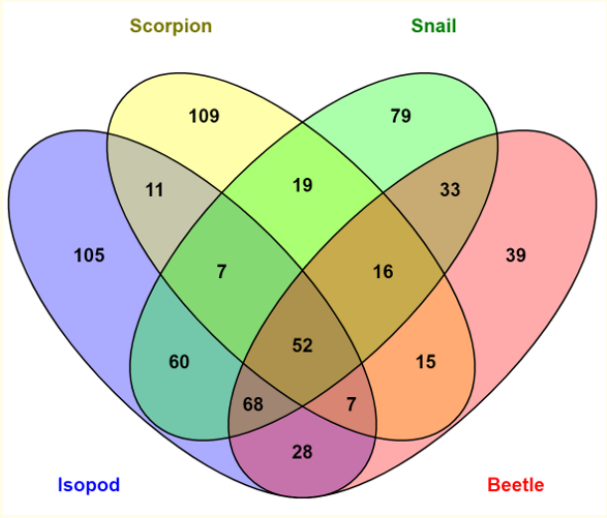

Figure 3: Venn diagram showing the distribution of bacterial OTUs between (a) b c d – individuals based on the 16rRNA pyrosequencing analysis represented as taxonomical order level. The numerical labels are the shared and individual OTUs for each individual invertebrate species.

We analyzed the number of OTUs shared among the four species. The intra-specific differences between the four invertebrates' gut community were found with the majority of OTU's being unique to *S. palmatus* (Figure 4), and only 52 OTUs (8%) being shared among the four invertebrates. The results indicate great bacterial variation among the species.

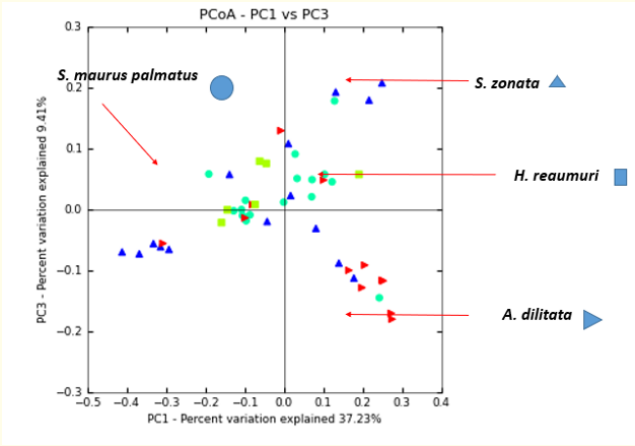

Figure 4: Principal coordinate analysis showing distinct clustering patterns of the gut microbiota of S. palmatus, A. dilitata, S. zonata and H. reaumuri of the 16s rRNA libraries, explaining the most of the variation in determining gut bacterial composition.

The Shannon-Weaver diversity index (H), which is commonly used to characterize species diversity in a community, evaluated *A. dilitata* as 1.73, *H. reaumuria* as 1.31, *S. maurus* as 2.15 and *S. zonata* as 2.16. Equitability (H/Hmax) yielded values lower than 1: 0.67, 0.50, 0.82 and 0.83 for *A. dilitata*, *H. reaumuri*, *S. maurus* and *S. zonata*, respectively. The obtained ratio emphasizes the randomness in the microbial gut community composition that is controlled by randomality of food source availability. Depicts the beta diversity of the bacterial communities associated with the four investigated invertebrates which was determined through a PCoA (Figure 4). The two components explain 46.64% of the variation (first component 37.23%; second component 9.41%). The grouped dots in the figure indicate that the distribution of the microbial community is not uniform. A wider dispersion is shown for *S. zonata* followed by *S. palmatus* in comparison to the other two invertebrates, indicating differences in their beta diversity.

Discussion and Conclusion

A significant number of studies on the diversity of invertebrates focused on the gut microbiome. However, most of these studies were dedicated to marine and humid terrestrial invertebrates. The present study is the first to investigate the gut microbiomes of four invertebrate species that share the same Northern Negev desert habitat. The driving force and the linkage between the four species are based on the unpredictability of the food source. The food source is unpredictable for all four species. Their gastrointestinal track must therefore perform efficient digestion and protective functions [26]. Two members of the group, *H. reaumuri* and *S. zonata*, share a similar diet. Both feed on soil crust and dry organic matter, whereas *A. dilitata* is a detritivore. These three species are a potential food resource for *S. maurus*, a predator. The variation among the feeding forms, the difference in the nutritional substrates and the variation in composition lead to variations in the gut-microbiome composition [27]. The gut microbiome of the four invertebrates, which was determined using molecular tools, was found to converge into three groups: 1. *A. dilitata*; 2. *H. reaumuri*; and 3. *S. palmatus* and *S. zonata* that can be differentiated by the bacterial diversity in the gut content. Improvements in the molecular information may allow a better characterization of the differences in digestive capacity.

Members of the phyla *Proteobacteria*, *Actinobacteria* and *Firmicutes* are the most abundant soil bacteria [28]. These phyla are the dominant members of the total gut microbiome, represented by 83% in *A. dilitata*, 63% in *H. reaumuri*, 93% in *S. palmatus* and 62% in *S. zonata*.

The diet of *A. dilitata* consists predominantly of dry organic debris (e.g., plants, fungi, animal and plant debris) [29]. *Enterobacteriales* were represented by over 33% of the total bacterial community. They are found in other beetle guts and are known to be involved in polysaccharides metabolism as well as in nitrogen-fixing processes [30,31]. Studies conducted by Rojas-Jimenez and Hernandez [32] on insect bacterial guts found that their guts contained several groups of bacteria: approximately 44% *Proteobacteria*, which are related to lignocellulolytic activities, 33% Firmicutes, and a relatively low percentage (less than 5%) of *Tenricutes*. According to Wang., *et al*. [5], the community structure of gut bacteria in Coleoptera larvae is dominated by *Proteobacteria, Firmicutes, Actinobacteria*, *Tenericutes*, *Bacteroidetes* and *Acidobateria*. The above results are similar to our findings that showed that the dominant bacteria in the A*. dilitata* gut were *Proteobacteria* (63.2%), *Actinobacteria* (21.7), *Tenericutes* (11.9%) and *Firmicutes* (3.3%).

H. reaumri that inhabit the desert terrestrial environment in the Negev desert are known to be effective herbivorous scavengers, feeding predominantly on soil crust and different plant materials [16]. They promote microbial activity by fragmentation of the feeding substrate and ingesting microorganisms. Their gut microbiota were represented by only three phyla: *Actinobacteria*, *Planctomycetes* and *Cyanobacteria*. The above representatives are known to be present in a wide range of cold, hot, terrestrial and aquatic habitats, are very abundant in soils and are known as a group that is involved in lignin degradation, in C, N, S and P cycling, and have the capability of oxidizing ammonium [33]. According to Rok [34], the food preference of isopods indicates the presence of cellulolytic microbiota in their digestive track.

S. palmatus, the most abundant scorpions of the loessial plains in the Negev desert highlands, prey on *H. reaumuri*, *A. dilitata*, ants and other invertebrates that cross their burrow entrance. They are known as extremely efficient eating machines which can increase their body weight by one-third during the feeding period. The metagenomics sequencing of microbial representatives in the food ingested by *S. palmatus* was represented by *Proteobacteria* (41.5%), *Actinobacteria* (31.1%), *Firmicutes* (21.1%), *Cyanobacteria* (4.7%) and *Sprirochaetes* (2.0%). A similar study conducted by Bolaños et al. [35] on gut microbials of two scorpion species obtained from South-Central Mexico, a temperate region, showed similar values to the ones in our study: *Proteobacteria* (59%), *Firmicutes* (13%) and *Actinobacteria* (12%). The *Spirochaetales* order, which in the current study is present only in *S. palmatus*, was represented by only 2.0%, whereas it is dominant in the hindgut of both lower and higher termite species, and appears to be responsible for most of the acetogenic activity, known as an energy generating mechanism [36].

Snails in general thrive in habitats rich in calcium and with a cool and moist soil, which is a necessity for reproductive purposes, e.g. egg laying and hatching. In desert ecosystems, snails need to fulfill their biological function in a very short period and be able to accumulate enough energy for the long (approximately 95% of the year) aestivation period. Their gut microbiota should therefore function as a very efficient "biotechnology industry" during the

short active period. Their feeding activity is related to soil crust rasping, which has a strong input on soil microflora and microbiota community succession and nutrient cycling [16,37]. Due to their feeding behavior, the desert snail *S. zonata*, as well as other desert snails feeding on soil crust, require a lignocellulosic and microbial digestion capability [38]. Dar., *et al*. [39] study on gut microbiome analysis of snails inhabiting marine, freshwater and terrestrial feeding habitats, and their use as food and medicine, showed that the snails' digestive functions are strongly related to spatial distribution and many metabolic properties.

The metagenomics sequencing detected in the gut of *S. zonata* was found to contain *Actinobacteria* (13.4%), *Proteobacteria* (47.6%) and *Tenerictutes* (29.3%). The *Cyanobacteria* phylum represented by Oscillatoriales (3.7%) was found only in the digestive track of *S. zonata* and not in the other invertebrates analyzed in the present study. These phyla are known to play an important role in nutrient cycling and comprise a biological consortium whose role is not well-understood [40].

As an overall interpretation of gut microbiota among the multifaceted environmental and food availability factors in the desert environment, the bacterial community was found to be highly representative of each of the invertebrates' feeding behavior. The differences in the bacterial gut community between the four invertebrates are represented by versatility in bacterial functions, e.g. lignin-ligninolytic activity, cellulose activity, toxin degradation activity, nutrient provisioning, N cycling and decaying activity.

Proper nutrition affects the longevity and successful performance of all biological functions of these desert-xeric invertebrates, which face extreme and unpredictable abiotic conditions, such as food source heterogeneity. Our hope is that this study will serve as a basis for future studies on the gut microbiomes of desert-xeric invertebrates.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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