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ORIGINAL ARTICLE

DNA metabarcoding of gut microbiota reveals considerable taxonomic differences among wild individuals of the dung beetle *Trypocopris pyrenaeus* (Coleoptera: Geotrupidae)

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Abstract. The gut microbiota of dung beetles comprises bacteria, archaea, and fungi. Most studies have only considered the bacterial element and focused on differences between species reared in the lab. This study considered microorganisms from the gut of *Trypocopris pyrenaeus* and concentrated on differences among wild individuals of an alpine Italian population. We revealed remarkable interindividual variation in the taxonomic composition of microbiota. Core taxa were few, while individuals harboured many exclusive taxa. Therefore, considering only a few individuals to describe the microbiota of a species is not sufficient. The study also revealed that the nMDS ordination of individuals based on prokaryotes was different from that based on fungi, meaning that both groups should be considered in microbiota analyses and that one microbic group cannot be considered a surrogate of the other. We identified many functions potentially provided by the microbiota and found the taxonomic richness of prokaryotes to be positively correlated with their functional richness. The analysis of the functions potentially provided by these microorganisms confirmed that the gut microbiota, in addition to being essential for the health of their host, may also contribute to the effective functioning of the ecosystems of which dung beetles are part.

INTRODUCTION

Dung beetles (Coleoptera: Scarabaeoidea) are ecosystem engineers that provide multiple functions and ecosystem services in many environments worldwide. As specialised decomposers that feed on vertebrate faeces, they are involved in numerous ecosystem processes such as dung removal and burial, nutrient cycling, plant growth enhancement, pest prevention and secondary seed dispersal (Losey & Vaughan, 2006; Nichols et al., 2008; Nervo et al., 2017; Piccini et al., 2018, 2020).

The growth and development of dung beetles, as well as their ecological and evolutionary success, depends on their gut microbiota, the characteristics of which allow them to cope with a nutritionally limited food source, namely mammal dung (Holter, 2016; Thiyonila et al., 2018; Winfrey & Sheldon, 2021; Macagno & Moczek, 2023). Recent studies clearly show the dung beetle microbiota to comprise a heterogeneous group of organisms, including bacteria, archaea and fungi (Franzini et al., 2016; Suárez-Moo et al., 2020; Ebert et al., 2021; Jácome-Hernández et al., 2023).

Part of the microbiota of dung beetles seems to have a parental vertical derivation (Estes et al., 2013; Ebert et al., 2021). In various dung beetle genera, multiple other factors shape the taxonomic composition of the gut microbiota in addition to parental vertical derivation; they include the insect's developmental stage (Suárez-Moo et al., 2020), phylogeny and the resulting differences in gut morphology (Ebert et al., 2021), the transmission of obligate and facultative symbiotic bacteria (Kucuk, 2020), their evolutionary history and local environmental conditions (Parker et al.,

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2019, 2020) and, of course, the specifics of their diet (Franzini et al., 2016; Shukla et al., 2016; Kolasa et al., 2019). Vertebrate dung contains many different microorganisms (Wan et al., 2021) that can be acquired by dung beetles during the consumption of different types of faeces. Therefore, in order to capture the natural variation in microbiota between individuals, it is essential that wild animals are studied, rather than those reared in the laboratory (Franzini et al., 2016).

The dung beetle bacterial microbiota includes Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria (Kolasa et al., 2019; Suárez-Moo et al., 2020). Archaea are not considered to be a fundamental component of dung beetle microbiota (Hammer et al., 2016; Ebert et al., 2021; Winfrey & Sheldon, 2021). The gut archaeome of dung beetles mainly includes Euryarchaeota taxa belonging to the class Methanobacteria (Ebert et al., 2021). Similarly, only a few studies have focused on the fungi colonising the dung beetle gut (Franzini et al., 2016; Kim et al., 2021; Nwaefuna et al., 2023). Ascomycota is the dominant phylum in the gut microbiota of dung beetles (Franzini et al., 2016).

Gut microbes facilitate digestion, and thus the provision of essential nutrients, vitamin production and protein hydrolysis, and protect the insect host from pathogens (Thiyonila et al., 2018; Van Arnam et al., 2018; Kolasa et al., 2019; Jing et al., 2020; Suárez-Moo et al., 2020; Wang et al., 2020). These organisms play a crucial role because they provide essential enzymes such as laccase, ligninolytic peroxidase, protease, lipase and glycoside hydrolase – enzymes required for the breakdown of otherwise unpalatable recalcitrant compounds such as cellulose, lignin and chitin (Estes et al., 2013; Shukla et al., 2016; Mabhegedhe, 2017; Schapheer et al., 2021; Lou et al., 2022). These gut microbes are thought to play two fundamental roles: on the one hand, they allow dung beetles to feed and survive, and on the other, by influencing dung beetle survival and physiology, they play a significant role in the material-degrading cycle of the ecosystem in which their hosts live. The importance of cellulose degradation by microorganisms has also been highlighted by several studies that have focused on investigating the dung beetle gut (and thus its microbes) as a bioreactor for biofuel production from cellulose (Mabhegedhe, 2017; Nwaefuna et al., 2021).

Although a small number of studies have now established the role of fungi as endosymbionts of various insect species, which benefit from these microorganisms in terms of nutrition, detoxification and nitrogen-recycling, most other studies addressing the dung beetle gut microbiota have solely focused on its bacterial component (Gibson & Hunter, 2010). However, due to the ability of fungi to degrade lignocellulose and act themselves as substrates for other microorganisms, they also play an important role in dung decomposition (Franzini et al., 2016; Sarrocco, 2016; Andlar et al., 2018; Dashora et al., 2023).

Studies on the gut microbiota of dung beetles have mainly focused on interspecific differences (Kumari et al., 2018; Parker et al., 2019, 2020, 2021; Ebert et al., 2021; Winfrey & Sheldon, 2021) or intraspecific differences between developmental stages (Shukla et al., 2016; Suárez-Moo et al., 2020). In some studies, in order to characterise the gut microbiota of just a few selected dung beetle species, numerous individuals of each species were sampled and homogenised before analysis (Estes et al., 2013; Hernández et al., 2015), effectively eliminating the possibility of analysing interindividual variability. Consequently, very little is known about the differences in microbiota among conspecifics of the same population. The only exception, to the best our knowledge, is Franzini et al. (2016), who observed marked interindividual differences in the gut-dwelling bacteria and fungi of *Pachysoma* dung beetles.

The goal of the present study was to describe and compare the gut microbiota of wild adult individuals of *Trypocopris pyrenaeus* (Charpentier, 1825), a dung beetle species widely distributed across Europe that feeds on the faeces of several vertebrate species (Dormont et al., 2007). To this end, we collected individuals from a single alpine population, preserved them in absolute ethanol and analysed the taxonomic and functional diversity of the bacteria, archaea and fungi present in their guts.

MATERIALS AND METHODS

Material collection and identification

Ten adult individuals (5 females and 5 males) of *T. pyrenaeus* were collected in the Western Italian Alps, Bocchetto Sessera, Piedmont (45.664°N, 8.062°E) on 10 Sept. 2021; specimens were detected by sight as they moved on the ground or by examining deposits of mammal dung. The Palearctic genus *Trypocopris* (Geotrupidae) comprises seven medium-sized coprophagous species of similar morphology (Schoolmeesters, 2023). For instance, *T. pyrenaeus* and *T. vernalis* (Linnaeus, 1758) are barely distinguishable (Baraud, 1992). Furthermore, *T. pyrenaeus* is polytypic, and subspecies that show subtle differences in body size, colour and punctuation of the pronotum possess concordant mitochondrial DNA differences (Carisio et al., 2004). Thus, following a preliminary morphological identification process based on external morphological traits (Baraud, 1992), we also tested for species identity and genetic structure by means of DNA barcoding techniques. Specific assignment and the genetic distance between the individuals collected from Bocchetto Sessera versus other alpine sites were also tested using the barcoding method (Figs S1, S2, Table S1).

Gut removal

Collected individuals were immediately euthanised by submersion in absolute ethanol. Once in the lab, dung beetles were dissected to extract the entire gut. The dissection tools were sterilized using a 30% sodium hypochlorite solution, then washed in distilled water. Once removed, the gut was preserved in absolute ethanol.

Microbiota DNA extraction

DNA was extracted using a CTAB method (Rogers & Bendich, 1985; Doyle & Doyle, 1990) subject to slight modifications. Samples were centrifuged at 14000 rpm for 10 min to remove the ethanol, then frozen in liquid nitrogen. Frozen samples were disrupted using the TissueLyser II (QIAGEN, Milano, Italy) (2 rounds of 2 min at 18 Hz). The disrupted tissues were mixed with 1 ml of pre-warmed (65°C) CTAB extraction buffer (2% w/v CTAB; 100 mM Tris- HCl; 20 mM EDTA; 1.4 M NaCl; 2% w/v polyvinylpyrrolidone K30) supplemented with proteinase K

(1 mg/ml) and RNase A (10 mg/ml), then incubated at 65°C for 1.5 h. Samples were then centrifuged at 10000 rpm for 10 min and the liquid phase transferred to a new 2 ml microfuge tube and an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) added, mixed several times by inversion, incubated at 4°C for 20 min and centrifuged for 10 min at 10000 rpm. The aqueous phase was transferred into a new 2 ml microfuge tube and an equal volume of chloroform : isoamyl alcohol (24 : 1) added, mixed several times by inversion and centrifuged for 10 min at 10000 rpm (this step was repeated twice). The upper aqueous phase was collected and transferred into a new 1.5 ml tube for DNA precipitation, achieved by adding 2/3 of the recovered volume of 2-propanol and incubating for 20 min at –20°C. Samples were then centrifuged and washed with 200 µl of ice-cold 70% ethanol (1 min at 14000 rpm). Next, samples were resuspended in 100 µl of sterile distilled water and stored at –20°C. Finally, DNA quality was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Courtaboeuf, France) before the samples were quantified using the Qubit dsDNA BR Assay kit and the Qubit Fluorometer 2.0, following the manufacturer's protocol.

A DNA metabarcoding approach was used to investigate the dung beetle microbiota: for the prokaryotic component, the 16S rRNA gene was amplified using the primer set 515fB (5′-GTGYCAGCMGCCGCGGTAA-3′) (Parada et al., 2016) and 806rB (5′-GGACTACNVGGGTWTCTAAT-3′) (Apprill et al., 2015); for the fungal component, the nuclear ribosomal ITS2 region was amplified using the primer pair fITS9 (5′-GAACGCAGCRAAIIGYGA-3′) (Ihrmark et al., 2012) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Gardes & Bruns, 1993). The following Illumina overhang adapter sequences were added to the primer pairs: forward overhang: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus specific target primer]; reverse overhang: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus specific target primer].

PCR reactions were performed using 0.4 U of Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific), 19 Phusion HF buffer, 0.5 µM of each primer, 0.2 mM of each dNTP and 1 µl of genomic DNA (20 ng) in a final volume of 25 µl. For the prokaryotic community, the PCR cycling programme was as follows: an initial step at 94°C for 3 min, 30 cycles at 94°C for 45 s, 57°C for 45 s, 72°C for 60 s and a final extension step of 72°C for 10 min. For the fungal community, the PCR cycling programme was as follows: an initial step at 95°C for 15 min, 35 cycles at 95°C for 35 s, 55°C for 35 s,72°C for 45 s, and a final extension step of 72°C for 7 min.

Extracted DNA was amplified in triplicate and pooled prior to purification using Wizard SV Gel and the PCR Clean-Up System (Promega, Madison, WI, USA). PCR purified products were quantified using the Qubit dsDNA BR Assay kit and Qubit Fluorometer 2.0 following the manufacturer's protocol and sent to IGA technologies (Udine, Italy) for Illumina MiSeq sequencing (2 × 250 bp).

Unfortunately, we had to exclude one individual (a male) from the analysis as the DNA amplification step failed. Therefore, the results refer to 9 individuals (5 females and 4 males).

Bioinformatic and statistical analyses

The following bioinformatic analysis was conducted for bacteria, archaea, and fungi. Sequencing adapters and primers were removed, and then the sequences were analysed by means of the microbiome bioinformatics platform QIIME2 (Quantitative Insights Into Microbial Ecology 2, v. 2019.7) (Bolyen et al., 2019). Denoising and quality control, including removal of chimeras, were achieved using the DADA2 (Callahan et al., 2016) plugin (qiime dada2 denoise-paired), and the chimera check was done by means of the method "consensus". Operational taxonomic units (OTUs) were identified using the qiime2 plugin "cluster-features-de-novo", and a percentage identity threshold of 97% was applied.

The taxonomic assignment of the prokaryotic community was achieved using the Silva 138 99% OTUs full-length sequences database (Bokulich et al., 2018; Robeson et al., 2021), whereas for fungi we used the UNITE Community (2019): UNITE QIIME release for fungi v.10.05.2021 (Abarenkov et al., 2021). Phylogenetic trees were generated by the QIIME2 plugin "qiime phylogeny align-to-tree-mafft-fasttree". The outputs of the QIIME2 pipeline "taxonomy.qza", "otu_table.qza" and "rooted-tree.qza", together with their metadata files, were then imported into Rstudio (RStudio Team, 2016) to create phyloseq objects using the R package qiime2R v.0.99.6 (Bisanz, 2018).

The phyloseq objects were used for the following diversity analyses. To allow for comparisons of samples with non-uniform coverage, the OTU tables were normalised by means of the *rarefy_even_depth* function of the R package phyloseq v.1.36.0 (McMurdie & Holmes, 2013). The function *rarecurve* of the R package vegan v2.6-2 (Oksanen et al., 2020) was adopted to obtain rarefaction curves of the rarefied OTU table. Biodiversity analyses were carried out by comparing the richness of microbial communities. Alpha diversity was evaluated using four estimators: "Observed OTUs" (i.e., comparable to the specific richness of insect communities), "Chao1", "Simpson" and "Shannon" by means of the functions *estimate_richness* and *plot_richness* of the R package phyloseq. The degree of correlation between these diversity indices was tested using a Spearman correlation matrix. The effect of sex on alpha diversity indices was tested separately on prokaryotes and fungi using *t*-tests.

For both prokaryotes and fungi, dissimilarity among individuals was visualised using non-metric multidimensional scaling (nMDS) based on a Bray-Curtis distance matrix of OTU composition. Stress was used as a measure of goodness of fit. The correlation between the nMDS of prokaryotes and fungi was calculated and tested for significance with a protest (Louca et al., 2016). nMDS and protest were performed using the R package vegan v2.6-2 (Oksanen et al., 2020). A permutational multivariate analysis of variance (PERMANOVA) using distance matrices was performed with the *adonis2* function of the same R package to evaluate the effect of sex on microbial community composition.

The trophic behaviours of the recovered prokaryotes and fungi communities were assessed using FAPROTAX: Functional Annotation of Prokaryotic Taxa v. 1.2.6 (Louca et al., 2016) and R package FUNGuild (Nguyen et al., 2016), respectively.

The correlation between taxonomic and functional richness was calculated using the Spearman's rank correlation coefficient for both bacteria and archaea at the OTU and family levels. The correlation was not calculated for Fungi because of the low inter-individual variability in trophic mode richness.

Figures were generated using the R package ggplot2 v. 3.3.6 (Wickham, 2016) and the plotrix package (Lemon, 2006). Flower plots were generated using a custom script, available upon request.

The detailed taxonomic and functional/trophic composition of prokaryotic and fungal OTUs for each individual was visualized using Krona tool (Ondov et al., 2011). Krona plots (Figs S6–S9) were generated using cpauvert/psadd library v.0.1.3 (Pauvert, 2022).

Defining core microbiota and abundance of each taxon

Because microorganisms constitute a large part of the dung beetle diet, it is important to distinguish between the microorgan-

isms stably associated with a beetle versus those that are transient and presumably environmentally acquired (Ebert et al., 2021). To this end, we identified core OTUs and core families of bacteria, archaea, and fungi as those OTUs and families shared by at least 7 out of the 9 individuals analysed. The number of core families and OTUs of prokaryotes and fungi were then divided by the total number of families and OTUs, respectively, to calculate the proportions that these taxa contributed to the overall gut microbiota.

The quantitative contribution (i.e. abundance, number of reads) of each bacterial, archaeal and fungal taxon was calculated as the ratio between the number of reads of each individual microbial taxon (a certain phylum, class, family, etc.), and the total reads of all taxa in the nine individuals.

In analogy to the taxonomy of the microbiota, cores and abundances were also calculated for prokaryotic functions and fungal trophic modes.

RESULTS

Individual identification via barcoding

DNA barcoding for species assignment confirmed that all the individuals collected were indeed *T. pyrenaeus*. DNA analyses showed the individuals to be closely related and distant from other samples collected in the Western Italian Alps, corroborating our assumption that they belonged to the same population (Fig. S1).

Microbiota taxonomic diversity

Alpha diversity

The bioinformatic analysis of prokaryotic DNA gave rise to 52,842 high-quality sequences. Following subsampling, 3676 sequences per sample were retained at a uniform sequencing depth, clustered in 398 OTUs (392 bacterial and 6 archaeal OTUs). For fungi, we obtained 108,111 high-quality sequences; sub-sampling identified 8579 sequences per sample at a uniform sequencing depth, clustered in 201 OTUs. We found large variations in the number of prokaryotic and fungal reads per individual, ranging from 3676 to 9241 for prokaryotes, and from 8579 to 17251 for fungi. However, rarefaction analysis (Fig. S3) and the Chao1 diversity index (Table 1) confirmed the sample coverage of *T. pyrenaeus* prokaryotic and fungal gut communities to be sufficient in each individual; specifically, rarefaction curves tended quickly to the asymptote and the Chao1 index showed values very close to or equal to the number of OTUs. Furthermore, the number of reads and observed OTUs per individual were not significantly correlated.

All bacterial, archaeal and fungal OTUs were assigned with certainty to kingdom and phylum, whereas taxonomic classification below the phylum level showed different rates of uncertainty depending on whether we considered prokaryotic or fungal OTUs (Fig. S4).

The alpha diversity indices obtained revealed great variation among the nine individuals studied (Table 1); for instance, the number of observed prokaryotic OTUs varied between 26 and 116, while the number of fungal OTUs varied between 14 and 49. Analogously, the Shannon diversity index of prokaryotes varied between 1.14 and 4.39, while that of fungi varied between 0.47 and 3.08. Alpha diversity indices were consistently high in certain individuals (e.g., F1) and consistently low in others (e.g., F4). In most individuals, the number of prokaryotic taxa at the different levels (i.e., OTUs, families and phyla) was higher than the number of fungal taxa (Table 1). The estimated contribution of archaea to the alpha diversity of each individual was negligible. Altogether, only 6 OTUs, 2 families and 2 phyla of archaea were identified versus 392, 108 and 21 of bacteria, respectively (see caption of Table 1).

The Chao1, Shannon and Simpson indices of prokaryotes were strongly and positively correlated (rho $\geq$ 0.85, p-value < 0.01). As for fungi, only the Simpson and Shannon indices were highly correlated (rho = 0.98, p-value < 0.001), while the remaining correlations with Chao1 were not significant (Chao1-Simpson rho = 0.16, p-value = 0.683; Chao1-Shannon rho = 0.28, p-value = 0.472). Alpha

Table 1. Individual alpha diversity. Numbers of reads, operational taxonomic units (OTUs), families, phyla, and diversity indices for prokaryotic and fungal gut communities of *T. pyrenaeus*. All values were calculated from rarefied reads (i.e., 3676 for prokaryotes and 8579 for fungi). For prokaryotes we identified 398 OTUs classified into 110 families and 23 phyla; of these, 6 OTUs from 2 families and 2 phyla belonged to the archaea. For fungi, we identified 201 OTUs classified into 85 families and 6 phyla. Individuals are sorted by sex (F – female, M – male).

	Individual	Number of reads	Observed OTUs	Families	Phyla	Chao1	Simpson	Shannon
Prokaryotes	F1	4706	116	54	18	119.00	0.98	4.39
	F2	4890	76	48	13	76.00	0.97	3.86
	F3	6742	60	32	13	60.00	0.73	2.62
	F4	3676	26	21	8	26.00	0.63	1.63
	F5	6991	67	38	9	67.33	0.97	3.81
	M1	7707	82	32	10	82.00	0.96	3.81
	M2	4507	32	19	4	32.00	0.91	2.93
	M3	4328	34	27	12	34.00	0.37	1.14
	M4	9241	87	34	8	87.00	0.98	4.07
Fungi	F1	17251	42	24	3	42.00	0.93	3.08
	F2	14097	35	23	2	35.00	0.80	2.37
	F3	12783	41	24	2	41.00	0.57	1.77
	F4	9634	14	8	2	14.00	0.16	0.47
	F5	11397	41	23	2	41.00	0.91	2.96
	M1	8579	49	30	4	49.00	0.83	2.82
	M2	10990	17	12	2	17.00	0.90	2.53
	M3	9821	32	23	3	32.00	0.92	2.97
	M4	13559	44	23	3	44.00	0.66	2.17

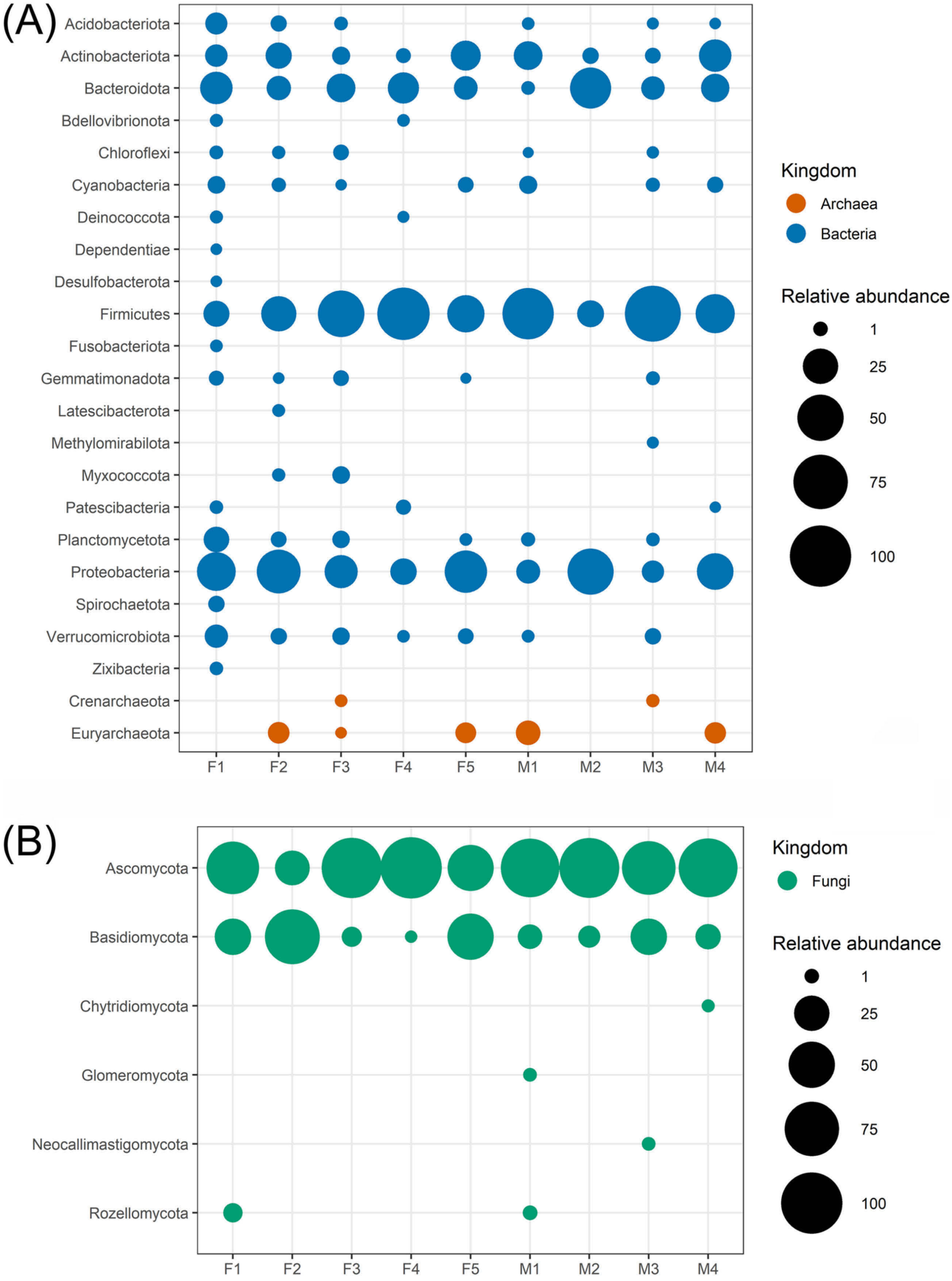

Fig. 1. Differences in the taxonomic composition of the phylum rank of the retrieved prokaryotic (A) and fungal (B) communities between wild individuals of *T. pyrenaeus*. Circle size represents the relative abundance of each phylum in each individual.

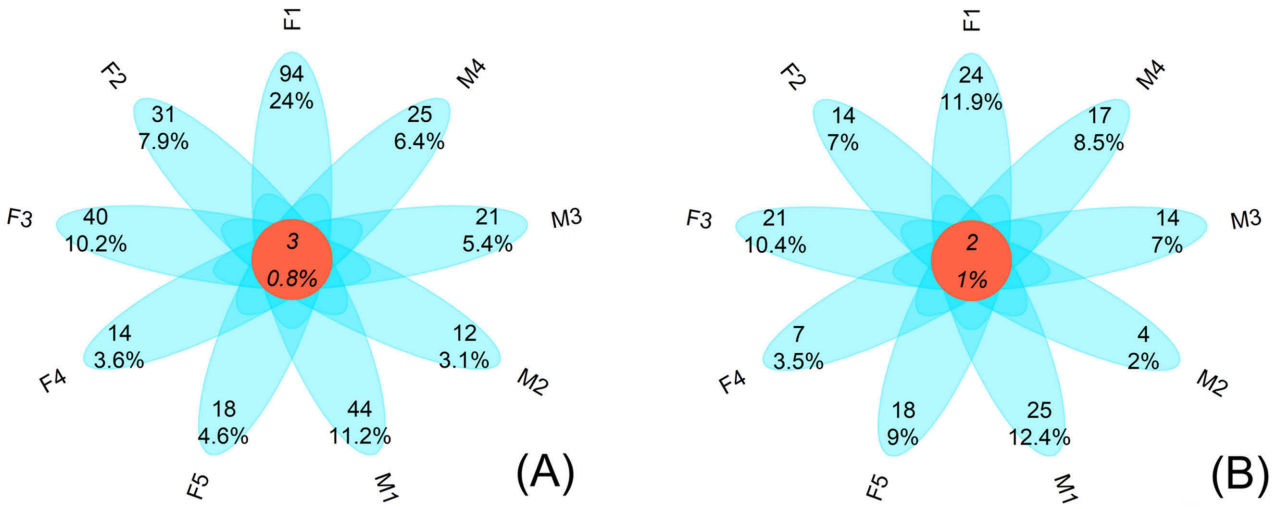

Fig. 2. Core and exclusive bacterial (A) and fungal (B) OTUs retrieved from individuals of *T. pyrenaeus*. Bacterial core OTUs account for 30.3% of retrieved sequences. Fungal core OTUs account for 17.6% of retrieved sequences.

diversity indices of females and males were similar for both prokaryotes and fungi *t*-test results (Table S3).

Taxonomic composition

The most abundant bacteria phyla were Firmicutes (42.45%), Proteobacteria (27.20%), Bacteroidota (synonym Bacteroidetes) (14.28%) and Actinobacteriota (synonym Actinobacteria) (8.33%), although the relative abundances varied between individuals (Fig. 1A). Some individuals showed very high phyla diversity (e.g., F1), whereas it was very low for others (e.g., M2) (Table 1; Fig. 1A). The dominant classes were Bacilli (33.06%), Gammaproteobacteria (21.5%), Bacteroidia (13.97%), Clostridia (9.27%) and Alphaproteobacteria (5.62%). The most abundant family was Enterococcaceae (28.43%). All other families showed an abundance of less than 5%. The most abundant genus was *Enterococcus* (35.12%), followed by other genera such as *Acinetobacter*, *Myroides*, *Pseudomonas* or *Dysgonomonas*, all of which did not, however, reach an abundance level of 5%. See Figs S6 and S7 for a complete taxonomic assignment for each prokaryotic and fungal OTU.

The preponderant archaea phylum was Euryarchaeota (96.14%), although the relative abundances varied between individuals (Fig. 1A). The dominant class was Methanobacteria (96.14%), and Methanobacteriaceae (96.14%) was the most dominant family. At the genus level *Methanobrevibacter* (66.89%) was dominant and *Methanosphaera* (33.11%) followed. However, three out of nine individuals did not possess archaeal OTUs.

The most plentiful phylum of Fungi was Ascomycota (76.00%), followed by Basidiomycota (23.27%) (Fig. 1B). The most abundant classes were Sordariomycetes (32.60%), Dothideomycetes (30.48%), Tremellomycetes (18.61%) and Leotiomycetes (6.68%). The dominant families were Nectriaceae (25.12%), Mycosphaerellaceae (18.07%) and Trichosporonaceae (12.59%). The most abundant genera were *Gibberella* (22.72%), *Mycosphaerella* (19.69%) and *Cutaneotrichosporon* (7.00%); the abundances of all other genera were below 5%.

Core and exclusive taxa

We found remarkable interindividual taxonomic differences in the gut prokaryotic and fungal communities of *T. pyrenaeus*. In general, very few core families and core OTUs were identified; by contrast, all individuals harboured exclusive families and exclusive OTUs.

At the family level, only 7 out of the 108 (6.48%) bacterial families identified were shown to be core to all individuals; we found no archaeal core families and only 3 core fungal families (out of the 85 identified, 3.53%) (Fig. S5, Table S2). At the same time, we observed a fair number of exclusive families for each individual, with percentages ranging from 1.9 to 13% for bacteria, and from 0 to 14.1% for fungi. Notably, the individual M2 had no exclusive families.

At the OTU level, we identified 3 core bacterial OTUs (out of 392, 0.77%), no archaeal core OTUs and 2 fungal core OTUs (out of 201, 1.00%). The low number of core OTUs was combined with a surprising and relatively high number of OTUs that were exclusive to each individual, with percentages varying from 3.1 to 24% for bacterial OTUs (Fig. 2A), and from 2.0 to 12.4% for fungal OTUs (Fig. 2B).

Only a single bacterial OTU (identified as a species of the genus *Enterococcus*) was shared by all nine individuals, and another two (identified as *Pseudomonas* sp. and Yersiniaceae sp.) were shared by 7 out of 9 individuals. The five preponderant bacterial OTUs were *Enterococcus* sp. (26.39%), *Myroides* sp. (2.82%), Yersiniaceae sp. (2.66%), *Escherichia-Shigella* sp. (2.18%) and *Dysgonomonas alginatilytica* (2.17%).

Only a single fungal OTU (identified as *Mycosphaerella tassiana*) was shared by all nine individuals, and one other (identified as *Nectriaceae* sp.) was shared by 7 out of 9 individuals. The five most abundant fungal OTUs were *Gibberella tricincta* (18.41%), *Mycosphaerella tassiana* (15.93%), *Cutaneotrichosporon jirovecii* (5.56%), *Vishniacozyma victoriae* (3.15%) and *Trichosporon ovoides* (2.77%).

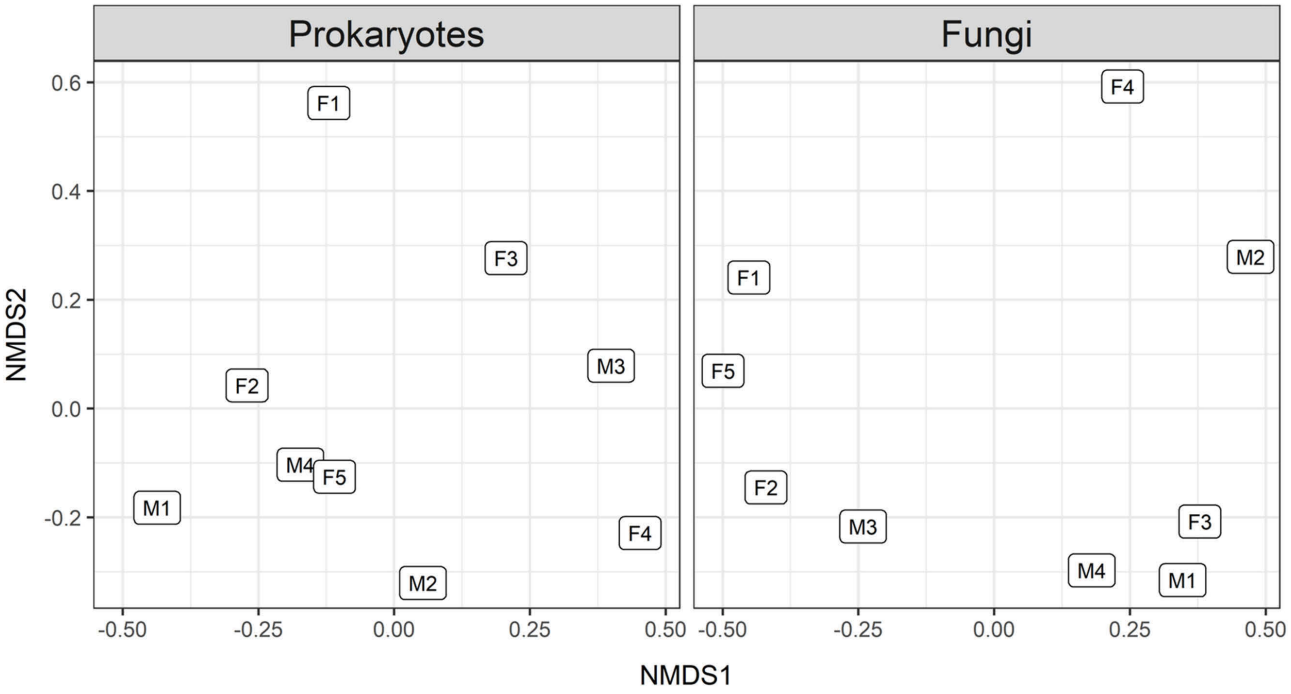

Fig. 3. Results of a non-metric multidimensional scaling analysis performed on 2 axes. Individuals that are more similar to one another are located closer together.

Dissimilarity among individuals and between microbial communities

Non-metric multidimensional scaling analysis for both prokaryotes and fungi resulted in the nine individuals being well scattered across the two-dimensional space (Fig. 3). Stress (a measure of goodness of fit) was 0.09 for prokaryotes and 0.08 for fungi, indicating a high-quality ordination. The two nMDS ordinations were not correlated ($r = 0.46$; p-value = 0.40), indicating that the distribution of individuals in the two-dimensional space changed depending on whether prokaryotes or fungi were considered. The PERMANOVA results suggested that the microbial community composition in males was like that in females in relation to both prokaryotes (p-value = 0.958, R2 = 0.07) and fungi (p-value = 0.460, R2 = 0.11).

Microbiota functional diversity

The functional annotation of ecological roles to the microbiota was fragmentary for bacteria and fungi as we were unable to attribute a function to 70.15% of the bacterial OTUs and 35.82% of the fungal OTUs. In contrast, we could assign all archaeal OTUs to at least one ecological role.

The most abundant bacterial potential functions retrieved were chemoheterotrophy (39.65%), fermentation (23.89%) and aerobic chemoheterotrophy (15.64%); all other functions retrieved realised abundances of less than 5%. Some individuals displayed a high number of OTU-associated bacterial functions (e.g., F2 and M4), while others presented much fewer (e.g., F4) (Fig. 4A).

The dominant functions associated with archaea were dark hydrogen oxidation (24.68%), hydrogenotrophic methanogenesis (24.68%), methanogenesis (24.68%), methanogenesis by CO_2 reduction with H_2 (16.50%), and methanogenesis by reduction of methyl compounds with H_2 (8.18%). Note, most functions associated with archaea (6 out of 7) were exclusive, and only one function (dark hydrogen oxidation) was shared with bacterial OTUs (Fig. 4A).

The preponderant fungal trophic modes were pathotroph-saprotroph-symbiotroph (34.71%), pathotroph-saprotroph (27.02%), saprotroph (13.75%) and pathotroph (13.69%) (Fig. 4B).

See Figs S8 and S9 for a complete functional and trophic assignment for each prokaryotic and fungal OTU.

Core and exclusive functions

Interindividual functional differences were less pronounced than taxonomic differences. When considering the functional profile of the prokaryotic community of each individual, we found that 6, out of the 31 bacterial functions identified (19.35%), to be core across all individuals, namely aerobic chemoheterotrophy, animal parasites or symbionts, aromatic compound degradation, chemoheterotrophy, fermentation, and photoautotrophy. No core functions were found for Archaea (Fig. 4A). In relation to fungi, 6 out of 7 trophic modes (85.71%) were core across all individuals (Fig. 4B).

Six bacterial functions were exclusive to three individuals: cellulolysis and iron respiration were exclusive to F1; nitrate ammonification, and nitrite respiration were exclusive to F2, and chitinolysis was exclusive to M3. No archaeal function or fungal trophic mode was exclusive to any individual.

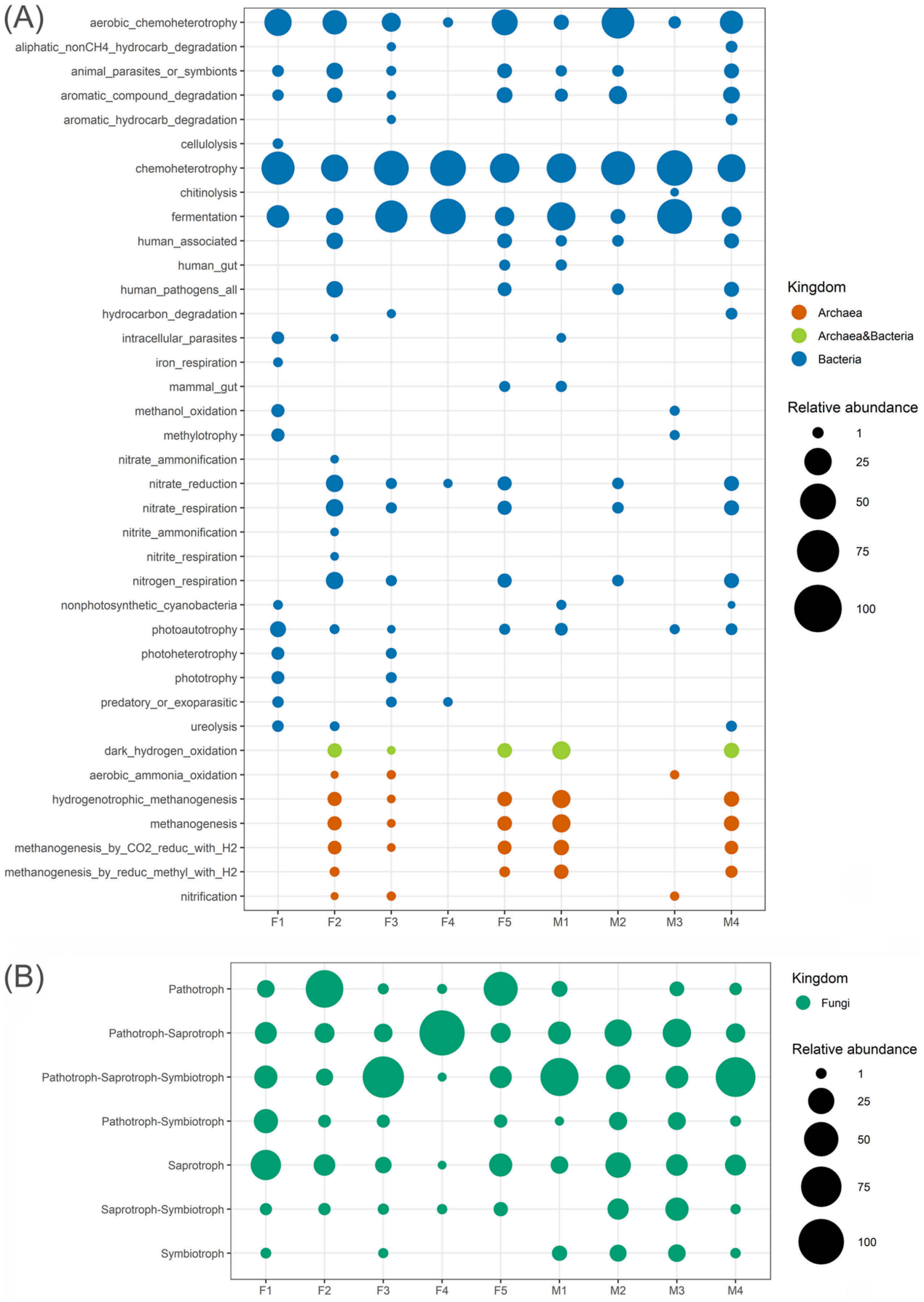

Fig. 4. Differences in functional composition and trophic mode assignment of the retrieved prokaryotic (A) and fungal (B) communities between wild individual specimens of *T. pyrenaeus*. Circle size represents the relative abundance of each function/trophic mode in each individual.

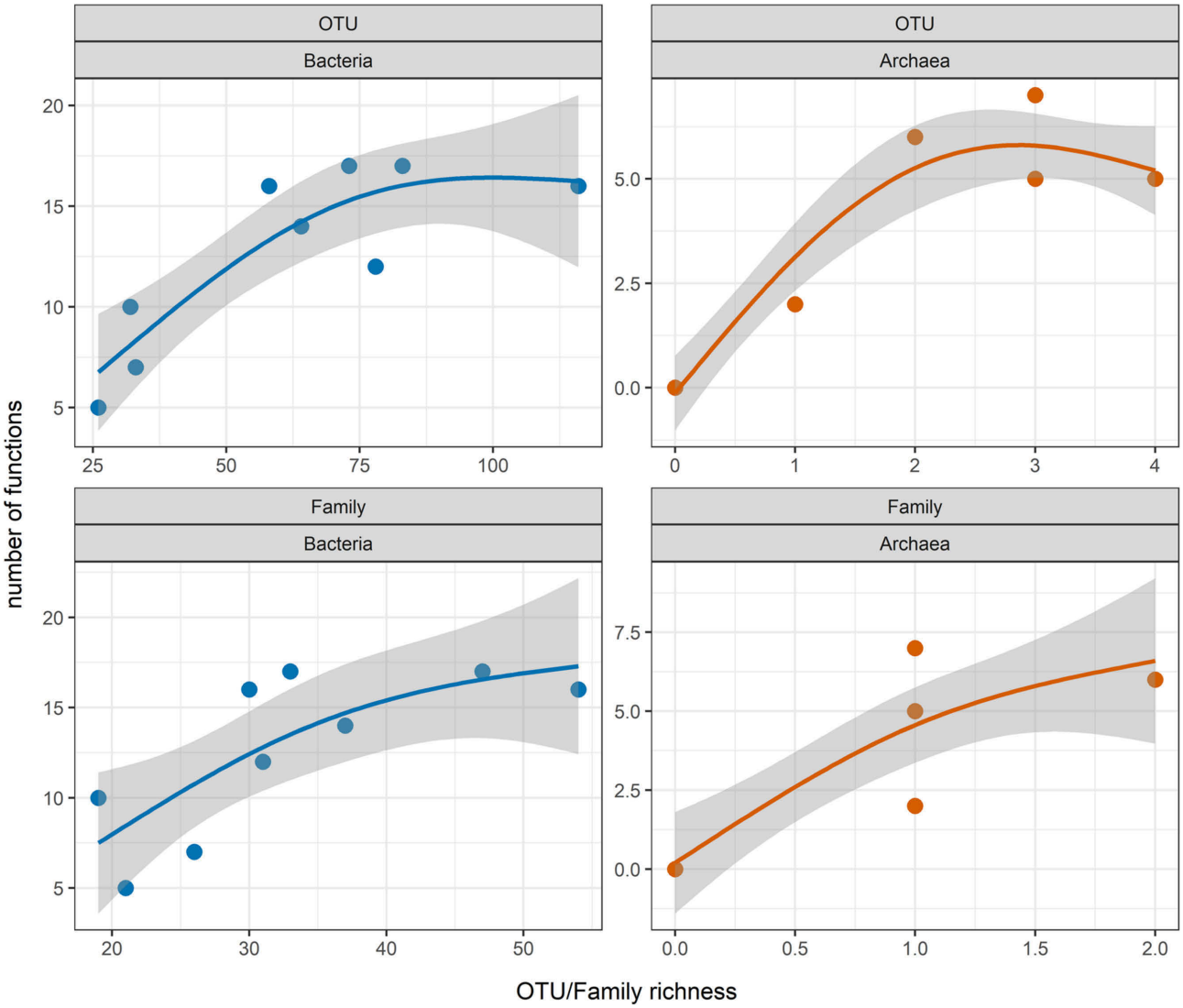

Fig. 5. Relationship between taxonomic richness and functional richness in bacteria and archaea. Trend lines were fitted using generalised additive models (GAM). The shaded area indicated the standard error.

Correlation between taxonomic and functional richness across individuals

The correlation between taxonomic and functional richness was positive and significant for both bacteria (OTU: rho = 0.748, *p*-value = 0.020; family: rho = 0.748, *p*-value = 0.020) and archaea (OTU: rho = 0.752, *p*-value = 0.019; family: rho = 0.868, *p*-value = 0.002). However, the slope of the correlation curves between functional and taxonomic diversity tend to plateau as the number of families or OTUs considered increases (Fig. 5).

DISCUSSION

DNA metabarcoding of the gut microbiota revealed considerable taxonomic differences among nine individuals of the dung beetle *Trypocopris pyrenaeus*. Functional differences were less pronounced, possibly because the assignment of the ecological roles to the microbiota was fragmentary. Individuals were collected in a natural pasture at the same alpine locality and were directly euthanised, then dissected in the lab on arrival. Thus, this study focused on wild individuals exposed solely to totally natural conditions.

Microbiota taxonomic diversity

The contribution of prokaryotes to alpha diversity was greater than the contribution of fungi, despite the negligible contribution of archaea. Prokaryotic and fungal alpha diversity varied greatly from individual to individual. The nine adults investigated hosted remarkably different numbers of phyla, families and OTUs, as well as yielding different alpha diversity index values (Shannon and Simpson), with certain individuals hosting a microbiota that was much more diverse than the others. *t*-test and PERMANOVA results showed that sex had no significant effect on alpha diversity, in keeping with a previous study (Suárez-Moo et al., 2020). The observed interindividual differences in microbiota alpha diversity are likely a result of different food items ingested by each animal. *Trypocopris* has a very varied diet (Dormont et al., 2007), and individuals have been observed to feed on the dung pats of a variety of

animals, including ungulates, canids and other mammals (personal observations of C. Palestrini and A. Rolando).

It is also worth mentioning that by focusing on bacteria, archaea, and fungi, we considered all the main components of the gut microbiota of dung beetles. To the best of our knowledge, very few previous studies have considered all these components at the same time (Franzini et al., 2016). Most studies surveyed bacteria (Gibson & Hunter, 2010) or only fungi (Nwaefuna et al., 2023).

Overall, Firmicutes was the most abundant bacterial phylum. Firmicutes was also found to be a very abundant phylum in the mushroom-feeding beetles *Onthophagus* and *Cephalodesmius* (Ebert et al., 2021), as well as in *Pachysoma striatum* (Franzini et al., 2016). The phylum Proteobacteria was also very abundant, in keeping with other studies on other dung beetle species (Estes et al., 2013; Suárez-Moo et al., 2020; Ebert et al., 2021). The most abundant bacterial family was Enterococcaceae, considered a core taxon in the mushroom-feeding beetles *Onthophagus dunningi* and *O. kumbaingeri* (Ebert et al., 2021) and an abundant taxon in *Aphodius sphacelatus* (Kolasa et al., 2019). Archaea are often overlooked in analyses of dung beetle gut microbiota. Our results show the dominant archaea class to be Methanobacteria, and not all individuals had evidence of archaea in their gut microbiota, confirming the results of the genus *Cephalodesmius* (Ebert et al., 2021) where archaea (Methanobacteria) were found only in small quantities in certain individuals. As for fungi, our results are in line with those reported in previous research: in a study on *Pachysoma striatum* (Franzini et al., 2016), Ascomycota was found to be the most abundant phylum, followed by Basidiomycota.

Of all the bacteria and fungi identified, very few families and OTUs were found in all nine individuals studied (7 families and 3 OTUs for bacteria; and 3 families and 2 OTUs for fungi); none of the identified archaea were found in all 9 individuals.

Previous studies report similar findings and have defined the phenomenon as a "minimal core model" (Hamady & Knight, 2009; Franzini et al., 2016), which states that any one individual may share significant parts of his microbiota with another individual, but very little is shared by all individuals (Hamady & Knight, 2009). The remarkable scarcity of core taxa may depend on the beetles' diet (Franzini et al., 2016). *Trypocopris pyrenaeus* feeds on the faeces of various vertebrate species, and this may lead to the establishment of different gut microbiota for each individual dependent on their diet and, in particular, on the type of food ingested in the hours preceding field collection. Indeed, repeated environmental perturbations, including variation in the food consumed, may prevent the establishment of a core microbiota in some individuals (Wong et al., 2013). The substantial interindividual variation observed could also be affected by the stochastic process of microbes entering the gut through the food source (Douglas, 2015) and the variable amount of food material contained in each individual's gut (Dillon & Dillon, 2004).

A remarkable result of the present study was that the very low number of core taxa was contextually associated with the presence of a relatively high number of taxa exclusive to each individual, which ranged from 2% to 24% of the OTUs identified. The great interindividual differences could be explained by the wide trophic preferences of *T. pyrenaeus* and/or by differences in the microbiota transmitted from mothers to offspring (Estes et al., 2013; Suárez-Moo et al., 2020).

Non-metric multidimensional scaling analysis for both prokaryotes and fungi evenly scattered the nine individuals across the two-dimensional space without any evident clustering, reinforcing the idea that individuals harbour distinct microbiota. Further information on interindividual differences could be obtained by analysing more populations of the same species, as well as from different areas. The degree of within-population variability could be greater or less than the degree of between-population variability. In addition, the two nMDS ordinations were not correlated, suggesting that the distribution of individuals in the two-dimensional space changed depending on whether prokaryotes or fungi were considered. This result reiterates the importance of considering both the prokaryotic and fungal components of the microbiota, as the two types of microbial communities provide different insights into the distinct microbiota of individual dung beetles.

Microbiota functional diversity

One previous study hypothesised that the absence of a consistent core microbiota at the taxonomic level may be compensated for by the presence of a core microbiota at the functional level, i.e., the presence of microbial communities of functional equivalence (Wong et al., 2013). This may also be the case in the present study as the results show a larger core at the functional level than at the taxonomic level. However, we were unable to assign a function to many OTUs, so this result could change with a more complete functional characterisation. Taxonomic diversity was remarkably variable among individuals due to the presence of few core taxa and many exclusive taxa. Conversely, functional diversity was less variable among individuals due to the presence of many core functions and few exclusive functions. However, for fungi, descriptions of the various trophisms are so broad that it is more likely that there are OTUs performing the same "task" in all individuals. For prokaryotes, however, the issue is certainly more complex than for fungi, since each OTU did not have a unique function associated with it, as in the case of trophism in fungi. In fact, the same prokaryotic OTU can also perform multiple functions, complicating the overall picture. In the case of archaea, this is particularly noteworthy because these organisms provide functions such as methanogenesis and nitrification, which are exclusive to this phylum.

We observed significant correlations between taxonomic and functional diversity, suggesting that greater taxonomic richness determines a greater number of metabolic pathways associated with dung degradation. This could, in turn, be related to the role of dung beetles in maintaining

multiple ecological functions and, more generally, to their role as ecosystem service providers (Nervo et al., 2017). In this regard, the prokaryote component of the dung beetle microbiota should be protected by limiting the treatment of cattle with antibiotics (Hammer et al., 2016), because their taxonomic diversity also guarantees optimal functional diversity.

Although several OTUs present in the *Trypocopris* microbiota may be important pathogens (Cannon & Kirk, 2007; Yun et al., 2014; Kolasa et al., 2019), molecular studies suggest that many other gut microbes may also be implicated in nitrogen fixation, iron uptake, uric acid metabolism, and plant cell wall degradation (Shukla et al., 2016; Kolasa et al., 2019; Suárez-Moo et al., 2020; Wang et al., 2020), which are useful both for host survival and for ecosystem functioning. *Pseudomonas* is one of the twenty most important contributors to plant cell wall (i.e., lignocellulose) degradation (Suárez-Moo et al., 2020; Dashora et al., 2023). *Dysgonomonas* is a crucial contributor to plant cell wall degradation in the gut of *Copris incertus* (Suárez-Moo et al., 2020) and *Pachisoma striatum* (Franzini et al., 2016). Fungi are also known to degrade lignocellulose (Andlar et al., 2018). This task is mainly performed by Ascomycota and Basidiomycota (Dashora et al., 2023), and our results show a clear predominance of these two fungal phyla in the microbiota of *T. pyrenaeus*.

Pseudomonas, *Acinetobacter* and *Nocardioides* are able to recycle uric acid, which is used as a nitrogen source (Shukla et al., 2016; Suárez-Moo et al., 2020). Archaea, on the other hand, can produce methane. In our work, the most common archaeal OTUs belong to the genus *Methanobrevibacter*, which is recognised as an important methanogen capable of producing methane through the reduction of carbon dioxide by hydrogen in insects (Ceja-Navarro et al., 2019) and arthropods (Horváthová et al., 2021). Certain species of the genus *Methanosphaera* are also methanogenetic, but they use hydrogen to reduce methyl to methane (Feldewert et al., 2020; Thomas et al., 2022). Our results also revealed the presence of non-methanogenic archaea, such as those belonging to the Nitrososphaeraceae family. They are capable of oxidising ammonia to nitrite, and are ubiquitous in soil, plant and water ecosystems (Jung et al., 2014). Their presence and predominance as soil archaea suggest that these taxa may have been ingested by accident, as previously reported in a wide range of animals (Thomas et al., 2022). Thus, given that methanogenetic taxa of the Nitrosospheraceae family are generally more abundant in the gut microbiota of carnivorous or insectivorous animals than in the gut of herbivorous animals, it is possible that the individuals of *T. pyrenaeus* in whom non-methanogenic archaea were found had recently fed on the excrement of carnivores. It is worth mentioning that the main ecological functions provided by the OTUs quoted above may be classed as either ecosystem services (as for cellulolysis) or ecosystem disservices (as for methanogenesis). Dual ecosystem behaviours have also been reported for dung beetles themselves (Piccini et al., 2017, 2018); for example, the dung beetle *Copris lunaris* contributes to multiple ecosystem services thanks to its high dung removal rate (Nichols et al., 2008; Nervo et al., 2017), but their activity is also responsible for the unexpectedly high emission of methane from dung pats (Piccini et al., 2017).

CONCLUSIONS

Interindividual variability in the microbiota of dung beetles has been largely overlooked in the literature. The present study focused on a wild population of the dung beetle species *Trypocopris pyrenaeus* and revealed notable taxonomic differences in the microbiota of the individuals examined, although the number of samples was limited. Core taxa were few, whereas the taxa exclusive to each individual were numerous. This has important implications for studies attempting to describe the gut microbiota of a species, as sufficient individuals of the species of interest should be examined in order to capture the full range of prokaryotes and fungi potentially harboured. The study also revealed that the nMDS ordination of individuals based on prokaryotes was different from that based on fungi, meaning that both groups should be considered in microbiota analyses and that one microbic group cannot be considered a surrogate of the other. Finally, our analysis of the potential functions provided by the harboured prokaryotes and fungi suggests that the gut microbiota, in addition to guaranteeing the health of the host, may also effectively contribute to the functioning of the ecosystems in which the individuals lived. Future studies that are based on metagenomics and/or transcriptomics would help to clarify the functional role of the microbiota in host physiology aspects such as nutrition or development and ecosystem services.

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DATA AVAILABILITY STATEMENT. The raw data used to generate the dataset for this study can be found in the NCBI Sequence Read Archive (SRA) under accession no. PRJNA949028 https://dataview.ncbi.nlm.nih.gov/object/PRJNA949028?reviewer=1430gnk8nhgk47k5v0moqoo0b and PRJNA949020 https://dataview.ncbi.nlm.nih.gov/object/PRJNA949020?reviewer=rih7f1bqmo8qonjt0jvhvtmj9r for prokaryotic and fungal communities respectively.

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Online supplementary files:

Supplement S1 (http://www.eje.cz/2024/007/S01.pdf): Figs S1, S2, Table S1.

Supplement S2 (http://www.eje.cz/2024/007/S02.pdf): Figs S3–S5, Tables S2, S3.

Supplement S3 (http://www.eje.cz/2024/007/S03.html): Fig. S6 (taxonomic assignment for prokaryotic OTUs).

Supplement S4 (http://www.eje.cz/2024/007/S04.html): Fig. S7 (taxonomic assignment for fungal OTUs).

Supplement S5 (http://www.eje.cz/2024/007/S05.html): Fig. S8 (functional and trophic assignment for prokaryotic OTUs).

Supplement S6 (http://www.eje.cz/2024/007/S06.html): Fig. S9 (functional and trophic assignment for fungal OTUs).