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Microbial Communities of the Gut and Nest of the Humus- and Litter-Feeding Termite *Procornitermes araujoi* (Syntermitinae)

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Abstract

The evolution of the symbiotic association with microbes allowed termites to decompose ingested lignocellulose from plant-derived substrates, including herbivore dung and soil humus. Representatives of the Syntermitinae (Termitidae) range in their feeding habits from wood and litter-feeding to humus-feeding species. However, only limited information is available about their feeding ecology and associated microbial communities. Here we conducted a study of the microbial communities associated to the termite *Procornitermes araujoi* using Illumina sequencing of the 16S and ITS rRNA genes. This species has been previously included in different feeding guilds. However, most aspects of its feeding ecology are unknown, especially those associated to its symbiotic microbiota. Our results showed that the microbial communities of termite guts and nest substrates of *P. araujoi* differed significantly for bacteria and fungi. Firmicutes dominated the bacterial gut community of both workers and soldiers, whereas Actinobacteria was found in higher prevalence in the nest walls. Sordariomycetes was the most abundant fungal class in both gut and nest samples and distinguish *P. araujoi* from the grass/litter feeding *Cornitermes cumulans*. Our results also showed that diversity of gut bacteria were higher in *P. araujoi* and *Silvestritermes euamignathus* than in the grass/litter feeders (*C. cumulans* and *Syntermes dirus*), that could indicate an adaptation of the microbial community of polyphagous termites to the higher complexity of their diets.

Introduction

Termites and wood-feeding cockroaches evolved from a common ancestor that gained the ability to digest lignocellulose through the symbiotic association with microbes [16, 30, 31, 45]. Gut symbionts in termites consist of several groups of cellulolytic flagellates and prokaryotes. Basal lineages of termites depend on flagellate protists and prokaryotes for lignocellulose digestion [24]. In contrast, the evolutionary success of Termitidae is attributed to the loss of cellulolytic flagellates and acquisition of a specialized bacterial flora, together with a highly dietary diversification and feeding strategies [5, 7, 22, 35]. Comparative studies have revealed a diet-related compositional convergence of termite gut microbiota from the same feeding guild [16, 51]. For example, gut symbiont composition in wood-feeding species consists mainly on Fibrobacteres and Spirochetes which are known to have a strong cellulolytic activity [28, 31]. Conversely, the microbiota of grass/litter-feeding species consisted mainly on Firmicutes [27, 32, 35, 44, 55], thus corroborating that the type of diet is related to the community structure of their bacterial gut symbionts.

The subfamily Syntermitinae is a monophyletic group of Termitidae comprising approximately one hundred species that range in their feeding habits from wood and litter-feeding to humus-feeding species [52]. Although basic aspects of the feeding ecology of the Syntermitinae remain poorly studied, the microbial communities of these termites can be very complex and diverse [35, 55]. *Procornitermes araujoi* (Emerson 1952) is a harvester Syntermitinae in savannas of central and southeastern Brazil [8, 9, 13, 23]. Its epigeal

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nests have rounded shape with the external walls made of a thin layer of loose soil and the dark carton core built mainly with fecal material [9, 14] (Fig. S1). This species has been previously included in different feeding guilds, specifically as grass/litter and humus feeder [26, 34] and wood/soil interface feeder [11, 12, 19]. However, most aspects of the feeding ecology of this species are unknown, especially those associated to its symbiotic microbiota. Here we conducted a detailed study of the microbial communities associated to the gut and the internal nest walls of *P. araujoi* using Illumina sequencing of the 16S rRNA and the internal transcribed spacer (ITS) region of rRNA gene. The present study first investigated the composition of microbial assemblages of termite guts and nest substrates of *P. araujoi*. Termite soldiers cannot feed themselves because their mandibles are modified or reduced and, therefore, depend on workers for nutrition [41], which could be reflected in the composition of the gut microbiota between workers and soldiers. In this context, we evaluated the variation in the microbial composition between the two castes. Since feeding habits of Termitidae are diverse, we also assessed how host diet influences bacterial assemblages by comparing the community structure of gut bacteria of workers of *P. araujoi* with representatives of other feeding guilds of Syntermitinae.

Materials and Methods

Termite Sampling

Colonies of *Procornitermes araujoi* (Emerson 1952) and the grass/litter feeder *Syntermes dirus* (Burmeister, 1839) [10] were sampled in pasture areas of Campinas, Sao Paulo State (22°54′3″S; 47°03′26″W) and Alfenas, Minas Gerais State (21°25′45″S; 45°56′50″W), southeastern Brazil. Termites used in this study also included the grass/litter feeder *Cornitermes cumulans* and the litter/wood feeder *Silvestritermes euamignathus* (Silvestri, 1901) [34, 53]. Three colonies of each species were used in this study. Sampling did not involve any endangered species and the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), a Brazilian Ministry of the Environment's enforcement agency, provided authorization for termite sampling (SISBIO no. 33269). Data regarding microbial gut microbiome of *C. cumulans* and *S. euamignathus* were obtained from Menezes et al. [35]. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. DNA extraction. microbial library preparation and sequencing were performed according to Menezes et al. [35] (Online Resource).

Sequence Filtering and Taxon Classification

The 16S libraries were processed using UPARSE pipeline [20]. Briefly, paired end reads were first merged using fastq_mergepairs from USEARCH package version 8.1.1803. Only reads with a minimum overlap of 250 bp and a maximum expected error of 0.5 were used for downstream analysis. After several filtering steps, reads were clustered into OTUs (operational taxonomic units) at 97% of sequence similarity using UPARSE-OTU algorithm. The identified OTUs were further compared to Gold database as reference to filter chimera sequences using chimera UCHIME [21], also implemented in USEARCH package. The OTU table was generated by mapping the reads from each sample back to the OTUs, and it was further filtered to remove potential spurious OTUs, i.e., OTUs that do not present more than one read in at least 10% of the samples were removed. ITS reads were processed in similar way to 16S reads, except for an additional filtering step using ITSx software [4], in order to keep only fungal ITS sequences. Reads were clustering at 97% of sequence similarity. Taxonomic assignment was performed using RDP classifier implemented in MOTHUR and sintax command as implemented in USEARCH version 10.0.240 softwares [57] using DictDB [38] and RDP Warcup training set v2 [15] databases for 16S and ITS sequences, respectively. Relative abundances were calculated as the number of reads per taxon. Only samples with >90 total reads at a clustering level were used to generate relative abundance tables. Downstream analysis, including α- and β- diversity analysis (see below), were calculated using the OTU tables rarefied to the smallest library size. Raw Illumina sequences were deposited in ENA with Accession no. PRJEB17080.

Statistical Analyses

We used R version 3.3.2 [50] to conduct statistical analyses using different software packages. Alpha-diversity estimates and community similarity among all the samples were obtained using the 'phyloseq' package. Plots were constructed with the packages 'ggplot2' [67], 'RcolorBrewer' [43], and 'phyloseq'. A permutational multivariate analysis of variance (PERMANOVA) [2, 46] was used to evaluate whether treatment groupings have a significant effect on community microbiota composition at OTU level. To identify microbial OTUs associated with termite gut and nest samples, we used linear discriminant analysis (LDA) coupled with effect size (LEfSe), a method for biomarker discovery, to detect taxa [59]. LEFSe scores measure the consistency in relative abundances between taxa in the groups analyzed (worker gut vs soldier gut vs nest wall samples). To assess LEFSe and differences in community composition,

we used Calypso web-server (Version 8.58), an online platform for evaluating multiple microbial community composition data [69]. The LDA score threshold was 4.0. We then identified variation in microbial taxa using Wilcoxon rank-sum tests (using Benjamini–Hochberg false discovery rate [FDR] correction procedure for multiple testing). Venn diagrams generated with the online program at http://bioinfogp.cnb.csic.es/tools/venny/index.html [47] were used to compare the distribution of OTUs in the samples.

Results

Sequencing Data from Microbiota of Guts and Internal Nest Walls of *P. araujoi*

We detected 2229 bacterial and 531 fungal OTUs in both gut and nest samples of *Procornitermes araujoi*, and considerable variation in the relative abundance (number of sequence reads) was noted between termite colonies (Tables S1, S2 and S3). Rarefaction curves indicated adequate sampling of bacteria for a valid comparison among nest and gut samples (Fig. S2). Diversity index of microbiota were not significantly different among samples (Figs. S3 and S4). Classification using the DictDb and Warcup databases successfully assigned 100% and 61.8% of the bacterial and fungal reads at phylum level (Tables S2 and S3).

Gut Microbial Communities of *P. araujoi* Exhibited a Similar Assemblage Pattern Between Workers and Soldiers

Gut samples of workers and soldiers of *P. araujoi* yielded 25 bacterial phyla representing 152 families, 151 genera, and 1660 OTUs (Table S2). The most abundant phyla (Firmicutes, Spirochaetes, Bacteroidetes, and Synergistetes), accounted for 96% of gut sequence reads. Firmicutes dominated the gut community, with an average abundance of 44% whereas Spirochaetes, Bacteroidetes, and Synergistetes accounted for 24, 12, and 6%, respectively (Fig. 1a). *Candidatus* Arthromitus (Firmicutes, Lachnospiraceae) was the most abundant genus with a relative abundance of 18%. The group *Treponema* (Spirochaetes) (the majority of the subclusters Ia and Ic), and Termite Cockroach Cluster of the family Synergistaceae (Synergistetes) showed a relative abundance of 14 and 8%, respectively (Fig. S5). Diversity and community composition were not influenced by the caste (Figs. S3 and S8). Approximately 63% of the bacterial diversity (1425 OTUs) overlapped between the gut of workers and soldiers (Fig. S10), with the majority belonging to the Firmicutes. Sordariomycetes and Dothideomycetes were the most abundant fungal classes in termite guts (Fig. S6). In total, 187 fungal OTUs were detected in gut samples of *P. araujoi* (Table S3). Sordariales (23%) and Pleosporales (16.8%) were the most abundant orders (Table S3; Fig. S7). Workers and soldiers shared approximately 40% of fungal reads (63 OTUs) (Fig. S11) and community composition was not different between castes (PERMANOVA, $F=0.76$, $R^2=0.15$, $P=0.60$) (Fig. S9).

Actinobacteria and Sordariomycetes are Abundant in the Internal Nest Walls of *P. araujoi*

The bacterial communities of the internal nest walls of *P. araujoi* harbored 23 phyla, 128 families, 184 genera and 1400 OTUs (Table S2). The majority of the OTUs belonged to the phyla Actinobacteria, Acidobacteria, Chloroflexi and Proteobacteria, accounting for 92% of the reads. The most abundant phyla were Actinobacteria (62% average abundance across samples), Chloroflexi (17%), and Proteobacteria (9%) (Fig. 1a). *Nocardioides* and *Acidothermus* (Actinobacteria) and uncultured lineage 1 of Proteobacteria were the most abundant genera, accounting for 13% of the sequence reads. (Fig. S5). The fungal community associated to the internal nest walls was represented by 493 OTUs, the majority of the class Sordariomycetes (Ascomycota) (Table S3; Fig.S6), of the orders Coniochaetales (18%) and Sordariales (17%) (Table S3; Fig. S8).

Gut and Nest Substrates of *P. araujoi* are Dominated by Different Microbial Assemblages

Analyses of variation in community structure (Figs. S8 and S9) confirmed that assemblages of termite guts and nest substrates differed significantly for bacteria (PERMANOVA, $F=6.97$, $R^2=0.70$, $P=0.005$) and fungi (PERMANOVA, $F=1.84$, $R^2=0.38$, $P=0.033$). Permutation tests for homogeneity of multivariate dispersions (*betadisper*) showed that variances of gut and nest substrate samples were not statistically different and therefore did not influence the results of PERMANOVA. Gut and nest samples shared 652 bacterial and 60 fungal OTUs, corresponding to 29.3 and 11.3% of the total diversity of bacteria and fungi, respectively (Figs. S10 and S11).

Five bacterial OTUs were significantly associated (FDR, $P<0.05$) to nest samples with LDA score >4 (Figs. 1b, 2a). OTU 12 of *Acidothermus* (Actinobacteria) (LDA = 4.68, $P=0.03$) (Fig. 2b) and four unclassified OTUs of Actinobacteria and Chloroflexi were significantly abundant in nest samples. On the other hand, Firmicutes and Bacteroidetes were found in higher abundance in termite guts (Figs. 1b, 2a). In particular, OTU 46 of *Treponema* Ic (Spirochaetes) (LDA = 4.16, $P=0.04$) was the most abundant taxa associated to workers, whereas OTU 90 of *Candidatus* Arthromitus (LDA = 4.12, $P=0.04$) and OTU 108 of the uncultured lineage 24 of Firmicutes (LDA = 4.31, $P=0.03$) were

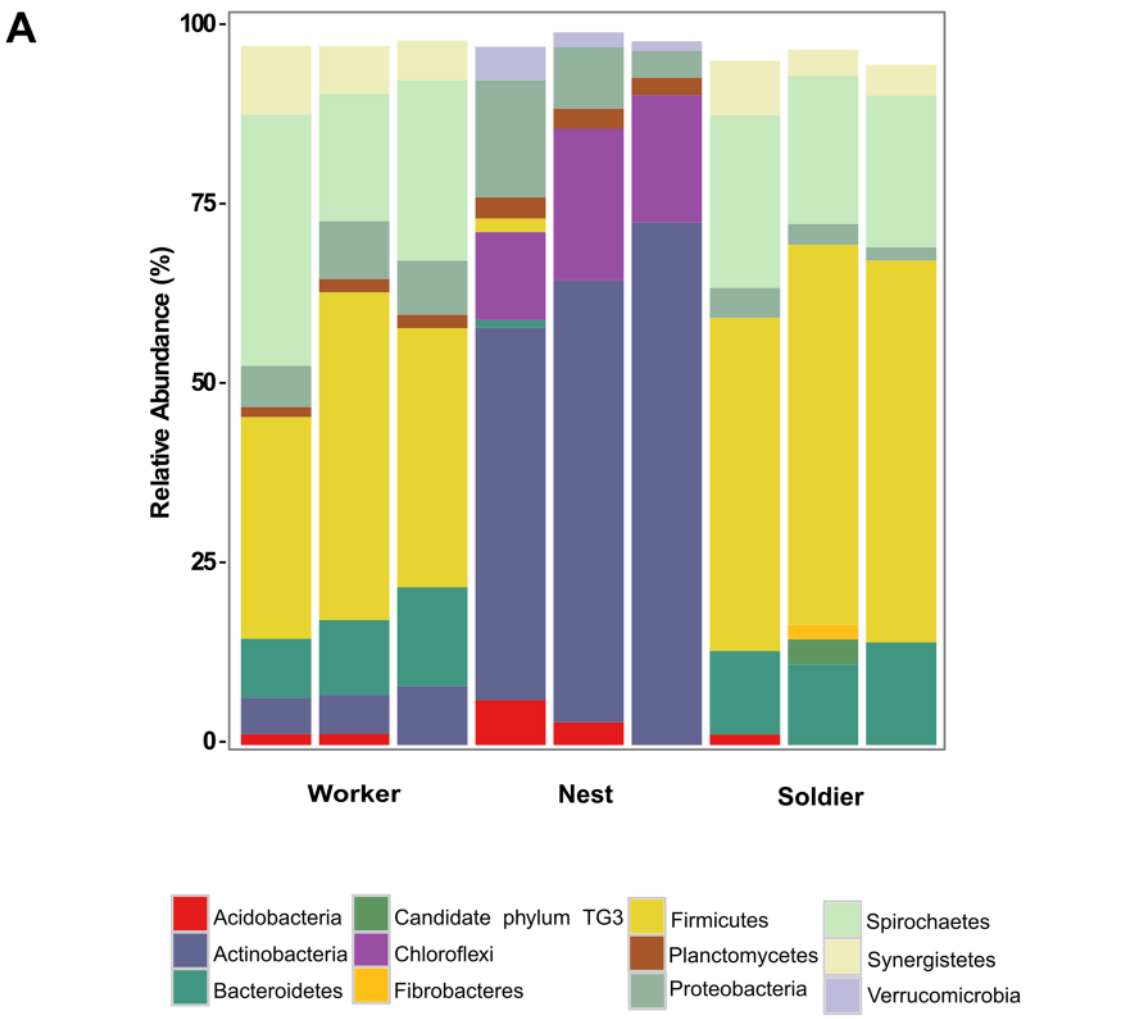

Fig. 1 **a** Taxonomic composition of the bacterial communities associated with the gut and nest substrates of *Procornitermes araujoi*. Relative abundances of the most abundant OTUs are shown at the Phylum level. **b** Comparison of the relative abundances (>1%) of bacterial phyla and genera of gut and nest substrates of *P. araujoi*

significantly associated to the gut of soldiers (Fig. 2b). No features with significant differences between the fungal communities were found between gut and nest samples of *P. araujoi*.

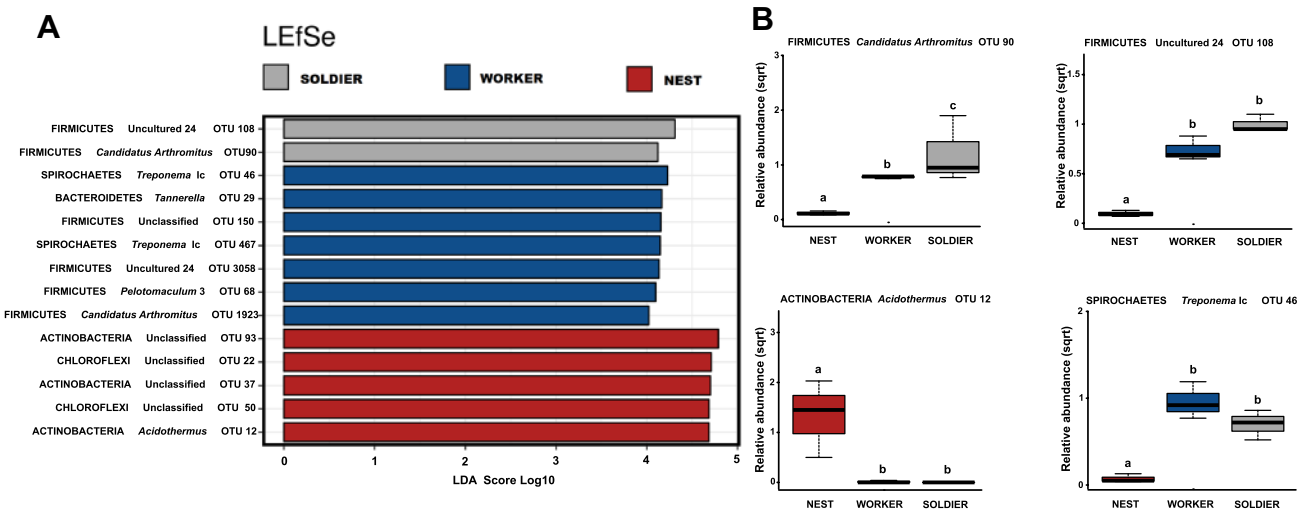

Fig. 2 Bacterial taxa discriminating between gut and nest substrates of *P. araujoi*. **a** Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) enable the identification of OTUs most responsible for the differences between gut and nest substrates. Relative abundances less than 0.01% of the total reads were omitted from further analysis. **b** Relative abundances of the best discriminant OTUs based on LDA analyses. Graphics report median, upper and lower quartiles, and maximum and minimum values. A *P*-Value of <0.05 and a score ≥4.0 were considered significant in Kruskal–Wallis and pairwise Wilcoxon rank tests with FDR-corrected p-values, respectively

Higher Diversity of Gut Bacteria in *P. araujoi* and *S. euamignathus*

Analyses of variation in community structure showed that bacterial assemblages of termite guts differed significantly among the species of Syntermitinae evaluated in this study (PERMANOVA, $F=3.90$, $R^2=0.59$, $P=0.001$). *P. araujoi* and *S. euamignathus* formed two clusters, clearly separated from *C. cumulans* and *S. dirus* (Fig. S12). Firmicutes, Spirochaetes, Bacteroidetes, and Synergistetes were the most abundant phyla present in the gut of all species. Spirochaetes was the most abundant phyla in *C. cumulans*. In contrast, Firmicutes predominated in the gut of *P. araujoi, S. euamignathus* and *S. dirus* (Table S4; Fig. S13). Twenty-two bacterial OTUs contributed most to the differentiation of gut microbiota among the Syntermitinae species. OTU30 of the subcluster Ia of *Treponema* (Spirochaetes) (LDA = 4.34, $P=0.04$) was strongly associated to *C. cumulans* (Fig. 3a, b). Interestingly, bacterial estimated richness and diversity of the gut of workers was significantly higher in *P. araujoi* and *S. euamignathus* (Fig. 3c) and a higher number of OTUs (the majority represented by the phylum Firmicutes, Spirochaetes, and Bacteroidetes) were significantly more abundant in these termites than in the grass/litter feeders *C. cumulans* and *S. dirus*.

Sordariomycetes Dominated Fungal Communities Associated to *P. araujoi*

Fungal community assemblages differed significantly between *P. araujoi* and the sympatric grass/litter feeding *C. cumulans* (PERMANOVA, $F=3.38$, $R^2=0.59$, $P=0.001$) (Fig. S14). Ten OTUs discriminated the gut fungal communities between the two species (Fig. S15). Two OTUs of Sordariomycetes were strongly associated to the gut of *P. araujoi* (OTU 105, LDA = 4.16, $P=0.01$ and OTU 35, LDA = 4.13, $P=0.02$), whereas three OTUs of Dothideomycetes were the most prominent taxa associated to *C. cumulans* (OTU 14, LDA = 4.55, $P=0.01$; OTU 9, LDA = 4.73, $P=0.01$; and OTU 228, LDA = 4.71, $P=0.01$). On the other hand, the most abundant fungal taxa that differentiated nest samples were OTU 6 of Sordariomycetes (Sordariales) (LDA = 4.93, $P=0.04$) and OTU 16 of Dothideomycetes (Pleosporales) (LDA = 4.30, $P=0.02$) for *P. araujoi* and *C. cumulans*, respectively.

Discussion

The gut and nest microbial community associated with termites is of interest due to their role in degrading lignocellulose and contributing to carbon mineralization and recycling of nutrients [7, 42, 54, 60]. However, for Syntermitinae the interaction with their associated microbiota is poorly understood. The most abundant taxa found in the gut of

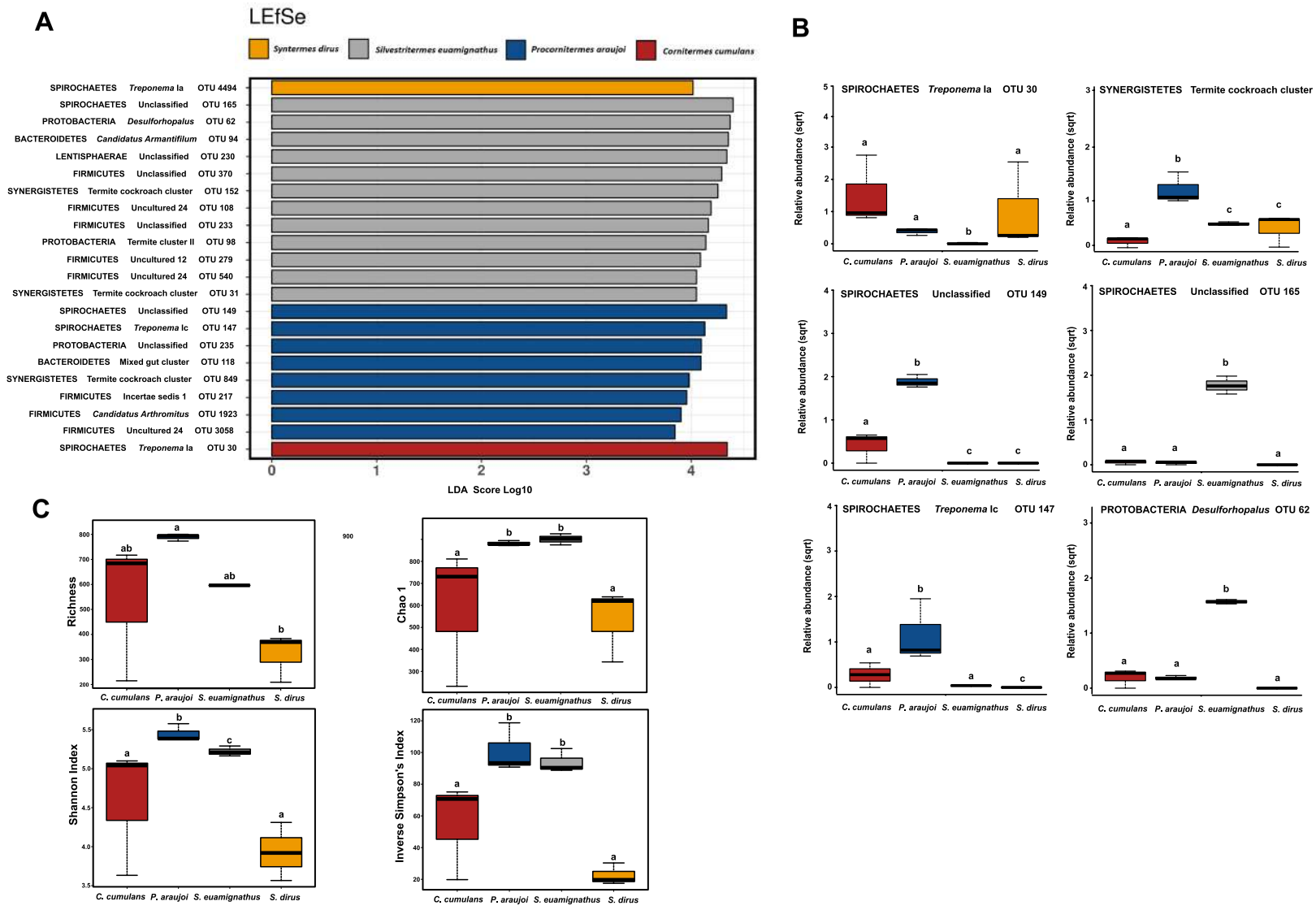

Fig. 3 Bacterial taxa discriminating the gut of Syntermitinae workers. **a** Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) enable the identification of OTUs most responsible for the differences between termite species. Relative abundances less than 0.01% of the total reads were omitted from further analysis. **b** Relative abundances of the best discriminant OTUs based on LDA analyses (score ≥4.0). **c** Alpha-diversity estimates for bacterial 16S amplicons of the gut of Syntermitinae workers. Graphics report median, upper and lower quartiles, and maximum and minimum values. A *P*-value of <0.05 was considered significant in Kruskal–Wallis and pairwise Wilcoxon rank tests with FDR-corrected p-values, respectively

workers and soldiers of *P. araujoi* where Firmicutes and Spirochaetes. At the genus level, *Candidatus* Arthromitus (Firmicutes, Lachnospiraceae), a filamentous bacterium commonly found on the gut wall of various arthropods [62], was the most abundant genus found in the gut of *P. araujoi*. The majority of Spirochaetes found in the gut of *P. araujoi* belonged to the subclusters Ia and Ic of *Treponema*. In the wood-feeding *Nasutitermes*, subclusters of *Treponema* have different affinities with the ingested food. In contrast with subcluster Ia, subcluster Ic is associated to wood particles in the termite gut Since members of subcluster Ia are not cellulolytic, it is possible that different subclusters have a different contribution to fiber degradation [39]. Furthermore, there is strong evidence that members of *Treponema* are involved in lignocellulose digestion [27], because the metagenome of some termites comprise many genes binned to *Treponema* that encoded for various types of cellulases [66].

Diet has been shown to affect bacterial assemblages in termites [37] and our results also showed clear differences among the species of Syntermitinae evaluated in this study. Firmicutes, Spirochaetes, Bacteroidetes, and Synergistetes were the most abundant phyla present in the gut of all species evaluated in this study. Firmicutes predominated in the gut of *P. araujoi, S. dirus*, and *S. euamignathus*, as found in other litter and humus-feeding species [13, 16, 35, 54]. In contrast, Spirochaetes of genus group *Treponema* was the most abundant phyla in *C. cumulans*, which in turn could be explained by the fiber-rich grass diet of this species. The bacterial profile found for *P. araujoi* differ in some extension to that of *Procornitermes* sp reported recently. In particular, Menezes et al. [35] profiles have higher proportions of *Ca*. Arthromitus, *Treponema* subcluster Ic, and the Termite Cockroach Cluster than the corresponding profiles in this study, which could reflect that different *Procornitermes* species were sampled or geographical variation on gut microbiota.

According to Mikaelyan et al. [36], *Ca*. Arthromitus is predominantly abundant in the enlarged first proctodeal segment (P1) of humus/litter and soil-feeders Termitidae; however, the abundance of this bacteria declines significantly in the posterior gut segments. Although the high alkalinity could select for bacterial lineages that are adapted to colonize the P1 compartment, other factors probably determine the distribution of bacteria in the termite gut. The diet of termite feeding groups seems to be a determinant of the community structure of symbiotic bacteria [37]; however, other factors such as the morphology and ultrastructure of the digestive tract could facilitate the presence of certain groups of bacteria. All the Syntermitinae have an enlarged P1 with a complex internal ornamentation that seems to be related to their feeding habits [53]. These structures may function as an abrasive and/or mixing surface for the food mass that enters from the midgut. However, another possible explanation for P1 ornamentation is microbial inoculation of ingested food before entry to the enteric valve, allowing the colonization of the food by bacteria [56]. This hypothesis is supported by the presence of numerous bacteria covering the P1 spines observed in several species of Syntermitinae [53]. It is possible that a spiny internal surface of P1 could favor the settlement of filamentous lineages of bacteria such as *Ca*. Arthromitus.

The gut microbiota of *P. araujoi* was dominated by a core set of bacterial lineages that were present across workers and soldiers, which is consistent with other species of Termitidae [17, 18, 48]. However, there is still little information about the mechanisms of transmission of gut symbionts among colony members in termites. It seems that the acquisition and maintenance of core gut microbiota in termite colonies rely on social interactions, allowing the transmission of gut bacteria from workers to other members of the colony.

Nevertheless, our more interesting finding was that richness and bacterial diversity of gut bacteria were higher in *P. araujoi* and *S. euamignathus*, than in the grass/litter feeders *C. cumulans* and *S. dirus*. Furthermore, a higher number of OTUs discriminated significantly *P. araujoi* and *S. euamignathus* from the grass/litter feeders. Although many aspects involving the feeding ecology of these species are unknown, our results could indicate an adaptation of the microbial community of these termites to the higher complexity of their diet as pointed out by several studies (see "Introduction" for references). *P. araujoi* workers are known to forage on grass/litter, humus [9, 26, 34], and even cattle excrement (personal observation). Moreover, species of *Silvestritermes* exploit several sources of food, including grass/litter, rotten logs, tree bark, and organic matter accumulated in the mounds of other termite species [34]. Termites are known to modulate their microbial community according to the feeding components of their diet [29, 61]. For instance, termites that exploit food substrates with more complex structures showed a higher diversity of gut microbes [40]. In contrast, termites with homogeneous diets may have a reduced microbiota diversity [65]. As the complexity of the diet increases, a greater repertoire of microbes may be required to utilize efficiently the nutritional components of the food. On the other hand, more homogenous diets may increase competition among gut symbionts and therefore, reduce their diversity [25].

Nests of termites are composed of soil and feces, which are incorporated into micro-aggregates by workers forming a microbial environment known as the termitosphere. The function of the termitosphere and the mechanisms by which it may complement the gut symbionts are not completely understood. According to Brauman [6], the organic matter in the termitosphere is more stable and protected from the intense mineralization that occurs in the tropics. In this

context, nest termitosphere could function as a supplementary reserve of lignocellulolytic microorganisms for termites or even acts as fermenting chambers in food storing termites [35]. Actinobacteria, Chloroflexi, and Proteobacteria dominated the microbiota assemblage of internal nest walls of *P. araujoi*, as found in the nests of other termite species [33, 35]. Specific lineages of Actinobacteria and Proteobacteria have been reported to constitute the largest groups of bacterial communities derived from a variety of soils [49]. These bacteria probably colonize nest substrates after their transit through the host gut since they were also found at lower proportions in workers' guts. Additionally, the dominance of these bacteria in termite nests could suggest that colonization conditions by these microorganisms are better in nest substrates than in guts. It has been suggested that Actinobacteria might play defensive roles in termite nests [63]. Therefore, another possibility worth further inquiry is that termites may benefit directly or indirectly from other nutrients or compounds, such as specific antibiotics provided by nest wall microbiota.

Except for the mutualistic association between *Termitomyces* (Basidiomycota) and fungus-growing termites (Macrotermitinae) [1], the interaction between fungus with non fungus-growing termites is poorly known. However, in contrast to the bacterial community, the fungal community was considerably less diverse. All the fungal OTUs found associated to the gut and the nest of *P. araujoi* belong to the phylum Ascomycota, which have been found in other insect gut microbiomes [58, 68]. Fungal assemblages of gut and nest substrates formed distinct assemblages. This dissimilarity may be associated with the ecological properties of the fungi as well as their ability to survive and reproduce in the termitosphere. Therefore, the availability of organic matter from the feces and the partially controlled microclimate within the termite nests favor the establishment and the proliferation of specific fungal species [3]. Interestingly, the fungi associated with the gut and nest walls of the sympatric grass/litter feeder *C. cumulans* was composed only by representatives of Dothideomycetes, suggesting some degree of host-specificity by fungi. Another possibility is that the association of fungi with termites is related to the decomposition of the ingested lignocellulose since some lineages, identified in nest and gut samples of both termite species are known to have lignocellulolytic properties [32, 64].

Our results provide new insights about the feeding ecology of Syntermitinae. While researchers are trying to understand the functions of the termite symbionts, fundamental biological aspects related to the diet of these insects and their associated microbiota are not known. The description of the gut microbiota of a litter-feeding termite species will help us to understand their feeding ecology and role in savannas by decomposing the lignocellulosic materials through the ingestion of various forms of plant substrates.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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