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HOST MICROBE INTERACTIONS

Food Storage by the Savanna Termite *Cornitermes cumulans* (Syntermitinae): a Strategy to Improve Hemicellulose Digestibility?

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Abstract

It has been suggested that food storage inside the nest may offer termites with a nutritional provision during low resource availability. Additionally, feces employed as construction material provide an excellent environment for colonization by microorganisms and, together with the storage of plant material inside the nest, could thus provide some advantage to the termites in terms of lignocellulose decomposition. Here, we conducted for the first time a comprehensive study of the microbial communities associated to a termite exhibiting food storage behavior using Illumina sequencing of the 16S and (ITS2) regions of rRNA genes, together with enzymatic assays and data collected in the field. *Cornitermes cumulans* (Syntermitinae) stored grass litter in nodules made from feces and saliva located in the nest core. The amount of nodules increased with nest size and isolation, and interestingly, the soluble fraction of extracts from nodules showed a higher activity against hemicellulosic substrates compared to termite guts. Actinobacteria and Sordariales dominated microbial communities of food nodules and nest walls, whereas Spirochetes and Pleosporales dominated gut samples of *C. cumulans*. Within Syntermitinae, however, gut bacterial assemblages were dissimilar. On the other hand, there is a remarkable convergence of the bacterial community structure of Termitidae nests. Our results suggest that the role of nodules could be related to food storage; however, the higher xylanolytic activity in the nodules and their associated microbiota could also provide *C. cumulans* with an external source of predigested polysaccharides, which might be advantageous in comparison with litter-feeding termites that do not display food storage behavior.

Keywords Gut microbiota · Stored food · Lignocellulose · 16S · ITS · Termites

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Introduction

For millions of years, termites have played an important role in ecosystems by decomposing most of the lignocellulose through the ingestion of various forms of plant materials, herbivore dung, and soil humus [1], thus contributing to carbon mineralization [2] and recycling of nutrients [3]. However, lignocellulose digestion is a complex process, and symbionts, along with endogenous enzymes, play an important role in the efficient degradation of plant-derived products [4].

The acquisition of cellulolytic symbionts and their transmission to siblings were one of the main events within termite evolution. Termites and their sister group, the wood-feeding cockroaches *Cryptocercus* (Cryptocercidae), evolved from a cockroach-like detritivore ancestor [5]. Both groups share

social behavioral traits [6] and depend on phylogenetically related microbial symbionts for lignocellulose digestion. Gut symbionts in termites consist of several groups of cellulolytic flagellates, bacteria, and archaea. Nontermitid termites (normally called lower termites), considered to represent basal lineages, consist typically of wood-feeding species and depend on flagellate protists and prokaryotes for lignocellulose digestion [7]. On the other hand, the evolutionary success of Termitidae (higher termites) is attributed to the loss of protists and acquisition of a specialized prokaryotic gut fauna, accompanied by a dietary diversification and a highly elaborate behavioral repertoire [8]. Food sources of higher termite lineages include wood, grasses, litter, microepiphytes, and even the mycelia of symbiotic fungi [9].

Dietary diversification of higher termites is reflected in the functional composition of their bacterial gut microbiota [1]. Recent comparative studies have revealed a diet-related convergence in bacteria community composition among species from the same feeding guild [5, 10]. In contrast, there are functional divergences of the symbiotic bacteria among termite-feeding guilds. For example, gut symbiont composition in wood-feeding higher termites consists mainly on Fibrobacteres and Spirochetes. Conversely, these bacterial lineages decreased in abundance in nonwood-feeding species [11], thus corroborating that the type of diet is determinant of the symbiotic community structure in termite-feeding guilds. Higher termites also employ an elaborate system of division of labor with fundamental differences among ages and castes. These differences in diet among castes and age of the individuals are also reflected in the community structure of their symbiotic bacteria [12].

The neotropical subfamily Syntermitinae contains species that range in their feeding habits from wood- and litter-feeding to humus-feeding representatives [13]. *Cornitermes cumulans* (Kollar, 1932) is a common mound-building Syntermitinae in pastures and savannas of center and south Brazil, Paraguay, and north of Argentina. Its nests (mounds) have a conical shape above ground (epigeal) and a rounded shape below ground (hypogeal). The external wall is hard and made mainly of soil, while the carton core is dark and soft, built mainly with fecal material and soil microaggregates. *C. cumulans* is a harvester termite and workers collect small pieces of living and dead grass to be stored inside the nest [14]. As far as we know, several species of Termitidae also store food in underground galleries or inside nest chambers [15]; however, workers of *C. cumulans* embedded small pieces of plant substrates on the walls of the nest core using their feces and saliva [16], forming ball-shaped nodules (Fig. S1). Feeding experiments performed in the laboratory, using different plant substrates, showed that the survival of workers was significantly higher when food nodules were offered (Costa-Leonardo, unpublished). It has been suggested that food storage may provide termites with a food supply enriched in nitrogen [17]; however, studies relying upon food storage in termites are scarce. In Brazil, *C. cumulans* is considered a keystone species because it is the most abundant mound-building termite in savannas and their nests promote the colonization by other organisms [18].

Here, we conduct for the first time a detailed study of the microbial communities associated to a termite exhibiting food storage behavior using Illumina sequencing of the 16S and the internal transcribed spacer (ITS) regions of rDNA genes, together with enzymatic assays and data collected in the field.

First, we evaluated food storage by *C. cumulans* in the field. Given the facts that the relationship between nest and population size of mound-building species is nearly linear [19] and that nest density could increase competition among colonies, we hypothesized that (1) larger nests will store a substantial amount of food nodules and (2) a higher nest density has a negative effect on food storage by reducing available food. Second, since the feces employed as nest construction material are an excellent environment for microorganisms [6], we predicted that plant materials stored in food nodules by *C. cumulans* would be colonized by nest microbes providing some advantage to the termites in terms of lignocellulose decomposition. We explored this issue through enzymatic analyses and characterizing microbial assemblages of the gut and nest substrates. In order to verify whether the enzymatic activity is characteristic for *C. cumulans*, we compared to patterns in other termite species that store and do not store food. Finally, we also evaluated how the community structure of gut and nest bacteria of *C. cumulans* compares to other feeding groups, representing the subfamilies Syntermitinae, Nasutitermitinae, and Apicotermitinae. Together with *C. cumulans*, the selected species included another termite that stores food inside the nest.

Methods

Termites and Study Site

Cornitermes cumulans (Kollar, 1982), *Procornitermes* sp., *Silvestritermes* sp. (Syntermitinae), *Velocitermes* sp. (Nasutitermitinae), and *Ruptitermes* sp. (Apicotermitinae) were collected in rural areas of Alfenas (21° 25′ 45″ S; 45° 56′ 50″ W)—State of Minas Gerais and Campinas (22° 54′ 3″ S; 47° 03′ 26″ W) and Rio Claro (22° 24′ 41″ S; 47° 43′ 31″ W)—State of São Paulo, Brazil. All the termite species were sampled in areas that were originally semideciduous forests but agricultural development has fragmented the vegetation, resulting in a largely deforested landscape with pastures interspersed with native forest fragments (Fig. S1). The climate is moderately humid; the annual mean temperature and rainfall are 23 °C and 1513 mm, respectively, with altitude ranging from 720 to 1350 m. 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). Sampled termites included representatives specialized on grass/litter (*C. cumulans* and *Velocitermes* sp.), leaf/litter (*Ruptitermes* sp.), and the intermediate feeders (*Silvestritermes* sp. and *Procornitermes* sp.) [13, 20]. The termites used in this study, except for the subterranean *Ruptitermes* sp., built epigeal nests with inner carton (fecal) structure [21]. In common with *C. cumulans*, the *Velocitermes* species collected in this study also store plant material; however, it is deposited diffusely inside nest cameras. Data regarding the relative abundance of gut bacteria in additional species of Termitidae were obtained from Mikaelyan et al. [11] which used Illumina MiSeq platform analyses. The selected species included litter feeders (*Cornitermes* sp. and *Velocitermes* sp2) and wood feeders (*Nasutitermes corniger* and *N. takasagoensis*).

Food Storage in the Nests of *C. cumulans*

Field observations were conducted on 30 nests located in three pasture areas dominated by *Brachiaria* grass between January and March of 2015. The nests were opened using hand tools and the food nodules were removed for ecological and nutritional analyses. The diameter and height of all nests were measured to calculate their volume. The nests were considered to have a cone shape structure. To evaluate the effects of nest size and density on food storage, the dry weight of food nodules and the distance to the nearest neighbor of each nest were measured. Analyses of nutrient composition were determined using 5 mg of food nodules from each nest. Food nodules were evaluated following AOAC [22] for moisture content (method 925.09), proteins (method 960.52, conversion factor 6.25), ash content (method 923,39, and lipids (method 945.16). Fiber content was determined using the method of Van Soest [23].

Assays of Cellulolytic and Hemicellulolytic Activities of Worker Guts and Stored Food

The activity against cellulose and hemicellulose substrates was assayed using crude enzyme extracts from worker guts of *C. cumulans*, *Procornitermes* sp., *Velocitermes* sp., and *Silvestritermes* sp. as well as from the food stored by *C. cumulans* and *Velocitermes* sp. The assays were conducted to evaluate the activity of the soluble fraction of protein extracts against natural polysaccharides and oligosaccharides that differed in monomer composition and branching. Three colonies of each species were used in this experiment. Crude protein extractions were performed from 50 workers as well as for 10 mg of stored food from each colony. The samples were homogenized in 2 ml of 100 mM sodium acetate buffer, pH 5.5. Afterwards, the crude extract was centrifuged at 20,100×*g* for 30 min at 4 °C, the supernatant was collected, and 1 μl of Protease Inhibitor Cocktail (Anresco) per ml of crude extract was added. The protein concentration from each crude extract was determined using the Bradford method [23].

The enzymatic assays followed the methodology previously described by Franco Cairo et al. [24], consisting of 10 μl of crude protein extract incubated at 37 °C for 40 min with 40 μl of 50 mM sodium acetate buffer pH 5.5 and 50 μl of 0.5% specific substrate (in water), in triplicate. Enzymatic assays were stopped after the addition of 100 μl of dinitrosalicylic acid (DNSA) and heated at 99 °C for 5 min. The measurement of color change was performed at 540 nm using a microplate reader. The enzymatic activity assay results were expressed in micromoles of glucose equivalents produced per milligram of protein. Blank reactions were performed as described above with 100 μl of dinitrosalicylic acid already added in the reaction before the incubation.

The enzymatic assays with *p*-nitrophenyl-monosaccharide (pNP-G) were performed as follows: 10 μl of crude extracts were incubated with 50 μl of 5 mM pNP-G and 40 μl of 50 mM sodium acetate buffer, pH 5.5. The reactions were stopped after the addition of 100 μl of 1 M sodium carbonate (Na_2CO_3). The measurement of color variation was performed at 412 nm using a TECAN M2000 plate reader. The enzymatic assays were done in triplicates and results were expressed in terms of millimolars of *p*-nitrophenyl released. Blank reactions were performed as described above with 100 μl of 1 M Na_2CO_3 already added in the reaction before the incubation. Glucose and *p*-nitrophenyl were used for standard curve construction. Substrates were purchased from Megazyme and Sigma-Aldrich: CMC (carboxymethyl cellulose low viscosity) (β-1,4-carboxymethylglucan), β-glucan from barley (low viscosity) (β-1,4-glucan), xylan from oat spelt (β-1,4-xylan), rye arabinoxylan (α-2,3-arabinose-β-1,4-xylan), pectin from *Citrus* sp. (β-1,4-D-galacturonic acid methyl ester), and pNP-G (4-nitrophenyl-β1,4-D-glucopyranoside).

DNA Extraction

Termites were anesthetized on ice for 5 min and dissected with fine forceps. For all the species collected in this study, the whole gut of 200 workers was placed in 2 ml tubes containing 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate (SDS)). All the samples were stored at − 20 °C until DNA extraction and purification using a bead-beating protocol [25]. DNA integrity was assessed by agarose gel electrophoresis (1.0 *w*/*v*), and the quantification was performed using a NanoDrop spectrophotometer by measuring the absorbance at 260 nm. For *C. cumulans*, the whole gut of 200 soldiers was also extracted. Samples (approx. 50 mg) from the carton nest core and the

food stored (together called hereafter as nest substrates) were collected and extracted as described above. Three colonies of each species were used in this study.

Bacterial and Fungal Library Preparation and Sequencing

To access the diversity and abundance of the bacterial communities, the V4 region of the bacterial 16S rRNA was targeted using primers 515F (5′ TCGTCGGCAGCGTC A G A T G T G T A T A A G A G A C A G G T G C CAGCMGCCGCGGTAA 3′) and 806R (5′ GTCT CGTGGGCTCGGAGATGTGTATAAGAGACAGGGAC TACHVGGGTWTCTAAT 3′) with addition of Illumina overhangs (underlined) [26]. The fungal community of the gut and nest substrates of *C. cumulans* was accessed by sequencing the internal transcribed spacer region 2 (ITS2) using primers ITS3 (5′ TCGTCGGCAGCGTCAGATGTGTATAA GAGACAG*GCATCGATGAAGAACGCAGC* 3′) and ITS4 (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AG*TCCTCCGCTTATTGATATGC* 3′) [27] with addition of Illumina overhang adapter sequences.

Bacterial and fungal libraries were prepared using two PCR steps. The first PCR step was performed using specific primers for each library using Phusion polymerase (Thermo Scientific) and 40 ng of template DNA from each sample. For 16S amplification, the thermocycler program was set at initial denaturing at 98 °C for 2 min, followed by 30 cycles of 98 °C (30 s), 60.1 °C (30 s), and 72 °C (40 s), ending with a final extension of 72 °C (5 min). For ITS2 amplification, the thermocycler was set as follows: 98 °C (2 min), followed by 25 cycles of 98 °C (30 s), 50 °C (1 min), and 72 °C (40 s), and a final extension of 72 °C (10 min). The second PCR step was necessary to add Illumina sequencing adapters and dual index barcodes (Nextera Index Kit, Illumina) to the amplified libraries using Phusion polymerase (Thermo Scientific) with 100 ng of purified PCR from a previous step as template and the indexing primers from Illumina. Each sample was amplified in triplicates and pooled for purification and quantification using Agencourt AMPure magnetic beads (Beckman Coulter) and Qubit Fluorometer 2.0 (Invitrogen). The second PCR step for 16S was set at initial denaturation at 98 °C for 3 min, followed by 5 cycles of 98 °C (30 s), 55 °C (30 s), and 72 °C (30 s), with a final extension of 72 °C for 5 min. The second PCR step for ITS2 was similar except that 8 cycles of amplification were performed. Libraries were quantified by real-time PCR using the KAPA Library Quantification Kit Illumina® Platforms (Kapa Biosystems). Sequencing was performed on the Illumina Miseq Platform (available at the Brazilian Bioethanol Science and Technology Laboratory - CTBE/CNPEM), using a Miseq Reagent Kits V3 (600 cycles) and V2 (500 cycles).

Sequence Filtering and Taxon Classification

The 16S libraries were processed using UPARSE pipeline [28]. 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 operational taxonomic units (OTUs) at 97% of sequence similarity using UPARSE-OTU algorithm. The identified OTUs were further compared to the Gold database as reference to filter chimera sequences using chimera UCHIME [29], 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 a similar way to 16S reads, except for an additional filtering step using ITSx software [30], 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 syntax command as implemented in USEARCH version 10.0.240 softwares [31] using DictDB [32] and RDP Warcup training set v2 [33] databases for 16S and ITS sequences, respectively. Relative abundances were calculated as the number of reads per taxon. Downstream analysis, including α- and β-diversity analysis (see below), was calculated using 'phyloseq' package [34], using the OTU table rarefied to the smallest library size.

Statistical Analyses

We used R version 3.3.2 [35] to conduct statistical analyses using different software packages. For field observations, we verified whether the amount of food nodules stored by *C. cumulans* was affected by nest size (volume) and the nearest-neighbor distance with generalized mixed effects models (GLMMs) ($\alpha = 0.05$) with normal error distribution and locality as random effect, using 'lme4' package [36]. We also explored the enzymatic activity of crude extracts using linear models (LM). The data was also transformed into log ($X + 1$) aiming to meet the assumptions of the test. We used the 'lsmeans' [37] and the 'multcompView' [38] packages to assess multiple comparisons of least-squares means (lsmeans).

Alpha-diversity estimates were calculated using the function *plot_richness* in the 'phyloseq' package, including observed OTU richness, Shannon's index, Simpson index, and Chao1 estimator of richness. ANOVA or Kruskal-Wallis tests with *p*-values adjusted for multiple comparisons via the false discovery rate (FDR) procedure [39] were performed to check for overall significant differences of α-diversity estimates among termite species, castes, and nest substrates. Plots were constructed with 'ggplot2' [40], 'RcolorBrewer' [41], and 'phyloseq'.

Community similarity among all the samples was visualized using principal coordinates analysis (PCoA) with 'phyloseq'. A permutational multivariate analysis of variance (PERMANOVA) was used to evaluate whether treatment groupings (termite species, caste, and nest substrates) visualized in PCoA plots have a significant effect on community microbiota composition at the phylum, genus, and OTU levels. PERMANOVA tests on log-transformed data were carried out via the *adonis* function in the 'vegan' package [42]. A multivariate analogue of Levene's test for homogeneity of group dispersions (function *betadisper*) was conducted to test for heterogeneity of community structure between treatments [43]. Taxa responsible for the differences in community composition between treatments were assessed using the function *multipatt* in the 'indicspecies' package [44] after significant results from PERMANOVA tests. Only samples with > 90 total reads at a clustering level were used to generate relative abundance tables.

Data Accessibility

Raw Illumina sequences were deposited in ENA with accession no. PRJEB17080.

Results

Food Storage in the Nest of *C. cumulans*

Food nodules were located at the lower portion of the carton nest core. A thin layer of termite feces covered the external wall of the nodules, and the interior contained small cut pieces of leaves, twigs, and seeds (Fig. S1). Size of nodules ranged from 1.1 to 3.5 cm with an individual dry weight of 4.21 ± 0.16 g (mean ± SD; $n = 10$). Basic analyses of nutrient composition revealed that food nodules contained fiber (38.40 ± 7.73%), minerals (27.36 ± 4.12%), nitrogen (6.16 ± 2.54%), and lipids (1.26 ± 0.74%). Water content in food nodules was determined in 27.56 ± 2.76%. Field observations showed that nests of *C. cumulans* contained an average of 145.69 ± 121.65 g of food nodules (dry weight; mean ± SD; $n = 30$) and the weight of stored food increased significantly with nest volume (GLMM; $t = 4.12$; $n = 24$; $p = 0.001$) (Fig. S2a). We also evaluated the impact of nest density on food storage by the termites, resulting in a significant positive effect on food storage as the nearest-neighbor distance increased (GLMM; $t = 3.87$, $n = 22$; $p = 0.001$) (Fig. S2b).

Increased Hydrolysis of Hemicellulose in the Food Stored by Termites

The soluble fraction of protein extracts from worker guts as well as from the food stored by *C. cumulans* and *Velocitermes* sp. was able to hydrolyze all the polysaccharides and oligosaccharides evaluated in this study. Cellulolytic and hemicellulolytic activities of gut crude extracts varied significantly among termite species, including those that do not store food (*Procornitermes* sp. and *Silvestritermes* sp.) ($F = 7.92$; $df = 15$; $p < 0.001$) with the highest values observed against cellulosic substrates (β-glucan and pNP-G). Interestingly, the food stored by *C. cumulans* and *Velocitermes* sp. showed a higher xylanolytic activity than gut extracts for all species evaluated ($F = 5.32$; $df = 5$; $p < 0.001$) (Fig. 1).

Sequencing Data from Microbiota of the Gut and Nest Substrates of *C. cumulans*

Worker gut samples generated between 132,452 and 190,827 16S rRNA gene reads and soldier guts between 92,211 and 160,473 reads. We obtained between 91,573 and 190,437 reads for the fecal material of the nest core and between 84,838 and 128,875 reads for food nodules. For the fungal ITS gene, gut sample reads ranged from 4613 to 15,853 and 4079 to 10,891 for workers and soldiers, respectively. On the other hand, the nest walls and the nodules generated considerably more reads, ranging from 185,409 to 299,514 and 53,181 to 765,419, respectively. Considerable variation in the number of bacterial and fungal sequence reads was noted between termite colonies (Table S1). A total of 2172 bacterial and 305 fungal OTUs were detected at gut and nest samples of *C. cumulans* (Tables S2 and S3). Rarefaction curves indicated adequate sampling of bacteria for a valid comparison among nest and gut samples (Fig. S3), and diversity indices of microbiota were not significantly different among samples (Fig. S4). In the fungal ITS gene region, the observed richness was significantly higher for nest wall and nodules, but the diversity was significantly lower ($p < 0.001$) in nodules (Figs. S3 and S5). Classification using the DictDb and Warcup databases successfully assigned 100 and 97.7% of the bacterial and fungal reads at the phylum level, decreasing with taxonomic depth (Tables S2 and S3).

Gut Microbial Assemblages of *C. cumulans* Are Represented by Abundant Spirochaetes and Pleosporales

Gut samples of workers and soldiers of *C. cumulans* yielded 23 bacterial phyla representing 98 families, 130 genera, and 1539 OTUs (Table S2). The most abundant phyla (Actinobacteria, Bacteroidetes, TG3, Fibrobacteres, Firmicutes, Proteobacteria, Spirochaetes, and Synergistetes) accounted for 96.7% of gut sequence reads. Spirochaetes dominated the gut community, with an average abundance of 51.5%, whereas Firmicutes and Bacteroidetes accounted for 17.2 and 7.7%, respectively (Fig. 2). The group *Treponema* (Spirochaetes), the majority of

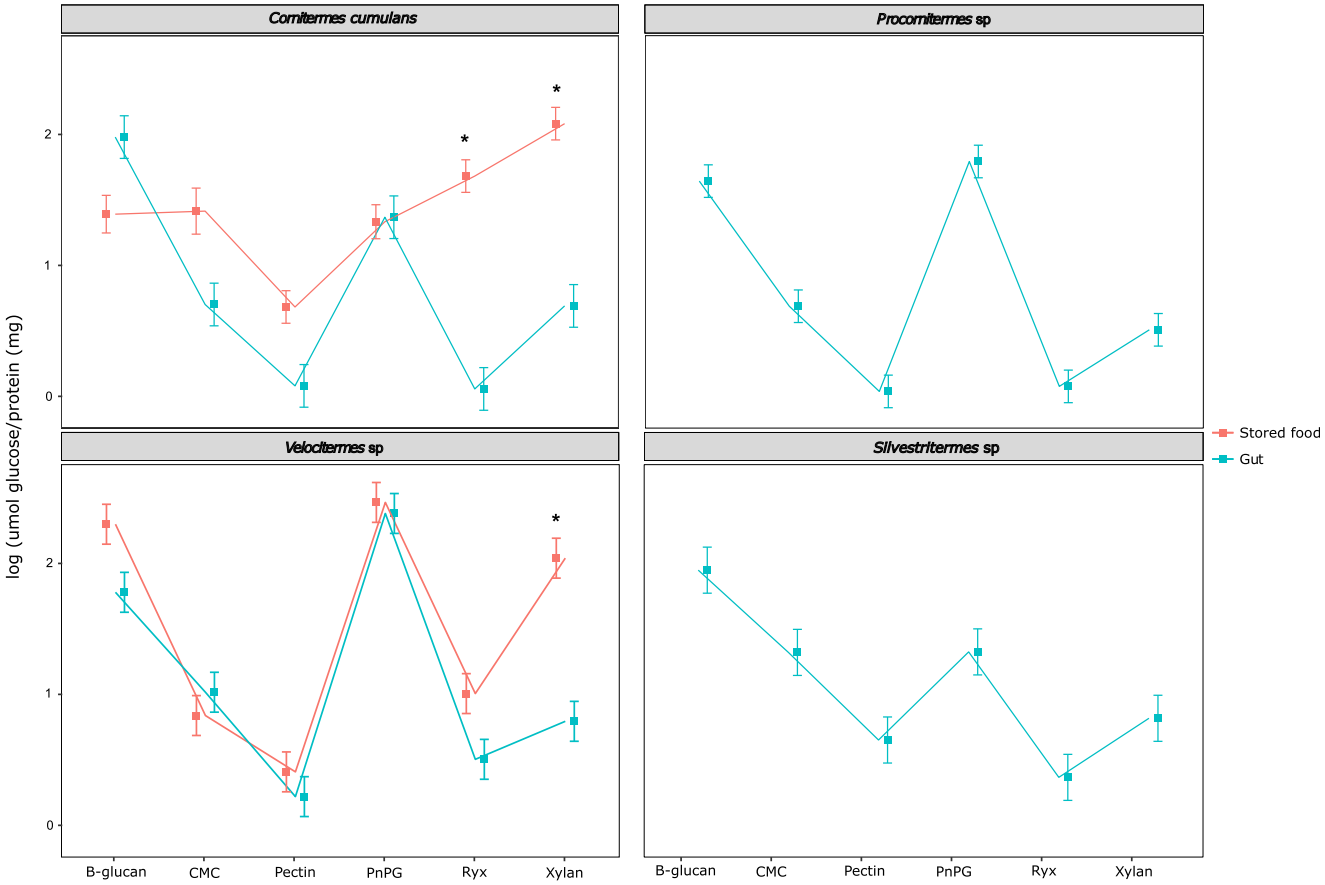

Fig. 1 Cellulase and hemicellulase activities of the soluble fraction of gut and stored food protein extracts. Displayed are the least-squares means (±SE) of each activity value from four termite species. Asterisks indicate significant differences after multiple comparison least-squares means test ($p < 0.05$). Substrate abbreviations: CMC (carboxymethyl cellulose) (β-1,4-carboxymethylglucan), β-glucan (β-1,4-glucan), xylan (β-1,4-xylan), rye arabinoxylan (α-2,3-arabinose-β-1,4-xylan), pectin (β-1,4-D-galacturonic acid methyl ester), and pNP-G (4-nitrophenyl-β1,4-D-glucopyranoside)

the subclusters Ia, Ic, and If, and *Candidatus Arthromitus* (Firmicutes, Lachnospiraceae) were the most abundant genera with 30.4 and 7.5% of relative abundance, respectively. Termite Cockroach Cluster in the family Synergistaceae (Synergistetes), the subcluster Ib of Fibrobacteres, the subcluster IIIb of the TG3 phylum, and *Tannerella* (Bacteroidetes) had a cumulative relative abundance of 10.4% (Table S2; Figs. 4 and S6). Alpha-diversity analyses showed that bacterial richness and diversity were similar between workers and soldiers (Fig. S4). Workers and soldiers shared approximately 73% of bacterial reads (1120 OTUs) (Fig. S7); however, similarity analyses showed that community composition was not affected by caste origin (PERMANOVA, $F = 1.00$, $R^2 = 0.20$, $p = 0.60$). At the phylum level, Actinobacteria and Proteobacteria were significantly more abundant in workers (FDR-corrected ANOVA test, $p < 0.05$) (Fig. 2); however, for lower-taxon-level groups, we did not detect differences of relative abundances between the castes.

In total, 159 fungal OTUs were detected in gut samples of *C. cumulans* (Table S3). Dothideomycetes (65%) and Sordariomycetes (30%) were the most abundant classes. Pleosporales (62%) and Sordariales (16%) were the most abundant order (Table S3; Fig. 2). Workers and soldiers shared approximately 60% of fungal reads (93 OTUs) (Fig. S7), and community composition was not different between these castes (PERMANOVA, $F = 0.76$, $R^2 = 0.15$, $p = 0.60$). We did not find differences of the relative abundance of class and order taxa between castes.

Actinobacteria and Sordariales Dominated Microbial Communities in the Nest of *C. cumulans*

The bacterial communities in the nest walls and food nodules of *C. cumulans* harbored 24 phyla, 134 families, 198 genera, and 1604 OTUs (Table S2). The majority of the OTUs belonged to the phyla Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes, Proteobacteria, and Verrucomicrobia, accounting for 98% of the reads. The most abundant phyla were Actinobacteria (63.2% average abundance across samples) and Proteobacteria (21.1%) (Table S2; Fig. 2). At the genus level, 52.9% of the reads were classified as unknown. The most

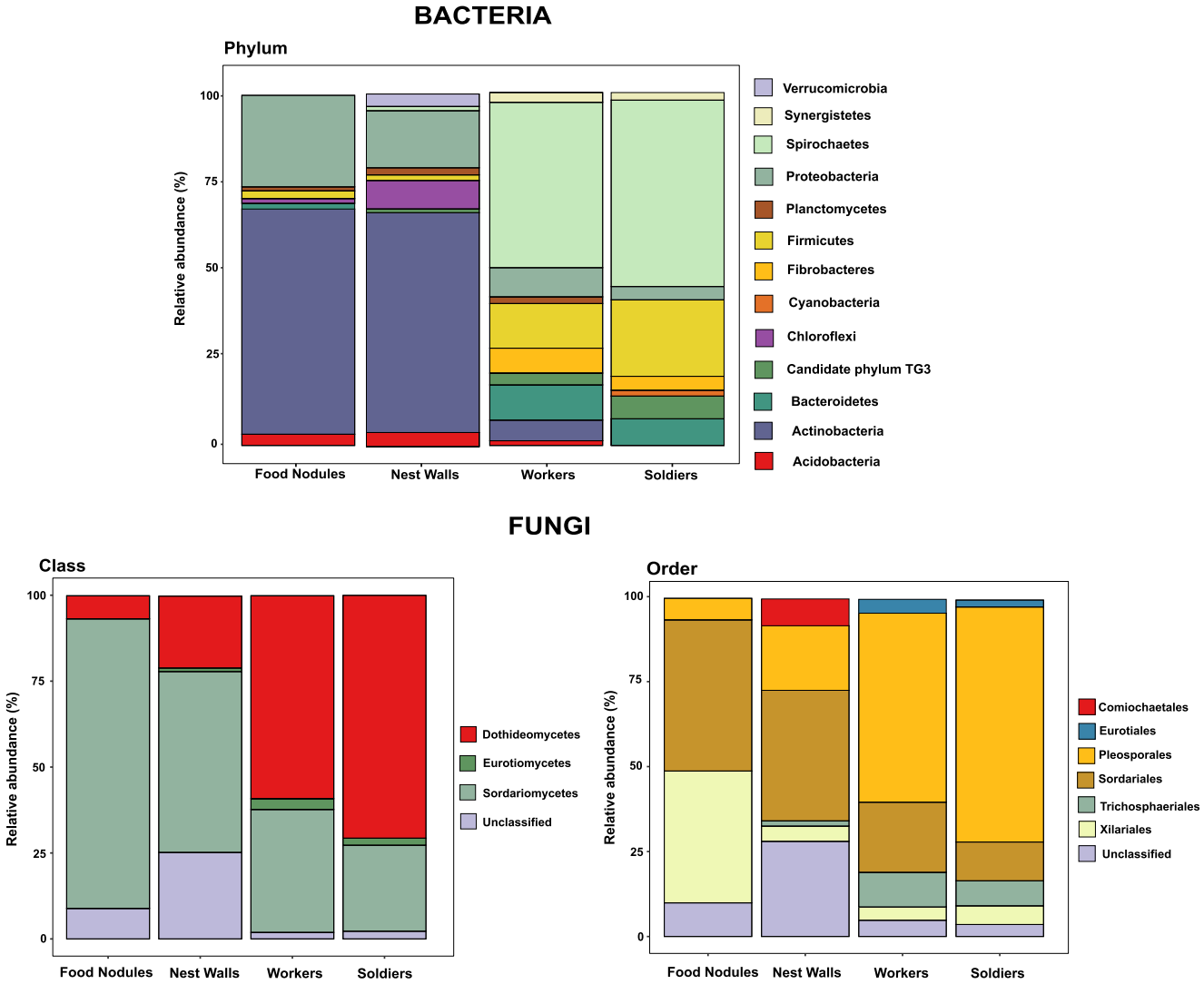

Fig. 2 Taxonomic composition of the microbial communities associated with the gut and nest substrates of *Cornitermes cumulans*. Relative abundances of the most abundant OTUs are shown at the phylum level for bacteria and at the class and order levels for fungi

abundant genera (> 1% relative abundance of reads) were *Nocardioides*, *Acidothermus*, *Curtobacterium*, and *Actinoallomurus* (Actinobacteria); uncultured lineage 6 of Sinobacteraceae (Proteobacteria); uncultured lineage 27 of Acidobacteriaceae (Acidobacteria); and lineage 12 of *Bradyrhizobium* which accounted for 33.4% of the sequence reads (Figs. 4 and S6). The genus *Nocardioides* (Actinobacteria) was the most prominent among samples (10.6%) (Table S2). Approximately 82% of bacterial diversity was shared between nest substrates (1129 OTUs) (Fig. S7). Analyses of variation in community structure corroborated that bacterial assemblages did not differ between nest substrates (PERMANOVA, $F = 1.18$, $R^2 = 0.23$, $p = 0.40$).

The fungal community of nest substrates was represented by 296 OTUs of the phylum Ascomycota (Table S3). Sordariomycetes (68%) and Dothideomycetes (14%) were the most abundant fungal classes, whereas Sordariales (41.8%), Xylariales (22%), and Pleosporales (13%) were the most abundant orders (Table S3; Fig. 2). Two hundred fifteen fungal OTUs (72% of the reads) were shared by food nodules and nest walls, the majority belonged to Sordariomycetes (Fig. S7). The community structure analysis revealed high similarities between nest substrates (PERMANOVA, $F = 4.09$, $R^2 = 0.51$, $p = 0.10$). We did not find any differences in the relative abundance of fungi between nodules and nest walls at the order and genera levels.

Bacterial and Fungal Communities Exhibited a Distinct Assemblage Pattern Between Gut and Nest Substrates of *C. cumulans*

Analyses of variation in community structure confirmed that microbial assemblages of termite guts and nest substrates differed for bacteria (PERMANOVA, $F = 8.66$, $R^2 = 0.40$, $p = 0.002$) and fungi (PERMANOVA, $F = 6.72$, $R^2 = 0.40$, $p = 0.003$) (Figs. S8 and S9).

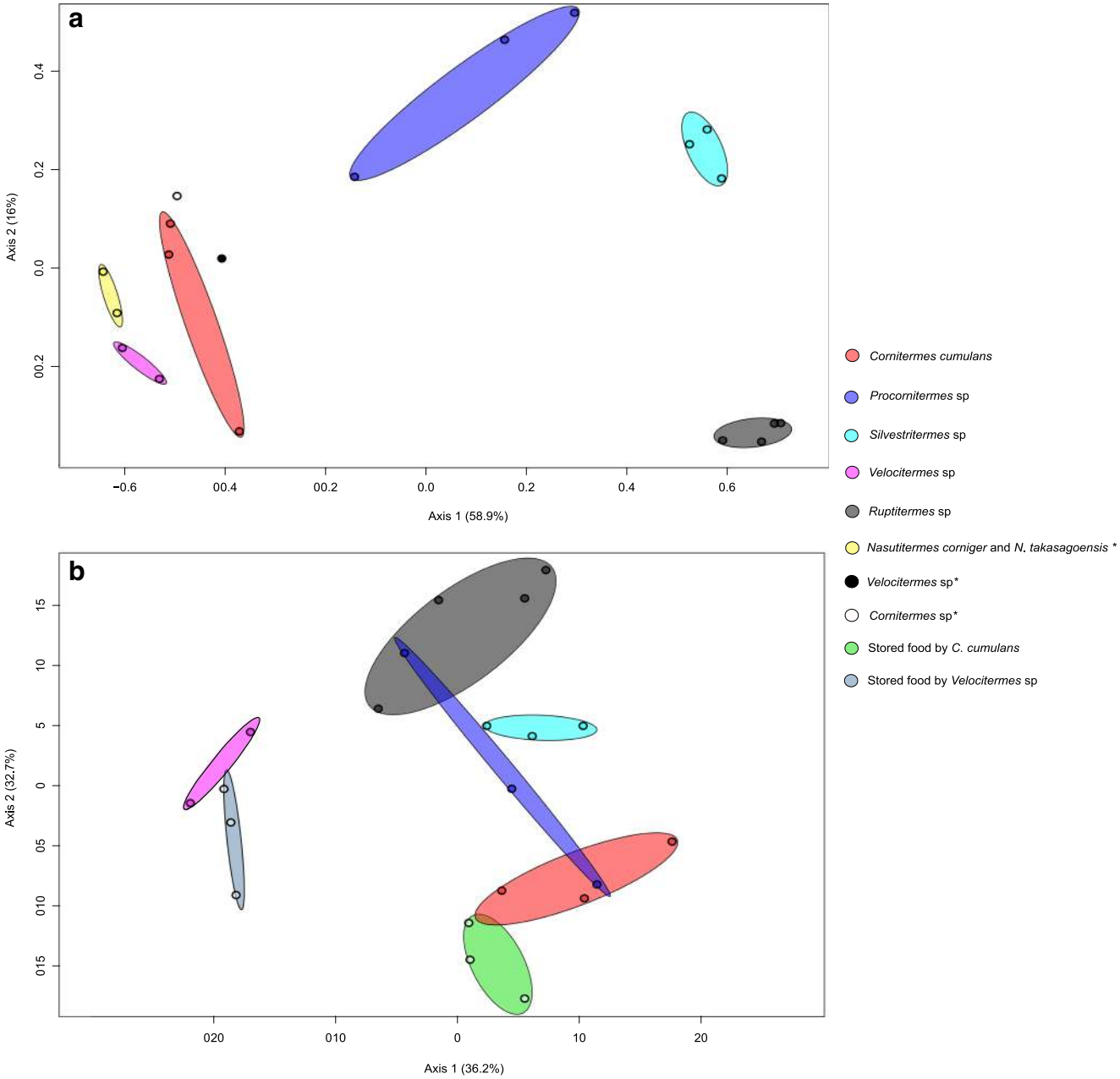

Fig. 3 Principal coordinates analysis (PCoA) of bacterial communities across termite guts (**a**) and nest substrates (**b**) of several species of Termitidae. Clustering was supported by PERMANOVA analyses ($p < 0.001$) at the genus level. Asterisks indicate termite species included from Mikaelyan et al. [11]

Permutation tests for homogeneity of multivariate dispersions showed that variances of gut and nest substrate samples were not statistically different and, therefore, did not influence the results of PERMANOVA. Indicator species analyses were then performed to identify the OTUs that reflected the differences. Taxon-sample association strength analyses (after 999 iterations) showed that some OTUs were significantly associated with gut samples. In particular, OTUs 636, 5021, 17,119, and 17,194 of *Treponema* Ia, Ic, and If were significantly associated to gut samples ($p = 0.001$) (Table S2). In contrast, OTUs 13,042 (*Xylanimonas*) and 19,596 (unclassified) of Actinobacteria, OTU 142 (*Burkholderia*) and OTU 12,258 (unclassified) of Proteobacteria, and the fungal OTU 129 (unclassified Dothideomycetes) were significantly associated with the food nodules and the nest walls ($p = 0.003$) (Tables S2 and S3). Only 2% of the bacterial diversity (480 bacterial OTUs) were shared between gut and nest substrates. In contrast, 26% of the fungal diversity (80 fungal OTUs) overlapped between gut and nest substrates (Fig. S7), with the majority belonging to Sordariomycetes.

Comparison with Other Termitidae

Ordination analyses at the genus level revealed that the gut community structure of the termites evaluated in this study formed distinct clusters (PERMANOVA, $F = 3.46$, $R^2 = 0.59$, $p = 0.001$). Apicotermitinae, represented by *Ruptitermes* sp., was clearly separated from Syntermitinae and Nasutitermitinae. Also within Syntermitinae, *Cornitermes*, *Procornitermes* sp., and *Silvestritermes* sp. formed separated clusters. Interestingly, grass/litter feeders *Cornitermes* and *Velocitermes* showed strong affinity with wood-feeding *Nasutitermes* species than with other litter-feeding species (Fig. 3a). Firmicutes, Spirochaetes, and Bacteroidetes were the most abundant phyla present in the gut of all species. Spirochaetes was the most abundant phyla in *Nasutitermes* species and in *Cornitermes* and *Velocitermes* sp. By contrast, Firmicutes predominated in the gut of other species, including the *Velocitermes* sp2 [11]. The group *Treponema* (Spirochaetes), subclusters Ia and Ic, was the most abundant genus in *C. cumulans*, *Velocitermes* sp., and *Nasutitermes*, whereas *Candidatus Arthromitus* (Firmicutes, Lachnospiraceae) was the most abundant genus in *Procornitermes* sp. and *Silvestritermes* sp. Subcluster IIIb of TG3 phylum was preferentially associated with *Cornitermes* sp. [11]. Termite Cockroach Cluster in the family Synergistaceae (Synergistetes) was the most prominent genus in the gut of *Ruptitermes* sp. (Table S4; Fig. 4).

In contrast, there is a remarkable convergence of the bacterial community structure of nest substrates (PERMANOVA, $F = 3.85$, $R^2 = 0.54$). Food stored by *Cornitermes* and *Velocitermes* showed strong affinity to conspecific nest wall substrates (Fig. 3b). Actinobacteria (52%) and Proteobacteria (16%) were the most abundant phyla present in the fecal material of nest substrates. Actinobacteria was the most abundant phylum in nest walls of all termite species and in the food nodules of *C. cumulans*. In contrast, Proteobacteria was the most abundant phyla in the food stored by *Velocitermes* sp. (Fig. 4). *Nocardioides* (Actinobacteria) was the most abundant genus in the food stored by *C. cumulans* and the nest walls of *C. cumulans*, *Procornitermes* sp., *Velocitermes* sp., and *Ruptitermes* sp. The lineage 9 of *Massilia* (Proteobacteria) was abundant in the nest substrates of *Velocitermes* sp., whereas *Acidothermus* (Actinobacteria) was abundant in the nests of *Silvestritermes* sp., *Procornitermes* sp., and *Ruptitermes* (Table S5; Fig. 4).

Discussion

Previous studies have suggested that food storage in termite nests is a facultative ability that could provide some advantages in terms of food provision during environmental constraints or by improving food quality after microbial decomposition [45]. In some termite species, plant material is usually stored in cells or chambers inside the nest [15]; however, workers of *C. cumulans* cut small pieces of plant litter and packed them with their feces against the internal nest walls, forming peculiar nodules that resemble those reported for two *Nasutitermes* species (Nasutitermitinae) [46]. It has been hypothesized that food nodules in those species could serve as a nutritional reserve because they have a high fiber content, similar to our findings in the nodules stored by *C. cumulans*. The nutritional reserve hypothesis could also explain why the amount of food nodules stored by *C. cumulans* increased when colony population demand is higher and decreased when foraging territories of colonies overlap. Food storage could also be advantageous during periods of low resource availability or when there is a higher production of alates and larvae. Additionally, the presence of stored food may be cyclic, and further work will be needed to elucidate if nodules of *C. cumulans* are present seasonally or during high colony demand.

Although we did not observe the feeding behavior of *C. cumulans* under natural conditions, the survival of workers in the laboratory was higher when food nodules were offered over other plant substrates (Costa-Leonardo, unpublished), suggesting that *C. cumulans* may benefit from specific nutrients or chemicals that result from the alteration of stored plant material inside the nodules. Aiming to explore this hypothesis, we evaluated the cellulolytic and hemicellulolytic activities of the food stored by *C. cumulans* and the co-occurring *Velocitermes* sp. (Nasutitermitinae) toward polysaccharide hydrolysis. Our results indicate the occurrence of cellulases and β-glucosidases in the stored food as well as in the termite gut, which is correlated with the expression of endogenous and symbiotic enzymes [47]. Grass litter constitutes the main food resource for the species evaluated in this study [21], and grass is known to have a higher content of hemicellulose, with xylan being its major constituent. Xylanases and β-xylosidases hydrolyze the xylan to release monomers of xylose, and the microbiome of higher termites contains abundant hemicellulolytic enzyme genes [48]. Thus, the xylanolytic activity observed in the gut is consistent with the grass-litter diet of these termites.

Food stored, instead, has a hemicellulolytic activity significantly higher than guts. Bacterial levels are known to be high in fecal material used for the construction of internal walls of mounds that sometimes are consumed by the termites [49]. According to Nalepa et al. [6], the cultivation and use of microbes within nest galleries is an important feature of termite nutritional ecology for the maintenance of gut microbiota. In addition, microbial assemblages in nest walls could also modify the physical or chemical properties of the stored food. Consequently, storing plant material inside the nest walls could be advantageous over other termite species since the microbiota from the feces might supply the termites with an

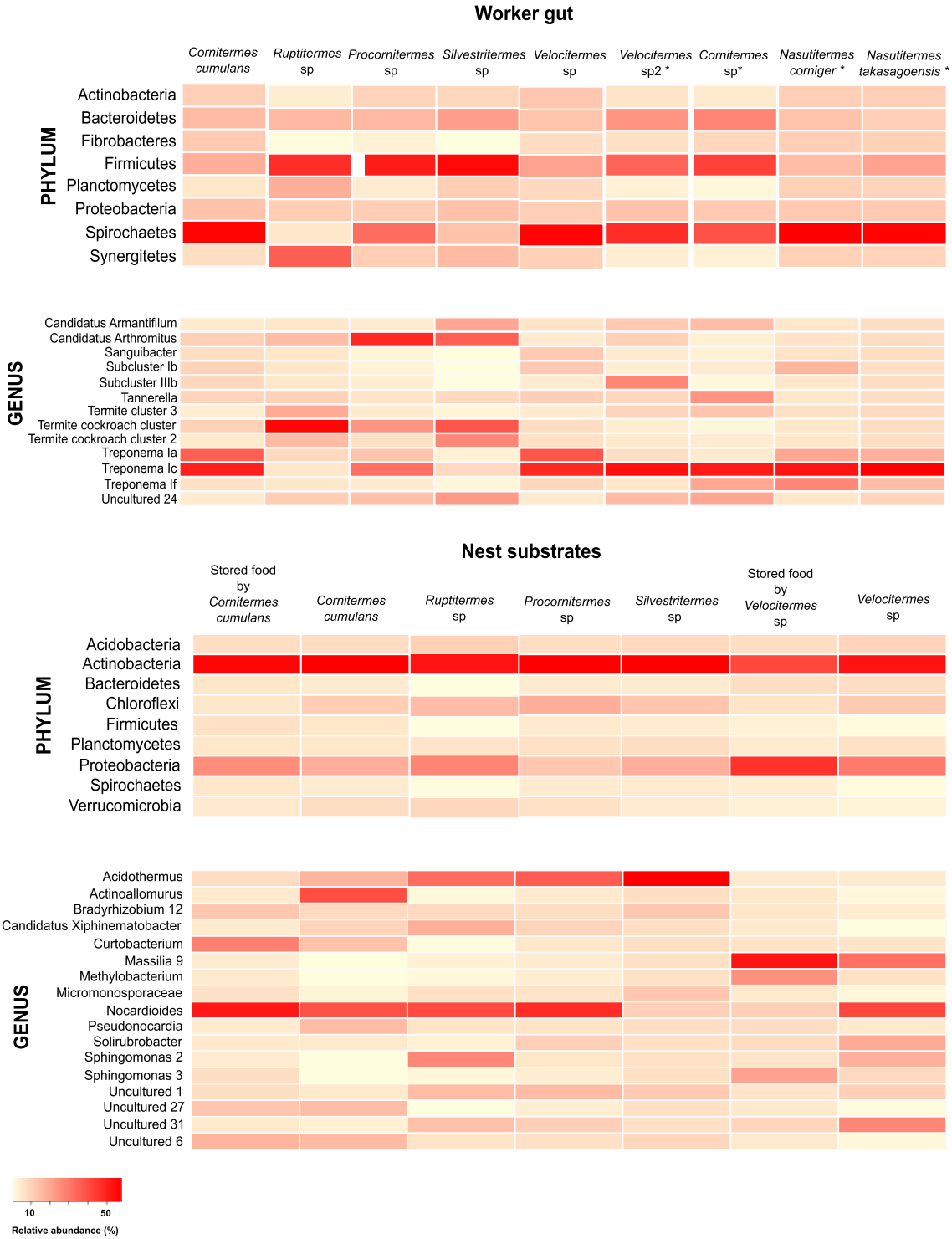

Fig. 4 Comparison of the relative abundances (> 1%) of bacterial phyla and genera of gut and nest substrates from several species of Termitidae. Asterisks indicate termite species included from Mikaelyan et al. [11]

external source of predigested polysaccharides. Furthermore, the xylanolytic activity in food nodules could overcome the hemicellulose barrier of the plant material and enhance the accessibility of cellulases and β-glucosidases to cellulose [50], resulting in additional fermentable monosaccharides. Litter-feeding termites are very abundant in central South America. It is possible that success of *C. cumulans* [18] was the result of its ability to predigest the grass litter by storing it inside nodules made with feces and saliva.

Even though the results of enzymatic activity were obtained from soluble crude extracts, cellulase activity could be affected by differences in the nature of solubility of enzymes associated

with hindgut fiber content [51]. Extraction protocols with surfactants showed that insoluble lignocellulases are abundant in the bacteria associated to wood particles [8]. However, some detergents are known to affect the enzymatic hydrolysis [52, 53] and the enzymatic pattern must be evaluated with caution. In order to determine whether the patterns observed in our assays are characteristic for *C. cumulans*, we compared them to the patterns in termites that do (*Velocitermes* sp.) and do not store food (*Procornitermes* sp. and *Silvestritermes* sp.). We obtained essentially the same results suggesting that the utilization of the soluble fraction for activity assays was adequate to compare the activity of lignocellulases.

Different nesting strategies in termites could lead to differences in the physicochemical properties influencing the structure of microbial communities [3, 54]. Except for *Ruptitermes*, that construct subterranean nests, the other termites are mound builders [20, 21]. However, there is a remarkable convergence of the bacterial community structure of nests among species. A possible explanation for this could be related to the morphogenesis of the mounds that initiates underground before being expanded to the soil surface [55] and would thus keep a similar bacterial community. Actinobacteria and Proteobacteria dominated the fecal material of nest substrates. These bacteria probably colonize nest substrates after gut transit since they were also found in lower abundances in the gut of termites. It is possible that colonization conditions by these microorganisms are better in the nest substrates than in the gut of workers, which may explain abundance divergences. Other studies have reported the presence of Actinobacteria in termite mounds [54]. It has been suggested that Actinobacteria might play defensive roles in the combs of some fungus-growing termites by controlling pathogens in the fungus gardens [56]. Therefore, another possibility worth further inquiry is that termites benefit directly or indirectly from other nutrients or compounds, such as specific antibiotics provided by the nest microbiota. Proteobacteria was also abundant in nest substrates. Some genera of Proteobacteria are known to display high rates of H_2-dependent oxygen reduction in the gut of termite species [57]. Actinobacteria and Proteobacteria also play important roles in the decomposition of hemicellulose because many strains are xylanolytic and some OTUs associated to stored food and nest walls of *C. cumulans* produce xylanases [58], which could explain the higher xylanolytic activity of the stored food.

Diet has been shown to affect the structure of bacterial communities in termites [11], and our results showed clear differences among the feeding groups of Termitidae evaluated in this study. However, gut bacterial communities also differed within the litter-feeding species of Syntermitinae. The gut of the grass/litter-feeding *C. cumulans* and *Velocitermes* sp. contains a higher abundance of Spirochaetes, which is in agreement with previous reports [11, 59]. Interestingly, bacterial assemblages of *Cornitermes* and *Velocitermes* species were closer to wood-feeding *Nasutitermes* than to other litter-feeding species. The majority belonged to the genus group *Treponema*, mainly the subclusters Ia and Ic. These bacteria are an important source of enzymes that act on the digestion of wood polysaccharides [60]. *C. cumulans* and *Velocitermes* sp. consume a greater quantity of fiber-rich grass previously stored in the nest, which in turn could explain the high abundance of Spirochaetes in the gut of these species.

In contrast, Firmicutes was the most abundant phylum in the gut of *Ruptitermes* sp., *Procornitermes* sp., *Silvestritermes* sp., and *Velocitermes* sp2 from Mikaelyan et al. [11]. Firmicutes are commonly found in high proportions in soil- and humus-feeding species, especially in the alkali gut compartments of higher termites [61] and in the combs of fungus-growing species [62]. *Candidatus Arthromitus* (Firmicutes, Lachnospiraceae) is a segmented filamentous bacteria commonly found on the gut wall of certain arthropods. The family Lachnospiraceae comprises many species with high xylanolytic and cellulolytic activities [63]; however, its function needs to be clarified.

Bacterial assemblages of *Procornitermes* sp., *Silvestritermes* sp., and *Ruptitermes* sp. formed distinct clusters. In contrast to grass/litter feeders, *Procornitermes* and *Silvestritermes* are intermediate feeders with a diet consisting of decomposed litter [64], whereas the *Ruptitermes* species used in this study feed on leaves and seeds [20]. Differences observed within Syntermitinae were also reflected within the same genus as found for the species of *Cornitermes* and *Velocitermes* from this study and those from Mikaelyan et al. [11]. Variations in the diet among the Syntermitinae are poorly known [21] and consequently, more studies within this subfamily will be needed in order to understand its feeding ecology.

Termite soldiers cannot feed themselves because their mandibles are modified or reduced and, therefore, depend on workers for nutrition via trophallaxis [65]. In termites, the proctodeal trophallaxis is the transfer of hindgut fluids and symbionts which could be reflected in the composition of the gut microbiota between workers and soldiers. Although we did not conduct experiments to evaluate trophallaxis behavior in *C. cumulans*, the similarity between the bacterial gut community profiles of workers and soldiers is consistent with the acquisition of gut symbionts from workers by trophallaxis [66]. This is compatible with the comparable richness and evenness indices of gut microbiota between workers and soldiers and by the large number of microbes shared by both castes.

Insects and fungi share a long history of association in various habitats, including lignocellulose decomposition [67]. The distinct fungal diversity between nest substrates and worker guts could be associated with their ability to survive and reproduce under different environmental conditions. Ascomycota was the most abundant fungal phylum sampled, which is typical for insect gut microbiomes. However, in contrast to the bacterial community, the fungal community was

considerably less diverse. Dothideomycetes and Sordariomycetes were the most numerous fungal classes in *C. cumulans*. Many wood-feeding insects maintain obligate external associations with fungi and directly inoculate fungal isolates into their food sources, where they facilitate predigestion of lignocellulose because these fungi are known to express hemicellulolytic enzymes including xylanases [68] and also express a number of lignin-degrading enzymes, such as laccases, thus being considered the most important biomass degraders. In this context, *C. cumulans* could benefit from the ability of fungi to digest lignocellulose in the food stored inside their nests. These strategies substantially reduce the carbohydrate complexity and lignin content of the food substrate prior to ingestion by the workers.

Our results provide new insights about the feeding ecology of Syntermitinae. This work showed the first evidence concerning the potential role of the stored food of a higher termite in relation to their bacterial and fungal communities. *Cornitermes cumulans* stored grass litter in nodules inside their nest. This strategy seems to be related with food provisioning as suggested by field observations. In addition, stored food in termite nests showed strong activity against hemicellulosic substrates and its associated microbiota was distinct in this regard from those of termite gut. We suggest that such specialization of the stored food microbial community could provide the colony with additional monosaccharides. Moreover, the gut microbiota of *C. cumulans* was consistent with the diet of fiber-rich substrates, and our results also confirmed that diet affects the gut communities among different subfamilies of Termitidae. Interestingly, we observed a remarkable variation of bacterial assemblages within litter-feeding Syntermitinae suggesting that the feeding patterns within this subfamily are very complex; consequently, future work will aim at elucidating basic aspects of the biology of additional members of this subfamily. While researchers are trying to understand the possible roles of termite symbionts, fundamental biological aspects of these insects and their associated microbiota are not well understood. Therefore, further studies of their ecology and its relation to the comparative metagenomics and metatranscriptomics of these microbial communities will be needed to improve our understanding of the feeding ecology of termites that store food in the nest.

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Compliance with Ethical Standards

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

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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