# Termites are associated with external species-specific bacterial communities

**Running title:** Termite external bacterial communities

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**Abstract**

All termites have established a wide range of associations with symbiotic microbes in their guts. Some termite species are also associated with microbes that grow in their nests, but the prevalence of these associations remains largely unknown. Here, we studied the bacterial communities associated with the termites and galleries of three wood-feeding termite species using 16S rRNA gene amplicon sequencing. We found that the composition of bacterial communities differs among termite bodies, termite galleries, and control wood fragments devoid of termite activities, in a species-specific manner. Termite galleries were enriched in bacterial OTUs belonging to Rhizobiales and Actinobacteria, which were often shared by several termite species. The abundance of several bacterial OTUs, such as *Bacillus*, *Clostridium*, *Corynebacterium* and *Staphylococcus*, was reduced in termite galleries. Our results demonstrate that both termite guts and termite galleries harbour unique bacterial communities.

**Importance**

As it is the case for all ecosystem engineers, termites impact their habitat by their activities, potentially affecting bacterial communities. Here, we studied three wood-feeding termite species and found that they influence the composition of the bacterial communities in their surrounding environment. Termite activities have positive effects on Rhizobiales and Actinobacteria abundance, and negative effects on the abundance of several ubiquitous genera, such as *Bacillus*, *Clostridium*, *Corynebacterium* and *Staphylococcus*. Our results demonstrate that termite galleries harbour unique bacterial communities.

**KEYWORDS:** *Coptotermes*, ectosymbionts, *Heterotermes*, *Nasutitermes*, symbiosis

## 1. INTRODUCTION

Termites harbour diverse communities of microbes in their hindguts that participate in lignocellulose digestion, nitrogen metabolism, and other functions (1–4). Gut microbes have been coevolving along with termites for tens of millions of years, and many species are found nowhere else other than in the termite gut (3–5). Consequently, termite gut microbial communities are unique in terms of composition, differing substantially among species (6–8) and differing from the communities present in soil, wood, and termite nest material (9, 10).

In addition to the microbes present in their guts, some termite species are known to partner with mutualistic symbionts that grow outside of their bodies, which we define here as 'external symbionts'. All species of Macrotermitinae cultivate the macroscopic fungus *Termitomyces* within their nests (11–13). *Termitomyces* species are only associated with fungus-growing termites (11–13) and, due to their prevailing horizontal transmission, have undergone a number of switches between species in this group (14, 15). Another putative example of nutritional external symbiosis is that between *Sphaerotermes sphaerothorax*, the only known species of Sphaerotermitinae, and bacteria of unknown taxonomic composition that are found inside specialized combs forming the core of *Sphaerotermes sphaerothorax* nests (16) No other nutritional external symbionts are known to be associated with termites.

Termites are known to host externally-associated symbiotic microbes that exhibit antifungal properties. Termites primarily feed on wood, sometimes in an advanced stage of decomposition, or on soil (17, 18), both of which are inhabited by a large number of microbes. In addition, termites are social insects that live in densely populated nests, potentially facilitating the transmission of diseases (19). Some termites harbour in their nests *Streptomyces* bacteria that display antifungal properties (20–22). External symbiotic

72 *Streptomyces* are not specific to termites, but are recruited from the soil surrounding the
73 faecal nest, and become abundant in termite-managed environments (22).

74 The diversity of microbes externally associated with termites is unlikely to be limited
75 to a handful of external symbionts with nutritional and defensive functions. Termite
76 activities are expected to have a significant effect on the composition of surrounding
77 microbial communities. For example, termites produce antifungal and antimicrobial
78 compounds that they release from their salivary glands and faecal pellets (23–27). Saliva and
79 faecal fluids are used as building material (28), and their biocide properties prevent
80 microbial colonization of the nest and galleries, which remain free of visible fungal
81 overgrowths (21, 29). Termites also tunnel into wood, and move vast amounts of soil (30–
82 32) , facilitating the spread of microbes and fungi (33). Lastly, termites maintain
83 microclimatic conditions within their nests and galleries (28), potentially favouring the
84 growth of certain microbes while supressing that of others. In consequence, the microbial
85 communities colonizing termite nests and galleries are expected to differ from that of
86 termite-free environments.

87 Several studies have shown that the bacterial communities thriving on termite-
88 modified materials differ from that of soil or wood (34–38) However, these studies provided
89 only limited insight into the composition of bacterial communities, and no insight into the
90 specificity of termite-bacteria associations. The few studies based on high-throughput
91 sequencing approaches, which allow taxonomic identification of bacteria, provided
92 conflicting results, either suggesting that microbial communities of termite nests are similar
93 to those of the surrounding soil (9), or showing that the fungal combs of each
94 Macrotermitinae species host unique bacterial communities (39).

In this study, we used high-throughput sequencing of 16S rRNA gene fragments to compare the bacterial communities of termite bodies, termite galleries, and control wood samples devoid of termite activities. We worked on three wood-feeding termite species abundant in French Guiana lowland tropical rainforests: *Coptotermes testaceus* (Linnaeus, 1758), *Heterotermes tenuis* (Hagen, 1858) (both Rhinotermitidae), and *Nasutitermes octopilis* Banks, 1918 (Termitidae: Nasutitermitinae). Using this dataset, we determined the influence of termites on the surrounding bacterial communities, and identified bacterial lineages with reduced abundance in the presence of termites, or those externally associated with termites.

## 2. MATERIAL AND METHODS

### 2.1. Study site and sampling

The fieldwork took place in November 2014 in the Nouragues Nature reserve (French Guiana; N 04°05', W 52°41'). All samples were collected within 50 m of the network of paths of the Nouragues Research Station. The full sampling area was about 100 hectares. We collected samples of three species: *Coptotermes testaceus*, *Heterotermes tenuis* and *Nasutitermes octopilis*. Upon encountering one of these species, we collected one series of samples, all collected in the same wood log, consisting of three termite samples (between 10 and 15 workers each), together with three samples of their feeding substrates (approx. 1 $cm^3$ piece of wood containing thin galleries), and three control samples (approx. 1 $cm^3$ of wood at least 10 cm away from the closest termite galleries). Sterile vials and flame-sterilised forceps were used for the sampling. Sample replicates were distant by more than 1 m. Occasionally, for small logs, only two samples of each type were collected. All samples were preserved in RNAlater®, stored at -20 °C within 8 hours following collection, and

shipped to Prague where they were stored at -80 °C until DNA extraction. In total, we sampled wood with foraging parties belonging to 10 colonies of *C. testaceus* and *N. octopilis*, and 11 colonies of *H. tenuis*.

**2.2. DNA extraction and PCR amplification**

Total DNA was extracted using the Macherey-Nagel NucleoSpin® Soil kit. For each termite sample, we homogenized whole individuals, including guts (hereafter termed 'bodies'), of up to ten workers using two sterile steel beads (3 mm diameter) and a Mixer Mill MM 400 set on 30 swings per second for two minutes. We carried out extractions as per the manufacturer's protocol, except for the lysis step that was shortened to 2 min of vortexing. Wood samples were placed in a sterile 2 mL tube, frozen in liquid nitrogen, mechanically crushed with five sterile steel beads for 1 min at 30 swings per second, and grinded with a Mixer mill Retsch MM 400 for 10 minutes. Following the first grinding step, we added 550 µL of SL2 extraction buffer to the homogenized material and repeated the grinding with the same settings. The lysis by vortexing was extended to 10 min, and precipitation of contaminants was carried out with 100 µL of SL3 buffer. Lysate was filtered with 650 µL of supernatant. Silica membrane was dried for 3 minutes in centrifuge. Finally, we added 50 µL of SE buffer to the silica membrane and centrifuged for 45 sec to elute the DNA. Each sample was handled with flame-sterilized forceps.

PCR reactions were performed using the Thermo Scientific DyNAzyme II DNA Polymerase kit. We used the universal primers 515F and 806R targeting the V4 region of the 16S rRNA gene (40), combined with an original combination of index reads. The PCR reactions contained 2.5 µL of 10× buffer for DyNAzyme II DNA Polymerase, 0.75 µL of BSA (20 mg/mL), 1 µL of each primer (0.01 mM), 0.5 µL of PCR Nucleotide Mix (10 mM each),

143 0.75 µL of polymerase (2 U/µL DyNAzyme II DNA polymerase), and 1 µL of template DNA.
144 DNA concentration ranged between 10.3 and 41.4 ng/µL. PCR reactions were performed
145 using an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany) nexus cycler, with
146 the following settings: initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 45 sec,
147 50 °C for 1 min, 72 °C for 45 sec, and a final extension step at 72 °C for 10 min. We carried
148 out three independent PCR amplifications for each sample, combined the three replicates,
149 and cleaned them using the MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany).
150 Pooled PCR products were mixed in equimolar concentration and paired-end-sequenced
151 with an Illumina MiSeq sequencer (Illumina Inc., USA) using the V2 chemistry to produce 250
152 bp paired-end reads. Sequence data are available on MG-RAST under the project accession
153 number: mgm4904347.3.
154
155 **2.3. Data filtering**
156 Raw paired-end reads were joined using fastq-join (41), and demultiplexed, filtered and
157 trimmed using SEED v 2.1 (42). Sequences with mean Phred quality score <30, as well as
158 sequences with mismatches in barcodes or ambiguous bases, were discarded. We also
159 discarded all bacterial sequences shorter than 200 bp or longer than 350 bp. A total of
160 5,863,706 bacterial sequences were obtained after initial quality-filtering.
161
162 **2.4. OTU clustering and classification**
163 Sequences were clustered into operational taxonomic units (OTUs) (3% sequence
164 dissimilarity) using UPARSE implemented in USEARCH version 8.1.1861 (43). Chimeric
165 sequences were identified during clustering to OTUs using UPARSE algorithm, and a total of
166 526,949 sequences were excluded from downstream analyses. To reduce the influence of

167 contaminations and to minimize the effect of barcode hopping (44), all OTUs with fewer
168 than five reads were discarded. We also used previous Illumina run data to estimate the
169 number of reads that potentially hopped among samples for all OTUs and removed those
170 reads.

171 The most abundant sequence from each OTU was used as a representative sequence
172 for taxonomic classification. Representative sequences were classified with the RDP classifier
173 from the RDPTools software version 2.0.2 using the 16S rRNA gene reference database (45).
174 Classification was verified using RDP Release 11 Update 5, accessed on September 30 2016
175 (46), that provided the closest BLAST hit for each OTU. We used rrnDB version 5.4 (47) to
176 estimate the relative abundance of each OTU, considering the variable number of 16S rRNA
177 gene copies per bacterial genome, as explained in Větrovský and Baldrian (48).

178

179 **2.5. Diversity of bacterial communities in termite bodies, termite galleries, and wood**
180 **controls**

181 We carried out all statistical analyses using a subsample of 3,000 sequences per sample. We
182 used the Chao1 (49), Evenness (50), and Shannon-Wiener (51) indices to characterise the
183 bacterial diversity of termite bodies, termite galleries, and wood controls. The values of the
184 three diversity indices were estimated using SEED v 2.1 (42) and visualized using the R
185 package ggplot2 (52). To test the null hypothesis of no effect of sample type and species on
186 diversity indices, linear mixed effect models were fitted using the function lme()
187 implemented in the R package *nlme* (53). A factor with seven levels, created by combining
188 termite species and sample types, was fitted as the fixed part of the model, and a random
189 structure of the form *~1|triplet/log* was included in each model to account for the fact that
190 measurements were grouped in triplets, which, in turn, were nested in logs. Pairwise

comparisons among groups were performed with Tukey *post-hoc* tests using the function lsmeans() of the R package *lsmeans* (54).

**2.6. Comparison of bacterial communities in termite bodies, termite galleries, and wood controls**

We visualized the relative abundance of bacterial phyla for each sample type (body, gallery and wood control) using the R package *ggplot2* (52). To test whether bacterial community composition differs among termite bodies, termite galleries, and wood controls, we performed PERMANOVA (55) using the adonis function from the R package *vegan* (56). The response matrix was calculated using the Euclidian distance on Hellinger-transformed bacterial composition, which resulted in a Hellinger distance matrix, commonly used as a measure of resemblance (57). We used sample type (body, gallery and wood control) as the explanatory variable. Since samples were collected in series of dependent triplets (or sometimes doublets) coming from a single log, with each triplet comprising three dependent samples (one termite body sample, one gallery sample, and one wood control sample) collected near to each other, the permutations were constrained to occur among samples of the same triplets, which were used as a blocking factor. As such, we used the formula "termite-species*sample-type" and the strata was set to "data$triplets". We compared termite species and sample types (body, gallery or wood control) using pairwise PERMANOVA implemented in the pairwiseAdonis R package (58). We used Bonferroni corrections to adjust p-values. Significance was assessed using 99,999 permutations.

We visualized the dataset using non-metric multidimensional scaling (NMDS) implemented with the metaMDS function of the R package *vegan* (56). NMDS analysis was



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carried out using community data regressed against logs and triplets. This procedure removed the effect of spatial variability inherent to the experimental design.

### 2.7. Identification of termite-associated bacteria

To identify the bacterial OTUs contributing to the separation between termite bodies, termite galleries and wood controls, we used partial redundancy analysis (partial RDA) (59). Each termite species was considered separately. For each RDA, we used Hellinger-transformed bacterial OTU composition as a response matrix, and sample type as fixed explanatory factor. The effects of triplets and wood logs were removed by using logs and triplets as conditioning factors in the partial RDA (see [59]). We focused our efforts on the identification of the main bacterial OTUs and considered those belonging to the $0.25^{th}$ and $99.75^{th}$ percentiles. Identified OTUs were classified in one of the following three categories: body-associated bacteria (OTUs predominantly found in termite guts), gallery-associated bacteria (OTUs predominantly found in termite galleries), and gallery-depleted bacteria (OTUs predominantly found in control wood samples). Note that generalist OTUs, showing a random distribution pattern, with no preference for termite bodies, termite galleries or control wood samples, are not considered further in this paper.

## 3. RESULTS

### 3.1. Bacterial diversity

We analyzed a total of 258 samples of termite bodies, galleries and wood controls in foraging areas of 10 colonies of *C. testaceus* and *N. octopilis* and 11 colonies of *H. tenuis*. After quality-filtering and removal of chimeras, we obtained an average of 20,685 sequences of the V4 region of the bacterial 16S rRNA gene for each of the 258 samples. 16S rRNA gene

238 sequences were clustered into 4,864 OTUs (3% sequence dissimilarity) represented by at
239 least five sequences (Table S1). The three diversity indices, Chao1, Evenness, and Shannon-
240 Wiener, were significantly higher for samples of termite galleries than for wood controls and
241 termite bodies (Figure 1). Chao1 indicated that termite bodies hosted the poorest bacterial
242 communities ($p < 0.05$), with no significant differences among termite species (Figure 1).
243 Evenness and Shannon-Wiener diversity indices were the smallest for *H. tenuis* bodies,
244 followed by *C. testaceus* bodies, and *N. octopilis* bodies ($p < 0.05$) (Figure 1).
245

246 **3.2. Comparison of bacterial communities in termite bodies, termite galleries, and termite-**
247 **free wood controls**

248 We found no significant difference among wood controls associated with *C. testaceus*,
249 *H. tenuis*, and *N. octopilis* (Table 1), and therefore pooled wood controls together. The
250 samples of termite galleries and wood controls had similar bacterial community composition
251 at the phylum level (Figure 2). The dominant phylum was Proteobacteria, which on average
252 made up over 40% of the bacterial reads of termite galleries and wood controls.
253 Acidobacteria and Actinobacteria were also abundant, and made up, on average, a minimum
254 of 10% of the bacterial sequences of termite galleries and wood controls. In comparison to
255 termite galleries and wood controls, Proteobacteria, Acidobacteria and Actinobacteria were
256 rare in termite bodies. Instead, the bacterial communities of *C. testaceus* and *H. tenuis*
257 bodies were heavily dominated by Bacteroidetes, which, on average, made up more than
258 75% of the bacterial reads. BLAST searches assigned most reads of Bacteroidetes in *C.*
259 *testaceus* bodies to *Candidatus* Azobacteroides and *Candidatus* Armantifilum, while the
260 Bacteroidetes reads of *H. tenuis* bodies mostly belonged to *Candidatus* Azobacteroides. The
261 bacterial communities of *N. octopilis* bodies were dominated by Spirochaetes and

262 Fibrobacteres, which, on average, made up 59.6% and 18.3% of the bacterial reads,
263 respectively. BLAST searches showed that the 16S rRNA gene sequences of Spirochaetes and
264 Fibrobacteres in *N. octopilis* bodies were mostly assigned to *Treponema* and putatively to
265 *Fibrobacter*, respectively. The PERMANOVA analysis yielded significant differences among
266 groups ($F = 22.33$, $p < 10^{-6}$), including significant differences among termite species ($F =$
267 $14.773$, $r^2 = 0.075$, $p < 10^{-5}$) and among sample types (body, gallery, and control wood) ($F =$
268 $34.636$, $r^2 = 0.175$, $p < 10^{-5}$). Figure 3 shows the NMDS plot calculated for all samples, and
269 represents the bacterial communities of *C. testaceus*, *H. tenuis*, and *N. octopilis* bodies as
270 three disjunct clusters. Termite galleries, as well as wood controls, also clustered by termite
271 species, although these clusters were more diffuse and largely overlapped. Pairwise
272 PERMANOVA indicated that the bacterial communities associated with *C. testaceus*, *H.*
273 *tenuis*, and *N. octopilis* bodies significantly differed from each other (Table 1). Similarly, the
274 bacterial communities of termite galleries significantly differed among termite species, and
275 significantly differed from the corresponding wood controls in the case of *C. testaceus* and *N.*
276 *octopilis*, but not in the case of *H. tenuis*, for which a Bonferroni correction made the
277 comparison only marginally significant (Table 1). Bacterial communities from bodies of *C.*
278 *testaceus*, *H. tenuis*, and *N. octopilis* significantly differed from communities colonizing
279 termite galleries and wood controls in all cases (Table 1).

280

281 **3.3. Identification of termite-associated bacteria**

282 We carried out RDA and considered OTUs from the $0.25^{th}$ and $99.75^{th}$ percentiles (Figure 4).
283 With this approach, we identified 97 bacterial OTUs associated with termites, or partly
284 excluded by termites, of which many were independently identified for two or three of the

studied termite species (Table S2). Of the 47 bacterial OTUs detected to have non-random associations with *C. testaceus* ( Figure 4A), 14 OTUs were body-associated bacteria and made up 68.1% of the bacterial community of *C. testaceus* bodies, 18 OTUs were enriched in termite galleries, making up 28.3% of the bacterial 16S rRNA gene sequences in termite galleries and 14.2% of the bacterial 16S rRNA gene sequences in wood controls, and 15 OTUs were partly excluded by *C. testaceus*, making up 24.8% and 3.2% of the bacterial 16S rRNA gene sequences in wood controls and termite galleries, respectively. *H. tenuis* and *N. octopilis* provided similar results. Of the 48 bacterial OTUs considered for *H. tenuis* (Figure 4B), 15 OTUs were body-associated bacteria and made up 80.8% of 16S rRNA gene sequences of *H. tenuis* bodies, 17 OTUs were gallery-associated bacteria, making up 27.7% of the bacterial community of termite galleries and 11.3% of the bacterial community of wood controls, and 16 OTUs were partly excluded by *H. tenuis*, making up 24.7% and 6.7% of the 16S rRNA gene sequences of the control and gallery samples, respectively. Lastly, of the 45 bacterial OTUs considered for *N. octopilis* (Figure 4C), 15 were body-associated bacteria and made up 60.3% of the termite bacterial community, 15 OTUs were gallery-associated bacteria and made up 25.6% of the bacterial community of *N. octopilis* galleries and 9.2% of the bacterial community of wood controls, and 15 OTUs were partly excluded by *N. octopilis* and made up 34.9% of the bacterial 16S rRNA gene sequences of wood control samples and 1.4% of the bacterial 16S rRNA gene sequences of *N. octopilis* galleries (Table S2).

## 4. DISCUSSION

In this study, we sequenced the bacterial communities associated with three termite species, *C. testaceus*, *H. tenuis*, and *N. octopilis*. We demonstrated that termite galleries host the

most species diverse bacterial communities, while termite bodies comparatively host species poor bacterial communities. We found that the composition of bacterial communities differs among termite bodies, termite galleries, and wood controls devoid of visible termite activities, in a species-specific manner. We also identified 97 abundant bacterial OTUs that are predominantly associated with termite bodies (referred to as body-associated bacteria), termite galleries (referred to as gallery-associated bacteria), or control wood samples (referred to as gallery-depleted bacteria). Consequently, our results show that termites not only shape the bacterial communities inside their gut (6, 7, 60), but also those in their environment.

The bacterial diversity indices calculated for the bodies of *C. testaceus* and *H. tenuis* closely match those previously calculated for the related species *Coptotermes niger* (6). Similarly, the bacterial diversity indices of *Nasutitermes octopilis* bodies closely match those of *Nasutitermes corniger* and *Nasutitermes takasagoensis* (6). These results indicate that our estimations of bacterial diversity are robust and reproducible. In addition, these results also suggest that the phylogenetic relationships among termites are predictive of the diversity of their bacterial communities.

The bacterial communities associated with termite galleries are more diverse than those found in termite bodies. Most OTUs found in termite bodies correspond to gut bacterial lineages identified in previous studies (5–7, 60), indicating that the majority of bacterial OTUs associated with termite bodies are gut specialists. The termite gut is a highly-specialised habitat, with extreme physicochemical properties, in some species having a pH >12 (61), and is largely populated by bacteria found nowhere else (3–5). Although termite gut hosts among the most diverse communities of microbes found in insects (62), the presence of a strong environmental filtering, preventing the colonisation of most bacterial

species, might explain the low bacterial diversity observed in termite guts when compared with termite galleries and wood controls.

We independently identified the 14-15 dominant body-associated bacterial OTUs for each of the three termite species (Figure 4, Table S2). These OTUs made up 60.3-80.8% of the total bacterial 16S rRNA gene sequences, and were, in most cases, known to be associated with termite guts. For example, the dominant gut symbiotic OTUs in *C. testaceus* were classified as *Candidatus* Azobacteroides and *Candidatus* Armantifilum, two bacterial lineages known to be associated with termite gut protists (63, 64). *Candidatus* Azobacteroides was also the dominant gut symbiotic OTU in *H. tenuis*. In *N. octopilis*, which belongs to Termitidae, the only termite lineage that lost their gut protists (4), the dominant gut symbiotic OTUs were assigned to *Spirochaeta* (Spirochaetes) and *Fibrobacter* (Fibrobacteres) genera. BLAST searches showed that our 16S rRNA gene sequences from these two genera corresponded to *Treponema* and the Fibrobacteres sequences previously found in the gut of other species of *Nasutitermes* (65, 66). Therefore, while our taxonomic identifications were imprecise in some cases, they matched bacterial taxa known to occur in termite guts and highlight the overwhelming dominance of a few bacterial groups.

We found that the bacterial communities associated with termite galleries are specific to termite species, and differ from that of termite bodies and wood controls. These results concur with previous studies that found that bacterial communities associated with nests differ from surrounding soil and wood samples (7, 34, 37, 38). Exclusion experiments have also shown that termites influence the bacterial communities in wood pieces (33). Importantly, our results show that the differences between galleries of different termite species and wood control samples are subtler than that found for gut bacterial communities, suggesting that the gallery-associated bacteria are loosely associated with termites. This

raises the possibility that termites established a symbiotic relationship with the bacterial communities associated with their galleries in absence of strict coevolution between the two partners, as is possibly common for many host-symbiont associations (67), including external symbionts of termites (21, 22).

The identification of the main gallery-associated bacterial OTUs confirmed their loose association with termites. We independently identified 15-18 bacterial OTUs classified as gallery-associated bacteria for each of the three termite species (Figure 4, Table S2). These OTUs made up 25.6-28.3% of the 16S rRNA gene sequences of termite galleries. However, in contrast to body-associated bacterial OTUs, many gallery-associated bacterial OTUs were shared among termite species, and out of 28 OTUs identified as gallery-associated bacteria, eight were shared by all three termite species, and six were shared by two termite species. In addition, gallery-associated bacterial OTUs were also present in wood controls, albeit in significantly lower abundances (only 9.2-14.3% of the 16S rRNA gene sequences). These results suggest that termite gallery-associated bacteria are recruited from the surrounding environment, as has been shown for *Coptotermes formosanus* and its externally-associated symbiotic *Streptomyces* (22). Lastly, we also found body-associated bacterial OTUs in termite galleries that probably originated from DNA of dead or inactive bacterial cells. One such OTU is *Candidatus* Azobacteroides, a bacterium known to be the intracellular symbiont of termite gut protists (63), and therefore clearly unable to live outside termite gut.

The gallery-associated bacterial OTUs identified in this study mostly belonged to Proteobacteria and Actinobacteria, which are known to dominate the nest bacterial communities of several Termitidae species (68). A total of 18 OTUs belonged to Proteobacteria, including seven OTUs assigned to Rhizobiales, five of which were identified as gallery-associated bacteria for each of the three termite species investigated in this study.



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Many Rhizobiales are able to fix atmospheric nitrogen and have developed symbiotic associations with plant roots (69). Whether they represent a source of nitrogen for termites, supplementing the low levels of nitrogen found the wood they consume, remains to be determined. We also identified four gallery-associated bacterial OTUs belonging to Actinobacteria, but none of them belonged to *Streptomyces*. Therefore, unlike previously found for *C. formosanus* (21, 22), *Streptomyces* spp. do not appear to be important gallery-associated bacteria of *C. testaceus*, *H. tenuis,* or *N. octopilis*. Several factors might be at the origin of the lower prevalence of *Streptomyces* in our study compared to that found in *C. formosanus* (21, 22), including the differences among the studied ecosystems (i.e tropical rainforest of French Guiana vs urban parks in Florida) and the sampling approach, based on visually-located wood items colonised by termites (French Guiana) and carton material sampled in bucket traps (Florida). However, because the low prevalence of *Streptomyces* was shared among the three studied termite species, it is unlikely for termite phylogenetic relationships to be at the origin of this pattern. Further studies are required to decipher the exact role of gallery-associated bacteria.

Several bacterial OTUs were partly excluded from termite galleries. The 15-16 gallery-depleted bacterial OTUs we identified for each termite species made up 24.7-34.9% of the 16S rRNA gene sequences in control wood samples, but only 1.4-6.7% of the 16S rRNA gene sequences in termite galleries. These results are indicative of the ability of termites to reduce the growth of some microbes in their direct environment, possibly through the production of antimicrobial and antifungal compounds, as it has been shown in several termite species (21, 29). External symbionts of termites are also known to produce antimicrobial compounds (20, 21), and it is possible that some of the gallery-associated bacteria we identified have this function. Finally, the microclimatic conditions of termite

404 galleries might also play a role in shaping bacterial communities, and reduce the abundance
405 of gallery-depleted bacteria.

406 As is the case for gallery-associated bacteria, a large fraction of the 27 gallery-
407 depleted bacterial OTUs were identified to have reduced abundance in the galleries of more
408 than one termite species, including five gallery-depleted bacterial OTUs with reduced
409 abundance in the galleries of the three studied termite species and nine gallery-depleted
410 bacterial OTUs with reduced abundance in the galleries of two of the three studied termite
411 species. Many of the gallery-depleted bacterial OTUs belong to ubiquitous genera, often
412 found in soil and wood, but that are also known to include animal pathogens, at least on a
413 facultative basis. This includes, among others, OTUs belonging to the genera *Bacillus*,
414 *Clostridium*, *Corynebacterium* and *Staphylococcus*. Whether they are excluded because they
415 represent potential threats to termite colonies remains to be determined. Fungus-growing
416 termites actively exclude fungal *Pseudoxylaria* pathogens from their *Termitomyces* fungus
417 garden (20, 70). Alternatively, modification of the physical and chemical properties of the
418 direct environment of termites, including that of their galleries (28), potentially affects
419 bacterial community composition by promoting the growth of some bacteria at the cost of
420 others. Additional investigations are required to determine how termites affect their
421 neighbouring bacterial communities. Our results show that as termites host specific
422 microbial communities inside their guts, specific microbial communities grow in their
423 galleries.

424

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**DATA ACCESSIBILITY**

The sequence data generated in this study are deposited in MG-RAST under accession numbers: mgm4904347.3

**AUTHOR CONTRIBUTIONS**

DSD, JŠ and TB conceived the study and carried out the fieldwork. PSo, PSt, KV and AC performed the lab experiments. TV, MK and IO analysed the data. PSo and TB wrote the paper with significant input from other co-authors. This study was supervised from inception to completion by JŠ.

652 **Table 1** Results of the pairwise PERMANOVA analysis.

653

654 **Table S1** List of bacterial OTUs found in this study.

655

656 **Table S2** Taxonomy and abundance of the bacterial OTUs identified by the Partial
657 Redundancy analysis represented in Figure 4.

658

659 **Figure 1** Box plot showing three diversity indices (Chao1, Evenness and Shannon-Wiener)
660 calculated for the bacterial communities associated with the bodies and galleries of the
661 termites *Coptotermes testaceus*, *Heterotermes tenuis* and *Nasutitermes octopilis*, and with
662 wood controls. Boxes indicate the first and third quartiles. The horizontal lines crossing
663 boxes are medians. Whiskers indicate the $5^{th}$ and $95^{th}$ percentiles, and black dots are outliers.
664 Groups that do not share at least one capital letter are significantly different (Tukey HSD
665 *post-hoc* test: $p < 0.05$).

666

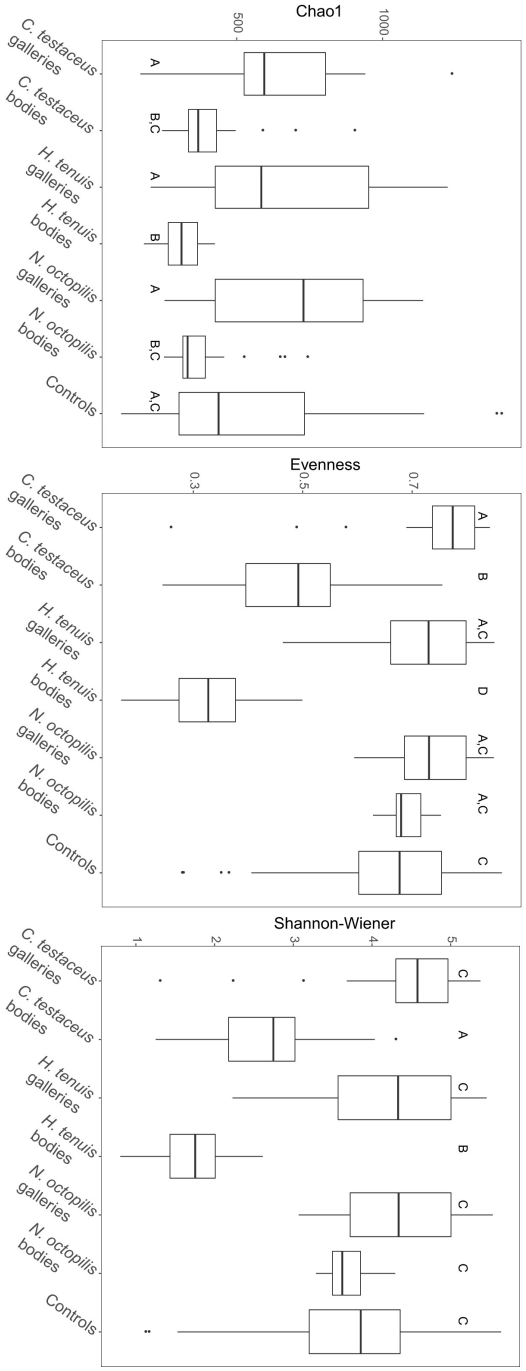
667 **Figure 2** Relative abundance of bacterial phyla associated with the bodies and galleries of
668 the termites *Coptotermes testaceus*, *Heterotermes tenuis,* and *Nasutitermes octopilis*, and
669 with wood controls.

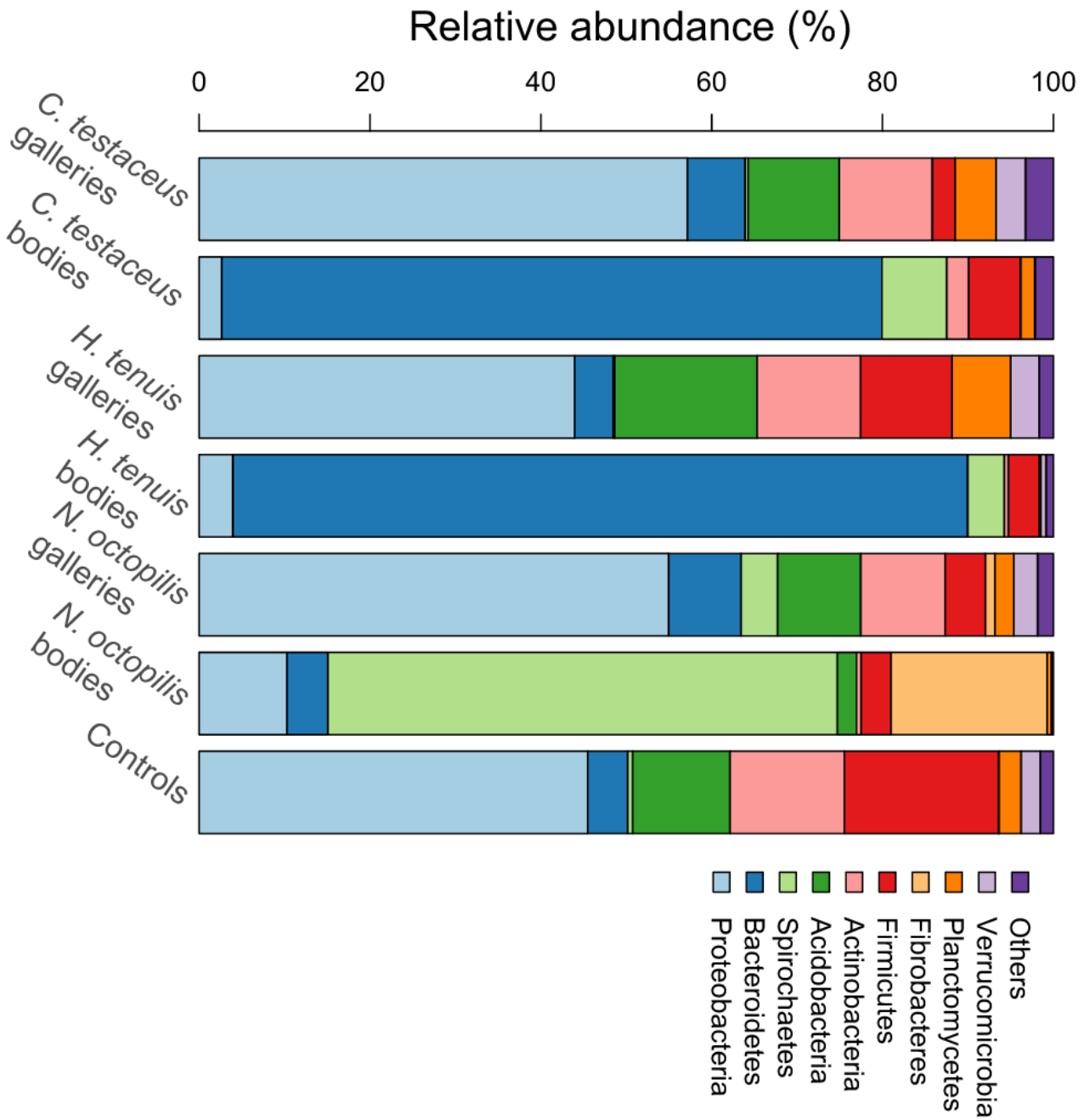
670

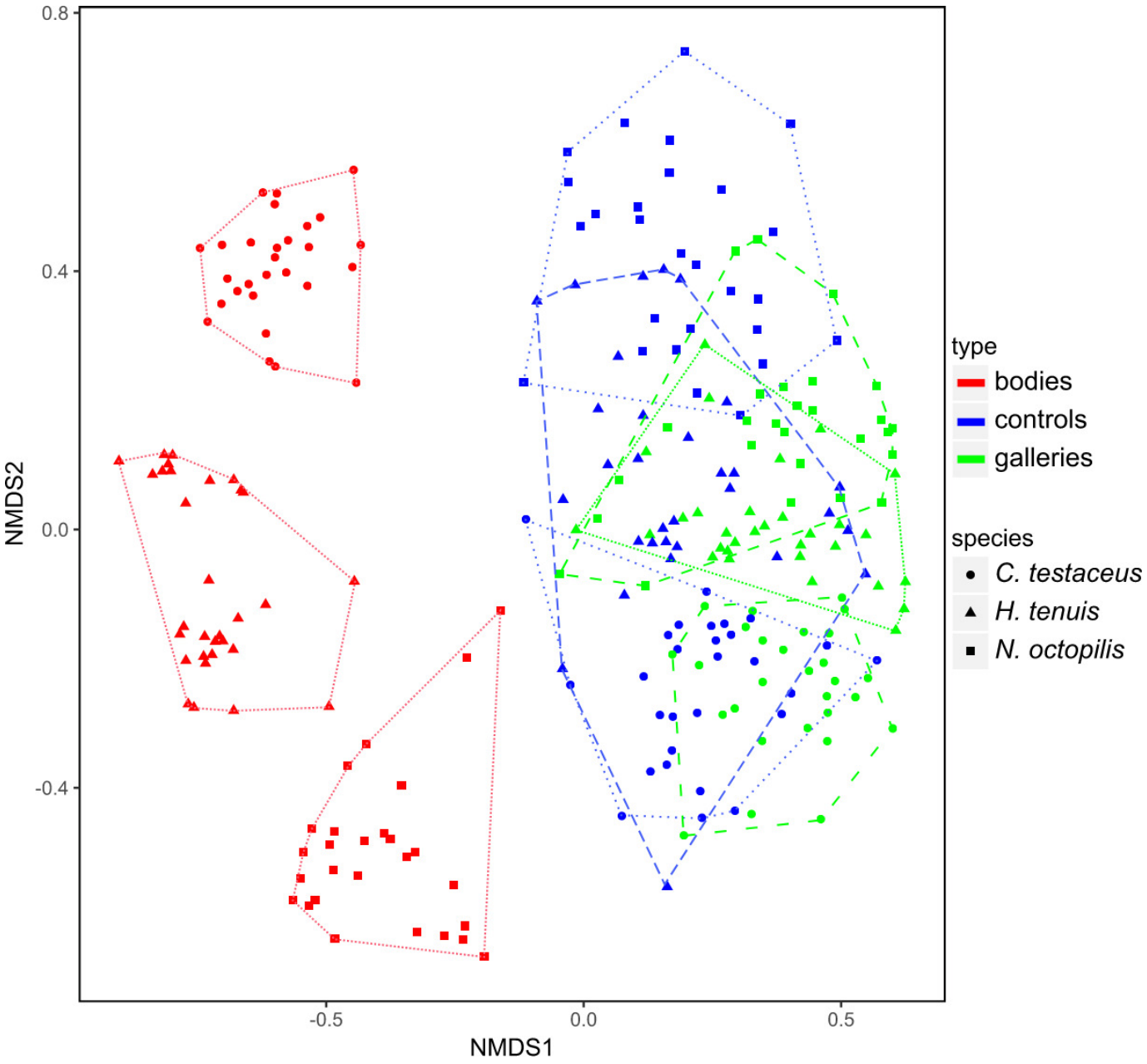
671 **Figure 3** Non-metric multidimensional scaling of bacterial communities associated with the
672 bodies and galleries of the termites *Coptotermes testaceus*, *Heterotermes tenuis,* and
673 *Nasutitermes octopilis*, and with wood controls.

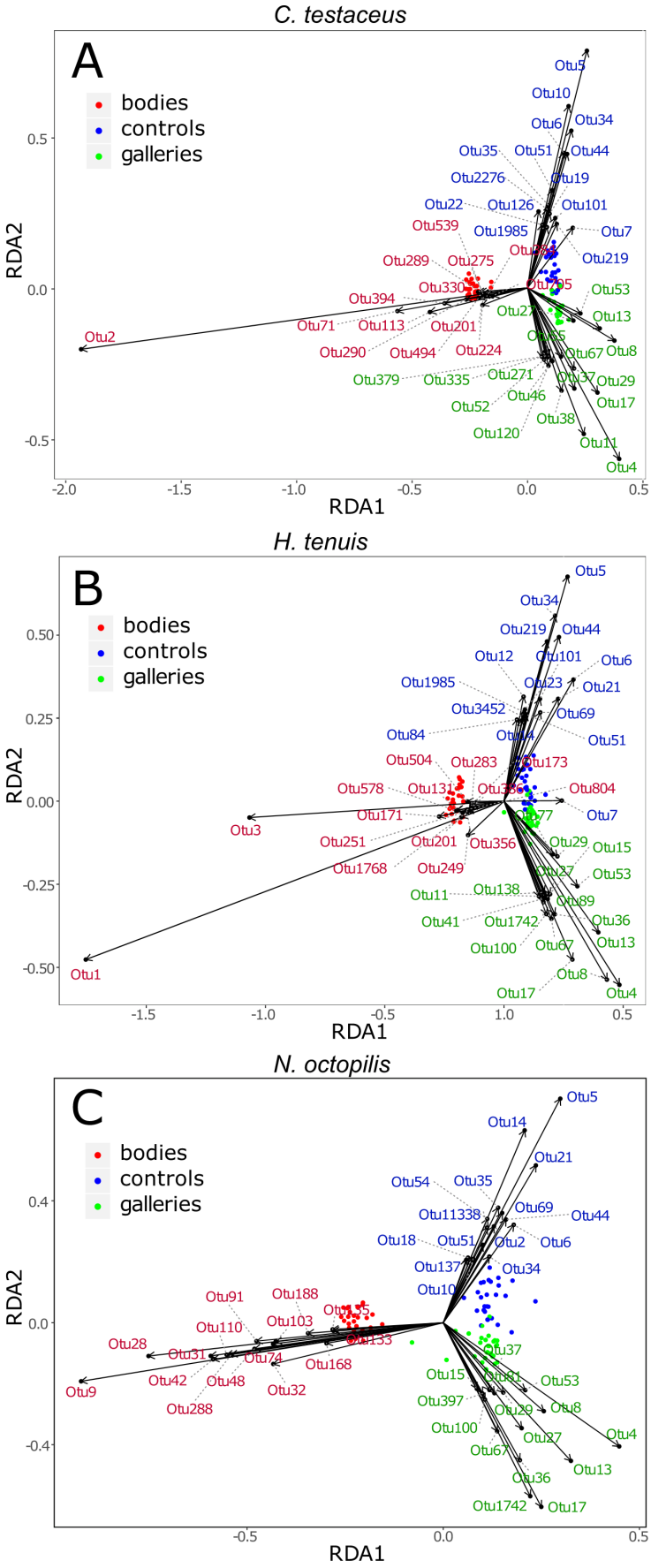
674

675 **Figure 4** Partial redundancy analysis of bacterial communities associated with termite bodies
676 and galleries and with wood controls. (A) *Coptotermes testaceus*, (B) *Heterotermes tenuis*,
677 (C) *Nasutitermes octopilis*. Taxonomic identification of OTUs is provided in Table S1.


Chao1
500
1000
C. testaceus galleries
C. testaceus bodies
H. tenuis galleries
H. tenuis bodies
N. octopilis galleries
N. octopilis bodies
Controls
A
B,C
A
B
A
B,C
A,C
Evenness
0.3
0.5
0.7
A
B
A,C
D
A,C
A,C
C
Shannon-Wiener
1
2
3
4
5
C
A
C
B
C
C
C


Relative abundance (%)
0
20
40
60
80
100
C. testaceus galleries
C. testaceus bodies
H. tenuis galleries
H. tenuis bodies
N. octopilis galleries
N. octopilis bodies
Controls
Proteobacteria
Bacteroidetes
Spirochaetes
Acidobacteria
Actinobacteria
Firmicutes
Fibrobacteres
Planctomycetes
Verrucomicrobia
Others





| | | | F | R2 | p-value | p-adjusted |
|---|---|---|---|---|---|---|
| *C. testaceus* bodies | vs. | *H. tenuis* bodies | 46.411 | 0.449 | <10-5 | <10-3 |
| *C. testaceus* bodies | vs. | *N. octopilis* bodies | 88.668 | 0.626 | <10-5 | <10-3 |
| *H. tenuis* bodies | vs. | *N. octopilis* bodies | 50.945 | 0.476 | <10-5 | <10-3 |
| *C. testaceus* galleries | vs. | *H. tenuis* galleries | 2.256 | 0.038 | <10-4 | 0.003 |
| *C. testaceus* galleries | vs. | *N. octopilis* galleries | 2.425 | 0.044 | <10-5 | <10-3 |
| *H. testaceus* galleries | vs. | *N. octopilis* galleries | 1.901 | 0.033 | <0.001 | 0.022 |
| *C. testaceus* galleries | vs. | *C. testaceus* controls | 2.929 | 0.052 | <10-5 | <10-3 |
| *H. tenuis* galleries | vs. | *H. tenuis* controls | 2.057 | 0.033 | 0.002 | 0.07 |
| *N. octopilis* galleries | vs. | *N. octopilis* controls | 3.443 | 0.062 | <10-4 | <10-3 |
| *C. testaceus* bodies | vs. | *C. testaceus* galleries | 34.076 | 0.387 | <10-5 | <10-3 |
| *H. tenuis* bodies | vs. | *H. tenuis* galleries | 22.625 | 0.274 | <10-5 | <10-3 |
| *N. octopilis* bodies | vs. | *N. octopilis* galleries | 25.984 | 0.333 | <10-5 | <10-3 |
| *C. testaceus* bodies | vs. | *C. testaceus* controls | 27.334 | 0.336 | <10-5 | <10-3 |
| *H. tenuis* bodies | vs. | *H. tenuis* controls | 19.262 | 0.243 | <10-5 | <10-3 |
| *N. octopilis* bodies | vs. | *N. octopilis* controls | 25.762 | 0.331 | <10-5 | <10-3 |
| *C. testaceus* controls | vs. | *H. tenuis* controls | 1.036 | 0.018 | 0.365 | 1 |
| *C. testaceus* controls | vs. | *N. octopilis* controls | 1.631 | 0.030 | 0.011 | 0.409 |
| *H. tenuis* controls | vs. | *N. octopilis* controls | 1.537 | 0.027 | 0.025 | 0.891 |

1