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Microbial colonisation patterns are more affected by plant substrate compositions than termite modulated environments

Short title: Termite effects on substrate colonisation

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Abstract

Background: Termites are dominant plant biomass decomposers and important ecosystem engineers that modify soil properties and microbial communities. In fungus-farming termites, mound building contribute to distinctions between three environments: fungal gardens, mound walls, and outside soil away from mounds. Here we determined the properties of these environments for 10 colonies of *Macrotermes bellicosus* termites and tested their consequences for fungal and bacterial colonisation of two novel substrates presented as compositionally-different types of tea. Lastly, capitalising on the mass loss of tea bags, we evaluated the decomposition rates in these environments across habitats (forest patch, woody savanna, and teak plantation) in central Côte d'Ivoire.

Results: We confirmed that *M. bellicosus* termite colonies create distinct local environments that are markedly different in both elemental (e.g., carbon and nitrogen content) and bacterial and fungal community compositions. Fungal colonisation of tea bags in outside soils, and to a lesser extent mound soil, reflected community compositions of soil sources, with dominant colonisers being opportunistic spore-forming saprophytes. Similar communities colonised tea bags within mounds, resulting in poor reflection of the termite gut and fungal comb source communities. Bacterial colonisation was also comparable across environments, but overall poorly reflected source communities, underlining that tea bag properties strongly select for coloniser taxa. Although decomposition rates were more consistent in mound walls and fungal gardens than outside soils, we did not detect significant differences between either of the three environments.

Conclusions: Our findings point to contrasting tea bag properties strongly selecting for opportunistic colonisers that are not necessarily abundant in source environments and across habitats. This canalisation was most prominent for fungi and likely contributed to the comparable decomposition rates across environments. The omnipresence of fast-colonising fungi to novel substrates in all three environments imply that they remain present and potentially threat termite farms, while termites actively suppress their proliferation within combs. Colonisation here thus best exemplified competitive release owing to reduced suppression of non-*Termitomyces* fungi. Even so, termite activities markedly shape environmental properties, demonstrating the importance of mound building in generating heterogeneous microbial niches in savannah ecosystems.

Key words

Macrotermitinae, teatime4science, soil microbiomes, *Termitomyces*, savanna, *Macrotermes*, stable isotopes, decomposition rates

Background

Microbial colonisation of new substrates is governed by community assembly processes, where dispersal, environmental filtering, and species interactions collectively determine which taxa successfully establish and proliferate (1, 2). When resident species differ in their ability to colonize and compete, colonisers will occupy patches where conditions best match their ecological requirements (3, 4). New niches for colonisation can arise from ecosystem engineering taxa, such as ants and termites, that increase landscape heterogeneity by manipulating their physicochemical environment (5-8).

Fungus-farming termites (subfamily Macrotermitinae) are important ecosystem engineers in tropical and subtropical ecosystems in Africa and Asia (9, 10). More than 400 described termite species in the subfamily engage in obligate mutualistic symbiosis with species of *Termitomyces* (Basidiomycota; Lyophyllaceae) (11-13). These fungi, along with gut and fungal garden (fungal comb) bacteria, convert plant polysaccharides into termite nutrition (14-16). Consequently, farming termites dominate as plant biomass decomposers in savanna ecosystems, restructuring habitats in ways that influence soil properties and microbial communities (15, 17, 18). At the landscape level, these effects can be stabilising as they contribute to the generation of islands of fertility that support a rich biodiversity of plants, animals, and microbes (19). Within termite mounds, the highly structured environment acts as an environmental filter that selects for microorganisms with traits that are adapted to unique gut and fungus comb physiochemical properties (20-23).

At the local spatial scale of a termite mound, farming activities contribute to the formation of three distinct environments. First, fungal gardens represent unique structures of fungal combs consisting of decomposing organic plant matter, a single fungal clone of the farmed

110 *Termitomyces* fungus (24, 25), and termite gut bacterial deposits (22) that break down plant
111 biomass under stable abiotic conditions (14, 15). Additionally combs include spores from other
112 fungi for which growth is suppressed. Second, mound wall soils that are densely packed contain
113 less microbial diversity and organic material than outside soils (26-29), and have more variable
114 abiotic conditions (5). Third, outside soils are less affected by termites but still impacted by
115 foraging and soil movement activities along with other abiotic and biotic factors. These
116 environments support distinct microbial communities (5), physicochemical and elemental
117 properties that reflect filtering properties of colony activities (30). Such differences should
118 predictably affect colonisation patterns and decomposition rates in available substrates.
119
120 We explore the consequences of distinct biogeochemical characteristics and microbial
121 properties of these niches on fungal and bacterial colonisation of compositionally-different
122 plant substrates: tea bags containing either Rooibos tea (complex polysaccharides and lignin-
123 rich) or Green tea (simpler polysaccharides and more soluble carbon) (31). We first
124 characterised the elemental and isotopic properties of the three environments (Fungal garden,
125 Mound wall, and Outside) by quantifying carbon and nitrogen content, stable isotope ratios
126 ($\delta^{13}C$ and $\delta^{15}N$), soil organic matter content (SOM), and bacterial and fungal community
127 compositions and densities in four source communities (Termite guts, Fungal combs, Mound
128 wall soil, and Outside soil). We then determined what fungi and bacteria from source
129 communities colonised tea bags and the consequences for overall decomposition rates. This
130 allowed us to test the following predictions: i) Colonisation patterns in tea bags in outside soils
131 should reflect the source bacterial and fungal community compositions of the soil they were
132 placed in, and be dominated by fast-colonising saprophytes; ii) We predicted tea bags in mound
133 walls to reflect source microbial communities of mound wall soil, but with lower colonisation
134 and slower decomposition than bags in outside soil because of anticipated lower diversity and

density of mound wall communities; and iii) Colonisation of tea bags placed in fungal gardens should least reflect source communities (guts and combs), since termites could not access the tea and since *Termitomyces* – even if vastly dominating fungal combs (25) – was not expected to effectively colonise plant substrates that had not been pre-processed by the termites. Instead, we expected fungal garden colonisation to be dominated by gut and fungus comb bacteria and fungi (22) and dormant (e.g., *Xylaria* (32)) and opportunistic (*Aspergillus* and *Trichoderma* (33)) fungi that are known to proliferate when termites are removed (33-35). In the fungal garden environment, we expected decomposition rates to be higher than in mound soil, given the specialized microbial communities and favourable growth conditions. An alternative to these predictions would be that tea bag properties canalise colonisation by the most prominent decomposition-related microbes, irrespective of source environment.

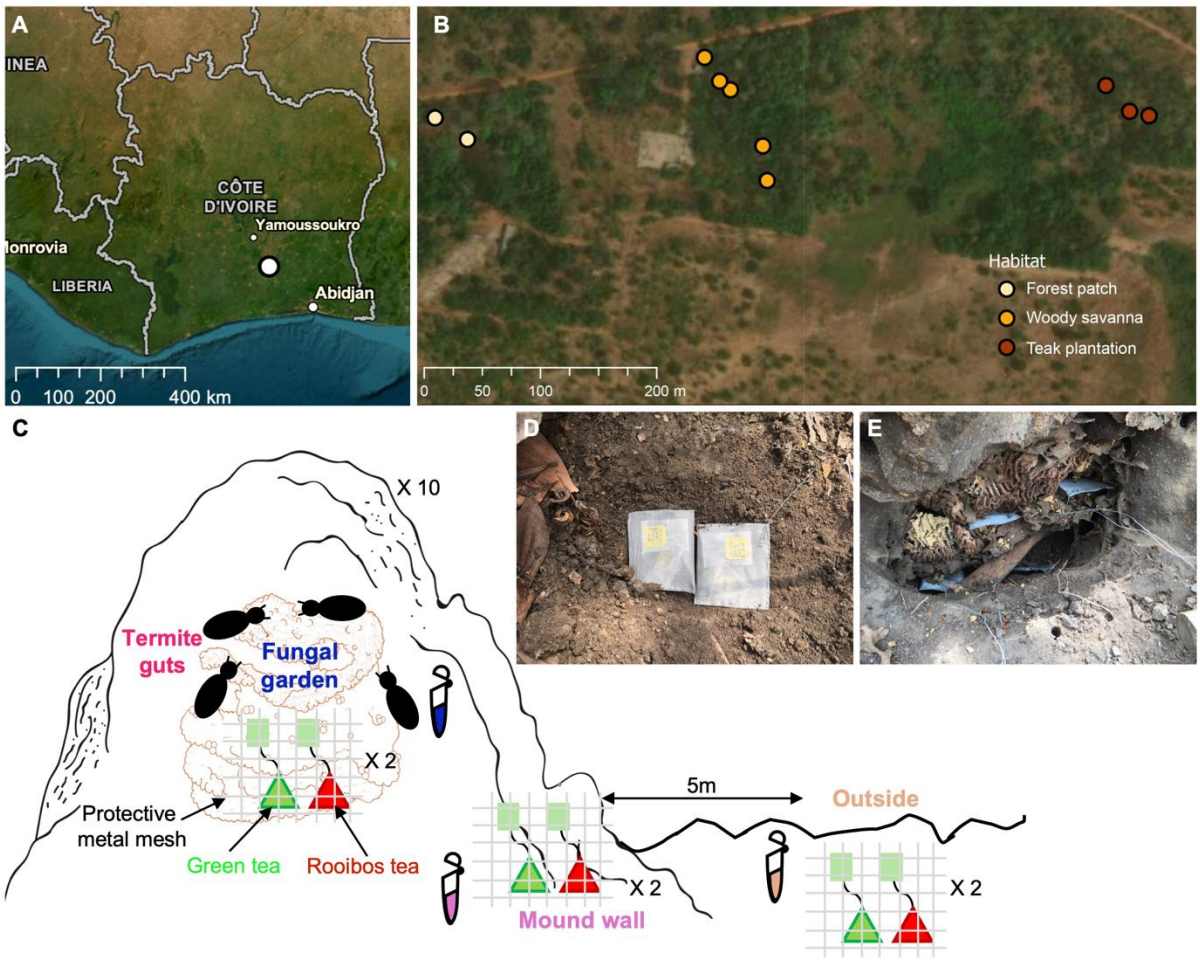


Figure 1. Field sites and sampling approach. A. Map of Côte d'Ivoire indicating the location

of the Lamto Ecological Research Station and **B.** Map of the sampling sites of 10 *Macrotermes bellicosus* mounds across three habitat types. The map was generated using ArcGIS PRO 3.1.0 (36). **C**. Illustration of a termite mound generating the environments that mesh bags were placed in and from which soils, combs, and termite source microbial communities were sampled. Images of pairs of Green and Rooibos tea bags enclosed in stainless steel metal mesh bags that were placed either in soil (**D**) or in fungus combs (**E**).

Materials and methods

Field site and sample collection

In February 2022, 10 *Macrotermes bellicosus* colonies from three different habitat types were identified: forest patch, woody savanna, and teak plantation in central Côte d'Ivoire, within the forest-savanna transition zone, at the southernmost tip of the "V Baoulé," near the Lamto Scientific Reserve (Table S1; Figure 1). These habitats were chosen to compare if putative differences in soil communities by habitat would impact tea bag colonisation. For each colony, two sets of Lipton Green tea and Rooibos tea bag pairs were placed directly within fungal gardens, and 6-10cm into termite mound wall (at ground level) and buried 6-10cm into outside soils (approximately 5m away from the mound). Each set of tea bags was placed in a 150-micron stainless steel wire metal mesh bag to avoid consumption by the termites. Each tea bag was weighed before deployment. Mound and outside soil samples, 10 workers per colony, and fresh fungus comb were collected in 2 ml sterile screw-cap tubes containing 500 µl of RNAlater®. Samples were kept at room temperature during fieldwork and –20°C until transport to Denmark. Tea bags were collected after 28 days in their metal mesh bags, along with soil from the mound wall and outside, and placed in individual Ziplock bags. At the same time, small pieces of fungus comb were collected in 50 ml falcon tubes for carbon and nitrogen analyses. All samples were transported frozen to Denmark and stored at –20°C until processing.

One set of tea bags was used to estimate decomposition rates, and a second set was used to determine microbial community compositions and densities. All analyses described below were conducted in R v.4.5.2 (37) and data were visualised using the ggplot2 package (37).

Microbiome characterisations

We characterised bacterial and fungal community densities and compositions on DNA extracted from 150-200 mg soil or comb sample, pools of five termite abdomens per colony (ca. 25-50 mg), and from Green and Rooibos tea using one set of tea bags using the Qiagen DNeasy® PowerSoil® kit (Hilden, Germany). In addition to experimental tea bags, we also extracted DNA from three tea bags that were brought to the field but not deployed per type as controls.

To determine microbial density, we performed quantitative PCR (qPCR) on the DNA extracts using the Femto Fungal (catalogue number E2007) & Bacterial (catalogue number E2006) DNA Quantification Kits from Zymo Research. Fungal and bacterial quantifications were conducted separately but following the same setup. For each DNA sample, we conducted two qPCR replicates, and all samples were processed in three qPCR runs. Both qPCR replicates of the same sample were included in the same qPCR run. In addition to the samples, each run included duplicates of seven DNA standards (provided with the kit) and a No Template Control. The qPCR mixes for each sample contained 18 µl premix and 2 µl of standards or No Template Control or DNA samples. Conditions for qPCRs were the same for fungi and bacteria. We ran the qPCRs using MxPro – Mx3005P qPCR system with the following conditions: initial 10 mins at 95°C; 45 cycles of 30s at 95°C for denaturation, 30s at 50°C for annealing, 1min at 72°C for extension, and a final extension of 7min at 72°C. Following qPCR, we used the Ct values of seven standards to generate a standard curve. We averaged the Ct values of sample

198 duplicates and used these averages and the standard curve to calculate bacterial and fungal
199 DNA concentrations per µl DNA.
200
201 We characterised microbiome compositions using amplicon sequencing of the 16SrRNA gene
202 (bacteria) and the ITS2 gene (fungi). For bacteria, we used a tagging approach (38), where each
203 forward (314F: 5′-CCTAYGGGRBGCASCAG-3′) and reverse (805R: 5′-
204 GGACTACNNGGGTATCTAAT-3′) (39) primers had a unique 7-neucleotide tags, allowing us
205 to label each sample during the first PCR. Each PCR reaction for bacteria included 0.25 µl of
206 Polymerase HIFI, 5 µl 5X buffer, 15.75 µl DNA/RNA free water, 1 µl tagged forward and
207 reverse primer, and 2 µl extracted DNA. PCR conditions were 95 °C for 1 min, followed by 30
208 cycles of 95 °C for 15s, 56 °C for 15s, 72 °C for 30s, and a final extension step of 72 °C for 5
209 min. PCR products were visualised on a 2% agarose gel and were pooled according to the band
210 strengths (bright bands = 4 µl, medium strength bands = 6 µl, weak or no band = 8 µl). The
211 pool was used to build Illumina libraries using the PCR-free Tagsteady library building
212 protocol (40). Purified libraries were then sequenced on ca. 3% of an Illumina NovaSeq 6000
213 SP lane (XP, v1.5,) at the GeoGenetics Sequencing Core, University of Copenhagen, Denmark.
214 For fungal community metabarcoding, we used gITS7 (5′-GTGARTCATCGARTCTTTG-3′)
215 (41) and ITS4ngs (5′-TCCGTAGGTGAACCTGCGG-3′) (42) primers without using the
216 tagging approach. The master mix and PCR conditions were identical to those for bacteria.
217 Library building was conducted following a two-step PCR approach, as described in Bodawatta
218 et al. (2024) (43). Libraries were sequenced on an Illumina MiSeq platform at the Section of
219 Microbiology, University of Copenhagen, using Reagent Kit v3 (2 × 300 cycles) (Illumina, San
220 Diego, CA, USA).
221

Bacterial and fungal sequences were processed using the QIIME2 pipeline (44). For bacterial sequences, we conducted demultiplexing using Cutadapt within QIIME2 (45). Denoising and merging of paired-end reads were done using the DADA2 (46) plugin. For bacterial sequences, we set p-trim-left-f and p-trim-left-r to 1 (as the primers were removed during the demultiplexing step) and p-trunc-len-f was set to 225, and p-trunc-len-r was set to 222. For fungal sequences we set p-trim-left-f and p-trim-left-r to 20, while p-trunc-len-f was set to 260 and p-trunc-len-r was set to 234. Output amplicon sequence variants (ASVs) were then assigned to taxonomy using SILVA 134 database for bacteria (47) and the UNITE database for fungi (48). We removed ASVs assigned to mitochondria, chloroplasts, and Archaea from the bacterial ASV table. ASV tables were further curated using the decontam package (49) along with the phyloseq package (50). We used extraction blanks and unused tea bag samples as controls, with the "prevalence" method in decontam to identify contaminant sequences (49). We detected a total of three bacterial and 87 fungal contaminant ASVs, that we excluded from further analyses as were samples with less than 2000 sequences (Tables S2-S3).

Elemental, isotopic, and microbial properties of environments

We determined carbon and nitrogen contents (%C and %N), stable isotope compositions ($\delta^{13}C$ and $\delta^{15}N$), and percentage Soil Organic Matter (SOM) in Fungal gardens, Mound wall soil, and Outside soil samples. Fungal garden samples were thawed and placed in paper bags, while soil samples were transferred to 50 ml Falcon tubes. All samples were dried for three days at 60°C. Samples were subsequently crushed with an IKA A10 Basic mill grinder, sieved to remove smaller stones, packed in tin capsules, weighed, placed in a 96 well plate and subject to Isotope Ratio Mass Spectrometry (IRMS) analyses using an Isoprime isotope ratio mass spectrometer (Elementar, Cheadle Hulme, Stockport, UK) coupled to a CN elemental analyser (Eurovector, Milan, Italy), at the Department of Biology, University of Copenhagen, as described by

247 Schmidt et al. (51). The detection limits were 0.100 % for C and 0.015 % for N. Isotope results
248 are reported as parts per mille (‰) deviation from the international standard V-PDB and
249 atmospheric N_2 for C and N, respectively, with drift correction using peach leaves (NIST 1547)
250 as internal standard. The standard deviation of isotope measurements of the standards was ±
251 0.1‰ for $\delta^{13}C$ and ± 0.2‰ for $\delta^{15}N$. SOM was determined by placing crushed samples in 50
252 ml Falcon tubes into crucibles that were weighed, placed on a furnace plate in a preheated
253 Nabertherm furnace, and then ashed at 550°C for 6hrs. SOM was determined as percentage
254 weight loss. We evaluated the statistical differences in soil properties using linear mixed effect
255 models (LMMs) in lmerTest package (52). Each model included the soil property as the
256 dependent variable, while source environment and habitat were included as independent
257 variables. We used colony ID as a random factor. The significance of independent variables
258 was evaluated using Type III ANOVAs. For significant variables, we further evaluated pairwise
259 differences using estimated marginal means with the emmeans package (52).
260
261 Microbiome alpha diversity indices (Chao1 richness, Shannon's diversity index, and Pielou's
262 evenness index) were estimated using microbiome (53) and phyloseq (50) packages. For source
263 environments (Fungal gardens, Mound wall soil, an Outside soil), we tested for differences in
264 alpha diversity and log transformed fungal or bacterial densities (from qPCR results) using
265 LMMs in the lmerTest package (54). Microbial community compositions of source samples
266 (Termite guts, Fungal combs, Mound wall soil, and Outside soil) were calculated using Bray-
267 Curtis dissimilarity indices and visualized in Non-metric Multidimensional Scaling (NMDS)
268 plots in phyloseq (50). We used Permutational Multivariate Analysis of Variance
269 (PERMANOVA) tests with the adonis2 function in the vegan package (55) to evaluate the
270 impact of environment on microbial community compositions in source samples. The models
271 used 10000 permutations, set "by" parameter to margin, and used colony ID as the strata

parameter. Pairwise differences in community composition were evaluated using the pairwiseAdonis package (56) with Bonferroni correction. Finally, to examine if there were links between microbial communities and soil nutrients, we used linear models to test for associations between %C, %N, C:N ratio, and SOM on Chao1 richness estimates, Shannon's diversities, and density.

Microbial recruitment from source communities to tea bags

To evaluate what microbial communities were recruited in tea bags, we first examined alpha diversities of the two tea types using LMMs, as for source samples. Here, models contained environment, tea type, and habitat as independent variables, and colony ID as a random factor. Similar to source samples, we calculated the microbial composition in tea bags using a Bray-Curtis dissimilarity matrix. We then conducted PERMANOVAs with environment, tea type, and habitat as independent variables and colony ID as a strata parameter. To evaluate the proportion of ASVs colonising tea bags at each environment, we conducted source tracking analyses using the FEAST package (57). These analyses were done individually by environment and by tea type. Source samples (Termite guts, Fungal combs, Mound wall soil, and Outside soil) were set to "Source" and tea was set to "Sink". We assigned matching source samples and sinks from each colony using the different_sources_flag = 1. We evaluated effects of environment on the proportion of ASVs in each tea type originating from difference sources by conducting zero-inflated generalized linear mixed models (GLMMs) in the glmmTMB package (58). The significance of the independent variables was evaluated using Type II Wald χ^2 tests. We visualised the 25 most abundant microbial genera at each environment in heat maps generated with the plot_heatmap function in the microeco package (57). We evaluated the differentially abundant microbial genera in each sample type (sources and tea types) in each

296 environment using the trans_diff function in the microeco package. Here we used the linear
297 discriminant analysis effect size (LEfSe) (59).
298
299 *Impact of recruited communities on tea decomposition*
300 We used the second set of tea bags to calculate decomposition rates (k) in the Fungal garden,
301 Mound wall soil, and Outside soil environments. Tea bags were removed from the metal mesh
302 bags and dried at 70°C for 48 hrs, after which the dried tea was taken out of the bag. Bags with
303 labels and the tea were weighed. Decomposition rates were then calculated using the initial
304 weight measures and weights after incubation using formulas provided by teatime4science.org
305 (31). We then evaluated the effect of environment using GLMMs in lmerTest package (52) and
306 tested if there were associations between alpha diversity or density on decomposition rates.
307 This was done using individual models for each diversity matrix and separate models for source
308 samples, Green tea, and Rooibos tea. R^2 values for each model were acquired using the r2
309 function in the performance package (60). Lastly, we evaluated using linear models if
310 decomposition rates were associated with %C, %N, C:N ratio, and SOM of the source
311 communities.
312

313 ## Results

314 *Elemental, isotopic, and microbial properties of environments*
315 The elemental properties of our source environments were markedly different, with %C, %N,
316 C:N, and SOM being significantly higher in fungal gardens than both mound and outside soils
317 (Figures 2A, S1, Tables S4-S6). This was unsurprising, as collected plant material is deposited
318 within fungal gardens after termite gut passage. $\delta^{15}N$ values were significantly lower in fungal
319 gardens than mound wall and outside soil (Figures 2A, S1), while $\delta^{13}C$ values were
320 significantly lower in fungal gardens and outside soil than mound soils (Figures 2A, S1, Table

S6). Habitat did not overall have a major effect on %N, C:N ratios, and $\delta^{15}N$, with a few notable exceptions for teak plantation samples (Table S5). Here, there was a trend towards lower N content than in the forest patch (Estimated Marginal Means (EMM) = -0.2352, t-ratio = -2.532, p = 0.0813), and higher C:N ratios than in the forest patch (EMM = 6.66, t-ratio = 3.178, p = 0.0317) and woody savanna (EMM = 4.85, t-ratio = 2.701, p = 0.0542) samples, along with significantly lower $\delta^{15}N$ (Table S6). The lower N content and $\delta^{15}N$ in the teak plantation aligns with expected effects of agriculture and silviculture on soil fertility (61).

After removal of contaminant sequences and retaining only samples with at least 2000 reads, we retained 1,352,806 bacterial sequences (average ± SD = 34,687 ± 23,679) and 1,905,359 fungal sequences (48,855 ± 15,890) in source samples (Tables S2-S3, S7). These sequences assigned to 19,773 bacterial and 6,026 fungal ASVs. All alpha diversity matrices and microbial densities differed significantly between source sample types (Table S8). Bacterial and fungal richness and Shannon diversity were significantly lower in gut and comb than mound and outside soil samples (Figures 2A, S1A,B, Table S9). Gut and fungus comb samples exhibited significantly lower Pielou's evenness than outside soils (Figures 2A, S1C, Table S9). However, as expected, bacterial densities in guts were significantly higher than comb and mound soil samples, and fungal densities in combs were significantly higher than all other sample types (Figures 2A, S1D, Table S9). Community compositions differed significantly between source samples for both bacteria (PERMANOVA: $F_3 = 5.448$, $R^2 = 0.3105$, $p < 0.0001$) and fungi ($F_3 = 5.473$, $R^2 = 0.3104$, $p < 0.0001$) (Figure 2B,C). Pairwise comparisons showed that all bacterial communities differed significantly between sources, except for mound wall and outside soil (Figure 2B, Table S10). Fungal community compositions differed significantly between all sources (Figure 2C, Table S10). We also found effects of habitat type on community compositions of bacteria ($F_2 = 1.668$, $R^2 = 0.0634$, $p < 0.0001$) and fungi ($F_2 = 1.726$, $R^2 =$

0.0652, $p < 0.0001$); however, pairwise analyses did not produce significant effects of habitat (Table S10).

When evaluating the link between source nutrients and microbial alpha diversities, only %C had a significant association with microbial (bacterial and fungal) Chao1 richness estimate, Shannon's diversity index, and microbial density (Figure S2; Table S11). Most of these associations were negative, except for that of the association between %C and fungal density. These associations are likely to primarily be driven by the very carbon rich fungal combs.

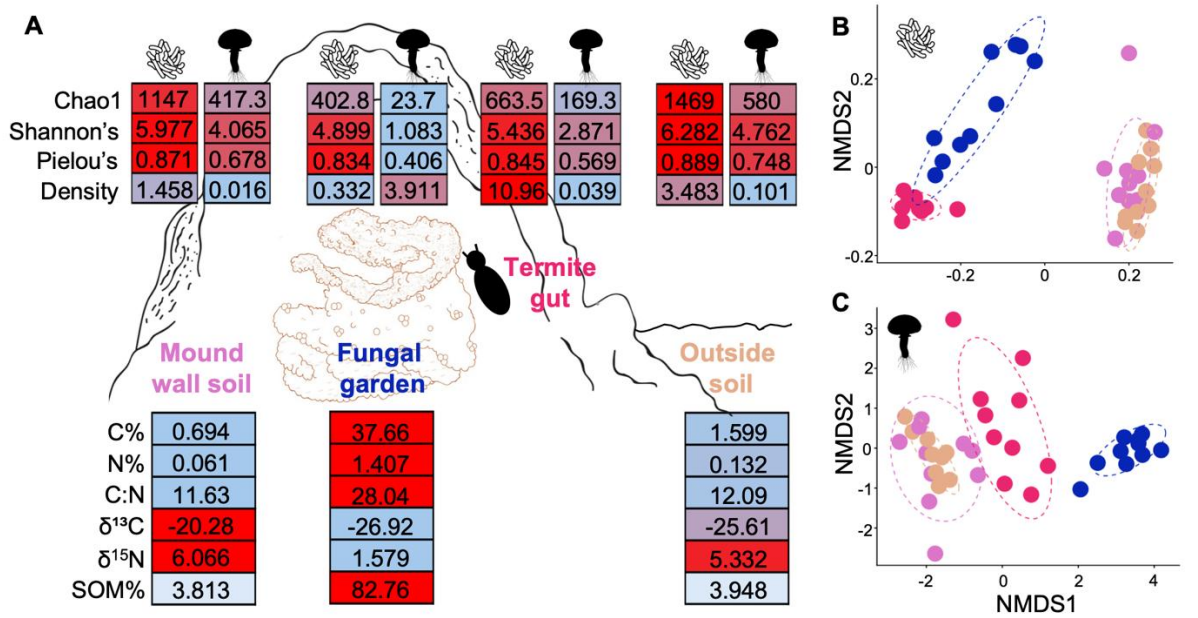


Figure 2. Termite mound environments have distinct nutritional and microbial properties. A. The top panel shows the average microbial (bacteria and fungi) Chao1 richness, Shannon's diversity index, Pielou's evenness index, and microbial density (per µl extracted DNA) across environments (Figure S3, Table S7). The bottom panel shows the average percentage Carbon, percentage Nitrogen, Carbon to Nitrogen ratio, $\delta^{13}C$ value, $\delta^{15}N$ value, and percentage Soil Organic Matter (SOM) in mound wall soil, fungal comb, and outside soil (Figure S1, Table S4). Values are only comparable across each microbiome and elemental property, and darker reds indicate higher while lighter blues represent lower values. NMDS

plots of bacterial (**B**: stress = 0.0973) and fungal (**C**: stress = 0.1404) community compositions by source environment (coloured as labels in **A**). Ellipses represent 95% confidence intervals. Bacterial and fungal illustrations from Phylopic.com.

Microbial recruitment from source communities to tea bags

Within tea bags, there were 1,965,882 bacterial (average ± SD = 40,120 ± 32,151) and 1,389,098 fungal (27,781 ± 14,204) sequences (Tables S2-S3, S7). These were assigned to 4,275 bacterial and 1,221 fungal ASVs. Overall, neither bacterial richness, diversity, nor density differed by tea type, but Pielou's evenness was significantly lower in Rooibos tea (Figure S4, Table S9). In contrast, fungal richness and diversity was significantly higher in Rooibos than Green tea, while density was higher in Green tea (Figure S4, Table S12). Bacterial richness and diversity in tea bags were affected by environment (Figure S4, Table S12), with tea bags in outside soils being richer and more diverse than those of mound wall soil and fungal gardens (Figure S4, Table S13). Similarly, fungal richness was higher in outside soil tea bags than those placed in fungal gardens and mound wall soil (Figure S4, Tables S12-S13). Habitat did not significantly affect alpha diversities of either bacterial or fungal communities within tea bags (Table S12).

Bacterial community composition differed significantly between tea types (Figure 3A; PERMANOVA: $F_1 = 4.938$, $R^2 = 0.0902$, $p < 0.0001$), and there were significant effects of environment ($F_2 = 2.154$, $R^2 = 0.0787$, $p < 0.0001$) and habitat ($F_2 = 1.261$, $R^2 = 0.0461$, $p < 0.0001$). Pairwise differences showed that bacterial communities in tea bags in outside soil differed significantly from those in fungal gardens and mound wall soil (Table S14). However, after Bonferroni correction for multiple testing, we did not see significant effects of habitat (Table S14). Fungal community composition also differed significantly between tea types (Figure 3B; $F_1 = 10.51$, $R^2 = 0.1682$, $p < 0.0001$), and they were overall significantly affected

386 by environment ($F_2 = 1.769$, $R^2 = 0.0566$, $p = 0.0330$) and habitat ($F_2 = 2.174$, $R^2 = 0.0696$, p
387 < 0.0001). However, pairwise differences again revealed no significant effects of neither
388 environment nor habitat (Table S14).
389
390 To determine the proportion of ASVs in tea bags originating from source samples, we
391 conducted source tracking analyses individually for each environment (Figure 3C,D, Table
392 S15). For bacteria, we detected a significant impact of environment on the proportions of ASVs
393 coming into tea bags from source samples (Type II Wald χ^2 test: $\chi^2 = 15.16$, $p = 0.0005$) but not
394 the tea type ($\chi^2 = 0.6173$, $p = 0.4321$). In fungal gardens, we detected significantly lower
395 contributions from source samples to both tea types (Green tea: comb – 0%, termite gut – 0.1%
396 ± 0.29%; Rooibos tea: comb – 1.1% ± 2.67%, termite gut – 0.02% ± 0.07%), with most
397 bacterial ASVs not having a discernible source that could be accounted for (Figure 3C, Table
398 S16). Contributions from source samples were higher in mound wall soil (Green tea: 7.5% ±
399 15%, Rooibos tea: 9.4% ± 22.4%) and outside soil (Green tea: 10.9% ± 18.1%, Rooibos tea:
400 11% ± 8.2%) (Figure 3C). For fungal communities, we detected a larger proportion of ASVs
401 in tea bags originating from source samples (Figure 3D). Similar to bacteria, we detected a
402 significant effect of environment on source contributions to tea bags ($\chi^2 = 16.48$, $p = 0.0003$)
403 and no effect of tea type ($\chi^2 = 2.618$, $p = 0.1057$). In fungal gardens, termite gut and fungal
404 comb samples contributed significantly lower proportions of ASVs to both tea types (Green
405 tea: comb – 0%, termite gut – 28.2% ± 36.6%; Rooibos tea: comb – 1.2% ± 3.1%, termite gut
406 – 17.8% ± 12.9%) than outside soil source communities did to tea bags placed in this
407 environment (Green tea: 58.2% ± 32.9%, Rooibos tea: 64.9% ± 26.4%) (Table S16). Overall,
408 this implies that source contributions of microbes to tea substrates is greater in outside
409 environments than the more controlled garden environment within termite colonies.
410

Aligning with our source tracking analyses, the 25 most abundant bacterial genera colonising tea bags were below detection rates in source microbiomes (Figure 3E). Many low-abundant bacterial genera in sources increased in abundance in tea bags in mound wall soils (Figure 3G) and in outside soil (Figure 3I), suggesting that niche openings (novel tea substrates) select for low-abundant bacteria to colonise. In contrast, many abundant fungal genera in tea bags were already relatively abundant in termite guts, while *Termitomyces* did not colonise tea bags to any appreciable extent – despite vastly dominating fungal combs (Figure 3F). Fungi colonising tea bags in both mound wall and outside soils better reflected source compositions, where e.g., *Aspergillus*, *Penicillium*, and *Talaromyces* dominated (Figures 3H, J).

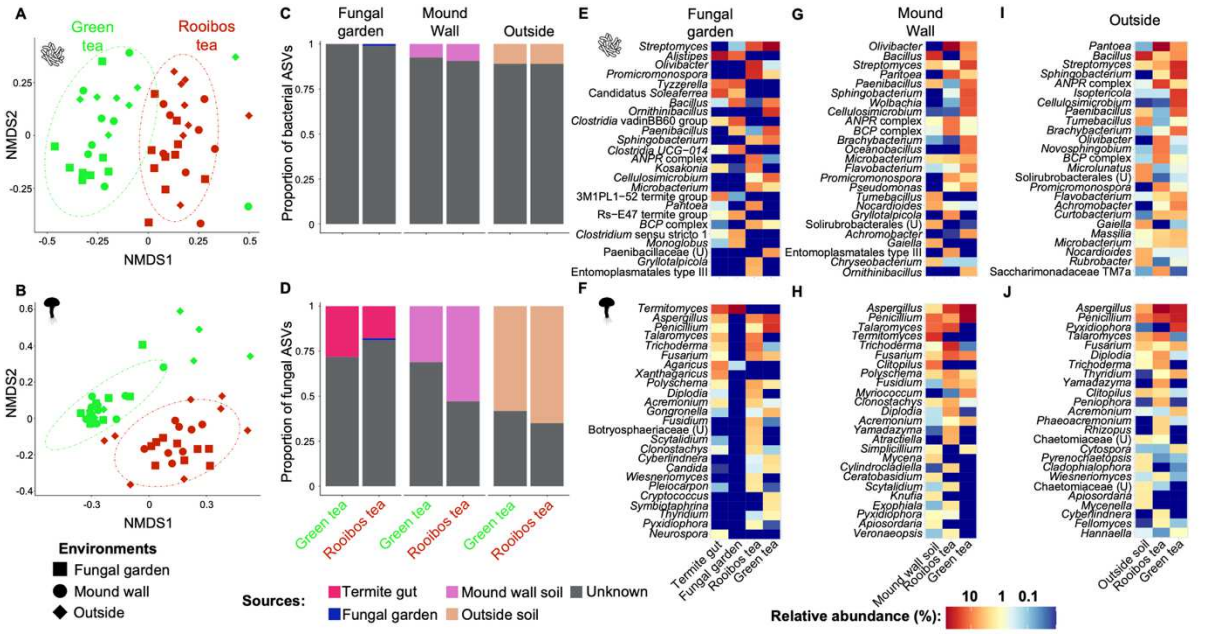


Figure 3. Microbial colonisation of tea bags. NMDS plots showing the bacterial (**A**: stress = 0.2206) and fungal (**B**: stress = 0.1854) community compositions in Green and Rooibos tea bags. Bar plots illustrate the results from source tracking analyses to identify the proportion of bacterial (**C**) and fungal (**D**) ASVs within tea bags that we could trace to their respective source communities. ASVs predicted to originate from unknown sources are labelled as "Unknown" (Table S15). Heatmaps of the 25 most abundant bacterial and fungal genera in source and tea bag samples in fungal gardens (**E** and **F**), mound walls (**G** and **H**), and outside (**I** and **J**). Genera

that could not be classified are indicated with closest taxonomic level (e.g., family) and (U). ANPR refers to the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus complex and BCP to the *Burkholderia-Caballeronia-Paraburkholderia* complex. Bacterial and fungal illustrations from Phylopic.com.

To pinpoint environment-specific microbial taxa that enriched in abundance within tea bags, we conducted differential abundance analyses for each environment. For bacterial communities in the fungal garden environment, 45 genera were significantly differentially abundant in fungal combs and 90 in termite guts. In the tea bag samples, 26 genera were differentially abundant in Rooibos and only 10 in Green tea (Figure 4A, Table S17). In the mound wall environment, 147 genera were differentially abundant in mound wall soil, while 12 and eight were differentially abundant in Rooibos and Green tea, respectively (Figure 4A, Table S17). In the outside environment, soil had 124 differentially-abundant bacterial genera, while Rooibos tea had 11 and Green tea had 12 (Figure 4A, Table S17). Of the differentially abundant bacteria in Green tea, three were shared across all environments (*Sphingobacterium*, *Paenibacillus*, and *Oceanobacillus*), and two were shared by fungal garden and mound wall environment (*Streptomyces* and *Ornithinibacillus*). In Rooibos, we detected three differentially-abundant bacterial genera that were shared between environments (*Pantoea*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus complex, and *Roseomonas*). Four bacterial genera were shared by fungal gardens and mound wall soil environments (*Olivibacter*, *Promicromonospora*, *Novosphingobium*, and *Ancylobacter*).

For fungal communities, only *Termitomyces* was significantly enriched in fungal garden samples (Figure 4B, Table S18). Twenty-six fungal genera were differentially-abundant in termite guts, three were differentially-abundant in Green tea, and 16 were differentially-

453 abundant in Rooibos tea (Figure 4B, Table S18). In the mound wall, 46 fungal genera were
454 differentially abundant in mound wall soil samples, contrasting only three in Green and eight
455 in Rooibos tea (Figure 4B, Table S18). In the outside environment, we detected 75 differentially
456 abundant genera in outside soil samples, four differentially-abundant genera in Rooibos tea,
457 and none in Green tea (Figure 4B, Table S18). Among all fungal genera enriched in Rooibos
458 tea, three were shared between environments (*Diplodia*, *Cyberlindnera,* and *Diutina*) and four
459 by fungal garden and mound wall environments (*Trichoderma*, *Scytalidium*,
460 *Phaeoacremonium*, and *Veronaeopsis*). For Green tea, three differentially-abundant genera
461 (*Penicillium*, *Aspergillus*, *Cryptococcus*) were shared by fungal gardens and mound wall
462 environments. Many differentially abundant fungal genera in tea bags in fungal gardens were
463 common weeds of termite fungus combs (e.g., *Fusarium*, *Aspergillus*, *Penicillium*, and
464 *Trichoderma*).
465

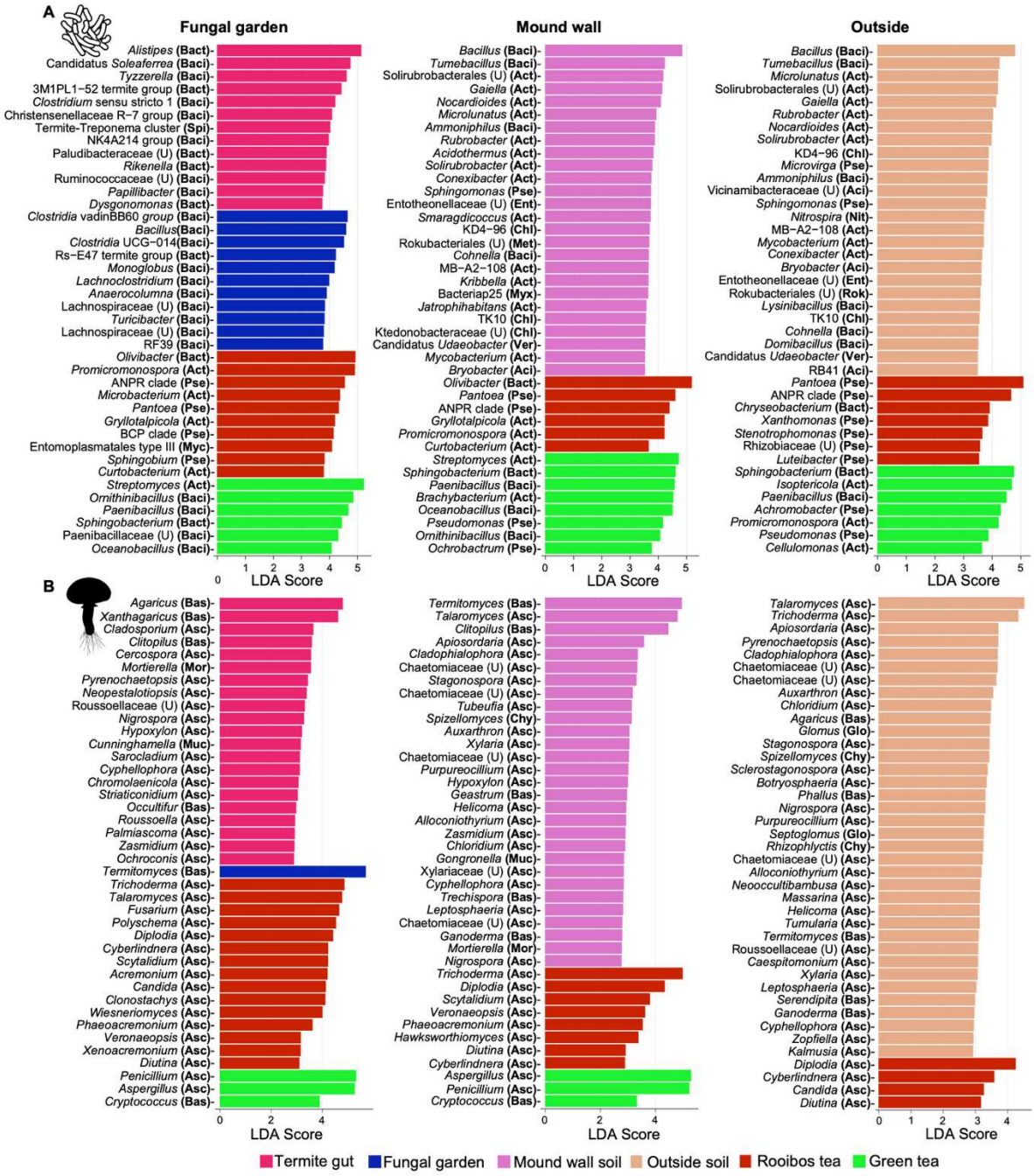


Figure 4. Differentially abundant bacteria (A) and fungi (B) in source and tea bag samples. Bar charts show the 40 most significantly differentially abundant genera at each sampling environment (Fungal garden, Mound wall, and Outside) based on LEfSe analyses (Table S17 and S18). Bars give LDA scores for each of the genus that were significantly more abundant in a given sample type than other sample types in that environment. Bold text in parentheses indicate the phylum of each genus (Bacteria: Bact = Bacteroidota, Baci = Bacillota,

Spi = Spirochaetota, Act = Actinomycetota, Pse = Pseudomonadota, Myc = Mycoplasmatota, Ent = Entotheonellaeota, Chl = Chloroflexota, Met = Methylomirabilota, Myx = Myxococcota, Ver = Verrucomicrobiota, Aci = Acidobacteriota; Fungi: Bas = Basidiomycetes, Asc = Ascomycota, Mor = Mortierellomycota, Chy = Chytridiomycota, Glo = Glomeromycota). Genera that could not be identified to genus level are indicated with closest taxonomic classification (i.e., family) and (U). ANPR = *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium* genera complex; BCP = *Burkholderia−Caballeronia−Paraburkholderia* genus complex. Bacterial and fungal illustrations from Phylopic.com.

Impact of recruited communities on tea decomposition

Decomposition rates were comparable across the three environments, and even though they were slightly higher (yet more variable) in outside soils, rates were not significantly different between environments (Type III ANOVA on GLMM: $F_2 = 2.526$, $p = 0.1139$) or habitats ($F_2 = 1.004$, $p = 0.4149$) (Figure 5A, Table S19). Neither bacterial nor fungal alpha diversity or density in source samples were significantly associated with the decomposition rate (Figure 5B, Table S20). There were also no associations between decomposition rates and source nutrients (Table S21). In Rooibos tea bags, bacterial Shannon diversity and Pielou's evenness tended ($p<0.1$) to be positively associated with decomposition rate (Table S20, Figure 5C), while Chao1 richness and bacterial density did not associate with the decomposition rate. The fungal Shannon diversity and Pielou's evenness in Rooibos were significantly negatively associated with decomposition rate (Table S20, Figure 5D). In Green tea bags, bacterial densities were significantly positively associated with decomposition rate (Table S20), while none of the fungal alpha diversity parameters or density were associated (Table S20).

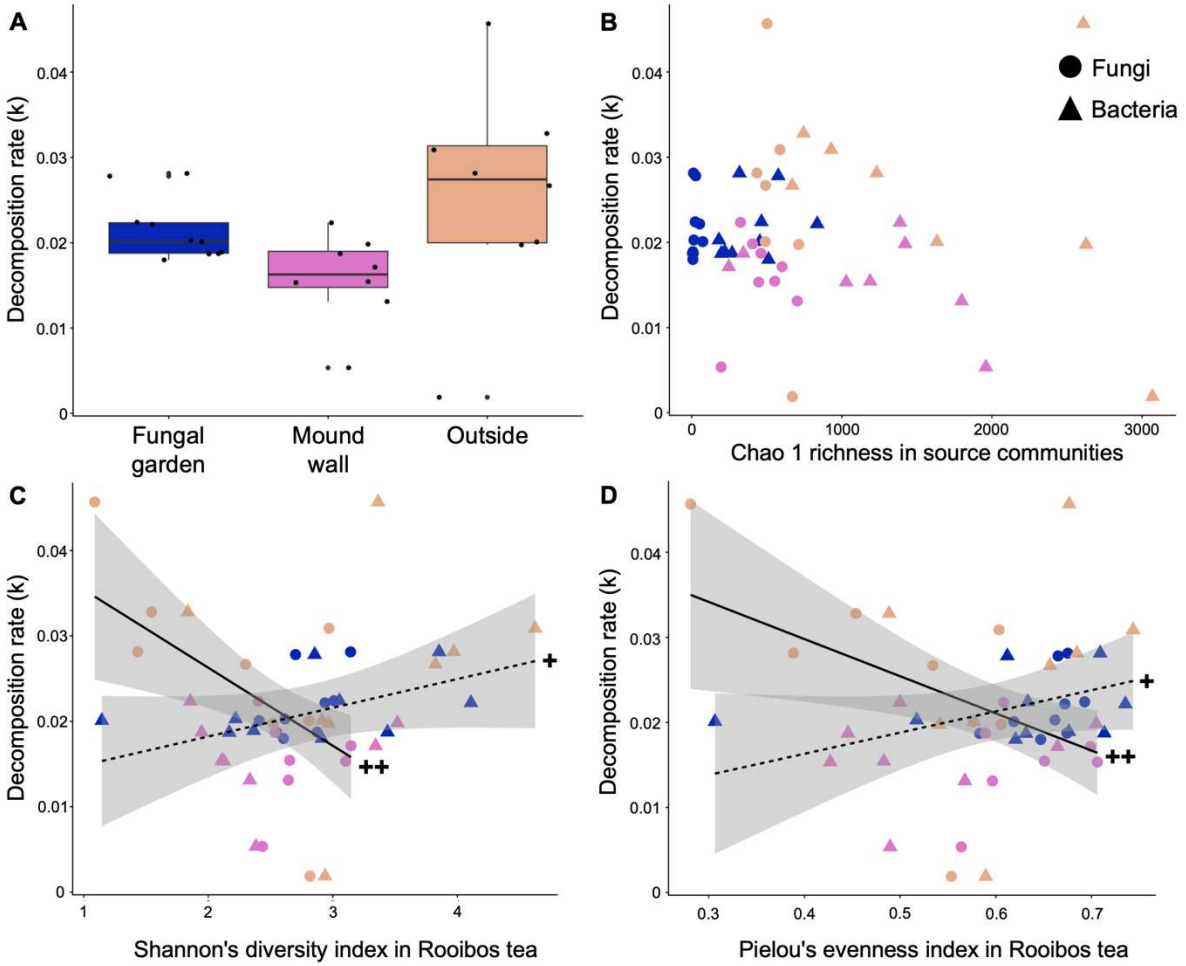


Figure 5. Associations between decomposition rates and microbial community properties. **A**. Box plots showing the decomposition rate (k) across environments. **B**. Scatter plots of decomposition rate as a function of Chao1 richness estimates in source samples. **C-D**. Scatter plots of decomposition rate as a function of Shannon's diversity index (**C**) and Pielou's evenness index (**D**) in Rooibos tea. Data points are coloured by sampling environment (as in panel **A**), grey shaded areas indicate standard error (C & D), with solid regression lines for fungi and dashed lines for bacteria. Marginally non-significant associations ($p<0.1$) are indicated with "+" and significant ($p<0.05$) associations are indicated with "++".

Discussion

We confirmed that *Macrotermes bellicosus* termite colonies create distinct local environments that are markedly different in both nutritional (carbon, nitrogen, and organic matter) and microbial community compositions. This finding is consistent with recent work demonstrating strong elemental differentiation across the termite–fungus farming system, with filtering occurring as resources move between soil, termite guts, and fungus comb (30). Interestingly, neither nitrogen content, SOM or decomposition rates were associated with bacterial or fungal community compositions, diversity, or microbial densities. The only associations were found between overall community properties and carbon content, which was notably driven by the carbon rich fungal combs. Against this backdrop of strongly differentiated source environments, we tested several predictions for the colonisation of "novel" niches in the form of tea types with distinct properties. Fungal colonisation of tea bags in outside soils reflected community compositions of soil sources, with dominant colonisers being opportunistic saprophytic soil genera. Similar taxa colonised mound wall tea bags, but compositions less strongly reflected source communities. Colonisation of tea bags in fungal gardens least reflected termite gut and fungal comb source communities, in part expected as we precluded termite and limited *Termitomyces* access. Colonisation here thus best exemplified competitive release owing to reduced suppression of non-*Termitomyces* fungi, with many fungi present in termite guts managing to colonise tea bags even if vastly outnumbered by *Termitomyces* in fungal combs. Bacterial colonisation generally poorly reflected source communities, suggesting that tea bag properties and colonisation ability shaped the resulting community compositions. This contrasts strong differentiation among source environments and suggests that filtering of microbial communities can occur at the level of the newly encountered substrate, overriding much of the source community structure.

532 Soil properties of the three environments reflected modulation by termites and symbionts.
533 Interestingly, mound soil had consistently lower C and N contents, and markedly higher $\delta^{13}C$
534 than outside soils. The simplest explanation for higher $\delta^{13}C$ values could be the incorporation
535 of relatively more C4-carbon during mound building, which could happen if mounds were built
536 during a time when grasses (with higher $\delta^{13}C$ values than trees using the C3-pathway for C
537 fixation) were more dominant in the environment than at the time of sampling. However, since
538 the pattern was extremely consistent across habitats, this is perhaps unlikely. Alternatively, the
539 difference could arise from substantial ^{13}C fractionation by *Termitomyces* within fungal gardens
540 (62). In colonies of congeneric *M. michaelseni* and *M. subhyalinus* in Kenya, asexual
541 *Termitomyces* feeding structures consumed by termites (nodules) are enriched in ^{13}C compared
542 to both fungal comb and foraged plant matter (62). If $\delta^{13}C$ levels in nodules in our *M. bellicosus*
543 colonies are comparable to those of Kenyan *Macrotermes* species (62), they would be 2-4 ‰
544 higher than combs and quite close to the levels we observe for mound soils. This would,
545 however, mean that most of the carbon within mound walls is routed through *Termitomyces*.
546 Fungus-farming termites primarily use soil mixed with saliva for mound building (63). While
547 the saliva component could incorporate some fresh, relatively ^{13}C enriched *Termitomyces*-
548 routed carbon to mound walls, this seems unlikely to alone explain the observed high $\delta^{13}C$
549 values of mound walls. Instead, these differences are more likely to reflect a vertical $\delta^{13}C$
550 gradient in soil carbon pools and preferential use of subsurface soils with typically higher $\delta^{13}C$
551 due to younger carbon deposited on the surface and microbial degradation removing ^{12}C (64,
552 65).
553
554 For nitrogen isotopes, baseline levels across the forest patch, woody savanna, and teak
555 plantation habitats were quite different, likely largely reflecting local $\delta^{15}N$-differences in
556 vegetation that drive local soil nitrogen pools (66). It is not uncommon that soil $\delta^{15}N$ levels

vary in African savannas, even between nearby locations (67). This can be due to vegetation differences driving variable contributions from ectomycorrhizal nitrogen uptake (68, 69) and root-associated nitrogen fixation. It can also stem from variation in the efficiency of soil processes and related N losses, which in turn, depend on e.g., N availability and soil moisture. The between-habitat and between-colony variation observed are consistent with this and thus likely due to local differences in vegetation and soil $\delta^{15}N$ baselines. Potential differences in atmospheric nitrogen fixation within combs and termite guts (70, 71) could, however, also affect $\delta^{15}N$ levels in colonies. Comb $\delta^{15}N$ – particularly for woody savanna colonies – was consistently much lower than soils, whereas the $\delta^{15}N$ levels were higher in mound than outside soils. As for $\delta^{13}C$ content, mound soil ^{15}N enrichment could reflect variance in source soil N pools (i.e., higher $\delta^{15}N$ in deeper soil layers) and ammonification, nitrification, and denitrification processes that also affect soil $\delta^{15}N$ values. Possible termite diet-derived N inputs from saliva could also play a role here. The relatively low $\delta^{15}N$ values in fungus combs could result from several factors. Selective foraging by the termites may lead to relatively ^{15}N-depleted N inputs to combs compared to average inputs to surrounding soils. These effects cannot be assessed without knowing precisely the plant input that termites provide the fungus, or the standing $\delta^{15}N$ variation in the local vegetation. Termite gut/comb processes may further transform N within colonies, through processes that increase (e.g., nitrification and denitrification) or decrease (N_2 fixation) $\delta^{15}N$. Lastly, fungal decomposition of plant biomass could affect $\delta^{15}N$ via trophic ^{15}N enrichment. In fungus-farming termites, this process is, however, difficult to separate from N cycling processes and particularly mixing effects with atmospheric nitrogen[69]. Thus, our inferences remain simplified models that will need testing, ideally by combining evaluations of soil substrates and plant inputs with termite and fungal processing, in different habitats.

Source communities were largely overlapping in compositions in mound and outside soils, and they were distinct from combs and guts, as expected based on past works (5, 21, 22, 25). Mound wall and outside soils contributed larger proportions of fungal communities to tea bags than combs, where only a fraction of source communities were detected. Consequently, the identities of fungal colonisers were consistent across the environments, and dominated by sporulating, fast-growing, opportunistic saprophytic moulds and yeasts. This suggests overall canalisation of fungal communities in new substrates, subsequently shaped by tea type, as Green tea communities were dominated by *Aspergillus, Penicillium,* and *Cryptococcus*, while Rooibos tea was dominated by *Trichoderma, Fusarium,* and *Talaromyces*. Similar fungal genera colonized tea bags within the fungal gardens, indicating that our exclusion of termite activities strongly affected colonisation patterns. The colonisers included a number of fungal genera that were not detected in comb sources but known to be suppressed by the termites, such as *Trichoderma*, *Aspergillus*, and *Fusarium* (25, 33). Their presence in the garden environment underlines past findings that a diversity of fungi survive termite gut passage of the plant substrates used to manure the fungal cultivar *Termitomyces* (34), and that termite hygiene behaviours are central to prevent them from rapidly proliferating (33, 35). We did not detect the fungal genus *Xylaria* that is frequently present, yet dormant, in fungal combs until conditions allow it to take over (32, 33, 35, 72, 73). Its absence in tea bags is perhaps unsurprising as the phylogenetic clade of *Xylaria* associated with combs is specialised (73) and with a reduced carbohydrate-active enzymes repertoire compared to free-living wood degrading *Xylaria* (74).

The colonisation patterns of novel plant biomass substrates by bacteria differed notably from fungi. The overall contributions of source communities to tea bags were low in bacterial communities, with gut and comb source environments contributing the least. However, similar

607 to fungi, tea substrates strongly canalised bacterial colonisations, even if enriched bacterial
608 genera in tea bags differed by environment to some extent. Several highly-enriched bacterial
609 genera in Green (*Paenibacillus, Oceanobacillus,* and *Ornithinibacillus*) and Rooibos
610 (*Pantoea*) tea are motile, which likely promotes their movement into new niches. Tea
611 enrichment also included a diversity of metabolic capabilities among enriched genera,
612 including for breakdown of hemicellulose and cellulose (*Olivibacter* and *Promicromonospora*)
613 (75, 76), lysis of fungal cell wall components (*Cellulosimicrobium*) (77), and genera that are
614 more substrate versatile (*Streptomyces* and *Paenibacillus*) (78, 79). Within the colony
615 environment, only very few gut and comb-residing bacteria colonise tea bags – notably
616 *Bacillus*, *Paenibacillus*, and *Pantoea*. The overall lower prevalence of bacteria that are
617 abundant in source communities, their more versatile carbohydrate metabolism, and a canalised
618 response for fungi, could indicate that bacterial colonisation is secondary to fungi, with fast-
619 colonising fungi creating niches for a fraction of the available (mostly motile) bacteria.
620
621 Given that tea bags ultimately hosted quite similar communities, particularly for fungi, it was
622 unsurprising that the decomposition rates were not significantly different across environments,
623 even if more variable in outside soils. Rates were comparable to estimates from past studies
624 across a range of soil systems, including in Africa (80-82), and we had expected microbial
625 density to exert direct effects on decomposition rates, but this was also not the case. It was also
626 somewhat puzzling that neither bacterial nor fungal alpha diversity in source samples
627 significantly affected decomposition. The only diversity effects we observed were in Rooibos
628 tea, where increasing bacterial diversity and evenness led to increasing decomposition rate and
629 the effect for fungal community properties were opposite. Given the relatively few samples and
630 the variation in estimates, we exert caution in the interpretation of these findings. Future work
631 to better associate recruited taxa, their diversity and densities to decomposition in these

environments are needed to understand how colonisation, interactions, and microbial properties affect decomposition rates. To this end, alternatives to the tea bag index should be explored, such as mesh bags with standardised substrates that allow comparisons of termite presence vs exclusion (83) these environments.

Conclusions

Our findings point to soil properties of three focal environments being affected by termite and symbioses activities. Excluding termites, and to a large extent also *Termitomyces*, access to tea bags resulted in canalised colonisation patterns across environments; strongest for fungi and less so for bacterial colonisers. Fungal colonisation was dominated by saprophytic sporulating moulds, with slight variation between tea types with simpler (Green) vs. more recalcitrant (Rooibos) plant compositions. Within fungal combs, colonisation thus best exemplified competitive release owing to reduced suppression of fungal proliferation. Bacterial colonisation reflected source communities less than fungi, were more variable in compositions, and taxa colonising were likely to be motile. The similarity in community compositions across environments ultimately led to similar decomposition rates, likely driven by these canalisation processes. Even so, termite activities markedly shape environmental properties, exemplifying the importance of mound building in generating heterogeneous microbial niches in savannah ecosystems.

Declarations

Ethics approval and consent to participate

Materials were exported under the permit 009/UNA/CRE/SREC, issued by Le Ministere de l'Enseignement Superieur de la Recherche Scientifique, Republique de Côte d'Ivoire.

Availability of data and material

The dataset for nutritional analyses is given in Table S4, bacterial and fungal densities estimates based on qPCR are given in Table S6, and tea bag weights and k calculations are given in Table S19. Bacterial and fungal amplicon sequences have been deposited to the SRA library in GenBank and are available under the accessions PRJNA1531751 (fungi) and PRJNA1531758 (bacteria). All analyses code will be uploaded to Zenodo upon acceptance of the manuscript. To make scripts available to reviewers, they have been uploaded as an additional file.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KHB: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. LAC: Investigation, Methodology, Formal analysis, Data Curation, Visualization, Writing - Original Draft, Writing - Review & Editing. SS: Investigation, Methodology, Data Curation. ELC: Investigation, Methodology, Data Curation. KSS: Investigation, Methodology. NAK: Investigation, Data Curation, Resources. RV: Formal

analysis, Validation, Writing - Review & Editing. AM: Investigation, Methodology, Formal analysis, Writing - Review & Editing. MP: Conceptualization, Methodology, Validation, Resources, Writing - Original Draft, Writing - Review & Editing, Project administration, Funding acquisition. All authors read and approved the final manuscript.

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- TableS2Bacteria.xlsx
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