



Household and Structural Insects

Bacterial communities of fresh and aged fecal pellets in western drywood termite (Blattodea: Kalotermitidae) and their potential use as biomarkers of recent or active infestations

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In addition to serving as a telltale sign of infestation, drywood termite fecal pellets can reveal information about the colony that produced them. In this study, the bacterial communities of fresh and aged fecal pellets of *Incisitermes minor* (Hagen) were investigated to test the hypothesis that patterns of bacterial succession can be used to distinguish fresh from aged pellets and therefore indicate an active infestation. Fecal pellets were collected from drywood termites that fed on either the wood they were collected from or Douglas-fir (D-fir) commercial lumber. Freshly produced, 3-mo, 6-mo, and 12-mo-old pellets underwent 16S rRNA gene sequencing to identify the bacteria present in the samples. Natural-wood pellets contained on average over five times the amount of bacterial DNA compared to D-fir pellets. Up to a 190-fold decrease in estimated bacterial DNA quantity was detected between fresh to 12-mo-old pellets. Comparisons of bacterial community compositions between the samples of different ages revealed diversity indices that were significantly different between fresh and aged pellets from D-fir. Furthermore, the current study identified five unique families of bacteria that were consistently present in all fresh fecal pellet samples from D-fir but completely absent in the fecal pellet samples that were aged for certain amounts of time. In addition to serving as a basis for the characterization of the microbiome of *I. minor* fecal pellets, the current findings suggest multiple candidate biomarkers which may be further investigated to develop a cost-effective method to distinguish freshly produced from aged fecal pellets.

Keywords: *Incisitermes minor*, drywood termite, microbiome

Introduction

The western drywood termite, *Incisitermes minor* (Hagen) (Blattodea: Kalotermitidae), causes significant economic damage in its native range of the southwestern United States and northwestern Mexico (Harvey 1934, Weesner 1965, Cabrera and Scheffrahn 2001). Beyond its native range, *I. minor* has spread to other parts of the United States, such as Hawaii and Florida, and it is also reported in Canada, China, Japan, and Australia (Cabrera and Scheffrahn 2001, Evans et al. 2013, Horwood and Lo 2022). Increased urbanization and globalization involving the movement of wood and wood-containing products worldwide, along with the cryptic lifestyle of *I. minor*, contribute to its status as an important structural pest in several parts of the world (Lewis et al. 2014). Western drywood termites typically nest and forage inside one piece of wood

(or pieces in continuous contact), with only winged reproductive (alates) leaving the nest for dispersal (Harvey 1934), making their detection and management challenging.

The cryptic nature of drywood termite colonies makes the detection of infestations difficult (Himmi 2017) and time consuming, with false positives and false negatives occurring with some regularity (Lewis et al. 2014). To inspect for drywood termite infestations, professionals perform a visual search of the structure for fecal pellets, live termites, and signs of drywood termite feeding damage, often accompanied by physical probing of the suspected wood members (Lewis et al. 2014). Many technologies and tools have been developed for the detection of drywood termite infestations, including: canine searches, fiber optic devices, acoustic detectors, infrared cameras, microwave motion detectors, and X-ray machines (Lewis et al. 2014,

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Hassan and Nanda 2024). These detection techniques demonstrate varying degrees of accuracy, and each of them comes with its own set of limitations. Due to these limitations, none of the detection techniques or technologies listed above are standard equipment of the pest management industry and visual inspection by an experienced inspector remains the most widely used and effective method for drywood termite infestation detection (Lewis et al. 2014, Hassan and Nanda 2024). Therefore, more research is required to improve existing techniques or develop a cost-effective method that is reliable and accurate enough to become an industry standard for drywood termite detection.

Drywood termites feed inside infested wood without breaking the wood surface, thus, aside from discarded alate wings or the alates themselves, fecal pellets (frass) are the primary sign of drywood termite infestations. Drywood termites expel fecal pellets from their gallery system through “kick-out” holes resulting in fecal pellets accumulating in piles underneath the “kick-out” holes (Lewis et al. 2014). Drywood termite fecal pellets are devoid of nearly all moisture, elongate with rounded ends and have flattened or depressed surfaces separated by six longitudinal ridges (Haverty et al. 2005). These features make fecal pellets diagnostic for drywood termites and can help to distinguish wood damage caused by other wood-destroying insects (Ebeling 1975, Moore 1992).

In addition to serving as a telltale sign of drywood termite infestations, drywood termite fecal pellets have been investigated as a potential source of information for the termites which produced them or the wood they have ingested. Haverty et al. (2005) demonstrated that unique, species-specific hydrocarbon compositions in fecal pellets can be used to identify the drywood termite species that produced them (Haverty et al. 2005). Similarly, species-specific primers can be used to sequence fecal pellet samples resulting in PCR products only being produced from the targeted termite species' pellets (Ide et al. 2016). Additionally, if there are limited number of drywood termite species in an area, subtle but consistent differences in fecal pellet size can be used to differentiate termite species (Rojo 2018). Size of the fecal pellets within a species can be useful to determine the cellulose content of the food source, with smaller fecal pellets being indicative of consumption of a high-cellulose food source, as more of the ingested material would be utilized without being excreted (Grace 2009, Grace and Yamamoto 2009).

Due to the importance of fecal pellets as evidence of drywood termite activity, estimating the age of fecal pellets is of particular interest to determine if the pellets were recently produced by termites. Two studies demonstrated it is possible to distinguish freshly produced from aged fecal pellets based on their chemical profiles. Lewis et al. (2010) showed that the relative quantities of individual hydrocarbons in *I. minor* fecal pellets change over time. Similarly, Haigh et al. (2024) demonstrated that the relative abundance of several chemical compounds, including hydrocarbons, changed over time in *Cryptotermes brevis* (Walker) fecal pellets. Both methods require the use of gas chromatography—mass spectrometry (GC-MS) for the chemical analyses. Therefore, this approach can be time consuming, and it would require training to operate and interpret results from the GC-MS. A previously unexplored aspect in determining the relative age of drywood termite fecal pellets is the microbial profiles of the fecal pellets at different time points since their production.

The symbiotic relationships of gut microbiota and termite are essential for the digestion of lignocellulose (Brune and Dietrich 2015). All three domains of life are represented in the termite gut: prokaryotes (Archaea and Bacteria), eukaryotic protists (lost in Termitidae), and fungi (Dar et al. 2022). The hindgut is the largest structure in the termite digestive system and harbors a diverse microbial community, some of which, particularly the bacteria, are found nowhere else on earth (Ohkuma and Brune 2010). In the hindgut of Kalotermitidae and Heterotermitidae, flagellates play a primary role in the digestion of cellulose while prokaryotes perform essential functions such as acetogenesis, fermentation, methanogenesis, and nitrogen fixation (Scharf and Tartar 2008). The gut microbial community structure of termites is largely maintained across generations through the exchange of hindgut fluids via proctodeal trophallaxis (Brune and Dietrich 2015). Given that bacteria-containing hindgut fluids and fecal pellets are direct products from the termite hindgut, it is likely that some bacteria from the drywood termite hindgut may be present in fecal pellets (Weiss 2006). Also, if their microbial profiles change over time (eg differences in bacterial composition or the presence/absence of some taxa at certain ages), this information may allow us to categorize fecal pellets according to their relative age.

The current study explored if there are any changes in the bacterial compositions of *I. minor* pellets over time. First, the stock of fecal pellets was obtained by keeping termites on either the natural wood they were collected from or on commercial Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco). Since Douglas-fir is a common building material in *I. minor*'s range in the United States, it was considered as a reasonable choice for standardization of the experiment.

Using 16S rRNA gene sequencing, the bacteria present in freshly produced, 3-, 6-, and 12-mo-old fecal pellets were identified and their relative abundances were quantified. The bacterial communities from these fecal pellet samples were analyzed to determine if there is any specific change in their microbial signatures over time. If so, this information can be potentially useful for distinguishing freshly produced fecal pellets from aged ones.

Materials and Methods

Termites

Western drywood termites, *I. minor*, were extracted from three separate pieces of infested wood collected in Riverside, California, representing three distinct colonies. Infested wood pieces were logs from trunks and branches of natural trees. Thus, it is unlikely that these logs were recently treated with any chemical preservatives or insecticides given their natural and aged state. The wood pieces termites were collected from were not identified. Each group of termites (≈ 500 individuals) was designated as a separate population. Each population was split in half with one half kept on four pieces ($9 \times 4 \times 0.75$ cm) of Douglas-fir lumber (The Home Depot, Atlanta, Georgia, United States), and the other half on similar sized wood pieces from the wood they were collected from. Termites and wood were kept in 473-mL plastic cups (PK16S-C; Frabri-Kal, Kalamazoo, Michigan, United States) with lids as containers with 2 small holes punctured in lids for ventilation. These units were kept in a growth chamber (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 26 °C and 20% to 26% RH in complete darkness for 3 d for complete passage of food material through the digestive tract. It typically takes 24 h

for a food bolus to pass through the termite intestinal tract (König 2006). After 3 d, each termite group was moved to a fresh container with new wood pieces (same type), indicating the start of the fecal pellet production period for this study.

Fecal Pellet Collection

Fecal pellets were collected for analysis 1 wk after the start of the fecal pellet production period. For a fecal pellet sample, 10 fecal pellets were randomly collected from a container. Three fecal pellet samples were collected from each container, yielding 18 samples in total (3 populations \times 2 wood types \times 3 samples). The collected fecal pellet samples were placed in sterile 1.5 ml microcentrifuge tubes (Fisher Scientific, Hampton, New Hampshire, United States), kept on dry ice, and sent for sequencing (see Bacterial 16S Sequencing below). These initial samples served as the “fresh” fecal pellets. The termites were allowed to feed on the wood in the containers for an additional 7 d for further fecal pellet production. After this period, termites were removed from the containers to stop the addition of new fecal pellets. This 14-d period established the fecal pellet stocks that would be used for the duration of this study. These fecal pellet stocks were kept in the same growth chamber under the same conditions as in the previous step. Each stock was subsampled three times and sent for bacterial 16S sequencing immediately after their collection 3, 6, and 12 mo after the fresh fecal pellets were sent in for sequencing.

Bacterial 16S Sequencing

DNA was extracted from raw fecal pellets using the ZymoBIOMICS-96 MagBead DNA Kit (Zymo Research, Irvine, California, United States) and eluted in 50 μ l of DNase/RNase-Free Water. The *Quick*-16S NGS Library Prep Kit (Zymo Research, Irvine, California, United States) was used for bacterial 16S ribosomal RNA gene targeted sequencing. Positive controls via The ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, California, United States) and negative controls (ie blank extraction and library preparation) were used. The V3-V4 region of the 16S rRNA gene was targeted for amplification using the following primers:

V3V4_341f_p1_n6: NCCTACGGGDGGCWGCAG, V3V4_341f_p2_n4: NCCTAYGGGGCGCWGCAG, V3V4_341f_p3_n1: NCCTACGGGGTGCAGCAG, V3V4_341f_p4_n1: GCC TACGGGAGGCTGCAG, V3V4_806r_p1_n24: NGACTACN VGGGTMTCTAATCC, V3V4_806r_p2_n4: NGACTACNAG GGTATCTAATCC, V3V4_806r_p3_n3: NGACTACDCAGGT CTCTAATCT, V3V4_806r_p4_n2: NGAMTACCGGGGTTT CTAATCC, and V3V4_806r_p5_n1: NGACTACCAGGG-TATCTAAGCC. PCR products were quantified using qPCR fluorescence readings and then pooled together based on molarity. The final PCR products were cleaned with Select-a-Size DNA Clean & Concentrator (Zymo Research, Irvine, California, United States), then quantified with TapeStation (Agilent Technologies, Santa Clara, California, United States) and Qubit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Samples were sequenced on Illumina MiSeq with a v3 reagent kit (600 cycles) with a 10% PhiX spike-in.

Sequence Filtering

Raw sequence data were imported into Qiime2 to derive unique amplicon sequence variants (ASVs) (Callahan et al. 2016). Raw reads were demultiplexed, denoised, dereplicated, filtered for chimeras, and singletons sequences were removed. Forward reads shorter than 321 bp and reverse reads shorter than 269 bp were removed. For taxonomy assignment, the Naïve Bayes classifier was trained using Silva 138 99% OTUs full-length sequences and 515F/806R region of sequences (Quast et al. 2013, Bokulich et al. 2018, 2023). Library sequencing depth normalization was performed by rarefaction (Sanders 1968). The examination of alpha rarefaction curves (Fig. 1) determined a 30,000-sequencing depth threshold for sufficient sequencing coverage. Three samples which did not meet the coverage criteria were removed from analyses. They were 3-mo, 6-mo, and 12-mo-old fecal pellet samples (one for each time point) from the same population on Douglas-fir.

Quantification of Total Bacterial DNA via qPCR

The bacterial DNA concentration was determined by quantitative real-time PCR. The same primers used for the bacterial

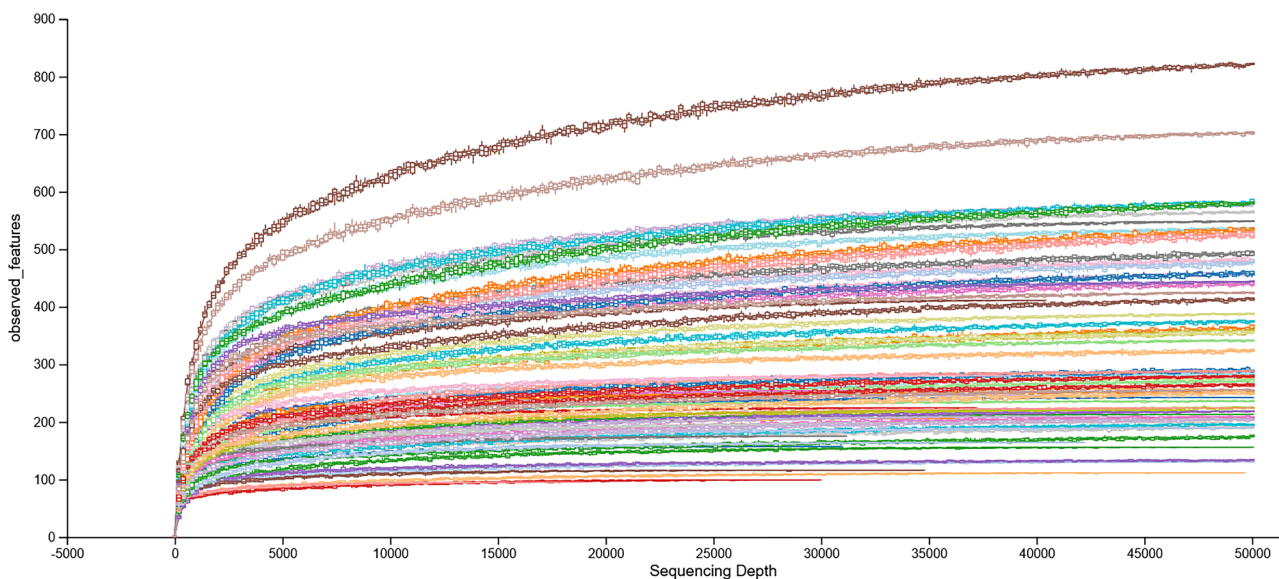


Fig. 1. Alpha rarefaction curves at 50,000 sequencing depth from *I. minor* fecal pellets samples of all types and ages.

16S sequencing (see above) were used. A standard curve was made with plasmid DNA containing one copy of the 16S gene prepared in 10-fold serial dilutions. The standard curve generated the equation used to calculate the number of gene copies for each reaction in each sample. The amount of DNA present in 1 μ l of DNA sample was calculated by the number of genome copies, assumed to be four 16S copies per genome, using the assumed genome size of *Escherichia coli*, 4.64×10^6 bp (Zymo Research, Irvine, California, United States).

Statistical Analyses

For the quantification of total bacterial DNA via qPCR reaction calculations, the DNA concentration found in the fecal pellets produced by the termites on natural wood (referred to as “natural-wood pellets” hereafter) of each age were compared to each other, separate from the fecal pellets produced by the termites on Douglas-fir (referred to as “D-fir pellets” hereafter). The DNA concentrations at different ages within each pellet type were compared with a blocked Kruskal-Wallis test, blocking by population to account for repeated measures within colonies. Posthoc pairwise comparisons were performed using permutation-based Wilcoxon rank-sum tests with population as a blocking factor. Benjamini-Hochberg false discovery rate was used to adjust *P*-values for multiple comparisons. At each time point, permutation-based Wilcoxon rank-sum tests with population as a blocking factor were used to compare DNA concentrations found in D-fir and natural-wood pellets. *P*-values were adjusted for multiple comparisons across time points using the Benjamini-Hochberg false discovery rate method. Filtered sequence data were imported into RStudio for analysis (v4.4.1, R Core Team 2024). The alpha-diversity metrics of the bacterial communities were calculated using Shannon index and analyzed with a blocked Kruskal-Wallis test using population as a blocking factor followed by blocked Wilcoxon rank-sum tests for pairwise comparisons between ages. The beta-diversity among groups was calculated using Bray-Curtis dissimilarity index (Bray and Curtis 1957) and statistically compared using PERMANOVA with 999 permutations. Differential abundance analyses were performed using ALDEx2 (Fernandes et al. 2014) to compare fresh samples to all other ages. From the ALDEx2 results, ASVs with the highest differential abundance between fresh and aged fecal pellets were used to calculate the log₂ fold change for each comparison $[(\log_2(x+1))/(\log_2(x+1))]$. Line plots were created using Sigma Plot ver.14.5 (Systat Software, San Jose, California, United States).

Results

Quantification of Total Bacterial DNA via qPCR

For natural-wood pellets, the estimated bacterial DNA concentrations based on 16S rRNA gene qPCR were 2.6 ± 0.5 , 2.8 ± 0.9 , $9.4 \times 10^{-3} \pm 4.5 \times 10^{-3}$, and $1.4 \times 10^{-2} \pm 3.7 \times 10^{-3}$ ng/ μ l (mean \pm SEM) for fresh, 3-mo, 6-mo, and 12-mo-old pellets, respectively. These DNA concentrations were significantly different among ages (blocked Kruskal-Wallis test: $H=28.60$; $P<0.0001$). Wilcoxon rank-sum tests showed no significant difference when comparing the DNA concentrations between fresh and 3-mo-old natural-wood pellets ($P=0.85$). All other time-point pairwise comparisons were significantly different from each other (Table 1).

Table 1. Bacterial DNA concentration comparisons among time points for natural-wood fecal pellets

Bacterial DNA Concentrations in Natural-Wood Pellets		
Timepoint Comparison	Statistic (Z)	P-value
Fresh vs 3 mo	0.25	0.84
Fresh vs 6 mo	3.66	0.0006
Fresh vs 12 mo	3.69	0.0006
3 mo vs 6 mo	3.63	0.0006
3 mo vs 12 mo	3.60	0.0006
6 mo vs 12 mo	2.67	0.006

Pairwise comparisons were performed using permutation-based Wilcoxon rank-sum tests with population as a blocking factor. Z statistics and FDR-adjusted *P*-values are reported.

Table 2. Bacterial DNA concentration comparisons among time points for Douglas-fir fecal pellets

Bacterial DNA Concentrations in D-fir Pellets		
Comparison	Statistic (Z)	P-value
Fresh vs 3 mo	2.45	0.011
Fresh vs 6 mo	3.40	0.0006
Fresh vs 12 mo	3.45	0.0006
3 mo vs 6 mo	3.27	0.0013
3 mo vs 12 mo	3.35	0.001
6 mo vs 12 mo	-3.19	0.001

Pairwise comparisons were performed using permutation-based Wilcoxon rank-sum tests with population as a blocking factor. Z statistics and FDR-adjusted *P*-values are reported.

For D-fir pellets, the estimated bacterial DNA concentrations based on 16S rRNA gene qPCR were 0.6 ± 0.2 , 0.2 ± 0.1 , $3.8 \times 10^{-4} \pm 9.8 \times 10^{-5}$, and $3.0 \times 10^{-3} \pm 8.5 \times 10^{-4}$ ng/ μ l for fresh, 3-mo, 6-mo, and 12-mo-old pellets, respectively (Table 2). These DNA concentrations were significantly different among the ages (blocked Kruskal-Wallis test: $H=27.46$; $P<0.0001$). Wilcoxon rank-sum tests showed DNA concentrations at each time point were significantly different from each other for all pairwise comparisons (Table 2).

The DNA concentrations found in natural-wood and D-fir pellets were significantly different from each other at all ages: fresh (Wilcoxon rank-sum tests: $Z=3.5$, $P=0.0004$), 3 mo (Wilcoxon rank-sum tests: $Z=2.7$, $P=0.004$), 6 mo (Wilcoxon rank-sum tests: $Z=3.02$, $P=0.002$), and 12 mo (Wilcoxon rank-sum tests: $Z=3.1$, $P=0.0006$).

Bacterial Diversity and Communities

The Shannon indices of fecal pellets changed significantly over time for both natural-wood pellets (Kruskal-Wallis test: $Z=12.01$; $P=0.004$) and D-fir pellets (Kruskal-Wallis test: $Z=16.40$; $P<0.001$). For natural-wood pellets, Wilcoxon rank-sum tests showed significant differences in Shannon indices for 12 mo and 3 mo ($P=0.008$) and 12 mo and 6 mo ($P=0.008$) comparisons (Table 3). For D-fir pellets, Wilcoxon rank-sum tests showed that the Shannon index of fresh pellets was significantly higher than those of 3-mo ($P=0.01$), 6-mo ($P=0.009$) and 12-mo-old pellets ($P=0.001$) (Table 4).

For natural-wood pellets, PERMANOVA analysis using Bray-Curtis dissimilarity index showed significant differences in bacterial community structure between different ages

Table 3. Differences in Shannon diversity indices among ages in natural-wood fecal pellets

Shannon Diversity Indices Comparisons in Natural-Wood Fecal Pellets		
Comparison	Statistic (Z)	P-value
Fresh vs 3 mo	1.87	0.10
Fresh vs 6 mo	2.15	0.07
Fresh vs 12 mo	0.06	1
3 mo vs 6 mo	-1.40	0.23
3 mo vs 12 mo	-2.96	0.008
6 mo vs 12 mo	-2.69	0.008

Pairwise comparisons were performed using permutation-based Wilcoxon rank-sum tests with population as a blocking factor. Z statistics and FDR-adjusted P-values are reported.

Table 4. Differences in Shannon diversity indices among ages in Douglas-fir fecal pellets

Shannon Diversity Indices Comparisons in D-fir Fecal Pellets		
Comparison	Statistic (Z)	P-value
Fresh vs 3 mo	2.58	0.01
Fresh vs 6 mo	2.80	0.009
Fresh vs 12 mo	3.16	0.001
3 mo vs 6 mo	0.72	0.53
3 mo vs 12 mo	1.55	0.17
6 mo vs 12 mo	1.88	0.1

Pairwise comparisons were performed using permutation-based Wilcoxon rank-sum tests with population as a blocking factor. Z statistics and FDR-adjusted P-values are reported.

(pseudo-F=4.55, $P=0.001$, $R^2 = 0.30$) (Fig. 2). The NMDS ordination of the natural-wood pellet community yielded a stress value of 0.1. Natural-wood pellets from all pairwise comparisons except for between 6 mo and 12 mo were significantly different from each other (Supplementary Table S1). For D-fir pellets, PERMANOVA analysis also showed significant differences in bacterial community structure between ages (pseudo-F=2.25, $P=0.001$, $R^2 = 0.19$) (Fig. 3). The NMDS ordination of the D-fir pellet community yielded a stress value of 0.09. D-fir pellets of all age groups were significantly different from each other (Supplementary Table S2).

For natural-wood pellets, the differential abundance analysis showed that the relative abundances of five unique phyla and 15 unique families were significantly different when comparing fresh to aged fecal pellets (Table 5). For fresh to 3 mo, significant differences were found in the relative abundances of ASVs belonging to the phyla Bacteroidota and Proteobacteria. Two of the ASVs from fresh to 3 mo, belonging to the families Rhizobiaceae and Burkholderiaceae, were found in none of the fresh pellet samples but all of the 3-mo-old pellets (Table 5). For fresh to 6 mo, significant differences were found in the relative abundances of ASVs belonging to the phyla Actinomycetota, Bacteroidota, Firmicutes, and Proteobacteria. Four ASVs from fresh to 6 mo, belonging to the families Bacteroidaceae, Dysgonomonadaceae, and Rhodocyclaceae (two different ASVs), were found in all fresh pellet samples but none of the 6-mo-old pellets (Table 5). Additionally, five ASVs from fresh to 6 mo, belonging to the families Burkholderiaceae, Cellulomonadaceae, Leuconostocaceae, Rhizobiaceae, and Sphingomonadaceae, were found in none of the fresh pellet samples

and all of the 6-mo-old pellets (Table 5). For fresh to 12 mo, significant reductions were found in the relative abundances of ASVs belonging to the phyla Bacteroidota, Cyanobacteria, Firmicutes, and Proteobacteria. Three ASVs from fresh to 12 mo, belonging to the families Rikenellaceae (two different ASVs) and Dysgonomonadaceae, were found in all fresh pellet samples but none of the 12-mo-old pellets.

For D-fir fecal pellets, the differential abundance analysis showed that the relative abundances of five unique phyla and six unique families were significantly reduced when comparing fresh to aged fecal pellets (Table 5). For fresh to 3 mo, a significant reduction was found in the relative abundance of an ASV belonging to the phylum Actinomycetota (Table 5). For fresh to 6 mo, significant reductions were found in the relative abundances of ASVs belonging to the phyla Actinomycetota, Bacteroidota, Firmicutes, Proteobacteria, and Spirochaetota. Three ASVs from fresh to 6 mo, belonging to the families Bacteroidaceae, Rhodocyclaceae, and Spirochaetota, were found in all fresh pellet samples but none of the 6-mo-old pellets (Table 5). For fresh to 12 mo, significant reductions were found in the relative abundances of ASVs belonging to the phyla Actinomycetota, Bacteroidota, Firmicutes, Proteobacteria, and Spirochaetota. Four ASVs from fresh to 12 mo, belonging to the families Anaerovoracaceae, Dysgonomonadaceae, Rhodocyclaceae, and Spirochaetaceae, were found in all fresh pellet samples but none of the 12-mo-old pellets (Table 5).

Discussion

The current study investigated the bacterial communities of *I. minor* fecal pellets. In general, the change in community structure between fresh and aged fecal pellets was driven by a decrease in some anaerobic bacteria, primarily belonging to Firmicutes and Bacteroidota, along with an increase in bacteria belonging to Proteobacteria and Actinomycetota (Figs 4 and 5). To our knowledge there is no earlier report on the fecal bacterial community in any other drywood termite (Kalotermitidae) species. This prevents us from discussing the current findings in a comparative context with other drywood termite species. However, gut microbiota of some other kalotermitids, including *Incisitermes spp.*, have been previously characterized (Brune and Dietrich 2015, Arora et al. 2022). Even though those reports did not survey fecal pellets specifically, they may provide valuable background information for the origin of some of the bacterial taxa identified in this study.

The microbial community of fecal materials has been studied in a few subterranean termite species. Instead of producing fecal pellets like drywood termites, some subterranean termite species use their fecal material to construct nesting structures, mud tubes, galleries, or cartons (Ebeling 1975). The galleries of *Reticulitermes flavipes* (Kollar) (Heterotermitidae) are dominated by the bacterial phyla Acidobacteria, Actinomycetota, Bacteroidota, Proteobacteria, and Verrucomicrobia (Aguero et al. 2021). The galleries of *Coptotermes testaceus* (L.) (Heterotermitidae) are dominated by Acidobacteria, Actinomycetota, Bacteroidota, Planctomycetes, and Proteobacteria (Soukup et al. 2021). The galleries of *Heterotermes tenuis* (Hagen) (Heterotermitidae) are dominated by Acidobacteria, Actinomycetota, Firmicutes, Planctomycetes, and Proteobacteria (Soukup et al. 2021). Our study found Firmicutes as the dominant bacterial phylum found in *I. minor* fecal pellets. Per-sample profiles of bacterial community composition over time of *I. minor* fecal

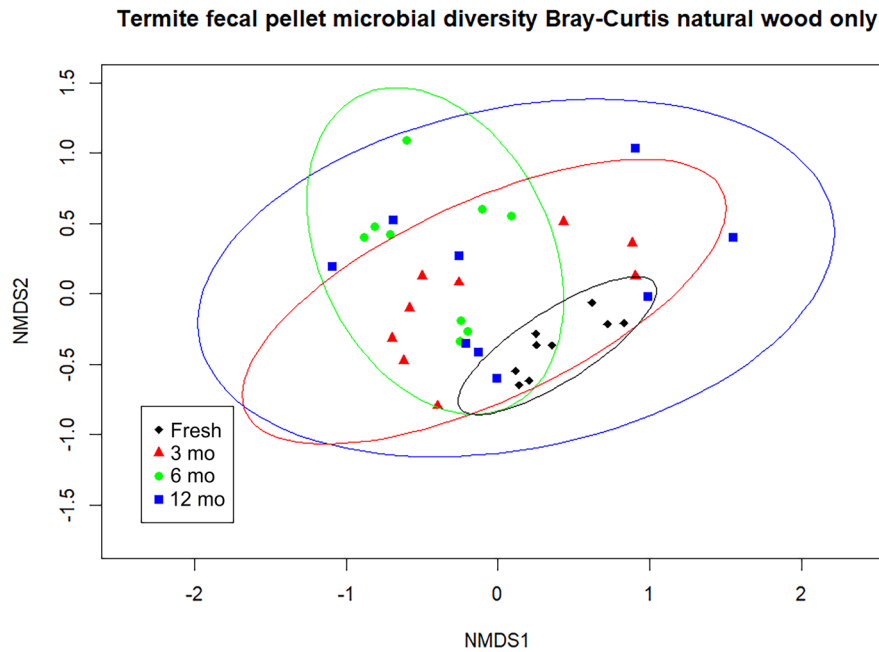


Fig. 2. Nonmetric multi-dimensional scaling of bacterial community structures found in natural-wood pellet samples at different ages calculated by Bray-Curtis dissimilarity distances.

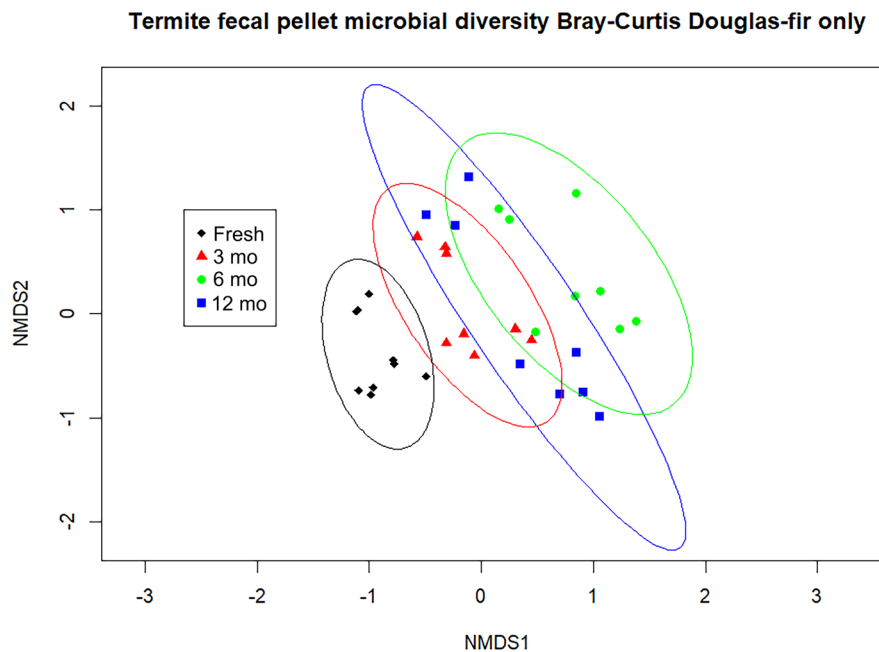


Fig. 3. Nonmetric multi-dimensional scaling of bacterial community structures found in D-fir pellet samples at different ages calculated by Bray-Curtis dissimilarity distances.

pellets in this study are provided in the supporting information (Supplementary Fig. S1). Averaged across all time points, Firmicutes accounted for 82 and 66% of all bacterial phyla found in natural-wood and D-fir pellets, respectively (Figs 4 and 5). Actinomycetota accounted for 5 and 10% of all phyla found in natural-wood and D-fir pellets, respectively (Figs 4 and 5). Proteobacteria accounted for 5 and 9% of all phyla found in natural-wood and D-fir pellets, respectively (Figs 4 and 5). Bacteroidota accounted for 3 and 7% of all phyla found in

natural-wood and D-fir pellets, respectively. Our findings show that the bacterial phyla present in *I. minor* fecal pellets and the galleries of some selected subterranean termites overlap significantly. In fact, many of these bacterial phyla mentioned above have been reported, albeit in different proportions, from the hindguts of many different termite species in the genera *Zootermopsis*, *Incisitermes*, *Coptotermes*, *Reticulitermes*, *Macrotermes*, *Nasutitermes*, *Cubitermes*, and *Microcerotermes* (Brune and Dietrich 2015).

Table 5. Highest differentially abundant bacteria found in *I. minor* fecal pellets

Comparison	Difference between	Log2 fold change	Phylum Family (f)
D-fir fresh to 3 mo	-7.60	-2.48	Actinomycetota Coriobacteriaceae
D-fir fresh to 6 mo	-9.54	-2.67	Bacteroidota Bacteroidaceae +>-
D-fir fresh to 6 mo	-9.19	-3.07	Bacteroidota Bacteroidaceae
D-fir fresh to 6 mo	-8.90	-2.55	Actinomycetota Coriobacteriaceae
D-fir fresh to 6 mo	-8.23	-2.57	Spirochaetota Spirochaetaceae +>-
D-fir fresh to 6 mo	-8.18	-2.51	Proteobacteria Rhodocyclaceae +>-
D-fir fresh to 6 mo	-7.94	-2.75	Firmicutes Anaerovoracaceae
D-fir fresh to 6 mo	-7.83	-2.49	Firmicutes Anaerovoracaceae
D-fir fresh to 12 mo	-8.43	-2.58	Bacteroidota Dysgonomonadaceae +>-
D-fir fresh to 12 mo	-8.31	-2.52	Spirochaetota Spirochaetaceae +>-
D-fir fresh to 12 mo	-8.25	-2.58	Proteobacteria Rhodocyclaceae +>-
D-fir fresh to 12 mo	-8.16	-2.99	Actinomycetota Coriobacteriaceae
D-fir fresh to 12 mo	-8.00	-2.91	Firmicutes Anaerovoracaceae
D-fir fresh to 12 mo	-7.88	-2.42	Firmicutes Anaerovoracaceae +>-
Nat-wood fresh to 3 mo	-8.32	-2.69	Proteobacteria Rhodocyclaceae
Nat-wood fresh to 3 mo	-8.10	-2.64	Bacteroidota Rikenellaceae
Nat-wood fresh to 3 mo	-7.83	-2.52	Proteobacteria Rhodocyclaceae
Nat-wood fresh to 3 mo	-7.78	-1.96	Bacteroidota Williamwhitmaniaceae
Nat-wood fresh to 3 mo	-7.69	-2.31	Bacteroidota Rikenellaceae
Nat-wood fresh to 3 mo	-7.37	-2.13	Bacteroidota Rikenellaceae
Nat-wood fresh to 3 mo	-6.86	-2.09	Bacteroidota Rs-E47 termite group
Nat-wood fresh to 3 mo	-2.09	-0.30	Bacteroidota Weeksellaceae
Nat-wood fresh to 3 mo	9.21	5.75	Proteobacteria Rhizobiaceae ->+
Nat-wood fresh to 3 mo	10.34	6.16	Proteobacteria Burkholderiaceae ->+
Nat-wood fresh to 6 mo	-9.00	-3.23	Bacteroidota Bacteroidaceae +>-
Nat-wood fresh to 6 mo	-8.53	-3.08	Proteobacteria Rhodocyclaceae +>-
Nat-wood fresh to 6 mo	-8.25	-3.15	Proteobacteria Rhodocyclaceae +>-
Nat-wood fresh to 6 mo	-7.01	-2.89	Bacteroidota Rikenellaceae
Nat-wood fresh to 6 mo	-6.57	-2.72	Bacteroidota Dysgonomonadaceae +>-
Nat-wood fresh to 6 mo	9.87	3.89	Proteobacteria Sphingomonadaceae ->+
Nat-wood fresh to 6 mo	10.07	4.14	Actinomycetota Cellulomonadaceae ->+
Nat-wood fresh to 6 mo	10.69	4.13	Firmicutes Leuconostocaceae ->+
Nat-wood fresh to 6 mo	10.73	3.91	Proteobacteria Rhizobiaceae ->+
Nat-wood fresh to 6 mo	11.63	4.58	Proteobacteria Burkholderiaceae ->+
Nat-wood fresh to 12 mo	-8.67	-3.20	Bacteroidota Rikenellaceae +>-
Nat-wood fresh to 12 mo	-8.65	-2.95	Bacteroidota Dysgonomonadaceae
Nat-wood fresh to 12 mo	-8.43	-2.72	Proteobacteria Rhodocyclaceae
Nat-wood fresh to 12 mo	-8.25	-2.72	Firmicutes Clostridia vadin BB60 group
Nat-wood fresh to 12 mo	-8.17	-2.88	Proteobacteria Rhodocyclaceae
Nat-wood fresh to 12 mo	-8.11	-3.07	Firmicutes Oscillospiraceae
Nat-wood fresh to 12 mo	-7.99	-3.12	Proteobacteria Rhodocyclaceae
Nat-wood fresh to 12 mo	-7.59	-2.97	Cyanobacteria Gastranaerophilales
Nat-wood fresh to 12 mo	-7.19	-2.97	Bacteroidota Rikenellaceae +>-
Nat-wood fresh to 12 mo	-6.93	-2.73	Bacteroidota Dysgonomonadaceae +>-

All comparisons are from fresh to aged fecal pellets. Negative values indicate a reduction while positive values indicate an increase in bacterial abundance. The +>- means that the bacteria were found in all fresh fecal pellet samples and none of the aged pellet samples for that comparison. The ->+ means that the bacteria were found in none of the fresh fecal pellet samples and all aged pellet samples for that comparison.

The phylum Actinomycetota is known to possess antimicrobial properties and is closely associated with termites (Waksman and Lechevalier 1962). In the fungal gardens of some higher termites (Termitidae), Actinomycetota inhibit the growth of unwanted microbes (Rouland-Lefèvre 2000, Nobre et al. 2010, Poulsen et al. 2014). Actinomycetota are also found in the carton nest of *Coptotermes formosanus* (Shiraki) (Heterotermitidae) where they provide additional disease resistance for the termite colony (Chouvenc et al. 2013). Furthermore, Actinomycetota are also found in the guts (Arango et al. 2016) as well as galleries of *R. flavipes*, suggesting that gut bacteria can move from the termite gut and colonize nest galleries (Aguero et al. 2021). Even though drywood termites inhabit

relatively dry and sound wood, which may not be particularly prone to propagation of bacteria or fungi (Ignoffo 1992), Actinomycetota were among the dominant phyla in *I. minor* fecal pellets. The antimicrobial properties of fecal pellets have not been reported for *I. minor*. However, based on a laboratory experiment, the fecal pellets of a dampwood termite *Zootermopsis angusticollis* (Hagen) (Archotermopsidae) were shown to reduce spore germination of the entomopathogenic fungus *Metarhizium anisopliae* (Rosengaus et al. 1998).

Our results show that freshly produced and 3-mo-old natural-wood pellets contain on average over five times the amount of bacterial DNA compared to D-fir pellets of the same ages. Although we did not sequence the wood samples directly,

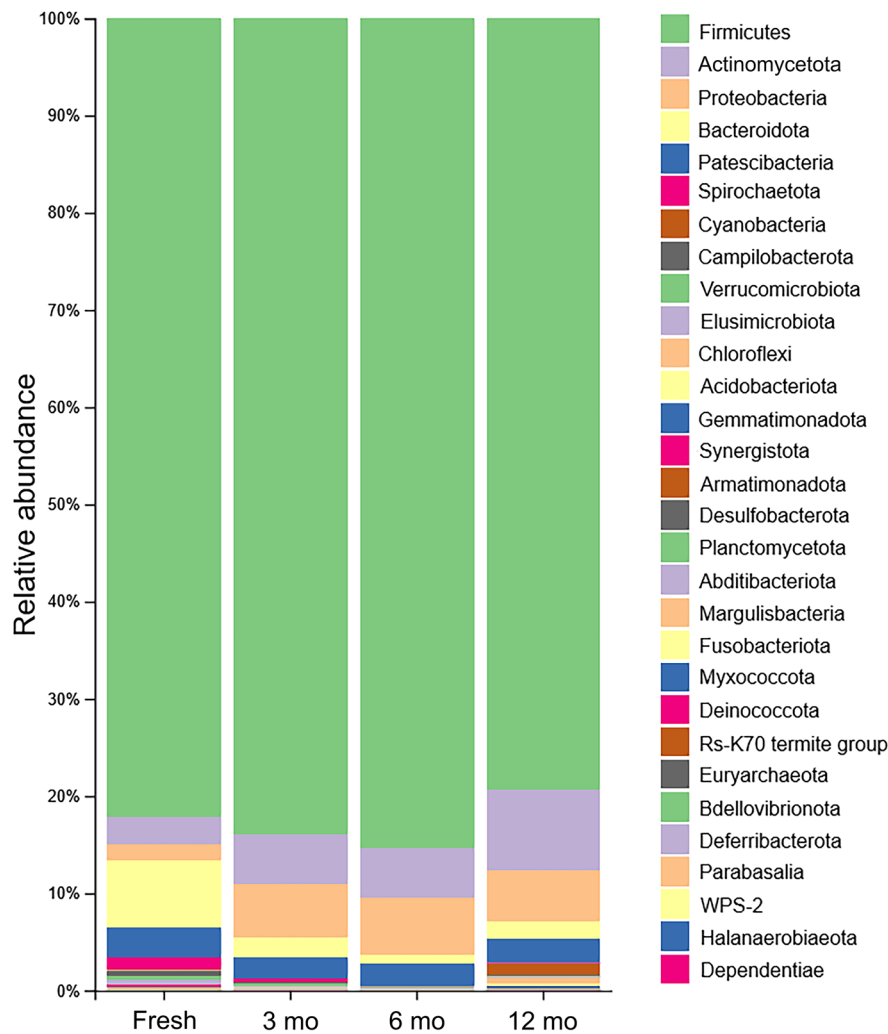


Fig. 4. Relative abundance of bacterial phyla found in *I. minor* natural-wood pellets at different ages.

the higher levels of bacterial DNA we observed in the natural-wood pellets may be due, in part, to the initially higher levels of bacteria in the natural wood compared to the processed D-fir lumber. The effects of wood extractives and hygroscopic properties of different wood types can influence the bacterial abundance found on wood (Milling et al. 2005). The D-fir commercial timber used in this study likely contained lower moisture levels than the natural wood due to drying steps in the manufacturing process (Kretschmann 2010). Furthermore, in both natural-wood and D-fir pellets, the amount of bacterial DNA was greatly reduced after 3 mo while maintaining similar bacterial phyla compositions (Figs 4 and 5). Since PCR-based identification methods do not discriminate between alive or dead bacterial cells (Josephson et al. 1993), it is not possible to determine if the bacteria identified were alive or dead at the time of sampling. However, the reduction of microbial DNA in the pellet samples is consistent with the findings of Young et al. (2007), where a 1,000-fold decay of bacterial DNA over 12 mo was reported after cell death based on 16S rRNA gene sequencing. We found a 190 and 184-fold decrease in the amount of bacterial DNA detected from fresh to 12-mo-old pellets for natural-wood and D-fir pellets, respectively (Tables 1 and 2). These differences in the amount of bacterial DNA present in pellets of different ages offer a potential method to distinguish

fresh from aged pellets. Future studies with more frequent sampling (eg every 2 wk) might provide higher resolution to understand the temporal dynamics of bacterial DNA concentration present in fecal pellets over time.

Interestingly, the Shannon diversity indices of both natural-wood and D-fir pellets significantly changed over time. These differences in alpha-diversity indices showed the diversity of bacterial taxa changing between these ages. The beta-diversity analysis revealed differences in the bacterial community composition of fecal pellets at different ages for both D-fir and natural-wood pellets. Fresh fecal pellets were tightly clustered while aged fecal pellets were more spread. Natural-wood pellets were nested inside pellets of other ages with a high degree of community overlap. D-fir pellets had slight overlap with 3-mo-old D-fir pellets but complete separation from 6-mo and 12-mo old D-fir pellets. The results from both analyses show a significant change in the bacterial community of D-fir pellets over time.

Information from the differential abundant analysis could offer important insights to identify potential biomarkers to distinguish fresh from aged D-fir pellets. The relative abundances of all taxa listed (Table 5) were significantly different between fresh and aged fecal pellets, indicating these taxa drove differences in the diversity indices between different time points. However, a reliable signal to distinguish between fresh and aged fecal pellets

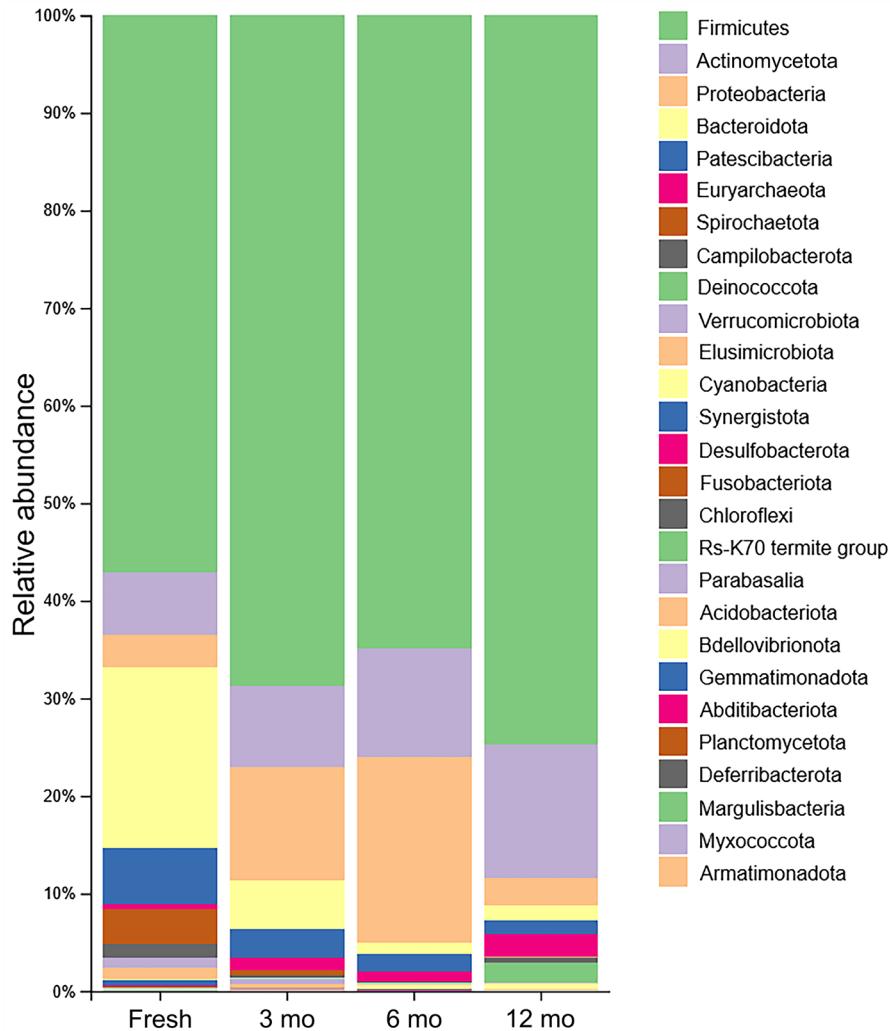


Fig. 5. Relative abundance of bacterial phyla found in *I. minor* D-fir pellets at different ages.

would need to be present or absent in all fecal pellets sampled of a time point comparison. Further investigation showed that bacterial families that were present in all fresh pellet samples and absent in all aged pellet samples for the “D-fir fresh to 6 mo” comparison were Bacteroidaceae and Rhodocyclaceae. Similarly, bacterial families that were present in all fresh pellet samples and absent in all aged pellet samples for the “D-fir fresh to 12 mo” comparison were Rhodocyclaceae (same ASV as “D-fir fresh to 6 mo” comparison), Anaerovoracaceae, Dysgonomonadaceae, and Spirochaetaceae. For further resolution, sequences were entered into Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). Only two sequences entered resulted in generic level identifications, *Bacteroides* (family Bacteroidaceae) and *Treponema* (family Spirochaetaceae), while the others remained unidentified with < 97% identity similarity (Schloss and Handelsman 2005). *Bacteroides* and *Treponema* were found in all fresh D-fir pellets, but none of the 6-mo-old pellets. *Treponema* were found in none of the 12-m-old D-fir pellets.

The genera *Bacteroides* and *Treponema* belong to the phyla Bacteroidota and Spirochaetota, respectively. *Bacteroides* are Gram-negative, non-sporulating, rod-shaped bacteria commonly found in termite guts as free-swimming symbionts or ectosymbionts of flagellated protists (Ohkuma et al. 2002, Desai et al. 2010). *Treponema* are Gram-negative, non-sporulating,

spiral or wave-shaped bacteria commonly found in termite guts as ectosymbionts of flagellated protists (Iida et al. 2000, Breznak 2002). Both genera have been described in the alimentary canals in both drywood and subterranean termites. *Bacteroides* comprise a major group of gut symbionts found across termites (Ohkuma et al. 2002, Desai et al. 2010). *Treponema* has been found in the guts of the drywood termites *Incisitermes tabogae* (Snyder) (Droge et al. 2008) and *I. minor* (Nicholas Poulos unpublished data). Additionally, diverse assemblages of *Treponema* have been reported in the alimentary tracts of subterranean termites (*R. flavipes* and *C. formosanus*) (Lilburn et al. 1999). Although both bacteria groups are incredibly diverse and widespread, those found in termite guts can be host-specific and are not found elsewhere in nature (Ohkuma et al. 2002, Graber et al. 2004, Sakamoto and Ohkuma 2013). Therefore, it is most likely that the *Bacteroides* and *Treponema* detected in fresh D-fir *I. minor* fecal pellets originated from the hindgut of *I. minor*, being incorporated into the fecal pellets during pellet formation. Future studies are warranted to further investigate the strains of *Bacteroides* and *Treponema* detected in this study, possibly describing them as novel obligate bacterial species associated with *I. minor*. It should also be noted that three additional candidates for potential biomarkers were reported in the current study: sequences associated with the families

Anaerovoracaceae, Dysgonomonadaceae, and Rhodocyclaceae. The current study focused on the genera *Bacteroides* and *Trepone* because they were the only two groups which resolved to the generic level. Further understanding of the taxonomy of these other bacteria, particularly at the species level, might lead to additional possible biomarkers.

Even though the current study did not explore the exact mechanisms by which the abundance of these bacteria quickly decreases as the fecal pellets age, a few possible mechanisms can be proposed. First, the obligate anaerobic nature of these bacteria may partially explain why they were not detected in the aged fecal pellets. The continuous aerobic exposure might have caused their reduction over time. Similar to the reduced diversity and changes in relative abundance of taxa we observed in D-fir pellets, aerobic exposure can cause microbiota dysbiosis which leads to a decrease in microbial diversity, a decrease in Bacteroidota, and an increase in Proteobacteria (Oladele et al. 2024). Second, lower moisture conditions as the pellets age may impact the bacterial communities (Alekhina et al. 2001). Third, the biological environmental changes driven by dominant groups of bacteria in the fecal pellets (Wang et al. 2014).

Development of rapid onsite detection tools for specific bacteria has been primarily motivated by the need of quick and onsite pathogen detection (Rajapaksha et al. 2019). Culture-based assays, PCR, GC-MS, and isothermal amplification are effective for pathogen detection. However, these methods take a relatively long time (2 to 3 d) and require highly trained personnel and costly equipment. These factors often make them unsuitable for the onsite detection of specific bacteria (Pan et al. 2024). In contrast, new biosensor methods explore the use of antigens, antibodies, aptamers, proteins, and artificial enzymes to detect specific bacteria (Nguyen and Kim 2020, Shang et al. 2023, Córdova-Espinoza et al. 2024). For wider adoption in the pest management industry, a viable tool for distinguishing fresh drywood termite fecal pellets from aged ones must be cost-effective, quick, and precise. One possible mechanism that fulfills these criteria is a colorimetric paper-based biosensor (Nguyen and Kim 2020). Further investigation is warranted to determine the feasibility of such a method with the candidate DNA-based biomarkers reported in the current study. Future studies can investigate the possible impact that drywood termite control methods (ie, fumigation, heat, residual insecticides, etc) have on the microbial community of fecal pellets. Furthermore, although *I. minor* is a significant economic pest in California, a successful technique to distinguish fresh from aged fecal pellets would have the greatest impact if applied to *Cryptotermes brevis* (Walker), one of the world's most destructive termites and the most widely distributed drywood termite (Evans et al. 2013).

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Nicholas Alexander Poulos (Conceptualization [equal], Data curation [lead], Formal analysis [lead], Investigation [lead], Methodology [equal], Visualization [equal], Writing—original

draft [lead], Writing—review & editing [equal]), Lyna Ngor (Formal analysis [equal], Visualization [equal]), Chow-Yang Lee (Conceptualization [equal], Methodology [equal], Supervision [equal], Validation [equal], Writing—review & editing [equal]), Quinn S. McFrederick (Formal analysis [equal], Methodology [equal], Validation [equal], Visualization [equal], Writing—review & editing [equal]), and Dong-Hwan Choe (Conceptualization [lead], Funding acquisition [lead], Methodology [equal], Project administration [lead], Resources [lead], Software [lead], Supervision [lead], Validation [lead], Visualization [equal], Writing—review & editing [lead])

Supplementary Material

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Conflicts of Interest

None declared.

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