Dysbiosis: A Potential Novel Strategy for Control of Subterranean Termites

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ABSTRACT

Although alternative control strategies using biocontrol agents have reduced populations of many pest insects, biocontrol of termites has met with limited success. Termite living conditions—crowded colonies in moist soil—are seemingly ripe for epizootic outbreaks, yet, termite colonies rarely succumb to disease. The source of their immunity comes from a multi-tiered defense system involving cellular, physiological, and behavioral mechanisms, the combination of which protects termites at individual and colony levels. Defeating termite immunity, however, may be possible by chemically inducing microbial imbalance or dysbiosis. In this study, treating solution (2% chitosan or control) and weather exposure (88 days or 0 days) were tested in a factorial design (four treatments total) to determine sublethal effects on hindgut bacterial diversity in the subterranean termite, *Reticulitermes flavipes.* The bioassay was set up according to the AWPA E1 single-choice feeding experiment. After four weeks, DNA was extracted from termite hindguts. Species abundance and richness were based on sequence analysis of the V3 and V4 region of the 16S ribosomal RNA gene using the Illumina 16S metagenomics workflow. After pre-processing the data by using data-driven methods to set thresholds for signal to noise ratio and removal of rare species, multivariate analysis was performed using the statistical package *PC-ORD.* Visualization of the phyla abundance data in a two-way cluster analysis indicated that the four treatments (CTW, chitosan-treated wood not exposed to weathering; CTW_E, chitosan-treated wood exposed to weathering; WTW, water-treated wood not exposed to weathering; and WTW_E, water-treated wood exposed to weathering) separated into two clusters, one uniquely comprised of CTW and the second formed by the other three groups. Association of CTW_E with the two controls (WTW and WTW_E) was presumably due to environmental leaching. PerMANOVA showed that treatment exerted a significant effect on phyla diversity with all four treatments being significantly different from each other. Indicator species analysis (Indicator Value ≥90) detected nine species specific to CTW. These species demonstrated both high relative abundance (>97% of total reads found were found in CTW) and constancy (species found in more than 90% of CTW DNA samples). Five have been isolated from human tissue infections or human tissue infections with comorbidities. Three species—*Mycobacterium abscessus, M. franklinii,* and *Sphingobacterium multivorum*—are ubiquitously found in nature and considered to be opportunistic pathogens. Based on these data, we hypothesize that antimicrobials like chitosan can chemically induce dysbiosis to allow establishment of disease-causing pathogens.

Keywords: dysbiosis, chitosan, antimicrobial, termite, hindgut, pathogen, metagenomics

OBJECTIVES

1. Treat wood with a sublethal concentration of chitosan to chemically induce hindgut dysbiosis in *Reticulitermes flavipes*.
2. Document changes in bacterial diversity using metagenomic sequencing of a portion of the 16S ribosomal RNA gene.
3. Differentiate shifts in bacterial community from disease-associated dysbiosis by performing an indicator species analysis.

INTRODUCTION

Cross-laminated timber (CLT) is an emerging market that shows strong U.S. growth in the tall building sector. In 2014, the map in Figure 1 was blank. Now in 2018, there are currently 12 CLT factories in various stages from announced to being completed and 20 notable CLT buildings that have completed construction. Because it is an emerging market, there are few pressure treatment options to protect CLT against termites for indoor applications. This is a major drawback for architects and clients who often prefer the sustainability and aesthetics of wood to concrete and steel, but fear the risks of termite infestations, especially drywood termites along coastal regions of CA and subterranean termites in the southeastern U.S.

Inspiration for new wood protection strategies often comes from research in medicine and agriculture. The human microbiome project, which ran from 2008 to 2017, was a major research initiative that grew from early reports that there were 100 trillion microbial cells living on and in the human body. The number of microbial cells in the human microbiome, which

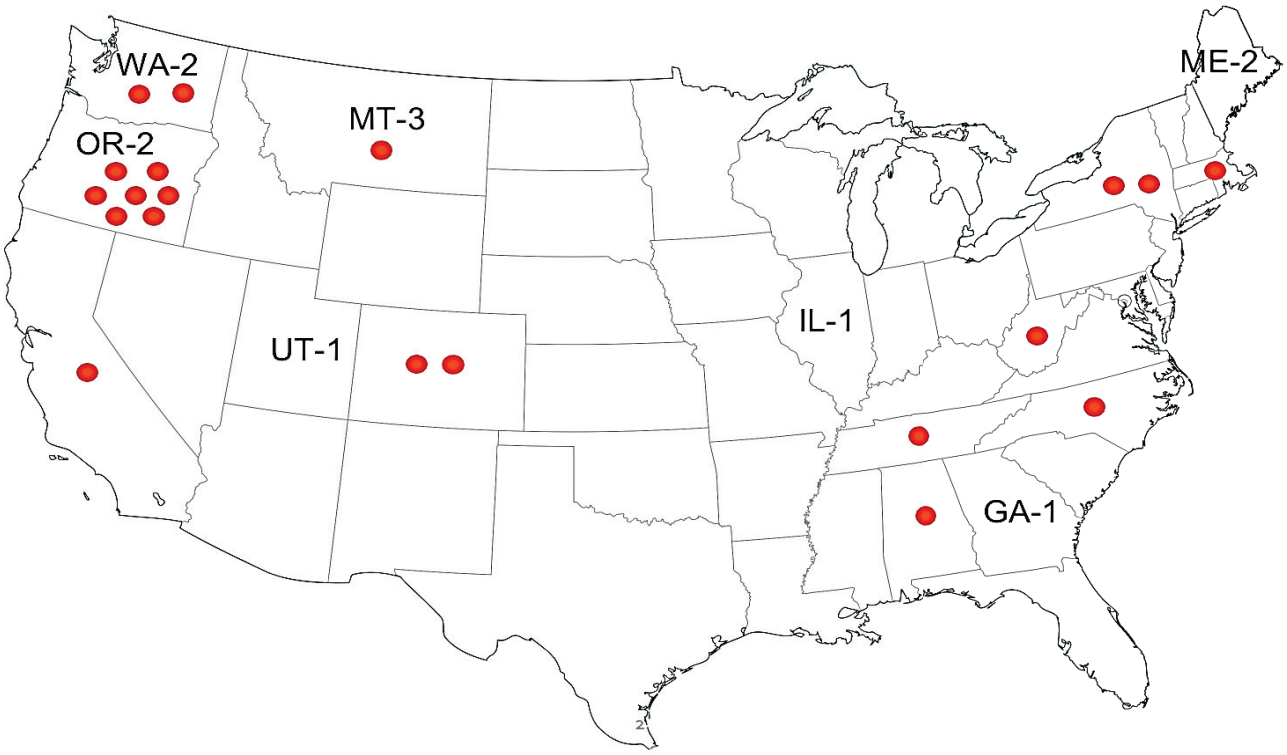

Fig. 1. Number of CLT factories (announced to completed, text) and notable buildings made entirely or partially from CLT (dots) across the U.S. Permission to use data given by Steve Marshall, USDA Forest Service, Wood Innovations.

accounts for 1.5-3 kg of our body weight, actually outnumber the human cell count, which begged the question, "What do they do?" There is now an ever-growing body of literature showing that microbes contribute significantly to our nutritional survival, emotional well-being, and health by prevention of disease. Moreover, these diseases, both intestinal and extra-intestinal, occur when there is long-term microbial imbalance or dysbiosis (Cho & Blaser, 2012).

In subterranean worker termites, the hindgut is 1/3 of its body weight, underscoring its tremendous importance to the health of the individual termite and colony. The hindgut functions as an anaerobic bioreactor, packed full of symbiotic microbes that digest wood fibers and contribute to the nutritional survival of the host. These microbes include unicellular protists (phylum Eukarya) and several hundred species of bacteria (domains Bacteria and Archaea). Together, this spatially structured, highly interdependent community orchestrates anaerobic digestion, the process that converts high molecular weight carbon from wood to CO_2 and CH_4. Anaerobic digestion occurs by (1) hydrolysis or conversion of cellulose and hemicellulose to simple sugars, (2) acidogenesis or conversion of sugars to short chain carbon acids with production of H_2 and CO_2, (3) acetogenesis, or assimilation of H_2 and CO_2 to produce acetate and H_2O, and (4) methanogenesis, conversion of acetate + H_2 to produce CH_4 and CO_2. Acetate is the major absorption product from the hindgut that the termite uses for energy and growth (Brune, 2014).

Review articles by Hongoh (2010) and Brune (2014) provide excellent summaries about the composition and function of the termite hindgut microbiome. There are at least 10-12 species of protists in the hindgut of *Reticulitermes.* At 10^3 to 10^5 cells per gut, they occupy about 90% of the hindgut space. Functionally, they are primarily responsible for hydrolysis and acidogenesis. Of the prokaryotes, the majority (99.9%) are bacteria with at least 3 million cells per gut. The bacteria can be found free-living, attached to the gut wall, or as ecto- and endo-symbionts of the protists. Their roles in anaerobic digestion include acidogenesis and acetogenesis, but bacteria also contribute to nitrogen assimilation (N_2 fixation to NH_3) and the sulfur cycle (sulfate-reducing bacteria). The remaining 0.1% of prokaryotes are Archaea. Although not as well studied, Boucias et al. (2013) reported that 94% of Archaea found in the termite hindgut were *Methanobrevibacter*, 4% *Methanothermobacter,* and 2% undescribed. Archaea occur as ecto-symbionts of protists or attached to the gut wall (Tokura et al., 2000).

Inducing disease-associated dysbiosis with biocontrol agents, however, is unlikely to be successful in termites. Termite living conditions—crowded colonies in moist soil—are seemingly ripe for epizootic outbreaks, yet, termite colonies rarely

succumb to disease despite 50 years of biocontrol efforts from more than 20 laboratories around the world (Chouvenc & Su, 2011, 2012). The source of their immunity comes from a multi-tiered defense system involving cellular, physiological, and behavioral mechanisms, the combination of which protects termites at the individual and colony levels (Chouvenc & Su, 2010). Defeating termite immunity, however, may be possible by chemically inducing dysbiosis with non-toxic, environmentally friendly antimicrobials like chitosan.

Chitosan has antibacterial and fungistatic properties and is derived from deacetylation of chitin. The first study to explore the termiticidal properties of chitosan was done by Raji et al. (2018). They showed a dose-dependent effect of chitosan on mortality of two species of subterranean termites, *R. flavipes* and *R. virginicus*, in an AWPA E1 single-choice test. This study continues our investigation of chitosan by investigating its effects on the termite microbiome and its ability to induce disease-associated dysbiosis. Our eventual goal is aimed at developing novel treatment strategies for protection of CLT used in indoor applications.

MATERIALS AND METHODS

Termites

Termites were collected from a fallen tree at the Sam D. Hamilton Noxubee National Wildlife Refuge, Brooksville, MS. The infested tree was cut into smaller sections, transported back to the lab in a covered metal trash bin, and held at room temperature until used for the bioassay within 6 months of collection. Species identification was based on morphological characteristics of soldiers (Scheffrahn & Su, 1994) and DNA sequence analysis of the mitochondrial AT-rich region (Foster et al., 2004).

Wood Treatment

Two solutions were prepared: 2% w/v chitosan (50-190kDA, Sigma-Aldrich) in 25% acetic acid and a control solution of 25% v/v acetic acid. Twenty blocks (25 x 25 x 6 mm, radial x tangential x longitudinal) were cut from southern yellow pine sapwood according to the AWPA E1-15 Standard (AWPA, 2015) and vacuum-treated at 24 inches Hg for 3 hours (10 blocks per solution). After drying, half of the blocks from each treatment group were placed outdoors (10 inches above ground) on a plastic mesh for 88 days of weathering. The resulting manipulations produced four treatment groups (5 blocks per treatment): CTW_E, chitosan-treated wood exposed to weathering; CTW, chitosan-treated wood not exposed to weathering; WTW_E, water-treated wood exposed to weathering; and WTW, water-treated wood not exposed to weathering.

Termite Single-Choice Feeding Bioassay

The wood feeding bioassay was performed according to the AWPA Standard E1-15 single-choice test (AWPA, 2015). Five jar replicates were set up per treatment (n = 5). After four weeks, surviving termites from each jar were removed, labeled, and stored at -80˚C until they were processed for DNA extraction.

DNA Extraction and Purification

Termite guts were freed from the termite body by cutting off the head and then using forceps to pull the abdominal tip while securing the anterior end of the thorax with another pair of forceps. The freed gut was rinsed in Trager U saline (Trager, 1934) and placed on ice in a 1.5 mL microcentrifuge tube. When the tube contained 5 guts, all guts were macerated with a clean, sterile, plastic pestle. DNA was then isolated using protocol and reagents provided by the MasterPure Complete DNA and RNA Purification Kit (Epicentre). A total of 80 guts (16 tubes of DNA) were collected per bioassay jar. The 16 tubes were pooled to reduce the number of DNA subsamples to 4 subsamples per jar. This procedure resulted in a total of 80 DNA sample units for the experiment, i.e., 20 DNA samples per treatment x 4 treatments.

Metagenomics Library Preparation and Reporting

Bacteria metagenomic libraries were prepared from the DNA samples following the protocol for the Illumina MiSeq as outlined in the 16S Metagenomic Sequencing Library Preparation Guide (Illumina Part # 15044223 Rev. B). The basic steps involve (1) using the polymerase chain reaction to amplify the hypervariable V3 and V4 regions of the 16S ribosomal RNA gene, (2) purifying the amplified product, (3) using the polymerase chain reaction to attach a unique index sequence to each end of the amplified product, (4) purifying the amplified product, now called a library, (5) validating library size, purity, and concentration, (6) diluting the libraries to the same concentration, (7) pooling the libraries into one sample, and (8) simultaneously sequencing the 80 libraries in one run on the Illumina MiSeq platform.

Details of the sequence report are described in the MiSeq Reporter Metagenomics Workflow Reference Guide (Illumina Part # 15042317 Rev. D). The software tool uses algorithms to execute the following steps: (1) demultiplex pooled libraries based on the index sequence, (2) match "words" or shorter subsequences along each read to the Illumina-curated version of the 16S ribosomal RNA Greengenes database, (3) assign a taxonomic classification to each read based on the number of word matches, (4) calculate the total number of reads matching a particular taxonomic classification, and (5) assign a unique

operational taxonomic unit (OTU) number to all reads showing >97% percent sequence similarity regardless of whether a taxonomic classification is made. For this study, the MiSeq Reporter was used to summarize read counts by taxonomic classifications at both phyla and OTU levels.

Data Pre-Processing

Three sequential steps were performed to pre-process the MiSeq reports. The first involved normalization of each sample unit library to 100,000 reads. Visualize a large matrix that has sample units in rows and OTUs in columns. During normalization, row sums (sums of OTUs per sample unit library) are calculated, the OTU read count is divided by the row sum to get the proportion of reads represented in the sample unit, then each proportion is multiplied by 100,000. This eliminates over- and under-representation of samples in the poolplex since methods of DNA quantitation are not exact. The second pre-processing step used data-driven methods to determine thresholds for signal to noise ratio and removal of rare OTUs. Signal to noise ratio was based on a histogram of the MiSeq count data and occurred where the plateau began. Rare OTU removal was based on a histogram of the binary version of the data matrix (1 for presence, 0 for absence) and also occurred where the plateau began. Pre-processing steps were done using R, a free statistical computing and graphics software environment (R Core Development Team, 2013).

Multivariate Statistical Analysis

Multivariate analysis was performed using *PC-ORD 6*, a statistical package developed for the analysis of ecological communities (McClune & Grace, 2002; Peck, 2010). After removing rare OTUs, different transformations were evaluated for their ability to improve normality. Concurrent analyses with the Advisor tool on both untransformed and transformed matrices was done to calculate distances between sample units in phyla space and distances between phyla in sample space. The number of outliers produced by each of the eight different distance measures was examined. An outlier was defined as having a distance value >2.0 SD from the mean. The final transformation and distance measure chosen had the lowest skewness and kurtosis values and produced the fewest outliers. A two-way hierarchical cluster analysis was done to classify sample units according to their degree of similarity in their phyla response patterns. Group testing was performed using distance-based PerMANOVA, a permutation-based non-parametric MANOVA test. A one-factor PerMANOVA of treatment at four levels (5000 permutations) was run using Euclidean distance measure to determine if treatment exerted a significant effect on phyla abundances (p <0.05). If treatment was significant, PerMANOVA was followed by pairwise t-testing to determine where significant treatment differences were found. Protection from false discovery during multiple hypothesis testing was not done. The last test run was an indicator species analysis on the OTUs. In this analysis, percent of reads and percent constancy of representation within a treatment were multiplied and divided by 100 to produce the indicator value (IV) for that particular combination of OTU and treatment. An OTU with treatment IV ≥90 was the threshold criteria used to identify an indicator species. Significance of the observed maximum IV value across the four treatments was evaluated by the Monte Carlo method using 5000 permutations.

RESULTS AND DISCUSSION

Termites

Soldier pronotal width, head length with mandibles, and curvature of the left mandible indicated that the termite collection was *Reticulitermes flavipes* (Kollar). DNA sequence comparison of the mitochondrial AT-rich region against the non-redundant nucleotide database in the National Center for Biotechnology Information showed greatest similarity with sequences for *R. flavipes*, thereby confirming the identification.

Wood Treatment

Although retentions of chitosan were not estimated in this study, a previous report showed that wood treatment with a 2% chitosan solution resulted in chitosan retention of 38 mg g^{-1} (Raji et al., 2018).

Termite No-Choice Feeding Bioassay

Mass loss and mortality were not measured in this study but based on the number of live termites retrieved, the 2% chitosan treatment exhibited about 40-45% mortality. A previous report showed 94% mortality for *R. flavipes* exposed to wood treated with a 2% chitosan solution in an AWPA E1 single-choice test (Raji et al., 2018). The latter study used termites collected from Saucier, MS, while these were from Brooksville, MS. The difference in mortality could be due to genetic differences in colony susceptibility, differences in chitosan retention since the blocks for the two studies came from different sticks, or from other uncontrolled factors, such as termite physiology.

Metagenomics Library Preparation and Reporting

About 37% of reads were given a species level assignment with 1722 species detected when aligned to the Greengenes

database. The Greengenes output file was a matrix that had 80 rows representing the DNA libraries x 1722 columns representing the OTU or species identifications. The range of total counts per library before normalization was 7133 to 131,236 with an average of 38,252.

Data Pre-Processing

After normalizing reads to 100,000 per sample unit, the data-driven methods showed that the MiSeq signal to noise ratio threshold was 40 read counts. Any MiSeq read count value <40 was considered to be noise and set to a zero value. The threshold for rare OTU removal was determined to be 3. In other words, any OTU present in fewer than 3 DNA libraries out of 80 was considered to be rare and was removed from the dataset. Removal of rare OTUs is especially important for metagenomic datasets, which can have as much as 80% zero values. Moreover, since the primary goal of this analysis was to find treatment effects on microbial biodiversity, removing rare OTUs helped reduce bulk and noise (McClune & Grace, 2002). After removal, the final number of OTUs was 306. The latter total was much closer to species estimates obtained through traditional cloning and Sanger sequencing methods. Fisher et al. (2007) found 261 OTUs for *R. flavipes*, while Hongoh et al. (2003) detected 268 OTUs from *R. speratus*. Depending upon the number of clones sampled, the traditional cloning method tends to underestimate total number of species, but sequences generated tend to be of high quality with few incorrect nucleotide or base calls. High throughput sequencing, on the other hand, provides more comprehensive sampling of population diversity but produces lower quality sequences with more inaccurate base calls.

Multivariate Statistical Analysis

The logarithmic transformation ln(x+1) was used because it reduced skewness and kurtosis for both phyla and OTU datasets. Plots and analyses were performed with the transformed normalized abundances, which henceforth will simply be referred to as relative abundances, unless otherwise specified. The distance measures that produced the fewest outliers for the phyla and OTU datasets were Euclidean and Jaccard, respectively. Euclidean is based on calculations of the shortest distance between two points while Jaccard is based on set theory, measuring similarity based on the intersection of two sets divided by their union. Both datasets had 4 outliers, all of which were in the CTW treatment. Outliers, however, were not removed because they did not appear to affect the overall outcome of each analysis.

Two-way cluster analysis (Euclidean distance measure, Ward's method of group linkage) based on similarity of phyla abundance patterns showed that the 80 DNA samples separated into two major clusters: one uniquely comprised of CTW and the second formed by the other three treatments, WTW, WTW_E, and CTW_E (Figure 2). Based on the position of the orange line, these two major clusters form at about 40% information remaining and account for 60% of the variance in distances. Similarity in phyla diversity of CTW_E with the two controls (WTW and WTW_E) rather than with CTW suggests that some amount of chitosan was lost from the wood during the 88 days of weathering. Loss of chitosan from weathering was consistent with previously published data showing that 60% of chitosan leached from wood during a 14-day AWPA E11 laboratory test. (Raji et al., 2018).

By examining the overall degree of shading in Figure 2 (darker shading means greater relative abundance), it appeared that the CTW treatment exhibited lower overall diversity than the other three treatments. Diversity is a measure of both number of taxa found (i.e., presence or absence) and their respective relative abundances. Based on presence and absence, CTW showed no difference from the other treatments because all detected phyla were present in all sample units. The conclusion of lower diversity was drawn from comparing the evenness of color shading across phyla. The shading was less uniform for the CTW cluster compared to the second cluster indicating that the relative abundances of phyla were less balanced for CTW. In a community of two species, A and B, a sample unit with 50% relative abundance of each species is, by definition, more diverse than a sample unit that has 99% of species A and 1% species B.

The normalized read counts for phyla grouped by treatment are depicted in Figure 3. The most abundant taxa were represented in eight phyla with the remaining assigned to one group called Other. The general rank order of abundance from highest to lowest for the assigned phyla was Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Thermotogae, Spirochaetes, Synergistes, and Verrucomicrobia. Exceptions to the rank order, however, were possible, depending upon treatment.

Results of the one-factor PerMANOVA using a Euclidean distance measure indicated that treatment had a significant effect on the observed relative abundances of phyla (Table 1). Pairwise t-test comparisons showed that all four treatments were significantly different from each other except CTW_E and WTW (Table 2). A significant difference indicated that the treatments being compared exhibited hindgut bacterial communities that were significantly different at the phylum level.

At this point in the analysis, it was unclear whether the differences in microbial communities were associated with disease or merely represented shifts due to differences in treatment effects. This was an especially important distinction since metagenomic analysis has demonstrated shifts in bacterial hindgut diversity when *R. flavipes* was fed grassy versus woody diets for six weeks (Huang et al., 2013). Complexity of carbon source has also been shown to affect the diversity of both protist and bacterial hindgut symbionts of *Coptotermes formosanus* Shiraki in 30-day feeding trials (Tanaka et al., 2006). Using visual

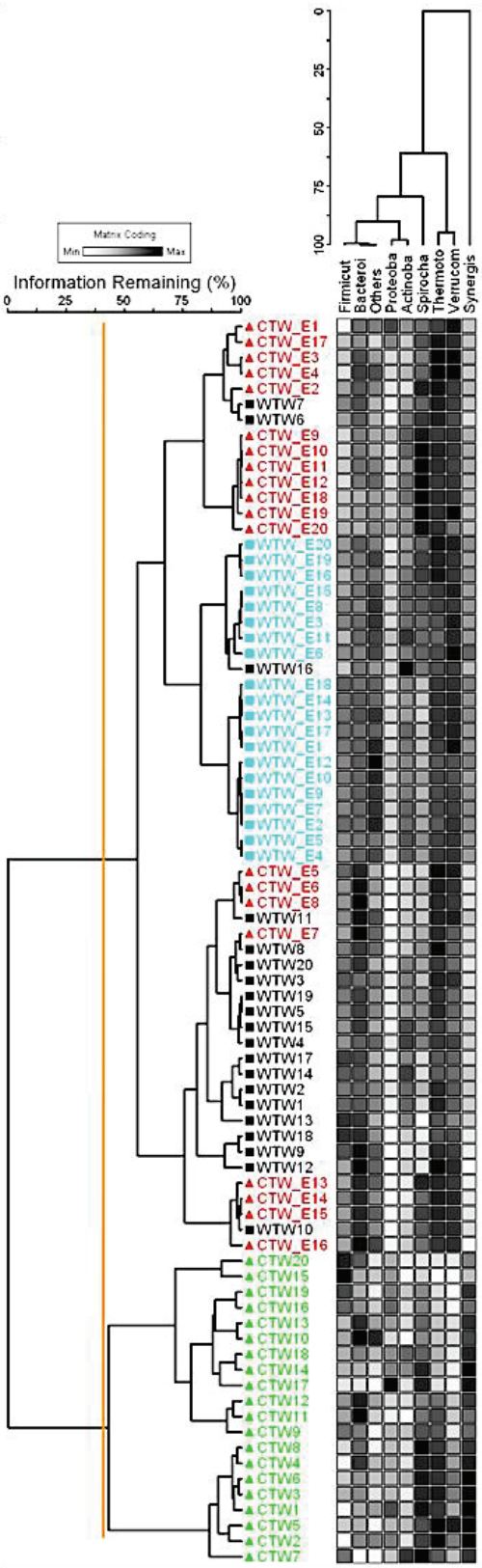

Fig. 2. Two-way cluster analysis showing how samples cluster based on pattern similarities of bacterial phyla relative abundances.

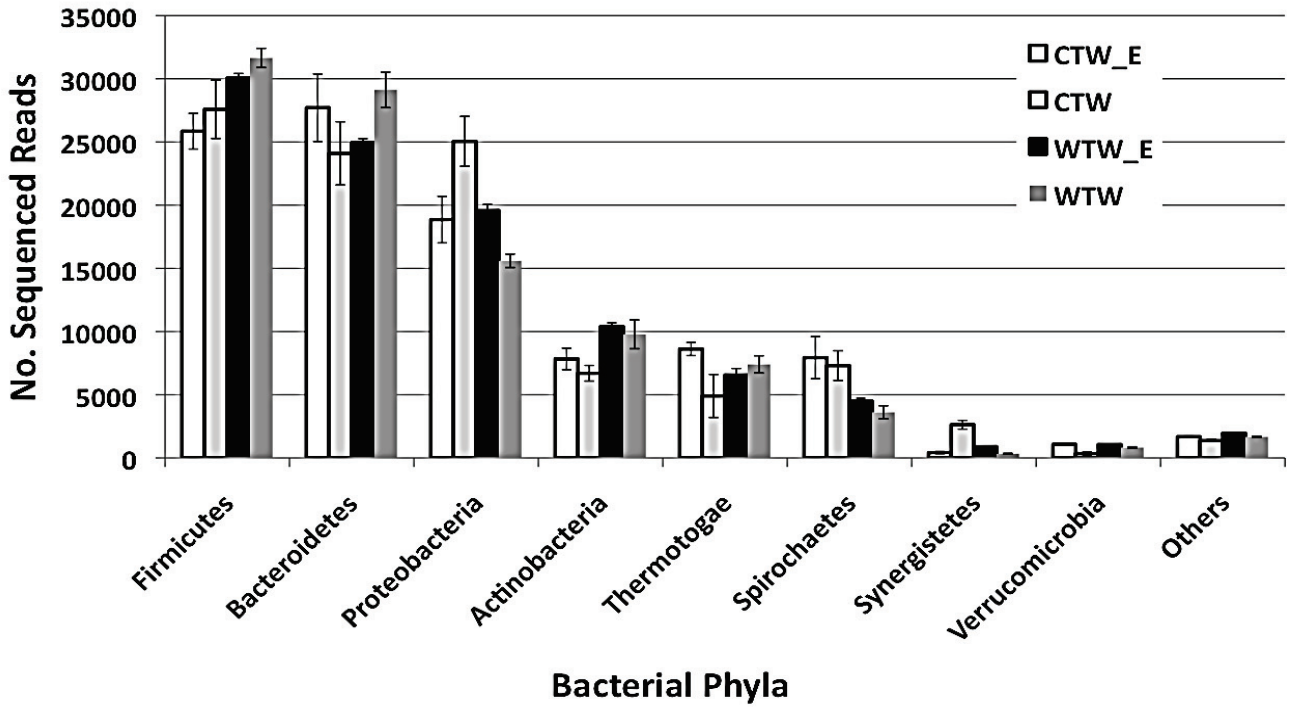

Fig. 3. Mean normalized abundances (untransformed) of bacterial phyla by treatment. Small bars denote ± standard error.

Table 1. PerMANOVA results for the effect of treatment on normalized bacterial phyla abundance.

Source	df	Sum of Squares	Mean Sum of Squares	F-statistic	*p*
Treatment	3	24.50	8.17	11.41	0.0002
Residual	16	11.46	0.72		
Total	19	35.96			

Table 2. Pairwise treatment comparisons for normalized phyla abundance.*

Level vs Level	*t*	*p*
CTW_E vs CTW	3.506	0.007
CTW_E vs WTW_E	2.622	0.008
CTW_E vs WTW	1.876	0.066
CTW vs WTW_E	3.202	0.007
CTW vs WTW	4.039	0.010
WTW_E vs WTW	4.152	0.008

* *p* values were not corrected for multiple hypothesis testing.

counts, Tanaka et al. (2006) found that two of three species of protists (*Pseudotrichonympha grassi, Holomastigoides hartmanni*) disappeared from workers fed low molecular weight carbon diets of cellobiose or glucose with the third species, *Spirotrichonympha leidyi,* barely represented compared to those fed high molecular weight carbon diets of powdered Japanese red pine or cellulose. Using polymerase chain reaction-denaturing gradient gel electrophoresis, similarities in the bacterial community between the low and high molecular weight diets were estimated at only 40% (Tanaka et al., 2006).

By drilling down from phyla to OTUs, species identifications that could separate community shifts associated with disease versus shifts due to differences in diet became clearer. Inspection of the IV for all 306 OTUs by treatment showed ten OTUs that had IV ≥90. Nine of these OTUs were indicator species for CTW while one was an indicator species for WTW_E (Table 3). These species demonstrated both high percent of reads (Table 4) and high percent constancy of presence across the 20 sample units of the indicated treatment (Table 5).

Table 3. Ten OTUs with IV values ≥90 for one of the four treatments.

OTU	Treatment	IV*
OTU_34	CTW	100.0
OTU_35	CTW	100.0
OTU_81	CTW	100.0
OTU_92	CTW	91.8
OTU_120	CTW	90.0
OTU_121	CTW	95.0
OTU_125	CTW	95.0
OTU_142	CTW	95.0
OTU_175	CTW	90.0
OTU_200	WTW_E	100.0

* $p = 0.0002$ for all IV values listed

Table 4. Percent of reads for indicator species found.

	Treatment			
	CTW_E	CTW	WTW_E	WTW
OTU_34	0	100	0	0
OTU_35	0	100	0	0
OTU_81	0	100	0	0
OTU_92	0	97	0	3
OTU_120	0	100	0	0
OTU_121	0	100	0	0
OTU_125	0	100	0	0
OTU_142	0	100	0	0
OTU_175	0	100	0	0
OTU_200	0	0	100	0

Table 5. Percent constancy of presence for indicator species found across sample units within a treatment.

	Treatment			
	CTW_E	**CTW**	**WTW_E**	**WTW**
OTU_34	0	100	0	0
OTU_35	0	100	0	0
OTU_81	0	100	0	0
OTU_92	0	95	0	5
OTU_120	0	90	0	0
OTU_121	0	95	0	0
OTU_125	0	95	0	0
OTU_142	0	95	0	0
OTU_175	0	90	0	0
OTU_200	0	0	100	0

The species identifications of the indicator OTUs along with their isolation sources, as determined by a literature review, appear in Table 6. Of the ten indicator OTUs found, four were harmless (above the bold line), three of which were indicator species for the CTW treatment (*Acinetobacter tjernbergiae, Acetobacter pasteurianus, and Sphaerisporangium rubeum*) and one for the WTW_E treatment (*Planctomyces limnophilus*). One, also an indicator species for CTW, was a probiotic with trade-offs of reduced fitness (Grau et al., 2017) (above the bold line). The remaining five (below the bold line) were all indicator species for the CTW treatment (in bold text) and were associated with human tissue infections (Lee et al., 2015; Nogueira et al., 2015; Tena et al., 2016) or human tissue infections with comorbidities (Renaud et al., 2007; Barahona & Slim, 2015), that is, suffering from more than one chronic disease or condition. It is also important to note that no pathogenic indicator species were detected for any of the other three treatments.

From these results, we infer that the antimicrobial properties of the ingested chitosan caused disease-associated dysbiosis in the termites from the CTW treatment. The shifts in bacterial community of the CTW_E, WTW_E, and WTW treatments, on the other hand, were community changes that led to invasion and establishment of disease-causing pathogens. Moreover, three species, *Mycobacterium abscessus, M. franklinii,* and *Sphingobacterium multivorum,* are ubiquitously found in nature where they are considered to be opportunistic pathogens. Another opportunistic pathogen, which has been documented from dead subterranean termites and shown to cause 100% mortality in forced feeding studies, is *Serratia marcescens* (Osbrink et al., 2001). *S. marcescens* was identified among the 306 OTUs of this study, but it did not meet the criteria to be an indicator species. Although 100% of the reads identified as *S. marcescens* were found in the CTW treatment, it only occurred in 30% of the CTW samples and, thus, had an IV value ≤90.

Table 6. Isolation sources of detected indicator species based on a review of the literature.*

OTU	Phylum	Species	Sources
34	**Proteobacteria**	***Acinetobacter tjernbergiae***	**wastewater treatment**
35	**Proteobacteria**	***Acetobacter pasteurianus***	**plants, industrial microbiology (vinegar, kefir)**
175	**Actinobacteria**	***Sphaerisporangium rubeum***	**sandy soil**
200	Other	*Planctomyces limnophilus*	aquatic environments
142	**Firmicutes**	***Enterococcus mundtii***	**lowers fitness and fecundity when fed to beetle larvae**
81	**Firmicutes**	***Streptococcus lactarius***	**lactational mastitis**
92	**Actinobacteria**	***Corynebacterium hansenii***	**severe thigh liposarcoma**
120	**Actinobacteria**	***Mycobacterium abscessus***	**infections of skin, soft tissue, and lung**
125	**Actinobacteria**	***Mycobacterium franklinii***	**pulmonary disease**
121	**Bacteroidetes**	***Sphingobacterium multivorum***	**lymphoma, peritonitis, septicemia, chronic lung infection**

* Indicator species are in bold font for CTW and regular font for WTW_E. Species associated with human infections and infections with comorbidities appear below the bold line.

The antimicrobial effect of chitosan may not merely be restricted to bacteria. Telmadarrehei et al. (2018) found that in an AWPA E1 single-choice test, chitosan-fed termites exhibited a dramatic loss of taxonomic diversity in the hindgut protist community of *R. virginicus*. Of the ten protist species residing in the hindgut, eight species were lost in termites fed wood treated with 1% and 2% chitosan solutions. The lost protist species were *S. flagellata, Trichonympha burlesquei, Trichonympha agilis, Spironympha kofoidi, Microjoenia* sp., *H. elongatum, Pyrsonympha minor,* and *Dinenympha fimbriata.* The only two species found in the 1% and 2% chitosan treatments were *Monocercomonas* sp. and *Trichomitus trypanoides.* As mentioned earlier, loss of protist species does not necessarily equate with death since termites fed low molecular weight carbon sources survived despite the disappearance of most of their protists (Tanaka et al., 2006). Thus, it would appear that the mortality factor in our chitosan studies was not the lack of protists but, more likely, chitosan-induced dysbiosis, which led to the establishment of pathogenic bacteria.

CONCLUSIONS

1. Data-driven methods to pre-process MiSeq metagenomics data were developed. After pre-processing, the total number of detected OTUs or species dropped from 1722 to 306, which was more in the range of values reported by traditional cloning and sequencing methods.
2. Two-way cluster analysis indicated that (a) phyla diversity of termites fed CTW in a single-choice E1 test was very different from the other 3 treatments, (b) the CTW cluster exhibited lower diversity, and (c) clustering of CTW_E with both control groups (WTW and WTW_E) suggested that chitosan had leached over the 88-day weathering period.
3. All four treatments exhibited significant differences in phyla diversity when examined by PerMANOVA indicating that shifts in the bacterial community had occurred.
4. Indicator species analysis identified five bacteria for the CTW group that were associated with human tissue infections and infections with comorbidities. Three of these species, *Mycobacterium abscessus, M. franklinii,* and *Sphingobacterium multivorum,* are ubiquitously found in nature where they function as opportunistic pathogens.
5. Taken together, these results suggest that the antimicrobial properties of the ingested chitosan caused disease-associated dysbiosis. The shifts in bacterial community of the CTW_E, WTW_E, and WTW treatments, on the other hand, were community changes that were not linked with pathogen invasion.

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DISCLAIMER

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