Effect of Chitosan on Diversity and Number of Protists in Subterranean Termites

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ABSTRACT

Although protist species composition in the hindgut of subterranean termites is known to vary among termite species, less is known about the effects of biocides on protist population dynamics within a single species. The goal of this study was to observe the potential effect of chitosan, an environmentally friendly antimicrobial compound, on protist communities harbored in hindguts of *Reticulitermes virginicus*. Workers of two termite colonies collected from different locations were exposed to treated wood with different concentrations of chitosan (0.5%, 1% and 2%) and two sets of control-treated (water and acetic acid-impregnated) wood specimens over a 14-day period. Protists were dissected from termite hindgut and loaded on a hemocytometer slide to count protist species under a light microscope at 400× magnification. Ten protist species were found in colonies exposed to the control and wood treated with 0.5% chitosan. The coexistence of *Trichonympha agilis* and *T. burlesquei* in *R. virginicus* is reported here for the first time. Only two protist species, *Monocercomonas* sp. and *Trichomitus trypanoides,* survived in colonies exposed to wood treated with higher chitosan concentrations (1% and 2%). The total raw protist counts in these higher chitosan treatments, however, were on average 12× less than in the controls and 0.5% chitosan. The results of this study indicate that chitosan may affect termites by acting on the protist symbionts. The species-specific response of protists to higher concentrations of chitosan can further advance the understanding of chitosan's mode of action.*Key words: chitosan, Reticulitermes virginicus, symbiotic protists, subterranean termites*

INTRODUCTION

Subterranean termites, an economically important wood destroying pest in North America, are lower termites belonging to the family Rhinotermitidae. They are ecologically important, as they contribute to lignocellulose decomposition and carbon recycling (Peterson *et al*., 2006; Hu *et al*., 2011). Termite colonies are divided into different castes and life stages including the workers, soldiers, and reproductives (kings, queens and alates). The workers form the majority of individuals in termite colonies and perform a pivotal role of feeding and lignocellulose digestion (Su *et al*., 2001).

The termite digestive tract (gut) is composed of three main parts: foregut, midgut and hindgut. The hindgut comprises microbial symbionts: bacteria, protists and archaea. Protists in the hindgut are anaerobic unicellular eukaryotes responsible for hydrolysis of cellulose, endocytosis and fermentation activities. The effectiveness of lignocellulose digestion in the termite gut relies upon collaboration between the host enzymes and microbial hindgut symbionts (Brune, 2014). The microbial symbionts are unevenly distributed through the termite hindgut and localized in distinct niches (Ohkuma, 2003).

The termite hindgut protist species are mainly classified into three orders: Oxymonadida (phylum Preaxostyla), Trichomonadida (phylum Parabasalia), and Hypermastigida (phylum Parabasalia). Species identification is based on host specificity and cell morphology, such as size, shape, flagellar number, axostyla, and the presence of an undulating membrane (Kirby, 1937; Honigberg, 1963). Cook and Gold (2000) discovered six protists in *Reticulitermes virginicus,* and identified them as *Dinenympha fimbriata* Kirby and *Pyrsonympha minor* Powell, both of which are in the order Oxymonadida, *Holomastigotes elongatum* Grassi, *Spironympha kofoidi* (Dubosq and Grassé), *Spirotrichonympha flagellata*, and *Trichonympha agilis* Leidy belong to order Hypermastigida. Lewis and Forschler (2004a, 2006) described nine protist species in *R. virginicus*, including three new genera reported for the first time *Microjoenia* (order Hypermastigida)*, Monocercomonas* (order Trichomonadida), and *Trichomitus* (order Trichomonadida).

Although the same protist community exists within a single species of termite, the frequency of protist species can vary among its castes (Huntenburg *et al*., 1986; Mannesmann, 1972; Lo Pinto *et al*., 2016). Additionally, factors such as geographical regions, diet, season, temperature, moisture, and starvation can influence protist relative abundance (Belitz and Waller, 1998;

Lo Pinto *et al*., 2016). Tang *et al*. (2018) found that dysbiosis or microbial imbalance of hindgut bacteria occurred after termites were exposed to a non-toxic, environmentally friendly chemical known as chitosan. Chitosan is a heterogeneous long-chain aminopolysaccharide of glucosamine and N-acetylglucosamine. The antimicrobial activity of chitosan on decay fungi has been studied (Alfredsen *et al*., 2004), but there is little information regarding the effects of chitosan on termite protists. Therefore, the aims of this study were to investigate effect of chitosan on the relative abundance of protist species and their diversity in termites exposed to chitosan treated wood.

MATERIALS AND METHODS

Termite species identification

Two colonies of *Reticulitermes* were collected from United States Department of Agriculture, Forest Service Harrison Experimental Forest, Saucier, Mississippi (Colony 1) and the other from Mississippi State University Dorman Lake Test Site, Starkville, MS (Colony 2). Both colonies were collected in May 2015, 10 days apart. Each colony came from one infested log. Each log was subsequently cut into smaller sections and placed into a 32-gallon metal container, covered, and brought back to the laboratory, where they were maintained at 24°C with adequate moisture in darkness and used within 6 months of collection. The Messenger (2001) identification guide was used to confirm termite species.

Wood sample preparation and treatment with chitosan

Defect free southern yellow pine samples with dimensions of 25 × 25 × 6 mm (tangential x radial x longitudinal) were chitosan-treated and used to test resistance of subterranean termites to chitosan according to the American Wood Protection Association (AWPA) E1-16 Standard (AWPA, 2016). Wood samples were oven-dried at 50°C to reach a constant weight and then treated with 0.5%, 1%, and 2% w/v solution of low molecular weight (50 – 190 kDA, Sigma-Aldrich) chitosan dissolved in water containing 25% acetic acid. In addition, 25% acetic acid and water-treated wood samples were prepared as controls. All treatments were performed under 29.8 mm Hg vacuum.

Termite no-choice exposure laboratory bioassay

The no-choice test was performed according to a modified American Wood Protection Association E1-16 Standard (AWPA, 2016). A total of 35 glass screw-top jars (8 cm dia, 10 cm tall) containing 120 g play sand (Quikrete Premium Play Sand) and 35 ml distilled water each were autoclaved for 45 minutes. Upon cooling, one test wood block and 0.5 g of termites, which contained approximately 150 workers and 2 soldiers, were added to each jar. Termites from Colony 1 were exposed to 4 wood replicates from each treatment, and termites from Colony 2 were subjected to 3 wood replicates due to the lower number of termites available.

Termite hindgut dissection and protists visualization

Fifteen termites from each replicate jar were collected after a 14-day exposure to the wood samples in order to estimate the protist counts per replicate jar. Three subsamples, composed of five termite hindguts each, were prepared for protist visualization. Two sharp forceps were used for hindgut extraction. One of the forceps grabbed the region that connected the termite head to thorax and the other gently pulled the tip of the abdomen in order to free the digestive tract from the surrounding exoskeleton. The forceps were also used to tease open the hindgut and release its contents in 100 µl of Trager U saline solution (Trager, 1934). Before the dissection, the saline was sparged with 99.99% nitrogen gas for 2 minutes. This method was a modification of sparging procedure of Lewis and Forschler (2004b), who used a nitrogen gas mixture (92.5% N_2, 2.5% H_2, and 5% CO_2) that was introduced into 6 ml Trager U saline at 1 liter per min for 5 min. Three aliquots of 10 µl of the hindgut-saline mixture were loaded onto Neubauer gridded cell-counting slide (hemocytometer) and analyzed separately under a Nikon Eclipse E600 microscope at 400X magnification. The pictures were taken by a ProgRes SpeedXT core 5 Microscope Camera. Protist species were identified according to their morphology using a nondichotomous key published by Lewis and Forschler (2006). All protist counts were made systematically. The protist cells were counted from four large squares and a central square of the hemocytometer, containing a total 0.5 µl of the solution. Cells touching the left line and the bottom line of the squares were not counted. The counts of each species in the observed area were recorded and used to estimate the abundance of protists from five termites in the 100 µl of Trager U saline solution using the following equation:

$$N_i = \frac{n_i \,.\, 100\ \mu l}{0.5\ \mu l} \qquad (1)$$

where N_i is the protist count estimated for 100 µl Trager U saline solution and n_i is the actual count in 0.5 µl of solution.

The average count (N_r) in 100 µl of the solution was calculated as:

$$N_r = \frac{\sum_{i=1}^{3} N_i}{3} \quad (2)$$

where *i* refers to the number of aliquots counted.

These values were then used to calculate the mean count of protists per replicate jar (R):

$$R = \frac{\sum_{r=1}^{n} N_r}{n} \quad (3)$$

where *n* is jar number, and equals to 4 for Colony 1, and 3 for Colony 2. Statistical analysis was performed on the mean counts of protists per replicate.

Multivariate statistical analysis

Differences in diversity of protist species among the treatments were compared using PC-ORD 6, a statistical package for multivariate analysis of ecological communities (McCune and Grace 2002; Peck 2010). Raw counts were normalized by calculating the relative proportion of each protist species in each sample unit and then multiplying by 25,000. Henceforth, normalized relative abundance will be referred to simply as relative abundance. In order to determine a suitable distance measure for some of the analyses, PC-ORD's Advisor tool was used on both untransformed and transformed relative abundance data to identify presence and absence of outliers that were more than two standard deviations from the mean. Two-way cluster analysis grouped sample units based on similarity of protist relative abundances and grouped protists based on similarity of their relative abundance across sample units. Another group testing method known as PerMANOVA, which is a permutation-based non-parametric multivariate analysis of variance (MANOVA), was used. One-way PerMANOVA determined whether there was a significant effect of treatment on relative abundance of protist species at p value < 0.05 using 4999 permutations. If treatment was significant, a pairwise comparison analysis was then performed. False discovery was not corrected in the pairwise analysis. A species analysis was run to describe protist distribution in terms of both percent relative abundance and percent relative constancy across replicates within a treatment.

RESULTS AND DISCUSSION

Termite species identification

Termite species were identified morphologically using the guide by Messenger (2001). Both of colonies were confirmed to belong to *R. virginicus*.

Termite hindgut dissection and protists visualization

Ten flagellate species were observed in hindguts of both *R. virginicus* colonies exposed to 0.5% chitosan treatment and controls. These were identified as *S. flagellata*, *T. agilis*, *T. burlesquei*, *S. kofoidi*, *Microjoenia* sp., *Monocercomonas* sp., *H. elongatum*, *T. trypanoides*, *D. fimbriata*, and *P. minor*. Some of them are shown in **Fig. 1.** In higher concentrations of chitosan treatment, only two protist species were observed, *Monocercomonas* sp. and *T. trypanoides*. All termites exposed to 1% and 2% chitosan-treated wood died after 16 days. Lewis and Forschler (2004a, 2010) detected nine of the same protist species in *R. virginicus*. The tenth species identified in our study was *T. burlesquei*. This species has also been reported in *R. virginicus* by James *et al*. (2013). The coexistence of *T. agilis* and *T. burlesquei* in *R. virginicus* is reported here for the first time.

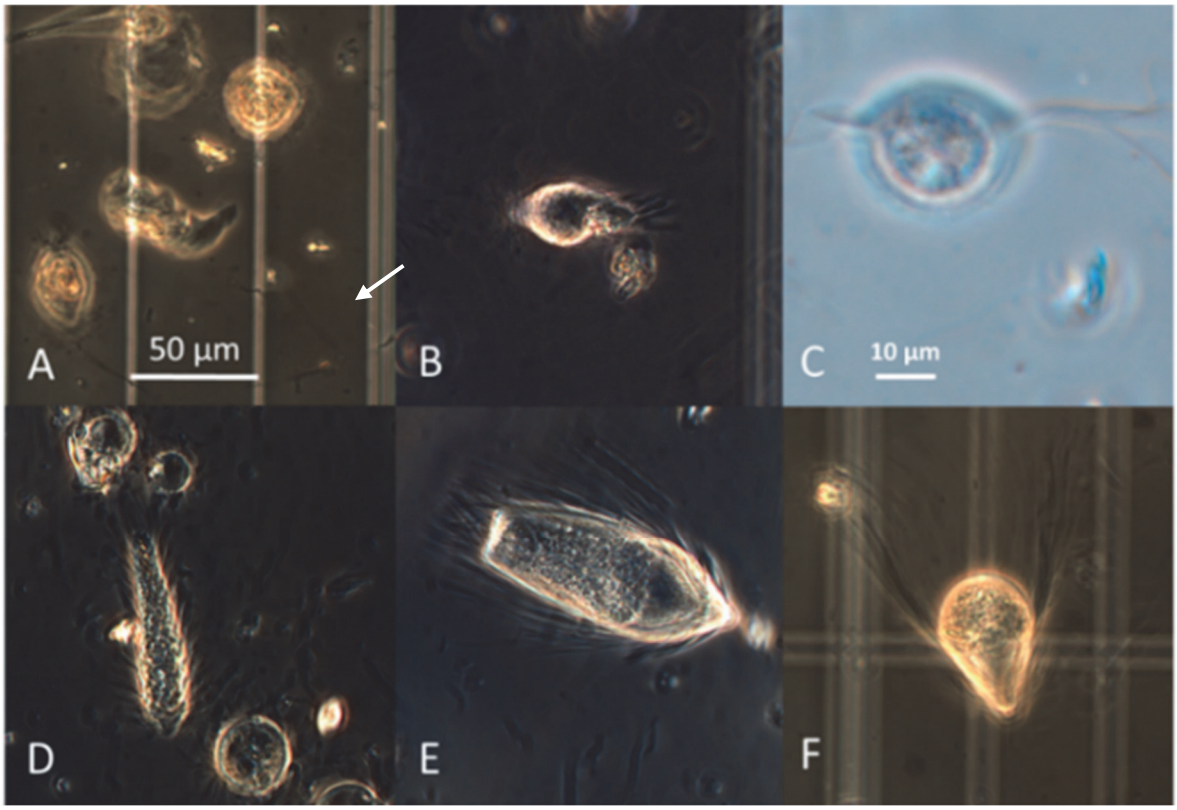

Fig. 1. Phase-contrast light microscopy of some of the protist species from *R. virginicus*; A) *P. minor*; B) *S. flagellata*; C) *Monocercomonas* sp.; D) *H. elongatum*; E) *T. agilis*; F) *T. burlesquei*. Scale bar for B, D, E and F same as in A.

Multivariate statistical analysis

Natural log transformation or ln (x+1) was determined to improve normality of protist counts. The chi-squared distance measure eliminated outliers in both Colony 1 and 2 datasets during two-way cluster analysis on the transformed relative abundance data. For Colony 1, the sample units formed two major clusters (**Fig. 2A**). One cluster included control (water and 25% acetic acid) and 0.5% chitosan treatments, where the 0.5% chitosan treatment lay on a separate branch from the control treatments. The second cluster included the 1% and 2% chitosan treatments with no difference between them (**Fig. 2A**). Protist species also fell into two major clusters (**Fig. 2B**). One cluster contained *Monocercomonas* sp. and *T. trypanoides,* while the second cluster was comprised of the remaining eight protists. The degree of shading of the cells in the heat map is a gross measure of protist relative abundance with darker shades corresponding to greater relative abundance. *S. flagellata*, *T. agilis*, *T. burlesquei*, *Microjoenia* sp., *H. elongatum*, and *S. kofoidi* showed similar relative abundance for the termites exposed to water, acetic acid, and 0.5% chitosan-treated wood. In addition, these species were absent in termites exposed to 1% and 2% chitosan-treated wood. *P. minor* and *D. fimbriata* were not present in the higher chitosan treatments and they were

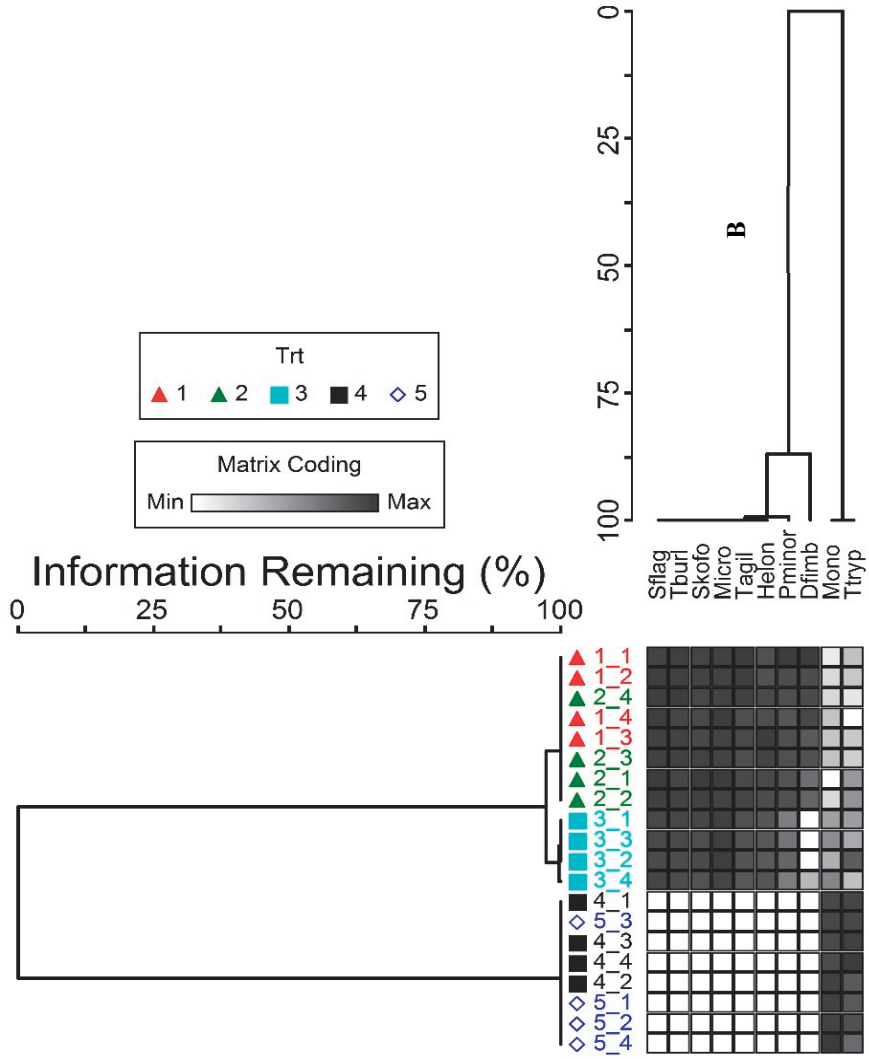

Fig. 2. Two-way cluster analysis of sample units (A) and protist species (B) based on relative abundance of protists in Colony 1. Treatments (Trt): water; 2, 25% acetic acid; 3, 0.5% chitosan; 4, 1% chitosan; 5, 2% chitosan. Sflag, *S. flagellata*; Tburl, *T. burlesquei*; Skofo, *S. kofoidi*; Micro, *Microjoenia* sp.; Tagil, *T. agilis*; Helon, *H. elongatum*; Pmino, *P. minor*; Dfimb, *D. fimbriata*; Mono, *Monocercomonas* sp.; Ttryp, *T. trypanoides*.

disproportionately lower in the 0.5% chitosan treatment compared to the control treatments (water and 25% acetic acid). On the other hand, *Monocercomonas* sp and *T. trypanoides* were observed in all chitosan treatments.

For Colony 2 sample units separated into two major clusters (**Fig. 3A**). One branch contained control (water and 25% acetic acid) and 0.5% chitosan treatments and other was composed of 1% and 2% chitosan treatments. Protists formed two major clusters, one branch included *Monocercomonas* sp. and *T. trypanoides* while the second branch contained the remaining eight protist species (**Fig. 3B).**

Small differences were found in the sub-branching of the sample units and species composition clusters when comparing Colonies 1 and 2 (**Fig. 2** and **3**). These differences were caused by a lower relative abundance of *D. fimbriata* in 0.5% chitosan compared to all controls in Colony 1 (shown by the lighter shading in the heat map of **Fig. 2**) than Colony 2 (**Fig 3**). Factors that could contribute to these small differences could be the different geographic origins, different dates of collection, or other inherent physiological, and genetic differences between the two colonies. In addition, the number of replicates for Colony 2 was lower than Colony 1.

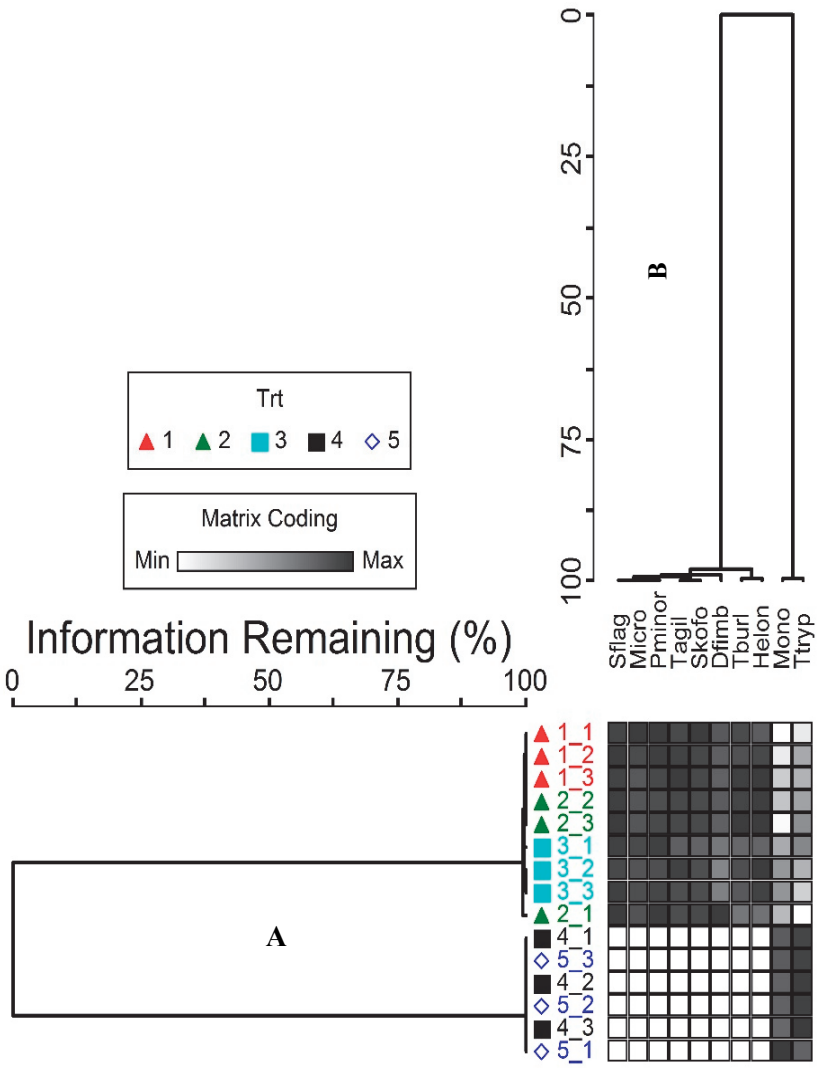

Fig. 3. Two-way cluster analysis of sample units (A) and protist species (B) based on relative abundance of protists in Colony 2. Treatments (Trt): 1, water; 2, 25% acetic acid; 3, 0.5% chitosan; 4, 1% chitosan; 5, 2% chitosan. Sflag, *S. flagellata*; Micro, *Microjoenia* sp.; Pmino, *P. minor*; Tagil, *T. agilis*; Skofo, *S. kofoidi*; Dfimb, *D. fimbriata*; Tburl, *T. burlesquei*; Helon, *H. elongatum*; Mono, *Monocercomonas* sp.; Ttryp, *T. trypanoides*.

In both colonies, *Monocercomonas* sp. and *T. trypanoides* were the only two protists surviving when termites were exposed to wood treated with 1% and 2% chitosan (**Fig. 2B** and **3B**). The total raw protist counts in these two treatments, however, were on average 12× lower than in the controls and 0.5% chitosan. These two species unlike the others do not exhibit vacuoles containing wood particles inside their cytoplasm and are not known to have cellulolytic functions (Boykin *et al.,* 1986; Huntenburg *et al.,* 1986; Brugerolle *et al.,* 2003). They are classified as saprophytic flagellates that survive on byproducts produced by other microbes and phagocytosis of bacteria (Brugerolle *et al.,* 2003). It is possible that these two species survived in our study because they did not ingest the wood fragments impregnated with chitosan. PerMANOVA with the chi-squared distance measure on the transformed relative abundance data showed that there was a highly significant effect of treatment on protist diversity for both colonies

Table 1. The effect of treatment on relative abundance of protists for both Colonies using PerMANOVA

Source	df	Sum of Squares	Mean Sum of Squares	F-statistic	*p*
Colony 1					
Treatment	4	1.26E-02	3.16E-03	1310.4	0.0002
Residual	15	3.61E-05	2.41E-06		
Total	19	1.27E-02			
Colony 2					
Treatment	4	1.23E-02	3.07E-03	728.01	0.0004
Residual	10	4.22E-05	4.22E-06		
Total	14	1.23E-02			

Table 2 displays the results of pairwise comparisons. For Colony 1, all pairwise comparisons were significantly different except for water vs 25% acetic acid and 1% chitosan vs 2% chitosan. These results indicate that protist diversity was significantly different between controls and all chitosan treatments and between 0.5% and higher percent chitosan treatments. For Colony 2, however, none of the pairwise comparisons were significant. The possible reason that Colony 2 failed to show any significant results for the pairwise comparison tests could be due to the fewer number of replicates and lower power compared to Colony 1. It is important to keep in mind that PerMANOVA and pairwise comparison analysis are different tests addressing different questions. Therefore, it is possible for the PerMANOVA to show a significant effect of treatment without any significant pairwise comparisons.

Table 2. Pairwise comparisons for factor treatments

Level vs Level	Colony 1		Colony 2	
	t	*p*	*t*	*p*
water vs 25% acetic acid	1.338	0.173	0.657	0.805
water vs 0.5% chitosan	7.082	0.028	1.555	0.200
water vs 1% chitosan	76.36	0.0346	54.996	0.098
water vs 2% chitosan	75.784	0.029	47.944	0.096
25% acetic acid vs 0.5% chitosan	6.35	0.029	1.423	0.195
25% acetic acid vs 1% chitosan	75.293	0.026	30.084	0.103
25% acetic acid vs 2% chitosan	74.737	0.028	28.731	0.095
0.5% chitosan vs 1% chitosan	33.923	0.031	43.616	0.098
0.5% chitosan vs 2% chitosan	33.866	0.029	39.673	0.099
1% chitosan vs 2% chitosan	1.641	0.171	1.187	0.303

**p* values were not corrected for multiple comparisons

Factors such as starvation have been shown to affect diversity of protists in termite hindgut. Several studies have reported temperature, flooding, and human activity as causes of termite starvation and decreased rate of metabolism (Forschler and Henderson, 1995; Marron *et al.*, 2003; Hu *et al*., 2011). Hu *et al.* (2011) found that starvation for forty days of *R. flavipes*

workers resulted in the reduction of protists to just five surviving species. Based on count numbers, two species (*T. trypanoides*, S. *flagellata*) were not affected by starvation, *D. fimbriata* and *S. kofoidi* were adversely affected by starvation, while *Monocercomonas* sp. proliferated.

Seasonal changes and insecticide can also affect protist diversity. Overall counts of protists in workers of *R. lucifugus* increased in summer with *S. flagellata* and *T. agilis* being the only species resistant to change (Lo Pinto *et al.*, 2016). Lewis and Forschler (2010) examined the influence of five commercial termite baits composed of chitin synthesis-inhibiting insecticide on protists in *R. flavipes*. Total protist population was reduced by ≥ 30% after termites were exposed to each treatment for three days. The most affected protist species were *D. fimbriata*, *D. gracilis*, *Microjoenia fallax*, *Pyrsonympha vertens*, and *T. agilis*.

Tables 3 and 4 show percent relative abundance of protists by treatment. In Colony 1 (**Table 3**), all species exhibited 10-64% relative abundance for the control (water and 25% acetic acid) and 0.5% chitosan treatments except for *D. fimbriata,* which had 0% relative abundance for the 0.5% chitosan treatment. At the 1% and 2% chitosan treatments, all species showed 0% relative abundance except for *Monocercomonas* sp. and *T. trypanoides,* which ranged from 28-32% relative abundance. In Colony 2 (**Table 4)**, similar results were found except all species had 8-61% relative abundance for control and 0.5% chitosan treatments, and at the higher chitosan treatments, all species showed 0% relative abundance except for *Monocercomonas* sp. and *T. trypanoides*, which showed 25-38% relative abundance.

Table 3. Percent relative abundance of the ten protist species by treatment for Colony 1

Protist species*	Water	25%acetic acid	0.5% chitosan	1% chitosan	2% chitosan
Sflag	36	38	26	0	0
Tagil	42	37	22	0	0
Tburl	36	38	26	0	0
Micro	34	33	33	0	0
Mono	11	11	17	30	31
Helon	38	40	22	0	0
Skofo	33	40	26	0	0
Pminor	51	38	10	0	0
Dfimb	64	36	0	0	0
Ttryp	10	13	17	32	28

* Sflag, *S. flagellata*; Tagil, *T. agilis*; Tburl, *T. burlesquei*; Micro, *Microjoenia* sp.; Mono, *Monocercomonas* sp.; Helon, *H. elongatum*; Skofo, *S. kofoidi*; Pmino, *P. minor*; Dfimb, *D. fimbriata*; Ttryp, *T. trypanoides*

Table 4. Percent relative abundance of the ten protist species by treatment for Colony 2

Protist species	Water	25%acetic acid	0.5% chitosan	1% chitosan	2% chitosan
Sflag	33	38	30	0	0
Tagil	42	31	27	0	0
Tburl	39	40	21	0	0
Micro	44	24	32	0	0
Mono	13	15	19	25	27
Helon	32	34	34	0	0
Skofo	44	33	23	0	0
Pminor	38	31	31	0	0
Dfimb	31	61	8	0	0
Ttryp	9	10	11	38	33

* Sflag, *S. flagellata*; Tagil, *T. agilis*; Tburl, *T. burlesquei*; Micro, *Microjoenia* sp.; Mono, *Monocercomonas* sp.; Helon, *H. elongatum*; Skofo, *S. kofoidi*; Pmino, *P. minor*; Dfimb, *D. fimbriata*; Ttryp, *T. trypanoides*

Percent constancy of protist presence for both colonies appears in **Table 5**. Values of 0 and 100% constancy mean that the protist was present in 0 and 100% of the sample units within a given treatment. At the two control and 0.5% chitosan treatments, all protist species exhibited 100% constancy for all treatments except *D. fimbriata* in Colony 1, which was present in 25% of the 0.5% chitosan sample units. At the 1% and 2% chitosan treatments, no protists were found in any of the sample units (0% constancy) except for *Monocercomonas* sp. and *T. trypanoides*, which were present in all sample units (100% constancy). Combining these results for percent relative abundance and percent constancy, it was evident that there were no obvious indicator species, *i.e.* a protist showing both high percent relative abundance in a single treatment and high percent constancy (present in all sample units) of the same treatment.

Table 5. Percent constancy of protist presence across sample units within a treatment for Colonies 1 and 2

Colony 1						Colony 2					
Protist species	**Water**	**25%acetic acid**	**0.5% chitosan**	**1% chitosan**	**2% chitosan**	**Protist species**	**Water**	**25% acetic acid**	**0.5% chitosan**	**1% chitosan**	**2% chitosan**
Sflag	100	100	100	0	0	Sflag	100	100	100	0	0
Tagil	100	100	100	0	0	Tagil	100	100	100	0	0
Tburl	100	100	100	0	0	Tburl	100	100	100	0	0
Micro	100	100	100	0	0	Micro	100	100	100	0	0
Mono	100	100	100	100	100	Mono	100	100	100	100	100
Helon	100	100	100	0	0	Helon	100	100	100	0	0
Skofo	100	100	100	0	0	Skofo	100	100	100	0	0
Pminor	100	100	100	0	0	Pminor	100	100	100	0	0
Dfimb	100	100	25	0	0	Dfimb	100	100	100	0	0
Ttryp	100	100	100	100	100	Ttryp	100	100	100	100	100

* Sflag, *S. flagellata*; Tagil, *T. agilis*; Tburl, *T. burlesquei*; Micro, *Microjoenia* sp.; Mono, *Monocercomonas* sp.; Helon, *H. elongatum*; Skofo, *S. kofoidi*; Pmino, *P. minor*; Dfimb, *D. fimbriata*; Ttryp, *T. trypanoides*

Raji *et al*. (2018) reported different levels of mortality of termite species to chitosan-treated wood. *R. flavipes* was more tolerant showing less than 50% mortality to wood treated with 0.5 and 1% chitosan and more than 90% mortality to wood treated with 1 to 5% chitosan. For *R. virginicus,* wood treated with all concentrations of chitosan produced 100% mortality. Raji *et al.* (2018), however, did not examine the likely causes of mortality. Tang *et al*. (2018) found that bacterial imbalance in *R. flavipes* fed chitosan-treated wood led to the establishment of three opportunistic pathogens (*Mycobacterium abscessus*, *M. franklinii*, and *Sphingobacterium multivorum*), and hypothesized that the pathogens were the causal agents of mortality. Even though the bacterial community was not examined in this study, it was determined that chitosan caused a drastic imbalance in protist diversity. Disappearance of eight protist species would have also eliminated their resident ecto- and endosymbiotic bacteria. Bacterial ectosymbionts from at least two different lineages have been found attached to exterior surfaces of the protist cell membrane. Spirochetes in the genus *Treponema* have been reported as ectosymbionts of *Dinenympha* and *Pyrsonympha* (Iida *et al*., 2000) and a new species of *Candidatus* from the order Bacteroidales has been found in both parabasalids and oxymonads (Hongoh *et al*., 2007). In the case of endosymbionts, the candidate genus "*Endomicrobia*" belonging to TG-1 or termite group I phylum were detected in the cytoplasm of both *T. agilis* and *Pyrsonympha vertens* (Stingl *et al*., 2005). Thus, several factors such as the lack of protist species, lack of the bacteria associated with protists, overall microbial imbalance, and/or antimicrobial action of chitosan could all have contributed to the observed termite mortality.

CONCLUSION

Analysis of two *R. virginicus* colonies showed the presence of ten protist species in hindguts of termites exposed to control (water and 25% acetic acid) and 0.5% chitosan-treated wood. Protist diversity, which is a function of species richness or number of species and relative abundance, however, was reduced in termites fed wood treated with higher concentrations of chitosan. Two-way cluster analysis of protist diversity showed that treatments fell into two groups: one group included controls and the 0.5% chitosan treatment, while the other was composed of the higher chitosan treatments (1 and 2%). Cluster analysis also partitioned the protists into two groups: those that survived at the higher concentrations of chitosan and those that did not. Eight of the protist species died in the 1 and 2% chitosan treatments, while *Monocercomonas* sp. and *T. trypanoides* were the only species that survived. Their raw counts, however, were reduced by 12× compared to controls and the 0.5% chitosan treatment. Factors that may have aided their survival are their lack of cellulolytic functions and vacuolar digestion of treated wood fragments. PerMANOVA determined that there was a significant effect of treatment on protist diversity for both colonies, although only Colony 1 showed significant differences in pairwise treatment comparisons. Controls were significantly different from all chitosan treatments and 0.5% chitosan was significantly different from the higher chitosan treatments. These results were consistent with the treatment groupings observed in the two-way cluster analysis. These results support the hypothesis that toxicity of chitosan is most likely due to the microbial imbalance caused by the missing protists and their resident endo- and ectosymbiotic bacteria.

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