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Study of Subterranean Termite Gut Symbionts as Affected by Chitosan Treatment of Wood

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The overall aim of this study was to investigate the potential influence of chitosan, a biodegradable and antimicrobial compound, on termite hindgut symbionts. For this purpose, a morphological quantifying technique was conducted on the protist community's hindgut after feeding termites on chitosan-treated wood. The aim was to characterize the diversity of protist species in the economically important dark southern subterranean termite, *Reticulitermes virginicus*. A molecular phylogenetic analysis of the V3 and V4 hyper-variable regions of 16S ribosomal RNA (rRNA) gene of the bacterial community in the hindgut of *R. virginicus* was performed on termites exposed to chitosan treatment.

Light microscopy visualization of protist species residing in the hindgut of workers showed presence of ten protist species both in the control sample and in termites fed a low concentration of chitosan. In this study, the coexistence of two species of the genus *Trichonympha* (*T. agilis* and *T. burlesquei*) is reported for the first time in *R. virginicus*. *Monocercomonas* sp. and *Trichomitus trypanoides* were the only two protists found in termites exposed to wood treated with higher chitosan concentration solutions

and the absence of wood fragments in their food vacuoles was clear. The results of this study indicated that the potential effect of chitosan caused elimination of the protist species in termite hindguts.

The genomic DNA of bacterial hindgut community of *R. virginicus* were profiled using sequences which amplified theV3-V4 sub-regions of 16S rRNA gene. Sequences were analyzed using a taxonomic analysis tool, Quantitative Insights Into Microbial Ecology (OIIME 2), in order to infer the effect of chitosan on the composition of the bacterial fauna in the hindgut. The richness and evenness results indicated that the most diversity was observed in the bacteria from termites not being exposed (UNX) to treatment compared to other treatment groups. On the other hand, the lowest richness and evenness were determined for chitosan-treated wood (CTE) and starved termites (STV). Of 28 bacterial phyla, Bacteroidetes, Firmicutes, Elusimicrobia, and Proteobacteria were the most dominant phyla across all the treatment groups. The results suggest that chitosan treated wood led to the microbial community shifts in *R. virginicus*.

Keywords: chitosan, *Reticulitermes virginicus*, protist diversity, hindgut bacteria, 16S rRNA gene, Illumina amplicon sequencing

DEDICATION

I dedicate this work to my beloved husband, Amir Rezazadeh, and parents, Maziar Telmadarrehei and Zohreh Nasiri. Without their love and support, this work would not have been possible. They have sacrificed a great deal of time and energy supporting me during my entire education. I would also like to dedicate my dissertation to my kind sister, Ghazaleh Telmadarrehei, and brother, Amin Nemati, for their encouragement.

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CHAPTER I

INTRODUCTION

1.1 Wood

Hardwoods (Angiospermae) and softwoods (Gymnospermae) are two broad classes of trees employed as raw materials in many industries and building construction. In the United States, softwood species are dispersed across the country. Pine is a popular species among softwoods for many construction projects. The high availability, treatability, strength, stiffness, and relatively affordability of pine species make them a preferred wood resource for construction (Wiemann, 2010).

Wood consists of cellulose, hemicellulose, lignin, and extractive compounds. Cellulose and hemicellulose are long carbohydrate molecules and, along with lignin, make up structural components of wood. Various wood species have differences in chemical composition. Pine has visually distinctive sapwood and heartwood and can be differentiated based on anatomical structure and chemical composition (Waliszewska *et al*., 2015).

There are disadvantages for using wood materials in construction. Fungi, insects, marine organisms and weathering conditions can shorten service life. Protection of wood involves mainly the use of preservative chemicals. Some of the preservatives may contribute to accumulation of chemicals in the environment, and may eventually reach harmful levels to certain species. For instance, decreased biodiversity in aquatic

organisms is the result of high levels of heavy metals in water such as copper, which is the major component of wood preservatives (Tarras-Wahlberg *et al*., 2001). Therefore, scientists have examined natural polymers and organic biocides that could potentially substitute for toxic biocides in wood preservatives in order to reduce damage to the ecosystem. A natural polymer that may serve as a potentially viable alternative to copper in wood preservation is chitosan (Liibert *et al*., 2011).

1.2 Chitosan

Chitin is natural polymer with a unique structure and function. It is a linear polymer of *N*-acetyl-D-glucosamine units linked by β-(1→4) glycosidic bonds. Chitin is biosynthesized as the most abundant homo-polysaccharide polymer in nature, and is a component of the integuments of insect, other arthropod's exoskeletons, shells of crustaceans, fungi and algae cell walls (Flach *et al*., 1992). It is commercially isolated from different sources, mainly as the outer exoskeleton of arthropods (including crustaceans and insects), marine diatoms, algae, fungi, and yeasts (Tharanathan and Kittur, 2003; Raafat and Sahl, 2009). To isolate chitin from crustacean shells, proteins and calcium carbonate is removed by deproteinization in a hot alkaline solution (sodium or potassium hydroxide) and demineralization with diluted acid. Afterwards, chitin is processed in concentrated sodium hydroxide (NaOH) to yield chitosan with different degrees of deacetylation and molecular weights (Synowiecki and Al-Khateeb, 2003). Instead of obtaining chitosan from the deacetylation of chitin, an eco-friendly method can be used to produce microbiological chitosan by growing a fungus species of Zygomycetes class in low cost culture media. The advantage of this method is the reduced acidic and basic residues (Batista *et al*., 2013). In addition to fungal-sourced

chitosan, it can also be produced by enzymatic hydrolysis, an alternative method to chemical isolation, which has been explored during the past few decades (Jung and Park, 2014).

Chitosan is a heterogeneous long-chain amino polysaccharide of D-glucosamine and *N*-acetyl-D-glucosamine linked by β-(1→4) glycosidic bonds. It is found in the cell wall of Zygomycetes fungi, Chlorophycean algae, and in insect cuticle (Hsu *et al*., 2012). Chitosan is soluble in dilute aqueous acid solutions including acetic and formic acids and it is insoluble in water and most organic solvents (Kumar, 2000). Chitosan's solubility relies upon its biological source, molecular weight, and degree of acetylation (Goy *et al*., 2009). Several studies on chemical modifications of chitosan were performed to improve its solubility and increase variety of its applications (Park and Kim, 2010; Zhang *et al*., 2010). Chitosan can be used either alone or in combination with other natural polymers. Additionally, it can be processed into different products such as flakes, fine powders, beads, fibers, membranes, sponges, cottons, and gels (Badawy and Rabea, 2011). The high biocompatibility, biodegradability, non-toxicity, antimicrobial, and adsorption properties of this natural polymer display its unique biological characteristics, making it valuable choice for many applications in food, pharmaceutical, biomedical, textile, agriculture, water treatment, and cosmetic industrial areas (Raafat and Sahl, 2009). Moreover, high-heat chitosan treatment of hardwood boards improved physical and mechanical properties of wood (Basturk, 2012).

Antimicrobial activity of chitosan has been demonstrated against bacteria, fungi, yeasts, insects, and subterranean termites (Raafat and Sahl, 2009; Badawy and El-Aswad, 2012; Raji *et al*., 2018). In spite of the high antimicrobial activities, it has lower toxicity

to mammalian cells and non-target organisms. Many factors such as microorganism species, pH, presence or absence of metal cations, pKa, molecular weight, and degree of deacetylation of chitosan influence the antimicrobial activities of chitosan (Kong *et al*., 2010). The effectiveness of the antimicrobial activity of chitosan is dependent on its molecular weight showed variable results. Some studies exhibited increased antimicrobial activity of high molecular weight chitosan in comparison to low molecular weight chitosan, while other studies showed opposite relationship (Meng *et al*., 2010; Kim and Rajapakse, 2005). For example, larval mortality and growth inhibition in cotton leafworm (*Spodoptera littoralis*) increased by exposure to lower molecular weight chitosan (Badawy and El-Aswad, 2012), while chitosan with high molecular weight was more efficient against wood decay fungi (Eikenes *et al*., 2005).

Chitosan is used as an alternative pesticide against some agricultural and ornamental pests. For instance, the efficacy of chitosan as an insecticide has been studied on cotton leafworm and oleander aphid (Badawy and El-Aswad, 2012). Many studies investigated antimicrobial properties of chitosan against plant pathogens, but minimal research has explored its properties against forest pathogenic, wood-inhabiting, and wood-decaying fungi (Laflamme *et al*., 1999; Alfredsen *et al*., 2004).

The researchers believe that chitosan can inhibit the fungal growth as a fungistatic agent, and acts as fungicide at higher concentrations (Reddy *et al*., 1998). Chitosan was shown to inhibit the fungal growth and the toxin produced by *Alternaria alternate* f. sp. *lycopersici* and *Aspergillus flavus*. Although chitosan at sub-lethal concentrations did not affect fungal mycelia growth, the toxin production was lower (Reddy *et al*., 1998; Cuero *at al*., 1991). The antifungal actions of chitosan at 1% (w/v) in nutrient agar medium

entirely inhibited the growth of brown rot (*Poria placenta* and *Coniophora puteana*) and white rot fungi (*Coriolus versicolor*). Moreover, 4.8% (w/v) chitosan in the impregnation solution was found to be the optimal preservative concentration for wood protection against brown rot fungi, with higher molecular weight of chitosan being more efficient against wood decay fungi (Eikenes *et al*., 2005).

Chitosan is considered to have bactericidal (killing bacteria) or bacteriostatic (inhibiting bacterium growth) properties, but the exact mechanism is still not completely known. Several factors are discussed regarding its antibacterial activity. Three models of the antimicrobial action have been suggested for chitosan in bacteria: 1) interaction between positively charged chitosan and negatively charged microbial cell membrane; 2) binding chitosan with microbial DNA; 3) chitosan chelation of metals essential nutrients for microbial growth (Goy *et al*., 2009). According to Wang *et al*. (2004), chitosan complexes with zinc showed better antibacterial than antifungal activity. Chitosan-Zn complex inhibited the growth of *Escherichia coli* and *Corynebacterium* on agar plates and showed excellent antibacterial action against both of them. The concentration of 0.02% low molecular weight chitosan in broth inhibited the growth of *E. coli* and *Pseudomonas aeruginosa* (gram-negative bacteria) and also *Bacillus subtilis* and *Staphylococcus aureus* (gram-positive bacteria) (Uchida, 1988; Takahashi *et al*., 2008). The comparison of antibacterial activities between chitosan and chitosan oligomers against four gram-negative (*E. coli*, *Pseudomonas fluorescence*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus*) and seven gram-positive bacteria (*Listeria monocytogenes*, *B. megaterium*, *B. cereus*, *S. aureus*, *Lactobacillus plantarum*, *L. brevis*, and *L. bulgaricus*) showed that longer-chain types have higher activities and considerably

inhibit most bacterial growth (No *et al*., 2002). In No *et al.* (2002), the effectiveness of 0.1% chitosan as a bactericide was higher against gram-positive versus gram-negative bacteria.

The termiticidal effects of chitosan aqueous solutions (0.5 to 5% concentrations) have been investigated against *Reticulitermes flavipes* and *Reticulitermes virginicus* (Raji *et al*., 2018). The higher concentrations of chitosan (≥ 2%) resulted in high termite mortality (≥ 94%) in *R. flavipes,* while in the case of *R. virginicus* termite mortality was 100% at all concentrations of the treatment. There is no information about the influence of chitosan on protists. Because the antimicrobial properties of chitosan are not fully understood, the susceptibility of the microbial community in termite hindguts exposed to chitosan treatment wood is investigated herein.

1.3 Termites

Termites are eusocial insects belonging to the order Blattodea. Termites are close relatives of cockroaches and are a sister group to the wood-feeding, *Cryptocercus*. Termites are classified into the order Isoptera. There were seven families of termites in the entire world including Mastotermitidae, Termopsidae, Kalotermitidae, Hodotermitidae, Rhinotermitidae, Serritermitidae, and Termitidae. Within these, there are approximately 281 genera and more than 2,600 known species (Kambhampati and Eggleton, 2000). Recently, molecular phylogenetic studies purposed that the order Blattodea consist of the termites (epifamily Termitoidae only), and all cockroach taxa. The epifamily Termitoidae is now comprised of nine families: Mastotermitidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Stylotermitidae, Rhinotermitidae, Serritermitidae, and Termitidae (Beccaloni and Eggleton, 2013; Krishna

et al., 2013). Termites are divided into two groups, lower and higher termites, depending on the presence and absence of protist symbionts respectively. Eight of the nine families of Termitoidae (epifamily) are lower termites and they possess cellulolytic protist symbionts, bacteria and archaea in their hindgut, while Termitidae is the only family belonging to higher termites and contain only bacteria and archaea in their hindgut (Hongoh, 2010; Matsui *et al*., 2009; Reid *et al*., 2014). There are 2929 known living species in the Termitoidae epifamily and among them 12 genera and 315 species have been identified as members of Rhinotermitidae family. The majority of living species (238 genera, 2072 species) are higher termites (Termitidae) (Beccaloni and Eggleton, 2013; Krishna *et al*., 2013).

Not all termites are considered pests. Termites are distributed across all continents, except Antarctica. The predominant species are found in tropical and subtropical regions and have remarkable ecological significance.

Termites have been grouped based on their feeding style into wood-feeders, fungus-growers and soil-feeders. In general, termites have unique ability to digest lignocellulose materials (Kudo, 2009). They decompose lignocellulose in collaboration with their associated symbionts and by their own digestive enzymes. Different diets influence not only termites' and symbionts' digestive enzymes, but also the diversity of symbiotic microorganisms in hindgut (Tanaka *et al*., 2006; Karl and Scharf, 2015; Benjamino *et al*., 2018).

Termites go through three distinctive developmental stages: egg, immature and adult life. They live in a colony and are classified into morphologically and physiologically distinct castes of individuals, which include queens, kings, alate forms

(reproductive adults), soldiers, workers and nymphs. These multiple castes make colonies a very organized and complicated system. Each caste plays important functions for the colony. The dispersal and reproduction are primary duties of reproductive caste. Winged reproductives (alates) are the main individuals that disperse colony. They vary in color, the length of wings, and season of swarms among different termite species. Colony's foraging region size is based on the workers' activity and the worker caste plays essential function to maintain colony, while the role of soldiers is to protect the colony and nest (Baker and Marchosky, 2005). Workers have an outstanding behavioral and ecological diversification. They perform particular tasks including foraging-related tasks, care of brood and the queen, burying corpses, alarm giving, phragmosis, and time spent stationary. The older workers carry out the majority of the nest pairing, foraging, and gallery building (Crosland *et al*., 1997). Some termite species workers do not molt into soldiers or reproductives, while other species allow workers to change based on the colony need (Roison, 2000). Soldiers are unable to feed themselves because of their large mandibles, which are used to protect colony from any predators and dangers. Thus, soldiers' nourishments completely depends upon the workers and is performed through trophallaxis.

Trophallaxis is the process of transferring food and nutrients within the colony through anus-to-mouth (proctodeal) or mouth-to-mouth (stomodeal) feeding. It also replaces and transfers the hindgut microbial symbionts among the castes. It is necessary for termites to regain the gut symbionts from worker nestmates very soon after losing the microbial contents upon molting.

Due to the importance in feeding the other castes and being the majority of individuals, biochemical and microbiological studies on termite workers is essential. Therefore, in the present study, it was hypothesized that workers feeding on chitosan would cause the change in the protists and bacterial community diversity or relative abundance in the hindgut (Figure 1.1).

Figure 1.1 Termite worker from *R. virginicus* colony (on ruler with single cm unit).

The termite gut is separated into three distinct sections: foregut, midgut, and hindgut (Figure 1.2). The foregut comprises of esophagus, crop, and salivary gland. The foregut passes food into midgut, where endogenous enzymes (endoglucanases and cellobiases) are excreted for lignocellulose digestion. The midgut section is aerobic and resorbs the digested products by its epithelium. In the case of lower termites, the salivary gland and midgut secrete endogenous enzymes and endoglucanases into gut, while higher termites produce endoglucanase in the midgut (Tokuda *et al*., 2004).

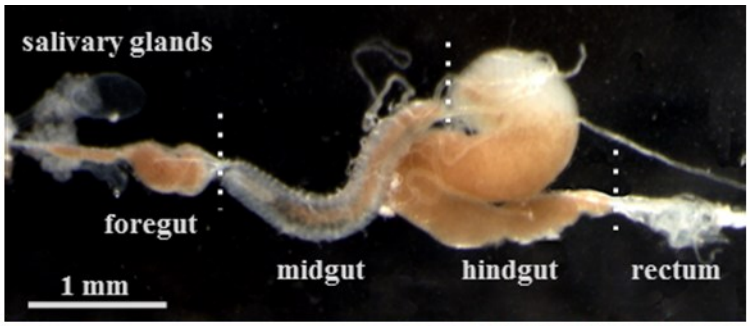

Figure 1.2 Different sections of the termite gut and rectum dissected from *R. flavipes*. Adapted from Tang, J. D. (USDA FPL).

The malpighian tubules, around the connecting area between the midgut and hindgut, eliminate the excreted waste and recycles nitrogen. The remaining material is received by the hindgut, where the symbiotic community inhabits and the most cellulose degradation, as well as fermentation, happens. There are four stages of anaerobic digestion in the hindgut (Odelson and Breznak, 1983; Breznak and Switzer, 1986; Spellman and Bieber, 2012):

1) Hydrolysis of cellulose : $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4\ H_2$,
2) Acidogenesis: simple monomers (products of hydrolysis)→fatty acids + H_2 + CO_2,
3) Acetogenesis: $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$, and
4) Methanogenesis: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$.

In general, the hindgut is an anaerobic chamber with micro-oxic zone around its periphery (Scharf and Tartar, 2008). Although oxygen continuously diffuse through the gut epithelia, facultative and obligate aerobic bacteria use up oxygen in the periphery of hindgut to create an anoxic zone. A bacterial center is maintained in the hindgut in order

to keep anoxic conditions. A steep oxygen gradient toward the hindgut periphery can be measured precisely with microelectrodes. Microorganisms nearby the hindgut wall receive higher partial pressure of oxygen (Brune *et al*., 1995). Hydrogen is highly concentrated in the center of hindgut and produced by strictly anaerobic protists, while low hydrogen concentrations are observed in the hindgut periphery. The major hydrogen sink relies upon methanogens (Brune, 1998). As previously described, several microhabitats can be found in the hindgut environment due to establishment of microbial community adopted to this environment. At the end of lignocellulose digestion, feces are released through rectum.

1.3.1 Subterranean termites

Subterranean termites (family Rhinotermitidae) are social insects and the most widespread pests in the United States and other parts of world. They are called "subterranean" because they dwell in moist soil habitats. They feed on wood above the ground in contact with soil, fallen logs, wooden structures (buildings, utility poles, fence posts, and wood by-products), paper, fiberboard, and fabrics (derived from cotton and other plants). In addition, termites can affect other non-cellulose materials such as plastics, thin metal, and cement while foraging food. Although these termite species significantly damage wooden structures, they are also beneficial in the ecosystem by increasing carbon recycling and improving the nutrient content of the soil (decomposing organic matter such as wood and vegetal litter). The increased amount of organic matter in soil leads to improved soil porosity and stability of aggregates, which results in higher soil aeration, increased water- holding capacity, and water filtration (DeSouza and Cancello, 2010). Likewise, the microbial community in the termite gut can be useful

toward converting plant biomass to energy for the production of biofuels (Brune, 2007). In order to forage food, termites construct tunnels and shelter tubes out of mud (Peterson *et al*., 2006). These mud tubes are used as runways for termites to protect themselves from dry environment and natural enemies, such as ants (Baker and Marchosky, 2005). Termite activity in the environment is based on availability of food, moisture and temperature.

Several genera and species have been identified as subterranean termites and among them the major termite pests belong to species of the genus *Reticulitermes* (Austin *et al*., 2002). This genus accommodates several species including *R. flavipes* Kollar, *R. virginicus* Banks, *R. hageni* Banks, *R. malletei* Clement, *R. hesperus* Banks, *R. tibialis* Banks, and *R. nelsonae* Lim and Forschler. According to Forschler and Lewis (1997), 90% of termite control industry is involved with the control of five primary subterranean species *R. flavipes*, *R. virginicus, R. hesperus*, *R. tibialis*, and *Coptotermes formosanus* Shiraki, which initiated the majority of termite damage in the United States.

The economic impact of subterranean species has been estimated to about US $23 billion of damage in the worldwide (Rust, 2014). Except Alaska, they are present everywhere in the United States, and they are the most common termite in southern areas of North America including Mississippi. In the United States, the annual economic losses caused by termite damage to wood is conservatively estimated at $1 billion but is sometimes as high as $7 billion (Peterson *et al*., 2006). Of the $2.2 billion annually spent to control termite in the United States, 80% is used for control of subterranean termites, most probably for the two principal species, *R. flavipes* and *R. virginicus* (Su *et al*, 2001).

1.3.2 *Reticulitermes virginicus*

Dark southern subterranean termites, *R. virginicus*, are lower termite species native to North America. The division of labor in the species is shared among distinct castes (workers, soldiers, reproductives). Workers are the most numbered individuals in the colony and are key castes to maintain their nest and colony alive.

The signs of damage of *R. virginicus* are not usually visible, but their presence can be detected by observing dispersal flight of alates or their mud tunnels. Alates are dark brown, and similar to alates of *R. flavipes*, except that they are smaller (Figure 1.3). Swarming for alates requires favorable environmental conditions such as higher temperature and increased humidity. Thus, they usually swarm on warm and moist days. The alates swarm relocate and ultimately attempt to develop a colony between early February and late May (Su *et al*., 2001).

Figure 1.3 Alates of *R. flavipes* and *R. virginicus*.
Adapted from Messenger (2001).

The length of soldiers of *R. virginicus* is 4.5-5 mm (Figure 1.4). In the past, *R. flavipes* was considered the only most economically important pest species in the United States while *R. virginicus* did not have significant economic impact. However, Su and Scheffrahn (1990) determined that the high rate infestations of wooden structures in Florida is due to *R. virginicus*. Recent claims suggest they are one of the five main species that responsible for the majority of termite damage to wooden structures and live plants (Forschler and Lewis, 1997; Szalanski *et al*., 2003).

Figure 1.4 Termite soldier of colony *R. virginicus* with length less than 4.5 mm including mandible.

Austin *et al*. (2004) investigated the distribution and genetic variation of *Reticulitermes* and their results indicated that there was no genetic variation in *R. virginicus*, although they identified several haplotypes among the other *Reticulitermes* species: *R. flavipes* (10 haplotypes), *R. hageni* (2 haplotypes), and *R. tibialis* (2 haplotypes). The hindgut of *R. virginicus* harbors protists, bacteria, and archaea like other subterranean termites. Although the composition of microbial community is different for each termite species, there are a certain similarity of protists and bacteria species between *R. virginicus* and other *Reticulitermes*. A feeding preference test revealed that *R.*

virginicus are susceptible to diet changes more than *R. flavipes* and *C. formosanus* (Smythe and Carter, 1970). Feeding *R. virginicus* on different cellulose sources (filter paper, birch, pine, and red oak) indicated that the cellulose diet changed the relative abundance of individual protist species (Cook and Gold, 2000). Lower termites are wood-feeders and changing their lignocellulose diets could affect the expression of enzymes in symbionts and hosts, which can be assessed by digestome microarrays (Tartar *et al*. 2009). In general, *R. virginicus* has not been widely studied and very little was published on the effects of different diets on hosts and their symbionts. Because of a lack of information on the effects of diets on bacterial symbionts in *R. virginicus*, this study assesses the bacterial community in *R. virginicus* exposed to chitosan-treated wood and evaluates the effect of chitosan on the protist community.

1.4 Protists symbiosis in termites' hindgut

Protists are considered single independent eukaryotic cells. The unicellular protists are not classified as plant, animal, and true fungi and their morphology are very diverse (Adl *et al*., 2005). Some of protists possess a specific characteristic that contributes to motion, while others are non-motile. Motile protists are able to move by one or multiple flagellates.

The hindgut of all lower termites is inhabited by anaerobic symbiotic protists, which are involved in the digestion of the lignocellulose diet of their hosts (Brune, 2014). Although endoglucanases and cellobiases secretions occur in the salivary glands and midgut of lower termites, they rely upon their symbiotic protists to digest wood and to survive. A dual cellulose digestion system between host and their associated protists was proposed for the wood-feeding termite, *C. formosanus* (Nakashima *et al*. 2002a) and this

notion was supported in the later study (Tokuda *et al*. 2007). Several studies indicated that different hindgut protists produced cellulases from glycoside hydrolase families such as GHF7 and GHF45, as well as xylanases (GHF8, 10, and 11) and β-glucosidase (GHF3), to aid termites to digest cellulose (Ohtoko *et al*., 2000; Nakashima *et al*., 2002b; Todaka *et al*., 2007). Cellulose is initially degraded in the midgut by the endogenous, termite-originated, cellulases. The undigested crystalline cellulose afterwards passes to the hindgut, where it is depolymerized by endoglucanases, exoglucanases, and β-glucosidases from various GHFs produced by symbiotic protists. The depolymerization is performed by endocytosis and fermentation processes (Ohtoko *et al*., 2000; Brune, 2014). As the result of protists fermentation of carbohydrates, short-chain fatty acids are produced and subsequently oxidized and absorbed by termite. Besides cellulose, protists also digest hemicelluloses, but lignin degradation remains controversial. Tartar *et al*. (2009) indicated that bacterial and protists symbionts do not have the capacity for lignin digestion, while fungal symbionts are able to digest it in the higher termites. Protists mainly function through breakdown of partially digested cellulose and glucose to acetate, carbon dioxide and hydrogen. Acetate serves as a source of energy and as a precursor for biosynthesis in termites (Odelson and Breznak, 1983).

Symbionts are limited to the hindgut, and the number of protists can reach 10^3 to 10^5 cells per termite (Yoshimura, 1995; Hongoh, 2010). Protist communities in termites are not uniform, but typical combinations of protist species exist in different termite species hindguts. *R. flavipes*, for example, has 12 cellulolytic protists in their hindgut, while *R. virginicus* has nine protist species (Lewis and Forschler 2004, Lewis and Forschler 2006). Lewis and Forschler (2004) have also reported the presence of 14,000

protist communities per *R. virginicus* worker. In the present study, protists species were identified according to morphological characters.

There are two distinct lineages of protists in the lower termites known as Parabasalia and Preaxostyla, both affiliated with Excavata (Adl *et al*., 2005). Each of these phyla comprises of different protists species (Kudo, 2009).

1.4.1 Preaxostyla

Preaxostyla is comprised of Oxymonadida and *Trimastix* Saville Kent (genus) that are sister taxa and not belonging to other eukaryotic lineages. *Trimastix* are free-living flagellates with small dense organelles instead of mitochondria, while oxymonads are gut symbionts without those organelles. The oxymonads lack classical mitochondria, hydrogenosomes, and parabasal apparatuses. It means they do not have any energy generating organelle. Oxymonads have seven genera including *Dinenympha*, *Monocercomonoides*, *Oxymonas*, *Polymastix*, *Pyrsonympha*, *Saccinobaculus*, and *Streblomastix*. *Dinenympha* and *Pyrsonympha* are found in the hindgut of wood-feeding lower termites (Adl *et al*., 2012). Both of these genera belong to the family Pyrsonymphidae (Stingl and Brune, 2003). Many genera (e.g. *Pyrsonympha*, *Streblomastix*, and *Oxymonas*) are attached to the cuticle of the hindgut wall of some lower termites using an anterior holdfast although this feature was not observed for the genus *Dinenympha* (Tamschick and Radek, 2013).

Due to their complexity, cultivation of oxymonads on media proved to be difficult, and only one strain (PA203), *Monocercomomonoides* from a vertebrate symbiont, has been successfully cultivated (Hampl *et al*., 2005). Hence, the metabolism of oxymonads is not completely clear. There are epibiotic or endobiotic bacteria

associated to many oxymonad species (Iida *et al*., 2000; Stingl *et al*., 2005). Distinct spatial distributions of different methanogens species in the hindgut of *R*. *speratus* and *Hodotermopsis sjoestedti* was described by molecular phylogeny of methanogenic archaea associated with *Dinenympha* that were phylogenetically different from the methanogens related to the hindgut epithelium (Tokura *et al*., 2000).

1.4.2 Parabasalia

The majority of protists principal in cellulolytic digestion of partially ingested wood particles in the lower termite hindgut belong to Parabasalia. Parabasalia, also known as parabasalids, comprise large, anaerobic, and very mobile cells (Brune, 2014) that are easily distinguishable from other flagellated protists through a presence of parabasal apparatus consisting of parabasal body (Golgi complex) and a parabasal filament (Ohkuma *et al*., 2005). Another distinctive feature of parabasalids is the microtubular pelta- axostyla complex. In some taxa, the ciliary apparatus is reduced or lost. All parabasalids exhibit a special type of close mitosis (cell division within intact cell nucleus) with an external spindle. Parabasalids possess a specialized organelle, hydrogenosomes in place of mitochondria in which anaerobic metabolism occurs (Tamschick and Radek, 2013). All species of Parabasalia produce hydrogen, which is a key metabolite in lower termite hindgut during digestion process (Brune, 2014). Traditionally, based on their morphology, Parabasalia have been divided into two classes: Trichomonada and Hypermastigia. Most of hypermastigids species are found in the digestive tract of termite hindgut and wood-eating cockroaches, while Trichomonads species are associated with the respiratory, digestive, and reproductive systems of vertebrates (Honigberg, 1978; Yamin, 1979; Viscogliosi *et al*., 1999). In recent molecular

phylogenetic studies, Parabasalia phylum are classified into six following classes: Trichonymphea, Spirotrichonymphea, Cristamonadea, Tritrichomonadea, Hypotrichomonadea, and Trichomonadea, with the former three classes belonging to morphologically classified hypermastigids. The protist species of Trichonymphea, Spirotrichonymphea, and Cristamonadea are unique to the hindgut of lower termites (Noda *et al*., 2012; Brune and Dietrich, 2015).

According to Adl *et al*. (2012), the species classification into Parabasalia classes are as follows:

1. Trichomonadea: *Hexamastix*, *Pentatrichomonas*, *Pseudotrichomonas*, *Tricercomitus*, and *Trichomonas*.
2. Hypotrichomonadea: *Hypotrichomonas* and *Trichomitus*.
3. Tritrichomonadea: *Dientamoeba*, *Histomonas*, *Monocercomonas*, and *Tritrichomonas*.
4. Cristamonadea: *Coronympha*, *Deltotrichonympha*, *Devescovina*, *Foaina*, *Joenia*, and *Mixotricha*.
5. Trichonymphea: *Barbulanympha*, *Hoplonympha*, *Staurojoenia*, and *Trichonympha*.
6. Spirotrichonymphea: *Holomastigotes*, *Holomastigotoides*, *Microjoenia*, *Spironympha*, and *Spirotrichonympha*.

1.5 Bacteria and archaea symbionts in termite hindgut

Bacteria are unicellular organisms and form a large domain of prokaryotes. Both lower and higher termites contain bacteria and archaea, in their hindgut. Bacteria colonize the majority of hindgut space, and archaea are present only in 0 to 10 % of the single

termite hindgut. A single termite contains approximately 10^6 to 10^8 bacteria cells, densely and unequally distributed within the hindgut (Yoshimura, 1995; Hongoh, 2010). Bacteria constitute large populations in the lower termite hindgut in comparison to eukaryotic protists. Bacteria in the hindgut exist freely in the lumen, attached to the wall, or associated with the protists. Although bacteria are not significantly involved in the cellulose digestion, they maintain the chemical environment through specific processes ascribed to acetogenic bacteria, spirochetes (homoacetogenic and oxygenase activity), nitrogen-fixing bacteria, lactic acid bacteria, sulfate-reducing bacteria, and uric acid-degrading bacteria (Odelson and Breznak, 1983; Potrikus and Breznak, 1977; Brune, 1998; Brun, 2014). There are six dominant bacteria phyla in *R. flavipes*: Proteobacteria, Spirochaetes, Bacteroidetes, Firmicutes, Actinobacteria, and Elusimicrobia (Fisher *et al*., 2007; Brune, 2014). All methanogenesis in the hindgut is performed by methanogenic archaea. In *R. flavipes*, methanogenic archaea are restricted to the hindgut wall, whereas in other termites, they are associated with the cytoplasm of protist cells. *Methanobrevibacter* species are dominant in the lower termites (Leadbetter and Breznak, 1996; Hongoh, 2010).

1.6 Objectives

The objective of this study is to examine the relationship between host termite and microbial symbionts as affected by chitosan intake. The specific goals of this study are to: 1) observe changes in protists relative abundance after exposure of termites to treated wood with a range of concentrations of chitosan; 2) examine the effect of chitosan treatment on bacterial diversity and frequency in *R. virginicus* as determined by 16S rRNA Illumina Miseq.

1.7 References

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CHAPTER II

EFFECT OF CHITOSAN ON DIVERSITY AND NUMBER OF PROTISTS IN SUBTERRANEAN TERMITES

2.1 Abstract

Although protist species composition in the hindgut of subterranean termites is known to vary among termite species, little 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 removed 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 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.

2.2 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). The genus *Reticulitermes* accommodates several species including *R. flavipes* (Kollar), *R. virginicus* (Banks), *R. hageni* (Banks), *R. malletei* (Clement), *R. hesperus* (Banks), *R. tibialis* (Banks), and *R. nelsonae* (Lim and Forschler). Among these species, *R. virginicus* is known as a dark southern subterranean termite. 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 that are responsible for hydrolysis of cellulose, endocytosis and fermentation activities. Effective lignocellulose digestion in the termite gut relies upon collaboration between the host enzymes and microbial hindgut symbionts (Brune, 2014). In the center of termite hindgut, less than 40% of the total hindgut volume constitutes an anoxic habitat for strictly anaerobic microorganisms, particularly cellulolytic protists. There is a micro-oxic zone around the

periphery of hindgut and its posterior region which comprises of aerobic and aerotolerant microflora that consume oxygen and create the anoxic region for anaerobic microorganisms (Brune *et a*l., 1995). Because of the anaerobic features of gut symbionts they are often laborious to culture in a laboratory. The microbial symbionts are unevenly distributed through the termite hindgut and localized in distinct niches (Ohkuma, 2003). The density of symbiotic microbes in the lower termite hindgut reaches around 10^{11} cells per mL (Ohkuma and Brune, 2010). These microbes, especially the protists in the hindgut, provide carbon and energy requirements for the termite host. Distinct and specific communities of protists exist in each termite species. These communities play different roles in lignocellulose degradation (Honigberg, 1970; Lewis and Forschler, 2004a).

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 which 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). The results of the previous study by Lewis and Forschler (2004a) revealed that the relative abundance of indicator protist species can be used to identify and differentiate subterranean termites. In order to count the number of protist cells outside of the host hindgut varying buffers are used to maintain cell viability through osmotically balanced saline solutions (Trager, 1934; Mannesmann, 1972; Cook and Gold, 2000; Lewis and Forschler, 2004b).

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.

2.3 Materials and methods

2.3.1 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 pine 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.

In order to identify termite species, a genomic DNA (gDNA) was isolated from soldier heads (5 heads from Colony 1 for each replicate (R=5) and 5 heads from Colony 2 (R=1)) using the GeneJET Plant Genomic DNA Purification Kit (ThermoFisher Scientific, Waltham, MA). The concentration and purity of soldier heads gDNA were analyzed on NanoDrop™ Spectrophotometer and agarose gel electrophoresis, respectively. Termite amplification primers (forward 5′-TGGGGTATGAACCAGTAGC-3′ and reverse 5′-CACTAAGGATAATCAATTATACGTC-3′) were designed by Foster *et al*. (2004) and targeted the mitochondrial DNA at the AT-rich region. After polymerase chain reaction (PCR) amplification, the amplified fragment was excised from agarose gel using QIAquick Gel Extraction Kit (Qiagen) and then ligated to the pGEM-T Easy Vector System II (Promega, Madison, WI). The recombinant clones were identified on LB/ampicillin/IPTG/X-Gal plates by blue and white color screening and transferred into JM109 High Efficiency Competent cells (*Escherichia coli* cells) according to manufacturer's instruction. Plasmid DNA was isolated from the competent cells (two clones from each termite colony) based on PureLink Quick Plasmid Miniprep Kit protocol (Invitrogen, Carlsbad, CA) and both strands of one clone was sent for sequencing to Eurofins Genomics (Louisville, KY). The returned DNA sequences were

edited by removing pGEM-T Easy vector sequences using Finch TV software version 1.4.0 (Geospiza). These sequences were analyzed against the NCBI non-redundant nucleotide database to find sequences with the greatest percent similarity to our colony-derived sequences. Phylogenetic tree analysis was also performed using MEGA7 to compare these termite colony sequences with NCBI reference sequences for *R. virginicus*, *R. flavipes* and *Coptotermes formosanus* (Foster *et al*. 2004). *Bacillus circulans* sequence was used as outgroup (Accession # KJ531945.1). In addition, the Messenger (2001) identification guide was used to confirm termite species.

2.3.2 Wood sample preparation and treatment with chitosan

Defect free southern yellow pine samples with dimensions of 25 × 25 × 6 mm (tangential × radial × 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 sample were treated at 29.8 mmHg vacuum for 3h. Chitosan retention (mg g^{-}1) was calculated based on oven dry mass of treated wood samples with chitosan and oven dry mass of wood samples before chitosan treatment.

2.3.3 Termite no-choice exposure laboratory bioassay

The no-choice test was performed according to a modified AWPA E1-16 Standard (AWPA, 2016). A total of 35 glass screw-top jars (8 cm dia, 10 cm tall) each

containing 120 g play sand (Quikrete Premium Play Sand) and 35 mL distilled water 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.

2.3.4 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), wherein 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 was used. 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 400× 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:

$$N_i = \frac{n_i \,.\, 100\ \mu L}{0.5\ \mu L} \tag{2.1}$$

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:

$$N_r = \frac{\sum_{i=1}^{3} N_i}{3} \tag{2.2}$$

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):

$$R = \frac{\sum_{r=1}^{n} N_r}{n} \tag{2.3}$$

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

2.3.5 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. Potential 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. In addition, Statistical Analysis Software version 9.4 (SAS 2013) was used to perform one-way ANOVA and assess homogeneity of variance within sample group through Levene's test. Differences in the mass loss values were statistically analyzed by treatments for each colony. If the assumption of homogeneity of variance was met, which means p value was greater than 0.05 significance level, ANOVA was performed to test whether there was a significant effect of treatments followed by the Tukey post hoc test for mean separation.

2.4 Results and discussion

2.4.1 Termite species identification

Concentration and quality of the genomic DNA isolations (R = 5 for Colony 1 and R=1 for Colony 2) are shown in Table 2.1. Concentration and the 260/280 absorbance ratios ranged from 15.80-26.15 ng/µL and 1.73 - 1.99, respectively, as measured by the NanoDrop™ Spectrophotometer. For pure DNA, the 260/280 ratio is approximately1.80. Although extractions from both Colony 1 and Colony 2 produced DNA with acceptable quality, it appeared that extractions from Colony 1 soldier heads yielded 1.5× to 1.75× higher concentrations of DNA compared to Colony 2.

Table 2.1 Concentration and quality of genomic DNA as determined by NanoDrop

Sample*	Concentration ng/µL	260/280
C1-R1	24.19	1.83
C1-R2	26.15	1.99
C1-R3	22.77	1.96
C1-R4	23.62	1.83
C1-R5	24.1	1.85
C2	15.8	1.73

*C1, Colony 1; C2, Colony 2; R, replicate.

The PCR amplicons were separated by electrophoresis on 1% agarose gel in 1× TAE (Tris-Acetate EDTA) buffer, and observed at the expected molecular weight of 400 bp (Figure 2.1). Alignment of our colony-derived sequences against the NCBI-nr nucleotide database through BLAST (Basic Local Alignment Search Tool) showed that the Colony 1 and Colony 2 exhibited 99% and 97% similarity to *R. virginicus* respectively. In addition, phylogenetic tree analysis showed that both colony 1 and 2 were more genetically related to *R. virginicus* than *R. flavipes* (Figure 2.2). Termite

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

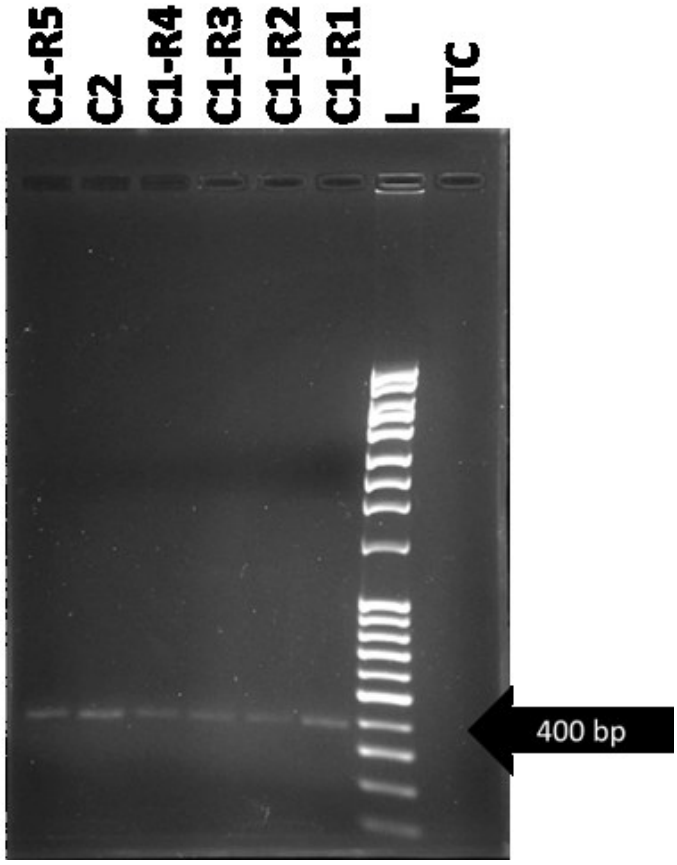

Figure 2.1 PCR amplification of genomic DNA of *Reticulitermes* sp. C1, Colony 1; C2, Colony 2; R, replicate; L, 1 Kb Plus DNA Ladder; NTC, non-template control.

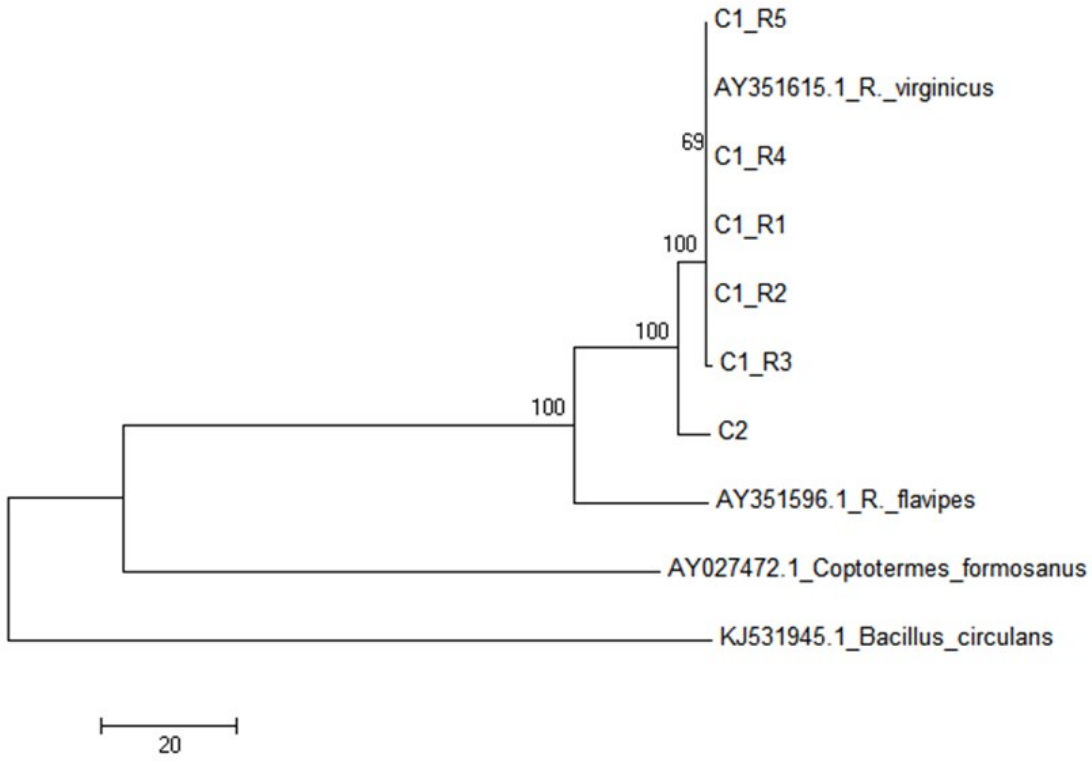

Figure 2.2 Neighbor-joining phylogenetic tree comparing the genetic relationship of our field-collected termites with NCBI reference sequences for *R. virginicus*, *R. flavipes*, and *C. formosanus*.

B. circulans was used as the outgroup. The reliability of tree was checked with bootstrap values 500. C1, Colony 1; C2, Colony 2; R, replicate.

2.4.2 Wood sample preparation and treatment with chitosan

In this study, the average retention of chitosan in treated wood samples was evaluated. The results showed a lower retention of 14 mg g^{-1} and 29 mg g^{-1} with 0.5% chitosan concentration solution for Colony 1 and Colony 2 respectively (Table A.1). In general, the lower concentration of chitosan solution indicated the lower retention while the average retention of chitosan increased with 1% and 2% chitosan concentration solutions. All retentions obtained in this study were slightly higher than retentions

calculated in Raji *et al*. (2018). They reported 11-15 mg g^{-1} treatment retentions for wood blocks that were treated with 0.5% and 1% chitosan solution and higher treatment retentions (≥ 38 mg g^{-1}) in the treated-wood with ≥ 2% chitosan solution.

2.4.3 Termite no-choice exposure laboratory bioassay

The average mass loss of treated-wood samples exposed to both termite Colonies 1 and 2 decreased with chitosan concentrations ≥ 1 (Table A.1), which was in agreement with the results of Raji *et al*. (2018). In Colony 1, there was no significant difference between mass loss of control (water and 25% acetic acid) and 0.5% chitosan-treated wood samples due to termite feeding. Instead, in Colony 2, the amount of mass loss in 25% acetic acid treatment was significantly higher than water and 0.5% chitosan treatment. On the other hand, the low percent mass loss was detected for 1% and 2% chitosan treatments in the both colonies (Figure 2.3). Termite mortality was not measured in this study.

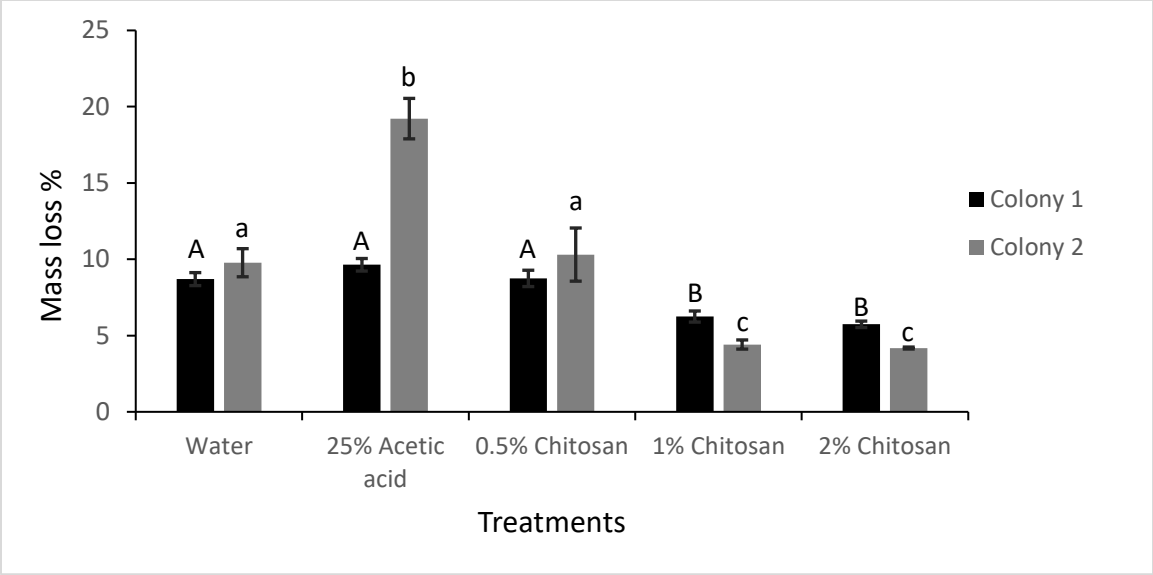

Figure 2.3 Effect of *R. virginicus* exposed to chitosan treatments on the average mass loss of wood samples (bars denote standard error).

Bars with different letters are significantly different ($p < 0.05$); uppercase letters for the Colony 1, lowercase letters for the Colony 2.

2.4.4 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 Figure 2.4. In higher concentrations of chitosan treatment, only two protist species were observed, *Monocercomonas* sp. and *T. trypanoides*. All termites that were 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.

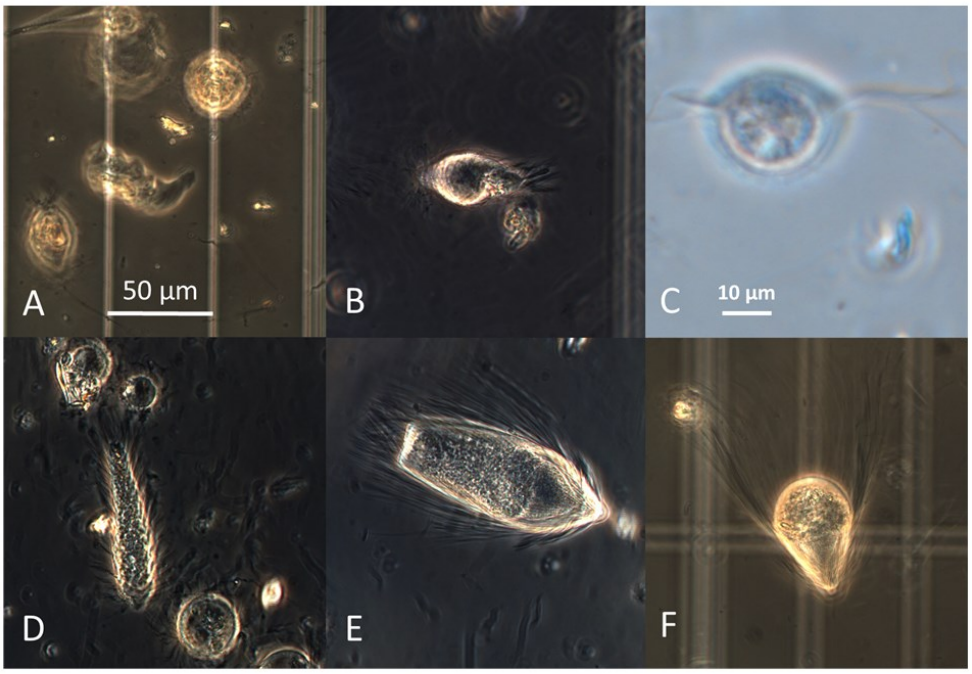

Figure 2.4 Phase-contrast light microscopy of some of the protist species from *R. virginicus*; A) *P. minor*; B) *S. flagellata*; C) *Monocercomonas* sp.; D) *H. elongatum*; E) *T. agilis*; F) *T. burlesquei*. Scale bar for B, D, E and F same as in A.

2.4.5 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 (Figure 2.5A). 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 (Figure 2.5A). Protist species also fell into two major clusters (Figure 2.5B). 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 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.

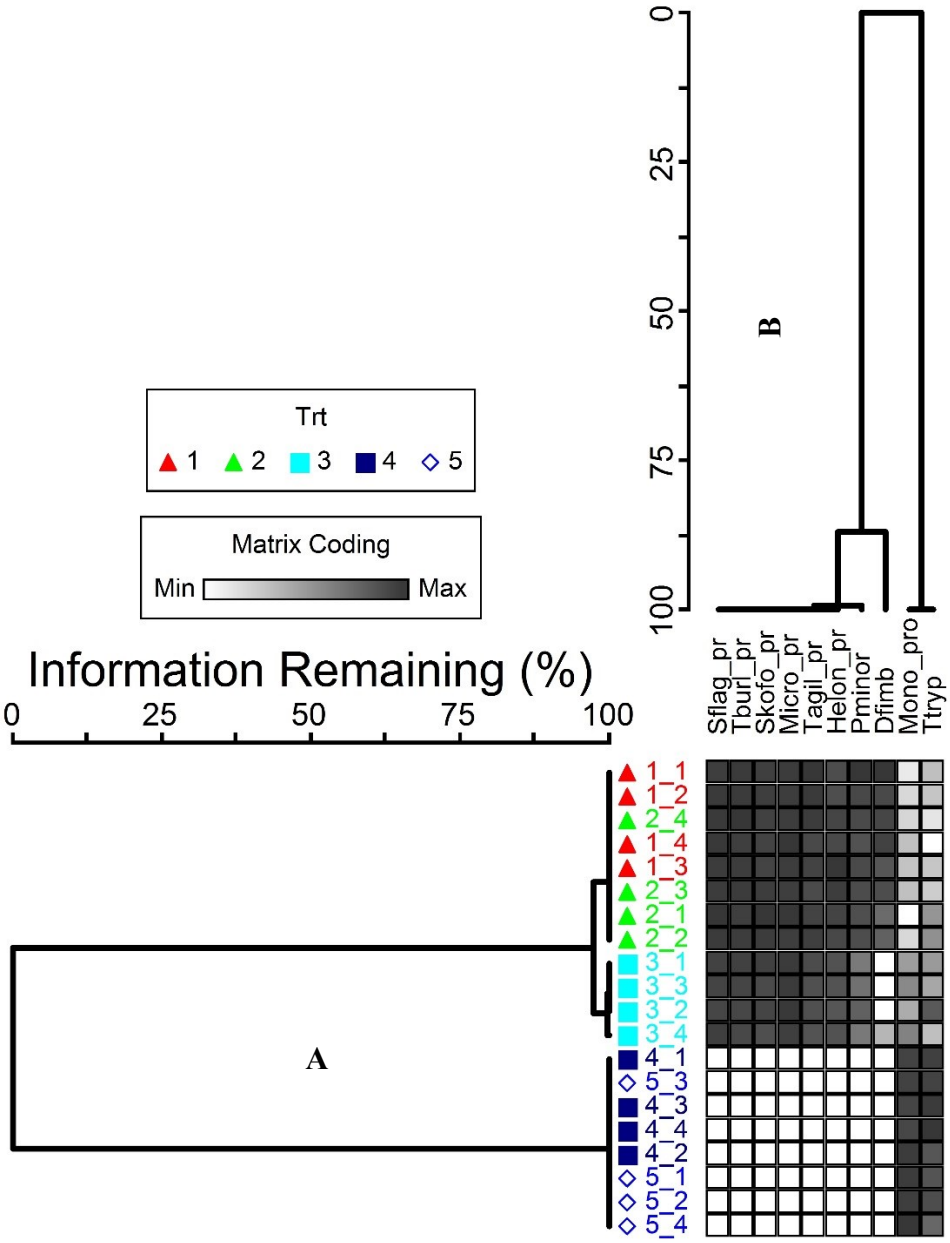

Figure 2.5 Two-way cluster analysis of sample units (A) and protist species (B) based on relative abundance of protists in Colony 1.

Treatments (Trt): 1, 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*.

For Colony 2 sample units separated into two major clusters (Figure 2.6A). 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 (Figure 2.6B).

Small differences were found in the sub-branching of the sample units and species composition clusters when comparing Colonies 1 and 2 (Figure 2.5 and 2.6). 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 Figure 2.5) as compared to Colony 2 (Fig 2.6). Factors that could contribute to these small differences may include 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.

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 (Figure 2.5B and 2.6B). 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.

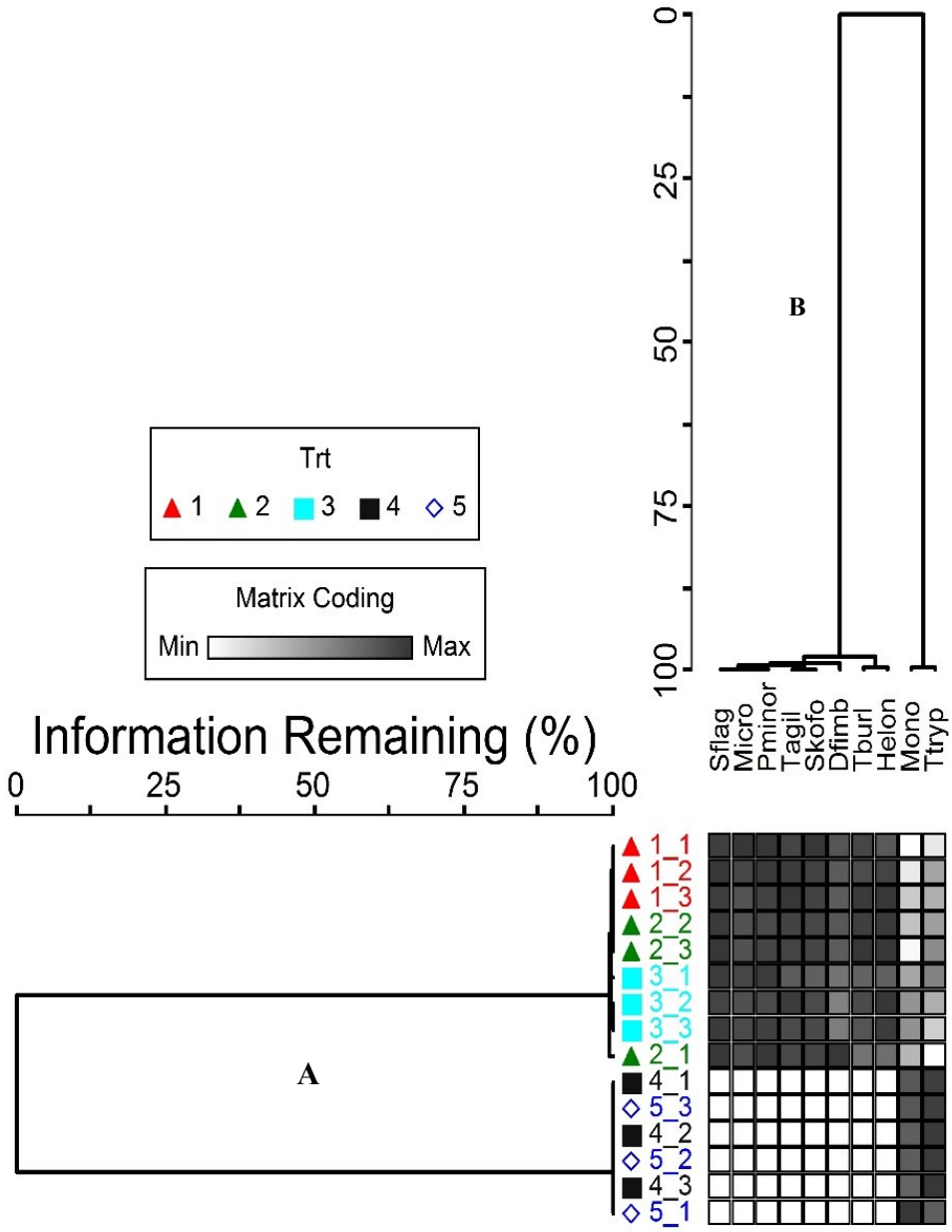

Figure 2.6 Two-way cluster analysis of sample units (A) and protist species (B) based on relative abundance of protists in Colony 2.

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*.

The average of normalized read counts within sample groups for each protist species congregated by treatment for both Colonies 1 and 2 are illustrated in Figure 2.7 and 2.8. The species distribution of Colony 1 almost had the same pattern as Colony 2. Lewis and Forschler (2004a) compared the relative abundance protist species among different casts of *R. virginicus* and showed that there were similar relative protist species abundances among workers, nymphs, and soldiers. Nevertheless, the presence of *D. fimbriata* and *P. minor* in workers were fewer than nymphs and soldiers. Although we did not compare the relative abundance among different casts, the average normalized read counts of these two species of workers were found in lower abundance for controls and 0.5% chitosan treatments in both Colonies 1 and 2. However, the presence of *D. fimbriata* was not observed in the 0.5% chitosan treatments for Colony 1 (Figure 2.7). In addition, *H. elongatum* in both colonies also displayed the low counts similar to two described species specifically to *P. minor*. *Monocercomonas* sp. between two colonies across the treatment revealed the most dominant species in *R. virginicus*. In the case of higher concentrations of chitosan (1% and 2%), *Monocercomonas* sp. showed the highest rank of abundance and followed by *T. trypanoides* (Figure 2.7 and 2.8). In all control and 0.5% chitosan treatments the highest abundance of species from both Colonies (1 and 2) were *Monocercomonas* sp. followed by *S. flagellata*, *Microjoenia* sp., and *T. trypanoides* while the relative abundance of *S. flagellata* from workers was dominant in a report by Lewis and Forschler (2004a), followed by *T. agilis*, *Microjoenia* sp., and *Monocercomonas* sp. Since pervious authors did not include the counts of *T. burlesquei* separately, the relative abundance of *T. agilis* showed the higher counts. In the current

study, the *T. burlesquei* and *T. agilis* had differences in the normalized read counts in both colonies 1 and 2.

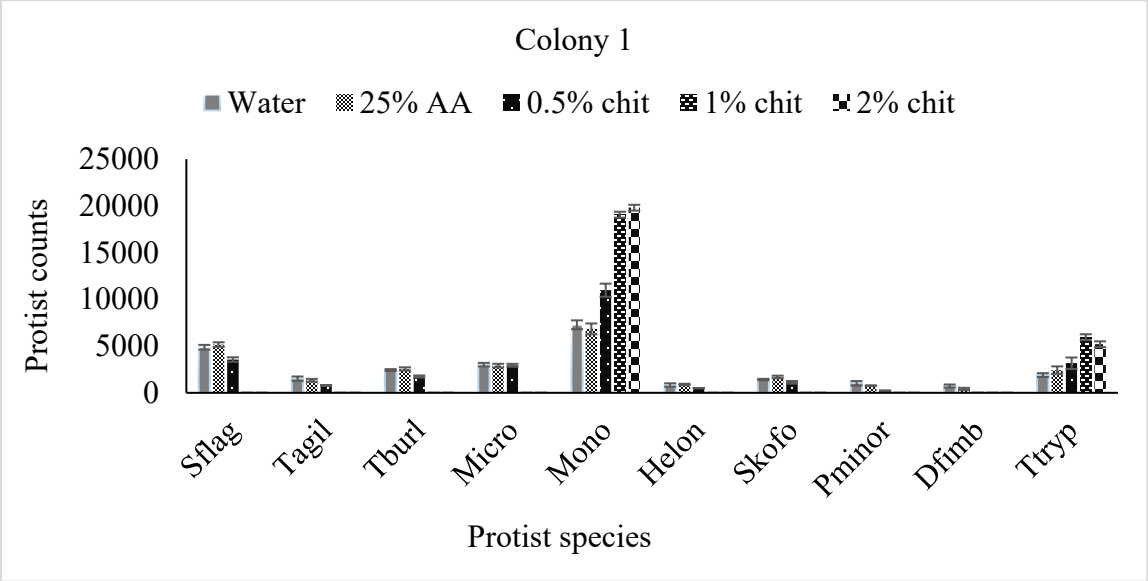

Figure 2.7 Mean normalized relative abundance (untransformed dataset) of protist species as affected by treatment from worker castes in *R. virginicus*.

Small bars indicate ± standard error. 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*. 25% AA, 25% acetic acid; chit, chitosan.

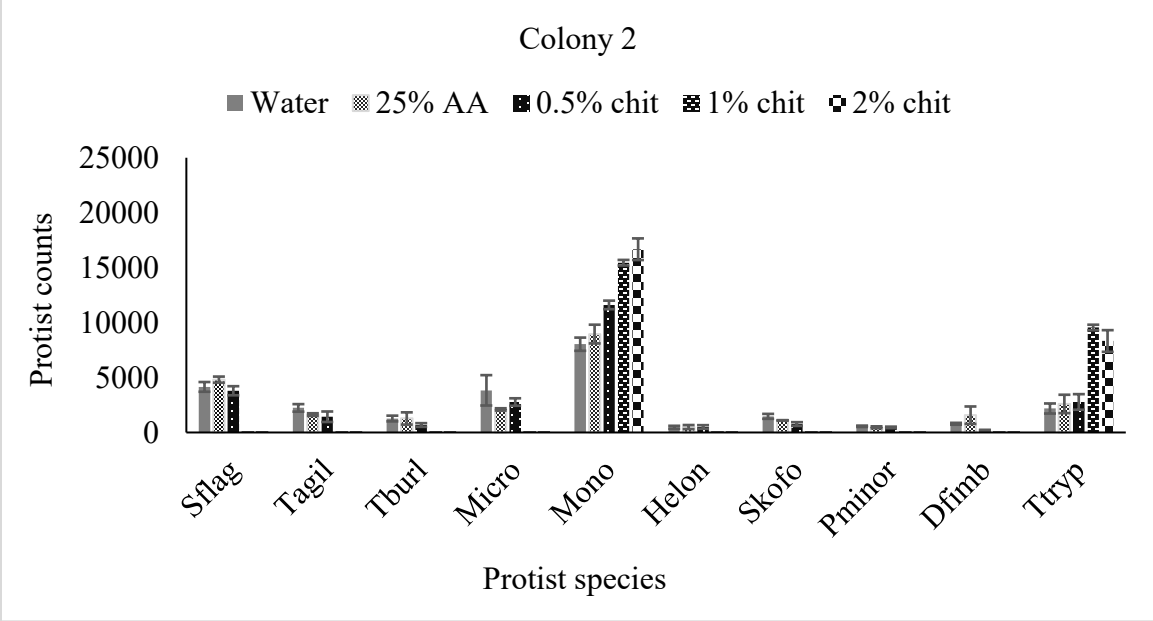

Figure 2.8 Mean normalized relative abundance (untransformed dataset) of protist species as affected by treatment from worker castes in *R. virginicus*.

Small bars indicate ± standard error. 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*. 25% AA, 25% acetic acid; chit, 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 2.2).

Table 2.2 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 2.3 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 2.3 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 inadequate food resources or starvation have been shown to affect diversity of protists in termite hindguts. Feeding preferences of *R. virginicus* on red spruce and white oak resulted in the elimination of total protist species (Mannesmann, 1972). The effect of different cellulose sources (filter paper, birch, pine, and red oak) on the protist community of *R. virginicus* was determined by Cook and Gold (2000). They indicated that except red oak, other cellulose sources changed the structure of protist community in the 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. Cleveland (1925) reported that three genera, *Microjoenia*, *Trichomitus*, and *Monocercomonas,* were not affected by termite starvation and not involved in termite nutrition (Lewis and Forschler, 2004a).

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 2.4 and 2.5 show percent relative abundance of protists by treatment. In Colony 1 (Table 2.4), 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 2.5), 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 2.4 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

Where: 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 2.5 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

Where: 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 2.6. 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 2.6 Percent constancy of protist presence across sample units within a treatment for Colonies 1 and 2

Colony 1						Colony 2					
Protist species	**Water**	**25% AA**	**0.5% chit**	**1% chit**	**2% chit**	**Protist species**	**Water**	**25% AA**	**0.5% chit**	**1% chit**	**2% chit**
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

Where: 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*; 25% AA, 25% acetic acid; chit, chitosan.

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 endo-symbiotic 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.

2.5 Conclusions

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 number (richness) 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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CHAPTER III

CHARACTERIZATION OF THE BACTERIAL COMMUNITY ASSOCIATED WITH HINDGUT OF TERMITE RETICULITERMES VIRGINICUS EXPOSED TO CHITOSAN TREATMENT OF WOOD

3.1 Abstract

Termite's digestion of lignocellulosic materials is aided by their hindgut microbial community, which includes protists, bacteria, and archaea. They play important roles in the termite's growth and environmental adaptation. A more thorough understanding of this community is needed to develop target-specific and environmentally benign wood protection systems. Majority of microbes in the termite hindgut are anaerobic and essentially difficult to culture on media. Thus, molecular analysis of microbial community has revealed more information about microbial diversity and their symbiotic relationship mechanisms. For this study, the composition of bacterial community from the southern subterranean termite *Reticulitermes virginicus* was examined through analysis of total genomic DNA isolated from the hindgut. Prior to the DNA isolation, the termites were subjected to five treatments: three groups of termites were fed on wood treated with 0.5% chitosan, 25% acetic acid, and water, one termite group was unexposed to treated wood, but was kept in the decayed wood logs collected, and one group of termites was starved over an 18-day period. 16S ribosomal RNA (rRNA) genes

containing hypervariable regions known as V1-V9, were studied to identify diversity among bacterial species. In present study, Illumina sequencing of 16S rRNA spanning V3 and V4 regions was performed and the data analyzed using QIIME 2 to understand how different treatments affect the dynamic of the hindgut microbiota. Twenty-eight bacterial phyla were classified, of which four phyla were dominant and included Bacteroidetes (34.4% total of reads), Firmicutes (20.6%), Elusimicrobia (15.7%), and Proteobacteria (12.9%). Weighted UniFrac beta diversity metrics showed the bacteria microbiota from unexposed and starved termites had similar separation from the other treatment groups. The significant effect of chitosan treatment (CTE treatment group) was only observed in four bacteria phyla; Firmicutes, Actinobacteria, Tenericutes, and Planctomycetes. The results of present study suggested that different treatment groups shifted the microbial composition of *R. virginicus* hindgut. Furthermore, the shifts of the bacterial communities revealed more complex relationship of microbiota in the hindgut. Finally, a wider range of variation in relative abundance of bacterial genera emphasized the influence of environment, lack of food sources, and treatments on the diversity of bacterial microbiota in the hindgut.

3.2 Introduction

The distribution of termites is reported across all continents especially in the tropical and subtropical regions, except in Antarctica. There are almost 3,000 identified termite species, and few of them make serious damage to wooden structures. There are about 45 termite species in the US, and among them, 30 species are identified as pests (Su and Scheffrahn, 1990). Termites are divided into lower termites (Mastotermitidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Stylotermitidae,

Rhinotermitidae, and Serritermitidae) and the higher termites (Termitidae). Diversity in feeding and nesting styles, which include wood, grass, litter, soil feeding, and fungus growing lifestyles in the higher termites is more prevalent than in the lower termites (Ottesen and Leadbetter, 2011). Another major difference between these two termite subgroups is the unique presence of symbiotic protists in the lower termite guts. However, Rahman *et al*. (2015) identified a low abundance of ciliate protist in the gut of higher termite, *Gnathamitermes,* as well. Fossil records of termite gut symbionts were discovered in 20-million-year-old amber (Wier *et al*., 2002). Lespes (1856) described the presence of microorganisms in the termites gut for the first time. Later, Leidy (1877) developed the idea of parasites existing in termite gut as he identified spirochetes and few protist species in *Termes flavipes* (*Reticulitermes flavipes*), although their capacity to digest wood was not determined. However, Cleveland (1925) described that symbionts were beneficial for feeding of different stages of termite castes. Symbionts and their synergistic relationship with termites have been studied over time, but a full understanding of their structure and function are still not established.

Different biological processes occurring in termite hindguts, including cellulose degradation, nitrogen fixation and recycling, acetogenesis, methanogenesis, and vitamin production are supported by termite symbionts (Ohkuma, 2003; Husseneder, 2010). Symbionts retain themselves in the hindguts swimming or attaching to particles to prevent being washed out from the hindgut during the digestion process (Brune and Dietrich, 2015). In lower termites, the prokaryotes (bacteria and archaea) are essential, as well as the eukaryotes (protists), for the host survival. Although 90% of hindgut is inhabited by protists (10^3 to 10^5 cells per single hindgut), which play important role in the

digestion of cellulose, the bacterial community are in the majority (10^6 to 10^8 cells per single hindgut), and essential for lignocellulose degradation (Hongoh, 2010). Although bacteria are not significantly involved in the cellulose digestion, they maintain the chemical environment through specific processes ascribed to acetogenic bacteria, spirochetes (homoacetogenic and oxygenase activity), nitrogen-fixing bacteria, lactic acid bacteria, sulfate-reducing bacteria, and uric acid-degrading bacteria (Odelson and Breznak, 1983; Potrikus and Breznak, 1977; Brune, 1998; Brun, 2014). Flagellated protists convert cellulose to acetate, H_2, and CO_2 in hydrogenosomes organelle, and symbiotic bacteria further consume H_2 and CO_2. The flagellated protists can also produce lactate, although the high rate of O_2 reduction in the hindgut periphery happens by the action of lactic acid bacteria. These bacteria quickly convert lactate to acetate thus preventing the lactate accumulation in the hindgut. These groups of bacteria are culturable and found in *R. flavipes* and other wood-feeding lower termites (Bauer *et al*., 2000). The O_2 can also be reduced in the presence of H_2 by several sulfate-reducing bacteria, *Desulfovibrio* spp., which are found in various termite species (Kuhnigk *et al.* 1996). Formate is produced in the hindgut of many wood-feeding termite species from H_2 and CO_2. It has three metabolic pathways, and it can be either accumulated, oxidized to CO_2, or reduced to acetate in the hindgut by homoacetogenic bacteria (Brune, 2014). The homoacetogenic bacteria consume most of H_2 from CO_2-reduction irrespective of the degree of accumulation in different termite species. Whereas, methanogenesis have only slight role in H_2 sink for most wood-feeding termites (Pester and Brune, 2007). Spirochetes catalyze the reductive acetogenesis and some of them have potential nitrogen fixation action (Lilburn *et al*., 2001). The spirochetes are highly diverse among different

termite species. *Treponema* is a common genus of spirochetes that has mutualistic relation with protists to carry out acetogenesis. There are non-homoacetogenic Treponema spp. in lower termites that use cellobiose (Brune, 2014). Since termites' diets have low ratio of nitrogen, symbiotic nitrogen-fixing bacteria are crucial in fixation, recycling, and upgrading of nitrogen. Enterobacteria (e.g. *Enterobacter agglomerans*) and spirochetes (e.g. *Treponema*) have been identified as symbiotic nitrogen-fixing bacteria in termite gut (Potrikus and Breznak, 1977; Lilburn *et al*., 2001). The potential nitrogen fixation by Spirochaetes, Clostridia, Bacteroidetes, and Fibrobacteres has been suggested upon identification of diverse nifH genes in termite gut (Brune, 2014). The consumption of atmospheric N_2 occurs by action of nitrogen fixing bacteria to synthesize amino acids. Uric acid is the major nitrogenous waste of wood-feeding termites, and it is excreted into hindgut via malpighian tubules. Uric acid provides another source of nitrogen which is produced by uric acid-degrading bacteria in the hindgut. Several uricolytic bacteria have been identified in *R. flavipes* gut including *Streptococcus* sp., *Sebaldella termitidis*, and *Citrobacter* sp. (Potrikus and Breznak, 1980). Thong-On *et al*. (2012) indicated 16 species of uric acid-degrading bacteria that are affiliated to Clostridia, Enterobacteriaceae, and low G+C Gram-positive cocci. These bacteria assimilate ammonia into microbial biomass. The ammonia is transferred into foregut and midgut through proctodeal trophallaxis for further enzymes digestion in order to resorb in another form of nitrogen (vitamins and amino acids) by termites. Methanogenesis in the hindgut is carried out by archaea. Archaea are low in density as compared to bacteria, and all methanogens in lower termites belong to the genus *Methanobrevibacter* (order Methanobacteriales). They utilize H_2 and CO_2 and generate methane. They are found on

the gut epithelium, as well as on or within symbiotic protists. The methanogenic archaea in the higher termites are highly diverse as opposed to those in lower termites (Tokura *et al*., 2000; Hongoh, 2010; Brune, 2014).

The symbiotic bacteria in the hindgut of lower termites can be found associated with the protists, or attached to the wall, or as free-living cells in the hindgut lumen. The hindgut lumen is only favorable for the free spirochete bacteria which can swim fast. Uneven distribution of bacterial community of *R. speratus* has been studied by Nakajima *et al*. (2005). They reported that Actinobacteria, Firmicutes, and Bacteroidetes were predominantly associated with the gut wall, and phylogenetically different from the bacteria in the hindgut lumen (such as Spirochaetes and Termite Group I). In general, most bacteria are associated with the surface (ectosymbionts), cytoplasm (endosymbionts), or nucleus (endosymbionts) of protist species (Brune and Dietrich, 2015).

Whereas protists are characterized based on their morphology, bacterial and archaeal communities living in the termite hindguts are mainly studied through molecular methods. Although several bacterial strains have been studied through culturing techniques, bacterial symbionts require strict cultivating environment for survival and reproduction (Fisher *et al*., 2007). Therefore, culture-independent methods make it possible to determine phylogenetic diversity of the termite gut bacteria (Fisher *et al*., 2007).

Heretofore, Ohkuma and Kudo (1996) studied amplified partial 16S ribosomal RNA (rRNA) genes from mixed microbial DNA of the *Reticulitermes speratus* hindgut. Most bacteria in the hindgut were affiliated with five groups of bacteria including the

Cytophaga-Flexibacter-Bacteroides group; the low-G+C Gram-positive bacteria; Proteobacteria; Spirochaeta; and Termite Group I. Later on, Hongoh *et al*. (2003) examined the phylogenetic diversity of bacteria in *R. speratus* through sequence analysis of near-full length 16S rRNA gene clones. The results indicated that spirochetes formed the most prevalent species in the hindgut (Hongoh *et al*., 2003). In their study, many phylotypes were found for the first time and classified into different bacterial phyla containing: Spirochaetes, Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Planctomycetes, Verrucomicrobia, Cyanobacteria, Acidobacteria, and other rare phyla. In addition, the diversity of hindgut bacteria of different termite species has also been examined by 16S rRNA clone analysis (Hongoh, 2010).

The sequence analysis showed that various termite species fed on distinct diets contained different hindgut bacterial community, although the major bacteria phyla remained consistent among termite species. The taxonomy comparison of wood-feeding bacteria in lower termites showed that phylum Spirochaetes were dominant in the hindgut of *R. flavipes* and *R. speratus,* while the phylum Bacteroidetes were the major bacteria species of *Coptotermes formosanus* (Fisher *et al*., 2007; Brune, 2014). However, the results of molecular cloning methods through Sanger DNA sequencing were inadequate for the reliable clarification of bacterial phylogenetic relationship and the species richness of the hindgut bacteria. With technological advancements, 454-pyrosequencing was used in several studies to evaluate the hindgut bacterial community and their changes as affected by different diets (Boucias *et al*., 2013; Arango *et al*., 2014). Nowadays, Illumina offers tremendous improvements that allow researchers to characterize the diversity and richness of microbial community. Therefore, in the present study, the

Illumina MiSeq system for small amplicon sequencing was employed to provide us with many insights into termite hindgut bacterial diversity.

The studies of effects of recalcitrant lignocellulosic diet on the bacterial hindgut of *R. flavipes* revealed that environment and/or termite genetics had more influence on bacterial community than diets across termite colonies (Boucias *et al*., 2013). Huang *et al*. (2013) indicated that the microbial composition of *R. flavipes* fed on woody diets and grassy diets, showed increase in some taxa, although the major bacterial taxa were consistent across all diets. However, the termites fed on corn stover had lower bacterial richness and diversity in comparison to other diets. They concluded that the degree of recalcitrance of diets may be the reason for the microbial community variations. Further investigation of Huang *et al*. (2016) showed that ingested blends of secondary metabolites (fumaric acid, citramalic acid, ethyl-4-hydroxybenzoate, and maleimide) had significant impact on shifting the bacterial community, with increase in abundance of Firmicutes and Spirochaetes. The influence of termiticidal treatments was examined on the bacterial community of *R. flavipes* and *R. tibialis* (Arango *et al*., 2014). The results revealed that among the bacterial groups, Bacteroidetes and Spirochaetes were not affected by termiticidal treatments, and formed the dominant group in *R. flavipes*. Moreover, there was no significant variation on the hindgut bacteria of *R. tibialis* as response to treatments. The bacterial communities change depends on termite ecology. Their variability in host ecology was higher over evolutionary time compared to protist communities in the hindgut (Waidele *et al*., 2017). As previously described, the major phyla of bacterial communities in the hindgut of wood-feeding termites do not change

with different diets, but the composition of these bacteria changes (Benjamino *et al*., 2018).

Chitosan is made from one of the most abundant amino polysaccharide polymers in nature, chitin, which is isolated mainly from the outer exoskeleton of arthropods (including crustaceans and insects), marine diatoms, algae, fungi, and yeasts (Tharanathan and Kittur, 2003; Raafat and Sahl, 2009). The high biocompatibility, biodegradability, non-toxicity, antimicrobial, and adsorption properties of this natural polymer display unique biological characteristics, making it a valuable choice for many applications. Despite the high antimicrobial activities, it has low toxicity to mammalian cells and non-target organisms. Chitosan can be considered as a bactericidal (killing bacteria) or bacteriostatic (inhibiting bacterium growth) compound, but the exact mechanism is still not completely known (Goy *et al*., 2009). Several studies have examined the effectiveness of chitosan on inhibiting the growth of gram positive and negative bacteria (Uchida, 1988; No *et al*., 2002; Takahashi *et al*., 2008). In the current study, chitosan as a non-toxic environmentally friendly treatment for wood was used to evaluate the bacterial community alternation in the termite hindgut fed on chitosan-treated wood.

Termites can be categorized based on their living habitat into three groups: subterranean, drywood, and dampwood. Subterranean termites are lower termites with a significant economic impact on wood structures in the United States. There are different species within subterranean termites containing *R. flavipes*, *R. hesparus, R. hageni*, *R. tibialis, R. virginicus*, and *C. formosanus*, which form the majority of termite damage (Su and Scheffrahn, 1990). Annual termite damages to construction and other wooden

structures has been estimated 1 to 7 billion US dollars by USDA Forest Service in Home and Garden Bulletin publication in 2006. Although these estimates may not include all treatments and repairs cost. *R. flavipes* is the most common species in the southeastern part of the United States and their colony structure varies in the number of termites (Howard *et al*., 1982). *R. virginicus* is known as a dark southern subterranean termite. Both *R. flavipes* and *R. virginicus* are dominant species in forest and residential area in Mississippi (Wang and Powell, 2001). The damage of *R. virginicus* was assumed insignificant until Su and Scheffarhn (1990) reported high rate of infested structures in Florida. Because of the morphological similarity between *R. virginicus* and *R. flavipes*, structures infested by *R. virginicus* have been identified incorrectly. As results of misidentifying, this species has attracted less attention. Most studies on *R. virginicus* examined protist diversity and abundance, and there are no studies investigated the influence of diets and treatments on bacterial community in *R. virginicus*. Therefore, *R. virginicus* was chosen in this study of characterization of the effect of chitosan treatments on the bacterial diversity and their abundance in the hindgut using Illumina MiSeq sequencing of the V3-V4 hyper-variable regions of 16S rRNA gene.

3.3 Materials and methods

3.3.1 Termite collection and species verification

One termite colony was collected from a decaying pine log from Mississippi State University Dorman Lake Test Site, Starkville, Mississippi (May 2017). The log was cut into small sections, which were placed into two covered metal containers (32-gallon per container). Subsequently, the containers were maintained at a room temperature in the dark. They were moistened with damp laboratory paper towels every 3 to 4 weeks as

needed. Termite workers and some soldiers were sampled after 2 months following the collection date, whereas some termite samples were obtained upon collection to examine their microbial community from their natural habitat.

To identify termite species, two methods (morphological observation and molecular identification) were employed. A species identification guide by Messenger (2001) was used to examine the morphological features of termite species, and accordingly, identify the species. In addition, genomic DNA from five termite soldier heads was sequenced based on the mitochondrial AT-rich region designed by Foster *et al*. (2004). MasterPur^{TM} Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA) was used to extract the genomic DNA, and subsequently the DNA was amplified in a polymerase chain reaction (PCR) using forward (5′-TGGGGTATGAACCAGTAGC-3′) and reverse (5'-CACTAAGGATAATCAATTATACGTC-3') primer sequences, as described previously (Foster *et al*., 2004). The amplified PCR product (~ 400 bp) was excised from 1% agarose gel using QIAquick Gel Extraction Kit (Qiagen), and then ligated to the pGEM-T Easy Vector System II (Promega, Madison, WI). The recombinant clones were selected on LB/ampicillin/IPTG/X-Gal plates by blue and white color screening. Afterwards, each white clone was transferred into JM109 High Efficiency Competent cells (*Escherichia coli* cells) as instructed by the manufacturer. Plasmid DNA was isolated from four clones according to PureLink Quick Plasmid Miniprep Kit protocol (Invitrogen, Carlsbad, CA). The plasmid DNA was sent for sequencing to Eurofins Genomics (Louisville, KY). The obtained DNA sequences were trimmed by removing pGEM-T Easy vector sequences using Finch TV software version 1.4.0 (Geospiza). The trimmed sequences were aligned against the NCBI non-redundant

nucleotide database to find sequences with the greatest percent similarity to the colony-derived sequences from this study to determine termite species identity.

3.3.2 Chitosan solution preparation and wood treatment

Low molecular-weight chitosan powder (50-190 KDa) purchased from Sigma-Aldrich (St Louis, MO, USA) was used for making 0.5% chitosan (w/v) solution. Chitosan (0.5 g) was dissolved in 100 mL of 25% aqueous acetic acid (w/v). The solution mixture at pH 1.85 was stirred vigorously using a laboratory magnetic stirrer at room temperature until no chitosan particles remained in the solution.

Defect free southern yellow pine, *Pinus* spp., sapwood samples measuring 25 × 25 × 6 mm (tangential × radial × longitudinal) were oven-dried at 50°C to reach a constant weight. The oven-dried wood samples were randomly chosen and submerged in 100 mL of different treatments including: 0.5% chitosan solution, 25% acetic acid solution (w/v), and distilled water, whereas the two latter treatments were used as controls. The samples treatments were vacuum treated at 29.8 mmHg vacuum for 3 h, and the samples were subsequently equilibrated in the solutions for 24 h (Figure 3.1). Samples were taken out from the solutions, and their surfaces were gently cleaned to remove the extra solutions. The samples were kept on a bench at room temperature for several hours to allow for air-drying and dried treated weights were obtained using a laboratory oven at 50°C until constant mass was reached.

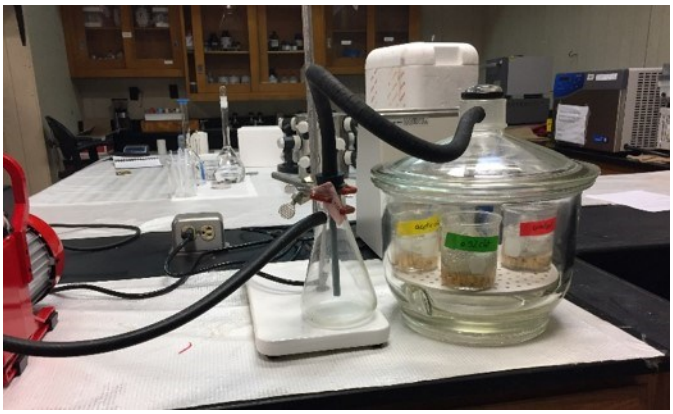

Figure 3.1 Desiccator containing three beakers of wood samples and their treatments under vacuum condition.

3.3.3 Termite no-choice exposure laboratory bioassay

A no-choice test was performed according to a modified American Wood Protection Association E1-16 Standard (AWPA, 2016). In this study, one of the modifications was the moisture content of soil that was lower compared to AWPA standard E1-16. Another minor change was decreasing the amount of worker termites used in this study (from 400 individual termites in AWPA E1 to 300 individual termites). A total of 20 glass screw-top jars (8 cm dia, 10 cm tall) each containing 150 g play sand (Quikrete Premium Play Sand) and 24 mL distilled water were autoclaved for 45 minutes. Upon cooling, one test wood block and 1 g of worker termites, which contained approximately 300 workers and 3 soldiers, were added to each jar. The experiment included five treatment groups: unexposed termites (termites sampled directly from the decayed log, UNX), 25% acetic acid-treated wood-exposed termites (ACE), water-treated wood-exposed termites (WE), 0.5% chitosan-treated wood-exposed termites (CTE), and starved termites (no test wood block in the replicate jar, STV). Each treatment group

comprised of five replicate jars. All termites (except UNX) were exposed to treated wood simultaneously and kept in a laboratory incubator (25°C) for 18 days (Figure 3.2). After 18 days, all survived workers were sampled, cleaned, and stored at -80°C for two days until the time of DNA isolation. Workers from UNX group were collected and stored in the freezer (-80°C) on the day of termite collection from the forest.

Figure 3.2 Termite jars exposed to treatments in the laboratory incubator at 25°C.

3.3.4 Termite dissection, DNA isolation, and RNA removal

Sixty termites were collected from each jar, and in total 300 termite guts were sampled for each treatment. Guts were removed from the termites by pulling the tip of the abdomen from thorax with forceps to release the digestive tract from the termite exoskeleton. Five guts were pooled and washed in a droplet of PBS buffer solution (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2). Guts of sixty termites from each replicate jar were processed in 12 microcentrifuge tubes (1.5 mL), each containing a pool of five guts. Briefly, the MasterPure^{TM} Complete DNA and RNA Purification Kit protocol was followed by adding 300 µL of Tissue and Cell Lysis Solution and 1 µL of

Proteinase K into a single microcentrifuge tube, and then the mixture of guts and solutions were homogenized by a sterilized pestle. The samples were incubated at 65°C for 15 minutes and inverted every 5 minutes. Upon cooling to 37°C, 1 µL of 5 µg/µL RNase A was added into each tube and incubated for 30 minutes. Before adding150 µL of MPC Protein Precipitation Reagent to the mixtures, samples were placed on ice for 5 minutes. Samples containing precipitation reagent were vortexed vigorously for 10 second and pelleted by centrifugation at 4°C (10.000× g for 10 min). The supernatant was transferred into new microcentrifuge tubes, and 500 µL of isopropanol was added to precipitate the DNA again by centrifugation (10.000× g for 10 min). The DNA was washed twice with 70% ethanol, allowed ethanol to dry off, and lastly suspended in 35 µL of TE buffer (10 mM Tris-HCL, pH 8.5). After resuspension, 4 microcentrifuge tubes of the extracted DNA were pooled into 1 subsample, so a total of 3 subsamples were obtained per replicate jar (i.e. 60 termites), 15 DNA subsamples per treatment, and a total of 75 DNA subsamples for the experiment, i.e. 5 treatment groups.

RNA from the extracted DNA was removed using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA). The minor changes were applied to the manufacturer protocol of the Qiagen kit. Three µL of RNase A (100 mg/mL) was added to 100 µL of each subsample. The mixture was gently inverted and incubated in water bath for 15 min. After incubation, 200 µL of buffer AL (Qiagen), and subsequently 200 µL of 100% ethanol were added into the mixture. The mixture was transferred to the DNeasy Mini spin column, centrifuged (6000× g), the supernatant decanted, and the pellet washed with 500 µL Buffer AW1, and subsequently with AW2 (Qiagen), according to manufacturer protocol. Finally, the column was placed in a clean 1.5 mL

microcentrifuge tube and 50 µL of TE buffer added directly onto the DNeasy membrane, followed by incubation at room temperature for 1 min, and centrifugation for 1 min (6000× g). The genomic DNA suspended in TE buffer was applied to the same DNeasy membrane to increase the maximum yield of DNA.

Concentration and quality of the genomic DNA subsamples were assessed by NanoDrop™ spectrophotometer and agarose gel electrophoresis, respectively. Genomic DNA concentration ranged from 16.56 – 94.57 ng/µL as shown in appendix B Table B.1.

3.3.5 Metagenomics library preparation

To prepare bacterial metagenomics library, the 16S Metagenomic Sequencing Library Preparation Guide (Illumina Part # 15044223 Rev. B) was used to followed from the DNA of subsamples, V3 and V4 hyper-variable region of the 16S rRNA gene were amplified using forward gene-specific primer sequence 5′-CCTACGGGNGGCWGCAG-3′ and reverse gene-specific primer sequence 5′-GACTACHVGGGTATCTAATCC-3′ developed by Klindworth *et al*. (2013). The Illumina adaptor overhangs nucleotide sequences were attached to the 5′ end of the gene-specific primer sequences as shown below:

Forward overhang: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[forward gene-specific sequence] and

Reverse overhang: 5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[reverse gene-specific sequence].

ReadyMix PCR Kit (Illumina, San Diego, CA) was used to amplify 16S rRNA gene. To increase amount of the PCR amplicon product, a minor change was applied. The volume of PCR reaction was increased from 25 µL to 28 µL. In addition, the microbial

genomic DNA concentrations was changed from 5 ng/µL of Illumina protocol to 10 ng/µL. Each PCR reaction included 2× KAPA HiFi HotStart ReadyMix (14 µL), 5.6 µM forward and reverse amplicon PCR primers, 10 ng/µL microbial genomic DNA, and dH_2O to the final volume of 28 µL. Amplification was conducted using with the following program: 95°C for 3 min, followed by 25 cycles of 95°C (30 s), 55°C (30 s), and 72°C (30 s), with a final extension step of 72°C for 5 min. The amplicon PCR were separated by gel electrophoresis on 1.5% agarose gel in 1× TAE (Tris-Acetate EDTA) buffer to observe purity and size of products (Figure C.1). All amplicon PCR products were purified by Agencourt AMPure XP (Beckman Coulter Inc., Brea, CA, USA) magnetic beads according to following protocol: (1) AMPure XP beads were brought to room temperature and vortexed to disperse beads evenly, (2) 20 µL of AMPure XP beads were added to each amplicon PCR reaction by pipette to combine entire volume, (3) the mixed solution were incubated at room temperature for 5 min without any movement, then (4) they were placed on 16-Tube SureBeads™ Magnetic Rack (Bio-Rad, Hercules, CA) for 2 min, (5) the supernatant was decanted and the beads were kept on the magnetic stand and washed twice with 200 µL of 80% ethanol, which was freshly made, (6) each wash required 30 second incubation at room temperature and the supernatant was decanted, (7) after removing excess ethanol, the amplicon PCR stayed on the magnetic stand for 10 min to air-dry completely, (8) the amplicon PCR was removed from the magnetic stand and 52.2 µL of 10 mM Tris-HCl buffer (pH 8.5) was added to the dried beads and mixed the entire volume well, (9) the mixed buffer and beads were incubated at room temperature for 2 min and placed on the magnetic stand for 2 min, and finally

(10) 50 µL of supernatant from the amplicon PCR clean-up was transferred into a new 1.5 mL microcentrifuge tube.

Index PCR reaction comprised a unique combination of Nextera XT Index Primer 1 (5 µL), Nextera XT Index Primer 2 (5 µL), 2× KAPA HiFi HotStart ReadyMix (25 µL), PCR grade water in the amount of 13 or 14 µL, depending on the concentration of amplicon PCR clean-up, and the amplicon PCR clean-up (DNA) to reach final volume of 50 µL. The index PCR program was followed: 95°C for 3 min, then 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension step of 72°C for 5 min. Index PCR clean-up from all samples were also purified with the magnetic beads. The protocol of magnetic beads cleaning in this step was similar to the amplicon PCR purifications, with three slight changes: in step (2), 56 µL of AMPure XP beads were added to each indexed PCR sample, in step (8), 27.5µL of 10 mM Tris pH 8.5 was added to the dried beads, and finally in step (10), 25 µL of the supernatant from the index PCR clean-up (that called a library) was transferred into a new 1.5 mL microcentrifuge tube. All 75 libraries were then quantified by Qubit dsDNA HS assay (Life Technologies: Molecular Probes32851 Rev. B) using Qubit 1.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). The following assay was performed for all 75 libraries: (1) Qubit working solution was prepared in a clean plastic tube by diluting Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer based on the number of libraries and standard solutions, (2) 190 µL of Qubit working solution was added into each Qubit tube that contained 10 µL of Qubit standard 1 and 2 respectively and vortexed for 2 seconds, (3) 198 µL working solution was added to each new Qubit assay tube that consisted of 2 µL of the 5× dilution of each library, and vortexed for 2 seconds, (4) all

prepared samples and standard solutions were incubated at room temperature for 2 min, (5) standards 1, 2, and samples were read respectively in each run. Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, CA) was used to verify the expected size of approximately 630 bp (Table C.1). Concentrations of each library were calculated and then diluted to 20 nM using 10 mM Tris-HCl (pH 8.5) plus 0.1% Tween-20. Subsequently, 2 μL of each diluted DNA library from all 75 samples was pooled in equimolar amounts for Illumina MiSeq sequencing. Bioanalyzer analysis and sequencing were performed by personnel at Institute for Genomic, Biocomputing and Biotechnology (IGBB) at Mississippi State University.

In summary, there were eight essential steps to make 16S library including: (1) amplifying 16S rRNA V3-V4 amplicons by PCR, (2) cleaning up the amplicon PCR product by AMPure XP beads, (3) using unique barcode combination by attaching specific barcode (Nextera XT Index Primer 1 and 2) sequence to each end of the amplicon PCR clean-up through PCR, (4) cleaning up the index PCR product now known as a library, (5) validating libraries by Qubit and Bioanalyzer, (6) making the libraries in equimolar amounts for sequencing, (7) pooling the libraries into one sample tube, and (8) Using Illumina MiSeq platform to sequence the 75 libraries in one run.

3.3.6 Sequencing, data processing, and analysis

The pooled libraries were sequenced on the Illumina-MiSeq, with paired read lengths of 300 bp and MiSeq v3 reagents, the ends of each read were merged to create full length of the V3 and V4 regions of the 16S rRNA gene (Illumina Part # 15044223 Rev. B). MiSeq outputs demultiplexed FASTQ sequence files corresponding to the initial sample libraries pooled, which were readily usable for secondary analysis.

Data analysis was performed using the open source software, Quantitative Insights Into Microbial Ecology (QIIME 2 version 2018.8) (Caporaso *et al*., 2011). Sequences were imported to QIIME 2 using Casava 1.8 paired-end demultiplexed fastq format to convert input data into a QIIME 2 artifact (.qza file format). The Casava 1.8 paired-end demultiplexed fastq format comprised of two files (fastq.gz, forward and reverse) for each sample, including sample identifier, barcode identifier, lane number, read number, and the set number each separated with an underscore. After visualization of reads quality scores, the reads displayed high quality scores (≥ 20) were chosen to trim all forward and reverse sequences from position 14 to 252 bp. The DADA2 plugin (Callahan *et al*., 2016) adopted into QIIME 2 was used for removal the PhiX (adapter-ligated library) control reads and chimeric sequences. In addition, the DADA2 plugin clusters unique sequence variants, which is essentially operational taxonomic units (OTUs) at 100% similarity level. Using different distance metrics to generate a rooted phylogenetic tree, to perform a multiple sequence alignment of the sequences and remove their highly variable positions in QIIME 2 package. Alpha diversity (Shannon and Observed OTUs) and phylogenetic diversity at a sequencing depth of 200,000 reads were performed in QIIME 2. Species diversity were calculated for each treatment community using Shannon and Observed OTUs methods to discern respectively the quantitative and qualitative biodiversity of the communities (richness), while also accounting for species evenness (Faith and Baker, 2006). As for species evenness, which explained how close in number bacteria species observed in the hindgut community and represented by Pielous's Evenness index (Pielou, 1966), the most even community approached a value of 1, while the least even community approached a value of 0. Rarefaction curves of sample grouped

by treatment was generated to estimate richness of the treatment communities at a sequencing depth of 200,000 reads. Beta diversity metric was calculated by applying Weighted UniFrac (Lozupone *et al*., 2011), which is a quantitative measure of community dissimilarity based on phylogenetic relationships between the OTUs. Principal coordinate analysis (PCoA) plot of Weighted UniFrac distance was also constructed using Emperor plug-in in QIIME 2 to visualize the relationships of the treatment groups based on the relative abundance of OTUs. To determine the significance of bacterial community differences among treatment groups, PERMANOVA (permutational multivariate analysis of variance) statistical analysis was performed using the Weighted UniFrac distance metric in QIIME 2 and then followed by pairwise comparison analysis between treatment groups. This analysis was performed over 999 permutations and provided a Pseudo-F (*f*) and *p*-value (*p*).

Representative sequence of each OTUs were then taxonomically classified using DictDb database. DictDb database (v. 3.0, 2015) is a curated database containing 16S rRNA sequences of bacteria associated with insect guts, including termites (Mikaelyan *et al.*, 2015). Heatmap was constructed using hierarchical clustering of sequence data to show the relative abundances of bacteria among treatment groups at the phyla taxonomic level.

The relative abundance of the hindgut bacterial community per treatment was computed at each taxonomic level (Phyla to Genera). Individual sample read in each treatment group was normalized to 650,000 reads. The 650,000 was the highest number of reads among samples obtained from sum of number of reads per sample unit across row. Then proportion of reads per sample unit was calculated by dividing the individual

sample read at a specific taxonomic level per total reads for that sample and then multiplied by 650,000. Average of the normalized sample read for each taxonomic level in each treatment group was computed to depict the relative abundance of bacteria. Statistical Analysis Software version 9.4 (SAS 2013) was used to perform One-way Analysis of Variance (ANOVA) to determine differences for each level across the treatment groups and calculate mass loss of treated wood samples exposed to termites.

3.4 Results and discussion

3.4.1 Termite species verification

Termite colony was identified as *R. virginicus* based on the termite identification guide and comparison of termite sequence against NCBI-nr nucleotides database with 86% query cover and 99% identity.

3.4.2 Termite no-choice exposure laboratory bioassay

Mass loss values of treated wood samples exposed to *R. virginicus* showed that there was no significant difference between mass loss of 0.5 % chitosan-treated wood and controls (water and 25% acetic acid). It could be that the low concentration of chitosan treatment did not prevent termites to stop eating wood. Raji *et al*. in 2018 indicated that the mass loss of wood treated with 0.5% chitosan solution and exposed to *R. virginicus* was not significantly different from the mass loss of 25% acetic acid-treated wood control and 1% chitosan-treated wood, while it was different from the water-treated wood control. However, there was no difference observed between mass loss of 0.5% chitosan-treated wood and controls in our experiment.

3.4.3 Metagenomics data analysis

Bacterial communities of *R. virginicus* were profiled through V3 and V4 regions of 16S rRNA Illumina MiSeq sequencing analysis. The total number of raw sequence reads resulting in approximately 19 million reads obtained from Illumina MiSeq sequencer (for the 75 samples) were initially demultiplexed from the paired-end libraries to 14,625,126 reads, which was 77.4% of total reads from the library preparation. All forward and reverse sequence reads were finally filtered and trimmed to 11,320,858 reads using DADA2 plugin in QIIME 2. As described in the 3.3.4 section of materials and methods, the 75 sample libraries originated from 15 samples of each treatment group, and each treatment group comprised of 5 replicate jars, each one having 3 subsamples. To reduce error in standard deviation, the reads from subsamples per replicate jar were grouped and then the 75 of samples converted to 25 samples. After filtering and removing chimeric sequences, for alpha diversity analysis and phylogenetic clustering, the selected sampling depth of 200,000 accounted for 44.17% of total processed reads in 100% of all 25 samples. QIIME 2 clustered unique sequence variants at 100% similarity into OTUs and 5,144 unique OTUs were identified. Although, 3,004 OTUs were identified as rare OTUs occurring in less than 3 samples out of 25 samples and a total frequency of less than 10. Among these rare OTUs, 11 OTUs occurred in less than 3 samples but with a frequency greater than 500. The BLAST of these rare OTUs against the NCBI nt database showed that 4 OTUs were assigned to Proteobacteria, 3 OTUs for phylum Firmicutes, and the remaining OTUs including Actinobacteria, Spirochaetes, Bacteroidetes, and Elusimicrobia with a range of 94 to 100 % identity (Table 3.1). The 11 rare OTUs had perfect BLAST matches with different organisms, which were isolated

from termite gut, environmental sample, sewage sludge, and soil. Therefore, there is a possibility that these 11 OTUs are not rare and they just have low abundance. The results of alpha diversity analyses are shown in Table 3.2 and a single value was presented for each treatment group by averaging the values of sample replicates within a treatment. Following similar trends for richness and evenness, UNX treatment was observed as the most diverse community when compared with other treatments. The UNX treatment group showed the most evenness of bacterial diversity (75%) among treatment groups.

Table 3.1 Number of rare operational taxonomic units (OTUs) present at high frequency and taxonomy classification against NCBI

OTUs ID	Frequency	Taxonomy classification	Organisms	Identity %
f574429038c574c1a6e62520881032b3	2,954	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae	*Stenotrophomonas acidaminiphila* strain ABG	100
eed09d3461cc53dd795f8e8a4bc6679b	579	Bacteria; Actinobacteria; Propionibacteriales; Nocardioidaceae	*Nocardioides daejeonensis* strain MJ31	100
a050414fe7a61665f7a0590f84e32bd3	530	Bacteria; Spirochaetes; Spirochaetales; Spirochaetaceae;	*Treponema* sp.	99
c04547b04b841348e0beaa5e59e22cc3	13,630	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae	*Elizabethkingia miricola* strain DSM _14571and *E. meningoseptica* strain C6-5	100
1a0294b1b23ba155cb73705c768466b0	5,088	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	*Lachnospiraceae bacterium* clone 290cost002-P3L-1430	94
e965f20b687a219f7d732a315f45cfaf	1,405	Bacteria; Elusimicrobia; Endomicrobia; Endomicrobiales; Endomicrobiaceae	Candidatus *Endomicrobium pyrsonymphae* clone Pv-1	99
410c9e67dff52f43b2f226f53a7f5dba	1,156	Bacteria; Firmicutes; Clostridia; Clostridiales; Eubacteriaceae	Eubacteriaceae bacterium	99
34e13b5900833ca42f32492a0fabbe21	1,016	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	Ruminococcaceae bacterium clone 4a_78715	96
eb4c82dc3ebb26c0fc04989c0b4e5c35	831	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	*Aquamicrobium lusatiense* strain XJ-3	100
14b219f1b5ac396f1b08d27347193154	696	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae	*Brevundimonas diminuta* strain 4H03 and *B. naejangsanensis* strain MS09	100
3097e3b819d2d8aac5992b0c8a65e2cd	573	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae	*Pandoraea* sp. OXJ-11	100

Table 3.2 Bacterial alpha diversity of *R. virginicus* hindgut within treatment groups based on the 16S rRNA amplicon

Treatment*	Shannon diversity index	Observed OTUs	Pielou's Evenness
UNX	8.16 ± 0.03	1823 ± 142	0.75 ± 0.01
ACE	6.67 ± 0.12	900 ± 114	0.68 ± 0.02
WE	6.76 ± 0.19	928 ± 184	0.69 ± 0.01
CTE	6.43 ± 0.27	889 ± 135	0.66 ± 0.02
STV	6.45 ± 0.34	798 ± 91	0.67 ± 0.03

* UNX, unexposed termites; ACE, 25% acetic acid-treated wood exposed to termites; WE, water-treated wood exposed to termites; CTE, 0.5% chitosan-treated wood exposed to termites; STV, starved termites. Average of bacterial diversity per treatment in each alpha diversity method ± SD (standard deviation).

Alpha rarefaction curves based on observed bacterial community richness showed that at a maximum sequencing depth of 200,000 reads, the richness of samples grouped by treatment was completely detected. The plot suggested that the bacterial diversity of the hindgut contents from UNX treatment group had the highest diversity, whereas STV treatment group displayed the lowest richness (Figure 3.3). The WE treatment showed more species diversity, after UNX treatment, compared to the other treatment groups.

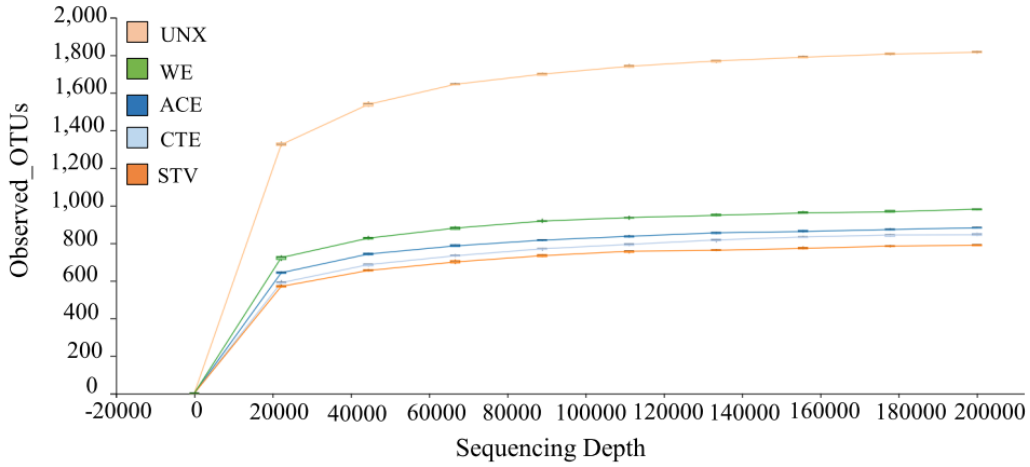

Figure 3.3 Rarefaction of sequencing depth to assess species richness.

To assess whether the microbial community in the hindgut was affacted after exposure of termites to different treatment groups, the Weighted UniFrac distance was implemented and used to create a PCoA plot of the five treatment groups (Figure 3.4). PCoA plot revealed a similar separation, with STV and UNX group also diverging from the other treatment groups.

PERMANOVA with Weighted UniFrac dissimilarity matrix for sample size 25 indicated that there were significant differences among five treatment groups (test statistic = 23.33 and $p = 0.001$). The relative abundance of microbial community of termites fed on CTE did not exhibit a significant change when compared to ACE and WE treatment group according to Pairwise PERMANOVA (CTE vs ACE: $f = 1.27$, $p = 0.3$; CTE vs WE: $f = 2.4$, $p = 0.098$). However, significant differences were observed in the relative abundance of bacteria between CTE versus STV (PERMANOVA: $f = 16.74$, $p = 0.011$) and CTE versus UNX (PERMANOVA: $f = 22.83$, $p = 0.006$). The relative abundance of bacterial community from termites exposed to ACE showed no significant difference with WE treatment group (PERMANOVA: $f = 1.48$, $p = 0.2$), while it differed significantly from STV ($f = 29.73$, $p = 0.008$) and UNX ($f = 74.24$, $p = 0.011$). The WE treatment group followed a trend of differences for STV ($f = 31.8$, $p = 0.012$) and UNX ($f = 73.82$, $p = 0.006$). In addition, the bacteria abundances were significantly different between UNX and STV treatment groups (PERMANOVA: $f = 52.9$, $p = 0.005$). Benjamino *et al*. (2018) described a temporal impact of dietary changes on the hindgut microbiota of *R. flavipes*. The microbial communities maintained without any changes for the first 7 days of experiment like day 0, but they were significantly changed later

according to PERMANOVA ($f = 4.18$, $p = 0.001$) using Bray-Curtis dissimilarity matrix in QIIME. Although we did not perform a temporal study on diet, the results of Benjamino *et al*. (2018) clarified that ACE, CTE, STV, and WE were significantly different from UNX. This can be explained by sampling termites after 18 days of exposure to treatment, except for UNX, which was sampled on the day of the log collection. Thus, higher microbial diversity is expected in UNX since termites in natural habitat forage different food sources and acquire new microorganisms from soil.

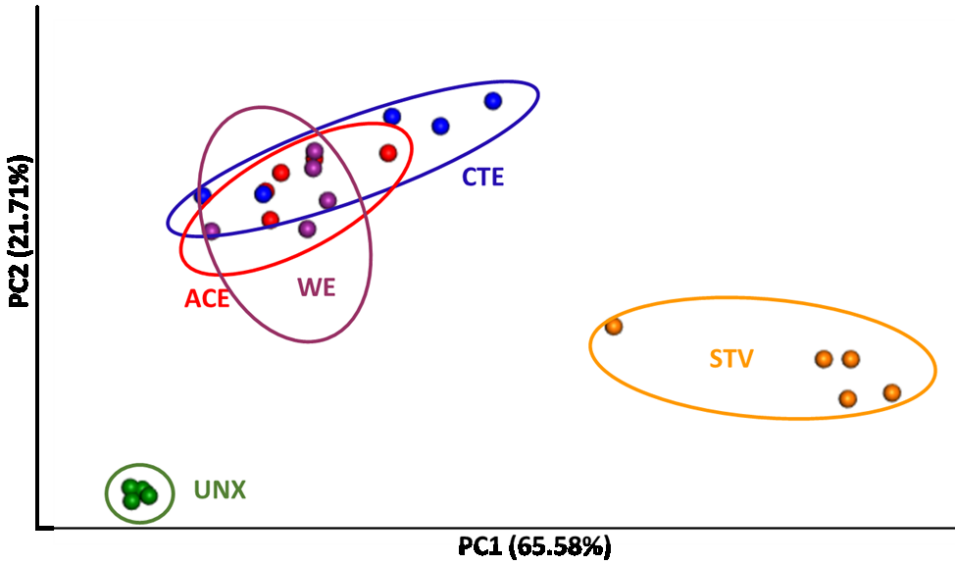

Figure 3.4 Principal coordinates analysis (PCoA) (Weighted UniFrac) based on the distance matrix for operational taxonomic units (OTUs) displaying differences among bacterial community of *R. virginicus* exposed to different treatment groups.

UNX, unexposed termites; ACE, 25% acetic acid-treated wood exposed to termites; WE, water-treated wood exposed to termites; CTE, 0.5% chitosan-treated wood exposed to termites; STV, starved termites. Ellipses correspond to different treatment groups and the similarity of microbial community within each treatment group.

All sequences were taxonomically identified and aligned against DictDb_v3 database. The results were classified to phylum, class, order, family, and genus levels at 100% identity level. A total 28 phyla, 50 classes, 101 orders, 190 families, and 409 genera were classified. In Figure 3.5, the composition of bacterial 16S rRNA sequences in *R. virginicus* at the phyla level was annotated to high percent sequence of reads. Of 28 phyla, 16 bacterial phyla, listed in descending order of frequency of reads, appeared in all 25 samples: Bacteroidetes (34.4% total of reads), Firmicutes (20.6%), Elusimicrobia (15.7%), Proteobacteria (12.9%), Spirochaetes (8.2%), Actinobacteria (2.3%), Candidate_phylum_TM7 (1.4%), Tenericutes (1%), Synergistetes (0.8%), Unassigned (0.7%), Verrucomicrobia (0.6%), Chlorobi (0.5%), Planctomycetes (0.3%), Candidate_phylum_SR1(0.15%), Candidate_phylum_BD1_5 (0.07%), and Candidate_phylum_OP11 (0.04%). The remaining 12 phyla (Deferribacteres, Cyanobacteria, Candidate_phylum_OD1, Lentisphaerae, Candidate_phylum_TG3, TA06, Acidobacteria, Fusobacteria, Fibrobacteres, Chloroflexi, Candidate_phylum_BRC1, and Armatimonadetes) were observed in less than 25 samples and formed 0.13% of the total number of assigned reads. The latter three phyla were detected in less than three total samples, and therefore, they were considered as rare phyla.

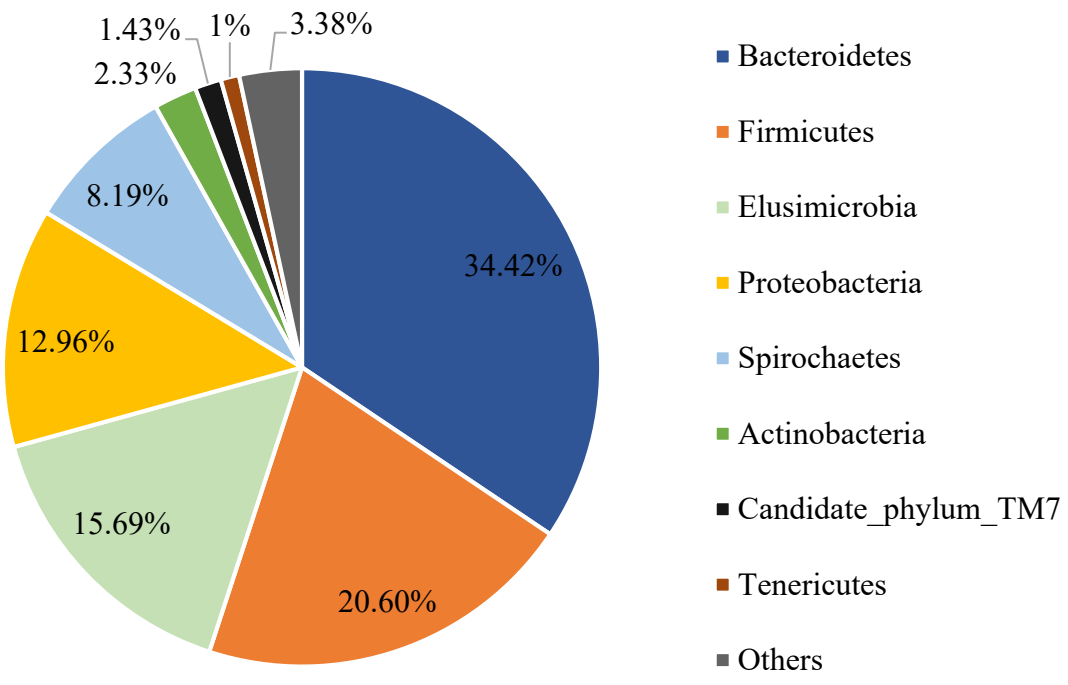

Figure 3.5 Observed composition of the bacterial community at phylum level.

To determine the presence and absence of the bacterial communities at the phyla level in each sample, a heatmap with weighted average hierarchical clustering of sequence data was generated in QIIME 2 (Figure 3.6). The lighter colored cells in the heatmap correspond to greater relative abundance. The treatment samples formed two major clusters, UNX cluster and the remaining treatment group cluster. The latter cluster included two major sub-clusters, where STV formed a separate branch from WTE, ACE, and CTE (Figure 3.6A, vertical scale). Bacteria phyla analysis also revealed two major clusters, and each one of them contained two main sub-clusters (Figure 3.6B, horizontal scale). One cluster contained 13 bacterial phyla of higher abundance than the second

cluster containing 15 phyla. The predominant bacterial phyla in the high-abundance cluster, were Bacteroidetes, Firmicutes, Proteobacteria, Elusimicrobia, and Spirochaetes.

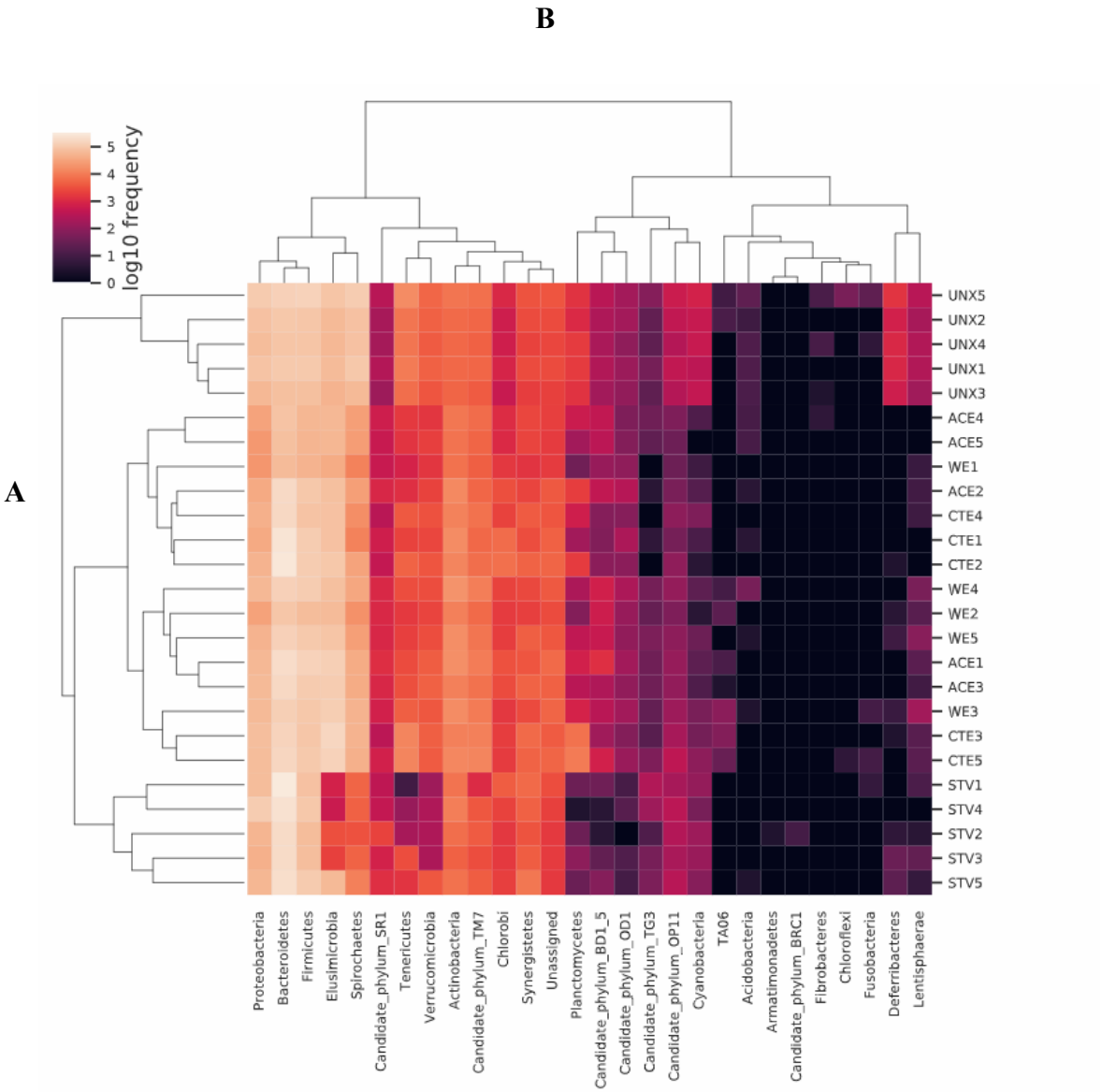

Figure 3.6 Abundance of bacterial phyla per treatment group in the hindgut of *R. virginicus* displaying with heatmap.

The treatment groups are labeled on the right and their clusters on the left (A). The bacterial phyla clusters on the top (B). UNX, unexposed termites; ACE, 25% acetic acid-treated wood exposed to termites; WE, water-treated wood exposed to termites; CTE, 0.5% chitosan-treated wood exposed to termites; STV, starved termites.

According to Fisher *et al*. (2007), the diversity of hindgut bacteria in *R. flavipes* as examined by 16S rRNA gene sequencing, revealed six dominant phyla Spirochaetes, Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria and Endomicrobia. In the present study, the relative abundance of the bacteria communities at the phyla level differed among treatments (Figure 3.7).

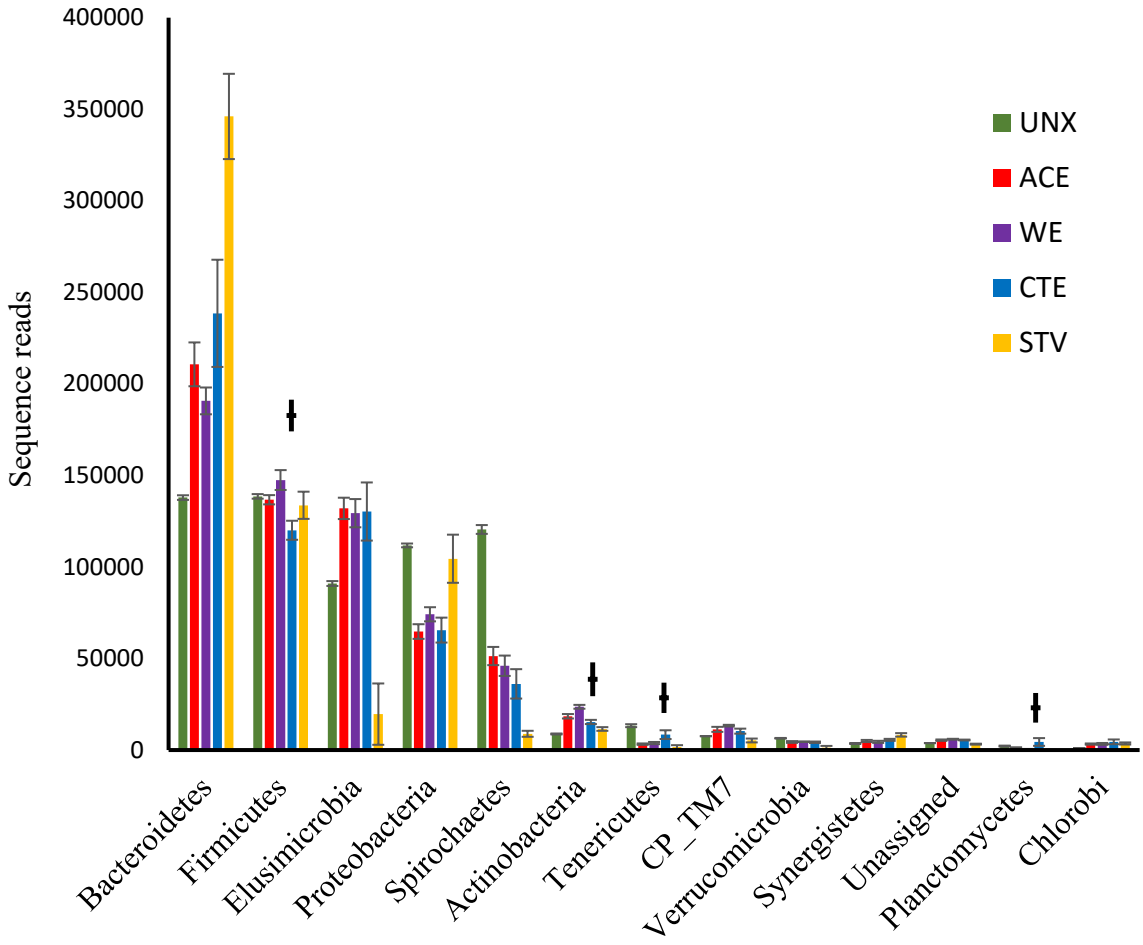

Figure 3.7 Relative abundance of 16S rRNA reads assigned to bacteria phyla in different treatment groups.

Bars represent averages ± SE (n = 5). Cross sign (†) above bars $p < 0.05$; indicates a specific phylum affected by CTE (0.5% chitosan solution) using ANOVA. CP_TM7, Candidate_phylum_TM7; UNX, unexposed termites; ACE, 25% acetic acid-treated wood exposed to termites; WE, water-treated wood exposed to termites; CTE, 0.5% chitosan-treated wood exposed to termites; STV, starved termites. Multiple comparisons were performed without correcting *p* values.

Most of the bacterial phyla belonged to Bacteroidetes, Firmicutes, Elusimicrobia, Proteobacteria, and Spirochaetes. Previous studies reported the consistency of bacterial

taxa in the hindgut of different termite species, but they also stated differences in relative abundance among taxa (Hongoh, 2010; Huang *et al*., 2013).

Several studies showed the effect of diet on the bacterial community in hindgut of subterranean termite species, specifically in *R. flavipes* and *C. formosanus*. Tanaka *et al*. (2006) reported that high molecular weight carbon diets share only 40% similarity of bacterial community with low molecular carbon diets of *C. formosanus*. Another study revealed that the microbial composition of *C. formosanus* shifted significantly between field-collected termites and termites fed on filter paper (Husseneder *et al*., 2009). The high-throughput 454 pyrosequencing of 16S V5-V6 amplicons of bacterial microbiota from *R. flavipes* exposed to lignocellulose diets indicated that the majority of bacterial phyla included Spirochaetes (24.9%), Elusimicrobia (Termite Group 1, 19.8%), Firmicutes (17.8%), Bacteroidetes (14.1%), and Proteobacteria (11.4%), while the amplification of 16S V1-V3 regions of bacteria in *R. flavipes* fed with grassy and woody plant substrates have showed the presence of majority phyla Spirochaetes (37%), Firmicutes (18%), Elusimicrobia (10%), and Verrucomicrobia (10%) (Boucias *et al*., 2013; Huang *et al*., 2013). In our study, Illumina sequencing of 16S rRNA V3-V4 region of bacterial microbiota in *R. virginicus* indicated that the majority of sequence reads were assigned to two taxa, Bacteroidetes (34.4%) and Firmicutes (20.6%). The difference in bacteria phyla between our study and others can be explained by the effect of different diets.

In our study, the relative abundance of Bacteroidetes phylum bacteria in termites exposed to STV treatment group was significantly higher when compared to UNX and other treatment groups. Moreover, slightly higher relative abundance of members of

phylum Bacteroidetes in CTE treatment was observed when compared to ACE and WE treatment groups. On the other hand, members of phylum Firmicutes had similar abundance among termites exposed to ACE, STV and UNX, while the relative abundance of this phylum was significantly lower in CTE treatment when compared to WE (WE was not significantly different from STV) and other treatment groups. A significant effect of chitosan treatment (CTE treatment group) was observed only on four bacteria phyla Firmicutes, Actinobacteria, Tenericutes, and Planctomycetes. Although, the significant effect of CTE treatment was not observed on Proteobacteria and Spirochaetes phyla, the analysis of the chitosan effect on genus level revealed that one OTU of Proteobacteria and two genera, unclassified and Treponema_Ib from Spirochaetaceae_Treponema_I family, were significantly affected by CTE ($p < 0.05$). ACE, CTE, and WE treatment group had significantly higher abundance of some taxa such as Elusimicrobia, Actinobacteria, Candidate_phylum_TM7, and Unassigned. In contrast, starvation (STV treatment) significantly reduced the population of Elusimicrobia, Spirochaetes, Tenericutes, and Verrucomicrobia phyla.

Our data showed that *R. virginicus* consisted of 409 bacteria genera. The influence of treatment among the genera were also evaluated. The high frequency sequence reads for genera in *R. virginicus* were shown in Figure 3.8.

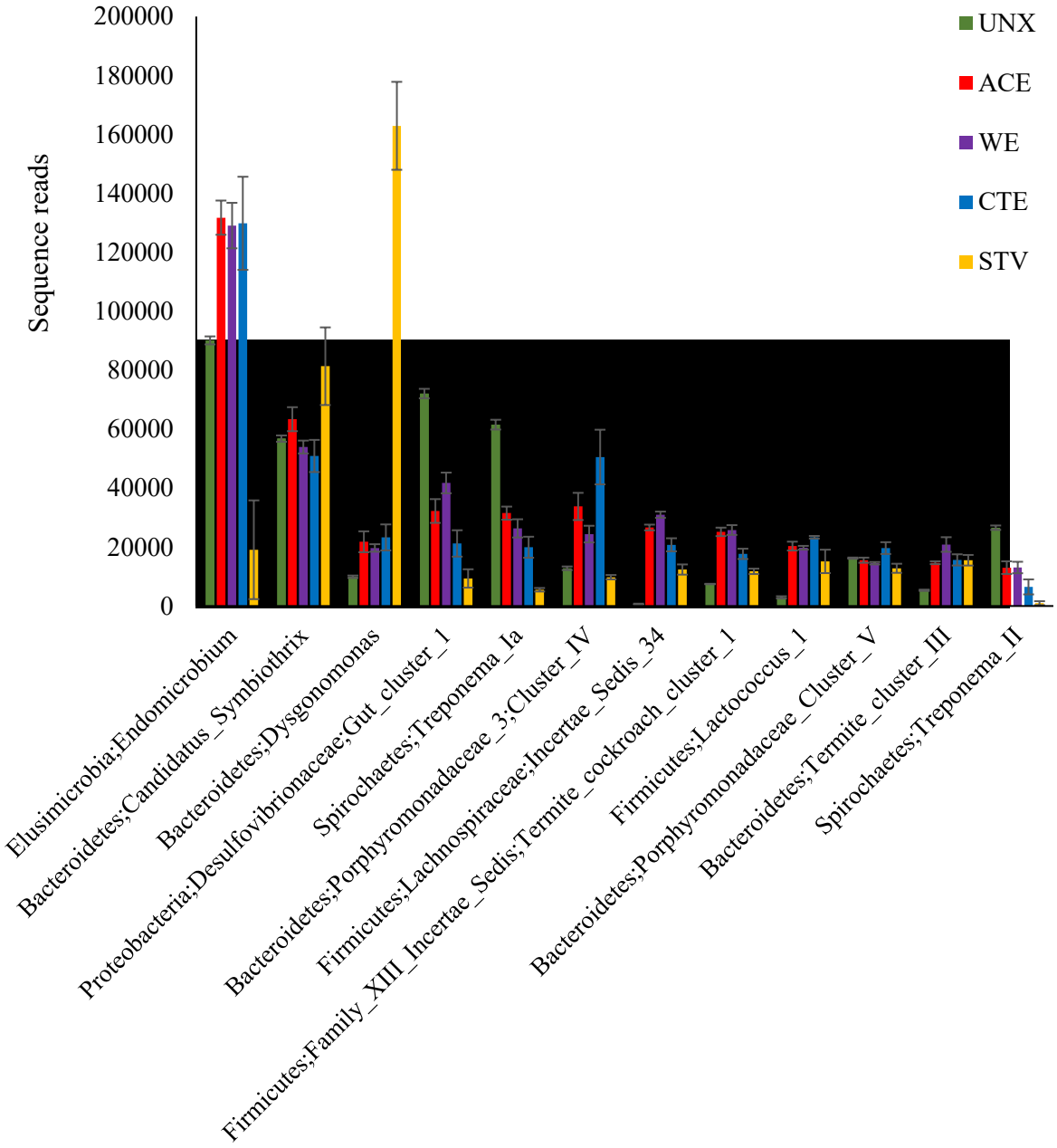

Figure 3.8 Relative abundance of 16S rRNA reads assigned to bacteria genera in different treatment groups.

Bars represent averages ± SE (n = 5). UNX, unexposed termites; ACE, 25% acetic acid-treated wood exposed to termites; WE, water-treated wood exposed to termites; CTE, 0.5% chitosan-treated wood exposed to termites; STV, starved termites. Multiple comparisons were performed without correcting *p* values.

The genus *Endomicrobium* accounted for 15.6%, and *Candidatus_Symbiothrix* and *Dysgonomonas* had an abundance of 9.2% and 7.1%, respectively. No significant differences were observed among treatment groups for some genera such as *Mycobacterium*, *Corynebacterium*_1, *Rhodococcus*_1, *Tsukamurella*, *Burkholderia*_1, and *Escherichia-Shigella*. However, 27 genera were affected by CTE and separated into different groups by color (Table 3.3). The majority of these genera belonged to Firmicutes (55.5%), followed by Proteobacteria (14.8%), Bacteroidetes (11.1%), Spirochaetes (7.4%), Tenericutes (7.4%), and Actinobacteria (3.7%). Few genera of the three phyla, Bacteroidetes, Proteobacteria, and Spirochaetes were also affected by CTE, although the difference was not obvious when analysis performed at phyla level. In addition, the effect of chitosan treatment was not shown in any genus of phylum Planctomycetes while the effect of CTE observed at phyla level. The effect of chitosan treatment group was significantly different and specific among twenty-seven genera. Among nine bacterial genera with red color, a family Porphyromonadaceae_3 of phylum Bacteroidetes, an unclassified genus of Spirochaetaceae_Treponema_I family, and a genus *Treponema*_Ib of phylum Spirochaetes had significantly higher relative abundance in CTE treatment group than in other treatment groups. The relative abundance of latter two genera were significantly lower in CTE treatment than UNX. The relative abundance of Gut_cluster_7, Uncultured_10, unclassified genus of Peptococcaceae_1 family of phylum Firmicutes, and an unclassified Proteobacteria showed significantly decreased in CTE treatment group compared to UNX, ACE, WE, and STV. The relative abundance of Termite_cockroach_cluster_1 of Family_XIII_Incertae_Sedis in CTE treatment were

lower than ACE and WE, while the bacteria abundance was higher in CTE than STV and UNX. In addition, an unclassified genus of Family_XIII_Incertae_Sedis showed reduction in the relative abundance in CTE treatment compared to other treatment groups, while it displayed higher relative abundance than STV treatment.

The relative abundance of another nine genera with blue color in CTE and STV treatment groups were significantly lower than other treatment groups. Although the abundance of *Desulfosporosinus* in CTE treatment was different from ACE and WE, there was no significant difference observed among CTE, UNX, and STV treatments.

Of nine identified genera with black color, seven bacterial genera including Cluster_IV, Mixed_gut_cluster, Gut_cluster_5, Gut_cluster_9, Gammaproteobacteria_1, Gut_cluster_1, and *Propionivibrio*, in termite hindgut were changed by CTE treatment, there were no significant differences between CTE and ACE treatment groups. The Cluster_IV of phylum Bacteroidetes, unclassified Gammaproteobacteia_1 (phylum Proteobacteria), and genus *Propionivibrio* (phylum Proteobacteria) showed significantly increase in relative abundance in CTE and ACE treatments compared to other treatment groups. In Uncultured_1 of Tenericutes, the relative abundance in CTE and UNX was higher than ACE, WE, and STV. The Subcluster_b of phylum Actinobacteria indicated the significant increase in ACE and WE compared to other treatment groups.

Table 3.3 Taxonomic classification of bacteria in termite hindgut affected by chitosan treatment group (CTE)

Phylum	Class	Order	Family	Genus
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae_3	Unclassified
Firmicutes	Clostridia_1	Clostridiales	Family_XIII_Incertae_Sedis	Termite_cockroach_cluster_1
Firmicutes	Clostridia_1	Clostridiales	Family_XIII_Incertae_Sedis	Unclassified
Firmicutes	Clostridia_1	Clostridiales	Ruminococcaceae	Gut_cluster_7
Firmicutes	Clostridia_1	Clostridiales	Ruminococcaceae	Uncultured_10
Firmicutes	Clostridia_2	Clostridiales_1	Peptococcaceae_1	Unclassified
Proteobacteria	Unclassified	Unclassified	Unclassified	Unclassified
Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae_Treponema_I	*Treponema*_Ib
Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae_Treponema_I	Unclassified
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae_2	Unclassified
Firmicutes	Clostridia_1	Clostridiales	Lachnospiraceae	*Catabacter*
Firmicutes	Clostridia_1	Clostridiales	Ruminococcaceae	Gut_cluster_6
Firmicutes	Clostridia_1	Clostridiales	Ruminococcaceae	Termite_group_aaa
Firmicutes	Clostridia_2	Clostridiales_1	Peptococcaceae_2	Uncultured_gut_Group_A
Firmicutes	Clostridia_2	Clostridiales_1	Peptococcaceae_2	*Desulfosporosinus*
Firmicutes	Clostridia_1	Clostridiales	Ruminococcaceae	Termite_cockroach_cluster
Firmicutes	Clostridia_1	Clostridiales	Ruminococcaceae	*Papillibacter*
Tenericutes	Mollicutes	RF9	Unclassified	Unclassified
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae_3	Cluster_IV
Firmicutes	Clostridia_1	Clostridiales	Lachnospiraceae	Mixed_gut_cluster
Firmicutes	Clostridia_1	Clostridiales	Ruminococcaceae	Gut_cluster_5
Firmicutes	Clostridia_1	Clostridiales	Ruminococcaceae	Gut_cluster_9
Proteobacteria	Gammaproteobacteia_1	Unclassified	Unclassified	Unclassified
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Gut_cluster_1
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae_3	*Propionivibrio*
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Uncultured_1
Actinobacteria	Actinobacteria	Actinomycetales	Termite_cluster_1	Subcluster_b

3.5 Conclusions

In summary, we characterized the composition of bacterial community from *R. virginicus* exposed to five treatments groups over 18 days: 0.5% chitosan-treated wood, 25% acetic acid-treated wood, and water-treated wood, plus one termite group isolated from nature (unexposed) and one starved termite group. Illumina sequencing of 16S rRNA V3-V4 amplicons generated approximately 11.3 million reads. Variation in diversity and richness of the hindgut composition confirmed the sensitivity of the termite species to the treatment. Treatment affected the overall composition of the bacteria microbiota and the relative abundance of bacteria community in the hindgut, as was observed at the phylum and genus levels. The low concentration of chitosan solution (0.5%, CTE) resulted in the identification of few bacterial genera. Using specific Dictyopteren sequence database (DictDb database) did not help identification of prokaryotes at species level. These results suggest that the microbial community shifts in *R. virginicus* differed from other reported bacterial taxa in subterranean termite species. The structure of the bacterial communities was affected by treatment groups, but not only due to chitosan treatment. Further analysis should be performed to examine the effect of low-abundant bacteria essential for maintenance of bacterial composition balance in the termite hindgut.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

Lower termites depend on their hindgut symbionts (protists, bacteria and archaea) to supply their nutrients. The involvement of each protist species, bacterial community, and their interaction in wood digestion are an interesting topic. To protect wood against lower termites, chitosan has potential for termiticide effect. Its biodegradability, environmental friendliness, and antimicrobial characteristics make it a great compound for use as a wood preservative. In this study, the economically important pest for wooden construction in Mississippi, *Reticulitermes virginicus*, was selected to evaluate protists and bacteria diversity as affected by chitosan-treated wood. The objectives of this study were:

- to determine the susceptibility of protists and changes in their abundance when termites exposed to chitosan treatment wood; and
- to assess the effectiveness of chitosan treatment on hindgut microbiome dynamics.

Low chitosan concentration solution yielded protists diversity in termite similar to control samples. This means all ten protists species associated with *R. virginicus* remained in termite hindguts. Except for the protist species *Monocercomonas* sp. and *Trichomitus trypanoides*, the remaining eight protist species disappeared when termites were fed wood treated to higher concentrations of chitosan. Two-way cluster analysis for both termite colonies in our experiment revealed that protists diversity led to treatment

divided into two groups: one group contained controls and lower chitosan concentration solution (0.5%), while the higher chitosan treatment (1% and 2%) grouped together. The same partitioning formed for protist species in the cluster analysis. The two surviving protists in termites exposed to higher chitosan-treated wood clustered in the same group and the other eight protists bundled together. The effect of treatment on protists diversity was significant for both *R. virginicus* colonies using PerMANOVA, although only Colony 1 showed significant differences in pairwise treatment comparisons. These results revealed the potential effect of chitosan on protists reduction and their elimination in the termite hindgut. These results support the hypothesis that chitosan may cause the microbial imbalance in hindgut which results in eliminating protists and their associated resident bacteria. In addition, monitoring termite's activity over time showed a high depression of worker activity after 14 days of exposure to the lower concentration. However, workers exposed to chitosan-treated wood with higher concentration solutions decreased intense attack to the wood after seven days.

The role of microbiota in the hindgut is critical and due to the complexity of the bacterial community and their variability in abundance are a challenging topic. Sequencing of 16S rRNA amplicon from the hindgut of *R. virginicus* generated about 11.3 million sequence reads after filtering and trimming via DADA2 plugin in QIIME 2. Among these reads, QIIME 2 identified 5,144 unique sequence variants known as operational taxonomic units (OTUs). Based on the alpha rarefaction curves, the highest observed OTUs belonged to termites unexposed to treatment (UNX) and the lowest to starved termites (STV). The bacterial compositions showed significant differences among five treatment groups using PERMANOVA with the Weighted UniFrac distance metric

method. Plot PCoA showed the separation of UNX treatment and STV from other treatment group. While not discussed earlier, removing UNX and STV treatment groups in our results showed that 0.5% chitosan treatment (CTE) formed a separate cluster from 25% acetic acid treatment (ACE) and water treatment (WE). Thus, the higher microbial diversity in UNX caused CTE to be grouped with ACE and WE. Since termites in natural habitat, forage different food sources and acquire new microorganisms from soil, UNX had the highest diversity compared to termites maintained in the laboratory. Taxonomic classification of representative sequences for each OUT identified 28 phyla with the four most abundant phyla being Bacteroidetes, Firmicutes, Elusimicrobia, and Proteobacteria. The effect of CTE resulted in the identification of few bacterial genera. The majority of them belonged to the Firmicutes phylum. We can assume the low concentration of chitosan led to detection of few genera and to not seeing better separation from two other treatment groups, ACE and WE. The analysis suggests that the structure of the bacterial communities was affected by treatment groups, ACE, WE, CTE, and STV, but not specifically with chitosan solution. In summary, diet shifts the composition of bacteria in the hindgut across all treatment groups and their frequency was detectable at phylum and genus levels.

APPENDIX A

TERMITE BIOASSAY DATA

Table A.1 Average treatment retention and mass loss for termite colony 1 and 2

Colony	Treatment	Retention (mg g^{-1})	ML%
1	Water	0	9
1	25% Acetic acid	0	10
1	0.5% chitosan solution	14	9
1	1% chitosan solution	22	6
1	2% chitosan solution	43	6
2	Water	0	10
2	25% Acetic acid	0	19
2	0.5% chitosan solution	29	10
2	1% chitosan solution	41	4
2	2% chitosan solution	60	4

ML, average mass loss after termite feeding on chitosan treatments.

APPENDIX B

DATA FROM GENOMIC AND ISOLATION

Table B.1 Concentration and quality of isolated genomic DNA, as determined by NanoDrop

Sample	Conc (ng/µL)	260/ 280	Sample	Conc (ng/µL)	260/ 280	Sample	Conc (ng/µL)	260/ 280	Sample	Conc (ng/µL)	260/ 280	Sample	Conc (ng/µL)	260/ 280
UNX1	19.88	1.87	ACE1	52.55	1.82	WE1	37.94	1.77	CTE1	32.41	1.95	STV1	40.26	1.78
UNX2	31.65	1.88	ACE2	46.99	1.86	WE2	61	1.63	CTE2	28.69	1.99	STV2	37.67	1.75
UNX3	50.32	1.84	ACE3	47.71	1.89	WE3	55.16	1.82	CTE3	42.86	1.8	STV3	34.18	1.88
UNX4	91.7	1.8	ACE4	48.15	1.89	WE4	67.44	1.83	CTE4	28.67	1.9	STV4	40.2	1.87
UNX5	94.57	1.8	ACE5	38.26	1.94	WE5	49.92	1.88	CTE5	32.57	2.13	STV5	45.7	1.92
UNX6	61.99	1.82	ACE6	38.48	1.83	WE6	42.83	1.87	CTE6	40.54	1.78	STV6	43.87	1.94
UNX7	68.34	1.85	ACE7	46.92	1.93	WE7	67.15	1.89	CTE7	94.05	1.73	STV7	56.16	2.01
UNX8	51.54	1.76	ACE8	48.87	1.96	WE8	68	1.66	CTE8	57.71	1.8	STV8	49.54	1.97
UNX9	54.36	1.75	ACE9	46.64	1.87	WE9	73.96	1.86	CTE9	75.12	1.81	STV9	55.02	1.98
UNX10	63.87	1.75	ACE10	70.6	1.8	WE10	72.54	1.87	CTE10	49.09	1.76	STV10	17.84	2.06
UNX11	56.74	1.77	ACE11	71.14	1.8	WE11	70.4	1.76	CTE11	46.82	1.84	STV11	23.78	1.95
UNX12	58.36	1.79	ACE12	65.68	1.85	WE12	84.48	1.89	CTE12	54.76	1.84	STV12	22.1	1.99
UNX13	45.13	1.74	ACE13	42.67	1.74	WE13	50.81	1.76	CTE13	68.39	1.82	STV13	16.86	2.18
UNX14	48.51	1.9	ACE14	44.73	1.82	WE14	47.02	1.78	CTE14	34.16	1.9	STV14	16.56	2.2
UNX15	58.04	1.78	ACE15	44.79	1.79	WE15	60.24	1.8	CTE15	37.84	1.77	STV15	17.42	2.22

Conc, concentration.

APPENDIX C

DATA FROM 16S LIBRARY PREPARATION

Table C.1 Qubit concentration and BioAnalyzer fragment size in indexed libraries

Sample	Qubit (ng/µL)	BioAnalyzer (bp*)	Sample	Qubit (ng/µL)	BioAnalyzer (bp*)
UNX1	25	627	ACE1	13.3	628
UNX2	13.6	626	ACE2	12	629
UNX3	13	626	ACE3	11.9	629
UNX4	14.3	627	ACE4	8.39	630
UNX5	15.7	628	ACE5	7.76	628
UNX6	14.2	629	ACE6	7.93	615
UNX7	20.9	629	ACE7	10.2	626
UNX8	12.7	629	ACE8	11	632
UNX9	7.97	615	ACE9	9.22	631
UNX10	11.7	626	ACE10	7.7	625
UNX11	12.4	629	ACE11	8.28	624
UNX12	16.3	628	ACE12	10.1	625
UNX13	16.8	625	ACE13	7.28	616
UNX14	15.3	626	ACE14	6.91	615
UNX15	12.7	627	ACE15	8.88	624

*bp, base pair.

Table C.1 (Continued)

Sample	Qubit (ng/μL)	BioAnalyzer (bp*)	Sample	Qubit (ng/μL)	BioAnalyzer (bp*)
WE1	8.13	615	CTE9	14.4	627
WE2	9.96	624	CTE10	10.1	630
WE3	7.73	601	CTE11	10.1	626
WE4	9.45	610	CTE12	11.9	616
WE5	13.4	629	CTE13	10.1	623
WE6	8.41	626	CTE14	12	632
WE7	10.6	622	CTE15	10.9	631
WE8	11.3	621	STV1	6.31	627
WE9	10.4	621	STV2	7.16	643
WE10	17	624	STV3	5.93	637
WE11	12.2	625	STV4	6.34	628
WE12	16.6	625	STV5	3.23	628
WE13	14.8	626	STV6	4.31	626
WE14	9.89	623	STV7	3.45	640
WE15	7.62	598	STV8	4.47	629
CTE1	8.01	623	STV9	3.23	611
CTE2	9.74	631	STV10	3.72	627
CTE3	9.79	629	STV11	4.41	635
CTE4	14.3	630	STV12	4.84	632
CTE5	10.5	629	STV13	4.07	638
CTE6	11.5	629	STV14	3.84	625
CTE7	10.7	627	STV15	4.41	623
CTE8	11.2	628			

*bp, base pair.

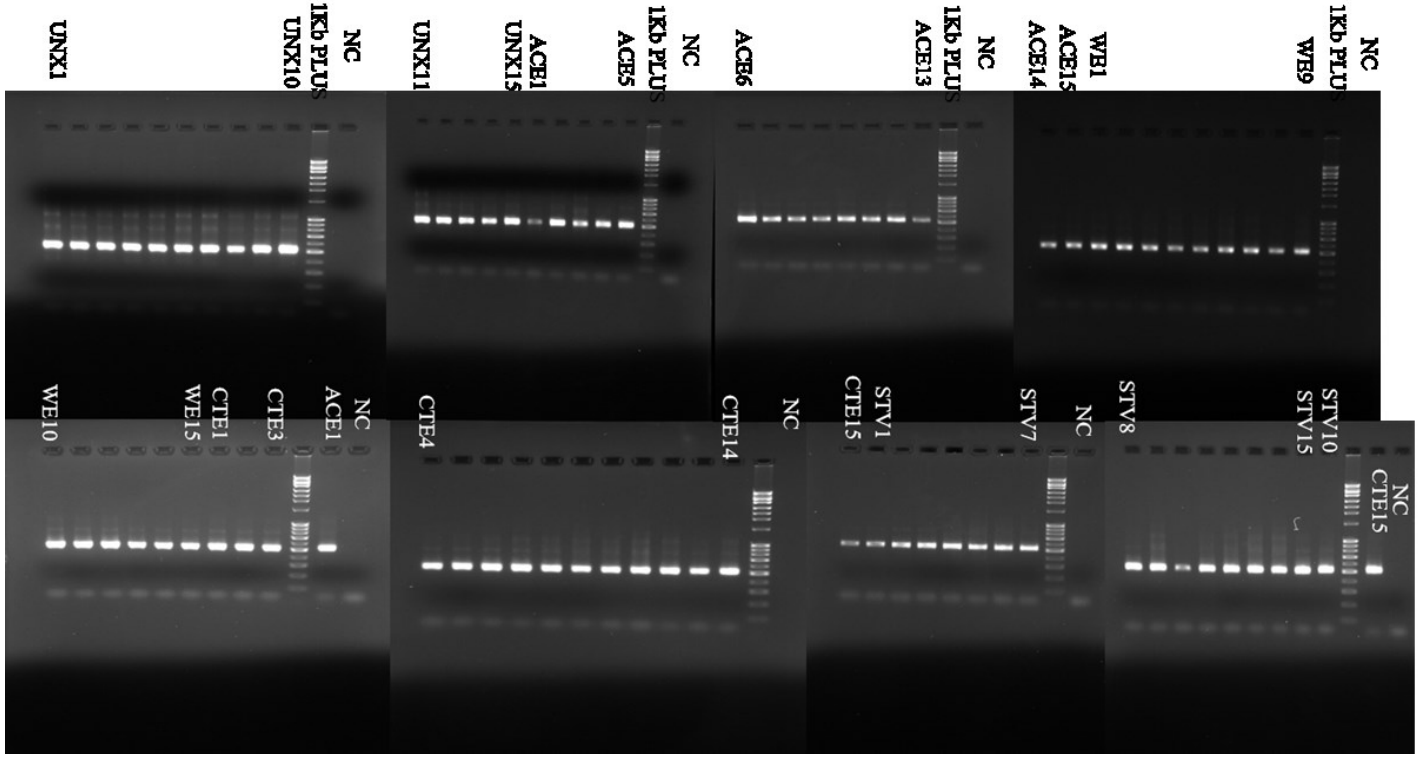

Figure C.1 PCR amplification of 16S rRNA gene of the bacterial hindgut with molecular size ~550 bp from 75 samples in *R. virginicus*.

UNX, unexposed termites; ACE, 25% acetic acid-treated wood exposed to termites; WE, water-treated wood exposed to termites; CTE, 0.5% chitosan-treated wood exposed to termites; STV, starved termites; NC, negative control; 1KbPLUS, ladder.