CHANGES IN GUT MICROBIOME COMPOSITION FROM LOWER TERMITES IN RESPONSE TO ENVIRONMENTAL AND COLONY-GENETIC FACTORS

by

Rajani Sapkota

A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Entomology

West Lafayette, Indiana

May 2022

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Michael E. Scharf, Chair

Department of Entomology

Dr. Douglas S. Richmond

Department of Entomology

Dr. Laramy Enders

Department of Entomology

Cindy H. Nakatsu

Department of Agronomy

Approved by:

Dr. Catherine Hill

ACKNOWLEDGMENTS

I want to express my sincere appreciation to my major professor: Michael E. Scharf, without whose support, encouragement and mentoring this project would not be possible. Because of his continuous guidance, I was able to see through the project I am passionate about. I thank him for believing and helping me out whenever I needed. I am especially thankful for his patience and expertise which helped me in finishing up this dissertation.

I would like to thank my dissertation committee, Drs Cindy H. Nakatsu, Douglas S. Richmond and Laramy Enders, for their direction during the development and execution of this project. Also, I am grateful for the time and suggestions they provided besides the committee meetings.

I would like to thank- Purdue graduate school for Ross and Bilsland dissertation fellowships, Purdue Pest management conferences and Pi Chi Omega for several scholarships they awarded throughout my PhD, and O. Wayne Rollins-Orkin Endowment at Purdue University for the financial support needed for this project.

I am grateful for my family for believing and supporting me while I was ready to take a giant step by moving several thousand miles away from them. To my family, thank you for all your unconditional love and support; without you I would not be standing on the shoes I am right now. And finally, to my husband- Manoj Pandey, thank you for always encouraging and being with me during the times I was ready to give up. I am indebted for your unconditional love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The relationship between termites and their gut microbiome is inseparable as the microbes provide plenty of physiological benefits such as digestion, acetogenesis, nitrogen fixation, caste differentiation and immunity. Therefore, it is very important to understand this relationship to attain innovations in pest management solutions. This project was undertaken to study the dynamics of termite gut microbiomes in response to environmental and colony-genetic factors. Here I investigated environmental variations under lab conditions by manipulating the social environment of termite workers via hormonal treatments (Chapter 2) and by exposing termites to commonly used insecticides (Appendix A). To investigate the interaction of gut microbes with natural environmental variation, I sampled field termites every month from May through October (Chapter 3). In addition, to study the impact of termite colony genetics, I reared termites originally from two different field colonies in a controlled environment in the lab (Chapter 4). The combination of lab and field investigations, and bioinformatic analyses have resulted in the following major findings- (1) gut microbes do impact the social environment within a colony, (2) seasonality has an impact on gut microbial abundances, (3) significant variation in microbiome follows genetics of termite colonies, and (4) insecticide applications do change the gut microbial loads specifically the protists load.

Results from these experiments support the idea that termite gut symbionts change their community structure possibly to help the host termite to be best fit to survive changing environmental conditions. For example, when termites were provided hormonal treatment to artificially induce caste differentiation, gut microbes were linked with this eusocial mechanism via differential selection. However, when termites were in the field experiencing change in the season, monthly temperature was correlated with several bacterial taxa, possibly selecting for taxa that help termite to thrive the changed environmental conditions. These results indicate that studying termite system without considering their gut microbiome would not provide a complete picture of physiological, biological or pest management studies. This dissertation altogether highlights the possible dynamics of gut microbiome to help a host adapt to changing conditions and emphasizes the importance of a holobiont approach while studying an insect system.

CHAPTER 1. LITERATURE REVIEW AND DISSERTATION OBJECTIVES

1.1 Introduction

Most animals live in close association with their gut microbial consortium, which provides plenty of physiological benefits (Petersen and Osvatic 2018). Even the human guts are associated with several type of microbes including bacteria, archaea, fungi, protozoa, and viruses, that impact the nutritional physiology, metabolism, neuromodulation and immune system of the host (Reviewed in Barko et al. 2018, Forsythe and Kunze 2013; Krishnan et al. 2015). A balanced animal-gut microbiome association is an important aspect of medical research as dysbiosis results in a number of gastrointestinal, systemic and neurodevelopmental disorders that impact animal physiology (Reviewed in Barko et al. 2018). For a balanced animal-microbe symbiosis, animal host provides a suitable cultivation media while in return the symbionts provide physiological benefits (Itoh et al. 2019). Likewise, insects are associated with several gut symbionts that interact with their immune system, digestive system, detoxification mechanisms, vitamin biosynthesis, metabolism, and pathogen defense, to enhance host fitness (Jing et al. 2020, Weiss and Aksoy 2011). Microbial communities are particularly prominent in the digestive tract of insects. Except for some insects, the insect gut bacteria are not as diverse as mammalian gut bacteria (reviewed in Engel and Moran 2013, Weiss and Aksoy 2011). Direct microscopic counts indicated that the number of bacterial cells vary widely among different insect species with the highest in wood feeders and detritivores (Cazemier et al. 1997). Nourishing of insects with essential nutrients is the main role of their gut symbionts followed by digestion and detoxification (Jing et al. 2020). Whether to improve insecticide tolerance levels in insects like stink bugs, or to provide a significant contribution to digestion, and the immune system; insect gut symbionts are important taxa housed by insects (Kikuchi et al. 2012, Peterson et al. 2015). In addition, gut bacteria also influence the pheromones, hemolymph proteins, hormones, survival, growth and egg production in insects (Coon et al. 2016, Engl and Kaltenpoth 2018, Lee et al., 2017). These works indicate a crucial role of gut symbionts in insect ecology, function and ultimately survival. This literature review highlights the interaction of proximate causes of eusocial mechanisms in termites with their gut microbiome.

1.2 Termite gut symbionts and their importance

The hind gut of lower termites is considered to be a sink of microbiota as they are densely populated with protists, Bacteria and Archaea (reviewed in Hongoh 2010). An important feature of lower termite guts is tripartite symbiosis, where protist symbionts live in association with several bacterial taxa (endo and ecto symbionts to protists) specifically in the hindgut region (Reviewed in Ohkuma and Brune 2011). The bacterial symbionts Spirochetes are ectosymbionts to Oxymonad protists, possibly providing motility to its protist host; while Bacteroidetes, Elusimicrobia and Firmicutes are reported as protist endosymbionts (Iida et al. 2000, Fröhlich and König 1999, Noda et al. 2007, Noda et al. 2005, Ohkuma et al. 2007, Stingl et al. 2005). Major groups of protists living in lower termite guts are Oxymonads and Parabasalids, while the higher termites are devoid of protists, but harbor Bacteria and Archaea in their hindguts (Reviewed in Ohkuma and Brune 2011, reviewed in Hongoh 2010). The protist communities of lower termite guts are well characterized for the digestion of lignocellulosic materials present in the woods (Wheeler et al. 2007). However, in a recent study, the gut protist- *Cononympha leidyi* was reported to degrade chitin for the assimilation of amino acids, which provides evidence of evolution and adaptation of species in the changing environment (Nishimura et al. 2020). Due to difficulty in cultivation of termite gut symbionts, their functions have not been characterized very well. However, the development of advanced microbial tools is helping characterize functional aspects of symbionts. And of course, there is still a need for more research to better understand the functions of termite gut symbionts.

The prokaryotic members in hindgut of Eastern subterranean termites (*Reticulitermes flavipes* Kollar) are 99.9% Bacteria and 0.11% Archaea. The bacterial phyla Spirochaetes, Elusimicrobia, Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are the most dominant phyla (Boucias et al. 2013). Termites feed on food sources poor in nitrogen content, and due to the nature of their food ecology, they harbor nitrogen fixing bacteria in their guts to meet nitrogen demand (Yamada et al. 2007). Gut microbiomes help termites survive in nitrogen poor diets and produce enzymes to digest cellulose from their food sources (Peterson et al. 2015, Zhou et al. 2007, Ohkuma 2008, Scharf and Peterson 2021). In addition, termites harbor antibiotic producing bacteria- *Actinomycetes* which may inhibit the growth of pathogens, possibly to provide resistance to attack by fungi like *Metarhizium anisopliae*, which is a common problem for organisms occupying subterranean habitats (Matsui et al. 2012, Arango et al. 2016, Sen et al. 2013,

Rosengaus et al. 2014, Chouvenc at al. 2009). Besides collaboration with the host to digest complex lignocellulose, gut symbionts in lower termites are involved in the defense against pathogens, nitrogen fixation, reductive acetogenesis, hydrogen formation and amino acid biosynthesis (Rosengaus et al. 2014, Chouvenc *et al.* 2013, Chouvenc at al. 2009, Peterson and Scharf 2016, Brune 2007). Since a major portion of termite gut bacteria are still uncultivable, the functions of entire microbial populations have not been well characterized. Previously, protists were only recognized as degraders of cellulose and hemicellulose, but a recent single-cell analysis approach has revealed that members of bacterial order Clostridiales can also degrade cellulose and hemicellulose in termite guts (Bourguignon et al. 2018). With newly emerged tools in molecular ecology, the functional characteristics of gut microbiomes are being unraveled, but still research gaps exist for several areas in understanding insect-microbiome interactions.

1.3 Termite's social environment and its impact on microbial counterparts

In social insects like termites, the unique gut microbes are transferred across generations, which may provide for specialized longer-term functions (Engel and Moran 2013). Termites are eusocial insects evolved from cockroaches and are equipped with a distinct division of labor among soldier, workers and reproductive castes. Moreover, termite colonies are also considered as superorganisms (Eggleton 2010). Due to their eusocial behaviors, like grooming of each other, termites are believed to be resistant to the attack of fungal pathogens like *Metarhizium anisopliae* in their natural habitat (Chouvenc at al. 2009). Termite workers also exhibit unique polyphenisms by retaining the ability to molt into soldier, worker, or reproductive castes depending on the need of a colony and several other environmental and physiological factors (Zhou et al. 2006). A study by Mao et al. (2005) reported Juvenile hormone (JH) to be responsible for a caste differentiation. JH titers in workers is regulated by the presence of soldiers, which are adults in a termite colony and do not undergo further molting process. In a study by Tarver et al. (2009), primer pheromone terpenes obtained from soldier head extracts of R. flavipes enhanced soldier differentiation on workers. On a genetic level, genes Hex-1 and Hex-2 coding for Hexamerin protein were responsible for polyphenism in termite workers (Zhou et al. 2006). Protein hexamerin as a caste regulating factor is also significantly impacted by the interaction between JH and temperature. Thus, the differentiation of soldiers from workers has a positive temperature correlation (Scharf et al. 2007, Tarver et al. 2012, Liu et al. 2005). Juvenile hormone homologs and synthetic juvenoids

can successfully mimic the action of JH in soldier differentiation (Scharf et al. 2003). However, soldier differentiation in a colony comes with a cost because the termite gut is involved during this process, and guts are disrupted/purged before a molt (Sen et al. 2013, Nalepa 2017). Hormonal treatment has been found to reduce gut symbionts in termite guts, suggesting that the natural molting phenomenon could result in detrimental dysbiosis of gut symbionts (Scharf et al., 2017). Several hindgut protists are significantly reduced with exposure to JH and/or methoprene (a juvenoid) (Sen et al. 2013, Howard 1983). In addition, several symbiont genes are impacted by the elevated JH titer in their hemolymph (Sen et al., 2013). Exposure of *R. flavipes* and *R. virginicus* to the juvenoids methoprene and hydroprene attained a lethal effect which could be due to water loss, starvation or loss of protist community (Haverty and Howard 1979). These works suggest that maintenance of social environment in a termite colony may come with a cost on their gut symbionts.

1.4 Factors influencing gut microbial composition and their relative abundances

The gut microbiome is impacted by host genetics and its interaction with the environment. The eusocial nature of termites promotes trophallaxis and coprophagy within a colony, which provides a suitable route for vertical (colony to offspring) transmission of microbes; however horizontal transmission can be from the environment, or other animals (Bourguignon et al. 2018). The eusocial behavior in termites is believed to result in a homogenous gut microbial population throughout a colony. Not all gut symbionts are transferred from parents to offspring. Some symbionts are opportunistic and can be acquired from an environment, and hence they can differ based on diet or environment (reviewed in Engel and Moran 2013). Based on the literature, vertical transmission was found to impact the composition of gut microbiomes; however, the relative abundance was impacted by environmental factors including the diet (Rahman et al. 2015, Huang et al. 2013, Boucias et al. 2013, Jiménez-Padilla et al. 2020). Similarly, the composition of the human microbiome was reported to be shaped by genetics after establishment in early age, while the relative abundances followed environmental selection or exposure (Tavalire et al. 2021).

In insects like oriental fruit flies, ants and honeybees, genetics impact the composition of their gut microbiomes (Wang et al. 2011, Segers et al. 2019, Vernier et al. 2020). Similarly, the identification of nest mates within *R. flavipes* colonies is believed to be due to the similar intestinal products produced by microbial communities. These products are unique to every colony,

suggesting that colony genetics has a role in shaping the microbiome in termites (Matsuura 2001). The relative abundances of bacterial taxa in honeybee were found to change with varying environmental conditions (Jones et al. 2018). In social insects like termites, gut microbial diversity was found more similar within than between colonies. These results suggest that termite genetics impact the microbial composition in the gut, while environment (dietary change in this case) impacts the relative abundances (Benjamino and Graf 2016, Auer et al. 2017, Benjamino et al. 2018, Matsuura 2001). In addition, seasonal temperature change is believed to impact relative abundances of gut bacterial taxa in termites, crickets, mosquitoes and honeybees (Arango et al. 2021, Kešnerová et al. 2020, Ferguson et al. 2018, Novakova et al. 2017). These works suggest a role of genetics and environment in shaping the gut community (compositions and abundances) in animals.

1.5 Impact of insecticides on gut microbiome and results of microbial dysbiosis

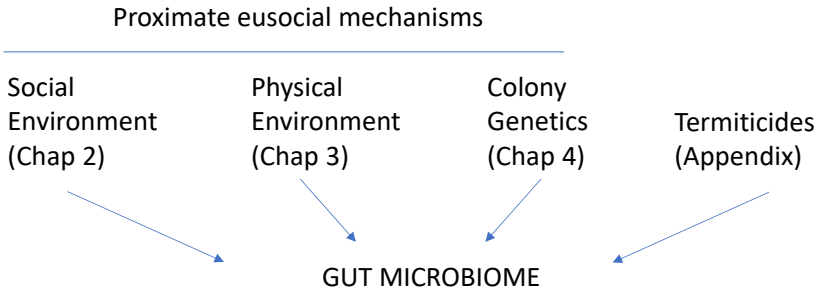
Slow acting insecticides and bait metrics are popular to manage subterranean termites in the U.S. The common bait metrics consist of chitin synthesis inhibitors like noviflumuron, hexaflumuron, diflubenzuron, and chlorfluzuron, while common slow-acting neurotoxins are fipronil and neonicotinoid based termiticides (Su 1994, Su 2005, Rojas and Morales 2001). Besides having different modes of action, these insecticides also impact on gut physiology. Microbial dysbiosis has been reported with imidacloprid and fipronil treatments that resulted in compromised social activity in termites, that ultimately increased sensitivity to fungal pathogens (Raza et al. 2019, Sen et al. 2015, Ramakrishnan et al. 1999). Feeding of slow acting insecticide was reported to cause microbial dysbiosis of gut microbiomes by decreasing the number of protists in the gut. Similar decreases in relative abundances of protist populations from the hindgut of *Coptotermes formosanus* were observed with several chitin synthesis inhibitors (diflubenzuron, hexaflumuron, lufenuron, noviflumuron, and novaluron) (Sen et al. 2015, Lewis and Forschler 2010). Also, differences in gut bacterial composition and relative abundances from insecticide resistant (to fipronil and chlorpyrifos) and susceptible Diamondback moths has been reported. Insecticide exposure is thus believed to change the gut microbial phenotype by causing a dysbiosis and/or differential selection (Xia et al. 2013).

A reduction or change in relative abundances of beneficial gut symbionts could be lethal to any animal. Therefore, it is very important to understand the physiological impacts resulting

from a change in microbial communities. To study the impact of microbial dysbiosis in the lab, antibiotics have been widely used. A dysbiosis of gut microbiomes in termites has been reported to impair physiological functions like digestion, and immunity against fungal pathogens (Peterson et al. 2015). A number of hindgut flagellates and bacterial symbionts are severely impacted by antibiotics, resulting into decreased digestive ability (Peterson et al. 2015). Similarly, the reproductive castes of Formosan subterranean termites fed with antibiotics, showed cannibalism of their progenies and later died of starvation, suggesting that they could not feed on or acquire nutrients from wood in absence of gut microbes (Raina et al. 2004). Gut microbial dysbiosis could be brought about by using insecticides, antibiotics or hormonal treatments in a termite; and this dysbiosis has been proven detrimental to their survival.

1.6 Dissertation Goals and Statement of Objectives

Research on termite gut symbionts started over a century ago but was halted or moved in different directions due to the complex microbial ecology of their guts. However, with the bloom and easy access to next generation sequencing platforms, our understanding of termite gut symbiont interactions has started to become untangled. The goal of this dissertation was to understand the environmental (social, seasonal and insecticide application) and colony-genetic selection of gut microbiomes in the termite *Reticulitermes flavipes* Kollar. To study my objective, termites were exposed to a combination of bioassays (JH, antibiotics, insecticides), enzyme assays, extensive rearing conditions (by controlling the environment including food and water sources) and next generation sequencing techniques. The following chart is the schematic of my dissertation chapters.



The <u>specific aims</u> of my dissertation were to (1) study the regulation of host phenotypic plasticity by gut symbiont communities in *R. flavipes*, (2) study the effect of seasonal variation on gut microbial communities in *R. flavipes*, (3) make inter-colony comparisons of gut microbial

communities between lab reared *R. flavipes* colonies, and (4) study the impact of commonly used termiticides on gut microbial load of *R. flavipes*. In sum, these objectives link termite holobiont's response to the environmental and genetic selections of their gut microbiome.

1.7 References

Arango, R. A., C. M. Carlson, C. R. Currie, B. R. McDonald, A. J. Book, Frederick Green, N. K. Lebow, and K. F. Raffa. 2016. Antimicrobial activity of actinobacteria isolated from the guts of subterranean termites. Environ. Entomol. 45: 1415-1423.

Arango, R. A., S. D. Schoville, C. R. Currie, and C. Carlos-Shanley. 2021. Experimental Warming Reduces Survival, Cold Tolerance, and Gut Prokaryotic Diversity of the Eastern Subterranean Termite, Reticulitermes flavipes (Kollar). Front. Microbiol. 12: 1116.

Auer, L., A. Lazuka, D. Sillam-Dussès, E. Miambi, M. O'Donohue, and G. Hernandez-Raquet. 2017. Uncovering the Potential of Termite Gut Microbiome for Lignocellulose Bioconversion in Anaerobic Batch Bioreactors. Front. Microbiol. 8: 2623.

Barko, P. C., M. A. McMichael, K. S. Swanson, and D. A. Williams. 2018. The gastrointestinal microbiome: a review. J. Vet. Intern. Med. 32:9-25.

Benjamino, J., and J. Graf 2016. Characterization of the core and caste-specific microbiota in the termite, Reticulitermes flavipes. Front. Microbiol. 7:171.

Benjamino, J., S. Lincoln, R. Srivastava, and J. Graf. 2018. Low-abundant bacteria drive compositional changes in the gut microbiota after dietary alteration. Microbiome. 6:1-13.

Boucias, D. G., Y. Cai, Y. Sun, V.U. Lietze, R. Sen, R. Raychoudhury, and M.E. Scharf. 2013. The hindgut lumen prokaryotic microbiota of the termite *Reticulitermes flavipes* and its responses to dietary lignocellulose composition. Mol. Ecol. 22:1836-1853.

Bourguignon, T., N. Lo, C. Dietrich, J. Šobotník, S. Sidek, Y. Roisin, A. Brune, and T. A. Evans. 2018. Rampant host switching shaped the termite gut microbiome. Curr. Biol. 28:649-54.

Brune, A. 2007. Woodworker's digest. Nature. 450:487–488

Cazemier, A. E., J. H. Hackstein, H. J. Op den Camp, J. Rosenberg, and C. van der Drift. 1997. Bacteria in the intestinal tract of different species of arthropods. Microb. Ecol. 33:189-97.

Chouvenc, T., C. A. Efstathion, M. L. Elliot, and N. Y. Su. 2013. Extended disease resistance emerging from the faecal nest of a subterranean termite. Proc. R. Soc. B 280, 1–9. doi: 10.1098/rspb.2013.1885

Chouvenc, T., N.Y. Su, and A. Robert. 2009. Inhibition of *Metarhizium anisopliae* in the alimentary tract of the eastern subterranean termite *Reticulitermes flavipes*. J. Invertebr. Pathol. 101:130-136.

Coon, K. L., M. R. Brown, and M. R. Strand. 2016. Gut bacteria differentially affect egg production in the anautogenous mosquito *Aedes aegypti* and facultatively autogenous mosquito *Aedes atropalpus* (Diptera: Culicidae). Parasite Vector. 9:375.

Eggleton, P. 2010. An introduction to termites: biology, taxonomy and functional morphology, Biology of termites: a modern synthesis (ed. by D.E. Bignel, Y. Roisin and N. Lo), pp. 1−26, Springer, Dordrecht, The Netherlands.

Engel, P., and N. A. Moran. 2013. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev. 37:699–735.

Engl, T., and M. Kaltenpoth. 2018. Influence of microbial symbionts on insect pheromones. Nat. Prod. Rep. 35, 386-397.

Ferguson, L. V., P. Dhakal, J. E. Lebenzon, D. E. Heinrichs, C. Bucking, B. J. Sinclair. 2018. Seasonal shifts in the insect gut microbiome are concurrent with changes in cold tolerance and immunity. Funct. Ecol. 32:2357-68.

Forsythe, P., and W. A. Kunze. 2013. Voices from within: gut microbes and the CNS. Cell. Mol. Life Sci. 70, 55-69.

Fröhlich, J. and H. König. 1999. Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator. Syst. Appl. Microbiol. 22, 249-257.

Haverty, M. I., and R.W. Howard. 1979. Effects of insect growth regulators on subterranean termites: induction of differentiation, defaunation, and starvation. Ann. Entomol. Soc. 72: 503-508.

Hongoh, Y. 2010. Diversity and genomes of uncultured microbial symbionts in the termite gut. Biosci. Biotechnol. Biochem. 74:1145-51.

Howard, R. W. 1983. Effects of methoprene on binary caste groups of *Reticulitermes flavipes* (Kollar)(Isoptera: Rhinotermitidae). Environ. Entomol. 12: 1059-1063.

Huang, X. F., M. G. Bakker, T. M. Judd, K. F. Reardon, and J. M. Vivanco. 2013.Variations in diversity and richness of gut bacterial communities of termites (*Reticulitermes flavipes*) fed with grassy and woody plant substrates. Microb. Ecol. 65: 531-536.

Iida, T., M. Ohkuma, K. Ohtoko, and T. Kudo. 2000. Symbiotic spirochetes in the termite hindgut: phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. FEMS Microbiol. Ecol. 34:17-26.

Itoh, H., S. Jang, K. Takeshita, T. Ohbayashi, N. Ohnishi, X. Y. Meng, Y. Mitani, and Y. Kikuchi. 2019. Host–symbiont specificity determined by microbe–microbe competition in an insect gut. Proc. Natl. Acad. Sci. U.S.A. 116:22673-82.

Jiménez-Padilla, Y., E. O. Esan, K. D. Floate, and B. J. Sinclair. 2020. Persistence of diet effects on the microbiota of *Drosophila suzukii* (Diptera: Drosophilidae). Can. Entomol. 152: 516–531.

Jing, T. Z., F. H. Qi, and Z. Y. Wang. 2020. Most dominant roles of insect gut bacteria: digestion, detoxification, or essential nutrient provision? Microbiome. 8:1-20.

Jones, J. C., C. Fruciano, F. Hildebrand, H. A. Toufalilia, N. J. Balfour, P. Bork, P. Engel, F. L. W. Ratnieks, and W. O. H. Hughes. 2018. Gut microbiota composition is associated with environmental landscape in honey bees. Ecol. Evol. 8: 441-451.

Kešnerová, L., O. Emery, M. Troilo, J. Liberti, B. Erkosar, and P. Engel. 2020. Gut microbiota structure differs between honeybees in winter and summer. ISME J. 14:801-14.

Kikuchi, Y., M. Hayatsu, T. Hosokawa, A. Nagayama, K. Tago, and T. Fukatsu. 2012. Symbiont-mediated insecticide resistance. Proc. Natl. Acad. Sci. U.S.A. 109: 8618-8622.

Krishnan, S., N. Alden, and K. Lee. 2015. Pathways and functions of gut microbiota metabolism impacting host physiology. Curr. Opin. Biotechnol. 36, 137-145.

Lee, J. B., K. E. Park, S. A. Lee, S. H. Jang, H. J. Eo, H. Am Jang, C. H. Kim, T. Ohbayashi, Y. Matsuura, Y. Kikuchi, and R. Futahashi. 2017. Gut symbiotic bacteria stimulate insect growth and egg production by modulating hexamerin and vitellogenin gene expression. Dev. Comp. Immunol. 69:12-22.

Lewis, J. L., and B. T. Forschler. 2010. Impact of five commercial baits containing chitin synthesis inhibitors on the protist community in *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Environ. Entomol. 39: 98-104.

Liu, Y., G. Henderson, L. Mao, and R. A. Laine. 2005. Effects of temperature and nutrition on juvenile hormone titers of *Coptotermes formosanus* (Isoptera: Rhinotermitidae). Ann. Entomol. Soc. 98: 732-737.

Mao, L., G. Henderson, Y. Liu, and R.A. Laine. 2005. Formosan subterranean termite (Isoptera: Rhinotermitidae) soldiers regulate juvenile hormone levels and caste differentiation in workers. Ann. Entomol. Soc. 98: 340-345.

Matsui, T., J. Tanaka, T. Namihira, and N. Shinzato. 2012. Antibiotics production by an actinomycete isolated from the termite gut. J. Basic Microbiol. 52: 731-735.

Matsuura, K. 2001. Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. Oikos. 92: 20-26.

Nalepa, C. A. 2017. What kills the hindgut flagellates of lower termites during the host molting cycle? Microorganisms. 5, 82.

Nishimura, Y., M. Otagiri, M. Yuki, M. Shimizu, J. I. Inoue, S. Moriya, and M. Ohkuma. 2020. Division of functional roles for termite gut protists revealed by single-cell transcriptomes. ISME J. 14: 2449-2460.

Noda, S., T. Iida, O. Kitade, H. Nakajima, T. Kudo, and M. Ohkuma. 2005. Endosymbiotic Bacteroidales bacteria of the flagellated protist *Pseudotrichonympha grassii* in the gut of the termite *Coptotermes formosanus*. Appl. Environ. Microbiol. 71, 8811-8817.

Noda, S., O. Kitade, T. Inoue, M. Kawai, M. Kanuka, K. Hiroshima, Y. Hongoh, R. Constantino, V. Uys, J. Zhong, J. and T. Kudo. 2007. Cospeciation in the triplex symbiosis of termite gut protists (*Pseudotrichonympha* spp.), their hosts, and their bacterial endosymbionts. Mol. Ecol. 16, 1257-1266.

Novakova, E., D. C. Woodhams, S. M. Rodríguez-Ruano, R. M. Brucker, J. W. Leff, A. Maharaj, A. Amir, R. Knight, and J. Scott. 2017. Mosquito microbiome dynamics, a background for prevalence and seasonality of West Nile virus. Front. Microbiol. 8:526.

Ohkuma, M. 2008. Symbioses of flagellates and prokaryotes in the gut of lower termites. Trends Microbiol. 16, 345-352.

Ohkuma, M., and A. Brune. 2011. Diversity, structure, and evolution of the termite gut microbial community. in *Biology of Termites: A modern synthesis,* eds. D. E. Bignell, Y. Roisin, and N. Lo. (New York City: Springer), 413-438.

Ohkuma, M., T. Sato, S. Noda, S. Ui, T. Kudo, and Y. Hongoh. 2007. The candidate phylum 'Termite Group 1'of bacteria: phylogenetic diversity, distribution, and endosymbiont members of various gut flagellated protists. FEMS Microbiol. Ecol. 60, 467-476.

Petersen J. M., and J. Osvatic. 2018. Microbiomes in natura: importance of invertebrates in understanding the natural variety of animal-microbe interactions. Msystems. 3: e00179-17.

Peterson, B. F., and M. E. Scharf. 2016. Metatranscriptome analysis reveals bacterial symbiont contributions to lower termite physiology and potential immune functions. BMC genom. 17: 772.

Peterson, B. F., H. L. Stewart, and M. E. Scharf. 2015. Quantification of symbiotic contributions to lower termite lignocellulose digestion using antimicrobial treatments. Insect Biochem. Mol. Biol. 59: 80-88.

Rahman, N.A., D.H. Parks, D. L. Willner, A.L Engelbrektson, S.K. Goffredi, F. Warnecke, R.H. Scheffrahn, and P. Hugenholtz. 2015. A molecular survey of Australian and North American termite genera indicates that vertical inheritance is the primary force shaping termite gut microbiomes. Microbiome. 3: p.5.

Raina, A. K., Y.I. Park, and A. Lax. 2004. Defaunation leads to cannibalism in primary reproductives of the Formosan subterranean termite, *Coptotermes formosanus* (Isoptera: Rhinotermitidae). Ann. Entomol. Soc. 97: 753-756.

Ramakrishnan, R., D.R. Suiter, C.H. Nakatsu, R.A. Humber and G.W. Bennett. 1999. Imidacloprid-enhanced *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) susceptibility to the entomopathogen *Metarhizium anisopliae*. J. Econ. Entomol. 92:1125-1132.

Raza, S., A. Riaz, and K. Z. Rasib. 2019. Synergistic Effect of Fungus *Metarhizium anisopliae* and Slow Acting Toxicants Fipronil and Imidacloprid against Subterranean Termites. Pak. J. Sci. 62: 88-96.

Rojas, M. G., and J.A. Morales-Ramos. 2001. Bait matrix for delivery of chitin synthesis inhibitors to the Formosan subterranean termite (Isoptera: Rhinotermitidae). J. Econ. Entomol. 94: 506-510.

Rosengaus, R. B., K. F. Schultheis, A. Yalonetskaya, M. S. Bulmer, W. S. DuComb, R. W. Benson, J. P. Thottam, and V. Godoy-Carter. 2014. Symbiont-derived β-1, 3-glucanases in a social insect: mutualism beyond nutrition. Front. Microbiol. 5: 607.

Scharf, M. E. and B. F. Peterson. 2021. A century of synergy in termite symbiosis research: linking the past with new genomic insights. Ann. Rev. Entomol. 66, 23–43

Scharf, M. E., C. R. Ratliff, J. T. Hoteling, B. R. Pittendrigh, and G. W. Bennett. 2003. Caste differentiation responses of two sympatric *Reticulitermes termite* species to juvenile

hormone homologs and synthetic juvenoids in two laboratory assays. Insectes Soc. 50: 346-354.

Scharf, M. E., C.E. Buckspan, T.L. Grzymala, and X. Zhou. 2007. Regulation of polyphenic caste differentiation in the termite *Reticulitermes flavipes* by interaction of intrinsic and extrinsic factors. J. Exp. Biol. 210: 4390-4398.

Scharf, M. E., Y. Cai, Y. Sun, R. Sen, R. Raychoudhury, and D. G. Boucias. 2017. A meta-analysis testing eusocial co-option theories in termite gut physiology and symbiosis. Commun. Integr. Biol. 10: e1295187.

Segers, F. H. I. D., M. Kaltenpoth, and S. Foitzik. 2019. Abdominal microbial communities in ants depend on colony membership rather than caste and are linked to colony productivity. Ecol. Evol. 9: 13450–13467.

Sen, R., R. Raychoudhury, Y. Cai, Y. Sun, V.U. Lietze, B. F. Peterson, M. E. Scharf, and D. G. Boucias. 2015. Molecular signatures of nicotinoid-pathogen synergy in the termite gut. PLoS One. 10: p.e0123391.

Sen, R., R. Raychoudhury, Y. Cai, Y. Sun, V. U. Lietze, D. G. Boucias, and M. E. Scharf. 2013. Differential impacts of juvenile hormone, soldier head extract and alternate caste phenotypes on host and symbiont transcriptome composition in the gut of the termite *Reticulitermes flavipes*. BMC Genom. 14: 491.

Stingl, U., R. Radek, H. Yang, and A. Brune. 2005. "Endomicrobia": cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. Appl. Environ. Microbiol. 71, 1473-1479.

Su, N. Y. 1994. Field evaluation of a hexaflumuron bait for population suppression of subterranean termites (Isoptera: Rhinotermitidae). J. Econ. Entomol. 87: 389-397.

Su, N. Y. 2005. Response of the Formosan subterranean termites (Isoptera: Rhinotermitidae) to baits or nonrepellent termiticides in extended foraging arenas. J. Econ. Entomol. 98: 2143-2152.

Tarver, M. R., C.B. Florane, D. Zhang, C. Grimm, A.R. Lax, and J. Traniello. 2012. Methoprene and temperature effects on caste differentiation and protein composition in the Formosan subterranean termite, *Coptotermes formosanus*. J. Insect Sci. 12: doi https://doi.org/10.1673/031.012.1801

Tarver, M. R., E.A. Schmelz, J.R. Rocca, and M.E. Scharf. 2009. Effects of soldier-derived terpenes on soldier caste differentiation in the termite *Reticulitermes flavipes*. J. Chem. Ecol. 35: 256-264.

Tavalire, H. F., D. M. Christie, L. D. Leve, N. Ting, W. A. Cresko, and B. J. Bohannan. 2021. Shared Environment and Genetics Shape the Gut Microbiome after Infant Adoption. Mbio. 12: e00548-21.

Vernier, C. L., I. M. Chin, B. Adu-Oppong, J. J. Krupp, J. Levine, G. Dantas, and Y. Ben-Shahar. 2020. The gut microbiome defines social group membership in honey bee colonies. Sci. Adv. 6: eabd3431.

Wang, H., L. Jin, and H. Zhang. 2011. Comparison of the diversity of the bacterial communities in the intestinal tract of adult *Bactrocera dorsalis* from three different populations. J. Appl. Microbiol. 110: 1390–1401.

Weiss, B., and S. Aksoy. 2011. Microbiome influences on insect host vector competence. Trends Parasitol. 27:514-22.

Wheeler, M. M., X. Zhou, M. E. Scharf, and F. M. Oi. 2007. Molecular and biochemical markers for monitoring dynamic shifts of cellulolytic protozoa in *Reticulitermes flavipes*. Insect Biochem. Mol. Biol. 37: 1366-1374.

Xia, X., D. Zheng, H. Zhong, B. Qin, G. M. Gurr, L. Vasseur, H. Lin, J. Bai, W. He, and M. You. 2013. DNA sequencing reveals the midgut microbiota of diamondback moth, *Plutella xylostella* (L.) and a possible relationship with insecticide resistance. PloS one. 8: e68852.

Yamada, A., T. Inoue, S. Noda, Y. Hongoh, and M. Ohkuma. 2007. Evolutionary trend of phylogenetic diversity of nitrogen fixation genes in the gut community of wood-feeding termites. Mol. Ecol. 16: 3768-3777.

Zhou, X., F. M. Oi, and M. E. Scharf. 2006. Social exploitation of hexamerin: RNAi reveals a major caste-regulatory factor in termites. Proc. Natl. Acad. Sci. U.S.A. 103: 4499-4504.

Zhou, X., J. A. Smith, F. M. Oi, P. G. Koehler, G. W. Bennett, and M. E. Scharf. 2007. Correlation of cellulase gene expression and cellulolytic activity throughout the gut of the termite *Reticulitermes flavipes*. Gene. 395: 29-39.

CHAPTER 2. REGULATION OF HOST PHENOTYPIC PLASTICITY BY GUT SYMBIONT COMMUNITIES IN THE EASTERN SUBTERRANEAN TERMITE (*RETICULITERMES FLAVIPES*)

This chapter has been published in Journal of experimental biology.
Sapkota, R., C. H. Nakatsu, and M. E. Scharf. 2021. Regulation of host phenotypic plasticity by gut symbiont communities in the eastern subterranean termite (*Reticulitermes flavipes*). J. Exp. Biol. 224: jeb242553.

Abstract

Termites are eusocial insects that host a range of prokaryotic and eukaryotic gut symbionts and can differentiate into a range of caste phenotypes. Soldier caste differentiation from termite workers follows two successive molts (worker-presoldier-soldier) that are driven at the endocrine level by juvenile hormone (JH). Although physiological and eusocial mechanisms tied to JH signaling have been studied, the role of gut symbionts in the caste differentiation process is poorly understood. Here, we used the JH analog methoprene in combination with the antibiotic kanamycin to manipulate caste differentiation and gut bacterial loads in *Reticulitermes flavipes* termites via four bioassay treatments: kanamycin, methoprene, kanamycin + methoprene, and an untreated (negative) control. Bioassay results demonstrated a significantly higher number of presoldiers in the methoprene treatment, highest mortality in kanamycin + methoprene treatment, and significantly reduced protist numbers in all treatments except the untreated control. Bacterial 16S rRNA gene sequencing provided alpha and beta diversity results that mirrored bioassay findings. From ANCOM analysis, we found that several bacterial genera were differentially abundant among treatments. Finally, follow-up experiments showed that if methoprene and kanamycin or untreated termites are placed together, zero or rescued presoldier initiation, respectively, occurs. These findings reveal that endogenous JH selects for symbiont compositions required to successfully complete presoldier differentiation. However, if the gut is voided before the influx of JH, it cannot select for the necessary symbionts that are crucial for molting. Based on these results we are able to provide a novel example of linkages between gut microbial communities and host phenotypic plasticity.

Keywords

Termite gut symbionts, juvenile hormone, methoprene, kanamycin, caste differentiation.

2.1 Introduction

Termites are eusocial insects living in colonies composed of three main caste phenotypes (workers, soldiers, and reproductives), each with their own well-defined socio-behavioral roles (Laine and Wright, 2003; Roisin, 2000; Wilson 1971). Termite workers further have the unique characteristic capacity to molt into other caste phenotypes through the process known as caste differentiation, which is a form of phenotypic plasticity. Caste differentiation and development in *Reticulitermes flavipes* can follow either apterous/neuter or imaginal routes (Scharf et al., 2003) and can have three different developmental potentials that include status quo molts into next-instar workers, differentiation into a presoldier that later molts into a soldier (Zhou et al., 2006a), or differentiation into a supplementary reproductive individual. Research on the worker-soldier molt process is of recent interest as a non-destructive form (soldier) develops from the most economically destructive life stage (worker). Several endogenous and exogenous factors have been reported to regulate the worker-soldier molt. At the endocrine level, the sesquiterpenoid-juvenile hormone (JH) acts as a hormone to differentiate workers into soldiers via an intermediate presoldier phenotype (Tarver et al., 2009; Zhou et al., 2007). In *R. flavipes* and most other insects, JH III is produced by a neuroendocrine gland known as the corpora allata (CA) closely associated with the brain (Yagi et al., 2005). The entire soldier differentiation process occurs in response to the elevated endogenous JH titers (Chan et al., 2011; Elliott et al., 2009; Elliott and Stay, 2008; Mao et al., 2005; Park and Raina, 2004). However, in other insects, elevated JH retains the immature phenotype through development resulting in extra-nymphal or larval instars (Nijhout et al., 2014; Smykal et al., 2014). In addition to regulating termite polyphenism, JH is also involved in vitellogenesis through inducing increased vitellogenin protein, insect diapause and other processes (Engelmann, 1984; Scharf et al., 2005; Yagi, 1976).

Besides JH, termite caste differentiation could be associated with their gut microorganisms. The termite gut is a habitat for numerous microorganisms from various taxa, including protists, Bacteria and Archaea, living in a consortium and contributing to termite physiology (Brune and Ohkuma, 2010; Ohkuma, 2008; Scharf and Tartar, 2008). Termite symbionts are crucial in cellulose/hemicellulose degradation, nitrogen fixation, acetogenesis and anti-fungal defense (Doolittle et al., 2008; Inoue et al., 2000; 1997; Peterson and Scharf, 2016a; Peterson and Scharf, 2016b). The literature is flooded with reports on the impact of gut symbionts on endocrine regulation in humans, mice and insects. The human gut bacterium Lactobacillus reuteri was

reported to up-regulate the neuropeptide hormone- oxytocin to enhance wound-healing properties through nerve-mediated pathways (Poutahidis et al., 2013). Studies on human and mouse models have supported the conclusion that gut symbionts can affect immune and endocrine modulation in a variety of signaling pathways (Chen et al., 2015). Similarly, in some insects, gut symbionts were observed to influence the pheromones released, social behavior, host development, egg production, expression of hemolymph proteins, and hormones produced (Engl and Kaltenpoth, 2018; Lee et al., 2017). In honeybees, gut symbionts were also reported to increase body mass by mediating changes in insulin and vitellogenin signaling pathways (Zheng et al., 2017).

Several aspects of bacteria-termite symbiosis are yet to be understood. No reports are yet available on the interaction of termite gut symbionts with presoldier differentiation, which is regulated by JH produced by the endocrine system. In female bean bugs (*Riptortus clavatus*), the gut symbiont *Burkholderia* was reported to modulate the JH titer (Lee et al., 2019). Association with commensal bacteria such as *Lactobacillus plantarum* in *Drosophila melanogaster* has also been correlated with hormone signaling, providing strong evidence for the influence of gut bacteria on insect endocrine functioning (Storelli et al., 2011), while in termites, JH was reported to alter the expression of hundreds of symbiont genes in the gut (Sen et al., 2013). Another study has demonstrated that presoldier phenotypes have biased expression of genes coding for vitellogenin, a protein thought to bind JH (Scharf et al., 2003), while in other insects, gut symbionts were reported to influence the expression of vitellogenin (Zheng et al., 2017). Several studies on other organisms have further demonstrated that gut symbionts can impact hormone signaling pathways.

The goal of this study was to investigate whether termite gut bacteria can influence JH-dependent presoldier differentiation in *R. flavipes*, with the overarching hypothesis being that specific gut symbionts are connected to the presoldier differentiation process. Our specific objectives were to (i) study the effects of JH and antibiotic treatments on presoldier emergence and/or survivorship and protist numbers, and (ii) seek to connect specific gut bacteria with the above conditions using 16S rRNA gene sequence surveys of the gut microenvironment. Our findings provide a novel example of the influences of gut microbial symbionts on host phenotypic plasticity.

2.2 Materials and Methods

2.2.1 Termite bioassays

This study is based on a preliminary experiment where JH III and kanamycin were used to induce presoldier differentiation and results were similar to those obtained in the present study. We opted to use methoprene in place of JH III because it is consistently available with high purity, whereas JH III is not. Termites [*Reticulitermes flavipes* (kollar 1837)] from colonies reared in the laboratory were used in bioassays. There were two phases for this experiment. In the first phase, paper towel sandwiches were pretreated with either kanamycin (50% w/v in water) or water alone and were then fed to replicate groups of termites for 2 days. For the second phase, termites were then moved to paper towel sandwiches that were treated with either acetone or methoprene (30µg per dish) in acetone (Chem Service Inc., West Chester, PA, USA) and dried in a fume hood prior to use. Methoprene and kanamycin were used, to induce soldier caste differentiation and manipulate gut microbial load, respectively. Based on previous studies, kanamycin significantly reduced and altered the gut microbial load with minimal impacts to the host (Peterson et al., 2015), while exogenous application of methoprene has been reported to induce the genes in JH-signaling pathways and similarly induce presoldier differentiation (Du et al., 2020; Tarver et al., 2012). Also, preliminary experiments with JH III demonstrated similar presoldier differentiation as seen in the present study with methoprene. Phase 1 pre-treatments lasted 2 days, which was identical for all the experiments; however, the duration of the final treatment was dependent on the type of experiment, as detailed in the following sections. Treatment abbreviations are as follows: Kan (kanamycin alone), Meth (methoprene alone), KanMeth (kanamycin in phase 1 and methoprene in phase 2), and UnCtl (untreated control), which received water in phase 1 and Acetone (evaporated) in phase 2.

2.2.2 Presoldier initiation and survivorship assessment bioassays

The assessment of presoldier emergence and survivorship was done to observe the differences among treatments at the whole-insect ‘holobiont’ level. There were four biological and three technical replicates each for this assessment (12 replicates total). For this bioassay, 25 worker termites were provided with kanamycin or water treated paper towel sandwiches in Petri dishes for 2 days (phase 1). After 2 days, 20 termites were transferred for final treatments as

detailed above (phase 2) and observed for 15 days. Termites emerging from molting with transparent elongated mandibles were scored as presoldiers. Termites that were immobile and could not move their appendages when disturbed by soft forceps were considered dead. Survivorship in untreated controls, even in smaller groups, is quite high and invariable. We have tried working with larger groups in the past, and treatment-induced mortality is still a problem, coupled with an inability to score assays quickly and without causing additional, confounding assay stress. Therefore, we used small groups of workers for this experiment.

2.2.3 Protist counts

Each bioassay treatment described above was replicated 6 times and 35 termites were placed in each Petri dish for pre-treatment (phase 1). For final treatments (phase 2), 30 termites were transferred to Petri dishes with methoprene- or acetone-treated paper towel sandwiches for 9 days, which was the day of emergence of at least one presoldier in the methoprene treatment. The hind guts from five live termites each from assay days 0, 2, 6 and 11 were dissected. The guts were squeezed gently into 500 µl phosphate buffered saline (PBS, pH 7.4). A 10 µl suspension was used to count the protist numbers using a phase contrast microscope at 20 × magnification and a Sedgwick Rafter counting cell (SPI Supplies, West Chester, PA, USA).

2.2.4 16S rRNA gene sequencing

DNA extraction

Bioassays with 25 worker termites were set-up for DNA extraction. There were four replications each for the treatments. Similar to the phenological study, 20 termites were taken for the second phase of the experiment. Live termite workers were sampled and dissected after at least one presoldier was sighted (on day 11) in methoprene bioassay treatments. We picked day 11 for sampling DNA because the survival of termites was high and only live termite workers were dissected to acquire sufficient high-quality DNA from gut tissues. Also, a histological study of fat body (Cornette et al., 2007) revealed that the production of fat body and protein granules starts within the first 3 days after hormonal treatment. These granules disappear within a few days of presoldier development, indicating that termite physiological changes can be documented up to the point of presoldier molting. Four replications of 10 guts per treatment were sampled for DNA

extraction. The ten guts from live termite workers were dissected, placed in 200 µl PBS and processed for DNA extraction using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Along with the experimental samples, water was also run through the DNA extraction protocol as an extraction control. The quality of DNA in the aliquots was assessed through gel electrophoresis and was quantified using a Nanodrop spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA) and later verified using fluorometric detection by the University of Minnesota Genomics Core. Thirty microliters (41.3-125.9 ngµl^{-1}) of extracted DNA was sent for library preparation and sequencing, and the remaining DNA was used for 16S rRNA gene quantitative PCR (qPCR) analysis.

16S quantitative PCR

The 16S rRNA gene copy number from different treatments detailed above was assessed using two independent primer sets that included (1) primers 338F and 518R and (2) primers 515F and 806R, flanking the V3 and V4 regions of 16S rRNA gene, respectively. For primer set 1 the total reaction volume was 15 µl, containing 7.5 µl buffer (SsoFast EvaGreen® Supermix, Bio-Rad Inc., Hercules, CA, USA), 1 µl reverse and forward primers each (10 µmol l−1 final concentration), 4 µl nuclease free water and 1.5 µl DNA template (0.31-1.48 ng). The cycling conditions used were initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s and extension at 72°C for 30 s followed by a melt curve from 65°C to 95°C with 0.5°C increments. The 16S rRNA qPCR with primer set 2 was performed by the University of Minnesota Genomics Core sequencing facility; the cycling conditions used were initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 98°C for 20 s, annealing at 55°C for 15 s and extension at 72°C for 60 s. Final extension was at 72°C for 5 min after which the samples were held at 4°C. DNA copy number was determined by comparison against a DNA standard curve (see equation below), with analysis and PCR efficiency determinations by the method 10^(-1/slope).

$$\text{Number of copies} = \frac{(X \times A)}{(N \times M \times C)}$$

where X is the amount of DNA (in ng) calculated using the equation of a standard curve, A is Avogadro's number (6.022×1023 molecules mole−1), N is the primer length, M is the average mass of one bp of dsDNA (660 g mole−1) and C is the conversion factor (109 ng g−1).

16S rRNA gene sequencing

The V3-V4 region of 16S rRNA gene was sequenced using bacterial universal primers 341F- CCTACGGGAGGCAGCAG and 806R- GGACTACHVGGGTWTCTAAT (Caporaso et al., 2011; Peterson et al., 2015). A two-step PCR was used for library preparation. The indexed PCR products were normalized to 10 ng µl−1, and 10 µl of each sample was pooled and concentrated to ~100 µl and cleaned using solid reverse phase immobilization (SPRI) purification. Cleaned library was quantified by Qubit and fragment analyzed by Agilent Tapestation and the library was diluted to 2 nmol l−1. The pooled samples were denatured with NaOH, diluted to 8 pmol l−1 in Illumina HT1 buffer, spiked with 15% PhiX and heat denatured at 96°C for 2 min immediately before loading. A MiSeq 600 (1/8th lane Stowaway) cycle v3 kit was used to sequence the sample by the University of Minnesota Genomics Center, MN, USA.

2.2.5 Sequence processing and community analysis

The paired end (301 bp) Illumina reads were processed and analyzed using the QIIME2 pipeline (Bolyen et al., 2019). Divisive Amplicon Denoising Algorithm 2 (DADA2) was used to obtain high-quality reads following the filtering, trimming, denoising, dereplicating, merging and removing chimera steps (Callahan et al., 2016). During this process, a total of 1,559,624 demultiplexed sequences were processed to obtain 1,234,554 non-chimeric high-quality sequences in QIIME2. The moving picture tutorial in qiime2 was followed to calculate the diversity metrics. To calculate the diversity metrics (alpha and beta), the sequences were subsampled from the feature table from phylogenetic diversity analysis at the sampling depth of 2000 sequences that removed one replicate from KanMeth and four replicates of extraction control, respectively. The alpha diversity measures used were observed operational taxonomic units (OTUs), Pielou's evenness, and the Shannon index. The beta diversities, phylogenetic Unifrac distances- [weighted and unweighted (Lozupone et al., 2011) and non-phylogenetic distances [Jaccard and Bray Curtis] were calculated and visualized using R and QIIME2. The reference database SILVA (version 132)

was customized following the instructions on training the classifier for the V3/V4 region of 16S rRNA gene. Taxonomy was assigned to the data using naïve Bayes trained Silva 132 99% OTU classifier bounded by the 341F/806R primers set as the reference reads (Quast et al., 2013). Bar graphs representing the relative abundance of amplicon sequence variant (ASV) at the phylum and family levels were generated using the R package phyloseq from QIIME2 generated phylotype tables and taxonomy files (McMurdie and Holmes, 2013). Finally, analysis of composition of microbiomes (ANCOM) was used to study differential microbiota composition at phylum and genus level in different treatments via the QIIME2 plugin (Mandal et al., 2015).

2.2.6 Trophallaxis experiment

A final trophallaxis experiment was conducted to understand the effect of different treatments in a eusocial context on donor and recipient termites. Prior to the experiment, 'donor' termites were treated with Nile Blue dye 0.5% w/v (Sigma-Aldrich, St. Louis, MO, USA) for 2 days. After 2 days, the donor termites were transferred into Petri dishes containing filter paper sandwiches treated with methoprene or kanamycin with an exposure duration of 2 days. The methoprene, and kanamycin treatments and the untreated controls were set on the same day. Similarly, some untreated termites were exposed to kanamycin in different Petri dishes, these termites later served as 'recipients' for methoprene-treated donors. The treated termite donors were then placed in new Petri dishes with untreated or kanamycin-treated recipients in the ratio of 1:1 with a total of 20 individuals. The donor-recipient combinations during the experiment were: kanamycin-treated donors + untreated recipients (Kan-D+ Untreated-R), methoprene-treated donors + kanamycin-treated recipients (Meth-D+ Kan-R), and methoprene-treated donors + untreated recipients (Meth-D+ Untreated-R). Controls for this experiment included kanamycin (Kan control), methoprene (Meth control) and untreated (Untreated control). Owing to the total mortality of termites in some treatments, the experiment was ended after 11 days. The data on termite mortality and presoldier initiation were taken every day until 11 days, with the cumulative data from day 11 of the treatment being presented. Each treatment combination was replicated three times.

2.3 Statistical analyses

Statistical analyses were performed in the QIIME2 interface and R (Bolyen et al., 2019; https://www.r-project.org/). The Kruskal-Wallis test was used to compare presoldier emergence, total mortality, average number of protists, and alpha diversities among the treatment groups and trophallaxis experiment. The pairwise comparisons were made using the Mann-Whitney U-test and were corrected for Benjamini-Hochberg false discovery rate. Beta-diversity metrics were subjected to permutational multivariant analysis of variance (PERMANOVA) and the PERMDISP test of homogeneity of dispersions to study statistical significance among groups. The graphs for 16S rRNA gene sequencing data were generated and visualized using R and Microsoft excel. Canonical correspondence analysis (CCA) (ter Braak, 1986) was conducted to study the relationship between the bacterial genera and other variables- protist number, presoldier initiation and total mortality. CCA was implemented with the R package- 'vegan'. For the CCA analysis, four replicates from the presoldier initiation, total mortality and protist numbers were used to study their association with the taxa abundance.

2.4 Results

2.4.1 Presoldier differentiation, survivorship and protist numbers significantly differed among treatments

The effect of treatments on cumulative percentage presoldiers initiated (H = 37.625, d.f. = 3, P < 0.001), cumulative percentage mortality (H = 27.027, d.f. = 3, P < 0.001) on day 17 and average protist numbers per gut (H = 19.048, d.f. = 3, P < 0.001) on day 11 were significant based on Kruskal-Wallis tests. Based on pairwise Mann-Whitney comparisons (P<0.05), the total percentage of presoldiers emerged was significantly higher in the Meth than in the KanMeth treatment, while no presoldiers emerged in the UnCtl and Kan treatments (Fig. 1A). From the same bioassays, survivorship of workers + presoldiers was also recorded. Total mortality in the KanMeth treatment was significantly higher than in any other treatments, and as expected, the UnCtl treatment had the lowest mortality. Mortality levels in the individual Kan and Meth treatments were not significantly different (Fig. 1B). Some variability in the mortality of termites from different replicates in the Kan, KanMeth and Meth treatments was observed, but this variability was not sufficient to overcome the statistically significant trends as reported. The variability could

be due to the variation in group response to the treatments. Similar group variation in the mortality of termites against JH analogue was observed by Hrdý et al. (2006) while treating different termite colonies with the same compound. The average number of protists per termite gut on day 11 was significantly higher in the UnCtl treatment in comparison with other treatments, while the Kan and KanMeth treatments had similar protist numbers (Fig. 1C). The average number of protists in the Meth treatment was significantly higher than in the Kan and KanMeth treatments, but still was greatly reduced in comparison with the UnCtl treatment. The average mortality of termites in all treatments on the day of protist and DNA sampling (day 11) was less than 50% (Fig. S1).

2.4.2 Confirmation of antibiotic impacts on gut bacterial populations

The two independent primer sets, 338F-518R and 515F -806R, used for qRT-PCR showed highly similar results, indicating that the Kan treatment had an expected result of reducing bacterial loads (~2.5x decrease compared with UnCtl), but also unexpectedly that the Meth (~1.2x decrease compared with UnCtl) and KanMeth (~2x decrease compared with UnCtl) treatments had reduced bacterial loads as well.

2.4.3 Bacterial alpha diversity significantly differed among treatments

Rarified 2000 sequence reads obtained from 16S rRNA gene sequencing were used to calculate alpha and beta diversity measures. Alpha diversity was estimated using observed OTUs for species richness (Fig. 2A), Pielou's evenness (Fig. 2B), and the Shannon diversity index (Fig. 2C). The effect of different treatments on observed OTUs (H = 12.829, d.f. = 3; P = 0.005), Pielou evenness (H = 12.567, d.f. = 3; P = 0.005) and Shannon index (H = 12.567, d.f. = 3; P = 0.005) were significant based on Kruskal-Wallis tests. Based on pairwise Mann-Whitney comparisons (P<0.05), the UnCtl treatment demonstrated significantly higher species richness and evenness, followed by the Meth treatment, whereas the Kan and KanMeth treatments were not significantly different from each other. Similar results were obtained for average numbers of protists estimated per gut (Fig. 1C), suggesting a close connection between some bacteria and protist symbiont communities.

2.4.4 Bacterial beta diversity significantly differed among treatments

PERMANOVA statistics show that the weighted UniFrac analysis was statistically significant (pseudo-F= 36.101, P = 0.001), which means the effect of treatments was significant in creating the dissimilarities among the different treatment groups (Fig. S2). Also based on the PERMDISP test, the bacterial community structures among the replicates were not determined to be significantly different (PERMDISP test, F= 0.7168, P = 0.224). Pairwise PERMANOVA results further indicate all treatments differed in terms of community structure for weighted Unifrac. Similar results were observed for beta diversity metrics (Bray-Curtis and Jaccard). However, for the unweighted Unifrac analysis, the Kan and KanMeth treatments were not significantly different, whereas other pairwise comparisons were significant.

2.4.5 Bacterial taxonomic composition differed among treatments

The taxonomic composition and relative abundance of major bacterial phyla at abundances greater than 0.1% varied greatly among treatments but was consistent among the replicates within treatments (Fig. 3). At the phylum level, in the UnCtl treatment, Spirochaetes, Firmicutes and Bacteroidetes were the most abundant (~70%). In the Meth treatment, the most abundant phyla were Proteobacteria and Bacteroidetes (~55%). In the KanMeth treatment, the most abundant phyla were Proteobacteria and Actinobacteria (~85%). Finally, the Kan treatment was dominated by the phylum Actinobacteria (~75%). Similar to phylum level results, family level relative abundances also varied widely among the treatments.

Based on the ANCOM test at the phylum level, the bacterial phylum Elusimicrobia was the most significantly abundant among treatments. In contrast, at the genus level, the following top 13 bacteria were significantly differentially abundant among treatments (centered log ratio, clr>200): uncultured delta proteobacterium (Proteobacteria), uncultured Synergistetes (Synergistetes), *Lactococcus* (Firmicutes), *Propionivibrio* (Proteobacteria), uncultured Epsilonproteobacteria bacterium (Epsilonbacteraeota), *Tyzzerella* 3 (Firmicutes), *Treponema* (Spirochaetes), *Treponema* 2 (Spirochaetes), Candidatus Endomicrobium (Elusimicrobia), unidentified Firmicutes (Firmicutes), *Stenoxybacter* (Proteobacteria), uncultured Bacteroidetes bacterium (Bacteroidetes) and *Cerasicoccus* (Verrucomicrobia). The observed log abundances tied

to different genera are shown in Fig. S3, and the list of significant genera based on ANCOM hypothesis test with their respective clr and W scores is shown in Table S1.

2.4.6 CCA confirms significant factors contributing to the differences among the treatments

The CCA model indicated that the variation in bacterial species abundance across treatments was significantly correlated with presoldier initiation, total mortality and protist numbers (chi-square, F3,11 = 1.7603, *P* = 0.006). The CCA plot shows that the number of protists and presoldiers initiated was strongly associated with the UnCtl and Meth treatments, respectively, whereas total mortality was strongly associated with Kan and KanMeth treatments (Fig. 4). Axes-1, 2 and 3 explain 38.6 %, 12.03%, and 7.8% of the total constrained variation, respectively.

2.4.7 Significant effects of trophallaxis on presoldier initiation and termite survivorship

The effect of trophallaxis 'rescue' treatments on cumulative percentage of presoldiers initiated (H = 10.692, d.f. = 3, P = 0.013) and cumulative percentage mortality (H = 16.111, d.f. = 5, P = 0.006) were significant based on Kruskal-Wallis tests. No presoldiers emerged in the Meth-D + Kan-R and untreated control treatments. All the other treatment combinations that did not result in presoldier emergence were removed from the presoldier initiation data analysis. Based on pairwise Mann-Whitney comparisons (P<0.05), the total percentage of presoldiers emerged in meth controls was significantly higher than the treatment combination Meth-D + Untreated-R termites (Fig. 5). From the same bioassays, survivorship of termites was also recorded. Total mortality in kan control and Meth-D + Kan-R were significantly higher than in other treatment combinations. The mortalities in Kan-D + Untreated-R and Meth-D + Untreated-R were not statistically different from one another (Fig. S4). Collectively these results show that presoldier emergence is higher in methoprene-treated individuals held with control individuals containing intact microbiomes, than in methoprene-treated individuals held with Kanamycin-treated individuals.

2.5 Discussion

This study experimentally quantified the effect of gut symbiont communities on the phenomenon of JH-mediated presoldier differentiation in termites. The study was conducted using methoprene, whose chemical structure closely approximates that of JH (Charles et al., 2011). Methoprene is also consistently available in a highly pure form, while JH III is not, which makes independent verification of findings for JH III difficult. These findings provide a novel example of gut microbial community influence on host phenotype. Four different treatments were used to investigate possible roles of bacterial and protist gut symbionts in the caste differentiation process. These treatments included kanamycin alone (Kan), kanamycin + the JH analog Methoprene (KanMeth), methoprene alone (Meth) and an untreated control (UnCtl). The effect of these treatments on presoldier formation, survivorship and protist abundance were observed through phenotypic bioassays and protist quantification. Treatment effects on bacterial abundance and composition were studied through the use of quantitative PCR and16S rRNA gene sequence analysis, which together were used to infer alpha, and beta diversities and differential abundance through data analysis procedures.

The JH analog methoprene demonstrates a similar effect as endogenous JH would on a termite worker, resulting in significant presoldier initiation (Zhou et al., 2006b). Kanamycin was previously shown to reduce both protist and bacterial symbiont load in the termite gut (Peterson et al., 2015), and a similar effect was observed in this experiment as well. Therefore, the higher percentage of presoldiers emerged in the Meth compared with the KanMeth treatment suggests that gut symbionts along with JH play a crucial role in presoldier initiation and their survival. In the survivorship assessment among different treatments, the higher percent mortality of termites in the KanMeth treatment could be linked to the double stress from antibiotic and methoprene/JH. That means exposure to kanamycin prior to methoprene reduced the gut microbial load (possibly the gut microbes needed to survive stress during the molting process), resulting in the significantly high mortality observed in phenological bioassays. Additionally, the mortality observed with the Meth-D +Kan-R treatment in our rescue experiment further supports that presoldier initiation within colonies is lethal to termites lacking intact gut microbiomes. The trophallaxis experiment further support that the microbiome is relevant for surviving the presoldier molt in termites.

The mortality observed in the Meth treatment was also higher than the mortality observed with JH III by Hrdý et al. (2006). The possible explanation of this higher mortality could be due

to the strong affinity of methoprene at the JH receptor or the stress caused during and after presoldier molting that is normally alleviated by the presence of specific gut symbionts and trophallaxis exchange with other colony members. Another cause of bioassay mortality could be the excessive proportion of presoldiers initiated in our study, which are unable to eat or be fed by workers exposed to methoprene in Petri dishes. Lastly, similar mortality percentages were observed by Howard and Haverty (1978) while using a similar concentration of methoprene as ours. Protist numbers were significantly reduced in termites prior to molting in Meth treatments (Nalepa 2017; Sen et al., 2013), while some protists were still intact. Termite gut undergoes defaunation during the molting process causing a reduction in the numbers of gut symbionts. This defaunation or reduced protist number could be another cause for the significant mortality we observed in Meth treated individuals. Similar to previous findings (Peterson et al., 2015), protist numbers were reduced in the Kan treatment, which corresponds well with the observed reduction of several bacterial taxa (Spirochaetes, Elusimicrobia, Firmicutes, Bacteroidetes) that are known to maintain symbiotic associations with protists.

Alpha diversity metrics from the 16S rRNA gene sequencing demonstrated that the UnCtl treatment had the highest species evenness and richness, which was expected. Other treatment groups, however, had reduced alpha diversity in ways that were not entirely expected. The reductions could be due to 'SPRY' genes which have been documented in association with reduced gut symbiont numbers in termites when challenged with hormones and antibiotics (Scharf et al., 2017). The significantly higher species evenness and richness in the Meth compared with Kan and KanMeth treatments strengthens our above conclusion, i.e. that certain gut bacteria are required for successfully completing the presoldier transition, which is well supported by both the presoldier differentiation and trophallaxis experiments. Even though the KanMeth treatment resulted in some presoldier emergence, the total presoldier emergence was reduced significantly in comparison with the Meth treatment. A likely explanation for this difference is that gut symbionts were selected at two levels by kanamycin and JH in the KanMeth treatment. Bacterial diversity was further investigated among treatments using beta-diversity metrics. Weighted UniFrac analysis demonstrated that different treatment groups had microbial communities different from each other. Similar results were observed for Bray-Curtis and Jaccard analyses, indicating that each treatment resulted in significantly different bacterial community structures (Lozupone et al., 2007). Overall, from the alpha and beta-diversity metrics, and supporting

statistical analyses, it is clear that different hormonal and antimicrobial treatments selected for different gut symbiotic load and content. This determination provided rationale to look further into the types of bacteria selected by the different treatments as detailed below.

Several protists, Archaea and Bacteria inhabit the small micro-environment inside a termite gut and provide synergistic physiological benefits to the host (Ohkuma, 2008; Scharf and Peterson, 2021). In this experiment, the major bacterial phyla observed were Spirochaetes, Firmicutes and Bacteroidetes, comprising more than 70% of abundance similar to the previous findings from 16S V5-V6 sampling of the *R. flavipes* gut lumen (Boucias et al., 2013). Shifts in both bacterial composition and abundance were observed among different treatments, following a hologenome theory of evolution, i.e. the artificially introduced stress was able to change the gut microbial community (Zilber-Rosenberg and Rosenberg, 2008). Most notably, Kan and KanMeth treatments lacked Spirochaetes and Elusimicrobia and had a reduction in Firmicutes. In wood-feeding termites, Spirochaetes are associated with acetogenesis and cellulose-degrading protists, whose absence could be detrimental to the survival of termite. Reduction in xylose released from lignocellulosic diet materials was also observed when the abundances of Elusimicrobia and Firmicutes were reduced (Leadbetter et al., 1999; Lucey and Leadbetter, 2014; Otani et al., 2014; Peterson et al., 2015). This evidence from prior studies highlights the involvement of these bacterial phyla in lignocellulose digestion and ultimately in the survival of termites, which is in agreement with the current findings. Spirochaete bacteria are ectosymbionts to flagellate protists (Iida et al., 2000), whereas Bacteroidetes, Elusimicrobia and Firmicutes are endosymbionts to protists (Fröhlich and König, 1999; Noda et al., 2007, 2005; Ohkuma et al., 2007; Stingl et al., 2005). Because the Kanamycin treatment eliminated or reduced the endosymbionts and ectosymbionts, the disruption of the bacteria-protist symbiosis likely resulted in significant reductions in protist counts and, ultimately, decreased termite survivorship (Peterson et al., 2015; Stephens and Gage, 2020; Treitli et al., 2019).

Based on ANCOM analysis, the genus *Cerasicoccus* (Verrucomicrobia) differed significantly by being completely absent from the Meth, KanMeth and Kan treatments. The taxa-*Propionivibrio* (Proteobacteria), uncultured Epsilonproteobacteria bacterium (Epsilonbacteraeota), *Tyzzerella* 3 (Firmicutes), *Treponema* (Spirochaetes), *Treponema* 2 (Spirochaetes), Candidatus Endomicrobium (Elusimicrobia), and unidentified Firmicutes (Firmicutes) demonstrated the highest abundance in the UnCtl treatment, while these taxa were

completely absent/or highly reduced in the Kan /KanMeth treatments and reduced in the Meth treatment; suggesting their importance in the survival and successful completion of the presoldier differentiation process. In contrast, the taxa- uncultured delta proteobacterium (Proteobacteria), uncultured Synergistetes (Synergistetes), and *Lactococcus* (Firmicutes) were equally abundant in the Meth and UnCtl treatments but were completely absent in the Kan and KanMeth treatments. And although the taxa *Stenoxybacter* (Proteobacteria) and uncultured Bacteroidetes bacterium (Bacteroidetes) were slightly reduced in the UnCtl compared with the Meth treatment, they were completely absent in the Kan and KanMeth treatments, suggesting their importance in the successful completion of the presoldier differentiation process. The significantly high mortality observed in the Kan and KanMeth treatments can be associated with gut stress owing to the disappearance or reduction of these taxa. Another possible explanation based on the trophallaxis experiment could be that when termites were treated with kanamycin and methoprene, they were not able to provide the symbionts to each other needed for survival resulting in high mortality; this possibility also explains the lower or lack of presoldier differentiation in these treatments and provides further eusocial context to our findings.

The CCA analysis also confirms the strong association of total mortality with the Kan and KanMeth treatments. The reduction of specific protist flagellate-associated bacterial groups (Elusimicrobia, Firmicutes, Spirochaetes) with the Meth treatment is in agreement with the fact that termite guts are voided prior to molting (Nalepa, 2017; Sen et al., 2013) and explains the mortality that might be present in the colony during the differentiation, which could be one of the reasons why there are typically under 3% soldiers in *R. flavipes* colonies. Based on the presoldier emergence results from the trophallaxis experiment, it is also possible that normally-faunated termites have inhibitory effects on presoldier differentiation, which may also contribute to the low numbers of soldiers present in *R. flavipes* colonies. In humans, bacterial symbionts are documented to impact host lipid metabolism, the immune system, and neuroendocrine pathways, which in turn can affect the nervous system and brain function through microbiota-gut-brain axis pathways (Forsythe and Kunze, 2013; Krishnan et al., 2015). Another study in early life stages of mice demonstrated that the acquisition of gut bacteria enhanced stress responses (Sudo et al., 2004). In the present study, the combined results from survivorship, protist numbers, 16S rRNA gene sequence data and the trophallaxis experiment indicated the reduced stress response capabilities of termites resulting from reduced gut symbiont abundance and diversity. In particular, the stress

owing to kanamycin and methoprene was reduced by holding treated individuals with faunated termites in the trophallaxis experiment, as would occur under natural colony conditions. Based on the tightly linked associations among different experimental outcomes, it is clear that gut symbionts and their relative proportions play important roles during the soldier caste differentiation process. Prior experimental evidence reveals how JH is able to select for a differential abundance of fat body related CYP4 and hexamerin genes and influence their expression during presoldier initiation (Zhou et al., 2006a, b, 2007). Subsequent studies have shown how JH dramatically reduces protist flagellate gene expression and populations specifically in the gut (Scharf et al., 2017; Sen et al., 2013). The current study builds on these prior findings by showing that JH selects for gut symbionts at certain abundance levels, which in turn contributes to successful completion of the presoldier differentiation process.

Lastly, the differential bacterial compositions we identified here between the Kan and KanMeth treatments are one of the advancements provided by this study. Specifically, we found that JH decreases protists and associated bacteria, while the remaining bacteria help emerging presoldiers survive the transition. However, if the gut microbial balance is disrupted before JH application, termites cannot withstand the physiological stress caused by the molting process. These impacts of JH on the gut microbial system are now disentangled by our findings. As the current study demonstrates, gut symbionts under the influence of JH, besides having importance in termite physiological functions such as digestion, reproduction, immunity, nitrogen fixation and acetogenesis (Cleveland 1923; Rosengaus et al., 2011; Scharf et al., 2011), are directly linked to presoldier differentiation and emergence and are important in the survival of termites under colony conditions. Moreover, the composition and abundance of these gut microbes may predict the phenotype of the termite host. However, the specific mechanisms involved await determination. Unraveling these mechanisms and the roles of specific bacteria is likely to provide important new insights into termite caste differentiation, and more broadly into the processes related to gut microbial control over host phenotypic plasticity.

2.6 Acknowledgements

We thank the University of Minnesota Genomics Center for library preparation and sequencing of 16S rRNA gene; Hannah Stewart, Ethan Hillman and Brittany Peterson for assay

development in earlier phases of this research; Dr. Timothy Johnson and Manoj Pandey for assistance with the data analysis; and Zachery Wolfe for laboratory assistance.

2.7 Competing Interests

The authors declare no competing or financial interests.

2.8 Author contribution

Conceptualization: M.E.S.; Methodology: R.S.; Validation: R.S.; Formal analysis: R.S., C.H.N.; Investigation: R.S.; Data curation: R.S., C.H.N.; Writing - original draft: R.S.; Writing – review & editing: C.H.N., M.E.S.; Visualization: R.S.; Funding acquisition: M.E.S.

2.9 Funding

This research was supported by a Ross Graduate Fellowship to R.S., USDA-NIFA-Hatch support to M.E.S. (project No.1010572), and the O.W. Rollins / Orkin Endowment in the Department of Entomology at Purdue University.

2.10 Data Availability

Sequences from this study are available through the NCBI Sequence Read Archive database under the BioProject accession number PRJNA693690 and the individual sequences are SRR13495705- SRR13495624.

2.11 Figures

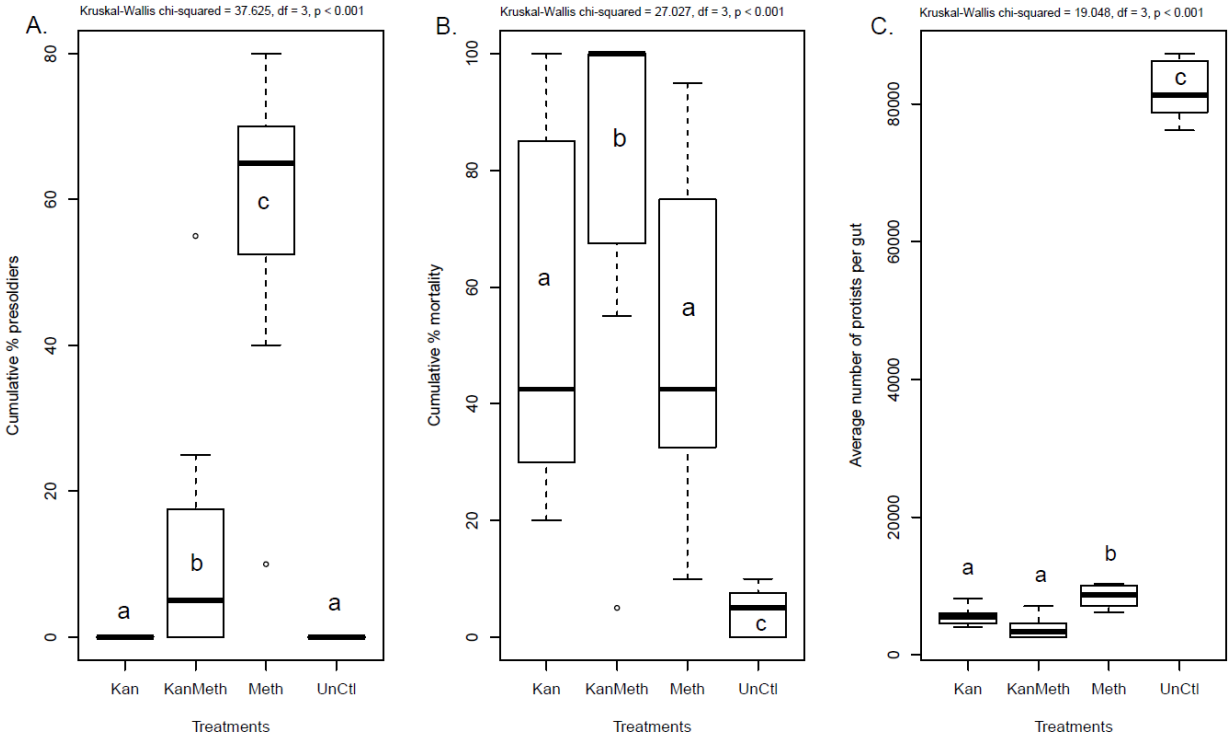


Fig. 2. 1. Bioassay and protist count results showing: Cumulative percent presoldiers initiated (A, 12 replications, sample size = 20), Cumulative percent mortality (B, 12 replications, sample size = 20) and Average number of protists per termite gut (C, 6 replications, sample size = 5). Boxplots showing the median (horizontal line in the box), interquartile range (IQR, the box), samples within 1.5 times IQR (vertical lines) and samples outside 1.5 times IQR are shown as small circles on the graph. P-values for global Kruskal-Wallis comparisons across treatments are shown at the top of each graph. Significance levels for pairwise comparisons are indicated by lowercase letters. The individual treatment groups in graphs with the same letters are not significantly different (Mann-Whitney U-test; $p<0.05$).

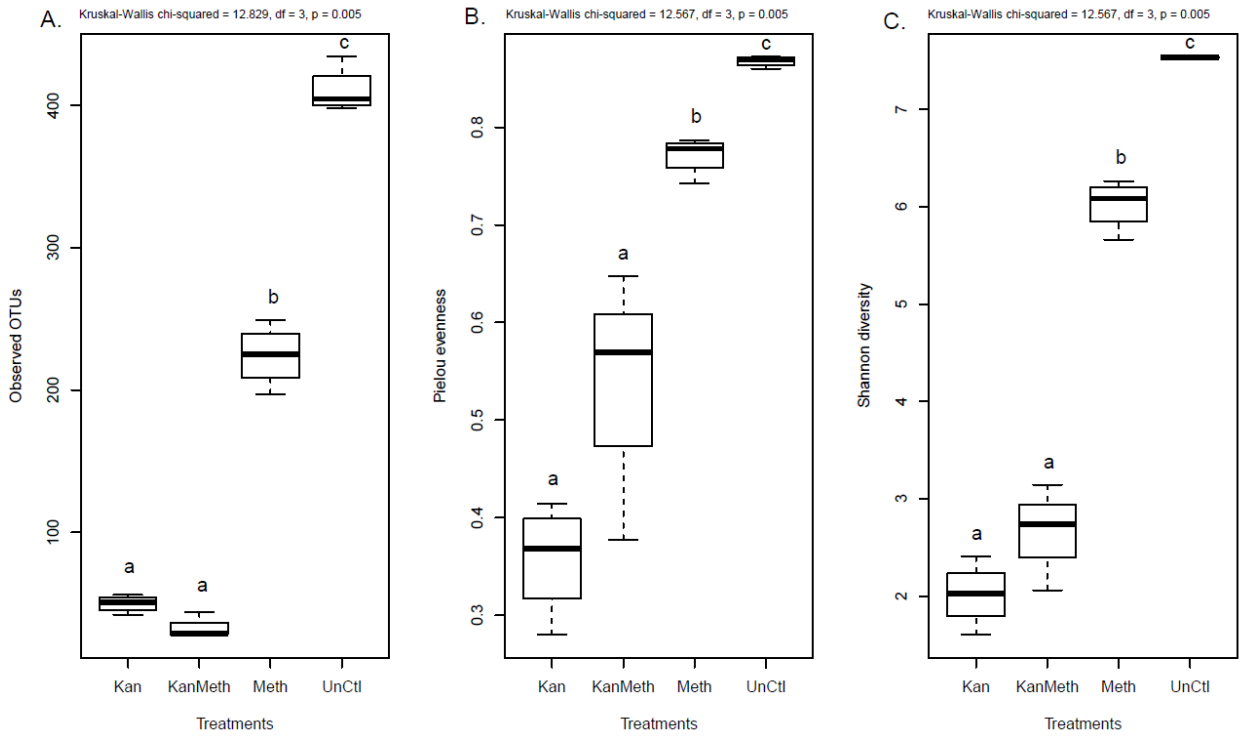


Fig. 2.2. Alpha diversity metrics from 16S surveys showing: Observed OTU numbers (A), Pielou evenness (B) and Shannon diversity (C). Boxplots showing the median (horizontal line in the box), interquartile range (IQR, the box), samples within 1.5 times IQR (vertical lines). Total replications for Kan, Meth and UnCtl were 4 while KanMeth had 3 replications (sample size = 10). P-values for global Kruskal-Wallis comparisons across treatments are shown at the top of each graph. Significance levels for pairwise comparisons are indicated by lowercase letters. The individual treatment groups in the graphs with the same letters are not significantly different ($p<0.05$).

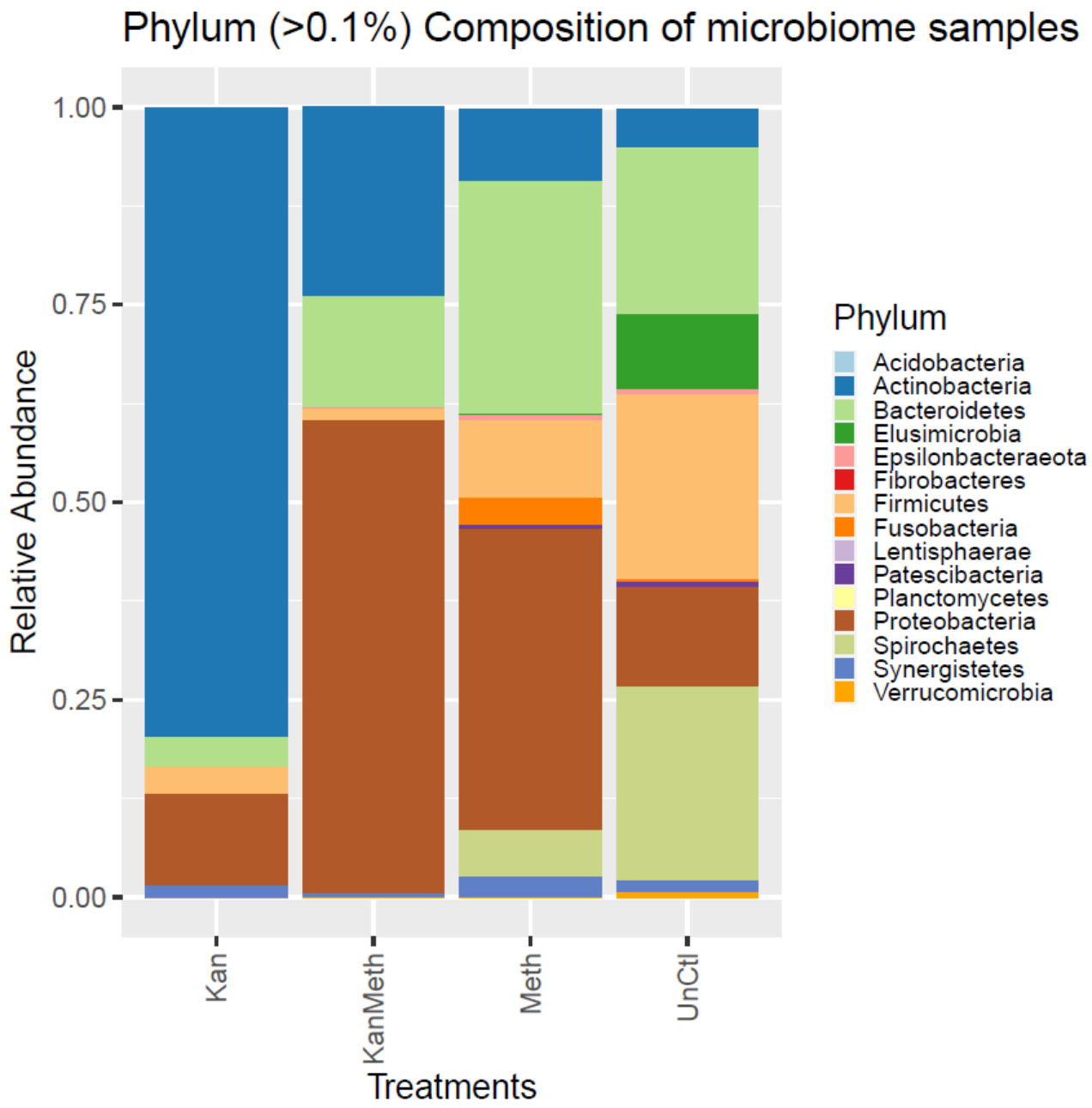


Fig. 2.3. Relative abundance (from abundance >0.001), measured as the percentage of the total bacterial abundance for each treatment. Total replications for Kan, Meth and UnCtl were 4 while KanMeth had 3 replications (sample size = 10). The index indicates the phylum level identification of bacterial species.

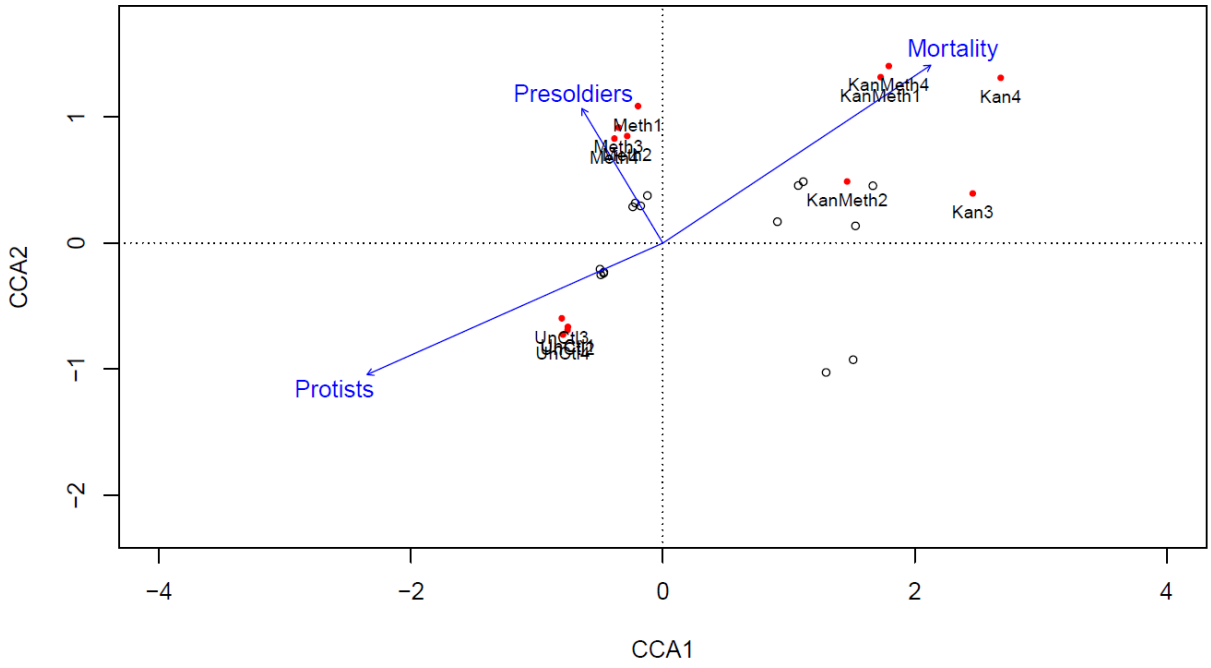


Fig. 2.4. Canonical Correspondence Analysis model performed with relative abundance of bacterial taxa and environmental variables- protist numbers, presoldier initiated and total mortality. Overall significance of the model: Chi-square, F(3,11) = 1.7603, P(>F) = 0.006, with first, second and third axes representing 38.6%, 12.03% and 7.8% of the total constrained variation. Total replications for Kan, Meth and UnCtl were 4 while KanMeth had 3 replications (sample size = N/A)

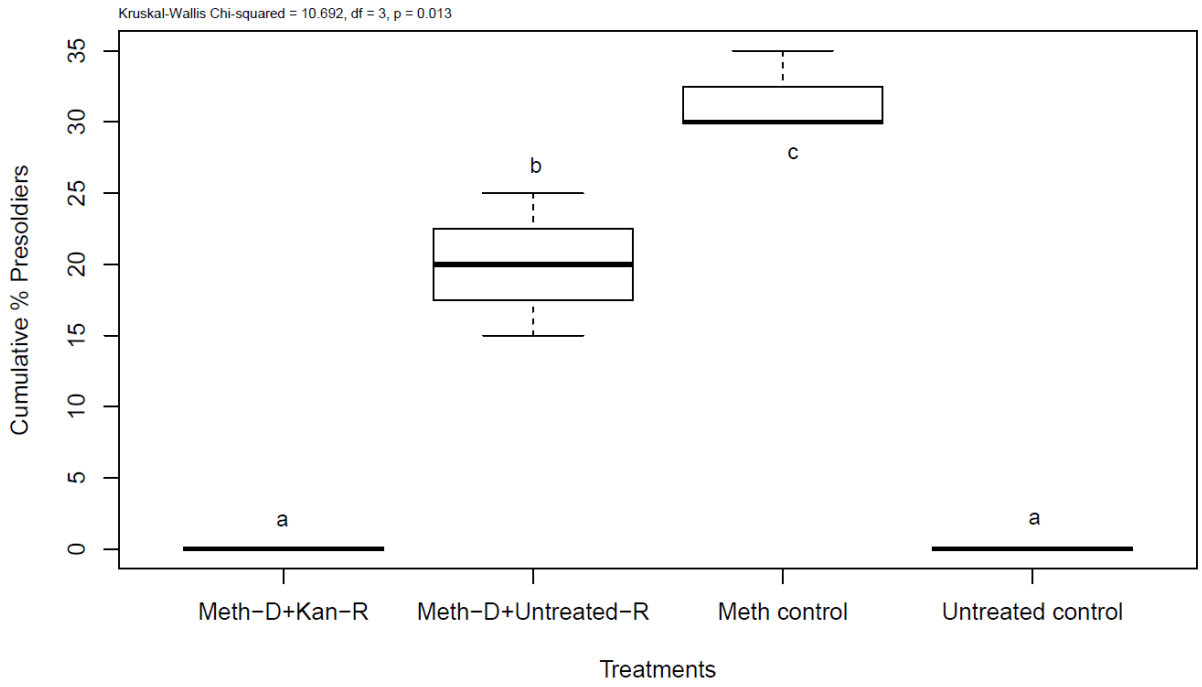


Fig. 2.5. Trophallaxis experiment on presoldier initiation study showing cumulative percent presoldiers initiated over the course of 11-day assays (3 replications, sample size = 20). Boxplots showing the median (horizontal line in the box), interquartile range (IQR, the box), samples within 1.5 times IQR (vertical lines). P-values for global Kruskal-Wallis comparison across treatments are shown at the top of the graph. Significance levels for pairwise comparisons are indicated by lowercase letters. The individual treatment groups in the graph with the same letters are not significantly different (Mann-Whitney U-test; $p<0.05$). Treatment abbreviations are defined in the Methods text.

2.12 References

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F. et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol*. 37, 852-857.

Boucias, D. G., Cai, Y., Sun, Y., Lietze, V. U., Sen, R., Raychoudhury, R. and Scharf, M. E. (2013). The hindgut lumen prokaryotic microbiota of the termite *Reticulitermes flavipes* and its responses to dietary lignocellulose composition. *Mol. Ecol.* 22, 1836-1853.

Brune, A. and Ohkuma, M. (2010). Role of the termite gut microbiota in symbiotic digestion. In Biology of termites: a modern synthesis (ed. D. Bignell, Y. Roisin and N. Lo), pp. 439-475. Springer, Dordrecht.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A. and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. methods*. 13, 581-583.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N. and Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci.* U.S.A. 108, 4516-4522.

Chan, K. K., Abel, D. S. and Stay, B. (2011). Fine structure of corpora allata of castes with different rates of juvenile hormone production in the termite *Reticulitermes flavipes*. *Arth. Struct. & Dev.* 40, 26-38.

Charles, J. P., Iwema, T., Epa, V. C., Takaki, K., Rynes, J. and Jindra, M. (2011). Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proc. Natl. Acad. Sci.* 108, 21128-21133.

Chen, J., Li, Y., Tian, Y., Huang, C., Li, D., Zhong, Q. and Ma, X. (2015). Interaction between microbes and host intestinal health: modulation by dietary nutrients and gut-brain-endocrine-immune axis. *Nat. methods*. 16, 592-603.

Cleveland, L. R. (1923). Symbiosis between termites and their intestinal protozoa. *Proc. Natl. Acad. Sci.* U.S.A. 9, 424.

Cornette, R., Matsumoto, T., & Miura, T. (2007). Histological analysis of fat body development and molting events during soldier differentiation in the damp-wood termite, *Hodotermopsis sjostedti* (Isoptera, Termopsidae). *Zoological science*. 24, 1066-1074.

Doolittle, M., Raina, A., Lax, A. and Boopathy, R. (2008). Presence of nitrogen fixing Klebsiella pneumoniae in the gut of the Formosan subterranean termite (*Coptotermes formosanus*). *Bioresour. Technol.* 99, 3297-3300.

Du, H., Tong, R. L., Huang, X., Liu, B., Huang, R. and Li, Z. (2020). Methoprene-induced genes in workers of Formosan subterranean termites (*Coptotermes formosanus* Shiraki). *Insects*. 11, 71.

Elliott, K. L., Chan, K. K., Teesch, L., Clor, O. and Stay, B. (2009). Identification of Phe-Gly-Leu-amide type allatostatin-7 in *Reticulitermes flavipes*: its localization in tissues and relation to juvenile hormone synthesis. *Peptides*. 30, 495-506.

Elliott, K. L. and Stay, B. (2008). Changes in juvenile hormone synthesis in the termite *Reticulitermes flavipes* during development of soldiers and neotenic reproductives from groups of isolated workers. *J. Insect Physiol.* 54, 492-500.

Engelmann, F. (1984). Regulation of vitellogenesis in insects: the pleiotropic role of juvenile hormones. In *Biosynthesis, metabolism and mode of action of invertebrate hormones* (ed. J. Hoffmann and M. Porchet), pp. 444-453. Springer, Berlin, Heidelberg.

Engl, T. and Kaltenpoth, M. (2018). Influence of microbial symbionts on insect pheromones. *Nat. Prod. Rep*. 35, 386-397.

Forsythe, P. and Kunze, W. A. (2013). Voices from within: gut microbes and the CNS. *Cell. Mol. Life Sci.* 70, 55-69.

Fröhlich, J. and König, H. (1999). Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator. *Syst. Appl. Microbiol*. 22, 249-257.

Hrdý, I., Kuldová, J., Hanus, R. and Wimmer, Z. (2006). Juvenile hormone III, hydroprene and a juvenogen as soldier caste differentiation regulators in three *Reticulitermes* species: potential of juvenile hormone analogues in termite control. *Pest Manag. Sci: formerly Pestic. Sci*. 62, 848-854.

Howard, R. W. and Haverty, M. I. (1978). Defaunation, mortality and soldier differentiation: concentration effects of methoprene in a termite. *Sociobiology*. 3, 73-77.

Iida, T., Ohkuma, M., Ohtoko, K. and Kudo, T. (2000). Symbiotic spirochetes in the termite hindgut: phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. *FEMS Microbiol. Ecol*. 34, 17-26.

Inoue, T., Kitade, O., Yoshimura, T. and Yamaoka, I. (2000). Symbiotic associations with protists. In *Termites: evolution, sociality, symbioses, ecology* (ed. T. Abe, D.E. Bignell and M. Higashi). pp. 275-288. Springer, Dordrecht.

Inoue, T., Murashima, K., Azuma, J. I., Sugimoto, A. and Slaytor, M. (1997). Cellulose and xylan utilisation in the lower termite *Reticulitermes speratus*. *J. Insect Physiol*. 43, 235-242.

Krishnan, S., Alden, N. and Lee, K. (2015). Pathways and functions of gut microbiota metabolism impacting host physiology. *Curr. Opin. Biotechnol*. 36, 137-145.

Lainé, L. V. and Wright, D. J. (2003). The life cycle of *Reticulitermes* spp.(Isoptera: Rhinotermitidae): what do we know?. *Bull. Entomol. Res*. 93, 267.

Leadbetter, J. R., Schmidt, T. M., Graber, J. R. and Breznak, J. A. (1999). Acetogenesis from H2 plus CO2 by spirochetes from termite guts. *Science*. 283, 686-689.

Lee, J.B., Park, K.E., Lee, S.A., Jang, S.H., Eo, H.J., Am Jang, H., Kim, C.H., Ohbayashi, T., Matsuura, Y., Kikuchi, Y. et al. (2017). Gut symbiotic bacteria stimulate insect growth and egg production by modulating hexamerin and vitellogenin gene expression. *Dev. Comp. Immunol.* 69,12-22.

Lee, J., Kim, C.H., Am Jang, H., Kim, J.K., Kotaki, T., Shinoda, T., Shinada, T., Yoo, J.W. and Lee, B.L. (2019). Burkholderia gut symbiont modulates titer of specific juvenile hormone in the bean bug Riptortus pedestris. *Dev. Comp. Immunol.* 99,103399.

Lozupone, C. A., Hamady, M., Kelley, S. T. and Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* 73, 1576-1585.

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J. and Knight, R. (2011). UniFrac: an effective distance metric for microbial community comparison. *The ISME journal*. 5, 169-172.

Lucey, K. S. and Leadbetter, J. R. (2014). Catechol 2, 3-dioxygenase and other meta-cleavage catabolic pathway genes in the 'anaerobic'termite gut spirochete *Treponema primitia*. *Mol. Ecol*. 23, 1531-1543.

Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R. and Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb. Ecol. Health Dis.* 26, 27663.

Mao, L., Henderson, G., Liu, Y. and Laine, R. A. (2005). Formosan subterranean termite (Isoptera: Rhinotermitidae) soldiers regulate juvenile hormone levels and caste differentiation in workers. *Ann. Entomol. Soc. Am.* 98, 340-345.

McMurdie, P. J. and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *Plos one*. 8, e61217.

Nalepa, C. A. (2017). What kills the hindgut flagellates of lower termites during the host molting cycle?. *Microorganisms*. 5, 82.

Nijhout, H. F., Riddiford, L. M., Mirth, C., Shingleton, A. W., Suzuki, Y. and Callier, V. (2014). The developmental control of size in insects. *Wiley Interdisciplinary Reviews: Dev. Biol*. 3, 113-134.

Noda, S., Kitade, O., Inoue, T., Kawai, M., Kanuka, M., Hiroshima, K., Hongoh, Y., Constantino, R., Uys, V., Zhong, J. et al. (2007). Cospeciation in the triplex symbiosis of termite gut protists (Pseudotrichonympha spp.), their hosts, and their bacterial endosymbionts. *Mol. Ecol*. 16, 1257-1266.

Noda, S., Iida, T., Kitade, O., Nakajima, H., Kudo, T. and Ohkuma, M. (2005). Endosymbiotic Bacteroidales bacteria of the flagellated protist *Pseudotrichonympha grassii* in the gut of the termite Coptotermes formosanus. *Appl. Environ. Microbiol*. 71, 8811-8817.

Ohkuma, M., Sato, T., Noda, S., Ui, S., Kudo, T. and Hongoh, Y. (2007). The candidate phylum 'Termite Group 1'of bacteria: phylogenetic diversity, distribution, and endosymbiont members of various gut flagellated protists. *FEMS Microbiol.Ecol*. 60, 467-476.

Ohkuma, M. (2008). Symbioses of flagellates and prokaryotes in the gut of lower termites. *Trends Microbiol*. 16, 345-352.

Otani, S., Mikaelyan, A., Nobre, T., Hansen, L.H., Koné, N.G.A., Sørensen, S.J., Aanen, D.K., Boomsma, J.J., Brune, A. and Poulsen, M. (2014). Identifying the core microbial community in the gut of fungus-growing termites. *Mol. Ecol*. 23, 4631-4644.

Park, Y. I. and Raina, A. K. (2004). Juvenile hormone III titers and regulation of soldier caste in *Coptotermes formosanus* (Isoptera: Rhinotermitidae). *J. Insect Physiol*. 50, 561-566.

Peterson, B. F., Stewart, H. L. and Scharf, M. E. (2015). Quantification of symbiotic contributions to lower termite lignocellulose digestion using antimicrobial treatments. *Insect Biochem. Mol. Biol*. 59, 80-88.

Peterson, B. F. and Scharf, M. E. (2016a). Lower termite associations with microbes: synergy, protection, and interplay. *Frontiers Microbiol*. 7, 422.

Peterson, B. F. and Scharf, M. E. (2016b). Metatranscriptome analysis reveals bacterial symbiont contributions to lower termite physiology and potential immune functions. *BMC Genom*. 17, 772.

Poutahidis, T., Kearney, S.M., Levkovich, T., Qi, P., Varian, B.J., Lakritz, J.R., Ibrahim, Y.M., Chatzigiagkos, A., Alm, E.J. and Erdman, S.E. (2013). Microbial symbionts accelerate wound healing via the neuropeptide hormone oxytocin. *Plos one.* 8, e78898.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590-D596.

Roisin, Y. (2000). Diversity and evolution of caste patterns. In *Termites: evolution, sociality, symbioses, ecology* (ed. T. Abe, D. E. Bignell and M. Higashi), pp. 95-119. Springer, Dordrecht.

Rosengaus, R. B., Zecher, C. N., Schultheis, K. F., Brucker, R. M. and Bordenstein, S. R. (2011). Disruption of the termite gut microbiota and its prolonged consequences for fitness. *Appl. Environ. Microbiol*. 77, 4303-4312.

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Scharf, M. E., Karl, Z. J., Sethi, A. and Boucias, D. G. (2011). Multiple levels of synergistic collaboration in termite lignocellulose digestion. *Plos one*. 6, e21709.

Scharf, M. E., Ratliff, C. R., Wu-Scharf, D., Zhou, X., Pittendrigh, B. R. and Bennett, G. W. (2005). Effects of juvenile hormone III on *Reticulitermes flavipes*: changes in hemolymph protein composition and gene expression. *Insect Biochem. Mol. Biol*. 35, 207-215.

Scharf, M. E. and Tartar, A. (2008). Termite digestomes as sources for novel lignocellulases. *Biofuel Bioprod. Biorefin.* 2, 540-552.

Scharf, M. E., Wu-Scharf, D., Pittendrigh, B. R. and Bennett, G. W. (2003). Caste-and development-associated gene expression in a lower termite. *Genome Biol*. 4, R62.

Scharf, M. E., Cai, Y., Sun, Y., Sen, R., Raychoudhury, R. and Boucias, D. G. (2017). A meta-analysis testing eusocial co-option theories in termite gut physiology and symbiosis. *Commun. Integr. Biol.* 10, e1295187.

Scharf, M. E. and Peterson, B. F. (2021). A century of synergy in termite symbiosis research: linking the past with new genomic insights. *Ann. Rev. Entomol.* 66, 23–43

Sen, R., Raychoudhury, R., Cai, Y., Sun, Y., Lietze, V. U., Boucias, D. G. and Scharf, M. E. (2013). Differential impacts of juvenile hormone, soldier head extract and alternate caste phenotypes on host and symbiont transcriptome composition in the gut of the termite *Reticulitermes flavipes*. *BMC Genom.* 14, 491.

Smykal, V., Daimon, T., Kayukawa, T., Takaki, K., Shinoda, T. and Jindra, M. (2014). Importance of juvenile hormone signaling arises with competence of insect larvae to metamorphose. *Dev. Biol*. 390, 221-230.

Stephens, M. E. and Gage, D. J. (2020). Single-cell amplicon sequencing reveals community structures and transmission trends of protist-associated bacteria in a termite host. *Plos one*. 15, e0233065.

Stingl, U., Radek, R., Yang, H. and Brune, A. (2005). “Endomicrobia”: cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. *Appl. Environ. Microbiol*. 71, 1473-1479.

Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J. and Leulier, F. (2011). *Lactobacillus plantarum* promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. *Cell Metab*. 14, 403-414.

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.N., Kubo, C. and Koga, Y. (2004). Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *J. physiol*. 558, 263-275.

Tarver, M. R., Schmelz, E. A., Rocca, J. R. and Scharf, M. E. (2009). Effects of soldier-derived terpenes on soldier caste differentiation in the termite *Reticulitermes flavipes*. *J. Chem. Ecol*. 35, 256-264.

Tarver, M. R., Florane, C. B., Zhang, D., Grimm, C., Lax, A. R. and Traniello, J. (2012). Methoprene and temperature effects on caste differentiation and protein composition in the Formosan subterranean termite, *Coptotermes formosanus*. *J. Insect. Sci*. 12, 18

Ter Braak, C. J. (1986). Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology*. 67, 1167-1179.

Treitli, S. C., Kolisko, M., Husník, F., Keeling, P. J. and Hampl, V. (2019). Revealing the metabolic capacity of *Streblomastix strix* and its bacterial symbionts using single-cell metagenomics. *Proc. Natl. Acad. Sci.* U.S.A. 116, 19675-19684.

Wilson, E. O. (1971). The insect societies. Cambridge, MA: Harvard University Press

Yagi, K. J., Kwok, R., Chan, K. K., Setter, R. R., Myles, T. G., Tobe, S. S. and Stay, B. (2005). Phe-Gly-Leu-amide allatostatin in the termite *Reticulitermes flavipes*: content in brain and corpus allatum and effect on juvenile hormone synthesis. *J.Insect Physiol*. 51, 357-365.

Yagi, S. (1976). The role of juvenile hormone in diapause and phase variation in some lepidopterous insects. In *The Juvenile Hormones* (ed. L. I. Gilbert), pp. 288-300. Springer, Boston, MA.

Zheng, H., Powell, J. E., Steele, M. I., Dietrich, C. and Moran, N. A. (2017). Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. *Proc. Natl. Acad. Sci.* U.S.A. 114, 4775-4780.

Zhou, X., Oi, F. M. and Scharf, M. E. (2006a). Social exploitation of hexamerin: RNAi reveals a major caste-regulatory factor in termites. *Proc. Natl. Acad. Sci.* U.S.A. 103, 4499-4504.

Zhou, X., Song, C., Grzymala, T. L., Oi, F. M. and Scharf, M. E. (2006b). Juvenile hormone and colony conditions differentially influence cytochrome P450 gene expression in the termite *Reticulitermes flavipes*. *Insect Mol. Biol.* 15, 749-761.

Zhou, X., Tarver, M. R. and Scharf, M. E. (2007). Hexamerin-based regulation of juvenile hormone-dependent gene expression underlies phenotypic plasticity in a social insect. *Development*. 134, 601-610.

Zilber-Rosenberg, I. and Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol. Rev*. 32, 723-735.

CHAPTER 3. SEASONAL IMPACTS ON GUT MICROBIAL COMPOSITION OF THE EASTERN SUBTERRANEAN TERMITE (*RETICULITERMES FLAVIPES*)

Abstract

Termite hindguts are inhabited by numerous gut symbionts that help with digestion, acetogenesis, nitrogen fixation, caste differentiation, immunity and more. The relationship between a host and its gut microbiome has been reported to vary with season, and more specifically, temperature. Changes in gut environment due to season can potentially impact the physiology of termites including their gut. Therefore, we studied the impact of seasonal changes on the composition and relative abundances of bacteria in the termite gut. Termites from cardboard-baited stations were obtained monthly, from May-October 2020 at a location in the central U.S. that typically experiences seasonal temperatures ranging from <0 to >30 °C. The guts of ten termites were dissected and frozen within 1-day after collections. DNA was extracted from frozen gut tissues and used for bacterial 16S rRNA gene sequence surveys. On processing the sequences, we observed alpha diversity (observed features, Pielou evenness and Shannon diversity) and beta diversity (weighted Unifrac and Bray-Curtis) metrics to be significant across months. Similarly, the average number of protists per termite gut were significantly different among months. Based on analysis of composition of microbiomes (ANCOM) at ASV (amplicon sequence variant) level, the taxa *Candidatus*, *Ancillula*, *Caproiciproducens*, uncultured Segniliparaceae, uncultured CR-115, *Corynebacterium*-1 and *Fusobacterium* were differentially abundant among sampling months. Based on spearman correlation analysis, a total of 63 bacterial taxa were significantly correlated with average monthly soil temperature. Out of these taxa 34 were significantly positively and 29 significantly negatively correlated. These results indicate that termite gut bacterial communities go through changes in relative abundances over time, possibly in response to seasonal temperature changes, although other seasonal effects cannot be excluded. Further, these results are potentially relevant to developing a better understanding of termite ecology and control and emphasize a need for more research or a meta-analysis of seasonal variation of termite gut microbiomes in diverse ecological niches.

3.1 Introduction

Animal guts are inhabited by numerous symbiotic microorganisms and their physiology is highly influenced by their relationships with gut microbiome (Petersen and Osvatic 2018). An animal host provides a suitable cultivation medium for bacterial symbionts to grow while in return the host gets physiological benefits (Itoh et al. 2019). However, this kind of symbiosis in the gut needs "checks and balances" to maintain a balanced system, i.e., under healthy conditions, the colonization and growth of any bacteria in an animal gut is selectively balanced by other bacteria that are present at the same time (Itoh et al. 2019). For example, a balanced symbiosis of bacteria in human guts can influence lipid metabolism, the immune system and neuroendocrine pathways through the microbiome-gut-brain-axis pathway (Forsythe and Kunze 2013; Krishnan et al. 2015), and in mice a balanced symbiosis can enhance responses to stress (Sudo et al. 2004).

Similar to other animals, insect-gut microbe symbiosis enables a myriad of physiological benefits. Insect-microbe symbiosis can enhance host fitness by modulating host digestive, immune and reproductive physiology, fat storage and social behavior (Bäckhed et al. 2004, Peterson et al. 2015, Peterson and Scharf 2016a, Peterson and Scharf 2016b, Maurice and Erdei 2018, Scharf et al. 2011). Gut microbiome may also change with host physiology, indicating the importance of a holobiont concept in interpreting insect-gut symbiont interactions (Davenport et al. 2014, Hou et al. 2021). Insects clearly obtain physiological benefits from a balanced gut symbiosis, and therefore a dysbiosis could be lethal. For example, gut microbial dysbiosis due to aging related expansion of Gamma proteobacteria in *Drosophila* results in intestinal dysfunction and ultimately death (Clark et al. 2015). Being ectotherms, insects also go through a daily fluctuation of temperature while they are dwelling in their habitat, and as a result their gut symbionts also experience the fluctuation (Paaijmans et al. 2013). Therefore, balanced symbioses within insects are likely highly influenced by their exposures to environmental temperatures (Iltis et al. 2021).

Among insects, termites are well renowned for their symbiotic relationship with microorganisms that include bacteria, archaea, protists (Scharf and Tartar 2008). The association of termites with symbionts is important for digestion, acetogenesis, nitrogen fixation, and immune functions (Zhou et al. 2007, Scharf et al. 2011, Peterson and Scharf 2016a, Peterson and Scharf 2016b). Therefore, symbiosis of termites with their gut microbes is crucial for their survival. Since termites are ectotherms, they face changes in seasonal temperatures ranging from extreme heat to extreme cold depending on where they are located. Elevated heat has been reported to reduce

bacterial symbionts in other insect systems including ants, whiteflies, and stinkbugs (Fan and Wernegreen 2013, Shan et al. 2017, Kikuchi et al. 2016). Alternatively, the symbioses of stored grain beetles with gut microbiome have been related to enhanced desiccation resistance (Engl et al. 2018). In contrast, temporal stability of the gut microbiome in warm blooded animals like humans has been reported (Davenport et al. 2014). Selection of symbionts, either replacement or colonization, in response to environmental conditions helps insects to better adapt in changing environments by enabling greater thermal adaptability (Sudakaran et al. 2017, Corbin et al. 2017, Lemoine et al. 2020).

Pattern of change in host microbiomes in crickets have been found to be driven by host physiological changes instead of temperature; while pre-exposure to high temperature has been associated with reduced gut bacterial taxa in termites (Ferguson et al. 2018, Arango et al. 2021). A prominent shift in gut microbiomes of honeybees has been reported during winter (Kešnerová et al. 2020). Even the endophytic microbial community of Mulberry trees undergo seasonal variation (Ou et al. 2019). Based on these findings in other study systems, a change in composition of termite microbiomes with changes in seasonal temperatures is expected. However, it remains unknown how microbial compositions might specifically change in association with different temperatures. Therefore, this study focused on the impact of seasonal temperature shifts on microbiome composition in the Eastern subterranean termite, *Reticulitermes flavipes*, at a location in the central U.S. Indiana that typically experiences seasonal temperatures ranging from <0 to >30°C. Our central hypothesis was that change in season will select for gut bacteria that might be beneficial for termites to thrive under specific environmental conditions. The specific goals of this project were to study gut protist numbers and associated exoglucanase activity over time (significant exoglucanase activity is found in hindgut region of *R. flavipes* termites; Zhou et al. 2007), as well as bacterial alpha and beta diversity metrics and the relative abundances of bacterial taxa throughout the collection period.

3.2 Material and Methods

3.2.1 Termite collection

Termites were collected monthly from May through October 2020 on the Purdue University campus (40.4163° N, 86.9310° W) from an active trap using cardboard paper roll bait.

The second collection of every month was used for this experiment and was collected between 9-9:30 AM throughout the collection period. Termites were brought to the lab, cleaned and sorted away from soil and cardboard, and their guts were dissected the next day. Ten dissected whole guts were kept in 200 uL PBS for DNA extraction, and 20 termite hindguts in 300 uL PBS for enzyme assay respectively. The guts in PBS were stored at -20°C until used later for DNA isolation.

3.2.2 Protist cell counts

To count the protist abundance in hindguts, five termite guts were dissected, trimmed, and the hindguts placed in 500µL PBS. Hindguts were homogenized and ten uL of this suspension was transferred to Sedgewick Rafter Counting Cell (SPI Supplies; West Chester, PA, USA) where protist cells were counted using a phase contrast microscope under 20x magnification. There were five replications each for every month's collection.

3.2.3 Exoglucanase activity

To quantify exoglucanase activity, hindgut supernatants were obtained since a significant production occurs in the hindgut region, where symbionts are housed (Zhou et al. 2007, Tartar et al. 2009, Sethi et al. 2013). The protocol for the exoglucanase assay was modified from Wheeler et al. 2007. The four replicates of extracted termite hindguts were stored at -20°C until used for enzyme assays. For the preparation of enzyme extract, homogenized hindguts were centrifuged at 15,000 rpm at 4°C for 15 minutes. The resulting supernatant was used as the enzyme source. The concentration of protein was estimated using the Bradford assay with Bovine Serum albumin as standard.

The exoglucanase enzyme (Glycosyl Hydrolase Family 7 [GHF 7]) acts on glycosidic bonds between glucose residues in reduced cellulose to release cellobiose (a glucose disaccharide). A cellulose polymer is cleaved by endoglucanase to create reduced cellulose, which is later acted on by exoglucanase. The action of exoglucanases on glycosidic bonds can be measured using the model substrate p-nitrophenol cellobioside (pNPC) (Zhou et al. 2007, Deshpande et al. 1984). Therefore, we used pNPC as a substrate. The substrate stock in acetonitrile (0.1 M, pH 6.5) was diluted in sodium phosphate buffer to make the final substrate concentration of 4mM. To determine enzyme activity, 96-well microtiter plates (Corning Inc., Corning, NY) and a microplate

spectrophotometer was used. The assay was carried out by using 10 uL of enzyme extract in 90 uL buffer-substrate solution in each well of 96-well microtiter plate. The Kinetic assay was read for 2 hours at an interval of 2 minutes at 420nm wavelength at 30°C. Specific enzyme activity (extinction coefficient = 0.6605 mM/OD) was calculated from the mean velocity of pNPC-exoglucanase reactions. Exoglucanase activity was obtained from May through September samples, as the October sample was not enough to conduct enzyme activity assays.

3.2.4 16S rRNA gene sequencing

DNA extraction

Termite guts were dissected in 200 uL PBS and stored at -20°C until DNA extraction was performed. There were four replications for each month's collection and 10 termite guts were used per replication. DNA from all gut samples (n=24) was extracted using the DNeasy® Blood and Tissue Kit (Qiagen; Valencia, CA, USA). Quality of extracted DNA was assessed through gel electrophoresis and was quantified using a Nanodrop spectrophotometer (Thermo-Fisher; Waltham, MA, USA). Twenty uL of the genomic DNA was sent to University of Minnesota Genomics Center, MN, USA for amplicon sequencing.

Bacterial 16S rRNA gene sequencing

For amplicon sequencing, we used the V3-V4 region of bacterial 16S rRNA gene. The bacterial universal primers used for PCR were 341F- CCTACGGGAGGCAGCAG and 806R-GGACTACHVGGGTWTCTAAT (Chapter 2 and Chapter 4). A two-step PCR was used for preparation of samples before Illumina sequencing. Amplification and "bar-code" indexing were done during the sample preparation. Then indexed PCR products were normalized to 10 ng/µl and 10 µl of each sample was pooled and concentrated to ~100 µl. The products were cleaned using SPRI (solid reverse phase immobilization) purification and quantification of cleaned libraries was done using Qubit and fragment analysis using Agilent Tapestation. Cleaned libraries were diluted to 2 nM. The pooled samples were then denatured with NaOH and further diluted to 8 pM in Illumina HT1 buffer to spike with 15% PhiX. The products were heat denatured at 96°C for 2 minutes before loading. A MiSeq 600 (1/8th lane Stowaway) cycle v3 kit was used to sequence the samples at the University of Minnesota Genomics Center, MN, USA.

3.2.5 Sequence processing and Community analysis

The Qiime2 pipeline was used to process and analyze paired end (301 bp) Illumina reads (Bolyen et al., 2019). The Divisive Amplicon Denoising Algorithm 2 (DADA2) was used for filtering, trimming, denoising, dereplicating, merging and removing chimera to obtain high-quality reads to process for further analysis (Callahan et al., 2016). During the DADA2 step, 2,014,585 demultiplexed sequences were processed to obtain 1,025,514 high quality reads. The moving picture tutorial was followed to calculate and analyze the diversity metrics. The diversity metrics were calculated using the rarified sequences from feature table at a subsampling depth of 13,000. The metrics calculated included: Observed features, Shannon diversity, Pielou evenness, and Faith phylogenetic diversity for alpha diversity and phylogenetic Unifrac distances- weighted and unweighted (Lozupone et al. 2011), and non-phylogenetic distances- Jaccard and Bray Curtis for beta diversity study. The reference database SILVA (version 132) was used to train the classifier bounded by V3/V4 region of 16S rRNA gene. Taxonomy assignments were made using the Naïve Bayes trained Silva 132 99% OTU classifier as a reference read (Quast et al. 2013). Bar graphs representing the relative abundances of ASVs at the phylum and family levels were generated using the R package phyloseq from QIIME2 generated phylotype tables and taxonomy files (McMurdie and Holmes 2013).

3.3 Statistical analysis

Statistical analyses were performed in the QIIME2 interface and R (Bolyen et al., 2019, R Core Team, 2020). Non-parametric Kruskal-Wallis tests were used to test the statistical significance of protist counts, enzyme activity and alpha diversity metrics. For pairwise comparisons of significant Kruskal-Wallis models, Mann-Whitney U-tests were used with “bh” (Benjamini-Hochberg) false-discovery-rate corrections. For statistical analysis of beta diversities, permutational multivariant analysis of variance (PERMANOVA) and PERMDISP tests of homogeneity of dispersions were used to test for statistical significance among and between groups. ANCOM was used to identify differentially abundant taxa among sampling months. The analysis of composition of microbiomes (ANCOM) was used to identify the significantly differentially abundant bacterial taxa (at genus and phylum levels) across months (Mandal et al. 2015). Lastly, I performed Spearman correlation analysis using relative abundances of ASVs obtained from

Qiime2 and average monthly soil temperature across sample collection months. The package "Hmisc" in R was used to determine the significance level and results were visualized using the package "corrplot".

3.4 Results

3.4.1 General weather trends

The soil temperature data from May through October in 2020 was acquired from Purdue Beck Center/ACRE farm https://ag.purdue.edu/indiana-state-climate/purdue-mesonet/purdue-mesonet-data-hub/. The fluctuation in daily soil temperature is provided in Fig. 3.1, while monthly average soil temperature ranged from 11.04°C (October) to 24.4°C (July) during termite collection period (Table C. S1). The monthly average soil temperature obtained from Purdue Beck Center/ACRE farm was not statistically significant with the temperature I recorded on the day of termite collection. Therefore, I used monthly average soil temperature for Spearman correlation analysis with bacterial taxa in order to identify the taxa that are correlated with average soil temperature.

3.4.2 Protist numbers and protist exoglucanase activity

The average numbers of protist in termite hindguts (Kruskal-Wallis chi-squared = 17.835, p-value = 0.001) and protist exoglucanase activity (Kruskal-Wallis chi-squared = 12.129, p-value = 0.016) were significantly different among sampling months (Fig. 3.2A and 3.2B). However, none of the pairwise comparisons of exoglucanase activity were significantly different from one another (Fig. 3.2B). In contrast, the number of protists during May were significantly different from other sampling months, but the average number of protists decreased during June and were not different from July, August, and September samples. Similarly, the average number of protists during September were not different from June and July samples (Fig. 3.2A).

3.4.3 Bacterial alpha diversities from 16S analyses

The quality sequences obtained from the DADA2 algorithm were rarified at a subsampling depth of 13,000 to generate diversity metrics. For estimating alpha diversity, we used the metrics

observed features, Shannon diversity, Faith phylogenetic diversity (pd) and Pielou evenness (Fig. 3.3). The observed features (H = 12.488; p = 0.028), Shannon diversity (H = 14.95; p = 0.010), and Pielou evenness (H = 14.17; p = 0.014) were significantly different, while Faith pd (H = 10.77; p = 0.056) was not significantly different among sampling months based on Kruskal-Wallis tests. In pairwise comparisons of Shannon diversity, September was significantly different from other months while October was significantly different from July, August and September samples, and had lowest Shannon index. Similarly, the lowest observed features and Pielou evenness were seen during October (Fig. 3.3 A-D). In contrast, there was increase in faith-pd from June through October.

3.4.4 Bacterial beta diversities from 16S rRNA gene sequence analyses

The beta diversity metrics we used included phylogenetic and non-phylogenetic distances. Phylogenetic distances were weighted and unweighted Unifrac, and non- phylogenetic distances were Bray Curtis and Jaccard. The weighted Unifrac (pseudo-F= 4.41, p = 0.001) and unweighted Unifrac (pseudo-F= 2.00, p = 0.001) were significantly different among months. The pairwise differences between months were not significant. With weighted Unifrac however, October samples were clustered away from other months in an ordination plot (Fig. 3.4), which means microbial diversity in the October sample was different than other samples (p <0.1). In addition, based on PERMDISP test of homogeneity weighted (PERMDISP test, F= 1.70, p = 0.075) and unweighted Unifrac (PERMDISP test, F= 0.699, p = 0.439) were not significant, which means the variation of samples within individual months were not significant. Similar significant overall model results were observed through Jaccard (Supplementary Fig. C. S1), with all the pairwise comparisons statistically significant among sampling months.

3.4.5 Bacterial taxonomic composition

For visualization of taxonomic composition at the phylum level, we selected the phyla with relative abundances greater than 0.1%. From the relative abundance plot, we found that phyla Proteobacteria, Elusimicrobia, Bacteroidetes and Actinobacteria dominated the samples from May through September; however, the phylum Fusobacteria increased noticeably in relative abundance during October (Fig. 3.5A). Based on NCBI taxonomy, the phyla Firmicutes, Bacteroidetes,

Proteobacteria and Actinobacteria are now named Bacillota, Bacteroidota, Psudomonadota and Actinomycetota, respectively. Similarly, the taxonomic composition of bacteria at the family level was computed at relative abundance greater than 1%. The relative abundance bar plot demonstrates that termite gut bacteria are more similar in the May through September samples (Fig. 3.5B). While during October, changes (increase) in taxonomic compositions can be observed at both phylum and family levels.

To identify bacterial taxa that significantly differed among sampling months, we performed Analysis of Composition of Microbiomes (ANCOM). Based on genus-level ANCOM results, the taxa *Candidatus Ancillula*, *Corynebacterium* 1, uncultured Segniliparaceae from phylum Actinobacteria, uncultured CR-115 from phylum Bacteroidetes, *Caproiciproducens* from phylum Firmicutes and *Fusobacterium* from phylum Fusobacteria were differentially abundant among sampling months. The log abundance of taxa *Corynebacterium* 1, uncultured CR-115 and *Fusobacteria* increased significantly with a decrease in seasonal temperature while other three phyla were reduced significantly during October. The change in log abundance of these taxa across the sampling months can be observed from graph (Fig. 3.6).

3.4.6 Temperature correlations

We next wanted to determine if monthly average soil temperature was associated with different bacterial taxa abundances observed across the sampling period. Spearman correlation tests were performed for the months of May-October to study the correlation between temperature and relative abundances of bacterial taxa. 63 bacterial taxa were significantly correlated with the temperature, among significant taxa, 34 were positively, and 29 negatively correlated (Table C. S2). The Spearman correlation coefficient (r) ranged from +0.885 to -0.680 (Table C. S2), suggesting that bacterial taxa were strongly both positively and negatively correlated with the temperature. The taxa with Spearman correlation coefficient, $r > 0.6$ are presented in Fig. 3.7. The relative abundances of taxa from *Candidatus Vestibaculum*, uncultured Ruminococcaceae bacterium, *Candidatus Ancillula, Nocardioides, Bradyrhizobium* and uncultured Coriobacteriales bacterium, significantly increased with temperature ($r > 0.65$). While the relative abundance of *Clostridioides, Blautia, Sebaldella* and unidentified Alphaproteobacteria, significantly decreased with increases in temperature ($r < -0.65$).

3.5 Discussion

This study investigated changes in termite gut microbial composition in response to seasonal changes in the state of Indiana, located in the central USA. Our study objectives included collecting termite samples monthly during the termite active season (May through October), along with 16S rRNA gene sequencing, protist counts and protist exoglucanase activity assays. The bacterial 16S rRNA gene sequencing enabled computation of diversity metrics, relative abundances of bacterial taxa, which were then compared in a correlation study of taxa with soil temperature. Our findings provide a novel example of seasonal variation in the abundance of bacterial taxa within a termite host. Specifically, we found that the autumn seasonal temperature shift correlates with a change in the gut microbiome composition of *R. flavipes*, possibly as a mechanism to counter the physiological challenges brought about by the decrease in temperature that signals the coming of winter. In addition, we found several taxa that were significantly correlated with temperature changes across the season, which supports our hypothesis that seasonal changes in temperature select for a composition of bacterial species that might be crucial during specific conditions.

Insects are ectotherms therefore their metabolic activities and energy usage decrease during the cold season (Sinclair 2015), but termite survivorship is higher with exposure to decreased thermophotoperiod, possibly due to reduced metabolic activities brought about by acclimation to lower temperatures (Cabrera and Kamble 2001). As evidence of reduced metabolic activity, protist numbers and protist exoglucanase activity were reduced in September samples during the onset of cooler temperatures. Based on 16S rRNA gene sequence data, observed features, Shannon diversity and Pielou evenness metrics, October sampling demonstrated lowest averages for these metrics in comparison to other sampling months. The trends for the above three metrics peaked in September followed by an abrupt reduction in October, which was right before termites disappeared from the trap site and apparently retreated underground. We hypothesize that a sharp increase in alpha diversities during September could be to prepare termites for overwintering and possibly to accumulate energy sources needed during overwintering period. *R. flavipes* are renowned for retreating into the soil before the onset of freezing temperatures to avoid freezing and related injury during the winter season (Cabrera and Kamble 2001, Clarke et al. 2013). In addition, the October samples were higher in average Faith phylogenetic diversity in comparison to hotter months, which agrees strongly with the relative abundance of bacterial taxa during

October sampling as noticeable change in bacterial taxa were observed via relative abundance bar plots. The significant results obtained from Jaccard and weighted Unifrac analyses further indicate that different months had differences in microbial communities at some level. However, statistically significant pairwise comparisons from Jaccard and statistically not significant pairwise comparisons from weighted Unifrac indicate that the taxa were phylogenetically closely related between months even though qualitative measure of community membership was significant. Overall, from alpha and beta diversity metrics, differences in richness, evenness and diversity of bacterial taxa were observed over time.

In terms of phylum-level relative abundance of bacterial taxa, there was only slight variation in relative abundance from May through September. However, a distinct shift of bacterial phyla along with appearance of a new phylum (Fusobacteria) was observed during October. A similar trend was followed at the family level, where the October sample demonstrated a distinct shift, which could be attributed to the sharp decline in soil temperature occurring at that time. Higher relative abundance of the phylum Bacteroidetes also was observed in October, while Actinobacteria decreased over time with lowest relative abundance occurring during October. This agrees with a prior study showing that consumption of complex carbohydrates correlates with higher abundance of Bacteroidetes and lower Actinobacteria (Davenport et al. 2014). In our case, termites might be getting to feed on more complex carbohydrates during late September to early October causing the observed increase in Bacteroidetes and decrease in Actinobacteria. The change in diet could be due to the fallen fall foliage during this month. We further performed ANCOM to identify significantly different taxa among sampling months. ANCOM results demonstrate that the taxa *Candidatus Ancillula*, *Corynebacterium* 1, uncultured Segniliparaceae, uncultured CR-115, *Caproiciproducens* and *Fusobacterium* were significantly different among sampling months. The abundance of uncultured Segniliparaceae and *Caproiciproducens* decreased through our study, with lowest abundance during October, while *Candidatus Ancillula* remained similar through time except for a sharp reduction in abundance during October. In contrast, the taxa uncultured CR-115 and *Fusobacterium* increased significantly in October. The abundance of taxa *Corynebacterium* 1 peaked during May, decreased through August and started to climb up with another similar peak during October. Based on these changes observed over time, we decided to look for correlation of taxa with soil temperatures.

From Spearman correlation analysis, we found several taxa were positively and negatively correlated with the average soil temperature. Positively correlated taxa increased with increase in temperature while negatively correlated taxa decreased with increase in temperature. Among the taxa significant from ANCOM analysis, *Fusobacterium* (Fusobacteria), uncultured bacteria from CR 115 (Bacteroidetes), uncultured Segniliparaceae (Actinobacteria) and *Corynebacterium* 1 (Actinobacteria) were negatively correlated with temperature, while the taxa *Candidatus Ancillula* (Actinobacteria) and *Caproiciproducens* (Firmicutes) were positively correlated with temperature. These results agree with the ANCOM results indicating increases or decreases of taxa abundance in specific months. Bacteria from the orders gamma (*Pragia*) and delta Proteobacteria (uncultured bacterium from Rs K70 termite group), Clostridiales (uncultured Clostridiales bacterium, *Clostridioides*, *Blautia*) and Fusobacteria (*Fusobacterium*, *Sebaldella*) were significantly negatively correlated with temperature in this study. The above findings seem particularly significant because these orders are typically reported as psychrophilic bacteria (Zhao et al. 2004), meaning they grow optimally at temperatures of 15°C or lower, with cessation of growth at 20°C and above (Moyer and Morita 2007, Bowman et al. 1997). Low winter temperature has been reported to select for psychrophilic bacteria by killing the bacteria that cannot thrive well during cold temperatures (Ferguson et al. 2018). These findings support the idea that environmental temperature influences the relative abundances of microbial species in an insect gut.

Gut microbial shifts in response to seasonal variation have also been reported in Giant Panda, North American wood frog, mosquitoes and wild mouse (Douglas et al. 2021, Maurice et al. 2015, Novakova et al. 2017, Wu et al. 2017). Even grave soil (with decaying carcasses) was higher in microbial content during summer than winter, suggesting the inevitable impact of seasonal temperature on soil microbiomes (Carter et al. 2015). An important mosquito gut bacterium, *Wolbachia*, was found to be negatively correlated with temperature (Novakova et al. 2017). Also, as similar to our results, changes in seasonal temperatures were correlated with the abundances of gut bacteria in crickets and mosquitoes (Ferguson et al. 2018, Novakova et al. 2017). Such a shift in abundance and composition of insect gut microbiomes in response to seasonal changes might be linked to cold tolerance physiology and antioxidant activity (Hou et al. 2021). In a lab experiment, termites were reported to retreat to warmer soil with decreases in temperature (Hu and Song 2014). Similarly, termites disappeared from our collection site after October sampling, likely in search of warmer or more optimal conditions. A change in gut microbiome in

response to seasonal temperature could be a type of adaptive phenotypic plasticity a host undergoes to thrive in changing temperature conditions. However, it is important to understand that a core microbiome might be providing essential physiological benefits to a termite, with the fluctuating portion of the microbiome helping with adaptation to changing conditions. Since functional redundancy is common in microbial ecology, it is also important to understand the functional consequences of microbial shifts over changing seasonal temperatures. In addition, understanding the functional role of insect gut taxa that are positively or negatively correlated with temperature could help us to better understand termite overwintering physiology from a microbial perspective.

3.6 Figures

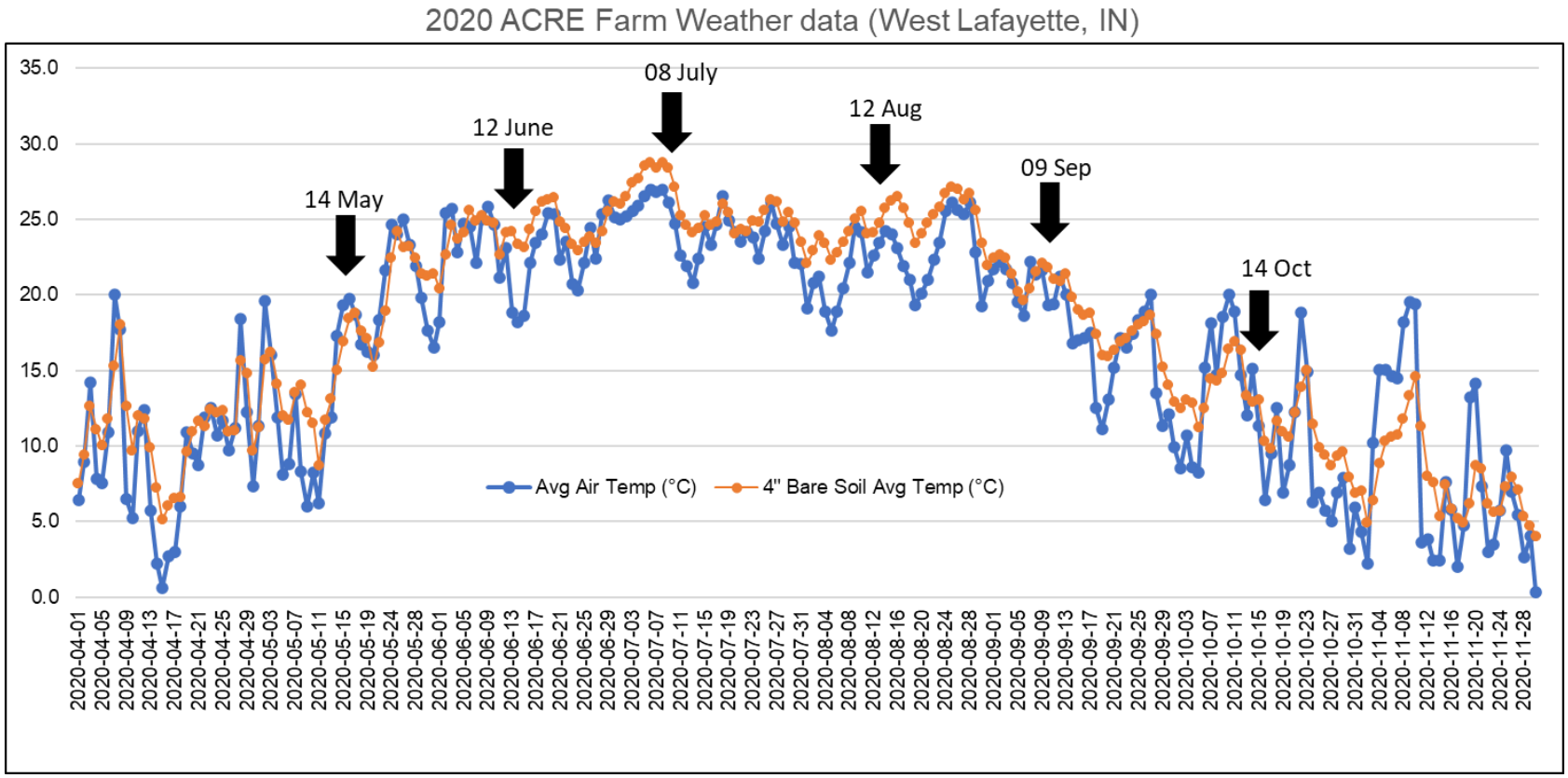


Fig. 3. 1. Daily average soil temperature from April to November 2020. Lines represent the average daily air and soil temperature (at 4" depth) recorded by Purdue Beck Center/ACRE farm, West Lafayette, Indiana. Note: The monthly average of the soil temperature from this data was used for Spearman correlation analysis.

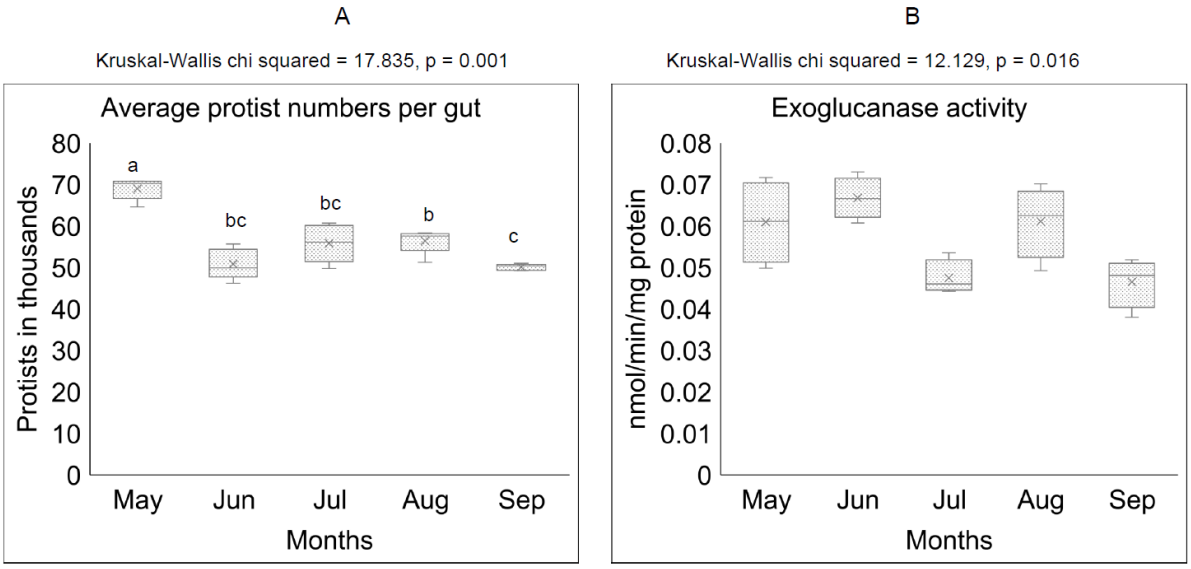


Fig. 3.2. The average number of protists per termite gut (A), exoglucanse activity measured using pNPC as a substrate (B). The overall models were significant based on Global Kruskal-Wallis chi square test. Significance levels for pairwise comparisons are indicated by lowercase letters, if not indicated, pairwise comparisons are not significant at $p<0.05$. Months with same letters are not significantly different ($p<0.05$).

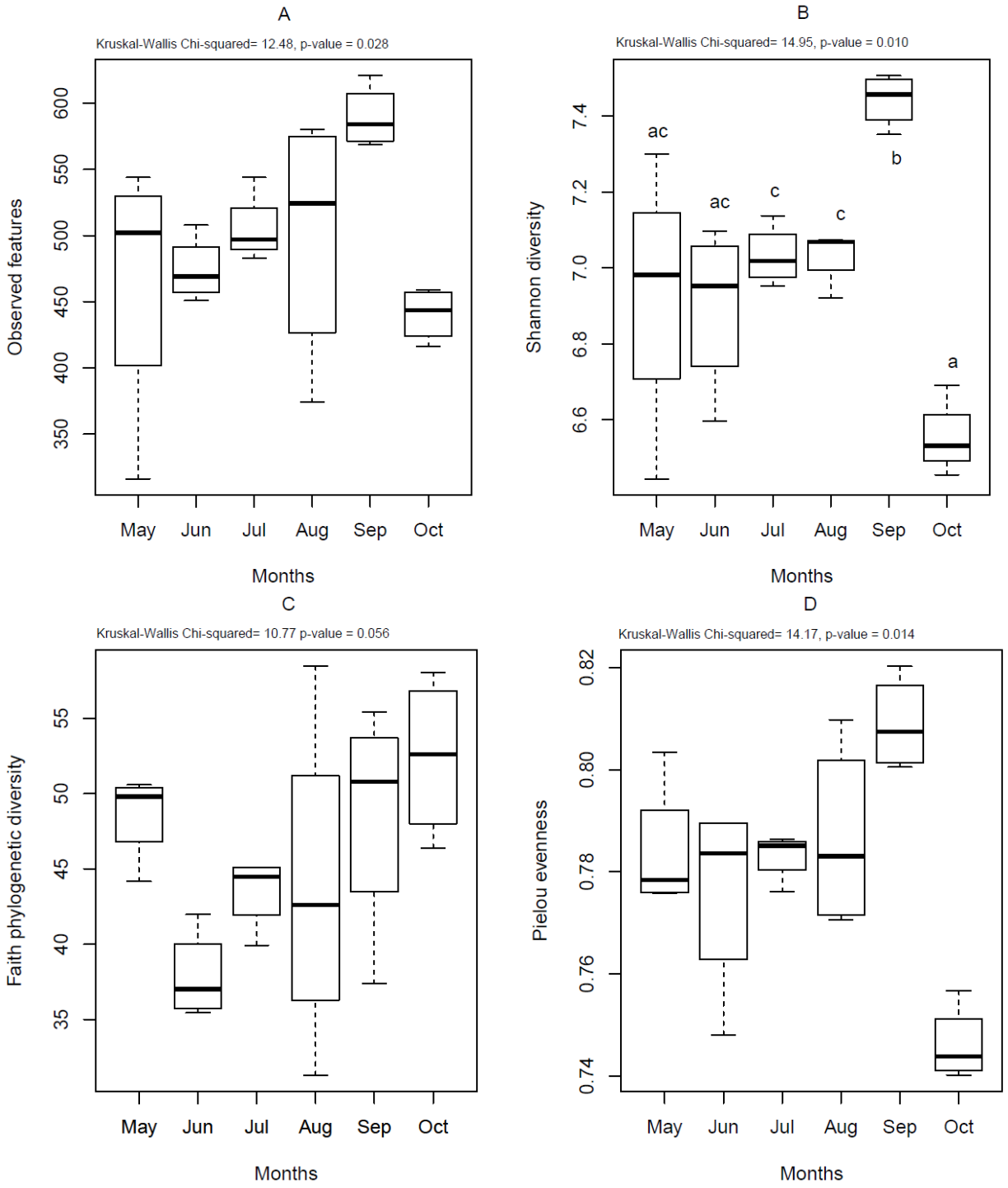


Fig. 3. 3. Alpha diversity metrics from 16S surveys showing: Observed features (A), Shannon diversity (B), Faith phylogenetic diversity (C) and Pielou evenness (D). P-values for global Kruskal-Wallis comparisons across months are shown at the top of each graph. Significance levels for pairwise comparisons are indicated by lowercase letters, if not indicated, pairwise comparisons are not significant at $p<0.05$. Months within graphs with the same letters are not significantly different ($p<0.05$).

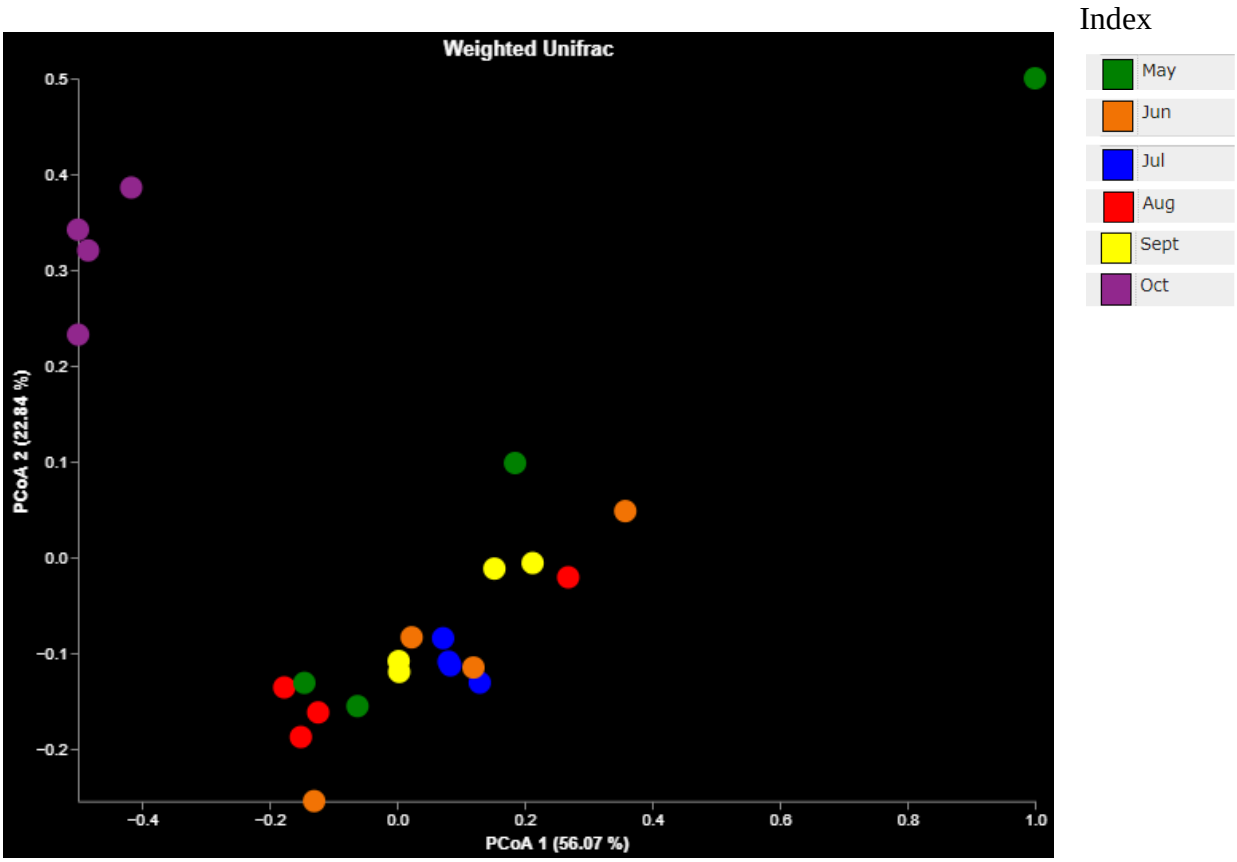


Fig. 3. 4. Principal coordinate plots from Weighted Unifrac (pseudo-F= 4.41, p = 0.001). Colors represent months and individual points represent individual replicates. Based on pairwise PERMANOVA, samples are not significantly different between months (p<0.05). Samples from each month did not show significant variation within replicates (PERMDISP, F-value= 1.70, p = 0.075)

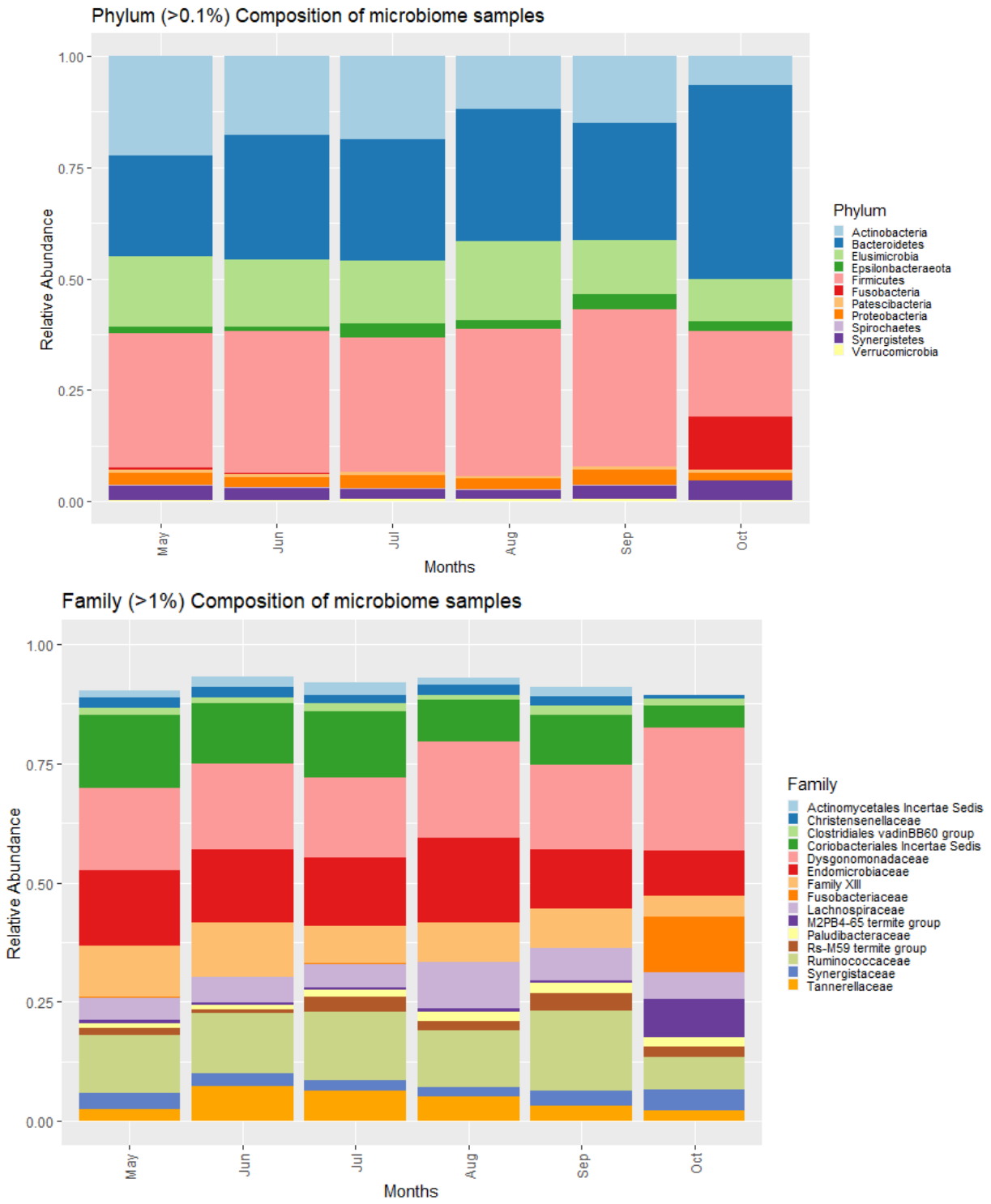


Fig. 3. 5. Relative abundance of bacteria (from abundance >0.001) at phylum level (A) and relative abundance of bacteria (from abundance >0.01) at family level (B). Data shown are the percentage of the total bacterial abundance for each month.

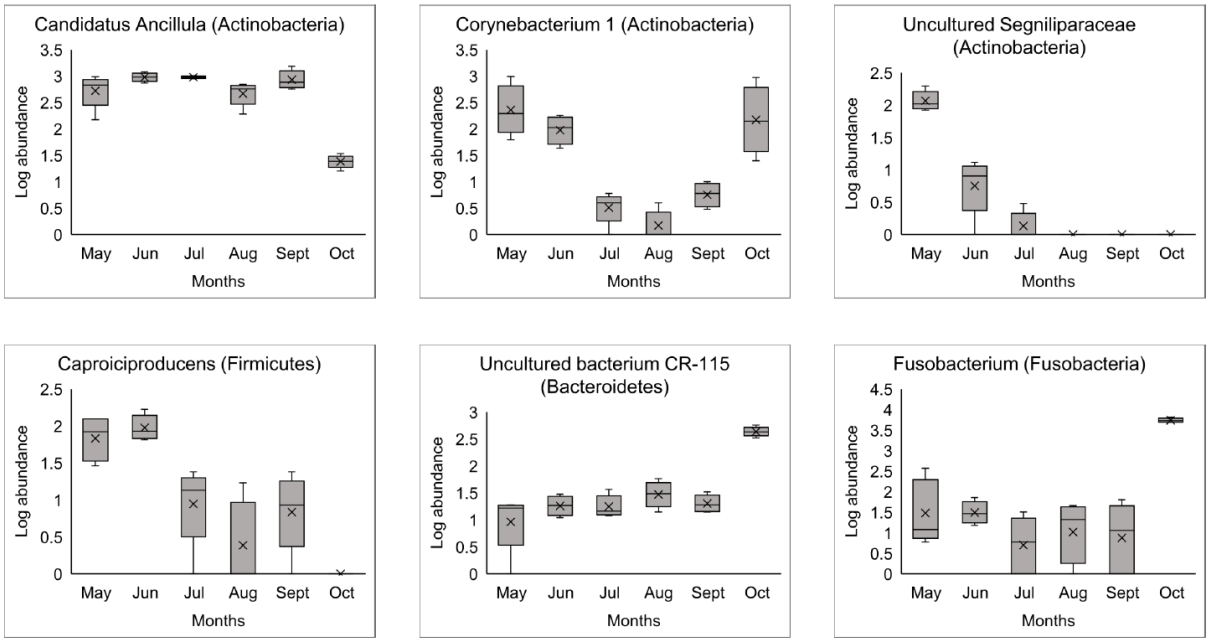


Fig. 3. 6. Boxplots of the log abundances of bacterial taxa (False Discovery Rate at 0.05). These taxa were significantly differentially abundant as measured by ANCOM among months.

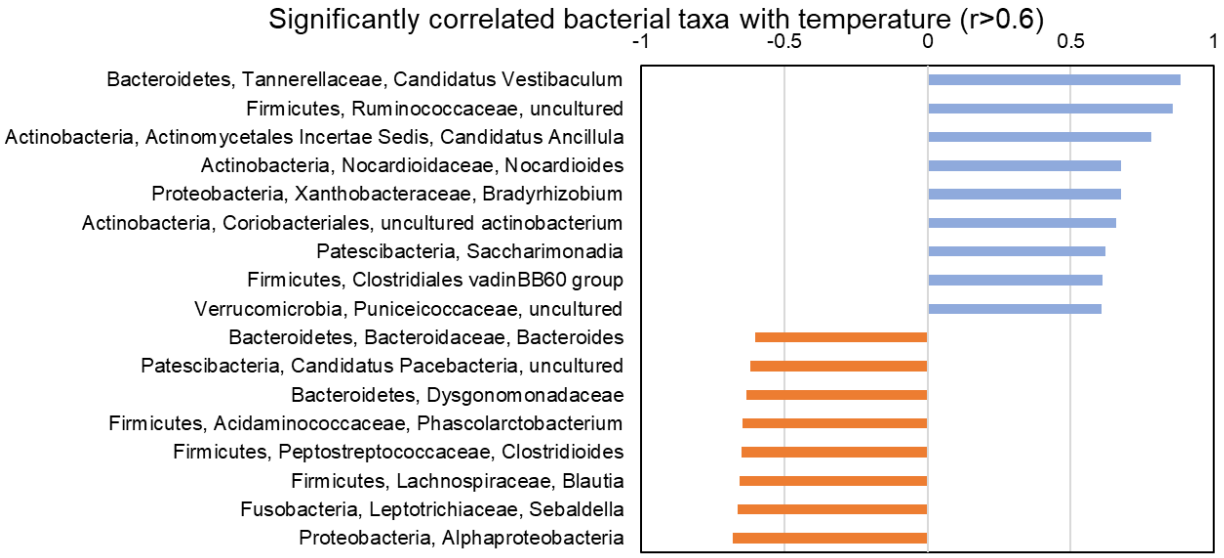


Fig. 3. 7. Significant taxa from Spearman correlation analysis with Spearman correlation coefficient (r) > 0.6. The taxa with positive values of 'r' are positively correlated while taxa with negative values of 'r' are negatively correlated with average soil temperature.

3.7 References

Arango, R. A., S. D. Schoville, C.R. Currie, and C. Carlos-Shanley. 2021. Experimental warming reduces survival, cold tolerance, and gut prokaryotic diversity of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar). Front. Microbiol. 2:1116.

Bäckhed, F., H. Ding, T. Wang, L.V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J. I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc. Natl. Acad. Sci. U.S.A. 101:15718-23.

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, F. Asnicar, and Y. Bai. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 37: 852–857.

Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin. 1997. Diversity and association of psychrophilic bacteria in Antarctic Sea ice. Appl. Environ. Microbiol. 63:3068-78.

Cabrera, B. J., and S. T. Kamble. 2001. Effects of decreasing thermophotoperiod on the eastern subterranean termite (Isoptera: Rhinotermitidae). Environ. Entomol. 30:166-71.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods. 13: 581–583.

Carter, D. O., J. L. Metcalf, A. Bibat, and R. Knight. 2015. Seasonal variation of postmortem microbial communities. Forensic Sci Med Pathol. 11:202-7.

Clark, R. I., A. Salazar, R. Yamada, S. Fitz-Gibbon, M. Morselli, J. Alcaraz, A. Rana, M. Rera, M. Pellegrini, W. J. William, and D. W. Walker. 2015. Distinct shifts in microbiota composition during *Drosophila* aging impair intestinal function and drive mortality. Cell Rep. 12:1656-67.

Clarke, M. W., G. J. Thompson, and B. J. Sinclair. 2013. Cold tolerance of the eastern subterranean termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae), in Ontario. Environ. Entomol. 42:805-10.

Corbin, C., E. R. Heyworth, J. Ferrari, and G. D. Hurst. 2017. Heritable symbionts in a world of varying temperature. Heredity. 118:10-20.

Davenport, E. R., O. Mizrahi-Man, K. Michelini, L. B. Barreiro, C. Ober, and Y. Gilad. 2014. Seasonal variation in human gut microbiome composition. PloS one. 9: e90731.

Deshpande, M. V., K. E. Eriksson, and L. G. Pettersson. 1984. An assay for selective determination of exo-1, 4,-β-glucanases in a mixture of cellulolytic enzymes. Anal. Biochem. 138: 481-487.

Douglas, A. J., L. A. Hug, and B. A. Katzenback. 2021. Composition of the North American wood frog (*Rana sylvatica*) bacterial skin microbiome and seasonal variation in community structure. Microb. Ecol. 81:78-92.

Engl, T., N. Eberl, C. Gorse, T. Krüger, T. H. Schmidt, R. Plarre, C. Adler, and M. Kaltenpoth. 2018. Ancient symbiosis confers desiccation resistance to stored grain pest beetles. Mol. Ecol. 27:2095-108.

Fan, Y., and J.J. Wernegreen. 2013. Can't take the heat: high temperature depletes bacterial endosymbionts of ants. Microb. Ecol. 66:727-33.

Ferguson, L. V., P. Dhakal, J. E. Lebenzon, D. E. Heinrichs, C. Bucking, and B. J. Sinclair. 2018. Seasonal shifts in the insect gut microbiome are concurrent with changes in cold tolerance and immunity. Funct. Ecol. 32:2357-68.

Forsythe, P., and W. A. Kunze. 2013. Voices from within: gut microbes and the CNS. Cell. Mol. Life Sci. 70, 55-69.

Hou, Z., Y. Dong, F. Shi, Y. Xu, S. Ge, J. Tao, L. Ren, and S. Zong. 2021. Seasonal Shifts in Cold Tolerance and the Composition of the Gut Microbiome of Dendroctonus valens LeConte Occur Concurrently. Forests. 12:888.

Hu, X. P., and D. Song. 2014. Behavioral responses of two subterranean termite species (Isoptera: Rhinotermitidae) to instant freezing or chilling temperatures. Environ. Entomol. 36:1450-6.

Iltis, C., K. Tougeron, T. Hance, P. Louâpre, and Foray V. 2021. A perspective on insect–microbe holobionts facing thermal fluctuations in a climate-change context. Environ. Microbiol. 24: 18-29.

Itoh, H., S. Jang, K. Takeshita, T. Ohbayashi, N. Ohnishi, X. Y. Meng, Y. Mitani, and Y. Kikuchi. 2019. Host–symbiont specificity determined by microbe–microbe competition in an insect gut. Proc. Natl. Acad. Sci. U.S.A. 116:22673-82.

Kešnerová, L., O. Emery, M. Troilo, J. Liberti, B. Erkosar, and P. Engel. 2020. Gut microbiota structure differs between honeybees in winter and summer. ISME J. 14:801-14.

Kikuchi, Y., A. Tada, D. L. Musolin, N. Hari, T. Hosokawa, K. Fujisaki, and T. Fukatsu. 2016. Collapse of insect gut symbiosis under simulated climate change. MBio. 7: e01578-16.

Krishnan, S., N. Alden, and K. Lee. 2015. Pathways and functions of gut microbiota metabolism impacting host physiology. Curr. Opin. Biotechnol. 36, 137-145.

Lemoine, M. M., T. Engl, and M. Kaltenpoth. 2020. Microbial symbionts expanding or constraining abiotic niche space in insects. Curr. Opin. Insect. Sci. 39:14-20.

Lozupone, C., M. E. Lladser, D. Knights, J. Stombaugh, and R. Knight. 2011. UniFrac: an effective distance metric for microbial community comparison. ISME J. 5, 169-172.

Mandal, S., W. Van Treuren, R. A. White, M. Eggesbø, R. Knight, and S. D. Peddada. 2015. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microb. Ecol. Health Dis. 26: 27663.

Maurice, C. F, S. C. Knowles, J. Ladau, K. S. Pollard, A. Fenton, A. B. Pedersen, and P. J. Turnbaugh. 2015. Marked seasonal variation in the wild mouse gut microbiota. ISME J. 9:2423-34.

Maurice, N., and L. Erdei. 2018. Termite Gut Microbiome, pp. 69–99. In Khan, Md.A., Ahmad, W. (eds.), Termit. Sustain. Manag. Vol. 1 - Biol. Soc. Behav. Econ. Importance, Sustainability in Plant and Crop Protection. Springer International Publishing, Cham.

McMurdie, P. J., and S. Holmes. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one. 8: e61217.

Moyer, C.L. and R.Y. Morita. 2007. Psychrophiles and psychrotrophs. eLS. 1(6).

Novakova, E., D. C. Woodhams, S. M. Rodríguez-Ruano, R. M. Brucker, J. W. Leff, A. Maharaj, A. Amir, R. Knight, and J. Scott. 2017. Mosquito microbiome dynamics, a background for prevalence and seasonality of West Nile virus. Front. Microbiol. 8:526.

Ou, T., W. F. Xu, F. Wang, G. Strobel, Z. Y. Zhou, Z. H Xiang, J. Liu, and J. Xie. 2019. A microbiome study reveals seasonal variation in endophytic bacteria among different mulberry cultivars. Comput. Struct. Biotechnol. J. 17:1091-100.

Paaijmans, K. P., R. L. Heinig, R. A. Seliga, J. I. Blanford, S. Blanford, C. C. Murdock, and M. B. Thomas. 2013. Temperature variation makes ectotherms more sensitive to climate change. Glob Chang Biol. 19:2373-80.

Peterson, B. F., and M. E. Scharf. 2016a. Lower Termite Associations with Microbes: Synergy, Protection, and Interplay. Front. Microbiol. 7: 422.

Peterson, B. F., and M. E. Scharf. 2016b. Metatranscriptome analysis reveals bacterial symbiont contributions to lower termite physiology and potential immune functions. BMC Genom. 17: 772.

Peterson, B. F., H. L. Stewart, and M. E. Scharf. 2015. Quantification of symbiotic contributions to lower termite lignocellulose digestion using antimicrobial treatments. Insect Biochem. Mol. Biol. 59: 80–88.

Petersen J. M., and J. Osvatic. 2018. Microbiomes in natura: importance of invertebrates in understanding the natural variety of animal-microbe interactions. Msystems. 3: e00179-17.

R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. Spec. Publ. 41, D590-D596.

Scharf, M. E., Z. J. Karl, A. Sethi, and D. G. Boucias. 2011. Multiple levels of synergistic collaboration in termite lignocellulose digestion. PloS one. 6: e21709.

Scharf, M. E., and A. Tartar. 2008. Termite digestomes as sources for novel lignocellulases. Biofuel Bioprod Biorefin. 2:540-52.

Sethi, A., E. S. Kovaleva, J. M. Slack, S. Brown, G. W. Buchman, and M. E. Scharf. 2013. A GHF7 cellulase from the protist symbiont community of *Reticulitermes flavipes* enables more efficient lignocellulose processing by host enzymes. Arch Insect Biochem Physiol. 84:175-93.

Shan, H. W., W. H. Deng, J. B. Luan, M. J. Zhang, Z. Zhang, S. S. Liu, and Y. Q Liu. 2017. Thermal sensitivity of bacteriocytes constrains the persistence of intracellular bacteria in whitefly symbiosis under heat stress. Environ. Microbiol. Rep. 9:706-16.

Sinclair, B. J. 2015. Linking energetics and overwintering in temperate insects. J. Therm. Biol. 54:5-11.

Sudakaran, S., C. Kost, and M. Kaltenpoth. 2017. Symbiont acquisition and replacement as a source of ecological innovation. Trends Microbiol. 25:375-90.

Sudo, N., Y. Chida, Y. Aiba, J. Sonoda, N. Oyama, X. N. Yu, C. Kubo, and Y. Koga. 2004. Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. J. physiol. 558, 263-275.

Tartar, A., M. M. Wheeler, X. Zhou, M. R. Coy, D. G. Boucias, and M. E. Scharf. 2009. Parallel metatranscriptome analyses of host and symbiont gene expression in the gut of the termite *Reticulitermes flavipes*. Biotechnol. Biofuels. 2: 1-9.

Wheeler, M. M., X. Zhou, M. E. Scharf, and F. M. Oi. 2007. Molecular and biochemical markers for monitoring dynamic shifts of cellulolytic protozoa in *Reticulitermes flavipes*. Insect Biochem. Mol. Biol. 37:1366-74.

Wu, Q., X. Wang, Y. Ding, Y. Hu, Y. Nie, W. Wei, S. Ma, L. Yan, L. Zhu, and F. Wei. 2017. Seasonal variation in nutrient utilization shapes gut microbiome structure and function in wild giant pandas. Proc. R. Soc. B: Biol. Sci. 284: 20170955.

Zhao, J. S., J. Spain, S. Thiboutot, G. Ampleman, C. Greer, and J. Hawari. 2004. Phylogeny of cyclic nitramine-degrading psychrophilic bacteria in marine sediment and their potential role in the natural attenuation of explosives. FEMS Microbiol. Ecol. 49:349-57.

Zhou, X., J. A. Smith, F. M. Oi, P. G. Koehler, G. W. Bennett, and M. E. Scharf. 2007. Correlation of cellulase gene expression and cellulolytic activity throughout the gut of the termite *Reticulitermes flavipes.* Gene. 395:29-39.

CHAPTER 4. INTER-COLONY COMPARISONS OF GUT MICROBIOME COMPOSITION FROM LAB REARED EASTERN SUBTERRANEAN TERMITES (BLATTODEA: RHINOTERMITIDAE)

This chapter has been published in Journal of Insect Science.
Sapkota, R., and M. E. Scharf. 2022. Inter-colony comparisons of gut microbiome composition from lab reared eastern subterranean termite (*Reticulitermes flavipes*). J. Insect Sci. 22:9.

Abstract

Termites are social insects living in colonies composed of worker, soldier, and reproductive castes. Termite hindguts are inhabited by all three domains of life- Eukarya (protists), Bacteria and Archaea. These gut microorganisms are horizontally and vertically transferred by nestmates and reproductives, respectively. Prior evidence suggests that every colony potentially has a different gut microbiome that was transferred vertically and horizontally over time. However, we do not know if different colonies reared in the laboratory on the same diet will ultimately demonstrate similar microbial composition and structure. Therefore, we looked at gut bacteria in Eastern subterranean termite (Reticulitermes flavipes) colonies that were reared in the laboratory with identical diets and rearing conditions. Based on16S rRNA gene sequencing, the observed features and Shannon's diversity were significantly different between the colonies while differences in Peilou evenness and Faith phylogenetic diversity were not statistically significant. In addition, the microbial community structures were significantly different between colonies. Based on ANCOM (Analysis of Composition of Microbiomes), the taxa Elizabethkingia (Bacteroidetes) and Chryseobacterium (Bacteroidetes) were differentially abundant between the colonies. These results suggest that providing the exact same diet and rearing environment for >2 years cannot result in identical gut microbiome between termite colonies.

Key words: Termite, Gut bacteria, Termite colonies, Lab rearing

4.1 Introduction

The lower termite gut possesses a unique symbiosis with different flagellates which themselves live in association with the prokaryotes (Ohkuma 2008, Scharf and Tartar 2008, Brune and Ohkuma 2010). Those symbionts demonstrate a mixed mode of transfer (horizontal and vertical) within termite colonies and most of which are unique and unculturable (Hongoh 2011,

Bourguignon et al. 2018). Termites can survive by feeding on dead plant materials by using the enzymes secreted by themselves and their microbial symbionts to digest complex lignocellulose (Peterson et al. 2015, Peterson and Scharf, 2016a, Peterson and Scharf, 2016b, Maurice and Erdei, 2018). Along with cellulose/hemicellulose degradation, termite gut microbes also contribute to termite physiology through nitrogen fixation, acetogenesis, anti-fungal defense, fecundity and fitness of reproductive castes, and caste differentiation (Inoue et al. 1997, Inoue et al.2000, Doolittle et al. 2008, Rosengaus et al. 2011, Peterson and Scharf 2016a, Peterson and Scharf 2016b, Sapkota et al. 2021).

Beginning in the past with symbiont identification and culturing efforts and progressing in the present to create microbial community databases, termite research on caste differentiation, digestion, pathogen defense and microbiomes has ramped up with new omics tools (Scharf 2015, Scharf and Peterson 2021). Specifically, next generation sequencing technologies have made culture-independent microbe identification, interaction and functional analyses much easier in present days. Key aspects of the association and influence of gut symbionts on termite holobiont physiology have been elaborated by using such techniques (Scharf 2020). The influence of gut microbiomes has been reported on host development and physiology including immunity, organ development and metabolism via inter-species homeostatic regulation between host and gut symbionts (Sommer and Bäckhed 2013). Along with this, bacterial symbionts have been reported to be dynamic over evolutionary time indicating their flexibility towards their host's changing physiology (Waidele et al. 2017). Physiological changes in a host termite could result from several other factors including temperature, hormones, life stages and diet (Ley et al. 2008, Scharf et al. 2017, Arango et al. 2021).

Change in diet has been reported to alter the abundance and diversity of eukaryotic and prokaryotic gut symbionts in *Drosophila* (Jiménez-Padilla et al. 2020). Beside insects, host diet has also been reported to influence the gut bacterial diversity in humans (from carnivory to omnivory to herbivory), and vertical transfer has been considered as a route of transfer of symbionts in mammals (Ley et al. 2008). Similarly, termite gut bacteria have been reported to change their community structure in response to dietary changes (Auer et al. 2017, Benjamino et al. 2018). However, a 7-day lab study on a variety of diets did not result in a significant change in termite gut bacterial species (Boucias et al. 2013). These differing results could be due to the length of restricted feeding, colony genetic differences, or rearing temperatures, e.g., high rearing

temperature has been reported to reduce gut bacterial abundance and diversity (Arango et al. 2021). Colonial differences in gut microbiomes have been reported in oriental fruit flies, ants and honeybees (Wang et al. 2011, Segers et al. 2019, Vernier et al. 2020). In addition, gut microbes in the termite are also believed to be, to a degree, colony specific (Matsuura 2001). Therefore, we planned an experiment to study the effect of identical diet and rearing temperature on the gut bacterial community from different colonies of termites with the following objectives: (1) to study the species richness, evenness and phylogenetic relatedness of gut bacterial populations from two colonies, and (2) to compare the similarity or differences in the gut bacterial composition between colonies.

4.2 Materials and methods

4.2.1 Termite collection and rearing

Termites were initially collected from two sites at the Purdue University (West Lafayette IN, USA) separated by approximately 200m: Whistler (Whslr) and Biochemistry (Biochem) colonies. The Biochem colony was collected once a week during the warm season in 2017 and entire collection was placed in one container in the laboratory. Similarly, the Whistler colony was collected in 2018 and placed in a separate container. The colonies were lab reared at 22°C and 24 hours of darkness and the gut DNA isolation was performed in 2020. Pine shims and brown paper towels were provided as food source and water was added on a need basis. Ten termite workers per replication were sampled for gut dissection and DNA extraction. There were four replications for each colony.

4.2.2 Sample preparation and DNA extraction

The whole gut from the termites from both colonies were extracted in 200 µL PBS (Phosphate Buffered Saline) and stored at -20°C until used later for DNA extraction. The DNeasy® Blood and Tissue Kit (Qiagen; Valencia, CA, USA) was used for DNA extraction with a slight modification in the protocol. The quantity of DNA in the aliquots were assessed using a Nanodrop spectrophotometer (Thermo-Fisher; Waltham, MA, USA), and the quality was assessed through the gel electrophoresis. Twenty µL of the extracted DNA was sent for 16S rRNA library preparation and sequencing and the rest was used to verify genetic relatedness of colonies. For this,

we PCR amplified the termite mitochondrial 16S rRNA gene using the primers Forward: 5′-TTACGCTGTTATCCCTAA-3′ and Reverse: 5′- CGCCTGTTTATCAAAAACAT-3′ (Austin et al. 2005, Boucias et al. 2013) amplifying ~ 428 bp region and sequenced them at the Purdue University Genomics core, IN, USA. The resulting sequences were blasted against NCBI database to study the genetic relatedness of colonies.

4.2.3 Bacterial 16S rRNA gene sequencing

The primers used for the 16S rRNA gene sequencing were 341F-CCTACGGGAGGCAGCAG and 806R- GGACTACHVGGGTWTCTAAT amplifying the bacterial V3-V4 region of the 16S rRNA gene (Caporaso et al. 2011, Peterson et al. 2015). A two-step PCR was used for amplification and indexing. The indexed PCR products were normalized to 10 ng/µl, and 10 µl of each sample was pooled and concentrated to ~100 µl and cleaned using SPRI (solid reverse phase immobilization) purification. Qubit was used to quantify the cleaned library and Agilent Tapestation for fragment analysis. The library was then diluted to 2nM. The pooled samples were denatured with NaOH, diluted to 8 pM in Illumina HT1 buffer, spiked with 15% PhiX and heat denatured at 96°C for 2 minutes before loading. A MiSeq 600 (1/8th lane Stowaway) cycle v3 kit was used to sequence the sample at the University of Minnesota Genomics Center, MN, USA. Sequences from this study are available through the NCBI Sequence Read Archive database under the BioProject accession number PRJNA782604 (SRR17005750 - SRR17005758) and Biosample id is SAMN23388497.

4.2.4 Sequence processing and community analysis

QIIME2 pipeline- moving picture tutorial (v. 2020.11, https://docs.qiime2.org/2020.11/tutorials/moving-pictures/) was used to analyze the resulting paired end illumina reads (Bolyan et al., 2019). Those sequences were filtered, trimmed to suitable depth, denoised, dereplicated, merged and chimeras removed using the DADA2 algorithm (Divisive Amplicon Denoising Algorithm 2) with the parameters p trim-left-f 15, p trim-left--r 20, trunc-len-f 275 and trunc-len-r 215 (Callahan et al. 2016). Those parameters for DADA2 algorithm were decided from interactive quality plots. During the DADA2 process, 726,212 demultiplexed sequences were processed to obtain 525,265 high quality reads. The moving picture tutorial in

qiime2 was followed to calculate the diversity metrics. Alpha and beta diversity metrics were calculated using the sequences subsampled from the feature table at the subsampling depth of 46,500. The alpha diversity metrics used are- Observed features, Shannon diversity, Peilou evenness, and Faith phylogenetic diversity; while the beta diversity metrics used are phylogenetic Unifrac distances- weighted and unweighted (Lozupone et al. 2011), and non-phylogenetic distances- Jaccard and Bray Curtis. The visualization of Unifrac distances were done in R (version 4.1.1, accessed on 2021-08-10) using the packages qiime2R, dplyr and ggplot. The reference database SILVA (version 132) was used to train the sequences after customization following the instructions on training the classifier for V3/V4 region of 16S rRNA gene. The Naïve Bayes trained Silva 132 99% OTU classifier, bounded by the 341F/806R primer set, was used as a reference read to assign taxonomy (Quast et al. 2013). Bar graphs representing the relative abundances of ASVs at the phylum and family levels were generated using the R package phyloseq from QIIME2 generated phylotype tables and taxonomy files (McMurdie and Holmes 2013). The analysis of composition of microbiomes (ANCOM) procedure was used to identify the significant differentially abundant bacterial taxa (at genus and phylum levels) using QIIME2 plugin (Mandal et al. 2015).

4.3 Results

4.3.1 Genetic relatedness among colonies

The partial sequence of host termite mitochondrial 16S rRNA gene was used to identify the isolates of termite colonies. Blasting of the sequences from colonies with each other resulted in 97.95% identity match which provides evidence for colonies being different isolates of *R. flavipes*, i.e., unique colonies. In addition, we used the sequences from closely related isolates of both the colonies to run Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm. MUSCLE algorithm was used to align multiple sequences and output was used to generate a phylogenetic tree (Madeira et al. 2019). The position of Whistler and Biochem colonies at different nodes of phylogenetic tree confirms that collected colonies were different isolates of *R. flavipes* (Supplemental Fig. S1).

4.3.2 Bacterial alpha diversity

Sequences rarified at the subsampling depth of 46,500 were used to generate alpha-diversity metrics. Alpha-diversity was estimated using four metrics: Observed features to estimate the number of unique features (Fig. 1A), Shannon diversity to estimate the relative abundance of species which is sensitive to species richness and evenness (Fig. 1B), Faith phylogenetic diversity to estimate species diversity using the number of phylogenetic tree-units within a sample (Fig.1C) and Pielou evenness to estimate the number of each species within an environment (Fig. 1D). Based on Kruskal-Wallis tests, the effect of termite colony on alpha diversity metrics resulted in mixed significant effects. The observed features (H = 5.33, df = 1; $p = 0.02$) and Shannon diversity (H = 5.33, df = 1; $p = 0.02$) were significantly different between colonies while the Faith phylogenetic diversity (H = 3.0, df = 1; $p = 0.08$) and Pielou evenness (H = 2.08, df = 1; $p = 0.14$) were not statistically significant. These results indicate colonies differed in terms of number of unique features and their relative abundance, while they were statistically similar in terms of number of unique features and their phylogenetic relatedness.

4.3.3 Bacterial beta diversity

The PERMANOVA statistics show that weighted UniFrac (pseudo-F= 12.99, $p = 0.02$) and unweighted UniFrac analyses (pseudo-F= 2.42, $p = 0.02$) were statistically significant between colonies, which means colonies differed from each other in terms of bacterial community structure (Fig. 2A, 2B). Additionally, the significant differences between colonies were not due to within-replication variation in weighted (PERMDISP test, F= 0.03, $p = 0.91$) and unweighted UniFrac (PERMDISP test, F= 0.29, $p = 0.46$). Similar significant results were observed for other beta diversity metrics- Bray-Curtis and Jaccard (Supplementary Fig. S2A, S2B).

4.3.4 Bacterial taxonomic composition

Based on relative abundance analysis, the taxonomic composition of dominant bacterial phyla at abundances greater than 0.01% varied slightly on their relative abundances between colonies (Fig. 3A). The major dominant phyla were Spirochaetes, Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. Based on NCBI taxonomy, the phyla Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria are now named as Bacillota, Bacteroidota, Psudomonadota and

Actinomycetota respectively. Similarly, the taxonomic composition of bacterial family at abundances greater than 1% was computed. Based on the relative abundance graph, 16 bacterial family were present at abundances greater than 1% (Fig. 3B). On computing Analysis of Composition of Microbiomes (ANCOM) at phylum level, the phyla RsaHF231 and Verrucomicrobia were found significantly differentially abundant between colonies (Fig. 4A). In addition, at genus level, the taxa *Elizabethkingia* and *Chryseobacterium* from phylum Bacteroidetes were differentially abundant between colonies (Fig. 4B).

4.4 Discussion

This study compares the effect of lab rearing of termite colonies on their gut bacterial composition. The study was conducted by rearing two *R. flavipes* colonies under identical conditions for 2-3 years, and the findings provide an example of colony variation among gut bacterial populations that was unaffected by diet and environment. Our experimental design cannot account for microbial changes due to lab rearing however changes in abiotic factors such as exposure to high temperature has been linked to lower gut microbial diversities in termite (Arango et al. 2021). The bacterial composition was studied using culture-independent 16S rRNA gene sequence analysis, from which alpha, beta diversities and differential abundance were calculated using several accepted data analysis procedures (Sapkota et al. 2021). Genetic relatedness between termite colonies was studied by sequencing the termite's mitochondrial 16S rRNA gene. We found that identical rearing conditions for 2-3 years could not completely eliminate the gut bacterial differences between independent termite colonies which were two different isolates (i.e., colony genotypes) of *R. flavipes*.

The number of unique bacterial species (Observed features) and their diversities (Shannon diversity) were significantly higher in the Whslr colony, but interestingly colonies were statistically similar in terms of abundance of each bacterial species (Pielou evenness) and their phylogenetic diversities (Faith phylogenetic diversity). Alternatively, in a culture dependent study, a group of researchers found that colonies of Formosan subterranean termites resulted statistically similar number of culturable gut bacteria (Husseneder et al. 2009). The differences in results between studies could be due to the large number of termite gut bacteria that are unculturable and/or due to difference in rearing conditions. Based on beta diversity indices, we also found a significant signal between overall variation of bacterial communities between termite colonies.

Similar to our results, distinct differences in metatranscriptome responses have been observed in termite colonies which could be tied to the gut microbial differences among them (Boucias et al. 2013, Scharf et al. 2017). The differences in gut bacteria from different colonies of oriental fruit flies and ants have been reported previously (Wang et al. 2011, Segers et al. 2019). Similarly, the composition of microbial flora in a termite gut has been believed to be colony specific (Matsuura 2001; Boucias et al. 2013). The composition of major bacterial phyla of wood feeding termites were found stable even though variation in relative abundance occurred with their diet (Van and Boopathy 2020). Interestingly, the overall functions within a termite gut apparently were not disturbed which indicates the occurrence of metabolic overlap; an important feature in microbial ecology (Van and Boopathy 2020). Furthering to these prior studies, our study demonstrates that the differences in gut bacterial structure among colonies are inevitable despite similar rearing conditions in the laboratory. The analysis of relative abundance of bacteria in our experiment indicates a similar composition of dominant bacterial phyla; however, a small but statistically significant difference was still prevalent suggesting that unique colonial signatures remain even after long-term feeding on identical diets. These unique signatures are likely maintained via vertical transfer of gut bacteria within colonies, leading to possible continued symbiotic associations which cannot be disrupted (Bourguignon et al. 2018).

A change in diet or overall termite "holobiont" physiology could be a cause for the slight differences in gut bacterial abundance among colonies/collecting sites (Benjamino and Graf 2016). The majority of gut bacteria in termites are believed to be coevolved with the host and are consistent among the termite genera with dominant bacterial phyla being remarkably similar within a genus. However, a small but significant difference in relative abundance of bacterial communities can be observed among termites from different collecting sites (Hongoh et al. 2005). Another study revealed similar dominating bacterial species among two different species of termites- *Cortaritermes fulviceps* and *Nasutitermes aquilinus* (Victorica et al. 2020). In agreement with previous studies, the major bacterial phyla between colonies remained similar in our study, while slight proportional differences and significant variation in some specific taxa were observed. The present study thus supports the idea that small differences in bacterial taxa among collecting sites/colonies do occur (Boucias et al. 2013, Benjamino & Graf 2016) and such differences cannot be eliminated by rearing termites with identical environments and diets for a seemingly long period of time. The remaining, persistent differences could be due to unique colonial signatures, which

supports the idea that specific termite colony isolates may have a uniquely co-evolved microbiota that cannot be eliminated, even with transfer to new environments. Our findings advance current research by demonstrating a small but significant colonial variation in the gut bacterial taxa after rearing termite colonies in lab. These findings have important implications for basic and applied termite science.

4.5 Figures

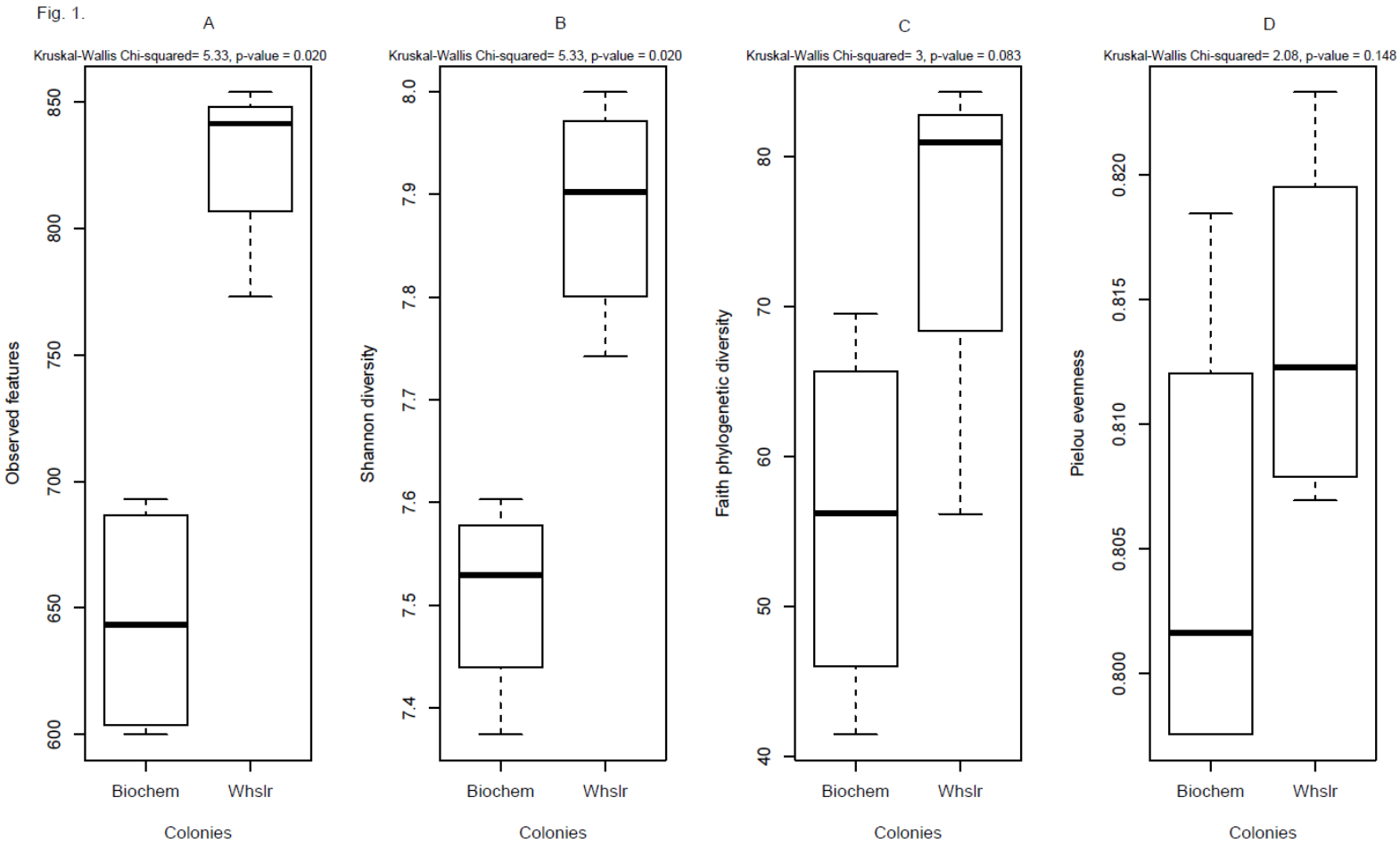


Fig. 4. 1. Alpha diversity metrics from 16S surveys showing: Observed features (A), Shannon diversity (B), Faith phylogenetic diversity (C) and Pielou evenness (D). P-values for global Kruskal-Wallis comparisons across colonies are shown at the top of each graph.

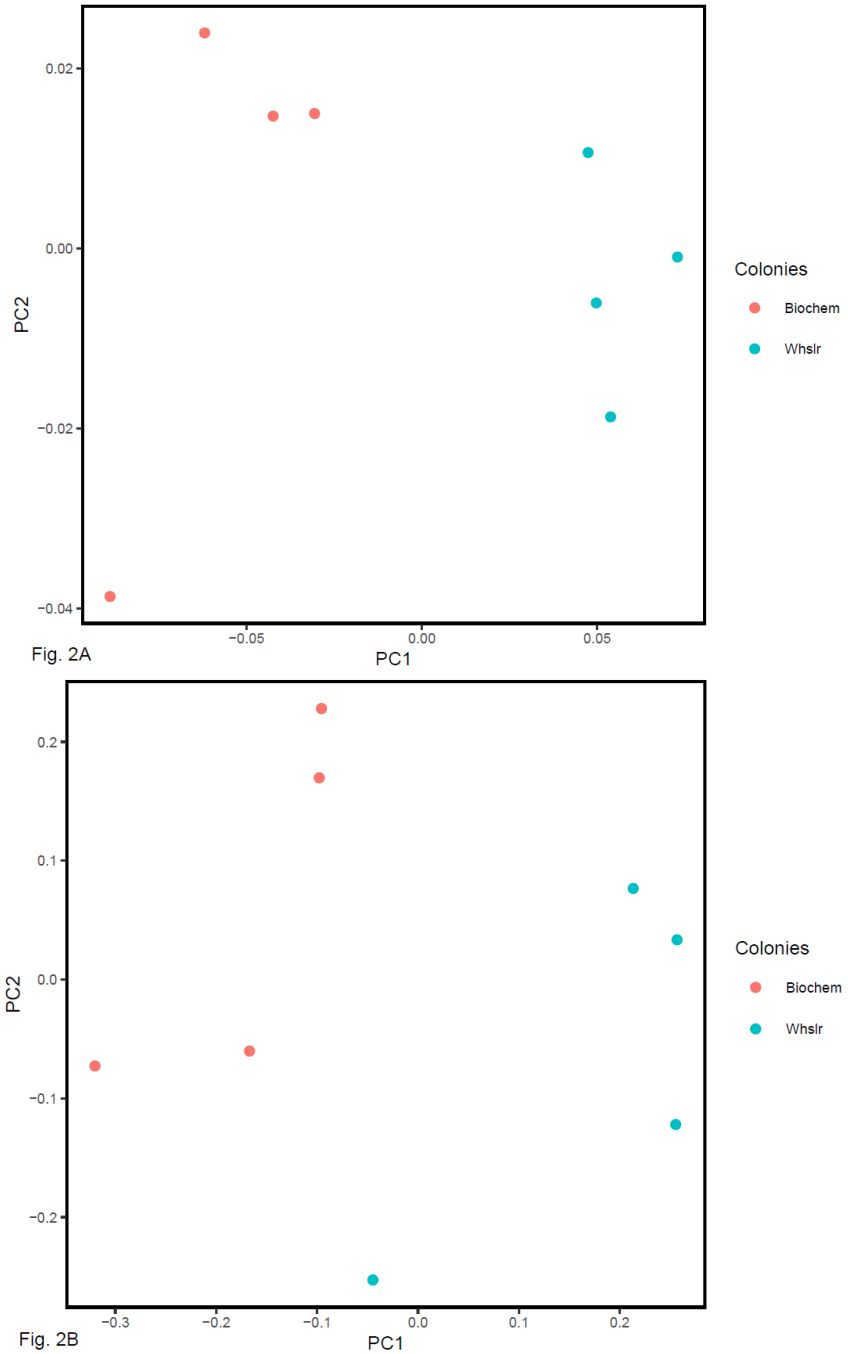


Fig. 4. 2. Beta diversity metrics- Weighted Unifrac (Permanova, Pseudo-F = 12.99, number of permutations = 999, p = 0.026) (A). Unweighted Unifrac (Permanova, Pseudo-F = 2.43, number of permutations = 999, p = 0.023) (B). Principal coordinate plots from bacterial 16S rRNA gene sequence surveys. Colors represent colonies and individual points represent individual replicates. Based on pairwise PERMANOVA, colonies are significantly different ($p<0.05$).

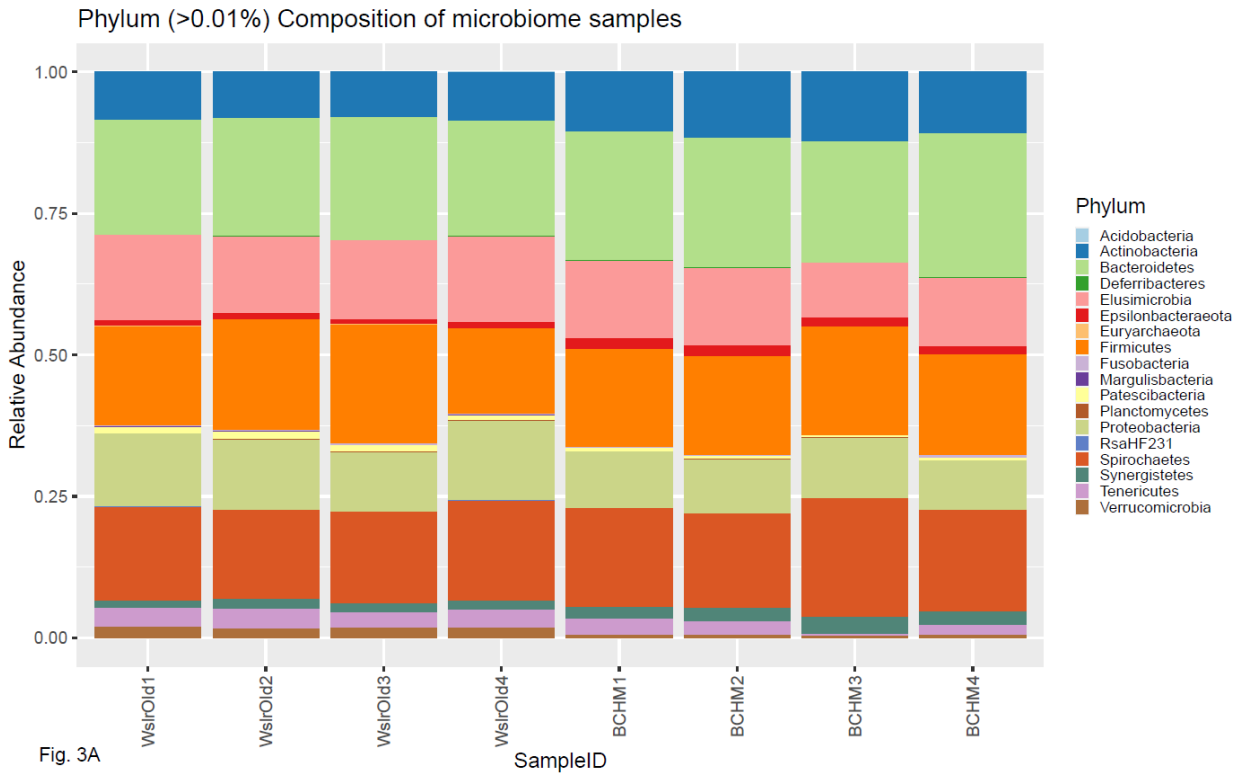


Fig. 3A

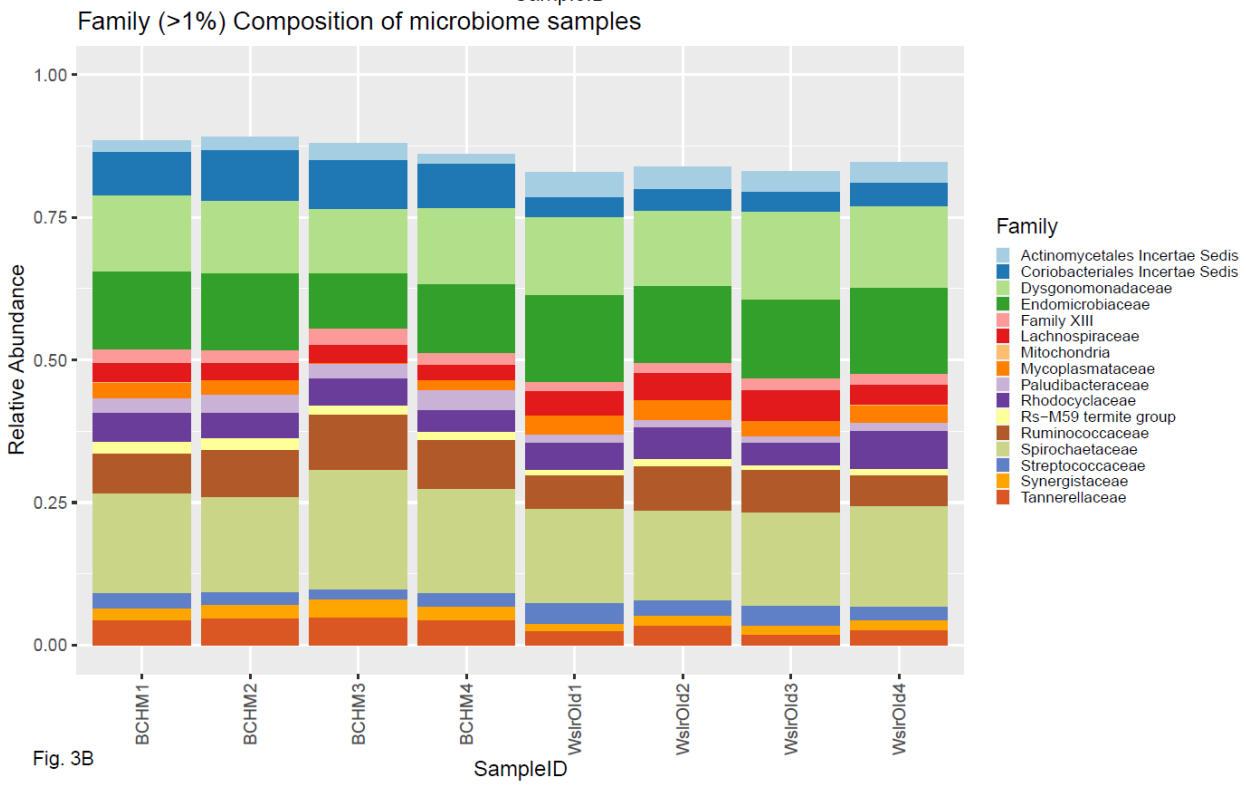


Fig. 3B

Fig. 4. 3. Relative abundance of bacteria (from abundance >0.0001) at phylum level (A) and relative abundance of bacteria (from abundance >0.01) at family level (B). measured as the percentage of the total bacterial abundance for each replication.

Fig. 4A.

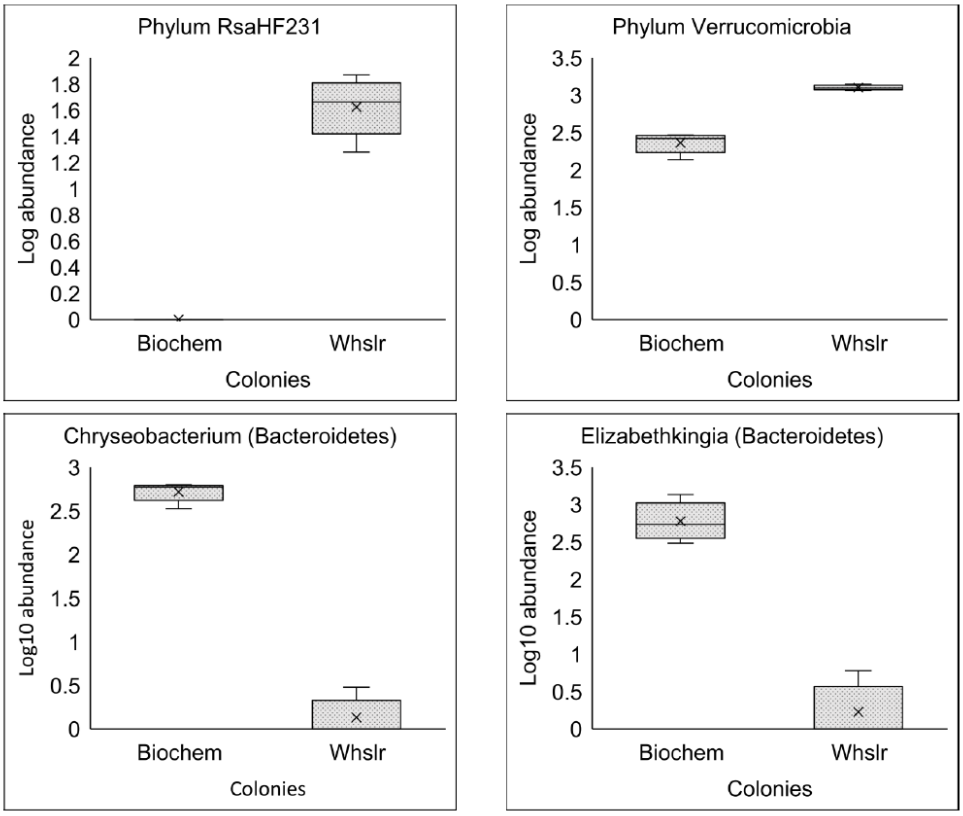


Fig. 4B.

Fig. 4. 4. Boxplots of the log abundances of bacterial phyla (A) and genera (B) (False Discovery Rate at 0.05). These phyla and genera were significantly differentially abundant as measured by ANCOM between the colonies Biochem and Whslr.

4.6 References

Arango, R. A., S. D. Schoville, C. R. Currie, and C. Carlos-Shanley. 2021. Experimental Warming Reduces Survival, Cold Tolerance, and Gut Prokaryotic Diversity of the Eastern Subterranean Termite, Reticulitermes flavipes (Kollar). Front. Microbiol. 12: 1116.

Auer, L., A. Lazuka, D. Sillam-Dussès, E. Miambi, M. O'Donohue, and G. Hernandez-Raquet. 2017. Uncovering the Potential of Termite Gut Microbiome for Lignocellulose Bioconversion in Anaerobic Batch Bioreactors. Front. Microbiol. 8: 2623.

Austin, J.W., A.L. Szalanski, R. H. Scheffrahn, M.T. and Messenger. 2005. Genetic variation of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) in North America applying the mitochondrial rRNA 16S gene. Ann. Entomol. Soc. Am. 98:980-988.

Benjamino, J., and J. Graf. 2016. Characterization of the Core and Caste-Specific Microbiota in the Termite, Reticulitermes flavipes. Front. Microbiol. 7: 171.

Benjamino, J., S. Lincoln, R. Srivastava, and J. Graf. 2018. Low-abundant bacteria drive compositional changes in the gut microbiota after dietary alteration. Microbiome. 6:1-13.

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander et al. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 37: 852–857.

Boucias, D. G., Y. Cai, Y. Sun, V.-U. Lietze, R. Sen, R. Raychoudhury, and M. E. Scharf. 2013. The hindgut lumen prokaryotic microbiota of the termite Reticulitermes flavipes and its responses to dietary lignocellulose composition. Mol. Ecol. 22: 1836–1853.

Bourguignon, T., N. Lo, C. Dietrich, J. Šobotník, S. Sidek, Y. Roisin, A. Brune, and T. A. Evans. 2018. Rampant host switching shaped the termite gut microbiome. Current biology. 28: 649-654.

Brune A., and M. Ohkuma 2010. Role of the Termite Gut Microbiota in Symbiotic Digestion, pp. 439-475. In: Bignell D., Y. Roisin, N. Lo (eds), Biology of Termites: a Modern Synthesis. Springer, Dordrecht.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods. 13: 581–583.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, C. A. Lozupone, P. J. Turnbaugh, N. Fierer, and R. Knight. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108: 4516–4522.

Doolittle, M., A. Raina, A. Lax, and R. Boopathy. 2008. Presence of nitrogen fixing Klebsiella pneumoniae in the gut of the Formosan subterranean termite (Coptotermes formosanus). Bioresour. Technol. 99: 3297-3300.

Hongoh, Y. 2011. Toward the functional analysis of uncultivable, symbiotic microorganisms in the termite gut. Cell. Mol. Life Sci. 68: 1311–1325.

Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, N. Noparatnaraporn, and T. Kudo. 2005. Intra- and Interspecific Comparisons of Bacterial Diversity and Community Structure Support Coevolution of Gut Microbiota and Termite Host. Appl. Environ. Microbiol. 71: 6590–6599.

Husseneder, C., J. M. Berestecky, and J. K. Grace. 2009. Changes in Composition of Culturable Bacteria Community in the Gut of the Formosan Subterranean Termite Depending on Rearing Conditions of the Host. Ann. Entomol. Soc. Am. 102: 498–507.

Inoue, T., K. Murashima, J.-I. Azuma, A. Sugimoto, and M. Slaytor. 1997. Cellulose and Xylan Utilisation in the Lower Termite Reticulitermes speratus. J. Insect Physiol. 43: 235–242.

Inoue, T., O. Kitade, T. Yoshimura, and I. Yamaoka. 2000. Symbiotic Associations with Protists, pp. 275–288. In Abe, T., Bignell, D.E., Higashi, M. (eds.), Termit. Evol. Sociality Symbioses Ecol. Springer Netherlands, Dordrecht.

Jiménez-Padilla, Y., E. O. Esan, K. D. Floate, and B. J. Sinclair. 2020. Persistence of diet effects on the microbiota of Drosophila suzukii (Diptera: Drosophilidae). Can. Entomol. 152: 516–531.

Ley, R. E., M. Hamady, C. Lozupone, P. J. Turnbaugh, R. R. Ramey, J. S. Bircher, M. L. Schlegel, T. A. Tucker, M. D. Schrenzel, R. Knight, and J. I. Gordon. 2008. Evolution of Mammals and Their Gut Microbes. Science. 320: 1647–1651.

Lozupone, C., M. E. Lladser, D. Knights, J. Stombaugh, and R. Knight. 2011. UniFrac: an effective distance metric for microbial community comparison. ISME J. 5: 169–172.

Mandal, S., W. Van Treuren, R. A. White, M. Eggesbø, R. Knight, and S. D. Peddada. 2015. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microb. Ecol. Health Dis. 26: 27663.

Matsuura, K. 2001. Nestmate recognition mediated by intestinal bacteria in a termite, Reticulitermes speratus. Oikos. 92: 20–26.

Madeira, F., Y. M. Park, J. Lee, N. Buso, T. Gur, N. Madhusoodanan, P. Basutkar, A.R. Tivey, S.C. Potter, R.D. Finn, and R. Lopez. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 47: W636-W641.

Maurice, N., and L. Erdei. 2018. Termite Gut Microbiome, pp. 69–99. In Khan, Md.A., Ahmad, W. (eds.), Termit. Sustain. Manag. Vol. 1 - Biol. Soc. Behav. Econ. Importance, Sustainability in Plant and Crop Protection. Springer International Publishing, Cham.

McMurdie, P. J., and S. Holmes. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one. 8: e61217.

Ohkuma, M. 2008. Symbioses of flagellates and prokaryotes in the gut of lower termites. Trends Microbiol. 16: 345–352.

Peterson, B. F., and M. E. Scharf. 2016a. Lower Termite Associations with Microbes: Synergy, Protection, and Interplay. Front. Microbiol. 7: 422.

Peterson, B. F., and M. E. Scharf. 2016b. Metatranscriptome analysis reveals bacterial symbiont contributions to lower termite physiology and potential immune functions. BMC Genomics. 17: 772.

Peterson, B. F., H. L. Stewart, and M. E. Scharf. 2015. Quantification of symbiotic contributions to lower termite lignocellulose digestion using antimicrobial treatments. Insect Biochem. Mol. Biol. 59: 80–88.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41: D590–D596.

Rosengaus, R. B., C. N. Zecher, K. F. Schultheis, R. M. Brucker, and S. R. Bordenstein. 2011. Disruption of the Termite Gut Microbiota and Its Prolonged Consequences for Fitness. Appl. Environ. Microbiol. 77: 4303–4312.

Sapkota, R., C. H. Nakatsu, and M. E. Scharf. 2021. Regulation of host phenotypic plasticity by gut symbiont communities in the eastern subterranean termite (*Reticulitermes flavipes*). J. Exp. Biol. 224: jeb242553.

Scharf, M. E. 2015. Omic research in termites: an overview and a roadmap. Front. Genet. 6: 76.

Scharf, M. E. 2020. Challenges and physiological implications of wood feeding in termites. Curr. Opin. Insect Sci. 41: 79–85.

Scharf, M. E., and A. Tartar. 2008. Termite digestomes as sources for novel lignocellulases. Biofuel Bioprod Biorefin. 2:540-52.

Scharf, M. E., and B. F. Peterson. 2021. A Century of Synergy in Termite Symbiosis Research: Linking the Past with New Genomic Insights. Annu. Rev. Entomol. 66: 23–43.

Scharf, M. E., Y. Cai, Y. Sun, R. Sen, R. Raychoudhury, and D. G. Boucias. 2017. A meta-analysis testing eusocial co-option theories in termite gut physiology and symbiosis. Commun. Integr. Biol. 10: e1295187.

Segers, F. H. I. D., M. Kaltenpoth, and S. Foitzik. 2019. Abdominal microbial communities in ants depend on colony membership rather than caste and are linked to colony productivity. Ecol. Evol. 9: 13450–13467.

Sommer, F., and F. Bäckhed. 2013. The gut microbiota — masters of host development and physiology. Nat. Rev. Microbiol. 11: 227–238.

Van Dexter, S. and R. Boopathy. 2020. Analysis of Termite Microbiome and Biodegradation of Various Phenolic Compounds by a Bacterium Isolated from the Termite gut in Louisiana, USA, pp. 163. In Z. A. Zakaria, R. Boopathy, and J. R. Dib (eds), Valorisation of Agro-industrial Residues–Volume I: Biological Approaches. p.163.

Vernier, C. L., I. M. Chin, B. Adu-Oppong, J. J. Krupp, J. Levine, G. Dantas, and Y. Ben-Shahar. 2020. The gut microbiome defines social group membership in honeybee colonies. Sci. Adv. 6: eabd3431.

Victorica, R. M., M. A. Soria, R. A. Batista-García, J. A. Ceja-Navarro, S. Vikram, M. Ortiz, O. Ontañon, S. Ghio, L. Martínez-Ávila, O. J. Quintero García, C. Etcheverry, E. Campos, D. Cowan, J. Arneodo, and P. M. Talia. 2020. Neotropical termite microbiomes as sources of novel plant cell wall degrading enzymes. Sci. Rep. 10: 3864.

Waidele, L., J. Korb, C. R. Voolstra, S. Künzel, F. Dedeine, and F. Staubach. 2017. Differential Ecological Specificity of Protist and Bacterial Microbiomes across a Set of Termite Species. Front. Microbiol. 8: 2518.

Wang, H., L. Jin, and H. Zhang. 2011. Comparison of the diversity of the bacterial communities in the intestinal tract of adult Bactrocera dorsalis from three different populations. J. Appl. Microbiol. 110: 1390–1401.

CHAPTER 5. CONCLUSION

R. flavipes termites and their gut symbionts act together as a holobiont while interacting with the environment they are exposed to. The gut symbionts along with an insect host are responsive to changes in environmental conditions. However, there might be distinct groups of core and dynamic gut microbiota. The core microbiome is resistant to changes possibly to support the basic physiology while dynamic members might have significance in adapting to changing conditions. The findings presented in this dissertation help better understand the microbial perspective of termite holobiont's response to environmental, colony and genetic factors. The environmental factors in this case were characterized by looking at- social environment (attained via artificially initiating a soldier molt in lab-reared termites), physical environment (attained via sampling termites from the field every month) and insecticidal exposure (attained by feeding termites insecticide-treated paper towel sandwiches). A colony genetic factor was studied using two genetically different colonies reared in the lab (with identical rearing conditions) for an extensive period. In other words, this dissertation established the presence of interactions between termite gut microbiome and proximate eusocial mechanisms which are social environment, physical environment, and colony genetics in our case. The combinations of several laboratory investigations and bioinformatic analyses have resulted in developing a better understanding of (1) impact of gut microbial taxa on termite's social environment, (2) impact of seasonality on gut microbiome, (3) variation brought about by colony's genetic differences in gut microbiome by controlling the environment (attained by rearing genetically distinct colonies for an extensive period of time), and (4) impact of commonly used termiticides on gut microbial loads.

Through combining hormonal and antibiotic treatments in standardized bioassays, we were able to connect gut microbiomes to the presoldier differentiation and survival of termites (Chapter 2). Significant changes in the composition of gut microbiome were observed when termites were ready for a presoldier molt. The maintenance of the social environment in termites (e.g., caste differentiation) resulted in a significant impact on termite's survival and gut microbial counterparts. From the next part of my dissertation, I found that as termites forage below the ground surface each month, there was significant variation in relative abundances of some bacterial taxa (Chapter 3). In addition, several taxa acquired by termites were correlated with temperature. However, when controlling environmental factors such as rearing temperature, food and water sources, two

bacterial taxa were significantly different between two genetically distinct colonies providing evidence for vertical transfer of these unique taxa (Chapter 4). For the applied part of this dissertation, I tested the impact of commonly used termiticides against gut microbial load (Appendix A). I found that insecticidal treatments reduced protist numbers in termite hindguts although bacterial load was not significantly affected. This final experiment calls for the further investigation to identify the remaining microbial taxa and their functions in the termite gut. This will help us characterize the non-target effect of commonly used termiticides, possibly leading to enhanced abilities to control termites in more environmentally friendly ways.

Through tying the above chapters together, the results presented herein show that termite gut microbial communities can vary in response to environmental, social and physical environments, and insecticidal exposure (Chapter 2, 3, Appendix A), and genetics of a colony (Chapter 4). From these results, I found that termite gut symbionts change their community structure with changing environmental conditions, possibly to help the host termite to best fit in the new environmental settings. However, alternatively if environment remains constant then the symbionts will apparently follow their "genetic selections" and vary slightly based on host genetic differences between two colonies. This dissertation altogether highlights the possible microbial dynamics to help a host adapt to changing conditions and emphasizes the importance of a holobiont approach while studying the termite gut symbionts.

I saw several significant trends in bacterial community composition and their relative abundances from my experiments. Overall, my dissertation supports the idea that both environment and colony genetics are important in shaping the gut microbial structures in termites. However, in a complex microbial environment like a termite gut, we should not forget about the functional redundancies that could be present among several taxa. Therefore, an important future direction from my project would be to understand the functional aspects of these taxa. In addition, the selection of primers for 16S rRNA gene sequencing can introduce another important limitation which can result in an incomplete story in studies that are designed for taxonomic identification. In spite of the above limitations posed by study design, this research also provides important new information to help us understand how microbiomes are a key component of termites and are constantly interacting with several aspects of the termite life cycle.

APPENDIX A. UNDERSTANDING THE INFLUENCE OF COMMONLY USED TERMITICIDES ON GUT MICROBIAL COMMUNITIES OF THE EASTERN SUBTERRANEAN TERMITE (*RETICULITERMES FLAVIPES* KOLLAR)

Introduction

Soil insecticides based on fipronil and imidacloprid, and bait metrics consisting of chitin synthesis inhibitors such as noviflumuron, hexaflumuron, diflubenzuron, and chlorfluzuron, are commonly used to manage subterranean termites (Su 1994, Su 2005, Rojas and Morales 2001, Oi 2022). Despite having variation in mode of actions, these insecticides are effective in managing termites. Since the association of termites and their gut symbionts is inseparable, there have been many attempts to connect insecticide treatments with the gut microbiome. The application of imidacloprid and fipronil has been reported to disrupt termite gut symbiotic stability and reduce immunity against fungal pathogens to which they are immune under normal conditions (Raza et al. 2019, Sen et al. 2015, Ramakrishnan et al. 1999). Insect gut symbionts are also reported to partially reduce the toxic effect of insecticides by providing essential amino acids to enhance insect immunity against the chemicals (Chen et al. 2020, Xia et al. 2018). Several insecticides have been reported to disrupt quorum sensing, which is a communication mechanism by colonizing bacteria in an insect gut (Gao et al. 2018, Gomes et al. 2020). Therefore, we were interested to look at the impact of insecticidal treatments on the abundance of termite gut microbes specifically protists and bacteria. The specific objectives of our study were (1) to quantify the number of protists and (2) to quantify bacterial copy numbers from insecticide treated termites.

Materials and Methods

Insecticide bioassays

Termites reared in the laboratory were used for termiticide bioassays. The termiticides used for experiments were fipronil (0.1 µg/mL), imidacloprid (0.001 mg/mL) and hexaflumuron (10 mg/mL) (Sen et al. 2015). The concentration of fipronil was decided based mortality data on Fig. A1 as 20 ng (0.1 µg/mL) resulted nearly 55% mortality in 5 days of exposure. Similarly, other concentrations used during diagnostic bioassays with fipronil were 10 ng (0.05 µg/mL), 5 ng

(0.025 µg/mL), 2.5 ng (0.0125 µg/mL) and 0 ng (0 µg/mL). Fifteen termites were used during these bioassays with fipronil and 4 replications each were set. However, sublethal concentrations for imidacloprid and hexaflumuron could not be determined in the lab due to the slow-acting nature of these chemicals, therefore the concentrations used were adopted from the literature (Sen et al. 2015, Sandoval and Scharf 2016).

For the insecticide treatments, brown paper towel sandwiches were treated with 200 µL of each insecticide solution and dried under fume hood. While for control, 200 µL of acetone was used to treat the paper towel sandwich. For all treatments, 20 termites were introduced into Petri dishes. For fipronil and imidacloprid, the bioassay lasted for 2 days while for hexaflumuron it lasted for 9 days due to the differences in their mode of action. Controls for day 0, day 2 and day 9 were set. The Petri dishes with experimental samples were kept at 26±2 °C in an incubator. There were four replications each for the treatments.

Protist cell count

The number of protist cells present in the hindgut of termites were counted using 5 hindguts. The termite guts from each treatment were dissected, trimmed to hindgut region and placed in 500 µL PBS. The dissected guts were homogenized, and ten microliter suspension was transferred to Sedgewick Rafter Counting Cell (SPI Supplies; West Chester, PA, USA) to enumerate using the phase contrast microscope under 20x magnification. There were four replication each for different treatments.

16S Quantitative PCR

There were four replications for each of the treatment for DNA extraction. Ten termite whole guts were dissected and transferred to 200uL PBS and stored at -20°C until DNA was extracted. The DNeasy® Blood and Tissue Kit (Qiagen; Valencia, CA, USA) was used to extract the DNA from whole guts. Extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo-Fisher; Waltham, MA, USA) while quality was assessed through gel electrophoresis. The extracted DNA was stored at -20°C until used later

The 16S rRNA gene copy number from different treatments was assessed using the primers 338F and 518R. The total reaction volume for qPCR was 15 µL, with 7.5 µL buffer (SsoFast

EvaGreen® Supermix, Bio-Rad Inc., Hercules, CA, USA), 1 µL reverse and forward primers each (10 µM final conc.), 4 µL nuclease free water and 1.5 µL DNA template. The following cycling conditions were adopted from Chapter 2- initial denaturation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 30 seconds followed by a melt curve from 65°C to 95°C with 0.5°C increments. DNA copy number was determined by comparison against a DNA standard curve (obtained using *E. coli* DNA as standard), with analysis and PCR efficiency determinations by the method 10^(-1/slope).

Results and discussion

The results from this experiment demonstrate most clearly that insecticidal application significantly reduces the gut protist abundance. The effect of different treatments on average protist abundance was significantly different (Kruskal-Wallis Chi-squared= 22.01, $p < 0.001$). The pairwise comparisons between treatments on protists showed that the number of protists on day zero was significantly different from the number of protists among other treatments, even with protist numbers on day 2 and day 9 (Fig. A2). The difference seen on protist abundance in control groups could be due to selection pressure introduced by transferring termites to small Petri dishes. Protist numbers from fipronil treatment were significantly different from imidacloprid treatment but were not significant with hexaflumuron treatment. However, the number of protists on all insecticidal treatments were significantly reduced in comparison to controls. Similar to our results, significant disruption in protist abundance in termite guts with neonicotinoid and chitin synthesis inhibitor treatments have been reported (Sen et al. 2015, Lewis and Forschler 2010). However, another chitin synthesis inhibitor insecticide, Noviflumuron, was not reported to impact termite gut protists (Xing et al. 2014).

In addition to protist count, we performed quantitative PCR to get bacterial copy numbers. Initial goal of this project was to conduct 16S rRNA gene sequence analysis to identify the bacterial taxa differentially selected by different insecticides. Therefore 20 µL of extracted DNA was sent to the University of Minnesota sequencing core for sequencing. However, we could not process 16S data obtained from the core, due to poor quality (the percentage of quality reads obtained after cleaning data was less than 30%, and even 0% recovery was obtained in some samples). Therefore, we decided to quantify the bacterial DNA present in our samples in the lab using qPCR. The quantitative PCR demonstrated a different story (than protist counts) on impact of insecticides on

gut bacterial abundances. The effect of treatment was not significant (Fig. A3). Similarly, in an experiment with honeybees, imidacloprid was not found to alter the gut bacterial composition (Raymann et al. 2018). When an insect is exposed to an insecticide, the gut microbes are suggested to be differentially selected to adapt to the selection pressure (Gomes et al. 2020). Therefore, our results could be suggesting that, instead of total abundances of bacteria, their composition might be more affected by insecticidal treatments. The next steps after these preliminary results presented above would be to look at the compositional changes in protist and bacterial taxa using the 18S and 16S rRNA gene sequencing, respectively. In addition, functional analysis of differentially selected protist taxa in response to an insecticide could result in new pest management insights.

Figures

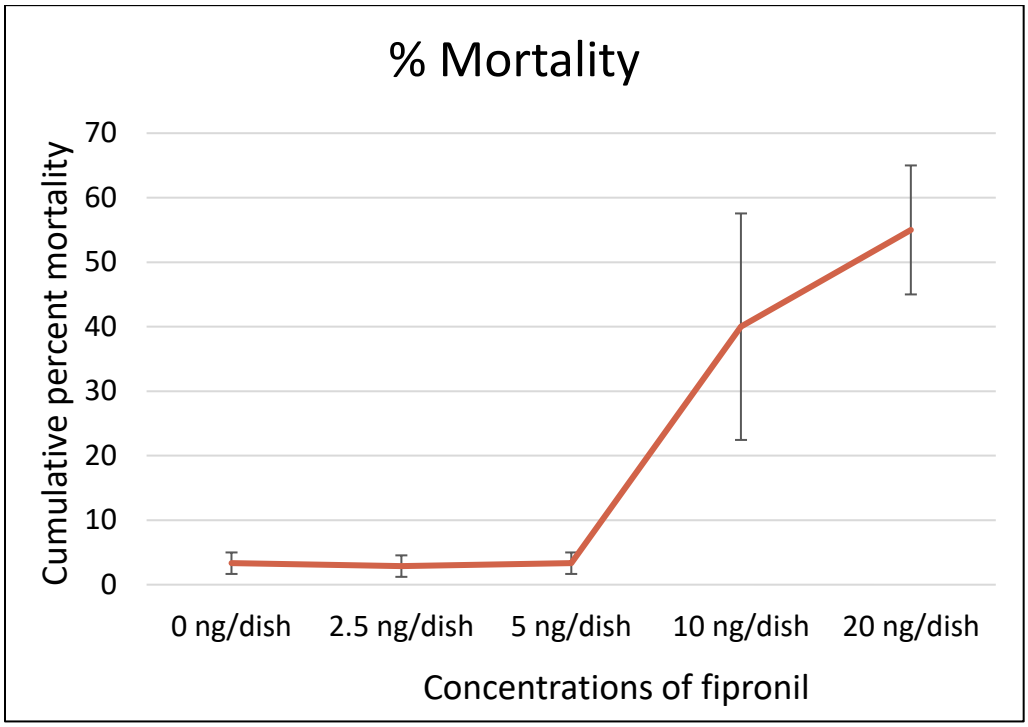


Fig. A1. Cumulative percent mortality (± SEM) of *R. flavipes* on different concentrations of fipronil after 5 days of exposure (n = 4).

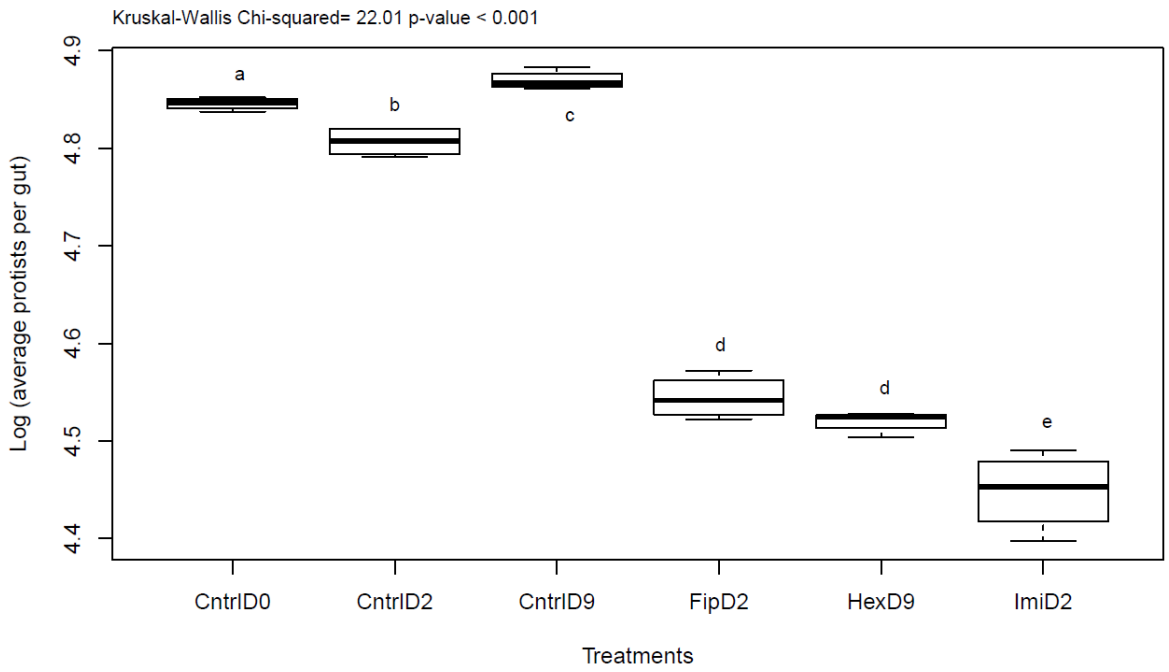


Fig. A2. Log average of protist per termite gut (n= 4). Significance level on pairwise comparisons is indicated by lowercase letters. Treatments with different letters are significant ($p < 0.05$). Note: The x-axis represents various treatments- abbreviations CntrlD0, CntrlD2, and CntrlD9 represent termites in control at day 0, 2 and 9 days of experiment, FipD2 represents fipronil treatment (results attained after 2 days of exposure), ImiD2 represents imidacloprid treatment (results attained after 2 days of exposure) and HexD9 represents hexaflumuron treatment (results attained after 9 days of exposure).

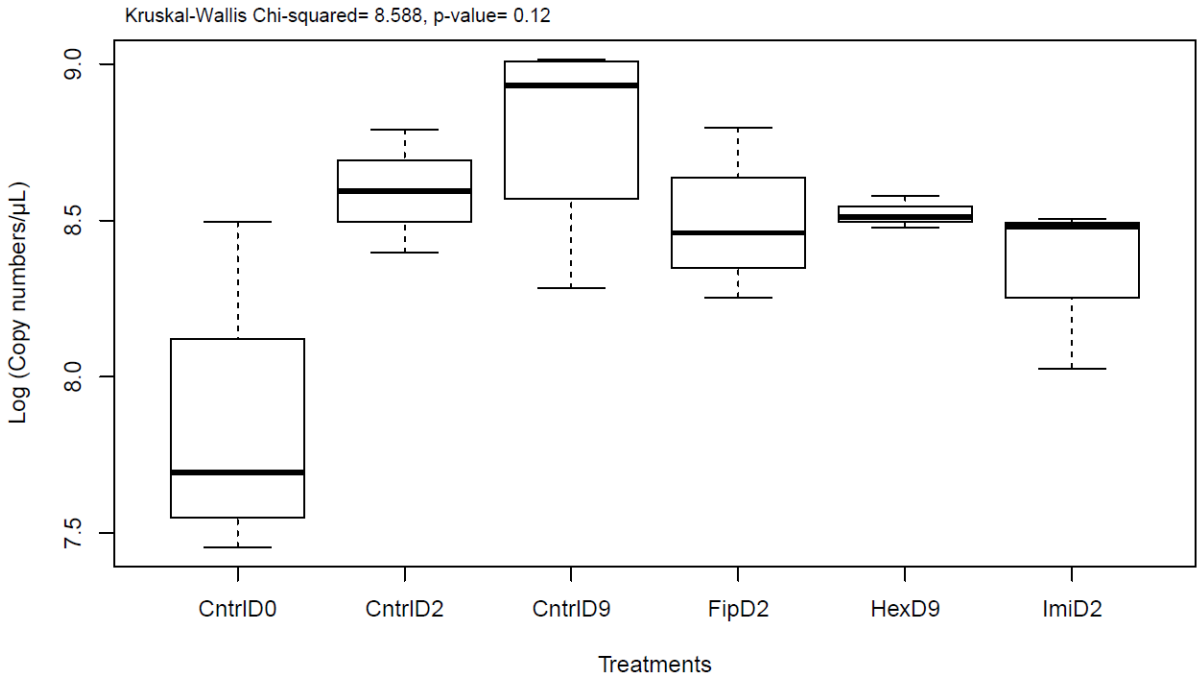


Fig. A3. Quantitative PCR result using primer sets 338F – 518R (equation; y = -1.2179x + 17.022, R^2 = 0.9945, PCR- efficiency= 2.2). Treatments were not statistically significant based on Global Kruskal-Wallis ($p>0.05$). Note: The x-axis represents various treatments- abbreviations CntrlD0, CntrlD2, and CntrlD9 represent termites in control at day 0, 2 and 9 days of experiment, FipD2 represents fipronil treatment (results attained after 2 days of exposure), ImiD2 represents imidacloprid treatment (results attained after 2 days of exposure) and HexD9 represents hexaflumuron treatment (results attained after 9 days of exposure).

References

Chen, B., N. Zhang, S. Xie, X. Zhang, J. He, A. Muhammad, C. Sun, X. Lu, and Y. Shao. 2020. Gut bacteria of the silkworm *Bombyx mori* facilitate host resistance against the toxic effects of organophosphate insecticides. Environ. Int. 143:105886.

Gao, B., L. Chi, P. Tu, X. Bian, J. Thomas, H. Ru, and K. Lu. 2018. The organophosphate malathion disturbs gut microbiome development and the quorum-Sensing system. Toxicol. Lett. 283:52-7.

Gomes, A. F., C. Omoto, and F. L. Cônsoli. 2020. Gut bacteria of field-collected larvae of *Spodoptera frugiperda* undergo selection and are more diverse and active in metabolizing multiple insecticides than laboratory-selected resistant strains. J Pest Sci. 93:833-51.

Lewis, J. L., and B. T. Forschler. 2010. Impact of five commercial baits containing chitin synthesis inhibitors on the protist community in *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Environ. Entomol. 39: 98-104.

Oi, F. A. 2022. Review of the Evolution of Termite Control: A Continuum of Alternatives to Termiticides in the United States with Emphasis on Efficacy Testing Requirements for Product Registration. Insects. 13: 50.

Ramakrishnan, R., D.R. Suiter, C.H. Nakatsu, R.A. Humber and G.W. Bennett. 1999. Imidacloprid-enhanced *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) susceptibility to the entomopathogen *Metarhizium anisopliae*. J. Econ. Entomol. 92:1125-1132.

Raymann, K., E. V. Motta, C. Girard, I. M. Riddington, J. A. Dinser, and N. A. Moran. 2018. Imidacloprid decreases honeybee survival rates but does not affect the gut microbiome. Appl. Environ. Microbiol. 84: e00545-18.

Raza, S., A. Riaz, and K. Z. Rasib. 2019. Synergistic Effect of Fungus *Metarhizium anisopliae* and Slow Acting Toxicants Fipronil and Imidacloprid against Subterranean Termites. Pak. J. Sci. 62: 88-96.

Rojas, M. G., and J.A. Morales-Ramos. 2001. Bait matrix for delivery of chitin synthesis inhibitors to the Formosan subterranean termite (Isoptera: Rhinotermitidae). J. Econ. Entomol. 94: 506-510.

Sandoval-Mojica, A. F., and M. E. Scharf. 2016. Silencing gut genes associated with the peritrophic matrix of *Reticulitermes flavipes* (Blattodea: Rhinotermitidae) increases susceptibility to termiticides. Insect Mol. Biol. 25:734-44.

Sen, R., R. Raychoudhury, Y. Cai, Y. Sun, V.U. Lietze, B.F. Peterson, M.E. Scharf and D.G. Boucias. 2015. Molecular signatures of nicotinoid-pathogen synergy in the termite gut. PLoS One. 10: e0123391.

Su, N. Y. 1994. Field evaluation of a hexaflumuron bait for population suppression of subterranean termites (Isoptera: Rhinotermitidae). J. Econ. Entomol. 87: 389-397.

Su, N. Y. 2005. Response of the Formosan subterranean termites (Isoptera: Rhinotermitidae) to baits or nonrepellent termiticides in extended foraging arenas. J. Econ. Entomol. 98: 2143-2152.

Xia, X., B. Sun, G. M. Gurr, L. Vasseur, M. Xue, and M. You. 2018. Gut microbiota mediate insecticide resistance in the diamondback moth, *Plutella xylostella* (L.). Front. Microbiol. 9:25.

Xing, L., T. Chouvenc, and N. Y. Su. 2014. Behavioral and histological changes in the Formosan subterranean termite (Isoptera: Rhinotermitidae) induced by the chitin synthesis inhibitor noviflumuron. J. Econ. Entomol. 107: 741-747.

APPENDIX B: SUPPLEMENTAL INFORMATION FROM SAPKOTA ET AL. 2021

Chapter-2: Regulation of host phenotypic plasticity by gut symbiont communities in the Eastern Subterranean termite (*Reticulitermes flavipes* Kollar)

Rajani Sapkota, Cindy H. Nakatsu, Michael E. Scharf

Figures:

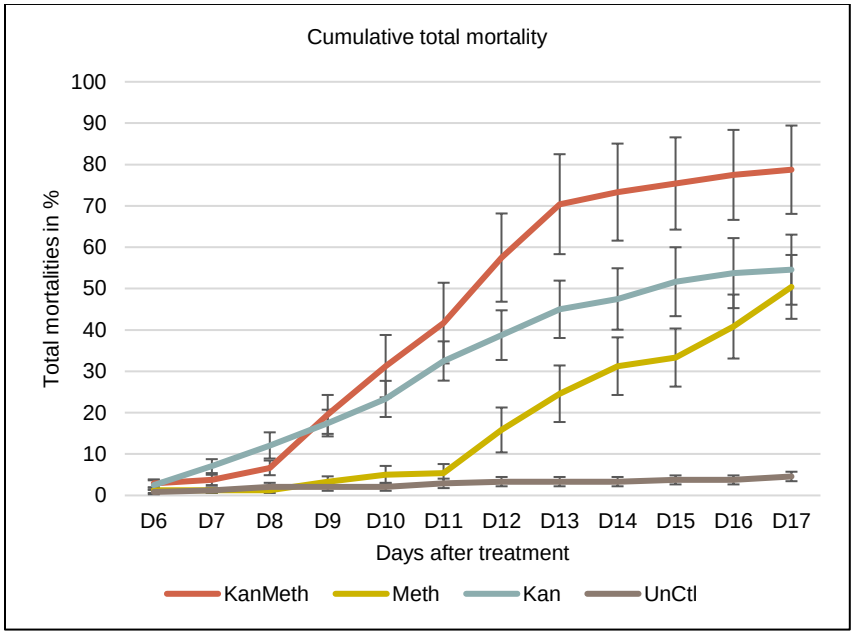


Fig. B. S1. Bioassay results showing the average everyday mortality ± SEM until day 17. The x-axis is days after treatment and y-axis is average percent mortality. Second phase of treatment with either methoprene or acetone started after 48 hours of either kan or water treatment (12 replications, sample size = 20).

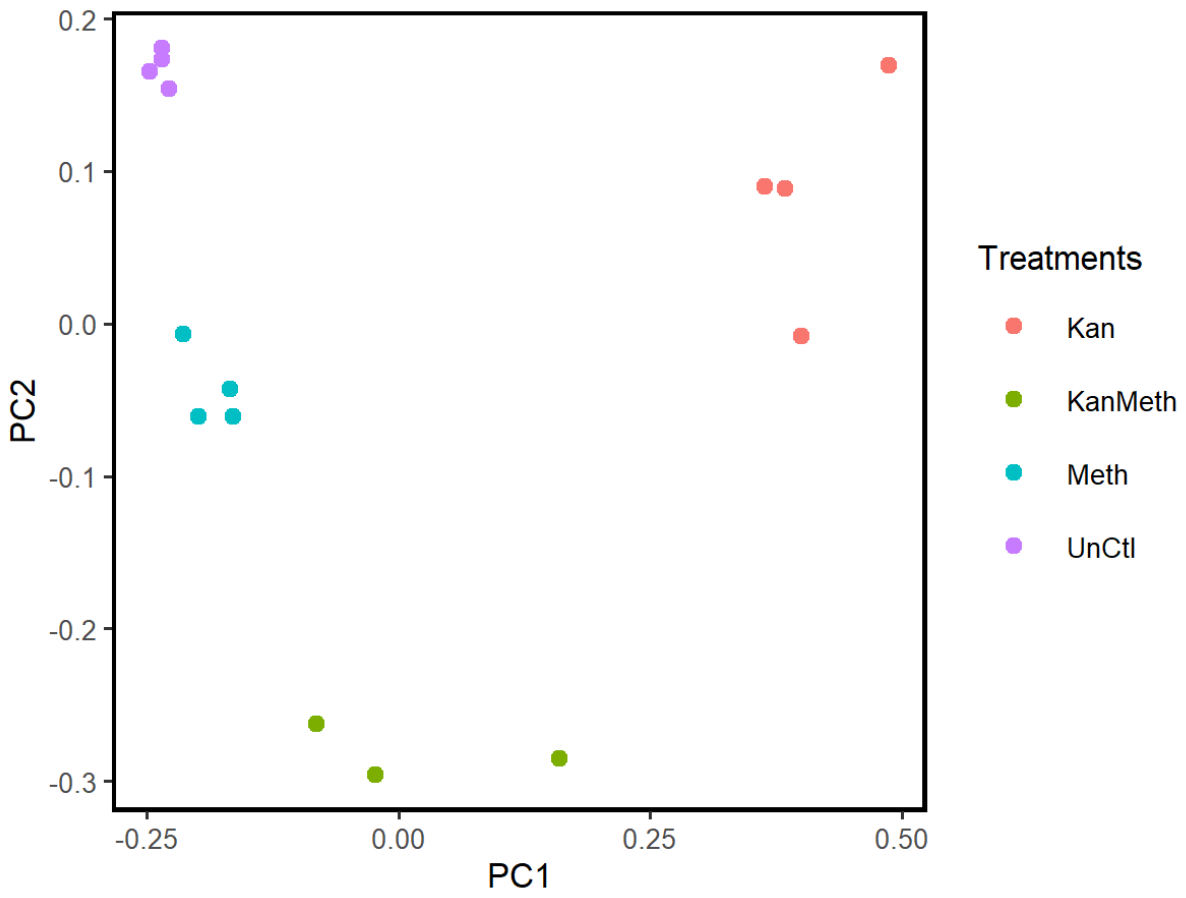


Fig. B. S2. Beta diversity- Weighted Unifrac, principal coordinate plots from 16S rRNA gene surveys. Colors represent treatment groups and individual points represent individual experimental replicates. Total replications for Kan, Meth and UnCtl were 4 while KanMeth had 3 replications (sample size = 10). Results as shown have strong statistical support (PERMANOVA, $p<0.001$). Based on pairwise PERMANOVA, each treatment is significantly different ($p<0.05$).

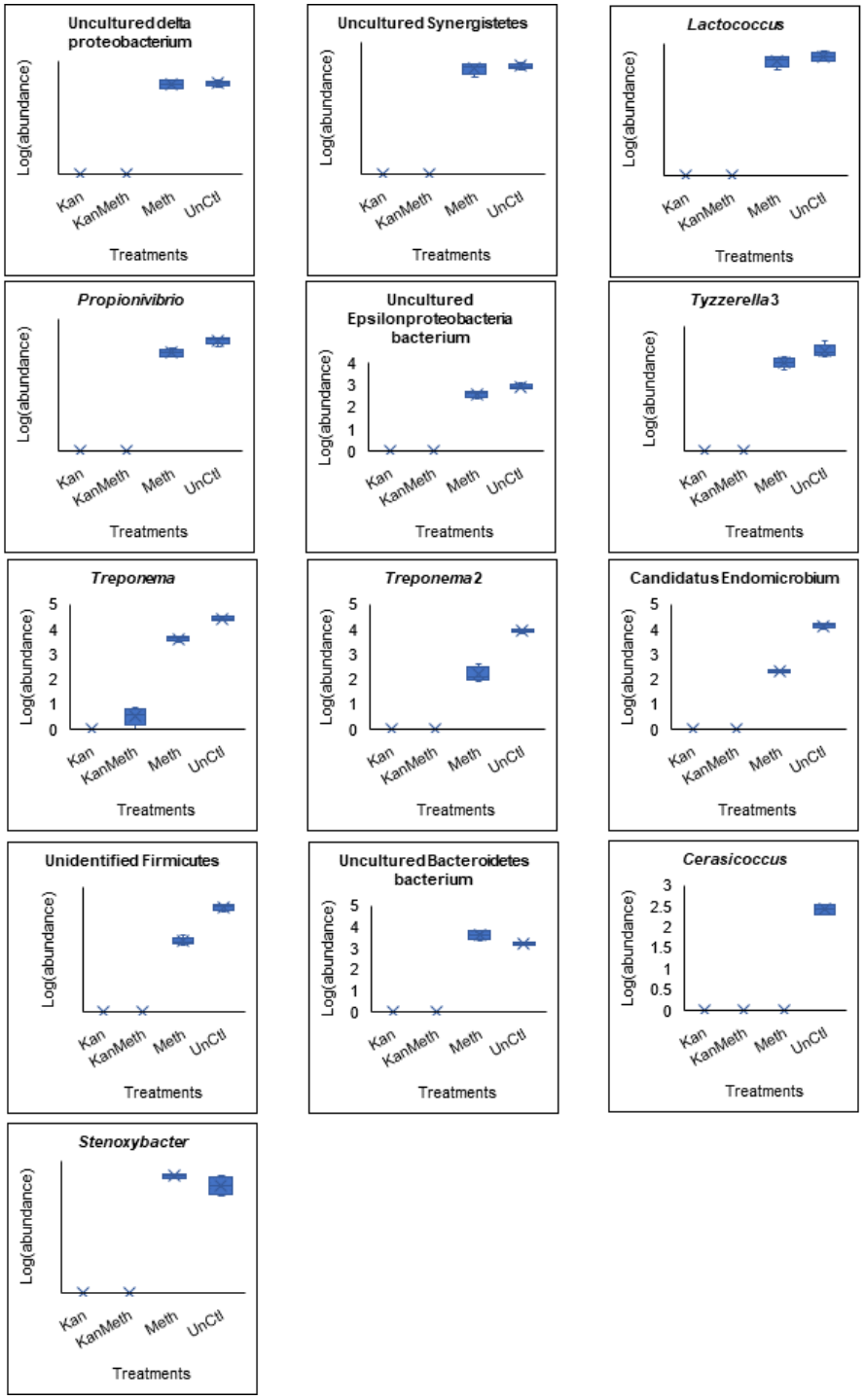


Fig. B. S3. Boxplots of the log abundances of top 13 bacterial genera (False Discovery Rate at 0.05). These genera were significantly differentially abundant as measured by ANCOM among all treatments (clr>200). Total replications for Kan, Meth and UnCtl were 4 while KanMeth had 3 replications (sample size = 10)

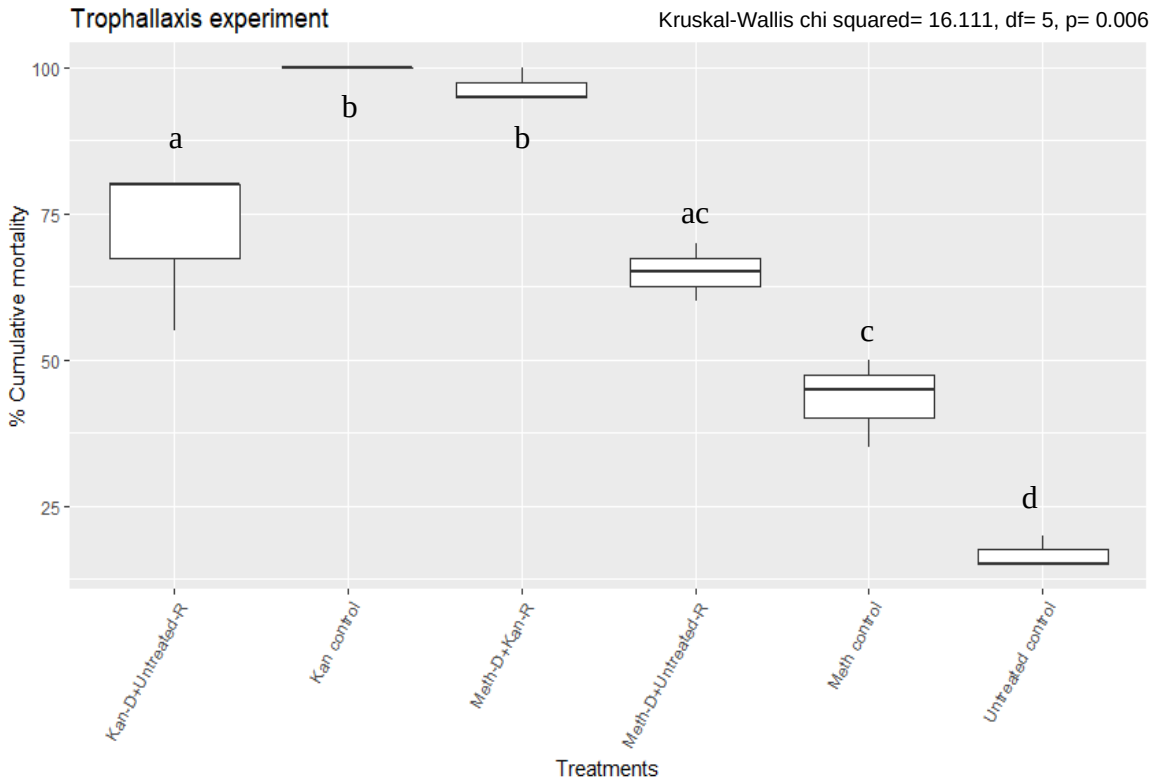


Fig. B. S4. Trophallaxis experiment on survivorship study showing: Cumulative percent mortality (3 replications, sample size = 20). Boxplots showing the median (horizontal line in the box), interquartile range (IQR, the box), samples within 1.5 times IQR (vertical lines) P-values for global Kruskal-Wallis comparison across treatments is shown at the graph. Significance levels for pairwise comparisons are indicated by lowercase letters. The individual treatment groups in the graph with the same letters are not significantly different (Mann-Whitney U-test; $p<0.05$). Treatment abbreviations are defined in the Methods text.

List of tables:

Table B. S1. Results from ANCOM analysis at genus level with W and clr- F statistics of significantly differentially abundant taxa based on the results from hypothesis testing. All the taxa that are listed True in hypothesis testing are significantly abundant.

Bacterial-ID (Genus level)	**clr**	**W**	**Reject null hypothesis**
Elusimicrobia; Endomicrobia; Endomicrobiales; Endomicrobiaceae; Candidatus Endomicrobium	1387.401	182	TRUE
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Rhodocyclaceae; Propionivibrio	1043.173	168	TRUE
Verrucomicrobia; Verrucomicrobiae; Opitutales; Puniceicoccaceae; Cerasicoccus	739.5076	159	TRUE
Synergistetes; Synergistia; Synergistales; Synergistaceae; uncultured	566.767	168	TRUE
Proteobacteria; Deltaproteobacteria; Rs-K70 termite group; uncultured delta proteobacterium; uncultured delta proteobacterium	476.9596	160	TRUE
Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Lactococcus	372.7378	174	TRUE
Epsilonbacteraeota; Campylobacteria; Campylobacterales; Rs-M59 termite group; uncultured Epsilonproteobacteria bacterium	307.831	156	TRUE
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae;_	292.9885	145	TRUE
Bacteroidetes; Bacteroidia; Bacteroidales; M2PB4-65 termite group; uncultured Bacteroidetes bacterium	286.3909	173	TRUE
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Neisseriaceae; Stenoxybacter	270.3162	172	TRUE
Spirochaetes; Spirochaetia; Spirochaetales; Spirochaetaceae; Treponema 2	261.4886	175	TRUE

Table B. S1. continued

Spirochaetes; Spirochaetia; Spirochaetales; Spirochaetaceae; Treponema	216.9411	184	TRUE
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Tyzzerella 3	201.104	152	TRUE
Verrucomicrobia; Verrucomicrobiae; Opitutales; Puniceicoccaceae; uncultured	189.9518	161	TRUE
Bacteroidetes; Bacteroidia; Bacteroidales; CR-115; uncultured bacterium	182.5917	159	TRUE
Bacteroidetes; Bacteroidia; Bacteroidales; Paludibacteraceae; uncultured	152.8	152	TRUE
Firmicutes; Clostridia; Clostridiales	145.769	142	TRUE
Bacteroidetes; Bacteroidia; Bacteroidales; Dysgonomonadaceae;__	118.601	144	TRUE
Firmicutes; Clostridia; Clostridiales; Clostridiales vadinBB60 group; uncultured Clostridiales bacterium	108.6716	143	TRUE
Actinobacteria; Coriobacteriia; Coriobacteriales; Coriobacteriales Incertae Sedis; uncultured	105.8418	150	TRUE
Patescibacteria; Saccharimonadia; Saccharimonadales; uncultured gamma proteobacterium; uncultured gamma proteobacterium	103.5559	140	TRUE
Bacteroidetes; Bacteroidia; Bacteroidales; Marinifilaceae; Odoribacter	87.32618	149	TRUE
Actinobacteria; Actinobacteria; Micrococcales; Micrococcaceae; Kocuria	80.4802	140	TRUE
Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae; Breznakia	70.22238	165	TRUE
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	65.52954	175	TRUE
Unidentified Bacteria	61.47229	156	TRUE

Table B. S1. continued

Unassigned group	61.15808	146	TRUE
Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas	60.35961	177	TRUE
Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; Alistipes	58.4699	146	TRUE
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Enterobacter	58.4645	152	TRUE
Bacteroidetes; Bacteroidia; Bacteroidales; Dysgonomonadaceae; Candidatus Symbiothrix	43.65309	139	TRUE
Bacteroidetes; Bacteroidia; Bacteroidales; Tannerellaceae; Parabacteroides	39.08342	138	TRUE
Bacteroidetes; Bacteroidia; Bacteroidales; Dysgonomonadaceae; uncultured	38.96364	154	TRUE
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; Cupriavidus	38.15444	167	TRUE
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Rhodocyclaceae; uncultured	34.65587	146	TRUE
Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; Ochrobactrum	33.7735	169	TRUE
Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetales Incertae Sedis; Candidatus Ancillula	32.1938	161	TRUE

APPENDIX C: SUPPLEMENTARY DOCUMENTS FOR CHAPTER 4

Supplementary Figure

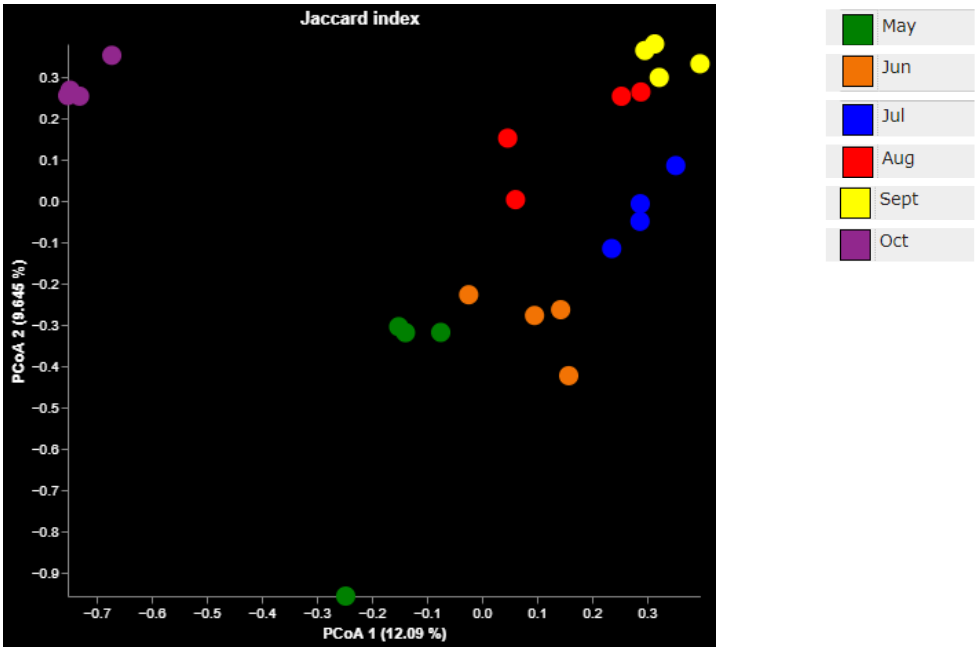


Fig. C. S1. Principal coordinate plots from Jaccard index (pseudo-F= 2.067, p = 0.001). Colors represent months and individual points represent individual replicates. Based on pairwise PERMANOVA, samples are significantly different between months ($p<0.05$).

Supplementary tables

Table C. S1. Monthly average of soil temperature data obtained from Purdue Beck Center/ACRE farm, West Lafayette, Indiana.

Sample collection date	Monthly average of soil temperature (°C)
May	15.91
June	23.06
July	24.4
August	22.18
September	17.84
October	11.04

Table C. S2. List of bacterial taxa that were significantly correlated with temperature via Spearman correlation analysis. The range of Spearman Correlation Coefficient (r) is -1 to +1. The strong positive association with temperature is described by +1 while strong negative correlation is described by -1.

Bacterial taxa	**r**	**p**
Bacteroidetes, Tannerellaceae, *Candidatus Vestibaculum*	0.885	<0.001
Firmicutes, Ruminococcaceae, uncultured	0.856	<0.001
Actinobacteria, Actinomycetales Incertae Sedis, *Candidatus Ancillula*	0.782	<0.001
Actinobacteria, Nocardioidaceae, *Nocardioides*	0.677	<0.001
Proteobacteria, Xanthobacteraceae, *Bradyrhizobium*	0.676	<0.001
Actinobacteria, Coriobacteriales, uncultured	0.659	<0.001
Patescibacteria, Saccharimonadales	0.620	0.001
Firmicutes, Clostridiales vadinBB60 group	0.610	0.002
Verrucomicrobia, Puniceicoccaceae, uncultured	0.606	0.002
Margulisbacteria, uncultured candidate division ZB3 bacterium	0.575	0.003
Actinobacteria, Coriobacteriales Incertae Sedis, uncultured	0.574	0.003
Firmicutes, Family XIII, *Anaerovorax*	0.574	0.003
Actinobacteria, Nocardioidaceae, *Aeromicrobium*	0.567	0.004
Firmicutes, Ruminococcaceae, Ruminococcaceae UCG 010	0.553	0.005
Proteobacteria, Rhizobiaceae, Allorhizobium Neorhizobium Pararhizobium Rhizobium	0.543	0.006
Patescibacteria, Saccharimonadaceae, uncultured bacterium	0.539	0.007
Actinobacteria, Micrococcaceae, Kocuria	0.532	0.007
Proteobacteria, Paracaedibacteraceae, uncultured	0.508	0.011
Firmicutes, Lachnospiraceae	0.497	0.013
Firmicutes, Ruminococcaceae	0.490	0.015
Firmicutes, Ruminococcaceae, Ruminococcaceae UCG 013	0.479	0.018
Planctomycetes, vadinHA49, uncultured planctomycete	0.472	0.020
Actinobacteria, Microbacteriaceae	0.471	0.020
Bacteroidetes, Paludibacteraceae	0.470	0.021
Bacteroidetes, Bacteroidales	0.465	0.022
Firmicutes, Family XIII, uncultured	0.451	0.027
Verrucomicrobia, Puniceicoccaceae, Verruc 01	0.448	0.028
Bacteroidetes, Sphingobacteriaceae, *Mucilaginibacter*	0.441	0.031
Bacteroidetes, Sphingobacteriaceae, *Parapedobacter*	0.441	0.031
Firmicutes, Lachnospiraceae, *Shuttleworthia*	0.434	0.034
Firmicutes, Family XIII	0.430	0.036
Elusimicrobia, Elusimicrobiaceae, *Elusimicrobium*	0.419	0.041
Firmicutes, Clostridiales vadinBB60 group, uncultured Thermoanaerobacterales	0.419	0.041
Actinobacteria, Ilumatobacteraceae, uncultured	0.405	0.050
Proteobacteria, Enterobacteriaceae, *Pragia*	-0.411	0.046

Table C. S2. Continued

Bacteroidetes, Marinifilaceae, *Odoribacter*	-0.420	0.041
Bacteroidetes, Rs E47 termite group, uncultured	-0.423	0.039
Synergistetes, Synergistaceae, uncultured	-0.423	0.039
Bacteroidetes, Bacteroidales, COB P4 1 termite group	-0.441	0.031
Bacteroidetes, Tannerellaceae, *Parabacteroides*	-0.441	0.031
RsaHF231, uncultured bacterium	-0.441	0.031
Firmicutes, Defluviitaleaceae UCG 011	-0.446	0.029
Synergistetes, Synergistaceae, *Candidatus Tammella*	-0.448	0.028
Actinobacteria, Coriobacteriales Incertae Sedis, *Raoultibacter*	-0.464	0.022
Fusobacteria, Fusobacteriaceae, *Fusobacterium*	-0.467	0.021
Firmicutes, Clostridiales vadinBB60 group, uncultured	-0.476	0.019
Synergistetes, Synergistaceae, *Fretibacterium*	-0.484	0.016
Actinobacteria, Corynebacteriaceae, *Corynebacterium 1*	-0.498	0.013
Bacteroidetes, COB P4 1 termite group, uncultured	-0.500	0.013
Bacteroidetes, Paludibacteraceae, F0058	-0.500	0.013
Proteobacteria, Rs K70 termite group	-0.507	0.011
Synergistetes, Synergistaceae	-0.508	0.011
Bacteroidetes, M2PB4 65 termite group, uncultured	-0.550	0.005
Firmicutes, Erysipelotrichaceae, *Breznakia*	-0.552	0.005
Bacteroidetes, Rikenellaceae, *Alistipes*	-0.567	0.004
Bacteroidetes, Bacteroidaceae, *Bacteroides*	-0.603	0.002
Patescibacteria, Candidatus Pacebacteria, uncultured	-0.621	0.001
Bacteroidetes, Dysgonomonadaceae	-0.634	0.001
Firmicutes, Acidaminococcaceae, *Phascolarctobacterium*	-0.645	0.001
Firmicutes, Peptostreptococcaceae, *Clostridioides*	-0.651	0.001
Firmicutes, Lachnospiraceae, *Blautia*	-0.656	<0.001
Fusobacteria, Leptotrichiaceae, *Sebaldella*	-0.664	<0.001
Proteobacteria, Alphaproteobacteria	-0.680	<0.001

APPENDIX D: SUPPLEMENTAL INFORMATION FROM SAPKOTA AND SCHARF 2022.

Inter-colony comparisons of gut microbiome composition from lab reared Eastern subterranean termites (Rhinotermitidae, Blattodea).
R. Sapkota and M. E. Scharf

List of Figures

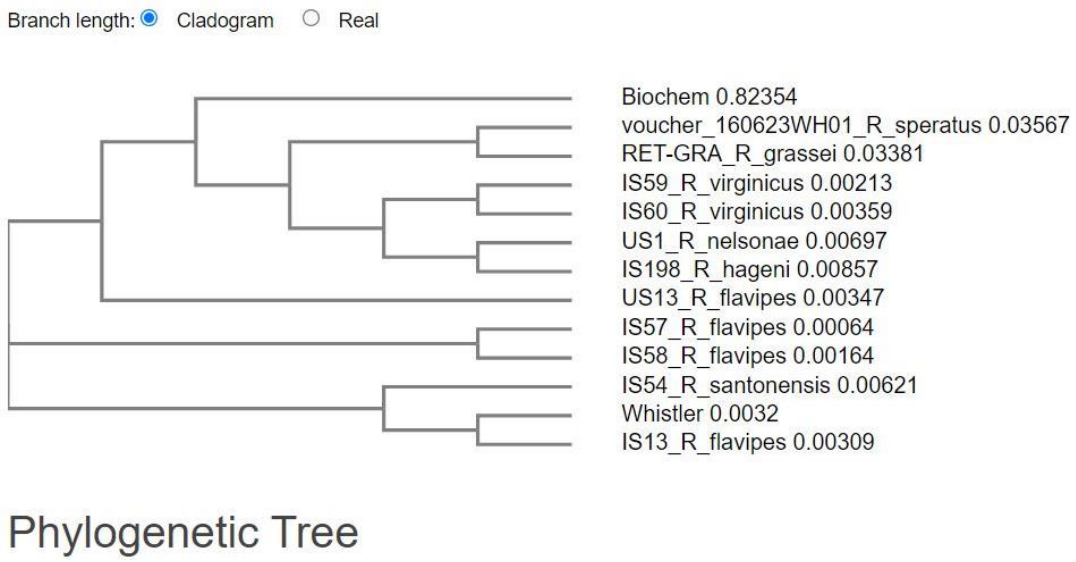
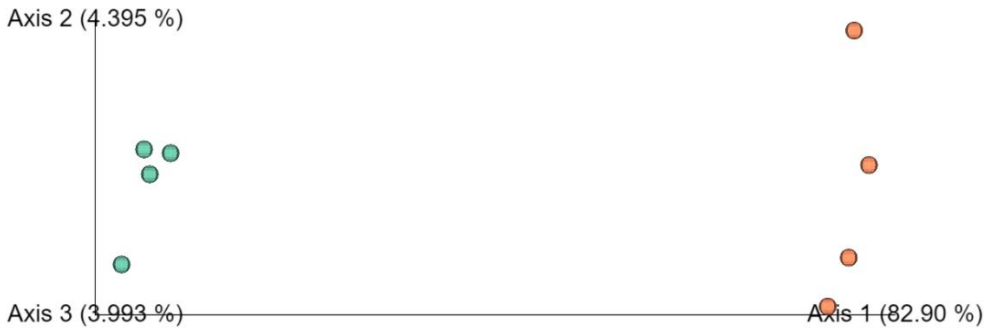
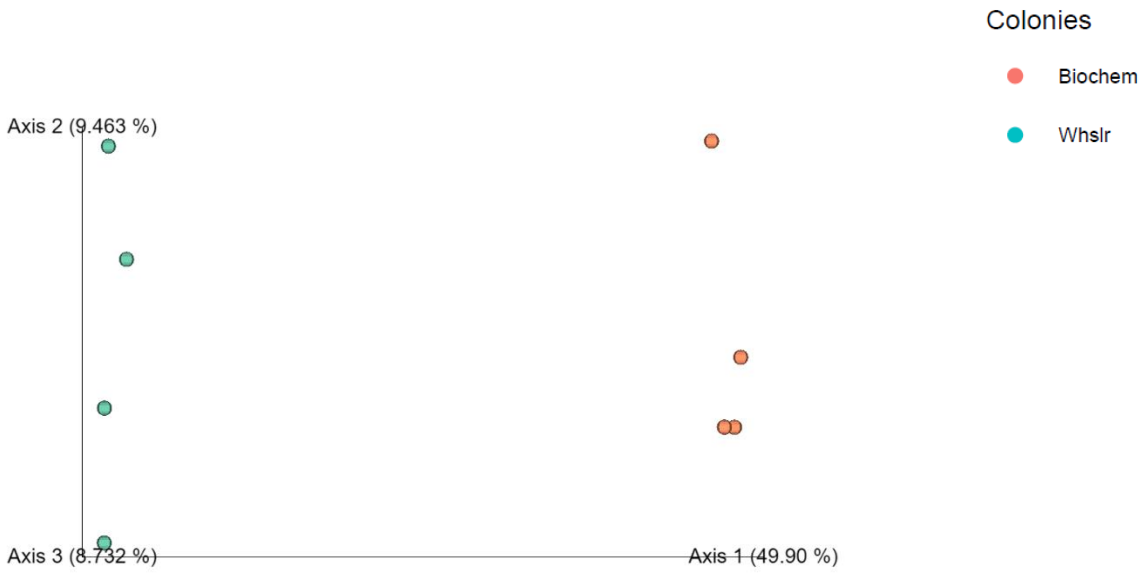


Fig. D. S1. Phylogenetic tree created by multiple sequence alignment 16S termite mitochondrial DNA through MUSCLE algorithm. The occurrence of Biochem and Whistler colony on different nodes of phylogenetic tree indicates that these are two different isolates of *R. flavipes*.



A.



B.

Fig. D. S2. Beta diversity metrics- Bray-Curtis, emperor plots obtained from QIIME2 visualization (PERMANOVA, pseudo-F= 28.755; p = 0.0028) (A). Jaccard index, emperor plots obtained from QIIME2 visualization (PERMANOVA, pseudo-F= 5.964; p = 0.031) (B). Colors represent colonies and individual points represent individual replicates. Based on pairwise PERMANOVA, colonies are significantly different ($p<0.05$).

VITA

RAJANI SAPKOTA

Education

- BS Agricultural Science, Institute of Agriculture and Animal Science Tribhuvan University, Nepal (2014).
- MS Entomology, Louisiana State University (August 2018)
- PhD Entomology, Purdue University (anticipated May 2022)

Honors and Awards

- 2022 Bilsland Dissertation Fellowship, Purdue University (Spring 2022)
- BASF Professional Pest Control Scholarship, Purdue University (January 2022)
- Duane Edwards/ Arab Pest Control Scholarship, Purdue University (January 2022)
- Terminix Scholarship, Purdue University (January 2022)
- Pest Management Professional Magazine Scholarship, Purdue University (January 2021)
- Pi Chi Omega Norm Ehmann Scholarship, Pi Chi Omega (2020)
- 2020 College of Agriculture's Dr. Dagnatchew Yirgou Endowment Travel Award, Purdue University (2020, Partially used)
- National Conference on Urban Entomology/ Student travel award, NCUE (2020, Not used)
- A-Mark Pest Management/Eli Lilly Scholarship, Purdue University (January 2020)
- 2019 College of Agriculture's Dr. Dagnatchew Yirgou Endowment Travel Award, Purdue University (2019)
- Ross fellowship, Purdue University (Fall 2018)
- Agriculture merit scholarship, Tribhuvan University, Nepal (2010-2014)

Publications

Peer reviewed

- **Sapkota, R.,** and M. E. Scharf. 2022. "Inter-colony comparisons of gut microbiome composition from lab reared Eastern subterranean termites (Blattodea: Rhinotermitidae)". (In press: Journal of Insect Science).
- **Sapkota, R.,** C. H. Nakatsu, and M. E. Scharf. 2021. "Regulation of host phenotypic plasticity by gut symbiont communities in the Eastern Subterranean termite (*Reticulitermes flavipes*)". Journal of Experimental Biology. 224: jeb242553

Direct Submission

- Wolfe Z., and **R. Sapkota**. July 2020. "Is gut bacteria a secret weapon?" Pest Control Technology. http://magazine.pctonline.com/article/july-2020/is-gut-bacteria-a-secret-weapon.aspx
- **Sapkota, R.**, and Z. Wolfe. April 2020. "Microbes and Management: The tiny world inside a termite may provide future pest management insights." Pest Control Technology. http://magazine.pctonline.com/article/april-2020/microbes-management.aspx