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INVESTIGATING PHYSIOLOGICAL COLLABORATIONS BETWEEN A LOWER TERMITE AND ITS SYMBIONTS

For the degree of Doctor of Philosophy

Is approved by the final examining committee:

Michael E. Scharf Chair	Jeffery J. Stuart
Peter E. Dunn	
Catherine A. Hill	
John Patterson	

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Approved by Major Professor(s): Michael E. Scharf

Approved by: Christine A. Hyrcyna 3/30/2016

Head of the Departmental Graduate Program Date

INVESTIGATING PHYSIOLOGICAL COLLABORATIONS BETWEEN A LOWER TERMITE AND ITS SYMBIONTS

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Brittany F Peterson

In Partial Fulfillment of the

Requirements for the Degree

of

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ABSTRACT

Peterson, Brittany F. Ph.D., Purdue University, May 2016. Investigating Physiological Collaborations between a Lower-Termite and its Symbionts. Major Professor: Michael E. Scharf.

This project was completed in an effort to better understand the contributions of symbiotic microbes to the biology of *Reticulitermes flavipes,* the eastern subterranean termite. Lower-termites, like *R. flavipes,* house symbionts from all three domains of life within their hindgut paunch. This intimate association is reflected in nearly every aspect of termite biology. Here, I investigate these physiological collaborations as they relate to digestion and immunity. My efforts focused on 1) quantifying the role of bacteria in wood digestion within the termite gut, 2) evaluating the role of symbionts in protection against pathogens, and 3) identifying gene products that bacterial symbionts contribute to naïve and pathogen-challenged termites. Bioassays coupled with *in vitro* enzyme assays, gene expression analysis, and symbiont population estimations show that termites with reduced gut fauna have less lignocellulolytic potential. Using a suite of antimicrobial compounds, bacterial contributions (direct or indirect) to wood digestion were calculated on average at ~23-50%. Apart from digestive potential, termite gut symbionts were also implicated in pathogen-resistance. Defaunated workers were 2-3X more susceptible to fungal infections and had significantly altered the expression of endogenous, immune-associated genes in response to challenge with the bacterial entomopathogen, *Serratia marcescens*.

Using recombinant enzymes, *in vitro* assays, and bioassays, two protist-derived glycosyl hydrolase family 7 (GHF7) enzymes showed promise as a potential mechanism for symbiont-derived, anti-fungal defense in *R. flavipes*. These signatures of symbiont-

mediated immunity/protection were further explored using RNAseq to capture a snapshot of the termite holobiont transcriptional response following pathogen challenge. This strategy served as a means of pinpointing critical taxa and physiological roles of microbes in this system by taking a global, metatranscriptomic approach. Differential expression analysis identified a bacterially-encoded amidohydrolase that may be important for anti-fungal defense.

Overall, this project has significantly expanded our perspective on the importance of microbes in termite physiology as a whole. With examples from digestion and immunity, this research lays the groundwork for future explorations of termite-symbiota collaborations including, but not limited to, the collaborations described herein.

Together, these results highlight the importance of a holistic “systems biology” approach to understanding termite biology from the perspective of the termite’s intimate associations with microbes. Assessing termite and microbe responses in isolation (as independent systems) may not provide an accurate account of the collaborative nature of this relationship. Additionally, my findings emphasize the importance of considering both organismal (termite and microbe) and sub-organismal (cellular and molecular) level processes when investigating physiology, symbiosis, and the link between them.

CHAPTER 1. LOWER TERMITE ASSOCIATIONS WITH MICROBES: SYNERGY, PROTECTION, AND INTERPLAY[†]

1.1 Abstract

Lower-termites are one of the best studied symbiotic systems in insects. Their ability to feed on a nitrogen-poor, wood-based diet with help from symbiotic microbes has been under investigation for almost a century. A unique microbial consortium living in the guts of lower termites is essential for wood-feeding, host and symbiont cellulolytic enzymes synergize each other in the termite gut to increase digestive efficiency. Because of their critical role in digestion, gut microbiota are driving forces in all aspects termite biology. Social living also comes with risks for termites. The combination of group living and a microbe-rich habitat makes termites potentially vulnerable to pathogenic infections; however, the use of entomopathogens for termite control has been largely unsuccessful. One mechanism for this failure may be symbiotic collaboration; i.e., one of the very reasons termites have thrived in the first place. Symbiont contributions are thought to neutralize fungal spores as they pass through the termite gut. Also, when the symbiont community is disrupted pathogen susceptibility increases. These recent discoveries have shed light on novel interactions for symbiotic microbes both within the termite host and with pathogenic invaders. Lower-termite biology is therefore tightly linked to symbiotic associations and their resulting physiological collaborations.

1.2 Introduction

The close association of lower termites with microbes is fundamental to their biology. For the last century, understanding the intricacies of the relationship between termites and their gut symbionts, i.e. the termite holobiont, has been a major focus of termite research.

[†]Parts of this chapter are published in, "Peterson, B.F., and Scharf, M.E., 2016. Lower termite associations with microbes: Synergy, protection, and interplay. Frontiers in Microbiol. 7, 422.

The majority of this work emphasizes both the complexity and novelty of functions carried out to process lignocellulose within the termite gut (*reviewed in* Brune 2014). For decades, termite wood digestion has been a quintessential example of symbiotic collaboration; however, symbionts have also been associated with a myriad of other functions in this system (*reviewed in* Ohkuma 2008). For example, in addition to synergistic digestive collaboration, symbionts of lower termites have also been shown to play protective roles against pathogens both *in vivo* and *ex vivo* (Rosengaus *et al.* 1998, Chouvenc *et al.* 2009, Chouvenc *et al.* 2013, Rosengaus *et al.* 2014). This interaction between the termite symbiotic consortium and potential pathogens adds a layer of interplay within this already-complex microbial community. Here we summarize the diversity and roles symbionts play in lower termites, highlight the broad implications of both topics for understanding termite biology and symbiotic evolution, and emphasize how a holistic approach to studying termite biology is necessary to encompass the impact of this obligate symbiotic association.

Lower termites are distinct from higher-termites in that they form relationships with both eukaryotic and prokaryotic symbionts within their digestive tracts (Eutick *et al.* 1978). While the diversity, abundance, and functionality of these symbionts fluctuates from species to species, an association with symbionts is ubiquitous and connected with much of the biology of termites. Fundamental defining aspects of lower termites, from eusociality to niche occupation, are impacted by their obligate association with microbes. Disruption of this community impacts termite physiological function, fitness, and survivorship (Cleveland 1924, Thorne 1997, Rosengaus *et al.* 2011, Rosengaus *et al.* 2014, Peterson *et al.* 2015, Sen *et al.* 2015). Lower termites house protists (unicellular eukaryotes), bacteria, and archaea all within the one-microliter environment of their hindgut, many of which are never found outside of this association. Restricted to their association with termites, these symbionts are exposed to and must tolerate a variety of chemical and biological stressors in the termite gut microenvironment. As the host termite feeds, forages, grows, and encounter pathogens, its symbiota are impacted. Thus, termites cannot be studied without also considering their symbionts. Characterizing and

cataloging these microbes poses many challenges because most are unable to be cultured with traditional techniques due to their fastidious nature. This gut microenvironment boasts organismal and metabolic diversity which rivals some of the better studied macro-ecosystems. Approaching the termite holobiont as a fully-functional, multifaceted ecosystem allows for concentration on individual species or processes and on the larger collaborative nature of the gut microenvironment.

1.3 Characterizing the Lower Termite Gut Consortium

The key division between lower and higher termite species is the respective nature of their symbiotic partners. While both retain prokaryotic symbionts, lower termites also have flagellated protists living in their guts which is an ancestral trait shared with wood-feeding cockroaches, *Cryptocercus* spp. (Stingl and Brune 2003, Lo and Eggleton 2011, Brune and Dietrich 2015). These protists belong to two groups: the oxymonads and the parabasalids. Originally described as parasites, protists were first found associated with termites over a century ago (Leidy 1877). Since this original observation, roughly 500 termite-associated protist species have been described (*reviewed in* Ohkuma and Brune 2011). As technology advances we are continually able to improve our understanding of the players and complexity of the termite gut community. In fact, new species of protistan symbionts are continually described from lower termite guts (Brugerolle and Bordereau 2004, Gile *et al.* 2012, James *et al.* 2013, Tai *et al.* 2013, Radek *et al.* 2014), and the breadth of their diversity is thought to be drastically underestimated in general (Harper *et al.* 2009, Tai and Keeling 2013). That being said, lower termites are thought to possess anywhere from a few to a dozen protist species as symbionts that maintain tight phylogenetic associations with their hosts (Tai *et al.* 2015).

As has happened with protist symbionts, our understanding of the bacterial consortium composition in lower termites is constantly evolving as methodologies and analyses improve. Early estimates from the eastern subterranean termite, *Reticulitermes flavipes*, numbered bacteria per gut in the millions, which seems to be a conservative

approximation at best (Schultz and Breznak 1978). Using culture-independent, cloning based methods, several groups have estimated the guts of lower termite species to contain anywhere from 222-1,318 ribotypes of bacteria (Hongoh *et al.* 2003a, Hongoh *et al.* 2003b, Shinzato *et al.* 2005, Yang *et al.* 2005, Fisher *et al.* 2007). With the onset of next-generation sequence technologies this number has only grown. More recently, the gut lumen content of *R. flavipes* workers was described to contain over 4,761 species-level phylotypes of prokaryotic symbionts, with over 99% being bacteria (Boucias *et al.* 2013). The majority of these identified phylotypes are unique to the termite gut, having never been reported elsewhere and not having close-relative sequences available in databases. *Coptotermes gestroi* has been estimated to house 1,460 species of bacteria using Illumina technology (Do *et al*. 2014). These estimates vary for a variety of possible reasons, including local environment, study locus, methodological limitations/caveats, sampling strategy, diet, genetic background, and termite species. While identifying the microbial players within this system is an important step, describing the functions and interplay between them will be equally necessary for understanding termite biology and evolution.

1.4 Symbiotic Collaboration in Termite Digestion and Nutrition

Apart from cataloging symbiont diversity, much of termite research has focused on their associations with the symbiotic microbes which aid in wood digestion. Feeding on this lignin-rich, nitrogen-poor diet requires a suite of enzymes both to catalyze its breakdown and supplement its nutritional deficiencies. Termites and their symbionts complement each other's capabilities in this way. Termites contribute several highly active enzymes important to this process including endogenous cellulases (β-1, 4-endoglucanase, β-glucosidase) and lignin/phenolic detoxifiers (aldo-keto reductase, laccase, catalase, cytochrome p450s) (Scharf *et al.* 2010, Zhou *et al.* 2010, Raychoudhury *et al*. 2013, Sethi *et al.* 2013a). Protists in the hindgut of lower termites have been credited with the contribution of several important glycosyl hydrolases (GHFs 5, 7, 45) which aid in cellulolytic activity and are important in hydrogen cycling (Ohtoko *et al.* 2000, Inoue *et al.*

2005, Inoue *et al.* 2007*,* Todaka *et al.* 2010, Sethi *et al*. 2013b). Based on transcriptomic studies, protists possess many more potentially important cellulases (Todaka *et al.* 2007, Tartar *et al.* 2009). Also, both the termite host and protist symbionts possess proteases which may be important for utilizing bacteria as sources of nitrogenous compounds (Sethi *et al.* 2011, Tokuda *et al.* 2014). Although both protists and bacteria possess many hemicellulases (Inoue *et al.* 1997, Tartar *et al.* 2009, Tsukagoshi *et al.* 2014), termite endogenous cellulases have been shown to have hemicellulase activity as well (Scharf *et al.* 2010, Scharf *et al.* 2011, Karl and Scharf 2015). However, despite this apparent hemicellulolytic redundancy, protists, bacteria, and archaea in the hindgut paunch clearly all contribute significantly to the overall efficiency of wood digestion (Peterson *et al.* 2015).

While protists are mainly responsible for lignocellulolytic activity, the prokaryotic community provides a more diverse subset of services in the termite gut. Spirochetes, the most conspicuous bacterial group in lower termite guts, are capable of diverse metabolic processes including acetogenesis, nitrogen fixation, and degradation of lignin phenolics (Lilburn *et al.* 2001, Graber and Breznak 2004, Lucey and Leadbetter 2014). The isolation and maintenance of pure cultures of several species of spirochetes from lower termite guts has been a powerful tool for describing their metabolic capabilities and collaborative potential within the community as a whole (Leadbetter *et al.* 1999, Lilburn *et al.* 2001, Salmassi and Leadbetter 2003, Graber *et al.* 2004, Graber and Breznak 2004, Graber and Breznak 2005, Dröge *et al.* 2006, Rosenthal *et al.* 2011).

Another major component of lower termite microbiota are the bacteria which are intimately associated with gut flagellates as intracellular endosymbionts (Stingl *et al.* 2005, Noda *et al*. 2009). There are four phyla of bacterial endosymbionts found within protist cells: Elusimicrobia, Bacteroidetes, Proteobacteria, and Actinobacteria (Hara *et al.* 2004, Noda, *et al.* 2005, Stingl *et al.* 2005, Strassert *et al.* 2012). These groups have been found to ferment glucose, synthesize amino acids, produce cofactors, fix nitrogen, and recycle nitrogenous wastes (Noda *et al.* 2007, Hongoh *et al.* 2008a, Hongoh *et al.* 2008b, Ohkuma and Brune 2011, Strassert *et al.* 2012, Zheng *et al.* 2015). *Methanobrevibacter*, a

methanogenic archaeal genus common across termite-associated flagellates, contribute methane to the gut environment using hydrogen that is present in copious amounts in the gut lumen as a product of cellulose metabolism (Shinzato *et al.* 1999, Tokura *et al.* 2000, Hara *et al.* 2004, Hongoh and Ohkuma 2011). This adds another level of complexity to termite gut ecology by creating a tripartite symbiosis: *prokaryotes within protozoa within termites*.

Apart from archaea associated with termite gut flagellates, representative Methanobacteriaceae are also associated with the microaerobic termite gut lining (Leadbetter and Breznak 1996, Ohkuma *et al.* 1999, Brune 2011). Together with the flagellate endosymbiota, the large amount of methane created by termite digestion can be attributed to archaea which are typically associated with the hindgut lining (Brune 2011, Hongoh and Ohkuma 2011). In sum, the microbes present in lower termite guts comprise a diverse ecosystem capable of nitrogen cycling, carbohydrate metabolism, methanogenesis, amino acid biosynthesis, hydrogen turnover, and consequently, complementing deficiencies of the host.

In addition to the contributions of individual organisms, the host fraction (foregut, midgut, and salivary glands) and the symbiont fraction (hindgut) of the termite digestive system have been shown to work synergistically (Scharf *et al*. 2011). While both fractions have lignocellulolytic activity, combining protein extracts from both the host and symbiont fractions results in more sugar release *in vitro* than the sum of the parts. Additionally, recombinant host and symbiont enzymes have been shown to work efficiently *in vitro* to liberate glucose and pentose sugars from wood (Sethi *et al*. 2013b). Hence, wood digestion is truly the result of successful collaboration between termites and their hindgut symbionts. This collaborative physiological functionality is a driver in termite success and niche occupation, and it should continue to be a major focus to understand termite holobiont biology and ecology as we go forward.

1.5 Symbiont-Pathogen Interplay

Social living and foraging in microbe-rich environments puts termite workers at risk to encounter pathogens and creates the potential for epizootic events within termite colonies. Though the relationship between termites and their symbionts is often perceived to be purely nutritional, there is growing evidence that gut microbiota have infection-buffering potential. However, termites also have evolved complex hygienic behaviors to mitigate the spread and persistence of pathogenic agents (i.e. fungal conidia) within colonies (Rosengaus *et al.* 1998, Rosengaus *et al.* 2011, Gao *et al.* 2012). Termites have been frequently observed to auto- and allogroom conidia from the bodies of themselves and nestmates. Passage through the alimentary canal and symbiont-filled hindgut effectively neutralizes fungal conidia (Chouvenc *et al.* 2009). Termites with perturbed gut microbiota, by oxygenation or chemical means, display a marked increase in susceptibility to fungal pathogens such as *Metarhizium anisopliae* and *Beauveria bassiana* (Boucias *et al.* 1996, Ramakrishnan *et al.* 1999, Rosengaus *et al.* 2014, Sen *et al.* 2015). One biochemical mechanism has been linked to this anti-fungal gut phenomenon in the form of symbiont-derived β-1, 3-glucanase activity (most likely protist) that is able to act on fungi and prevent their germination (Rosengaus *et al.* 2014). Similarly, the inhibition of this antifungal enzyme activity, β-1, 3-glucanase, results in a marked increase in termite susceptibility to a variety of pathogens (Bulmer *et al.* 2009) and is conserved evolutionarily from woodroaches to termites (Bulmer *et al.* 2012).

As mentioned above, grooming and hygienic behavior play an important role in termite immunity. Termites also participate in proctodeal trophallaxis as a means to replenish symbionts, nutrients, and chemical signals amongst individuals in the colony (Suarez and Thorne 2000, Machida *et al*. 2001). This is another means by which symbionts and potential pathogens may interact, but it does not seem to play an important role in immune priming (Mirabito and Rosengaus 2016).

Lastly, outside of the termite body, symbiotic bacteria provide additional protection. Termites build elaborate nest structures from fecal material to house their colonies. As

with hindgut populations, these nest materials contain varying degrees of microbial abundance and richness dependent upon the species of termite (Rosengaus *et al.* 2003). This material contains diverse kinds of bacteria but has comparatively less fungus (Rosengaus *et al.* 2003, Manjula *et al.* 2015). The nests of one species of subterranean termite, *Coptotermes formosanus*, are commonly laden with symbiotic Actinobacteria demonstrated to have antifungal activity *ex vivo* in nest walls (Chouvenc *et al.* 2013). This finding extends symbiont-mediated protection from the termite gut outside into the nest material, in at least one species.

1.6 Concluding Remarks

Lower termite symbioses with microorganisms are unmistakably integral to termite biology. Hindgut microbial communities are tightly linked with termite digestion of wood and play important roles in supplementing this nutrient-poor food source. Symbionts catalyze reactions involved in the breakdown of all three major components of wood (cellulose, hemicellulose, and lignin phenolics) and supplement this diet by synthesizing other important nutrients. However, outside of the classic role for termite symbionts in digestion and nutrition, there is increasing recognition that they buffer the impacts of environmental stressors to their hosts. In particular, both protists and bacteria have been found to provide anti-fungal defenses in lower termites (Chouvenc *et al*. 2013, Rosengaus *et al.* 2014). Even fitness is impacted by the interconnectivity between termites and their symbionts (Rosengaus *et al.* 2011). Recent discoveries emphasize that despite nearly a century of studying the obligate relationships between lower termites and microbes, there are still many facets of this complex association which are yet to be understood. Lower termites provide an important model for studying persistent, multi-layer symbioses.

It is also important to consider the role that symbiota play in other animal systems for the purpose of formulating relevant questions to probe, interrogate and eventually understand the termite holobiont. Recent discoveries in other models highlight microbiota as playing more active roles in host physiology, development, and behavior.

These roles extend further than the bounds of the intestinal walls, affecting a range of processes from immune system development/maturation to mood and pain perception (Sommer and Backhed 2013). The broad influence of gut microbiota found in these other systems can serve as an excellent guide to generate hypotheses for testing in the termite system.

Moving forward, based on recent and emerging trends, it will be imperative to consider all components of the termite holobiont when investigating aspects of termite biology. Understanding the role of symbiotic microbes in the physiological processes of digestion and immunity represent some of the first steps toward a better understanding of the broader functionality of the lower termite consortium. Viewing any of these interactions within the termite holobiont as discrete may be an oversimplification. However, as methodologies and analyses advance, our ability to understand the functions of the consortium as a whole will continue to improve, as will our understanding of the roles of individual taxa in the system, and collaborations between host and symbiota. Efforts to characterize the holobiont in the presence and absence of stressors, both biotic and abiotic, using comprehensive omics-based approaches are likely to be major hypothesis-generating endeavors. However, the key to doing this successfully will involve careful sample preparation and carefully constructed analysis pipelines to limit taxonomic biases whenever possible. These big data approaches will in turn become a springboard into understanding symbiotic association, trends and commonalities, which may help to begin building models for the compartmentalization, complementation, and collaboration between lower termites and their symbiota.

Understanding the extent, bounds, and ramifications of these associations will be necessary to move towards a fuller appreciation of lower termite biology. Ultimately, studying the collective function and interplay between all members of this symbiosis in response to environmental challenges and in periods of stasis will shed light both on the micro-ecosystem that is a termite gut and the super-organism that is a termite colony.

1.7 Dissertation Scope and Statement of Objectives

Recently, advances have been made toward understanding lower termite-microbe interactions. The *central goal* of this dissertation was to develop a better understanding of the collaborative nature of the termite symbiotic consortium. My *central hypothesis* was that *R. flavipes* and their symbiotic partners collaborate physiologically to accomplish basic tasks such as digestion and immunity. Taking a more holistic view of the termite system in this way, while making certain questions more challenging to address, has the payoff of being able to capture the true nature of this symbiosis as a whole. To accomplish my overall goal, the termite holobiont was interrogated using a combination of bioassays, enzyme assays, classic microbiology, and gene expression analyses (including next-generation sequencing). Because it is a classic model of symbiosis, investigating the intricacies of termite-microbe associations can lead to transformative advances in our understanding of animal-bacterial mutualisms. Ultimately, characterizing the diverse roles of symbionts in the termite hindgut provides the potential for development of novel pest-control approaches, creative applications to the biofuel industry, biomedical advances, and a deeper appreciation for the evolution of symbiosis.

The *specific aims* of this dissertation were to 1) determine the impact of prokaryotic symbionts on termite digestion efficiency, 2) investigate pathogen specificity and potential mechanisms of symbiont-mediated immune defense, and 3) identify symbiotic contributions in response to pathogen challenge using metatranscriptomics. In sum, these objectives link the mechanistic contributions of different symbiotic groups to termite homeostasis and biology, and also emphasize the collaborative nature for the termite gut consortium with its host. Using a variety of organismal, molecular, and "omics" methods, this work takes an interdisciplinary approach to clarifying the diversity of functions microbes carry out within the termite holobiont.

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CHAPTER 2. QUANTIFICATION OF SYMBIOTIC CONTRIBUTIONS TO LOWER-TERMITE LIGNOCELLULOSE DIGESTION USING ANTIMICROBIAL TREATMENTS‡

2.1 Introduction

The wide variety of microbial niches on Earth has led to a tremendous diversity of prokaryotes that essentially occupy and exploit all available microenvironments. As a result, bacteria and archaea are frequently found living in symbiosis with other organisms. Animal-microbe symbioses are ubiquitous throughout nature. From the vectoring of plant and animal pathogens to housing co-evolved, intracellular mutualists, insects (the most diverse and populous group of animals) form a wide variety of symbioses with microbes. The eastern subterranean termite, *Reticulitermes flavipes* (Kollar), which hosts gut symbionts from all three domains of life, is a unique model system in which to study complex symbiotic interactions. This tripartite symbiotic system serves as an important model for understanding the co-evolution of interactions, physiologies, and specialization of animal-microbe relationships.

The termite holobiont, consisting of the host and its associated microbes, is an obligate, synergistic system (Brune and Ohkuma, 2011; Scharf *et al.*, 2011). Together these organisms work to digest lignocellulose, a complex, nitrogen-poor food source. Liberating monosaccharides from wood requires a cocktail of cellulases, hemicellulases and accessory enzymes (Sethi and Scharf, 2013). Metabolic collaboration between the termite host and its symbionts makes the hindgut an efficient bioreactor, capable of efficiently liberating sugar from cellulose and hemicellulose sequestered in lignocellulose (*Reviewed in:* Breznak and Brune 1994; Brune 2014; Brune and Ohkuma 2011; Ohkuma 2003).

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Though the co-evolution of these symbionts with their host is still unclear (Dietrich *et al.*, 2014), it is well established that all termites are closely associated with a consortium of microbes which augment endogenous host physiology. Bacteria have been credited with a myriad of functions in the termite gut, from nitrogen fixation to fermentation (Breznak and Leadbetter, 2007; Lucey and Leadbetter, 2014; Stanton and Canale Parola, 1980; Warnecke *et al.*, 2007; Wyss *et al.*, 1997). Given that there are over 4,000 species-level OTUs of bacteria in the *R. flavipes* gut (Boucias *et al.*, 2013), the potential for functional redundancy and interdependence is high.

Although the diversity of the *R. flavipes* hindgut has been elucidated with the development of sequencing strategies, the importance of prokaryotic symbionts in lignocellulose digestion remains unclear (Boucias *et al.*, 2013; Yang *et al.*, 2005). This diversity is dominated by Spirochetes (25-55%), Elusimicrobia (11-25%), Firmicutes (10-20%), and Bacteroidetes (5-15%) (Boucias *et al.*, 2013; Yang *et al.*, 2005). Spirochetes, the most abundant phylum in the hindgut, are credited with an array of metabolic capabilities including carbohydrate and aromatic ring metabolism, both of which could directly contribute to lignocellulose degradation (Lucey and Leadbetter, 2014; Stanton and Canale Parola, 1980; Wyss *et al.*, 1997). Elusimicrobia are important endosymbionts of cellulolytic protists in the hindgut of *R. flavipes* and other lower termites (Brune, 2012). Genomic sequencing efforts have shown that the Elusimicrobia are important for nitrogen fixation, but lack lignocellulase coding sequences (Hongoh *et al.*, 2008). Additionally, cellulolytic Firmicutes have recently been isolated from the guts of other xylophagous insects (Hu *et al.*, 2014, Mikaelyan *et al.*, 2014). To date, the prokaryotic contribution to other metabolic processes such as acetogenesis, nitrogen fixation, fermentation, methanogenesis and amino acid synthesis within the termite holobiome has been empirically supported (Breznak, 2002; Graber and Breznak, 2004; Hongoh *et al.*, 2008; Ohkuma *et al.*, 1996; Wertz *et al.*, 2012). Cultured bacteria from termite guts have also shown genomic evidence of vitamin synthesis capabilities (Graber and Breznak, 2004). A recent effort to mine the *Coptotermes gestroi* holobiome for cellulases shows the potential cellulytic roles for prokaryotes in lower termite systems (Do *et al.*, 2014).

Despite their abundance, as well as phylogenetic and metabolic diversity, the relative importance of *R. flavipes* gut bacteria to wood digestion by the gut holobiome remains unknown.

Proportionally greater research has focused on the eukaryotic members of the gut consortium, the protists. Termite gut protists are known to contribute many important enzymes like exoglucanases, endoglucanases and hemicellulases to the digestive process (Tartar *et al.*, 2009; Todaka *et al.*, 2007, 2010). These protist enzymes synergize with a number of highly-expressed enzymes, cellulolytic and otherwise, from the termite host (Coy *et al.*, 2010; Scharf *et al.*, 2010, 2011; Sethi *et al.*, 2013a, 2013b; Zhou *et al.*, 2010). However, the contributions of the prokaryotic symbionts to this process remain unclear.

Antimicrobial treatments have been an important tool for investigating other functions of microbiota in termites. Treatment with metronidazole in the higher termite *Nasutitermes exitiosus* showed that the removal of Spirochetes resulted in a reduction in lifespan (Eutick *et al.*, 1978). In *R. flavipes*, antibiotics were used to demonstrate the uricolytic activity of gut bacteria (Potrikus and Breznak, 1981). Nestmate recognition can be impeded by antibiotic treatment in lower termites (Matsuura, 2001; Kirchner and Minkley, 2003). Primary reproductives from *R. flavipes* and *Zootermopsis angusticollis* were found to have significant reductions in longevity, fecundity, and weight when treated with rifampin (Rosengaus *et al.*, 2011). Finally, in the higher termite *Nasutitermes takasagoensis*, antibiotic treatment clarified the role of bacteria in lignocellulose digestion in a system lacking protists (Tokuda and Watanabe, 2007).

The goal of this research was to quantify the importance of bacteria in lignocellulose digestion within the *R. flavipes* holobiont. We used antimicrobial compounds as a subtractive tool to test the hypothesis that, in addition to endogenous termite and protist-contributed enzymes, prokaryotic symbionts play a role in the lignocellulase potential of the *R. flavipes* hindgut. Four commercially available antimicrobials were used: 1) ampicillin, a cell wall synthesis inhibitor of gram-positive bacteria; 2) kanamycin, a broad-spectrum antibiotic causing misreading of mRNAs during translation; 3) metronidazole, an anti-protozoal/anti-anaerobe which binds DNA

preventing nucleic acid synthesis/replication; and 4) tetracycline, a broad-spectrum antibiotic which interferes with translation by preventing tRNA binding at the ribosome (Walker 1996). Treatment with each of these compounds resulted in distinct fluctuations in symbiont abundance, prokaryotic diversity, and lignocellulose saccharification potential. Most importantly, these findings show that removal of certain bacterial taxa lead to shifts in community composition that negatively and differentially impact the overall efficiency of lignocellulose breakdown. Specifically, disruption of the synergistic, tripartite symbiosis by antimicrobial treatment leads to a reduction of holobiont metabolism by 25-50%.

2.2 Materials and Methods

2.2.1 Termite and Bioassay Setup

R. flavipes termite colonies were collected from West Lafayette, IN and maintained in the laboratory with 24 hours of darkness on a diet of pine wood shims and brown paper towels. Three individual colonies were used as biological replicates in all studies. Large termite workers (third instar or later) were used in this study; workers lacked wing-buds and large mandibles. Sixty termites were placed in small, Petri dishes (Nunc, 33mm) sanded with 200 grit sandpaper. They were fed with a ~1cm disk of diet consisting of pine wood sawdust and shredded brown paper towel (50/50 w/w). Initially, the diet was treated with 200µL of one of 5 solutions based on treatment group: water (untreated group), 5% ampicillin (w/v), 5% kanamycin (w/v), 5% metronidazole (w/v), or 2.5% tetracycline (w/v). These concentrations were determined to be sublethal to termites based on extensive preliminary optimization studies. Diet disks were rewetted every other day with 100µL of the appropriate solution for a total holding period of seven days. After the seven day holding period, whole guts were dissected from the termites in sodium phosphate buffer (0.05M, pH 7.0).

2.2.2 Bacterial Enumeration and Culturing

To estimate bacterial abundance after antimicrobial treatment, twenty-five whole termite guts from each treatment were dissected, pooled, and homogenized in 750µL of

sodium phosphate buffer (0.05M, pH 7.0). Of this homogenate, 100µL was used for serial dilutions which were plated in sextuplicate onto brain heart infusion agar and incubated at 37ºC either aerobically or anaerobically to determine the number of colony forming units (CFU) per treatment. Anaerobic conditions were made using a glass containing one BD GasPak EZ Anaerobe System with Indicator sealed with modeling clay (Franklin Lakes, NJ). Raw CFU counts were Log10 transformed prior to data analysis.

2.2.3 Protist Cell Counting

In order to estimate protist abundance, bioassays were repeated as described above with fifteen termite workers per assay dish. From each antibiotic treatment, 10 guts were dissected, trimmed to only the hindgut, and placed in 1mL of sodium phosphate buffer (0.05M, pH 7.0). Guts were then homogenized and the homogenate was transferred to a Sedgewick Rafter Counting Cell (SPI Supplies; West Chester, PA) for enumeration. Cells were counted using a phase contrast microscope under the 20X objective. Protist cell concentration in the hindgut was determined as described previously (Wheeler *et al.*, 2007).

2.2.4 16S rDNA Clone Library Construction and Analysis

Twenty-five whole guts were used for DNA extraction (Qiagen DNeasy Blood and Tissue Kit; Valencia, CA). The V3 hypervariable region of the 16S rRNA gene was amplified using universal primers: U341F-CCTACGGGRSGCAGCAG and U519R-GWATTACCGCGGCKGCAG (modified from: Wang and Qian, 2009). Pools of 6 PCR reactions (20µL/reaction = 120µL total) per treatment per colony were created and concentrated using a standard sodium acetate/ethanol DNA precipitation protocol. The resulting ~180bp fragments were then cloned into the pGEM-T easy vector using the pGEM-T easy Vector System I (Madison, WI). White colonies were used for PCR screening with GoTaq Green Master Mix (Promega; Madison, WI) and standard M13 primers (Table S1). Clones containing the correct-sized insert were grown individually overnight in LB broth with ampicillin to make glycerol stocks for future sequencing. Ninety-six clones were picked for each treatment/colony. In total, 1,536 clones were sequenced.

Deep-well glycerol plates were inoculated from individual clone glycerol stocks and submitted for high-throughput Sanger sequencing by the Purdue University Genomics Core Facility. Resulting sequences were trimmed and compared to NCBI's nucleotide sequence database to ascertain clone identity. Sequences were then compared across the treatment groups and replicates to identify unique sequences. An unexpected artifact of using degenerate 16S rDNA primers was the amplification of host 18s rDNA. As a result, the clone library from each treatment contained clones identifying as host. These host sequences were filtered out leaving only prokaryotic clones in the library for subsequent analyses. A total of 379 unique sequences were identified from an entire library of 1,475 clones across all five treatments groups.

To determine the completeness of clone libraries, data from each treatment were subjected to rarefaction analysis using Analytic Rarefaction freeware (version 1.3; S.M. Holland, University of Georgia, Athens, GA, USA, available from: http://strata.uga.edu/software/Software.html).

2.2.5 Post-Hoc Determination of Bacterial: Host Genomic DNA Ratio

qPCR was performed to determine the relative amount of bacterial and host genomic DNA (gDNA) in DNA samples used for clone library preparation. Primers amplifying a 291bp fragment containing the V4 hypervariable region of the 16S rRNA gene were used as a proxy for bacterial gDNA abundance (Rubin *et al.,* 2014; Table S1). Host DNA was quantified using primers specific to an apparent single-copy host gene, Actin 5C-1 (Table S1). Data were normalized to the Actin 5C-1 gene to determine the relative abundance of 16S amplicons in each antimicrobial treatment-gDNA preparation. This relative abundance was then regressed against the percent prokaryote composition of each antimicrobial treatment's clone library.

2.2.6 RNA Extraction and qPCR

After the 7-day bioassay, 20 whole guts including salivary glands were dissected from termite workers and stored at -80ºC in 150µL of RNAlater (Life Technologies; Grand Island, NY). Subsequently samples were thawed and RNAlater removed. Total RNA was extracted

using the SV Total RNA Isolation Kit (Promega; Madison, WI.) cDNA was synthesized from 1µg of total RNA using the iScript cDNA synthesis kit (BioRad; Berkley, CA).

Quantitative PCR (qPCR) was used to determine the expression level of various host, protist and candidate bacterial cellulase genes across treatments (Table S1). qPCR reactions were performed in a 20µL reaction containing: 1µL of cDNA template, 1µL each of forward and reverse gene specific primers (10µM), 10µL of iTaq Universal SYBR Green Supermix (Bio-Rad; Hercules, CA), and 7µL of nuclease-free water. Gene expression across treatments was normalized to the reference gene and to the untreated control.

2.2.7 Termite Gut Protein Preparations

After being subjected to the antimicrobial bioassay described above, twenty-five termite whole guts from each treatment were dissected, pooled, and homogenized in 750µL of sodium phosphate buffer (0.05M, pH 7.0). The homogenate was centrifuged for 15 min at 14,000rcf at 4˚C and the supernatant collected for use in assays described below. This procedure was repeated across treatments for each of three termite colonies which served as biological replicates.

2.2.8 *In Vitro* Saccharification Assays

For these assays, 600µL of 100mM sodium acetate containing 10mM calcium chloride pH 7.0, 150µL of native termite gut homogenate (5 gut equivalents) and pine sawdust (2% w/v) were combined in a 1.5mL microcentrifuge tube with a small pin hole in the lid. Tubes were incubated at 37˚C with shaking at 220rpm for 18hr, after which they were stopped with the addition of 15µL of 0.2M EDTA (Scharf *et al.*, 2011).

2.2.9 Monosaccharide Detection

Monomeric glucose detection was performed as described previously, using a commercially available glucose detection kit (Wake Chemical; Richmond, VA.; Scharf *et al.*, 2011). To detect D-xylose release from the degradation of hemicellulose, we used a commercially available kit, modifying the protocol for a microplate format (Megazyme; Wicklow, Ireland). Supernatant from *in vitro* assays (50µL) was combined with 242µL freshly mixed kit mixture (as prescribed in the manufacturer's protocol) in each well and

an initial absorbance reading at 340nm was read after 5 minutes of orbital shaking using a microplate reader (BioTek PowerWave 340). Next, 5µL of solution 4 from the kit was added to each well, the plate was subjected to 6 addition minutes of orbital shaking, and a final absorbance reading was taken at 340nm. Three technical replicates were done for each treatment and assays were always run in parallel with buffer blanks that contained all reaction components except the protein supernatant. The concentration of D-xylose was then ascertained using the extinction coefficient of NADH at 340nm (6300/M/cm).

2.2.10 Cellulase Activity Assays using Model Substrates

Native gut homogenate described above was used in kinetic cellulase activity assays using p-nitrophenol-β-D-cellobioside (pNPC) and p-nitrophenol-β-D-glucopyranoside (pNPG) as substrates. For these assays, 10µL of native gut homogenate was combined with 90µL of 6mM pNP(C or G) in 0.1M sodium phosphate (pH 7.0). Reactions were repeated in triplicate. Enzyme activity was read kinetically at 420nm every minute for 3 hours. Cellulase specific activity per termite was estimated using mean velocity for each treatment on both substrates.

2.2.11 Antimicrobial Interference Assays

To assess the extent to which antimicrobial compounds might inhibit termite gut cellulase activity *in vitro* (and interfere with colorimetric monosaccharide detection assays), additional assays were performed. To determine if antimicrobials interfered with native, control gut homogenate enzyme activity, twenty-five naive termites per treatment were dissected, their whole guts homogenized, and *in vitro* sawdust assays were set up as above except the buffer contained 5% (v/v) spike-in of a given concentrated antimicrobial solution. Ampicillin, kanamycin, and metronidazole concentrations tested include the following volume/volume spike-in concentration: 50%, 25%, 5%, 0.5%, 0.05%, 0.005%, 0.0005%, and 0.00005%. Tetracycline concentrations tested were: 25%, 12.5%, 2.5%, 0.25%. 0.025%, 0.0025%, 0.00025%, 0.000025%. After overnight incubation, glucose release was detected as before and percent inhibition calculated for each tested concentration relative to the water control.

Assays were also performed to determine the level at which antimicrobial compounds might potentially interfere with absorbance readings at 505nm. To do this, 50µL of a given antimicrobial concentrated stock was combined with 200µL of glucose detection reagent and absorbance at 505nm was recorded for each of the concentrations listed above. No interference was detected in either case.

2.2.12 Statistical Analyses

Three biological replicates were performed for all studies using three individual termite colonies. All statistical analyses were performed using JMP 8. Differences across treatments and termite colonies (biological replicates) were evaluated using ANOVA (df=6, α=0.05) with Tukey HSD post-hoc analyses and an FDR-corrected α-value (α=0.002). Though not all data were normally distributed, ANOVA was still used due to its robust characteristics (Table S2). All error bars represent standard error of the mean (SEM).

To assess which variables were most significantly correlated with saccharification, each measured variable (bacterial CFUs, protist cell counts, abundance of bacterial taxa in the clone library, gene expression of cellulases, and enzyme activity) a series of pairwise regressions were performed for glucose and xylose release individually (α=0.05).

2.3 Results

2.3.1 Antimicrobial treatments significantly reduce symbiont load in the termite gut

Four antimicrobial feeding treatments (ampicillin, kanamycin, metronidazole, and tetracycline) were used to experimentally induce dysbiosis in the termite gut and quantify changes in community ecology and digestion. To first determine if these antimicrobials could reduce bacterial populations, gut bacteria were isolated and quantified using traditional culturing techniques. The two broad-spectrum antimicrobials, kanamycin and tetracycline, significantly reduced the culturable community of bacteria isolated from the termite gut (Fig. 1A). Whereas, ampicillin, which targets gram-positive bacteria, and metronidazole, an antiprotozoan, did not significantly reduce the culturable bacterial community. Antimicrobial treatments also resulted in non-significant changes to the culturable, anaerobic community (Fig. S1). In addition to reductions in bacterial load,

kanamycin, metronidazole, and tetracycline treatments also impacted protist abundance significantly (Fig. 1B). Overall, all antimicrobial treatments significantly reduced the symbiont load, both bacteria and protists, in a seven-day feeding period (Fig. 1).

2.3.2 16S sequencing captures antimicrobial impact on termite gut bacterial diversity

Although results above show that the culturable community was reduced to some extent, the impact of antimicrobial treatments on community diversity was also verified. To characterize prokaryotic diversity, the community was surveyed using a 142 bp fragment of the 16S rRNA gene as a barcode (U341F-U519R; modified from (Wang and Qian, 2009)) and a library of 1,475 clones was assembled. Given the length of this fragment, only phylum-level identification of 16S sequences was possible. Biodiversity statistics demonstrate that the untreated group shows the most unique sequences (richness), prokaryotic sequence diversity (H'), and evenness (J = H'/Hmax) (Shannon and Weaver, 1949; Morin, 1999; Fig. 2A). Despite having an intermediate number of unique sequences, the broad-spectrum antibiotic kanamycin has the least diversity and evenness (Fig. 2A); whereas, the tetracycline treatment has the fewest unique sequences, but moderate diversity and evenness (Fig. 2A). The kanamycin treatment clone library is dominated by a single, highly-abundant sequence belonging to the Bacteroidetes. Contrastingly, the tetracycline library is smaller, but more diverse than that of kanamycin. These trends are echoed by the modest slopes of rarefaction curves for the antimicrobial treatments. The chosen 16S fragment and cloning strategy were able to capture the majority of diversity remaining in the gut (Fig. 2B). Unlike all of the treated groups, the untreated rarefaction curve has a steeper slope implying that there is remaining diversity that was not captured (Fig. 2B). The trends suggested by these metrics are further supported when the prokaryotic reads from each treatment are compared side by side (Fig. 3). Thus, each antimicrobial treatment alters prokaryotic abundance and diversity uniquely, resulting in distinct phylum-level microbial profiles.

As exhibited in the literature, Spirochetes, Elusimicrobia (TG1), Firmicutes and Bacteroidetes are the dominant prokaryotic groups in the *R. flavipes* gut, comprising

approximately three-quarters of the community (Boucias *et al.*, 2013). There are shifts in all of these major groups in at least one of the four antimicrobial treatments. Most strikingly, our results show no clones belonging to the Spirochetes in any of the four antimicrobial treatments (Fig. S2A). Despite having relatively few prokaryotic clones, there is a significant increase in the proportion of Elusimicrobia in the tetracycline treatment (Fig. S2B), though there was not a corresponding increase in protist abundance (Fig. 1B). Both kanamycin and metronidazole groups have a reduction in the abundance of Firmicutes and an increase in Bacteroidetes clones, compared to the untreated, ampicillin and tetracycline groups (Fig. S2C, D). Other phyla fluctuated to differing degrees but lacked statistical significance (Figs. S2E-H). One key group that is absent, the Fibrobacters, was not detected in our dataset.

Due to the promiscuous nature of universal primers, some antimicrobial treatments had a significant amount of termite 18s rDNA artifact in their libraries. This is likely a product of variable levels of bacterial DNA relative to host DNA in each individual sample (Fig. S3). This finding, although unanticipated, provides additional independent evidence of antimicrobial impacts on decreasing prokaryotic abundance and causing shifts in community composition.

2.3.3 Termite saccharification potential and efficiency are reduced in association with antimicrobial induced microbiota shifts

In vitro enzyme assays have been used previously to determine the lignocellulolytic potential of termite guts (Scharf *et al.*, 2011; Sethi *et al.*, 2013a, 2013b). In this study, following *in vitro* assays, glucose and xylose monosaccharide liberation were measured as indicators for cellulose and hemicellulose degradation, respectively. Glucose release was reduced by 23-47% across the antimicrobial treatments (Fig. 4). Xylose assays, alternatively, showed non-significant reductions of 30-52%, with kanamycin and metronidazole having the most dramatic affects (Fig. S4). These reductions in saccharification potential are due to the removal of prokaryotic and protist symbionts,

rather than inhibition or interference by antimicrobials with enzyme assays or colorimetric readings (Figs. S5).

Cellulase activity was assessed using native gut homogenates in combination with the model substrates pNPC and pNPG. Exoglucanase activity (a protist-derived activity) was significantly impacted by kanamycin, metronidazole, and tetracycline treatments as indicated by activity on the pNPC substrate (Fig. 5). However, β-glucosidase activity (a host-derived activity), as measure by activity on pNPG, was not significantly impacted by antimicrobial treatment (Fig. 5).

In addition to enzyme activity assessment, gene expression of several glycosyl hydrolase family (GHF) cellulases was evaluated following antibiotic treatment. Of the host and protist enzymes that have been previously characterized (Scharf *at al.,* 2010; Sethi *et al.,* 2013; Zhou *et al.,* 2010), the transcript levels of the two host enzymes, GHF-1 (β-glucosidase) and GHF-9 (β-1, 4-endoglucanase), do not change significantly with antimicrobial treatment (Fig. 6). GHF7-3, a protist exoglucanase, had a significant reduction in expression in the metronidazole and tetracycline treatments. We also selected four bacterial cellulase candidates: GHF-2 (β-galactosidase), GHF-26 (β-mannosidase), GHF-42 (β-galactosidase), and GHF-43 (β-xylosidase) based on published metatranscriptomic data (Tartar *et al.*, 2009). Each of these genes have unique expression patterns depending on antimicrobial treatment, further evidencing the impact that these treatments have on the cellulolytic potential present in the termite hindgut.

2.4 Discussion

2.4.1 Key variables associated with efficient lignocellulose digestion

The experiments presented here emphasize the roles and relative importance of each symbiotic taxon in the termite gut. Synergy between the host and the collective symbiont consortium has been demonstrated previously, as has the functionality of some key host and protist digestive enzymes, but the importance of prokaryotes in lignocellulolytic potential in the lower termite, *R. flavipes,* has been unclear (Scharf *et al.*, 2011; Sethi *et al.*, 2013a, 2013b; Tartar *et al.*, 2009). In wood-feeding higher termites, which lack protists,

there is clear functional evidence of saccharolytic bacteria in the hindgut (Tokuda and Watanabe, 2007). Despite striking phylum-level similarities in the prokaryotic composition of the symbiont community between wood-feeding higher and lower termites (Boucias *et al.*, 2013; Hongoh *et al.*, 2003, 2006), the cellulolytic potential of bacteria in lower termites has been mostly unexplored relative to their eukaryotic counterparts.

This study aimed to experimentally quantify the role of prokaryotic symbionts in lignocellulose degradation by a lower termite, as part of a tripartite symbiosis. We used a variety of antimicrobial compounds with diverse modes of action and target organisms in an effort to garner as much information about important symbiotic groups in this system as possible (Walker 1996). Impacts of these antimicrobials were measured via cellulase gene-expression levels, enzymatic activity, symbiont population shifts, and saccharification potential. In an effort to identify the variables which were most significantly impacting glucose liberation from complex lignocellulose, we performed a series of pairwise regression analyses (see Methods 2.12; Table 1). This evaluation showed that six tested variables were significantly correlated with glucose release, and from this information we are able to begin to elucidate prokaryotic roles in *R. flavipes* digestion. These six variables are abundance of all symbiont groups (aerobic CFUs, anaerobic CFUs, protist abundance), Spirochete presence in the 16S clone library, enzyme activity on the pNPC substrate, and gene expression levels of the candidate bacterial cellulase GHF-43.

Most notably, as a result of antimicrobial-induced dysbiosis, we observed 23-47% reductions in glucose and 30-52% reductions in xylose liberated from lignocellulose in *in vitro* saccharification assays (Figs. 4 & S4). Glucose release was significantly reduced in all treatment groups (Fig. 4). The most striking finding is in the metronidazole treatment. After treatment with metronidazole, termite guts had an estimated 1 protist cell/mL but had intermediate amounts of culturable bacteria compared to the other treatments, which is to be expected given metronidazole's target organisms (Walker 1996; Fig. 1). However, despite being almost devoid of protists, glucose liberation was 74% of the

control which is significantly more than the kanamycin treatment, where termite guts were devoid of almost all symbionts (Figs. 1 & 4). The primary difference between these two treatments are their bacterial abundance and the diversity of residual bacteria (Figs. 1, 2, 3, S1). This sheds the best light on the potential bacterial role in saccharolysis within the *R. flavipes* gut*,* and thus represents potentially seminal documentation of such a niche for bacterial symbionts in a lower termite. This also suggests that bacteria which dominate in metronidazole treated guts (i.e., Bacteroidetes, Proteobacteria, and Actinobacteria) would be the best candidates to consider for potentially useful cellulases. Gene expression levels of four candidate, bacterial cellulases highlight the complexity of this relationship. All four of these genes have significant fluctuations following antimicrobial treatment, which mirror the changes seen in the 16S library but still provide only limited resolution of the key contributors.

In the antimicrobial treatments where monosaccharide release was not significantly impeded, symbionts not affected by a given treatment may be able to compensate for the removal of other consortium members due to functional redundancy within the remaining consortium. Most notably, niches previously occupied by dominant groups like Spirochetes are evacuated with antimicrobial treatment. This evacuation has the potential to make resources and nutrients available for other antimicrobial-tolerant members of the consortium with similar metabolic capabilities. This phenomenon is common in biofilms outside of the termite gut, and such redundancy is often thought to provide microbial communities with increased resilience in the face of natural perturbations (Yannarell *et al.*, 2007). Evidence of this possibility may be found in tetracycline and metronidazole treatments, which express higher levels of some candidate bacterial cellulase genes (Fig. 6).

It is important to note that saccharification is not completely abolished with any antimicrobial treatment, which emphasizes the importance of host contributions to wood digestion. This agrees with the previous findings in *Nasutitermes* and *Coptotermes* that treatment with tetracycline did not eliminate cellulolytic activity on the model substrate carboxymethylcellulose (O'Brien *et al.*, 1979). This idea is supported by stable expression

of a highly active host cellulase (Fig. 6) and accessory enzymes (laccase, catalase, p450s, aldo-keto reductase, etc.) (Coy *et al.*, 2010; Scharf *et al.*, 2010; Sethi *et al.*, 2013b; Zhou *et al.*, 2010). Together the host and symbionts work together with at least 1.6-fold synergy to saccharify complex lignocellulose (Scharf *et al.*, 2011). Further, host cellulolytic activity following antimicrobial treatment is stable as evidenced by primarily host-derived endoglucanase activity (Figs. 5). Though the expression of candidate bacterial cellulase genes (GHF-2, 26, 42, and 43) is not eliminated, it is important to note that the identity of these genes is based on bioinformatic analysis (Tartar *et al.,* 2009). There may be several organisms contributing to these contigs that may be responding differently to our treatments, and that cannot be detected using the present methods. Ultimately, the preservation of lignocellulolytic activity following antimicrobial treatment emphasizes that the remaining contributors to this process are still functioning.

2.4.2 Symbiotic prokaryotes of note

In addition to symbiont abundance and cellulase activity, cases of low abundance of Spirochetes in the clone library were significantly correlated with reduced monosaccharide liberation (Tables 1 & S3). Spirochetes are the dominant bacterial group in *R. flavipes*, making up about 40% of the native microbiota, and they are known to be saccharolytic, fermentative, and aromatic-metabolizing in other systems (Lucey and Leadbetter, 2014; Stanton and Canale Parola, 1980; Warnecke *et al.*, 2007; Wyss *et al.*, 1997). Spirochetes are not strictly associated with lower termites, but appear to be tightly associated with xylophagous lifestyles (Otani *et al.* 2014). Additionally, there is evidence in lower termites that Spirochetes are closely associated with cellulolytic protists and may perform acetogenesis (Breznak and Leadbetter, 2006). Thus, a loss of these activities would be detrimental to the efficiency of the consortium as a whole. Whether the decrease in monosaccharide release observed in this study is directly or indirectly dependent on Spirochetes remains to be determined; however, the liberation of specific hindgut niches caused by the elimination of Spirochetes is definitely a contributor to the overall dysbiosis observed in the gut (Fig. 3). Ultimately, it is important to note that the

gram-positive, anaerobic Spirochetes are vulnerable to all the selected antimicrobial treatments (Walker 1996; Fig. 3). In addition to Spirochetes, we observed significant differential shifts in Elusimicrobia, Firmicutes, and Bacteroidetes following antimicrobial treatments (Fig. S2B-D, Fig. 3).

In light of recent discoveries (Hu *et al.*, 2014), Firmicutes may also be involved in glucose liberation particularly when considering their reduction in the kanamycin treatment (Fig. S2C). Reductions in xylose release, while not statistically significantly, coincide with reductions in the abundance of Elusimicrobia and Firmicutes in the gut (Figs. S2B, C & S3). All hemicellulase genes in *R. flavipes* have been attributed to hindgut symbionts (protists and bacteria) (Tartar *et al.*, 2009), which agrees with the observed fluctuations in hemicellulose digestion with antimicrobial treatments seen here (Fig. 6). Additionally, microbiota composition has been found to vary significantly among termite colonies (Boucias *et al.*, 2013), which may also be obscuring our interpretations of changes in hemicellulytic activity.

One group not detected in this study, the Fibrobacters, was previously estimated as ~6% of the *R. flavipes* hindgut lumen bacterial community by cloning-independent 16S pyrosequencing (Boucias *et al.*, 2013). However, another cloning-based project, using the synonymous species *Reticulitermes santonensis*, also failed to detect Fibrobacters (Yang *et al.*, 2005). This suggests that detection of Fibrobacters is either method-dependent or their presence is influenced by environment (Boucias *et al.*, 2013). Due to this potential limitation, we are unable to account for antimicrobial impacts on Fibrobacter populations. This is a possible caveat to our approach, i.e., due to the known cellulolytic nature of Fibrobacters in other systems (Lin and Stahl, 1995; Mikaelyan *et al.* 2014; Qi *et al.*, 2007; Stewart and Flint, 1989; Tokuda and Watanabe, 2007). Conversely, this finding may suggest Fibrobacters as a potentially variable, facultative component of lower termite gut microbial consortia.

In broader terms, this research demonstrates a significant role for bacteria in lower-termite wood-digestion. Lignocellulytic activity in the termite holobiont is a collaborative effort with all members (termite-host, protists, and prokaryotes)

contributing factors which maintain bioreactor efficiency. The host and protists both produce highly active digestive and accessory enzymes that collaborate in lignocellulose saccharification (Scharf *et al.*, 2011; Sethi *et al.*, 2013a, 2013b). The current study adds to our understanding of this system by showing the relative importance of prokaryotes to the digestive process, and highlights the potential for undiscovered, cellulolytic prokaryotes in the *R. flavipes* gut.

2.4.3 Conclusions

Although we cannot ascertain whether prokaryotic contributions to lignocellulolytic activity are direct or indirect based on these data, we can nonetheless conclude that prokaryotes are important to the digestive process. In light of these findings and work done in other organisms, we hypothesize that bacteria can directly metabolize carbohydrate and phenolic components of lignocellulose and indirectly supply the termite host and protists with essential nutrients absent in lignocellulose (i.e. nitrogen, vitamins, essential amino acids, etc.) (Lucey and Leadbetter, 2014; Rosengaus *et al.*, 2011; Tartar *et al.*, 2009; Tokuda and Watanabe, 2007). Finally, our cloning-based 16S strategy lays the groundwork for more comprehensive high-throughput metagenomic and metatranscriptomic studies that can specifically investigate the impact that the microbiome has on host physiology. Going forward, it will be critical to approach termite, and other animal symbioses from a collaborative perspective that considers all the key players (host, protozoa, prokaryotes, etc.) within the holobiont as contributors. The tripartite symbiotic system of lower termites provides an important example and model system for such investigations.

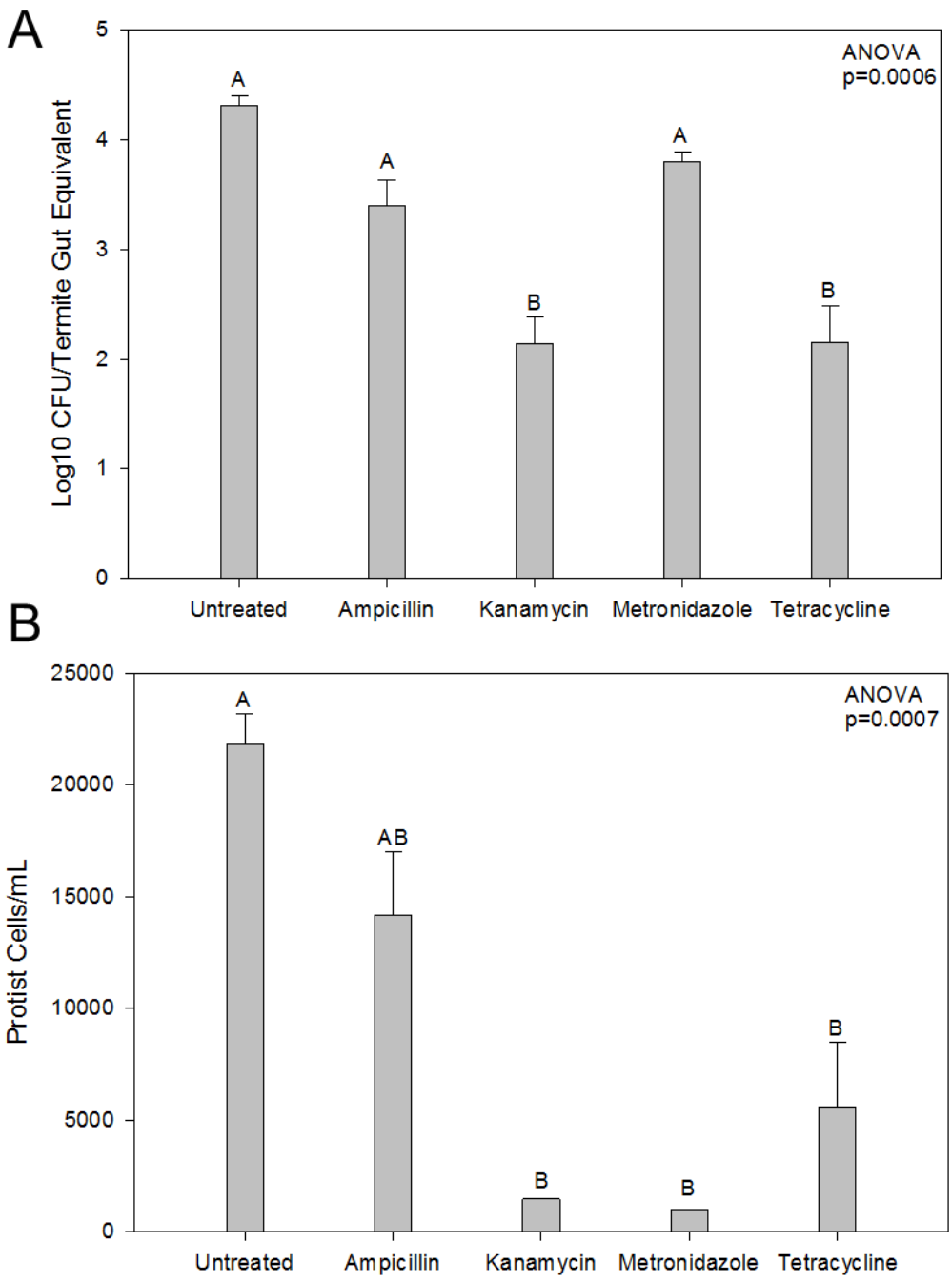

Figure 2.1 Symbiont abundance 7-days post antimicrobial treatments

A. Antimicrobial treatments impact the abundance of culturable bacteria per termite gut in aerobic growth conditions B. Protist cell counts are reduced following antimicrobial treatment. Bars within graphs labeled with the same letters are not significantly different (Tukey HSD α=0.002). Error bars represent standard error (SEM).

A

B

Treatment	Richness[a]	Diversity[b]	Evenness[c]
Untreated	131	4.52	0.81
Ampicillin	68	3.84	0.72
Kanamycin	44	2.51	0.47
Metronidazole	58	3.76	0.77
Tetracycline	47	3.70	0.82

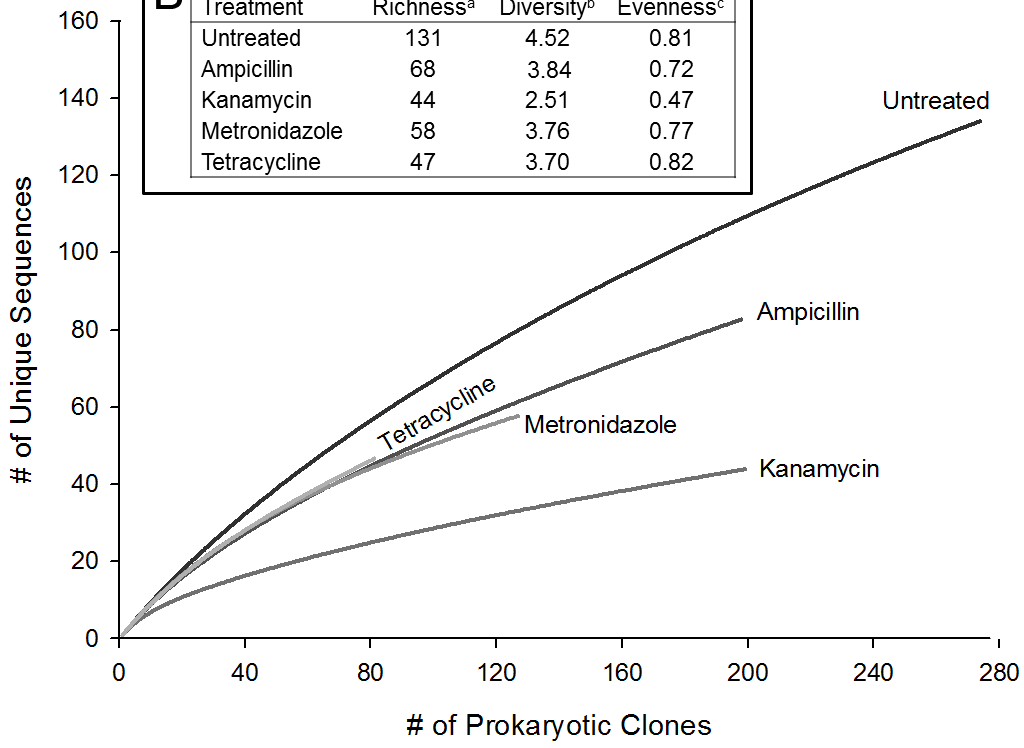

Figure 2.2 Biodiversity metrics from sequencing

A. Biodiversity metrics indicating the impact of antimicrobial treatments on termite gut prokaryote diversity: [a]Number of unique sequences, [b]Shannon-Weaver index (H'), [c]Evenness (J = H' / Hmax). B. Rarefaction analysis of clone libraries created from each of the antimicrobial treatment groups pooled across three biological replicates. The slopes of the curves indicate the rate at which unique sequences were being discovered at the exhaustion of sampling efforts.

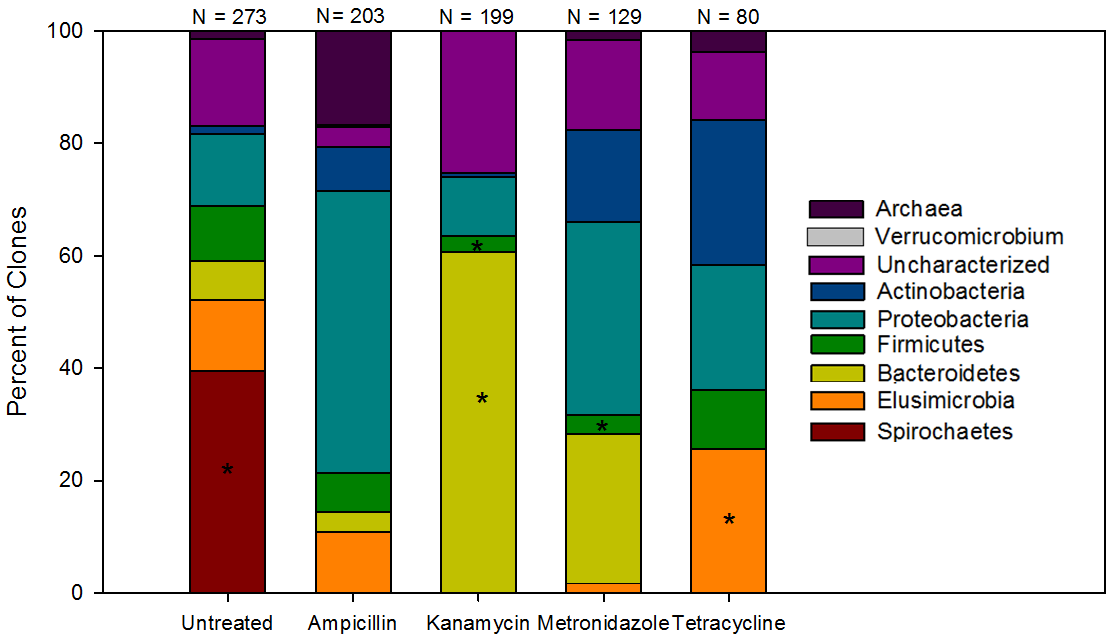

Figure 2.3 16S profiles of bacteria following antimicrobial treatments

The average phylum level prokaryotic profile of each treatment based on clone libraries prepared using the V3 hypervariable region of the 16S rRNA gene. Asterisks (*) indicate a significant fluctuation ($p < 0.05$) in the indicated phylum in the given treatment group. "N" indicates the number of clones representing each treatment group.

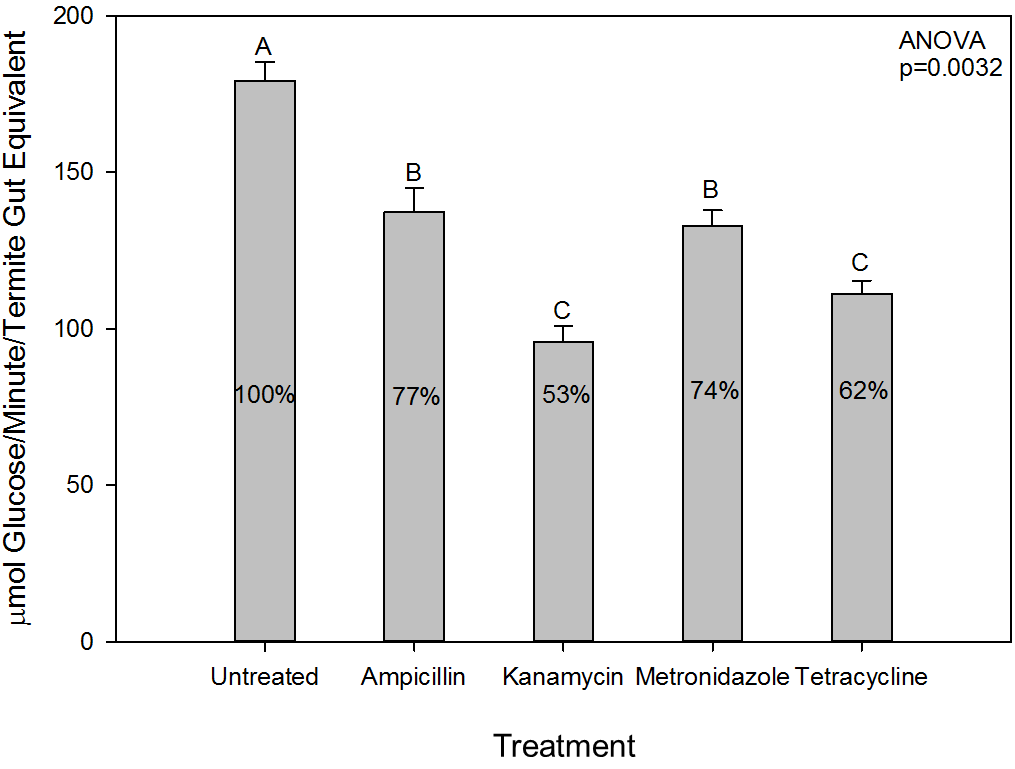

Figure 2.4 *In vitro* glucose liberation from wood lignocellulose by control (untreated) and antimicrobial treated guts

Glucose liberation from pine sawdust by antimicrobial-treated termite guts in *in vitro* saccharification assays, as detected by colorimetric tests. Error bars represent standard error (SEM).

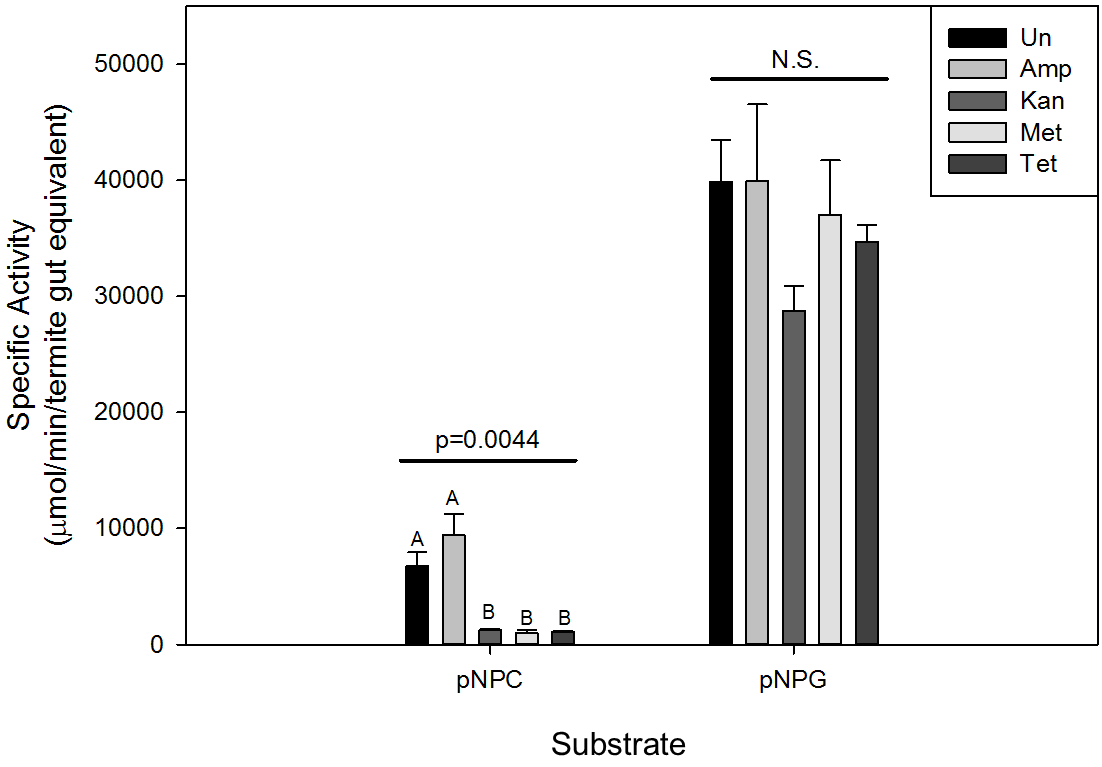

Figure 2.5 Cellulase activity on model substrates

Cellulase activity following antimicrobial treatment using the model substrates pNPC and pNPG. Error bars represent standard error (SEM). Treatments abbreviated as untreated (Un), ampicillin (Amp), kanamycin (Kan), metronidazole (Met), and tetracycline (Tet). N.S. indicates no significant difference at $\alpha = 0.05$.

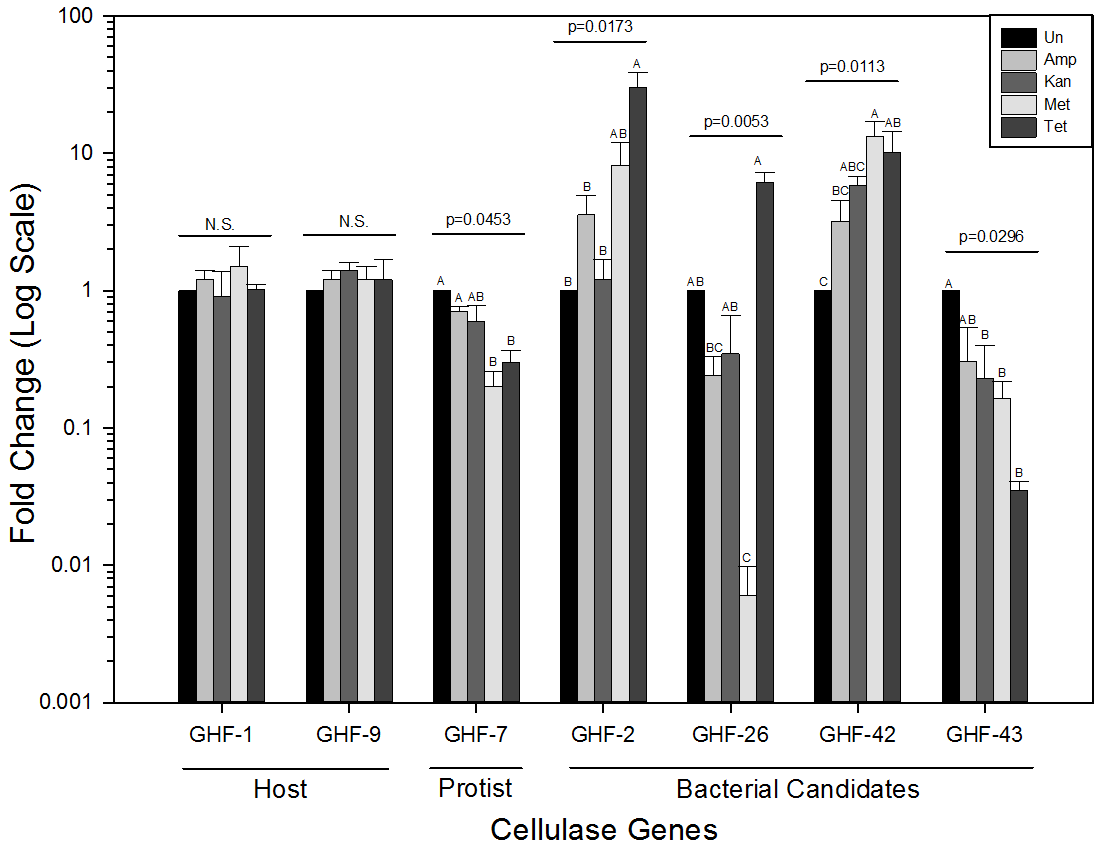

Figure 2.6 Gene expression profiles of cellulase genes 7-days post antimicrobial treatment

Gene expression analysis of three previously characterized cellulase genes from the termite host (GHF-1 and GHF-9) and protist symbionts (GHF-7) and four candidate bacterial cellulases (GHF-2, GHF-26, GHF-42, and GHF-43). Transcript levels are presented as fold-change relative to a reference gene and the control treatment group for each gene. Error bars represent standard error (SEM).Treatments abbreviated as untreated (Un), ampicillin (Amp), kanamycin (Kan), metronidazole (Met), and tetracycline (Tet).

Table 2.1 Regression analysis of factors associated with glucose liberation.

Pairwise linear regressions of glucose liberation (X-variable) with each of the tested variables (Y-variables) from this study, with respective p-value and R2 values shown. Variables above the line show a significant correlation with glucose liberation.

X-Variable	Y-Variable	p-Value	R^2	Adjusted R^2
Glucose	Log CFU-Aerobic	0.0004	0.6349	0.0680
	Protist Abundance	0.0004	0.6345	0.6064
	Spirochetes	0.0030	0.5035	0.4653
	Gene Expression-GHF-43	0.0097	0.4139	0.3688
	Enzyme Activity-pNPC	0.0202	0.3501	0.3501
	Log CFU-Anaerobic	0.0218	0.3931	0.3379
	Bacteroidetes	0.0581	0.2493	0.1915
	Enzyme Activity-pNPG	0.0775	0.2203	0.1037
	Gene Expression-GHF-42	0.0846	0.2114	0.1508
	Gene Expression-GHF-2	0.1463	0.1552	0.0902
	Gene Expression-GHF-9	0.2450	0.1024	0.0333
	Gene Expression-GHF-7	0.3883	0.0577	-0.0147
	Actinobacteria	0.4142	0.0519	-0.0210
	Uncultured-Unknown	0.4182	0.0510	-0.0220
	Verrucomicrobia	0.4560	0.0434	-0.0301
	Firmicutes	0.4599	0.0427	-0.0309
	Gene Expression-GHF-26	0.5414	0.0294	-0.0453
	Proteobacteria	0.5935	0.0225	-0.0527
	Elusimicrobium	0.6246	0.0190	-0.0565
	Gene Expression-GHF-1	0.7608	0.0074	-0.0690
	Archaea	0.8271	0.0038	-0.0728

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CHAPTER 3. SYMBIONT-MEDIATED PATHOGEN DEFENSE IN *RETICULITERMES FLAVIPES*

3.1 Introduction

All animals form intricate associations with microorganisms present in their environment. These relationships range across a spectrum from beneficial for both parties (mutualism) to detrimental to one party while benefitting the other (parasitism). The most diverse group of animals, the insects, forms a myriad of relationships with microbes spanning this spectrum. Some of these relationships allow for animals to occupy specialized niches as seen in galling midges (Joy 2013), while others may be used as biocontrol against pests in agro-ecosystems like fungal control of whiteflies (Wraight *et al.* 2000, Santiago-Alvarez *et al.* 2005). Despite the importance and potential exploitation of these relationships, there is still much to understand about the evolutionary and physiological basis of these interactions. The interplay between insect hosts, symbionts, and physiological processes have co-evolutionary implications, and have recently been highlighted as a means to identify potential biomaterials for applied purposes, such as pest control (Ponton *et al.* 2012).

Understanding the interplay between beneficial and detrimental microbes within a single insect is especially interesting from an ecological perspective. Documentation of symbiont-mediated protection against pathogenic and parasitic infection is widespread in insects (Scarborough *et al.* 2005, Haine 2008, Hedges *et al.* 2008, Brownlie and Johnson 2009, Feldhaar 2011, Hughes *et al.* 2011, Koch and Schmid-Hempel 2011). The classic example of this is found in the interaction between aphids, their facultative symbiont *Regiella insecticola*, and a fungal pathogen. Infection with *R. insecticola* increased aphid fitness, reduced susceptibility to a fungal pathogen,

and reduced pathogen transmission (Scarborough *et al.* 2005). In mosquitoes, the acquisition of the intracellular symbiont *Wolbachia* interferes with vectors of the human malaria parasite (Hughes *et al.* 2011). Gut symbionts can also play an important role in insect immunity; e.g., socially transferred symbiotic gut bacteria are known to protect bumble bees from the intestinal parasite *Crithidia bombi* (Koch and Schmid-Hempel 2011). These examples emphasize the relevance of the resident microbes of insects for mitigating pathogen effects.

Termites house a complex consortium of microorganisms in their guts, and as soil-dwelling insects, live in very close contact to potential pathogens. To decrease the likelihood of disease outbreaks, termites use a combination of hygienic, social behaviors and innate immunity (Traniello *et al.* 2002, Cremer *et al.* 2007, Fefferman *et al.* 2007, Yanagawa *et al.* 2007, Chouvenc and Su 2010). The combination of these defenses likely contributes to failures in the use of fungal pathogens, like *Metarhizium anisopliae,* for control of termites except in combination with neurotoxins and/or pesticides, like imidacloprid (Ramakrishnan *et al.* 1999, Thorne and Breisch 2001, Sen *et al.* 2015). The primary route of entry for entomopathogenic fungi (i.e. *M. anisopliae* and *Beauveria bassiana*) is through the cuticle, so termites have evolved complicated grooming behaviors to remove spores from themselves and their nestmates (Yanagawa *et al.* 2008). Although termite symbionts are primarily thought to serve a nutritional function, there is compelling evidence that suggests the gut environment aids in pathogen neutralization/defense (Rosengaus *et al.* 1998, Yanagawa *et al.* 2008, Chouvenc *et al.* 2013, Rosengaus *et al.* 2014). Specifically, in *Zootermopsis angusticollis* protist symbionts appear to contribute a β-1, 3-glucanase which is active against *M. anisopliae* conidia and when β-1, 3-glucanases are inhibited termite susceptibility to the pathogen increases significantly (Hamilton *et al.* 2011, Rosengaus *et al.* 2014). Treatment with imidacloprid results not only in 100% termite mortality, but in drastic reductions in protist populations and in the 1000-fold down regulation of a protist enzyme, GHF 7-5 (Sen *et al.* 2015). It appears that symbiota likely

contribute to anti-fungal defenses in many termite species, and that β-1, 3-glucanase activity is linked with termite protection.

In order to investigate the potential protective role mediated by termite symbiota, I aimed to determine if 1) symbiont-mediated immunity was dependent on pathogen type, 2) termite endogenous anti-microbial pathways were impacted by defaunation, and 3) protist-derived GHF7s serve as a mechanism for symbiont-derived, anti-fungal activity. In bioassay experiments, I challenged both normally faunated and antibiotic treated termites with bacterial and fungal pathogens and observed signatures of symbiont-mediated immunity in both to varying degrees. By measuring changes in immunocompetence from molecular to the whole-organism levels, I found that termite immune status is affected on a variety of levels by faunation state and pathogen type. Termites are more susceptible to fungal pathogens when symbiota (both protists and prokaryotes) are removed using the antibiotic kanamycin, and building upon previous works, I found that protist GHF7 enzymes reduce fungal germination *in vitro* and show potential to rescue the effects of defaunation on fungal susceptibility by termites *in vivo*. Together, these results corroborate previous findings that termite gut symbionts perform important functions outside of their stereotypical nutritional/digestive roles (Rosengaus *et al.* 2014), and supports the existence of a novel mechanism for anti-fungal, symbiont-mediated protection via protist GHF7s.

3.2 Materials and Methods

3.2.1 Termites, Pathogens, and Bioassay Setup

R. flavipes termite colonies used in bioassay experiments were collected from West Lafayette, IN and maintained in the laboratory with 24 hours of darkness on a diet of pine wood shims and brown paper towels prior to bioassay. Three individual colonies were used as biological replicates in this study. Seventy termite workers were placed in small, Petri dishes (Techno Plastic Products Tissue Culture Dish 40) with a Whatman filter paper saturated with either 200µL of either water (control) or 5% kanamycin (w/v). Termites were fed on this diet for 48 hours. Kanamycin is a broad-spectrum antibiotic and has been

shown to reduce termite gut microbiota (Peterson *et al.* 2015). Termites were then transferred to new dishes for pathogen challenge.

Pathogens used in bioassays were *B. bassiana* isolate #5477 and *S. marcescens*. *S. marcescens* was verified with 16S sequencing prior to use. For bacterial pathogen challenges, termites were fed on agar-based termite diet disks soaked in either 150µL of sterile 0.85% NaCl (control) or 8 x 10^8 cells /mL *Serratia* in 0.85% NaCl. For fungal pathogen challenges, termites were submerged in either 150µL 0.5% Tween 20 (v/v; control) or 1.25 x 10^4 *B. bassiana* conidia suspended in 150µL 0.5% Tween 20. Pathogen viability was verified by plating on Luria-Bertani agar (*Serratia*) or potato dextrose agar (*Beauveria*) at the time of inoculation. 50 termites were collected from each pathogen group 48-hours post-inoculation. These were dissected to provide whole-guts for bacterial enumeration, hemolymph for hemocyte enumeration, and carcasses for protein and RNA extraction. The remaining 20 workers were left in bioassay for an additional 5 days, and mortality was recorded 7-days post inoculation (DPI).

3.2.2 Gut Bacteria Enumeration

To determine bacterial load of termites following antibiotic treatment and/or pathogen challenge, culturable bacteria were quantified. Whole-guts (25) dissected from termites were homogenized in 750µL of 50mM sodium phosphate buffer (pH 7.0). The gut homogenate was then diluted in water (10^{-1} -10^{-3} depending on treatment) and 100µL plated in triplicate onto brain heart infusion agar. These plates were grown at 37˚C overnight and the resulting colonies counted to calculate colony forming units (CFU) per termite gut equivalent. Additionally, triplicate plates from each treatment were grown at 25˚C to allow us to calculate the percent of pathogen being cultured in pathogen challenged treatments, as *S. marcescens* has a red pigment when grown at 25˚C which distinguishes it from other normal, culturable gut flora.

3.2.3 Hemocyte Enumeration

Hemocytes were counted as a metric for cellular immune response. Hemolymph was bled from 10 workers by puncturing the lateral meta-thoracic segment with an insect pin. To

collect the hemolymph, a 5µL glass capillary tube was pre-loaded with 1µL of anticoagulation buffer (Willott *et al.,* 1994) and touched to the bubble of hemolymph produced at the wound site. On average 0.2µL of hemolymph was collected from each termite resulting in a final dilution of 1:5 ratio of hemolymph to anticoagulation buffer. To image hemocytes, 10µL of diluted hemolymph was transferred on a hemocytometer (Hausser Scientific Co.; Horsham, PA) and visualized using a phase contrast microscope. Still images were captured using a camera apparatus connected to the microscope and the Olympus MicroSuite B3 Biological Suite software (Olympus; Melville, NY). Hemocytes were then counted using ImageJ freeware (available from:http://imagej.nih.gov/ij/). Hemocyte counts were compared between treatments using ANOVA with Tukey HSD post-hoc mean separation (α=0.05).

3.2.4 Protein Analysis

To look at global protein expression, twenty carcasses (thorax and abdomen minus gut) were homogenized in 100µL 50mM sodium phosphate buffer (pH 7.0). To extract total protein, homogenate was spun at 15,000 G for 10 minutes at 4^{o}C and the supernatant collected. Protein was quantified using the Bradford method (ThermoScientific; Wilmington, DE). Protein samples (20µg) were then analyzed using 15% acrylamide SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

3.2.5 Quantitative Real-Time PCR

As a means to quantify expression of a number of immune-associated genes, I used quantitative real-time PCR. Carcasses (thorax and abdomen minus gut) were stored in 150 µL of RNAlater RNA Stablization Reagent (Qiagen; Germantown, MD) at -80^{o}C until RNA was extracted. RNAlater was removed from samples by brief centrifugation and pipetting. RNA was extracted from 25 worker carcasses from each treatment group using the Promega SV Total RNA Isolation System and manufacturer's instructions (Madison, WI). The quality and quantity of RNA was assessed using the ThermoScientific NanoDrop2000 (Wilmington, DE). Then 1µg of good quality RNA from each sample was reverse transcribed using the Bioline SensiFAST cDNA Synthesis Kit (Taunton, MA).

Quantitative Real-Time PCR (qRT-PCR) was performed using 20µL reactions with 0.5mM final primer concentration per the manufacturer's protocol (Bioline; Taunton, MA). Primer-pairs were designed against *R. flavipes* homologs of known immune-associated genes (Hussain *et al.*, 2013; Table B.1). Cycle threshold values were recorded using the BioRad CFX96 Real-Time PCR Detection System (Hercules, CA). Gene expression was normalized to the reference gene, β-actin, (ΔCT) and then to the unchallenged treatment for each group (CC for control groups and AC for antibiotic treated groups for each colony; ΔΔCT). Fold changes between treatment groups were compared using a t-test for each gene (α=0.05).

3.2.6 Data Normalization

In order to determine the effect of faunation status on termite response to pathogens data from pathogen challenged groups in the above assays were normalized to their respective controls. For example, treatment effect was expressed as a proportion of the control effect or: Measured treatment effect ÷ Measured control effect = Normalized treatment effect. This normalization was done to data collected in paired sets per the experimental design. This includes termite mortality, hemocyte enumeration, immune gene expression, and protein profiles.

3.2.7 Biochemical Characterization Enzyme Activity Assays

A previous study tested the activity of these two enzymes, GHF7-5 and 7-6, on a variety of β-1, 4 glycosidic bonds and determined very weak activity (Sethi *et al.* 2013). I tested these enzymes against a variety of substrates containing β-1, 3 and β-1, 6 glycosidic bonds, which are present in fungal cell walls. In all I tested laminarin (β-1, 3 and β-1, 6), pustulan (β-1, 6), carboxymethylcellulose (β-1, 4), and carboxymethylcurdlan (β-1, 3) at a range of concentrations using 100mM, filter sterilized HEPES buffer pH 7.0. Enzymes (0.5µg) and substrates (0-1.25%) were incubated for 1 hour at 30^{o}C. Then 10µL of enzyme/substrate solution was combined with 90µL of 3, 5-dinitrosalicylic acid (DNSA) stop solution in a microplate, accompanied by a glucose standard curve. The plate was submerged in boiling water for 10 min and then an endpoint reading was taken at 540nm.

Additionally, these enzymes were tested for activity against four nitrophenol model substrates: o-nitrophenol-glucoside (oNPG), o-nitrophenol-cellobioside (oNPC), p-nitrophenol-glucoside (pNPG), and p-nitrophenol-cellobioside (pNPC) at 6mM concentrations in 100mM, pH 7.0 HEPES buffer. These assays contained 95µL of substrate in buffer and 5µL (1µg/µL) enzyme and were read kinetically for 1 hour at 30^{o}C.

GHF7-5 and 7-6 were also tested with 0.75% laminarin in 100mM sodium phosphate buffer at pH 7.0. Unlike the previous laminarin assay, 0.5 µg of each enzyme and the substrate were incubated for 10 min at 50^{o}C. Then the samples were developed and measured as with the previous DNSA assay.

3.2.8 Conidia Viability Experiments and Potency Bioassays

To test the efficacy of recombinant GHF7-5 and GHF7-6 as antifungal enzymes, 1500µL of 1 x 10^4 conidia/mL of either *B. bassiana* or *M. anisopliae* were suspended 0.5% Tween 20. These conidia were combined with 300 µL of 0.03µg/µL recombinant protein in HEPES (no enzyme controls contained only buffer) and 200µL 5mg/mL ampicillin in a 5mL Eppendorf tube with the cap sealed with Parafilm® (Beemis) and each sample type was repeated in triplicate. These suspensions were incubated a room temperature (25±2^{o}C) for 24 hours shaking at 50 rpms. Then 100µL of each suspension plated on to potato dextrose agar plates containing 5mg/mL ampicillin in triplicate. After 96 hours at room temperature in the dark, plates were counted for CFUs. Supernatants of these assays were also subjected to DNSA assays to assess presence/abundance of reducing sugars in assays following incubation with GHF7s. For these assays, a mixture 100µL of conidia-free *in vitro* assay supernatant and 100µL of DNSA reagent was incubated and analyzed as described above. Three technical replicates were performed for each sample type.

Additionally, conidia suspensions were used to challenge termites to determine conidial potency following incubation. Groups of 10 termite workers, pre-treated with 5% kanamycin or water for 48-hours, were submerged in 150 µL one of the suspensions. Termite mortality was scored at 7-days post-inoculation.

3.3 Results

3.3.1 Whole organism bioassays show pathogen specific effects of symbiont removal

For this project I was interested in the role for symbiota in pathogen defense. To do this I evaluated the effect of symbiont removal on termite survival after treatment with three entomopathogens. The experimental design allowed us to account for treatment specific mortality (i.e. antibiotic treatment) and assess the role of symbiont-mediated protection. Overall, removal of symbionts increases termite susceptibility to *B. bassiana* but does not significantly impact termites challenged by *M. anisopliae* or *S. marcescens* (Figure 3.1). Though, it should be noted that the biological trend suggests symbiota are more important for fungal pathogen defense than for protection against *S. marcescens*.

Although, *S. marcescens* treated termites do not suffer increased mortality, I did observe *S. marcescens* in the guts of both control and antibiotic treated termites, 48-hours post-inoculation (Figure B.1). Regardless of treatment, pathogen challenged termites had increased CFUs/gut compared to their paired controls at levels higher than can be accounted for by the presence of *S. marcescens* (Figure B 1).

3.3.2 Stereotypical host immune responses appear inconsistent

Here it was hypothesized that removal of gut symbiota would result in a compensation effect from the endogenous host immune system. To test this hypothesis, I investigated the effect of antibiotic treatment on various typical immune system parameters such as: circulating hemocytes, increased protein-level immune responses, and corresponding increases in immune-associated gene expression. Overall, termite immune responses assessed here-in followed no discernable pattern.

Termites with reduced microbiota due to antibiotic treatment did not experience a rise in circulating hemocyte numbers in response to pathogen challenge (Figure 3.2). Both pathogens tested, *B. bassiana* and *S. marcescens*, elicited an increase of 2-2.5X in hemocytes in faunated termites (Figure 3.2). In contrast, defaunated termites only possessed ~1.5X increases in hemocytes when exposed to *B. bassiana* and even less when dosed with *S. marcescens* (Figure 3.2).

At the protein level, I investigated the total protein profiles of my four treatment groups via SDS-PAGE to identify treatment or pathogen specific patterns of protein expression. Overall, no obvious patterns emerged and protein profiles seemed to vary more by colony than by treatment or pathogen type (Figure B.2).

In contrast, some host immune-associated genes were impacted by pathogen type. Defaunated termites challenged by *B. bassiana* had significant increases in expression of seven genes previously described to be associated with pathogen challenge (Hussain *et al.* 2013). Of the tested genes, asparaginyl endopeptidase-like cysteine peptidase (AEP), cathepsin O, calpain B, and metacaspase-like cysteine peptidase (MCP) all shared a similar pattern of being down-regulated in faunated termites and up-regulated in defaunated termites (Figure 3.3A). Termicin and 14-3-3 protein 1 (14-3-3) were induced regardless of faunation status; and hexamerin 2 (Hex2) was only differentially regulated in defaunated, *B. bassiana* challenged termites (Figure 3.3A). In contrast a pathogen-specific response to *S. marcescens* was only detected in AEP, where it was again up-regulated in defaunated and down-regulated termites but to a lesser degree than in the fungus treatment (Figure 3.3B).

Three genes shared common expression patterns regardless of pathogen type. The host-derived heat shock protein 90 (HSP90) and glycosyl hydrolase family 7-3 (GHF7-3) were up-regulated only in pathogen-challenged, defaunated workers (Figure 3.3C). Gram-negative bacterial binding protein, a key antimicrobial recognition protein, was up-regulated in all treatments but to a greater extent in symbiont-depleted termites (Figure 3.3C).

3.3.3 GHF7-5 and 7-6 activity against β-1,3- and/or β-1,6-glycosidic bonds

Using pure recombinant enzyme I tested GHF7-5 and 7-6 activity on a variety of substrates with varying incubation times and buffers. Ultimately, the best activity of this enzyme was with sodium acetate buffer at pH 7.0 incubated at 50^{o}C for 10 min on 0.75% laminarin (Figure 3.4). The other reducing sugar assays did not show enzyme activity scaled with substrate concentration (B.3). Nitrophenol assays with HEPES buffer also failed to show activity.

3.3.4 Testing two protist glycosyl hydrolases for anti-fungal activity

Again using recombinant enzymes for two protist-derived GHF7s, I tested the capacity of these enzymes to impede fungal development and in turn confer fungal protection to their host termites. Following *in vitro* incubation of fungal conidia with these enzymes, *M. anisopliae* show significant reductions in fungal colony forming units relative to a buffer control (Figure 3.5). *B. bassiana* showed some decrease in conidial success, however, there was a large amount of variation across replicates (Figure 3.5). Additionally, supernatant from *in vitro* assays of all enzyme-fungus combinations had detectable levels of reducing sugars present which were absent from buffer controls. This indicates the breakdown of polysaccharides in GHF-positive *in vitro* assays (Figure 3.6).

3.3.5 Pre-treatment of fungal pathogenic agents with GHF7s attenuates some antibiotic-induced termite susceptibility

The effect of pre-treatment of fungal conidia with GHF7s on subsequent termite survival was also evaluated. Defaunated termites which are subsequently challenged with GHF7 pre-treated *B. bassiana* had higher survival than those challenged with buffer pre-treated *B. bassiana* conidia (Figure 3.7). Unfortunately, termite colony-to-colony variation was high in this experiment and limited my ability to draw conclusions from the *M. anisopliae* data.

3.4 Discussion

3.4.1 Symbiota Serve a Protective, Anti-Fungal Role in Termites

The goals of this study were multifaceted. First, I wanted to test the hypothesis that reduction in *R. flavipes* gut symbiota will increase pathogen susceptibility. My data show that, while this may be supported in regard to the fungal pathogens tested, antibiotic pre-treatment did not influence termite susceptibility to *S. marcescens* (Figure 3.1). This corroborates results found in two similar studies. In *Zootermopsis angusticollis,* termites subjected to oxygenation treatment (to reduce symbiota) suffered higher mortality than their faunated counterparts when exposed to *M. anisopliae* (Rosengaus *et al.* 2014). *R.*

flavipes have also been shown to have increase susceptibility to low doses of *M. anisopliae* following treatment with imidacloprid, which reduces protist abundance in the hindgut (Sen *et al.* 2015). Imidacloprid did not increase *S. marcescens* susceptibility to the same extent, however (Sen *et al.* 2015). Thus, termite symbiota may serve an immuno-protective role for their hosts when specifically challenged with fungal pathogens.

3.4.2 Termites with Reduced Microbiota Are Able to Mount Some Immune Responses, While Others are Attenuated

We measured a number of conventional immune responses in the termite following pathogen challenge with and without symbiota to determine if the host immune system compensates for any lost symbiont-mediated protection. Circulating hemocytes are often used as a barometer for the induction of cellular immunity (Lu and St. Leger 2016). In the present study, I observed a failure of defaunated, pathogen-challenged termites to induce increased circulating hemocytes compared to symbiota-intact controls (Figure 3.2). This result may indicate a couple of scenarios. One option could be that defaunated termites rely on cues from their symbiota to initiate cascades leading to increased hemocyte production, so removal of the symbionts and, thus this cue, slows or inhibits hemocyte induction/proliferation. In fact, in some insects host immune system maturation and circulating hemocyte abundance have been linked with symbiotic microbiota (Weiss *et al.* 2011, Schmitz *et al.* 2012*,* Weiss *et al.* 2012). Another possibility is that the act of defaunation preemptively triggers an immune response. Hemocyte numbers are known to fluctuate throughout the lifecycle of insects (Jones 1962, Wheeler 1963). During the molting process insects shed their cuticle which includes the lining of their fore- and hindgut, also eliminating symbiota (Douglas 2011). Therefore, the loss of symbiota in the hindgut may trigger a pre-programmed response to up-regulate cellular and humoral immune mechanisms. In the present case, it seems to be the latter. Termites treated with antibiotics have more circulating hemocytes than control termites, showing that this is an antibiotic effect. The failure to induce greater circulating hemocytes seen in pathogen-challenged, defaunated termites may be due to an already higher-than-normal titer.

In addition to cellular immune response, I measured the relative expression of several endogenous, immune-associated genes in my treatment groups. The two pathogens tested shared consistent results across pathogens for two termite genes: GNBP1 and HSP90 (Figure 3.3C). GNBP1 is an enzyme which degrades pathogen cell walls by way of hydrolysis (Hamilton *et al.* 2011). Though it is usually associated with bacterial pathogens, there is parallel evidence of its up-regulation in response to fungal pathogen challenge in previous studies (Gao *et al.* 2012, Hamilton and Bulmer 2012, Hussain *et al.* 2013). HSP90 is a chaperonin associated with many kinds of stress responses in termites, including pathogen challenges (Hussain *et al.* 2013, Sen *et al.* 2015). *B. bassiana* treatment induces the most numerous (7) pattern of predictable gene expression (Figure 3.3A). Termicin and 14-3-3 are genes which have been associated with mediating direct interactions with pathogens (Bulmer *et al.* 2009, Ulvila *et al.* 2011, Hamilton & Bulmer 2012). AEP, calpain B, cathepsin O, and MCP are all peptidases which have- previously been reported to be induced by pathogen challenge in *Coptotermes formosanus* (Hussain *et al.* 2013). These genes all follow the pattern of being down-regulated in control, *B. bassiana* treated termites and up-regulated in defaunated termites.

S. marcescens treatment also caused an up-regulation in AEP expression, but to a lesser extent than in *B. bassiana* treated termites (Figure 3.3B). Again, faunated termites show down-regulation of this gene in pathogen-challenged termites and up-regulation in defaunated, pathogen-challenged termites. This discontinuity between my study and others could be due to my use of carcasses (thorax and abdomen minus gut) as my tissue sample for RNA extraction. The use of whole insects may have produced results more consistent with other studies.

Despite these methodological pitfalls, I can say that defaunation is impacting termite immune functionality. However, both the induction of hemocytes upon removal of symbionts (even in the absence pathogen challenge) and the up-regulation of immune-associated genes are consistent with my hypothesis that termites rely on endogenous immunity when symbiota are disrupted.

3.4.3 GHF 7-5 and 7-6 as Potential Symbiont-Derived, Anti-Fungal Enzymes

Following the prior-identification of symbiont derived β-1, 3-glucanase activity as a means of anti-fungal defense (Rosengaus *et al.* 2014) and the result that the combination of *M. anisopliae* + imidacloprid treatment effects termite survival and down-regulation of GHF7-5 by 1000-fold (Sen *et al.* 2015), I decided to investigate the role of two protist GHF7s in anti-fungal defense. These particular enzymes, GHF7-5 and 7-6, had been previously characterized as having weak β-1, 4-glucanase activity, and as such were concluded as not likely to be not important for lignocellulose digestion (Sethi *et al.* 2013). While I tested a number of assay conditions and substrates, GHF7-5 and 7-6 only had strong activity with the substrate laminarin with sodium acetate buffer (Figure 3.4). Activity of these enzymes on the substrate laminarin is promising due to its homology to fungal cell walls (having β-1, 3 and β-1, 6-glucan linkages), in fact it has been used as an analog to trigger immune responses *in vivo* (Mullin and Goldsworthy 2006). However, this result may be considered dubious because of sodium acetate's limited buffering capacity at pH 7.0, though other enzymes from the *R. flavipes* gut environment also operate optimally in this buffer (Scharf *et al.* 2011). Additionally, the reaction temperature (50^{o}C) is not physiologically relevant for the termite gut.

That being said, the result that reducing sugars are being liberated from fungal conidia *in vitro* shows promise (Figure 3.6). Based on the contents of this *in vitro* assay, the only substrate with complex polysaccharides would be the fungal cell wall. Fungal cell walls often have varied arrangements of primarily β-1, 3 and β-1, 6 glucans latticed atop of a chitinous layer (Free 2013). Additionally, no reducing sugars were detectable in no-enzyme control assays, strengthening the argument that GHF7-5 and 7-6 are responsible for this breakdown. Finally, the marked difference in abundance of reducing sugars between species of fungi may reflect differences in their cell wall glucan composition, which is known to vary drastically across fungi (Free 2013).

GHF7-5 and 7-6 also had varying impacts on the viability of conidia. Fungal conidia from both tested fungi exhibited some reduction in germination following incubation,

however as with the reducing sugars assay, *M. anisopliae* was significantly more inhibited by GHF7 pre-treatment than *B. bassiana* (Figure 3.6).

Perhaps contrary to these data, *B. bassiana* pre-treatment with GHF7-5 and 7-6 seemed to more positively impact defaunated termite survival; whereas, there is no statistical effect of pre-treatment in *M. anisopliae* (Figure 3.7). There is considerable variability across termite colonies that may be impeding my ability to interpret these results. Colony-to-colony variability in termites is a confounding factor due to differences in genetic background, age, time in culture, and symbiont populations (Matsuura 2003, Boucias *et al.* 2013).

Overall, my efforts to pinpoint a role for GHF7-5 or 7-6 in protist-mediated anti-fungal defense in *R. flavipes* proved difficult. While there are several interesting data points in this study, more work is required to elucidate the level of necessity and mechanism of GHF7-5 and/or 7-6 action on entomopathogens like *M. anisopliae* and *B. bassiana*.

3.4.4 Conclusions

The aims of this project were to 1) investigate symbiont roles in pathogen defense in *R. flavipes* workers, 2) determine if termite endogenous immune responses are sufficient for pathogen defense in the absence of symbiota, and 3) evaluate two protist-derived GHF7 enzymes as anti-fungal enzymes. I found that symbiont removal is more detrimental in the face of fungal pathogen than the tested bacterial pathogen. Endogenous termite immune responses also play a role in compensating for defaunation in pathogen-challenge termites, though tissue selection for gene expression studies may have influenced these results. Finally, two protist glycosyl hydrolase enzymes, GHF7-5 and 7-6, show some signs of promise as potential mechanisms for anti-fungal activity against *M. anisopliae* and *B. bassiana*. *In vitro*, both enzymes impact *M. anisopliae* conidial viability and liberate reducing sugars, likely from digested fungal cell walls. In contrast, pre-treatment with these enzymes seems to have more impact on termite survival in *B. bassiana* treatments. These results suggest that perhaps fungus-specific cell wall arrangements influence the impact of GHF7 activity. In general, these data support the

concept of symbiont-mediated pathogen defense in *R. flavipes*. GHF7-5 and 7-6 show potential as a means for this protection, but further study, including more complete biochemical characterization, is needed.

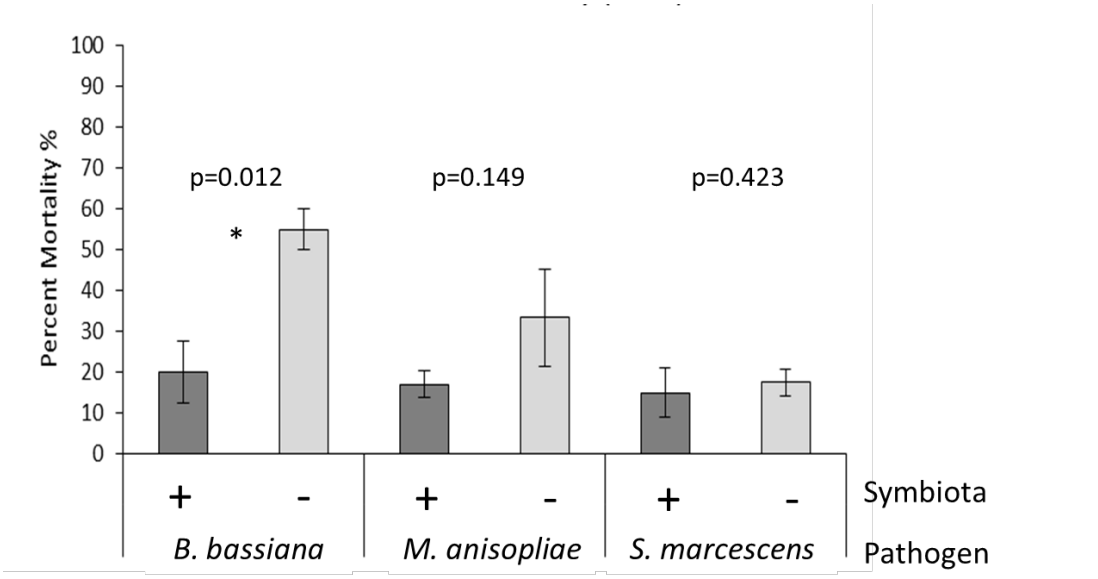

Figure 3.1 Changes in termite mortality following pathogen challenge

Dark bars represent termites with intact gut fauna and light bars represent termites with reduced gut fauna following kanamycin treatment. Error bars represent SEM. Each paired treatment was compared using a t-test to determine the impact of symbiont remove on individual pathogen susceptibility (α=0.05).

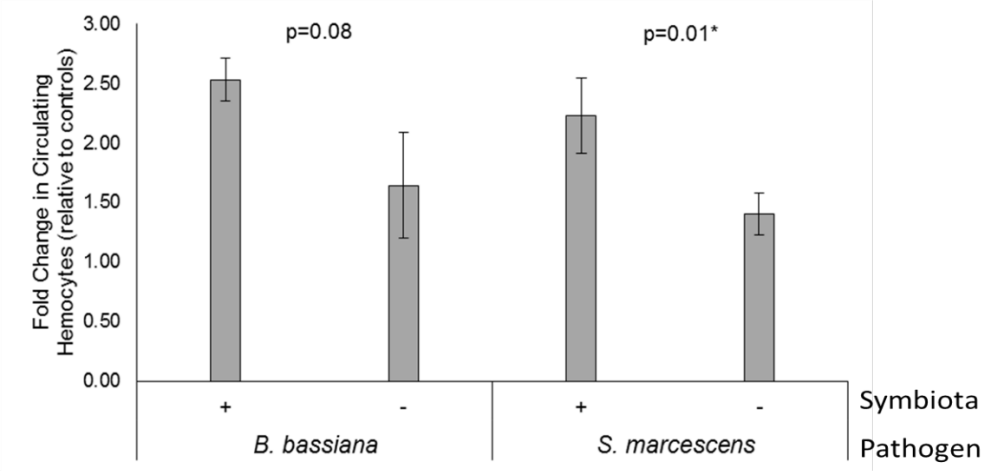

Figure 3.2 Fold-change in circulating hemocytes

Fold-change in hemocytes following pathogen challenge with *B. bassiana* (right) or *S. marcescens* (left). Error bars represent SEM.

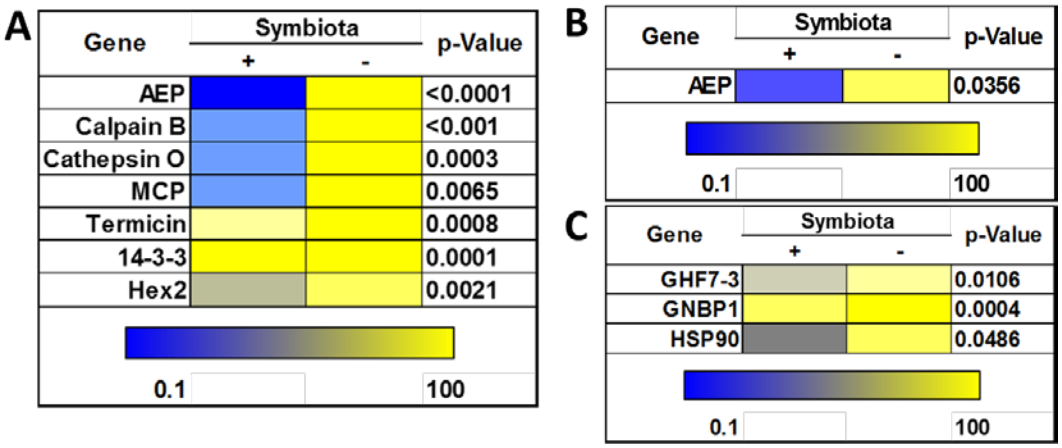

Figure 3.3 Heat maps showing patterns of differential gene expression among treatments

Gene expression patterns following pathogen challenge with either **A**) *B. bassiana,* **B**) *S. marcescens,* or **C**) common among both treatments. Each row represents a different immune-associated gene. Blue represents down-regulation of a gene and yellow colors represent up-regulation, the intensity of the color is indicative of the degree of differential expression. Results from t-test comparison of normal and antibiotic treated termites are listed to the right of each row (α=0.05).

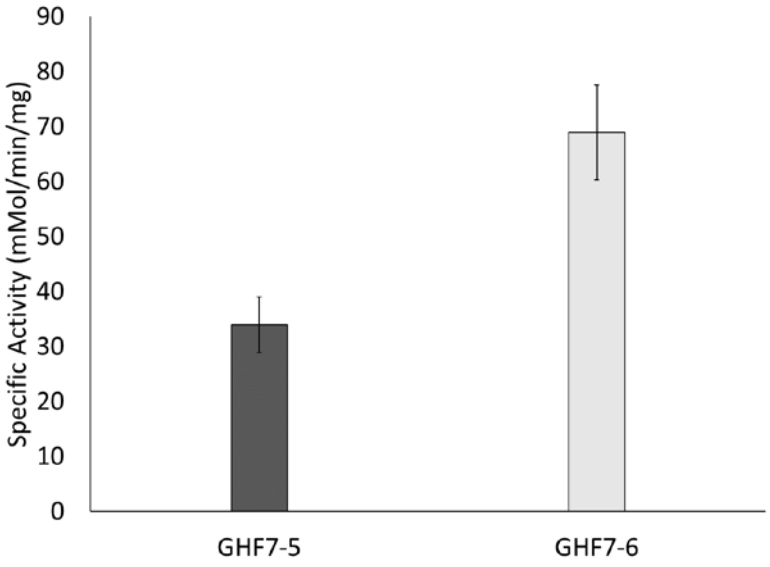

Figure 3.4 GHF7-5 and GHF7-6 activity on the substrate laminarin

Activity of GHF7-5 (dark bar) and GHF7-6 (light bar) on 0.75% laminarin producing reducing sugars detected by DNSA. Error bars represent standard error across six technical replicates.

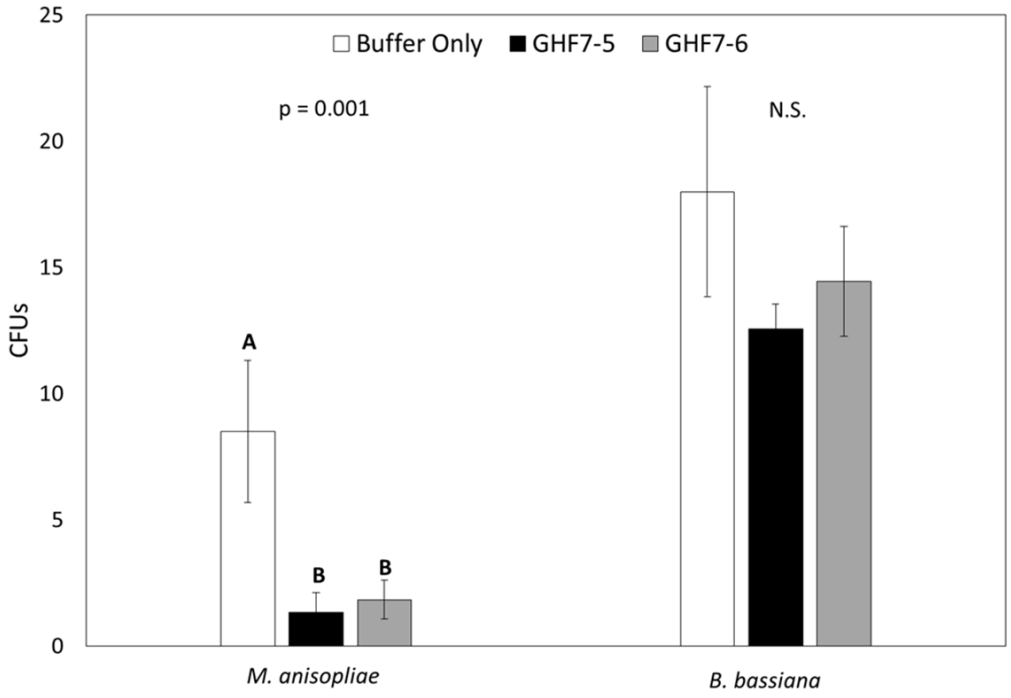

Figure 3.5 Fungal CFUs after pre-incubation with GHF7s

Colony forming units counted 96-hours post-incubation with potential anti-fungal enzymes. White bars represent buffer only controls, black bars represent GHF7-5 treated conidia, and gray bars represent GHF7-6 treated conidia. Error bars represent SEM. Bars with the same letter are not statistically different ($\alpha = 0.05$).

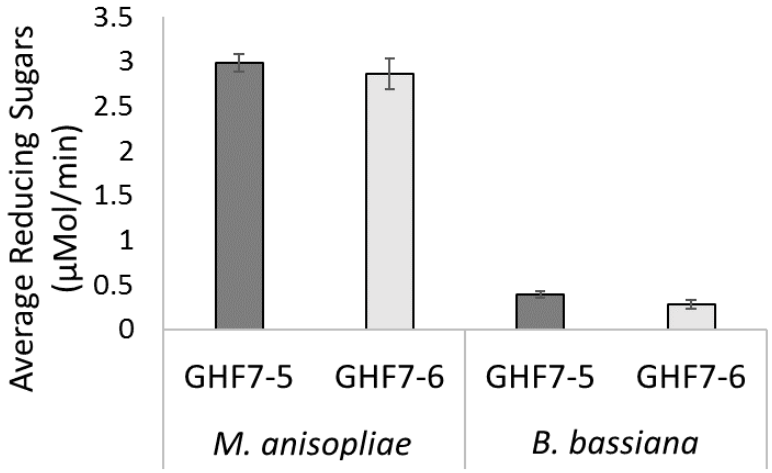

Figure 3.6 Reducing sugars detected in *in vitro* assays

Average reducing sugars released during *in vitro* assays detected with DNSA. Note no reducing sugars were detected (<0.000 mMol total at endpoint) in no enzyme controls.

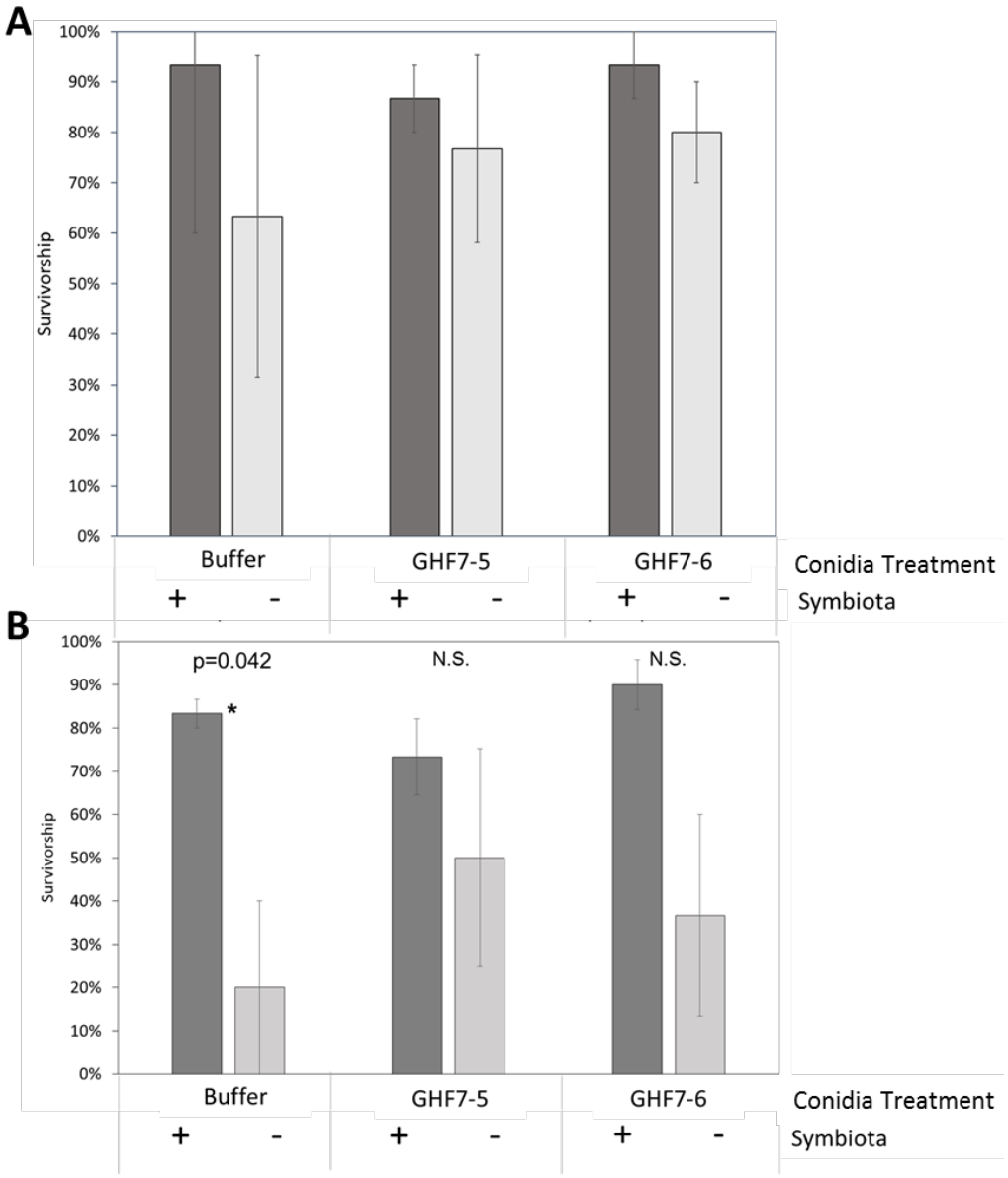

Figure 3.7 Termite survival following challenge with fungal conidia

A is *M. anisopliae* and **B** is *B. bassiana* pre-treated with buffer (control), GHF7-5, or GHF7-6. Dark bars represent termite workers with intact gut fauna and light bars represent termite works pre-treated with kanamycin. Error bars represent SEM. Each paired treatment was compared using a t-test to determine if conidial pre-treatment would rescue symbiont depletion (α=0.05).

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CHAPTER 4. NOVEL METATRANSCRIPTOME ANALYSIS REVEALS BACTERIAL SYMBIONT CONTRIBUTIONS TO LOWER TERMITE PHYSIOLOGY

4.1 Introduction

The intimate association of termites and microbes is so tightly linked that often what it means to be a termite cannot be discussed without describing their symbiotic relationships. In the 1920's L.R. Cleveland described this association and the necessity of these 'parasites' to termite survival (Cleveland 1923, Cleveland 1924). The discovery of protist-produced cellulases in lower termites and wood roaches as a means to thrive on a nitrogen poor, recalcitrant diet such as wood forever solidified termite symbiosis as the quintessential example of insect-microbe collaboration (Trager 1932). For decades, termites were thought to rely entirely on symbiota for the digestion of their food until a highly-active, highly-expressed, endogenous β-1, 4-glucanase was identified in a lower termite species (Watanabe *et al.* 1998). This finding shifted the perspective of this symbiosis from unidirectional to collaborative. As tools in molecular biology advanced, more and more cellulytic enzymes were identified from the symbiotic partners and hosts in all termite symbioses (Ohtoko *et al.* 2000, Tartar *et al.* 2009, Todaka *et al.* 2010, Zhou *et al.* 2010, Do *et al.* 2014, Yuki *et al.* 2015). The synergy demonstrated by these enzymes from lower termites begins to explain how the efficiency of this system led to such success (Scharf *et al.* 2011).

Termite research consistently addresses digestive symbioses, but lower termite literature has focused on protist-termite collaborations. Until recently, bacterial contributions to wood digestion in lower termites were largely disregarded (Brune and Dietrich 2015). However, bacteria are now known to play important roles in

nitrogen cycling, hemicellulose and aromatic compound degradation, and acetate metabolism which likely contribute to the maintenance of efficient cellulose digestion in the termite gut (Hongoh *et al.* 2008a, Hongoh *et al.* 2008b, Tartar *et al.* 2009, Lucey and Leadbetter 2014). Reduction in bacterial number and diversity have been linked to reduction in lignocellulytic activity (Peterson *et al.* 2015, Chapter 2).

Though the traditional, intuitive role for gut bacteria may be nutritional, gut microbiota can have profound impacts on their insect hosts. Symbionts of stinkbugs have been shown to confer pesticide resistance to their hosts, Colorado potato beetles circumvent plant defenses with help from bacterial symbionts, and microbes appear to be at least partially responsible for rotation resistant populations of western corn rootworm (Kikuchi *et al.* 2012, Chu *et al.* 2013, Chung *et al.* 2013). These examples suggest that insect associated microbes may play more diverse roles than previously thought. Indeed, lower termite symbionts have recently been credited with contributing important anti-fungal enzymes which extend increased pathogen defense to their insect hosts. Beta-1, 3-glucanases from protist symbionts have been identified as a source of fungal deactivation in the lower termites (Rosengaus *et al.* 2014). While symbiotic actinobacteria have been shown to provide anti-fungal functions within the nest walls of some subterranean termites (Chouvenc *et al.* 2009), there has been no mechanistic link between symbiotic gut bacteria and pathogen defense in lower termites. However, bacteria play important roles in ant and termite fungus farming mutualisms (Barke *et al.* 2010, Visser *et al.* 2012). So it is reasonable to postulate that lower termite-associated gut bacteria are contributing to host physiology in more ways than just nutrition/digestion.

This idea of “collaborative physiology” represents a joint effort by the members of the holobiont to accomplish basic physiological tasks, like digestion and immunity. Steps in assessing the extent and mechanisms of these collaborations require approaches which encompass the entire micro-ecosystem that is the termite gut. Assessing the holobiont allows for a more complete picture of functional capacity of individual members of the consortium but also sheds light on interspecific collaborations (Peterson and Scharf 2016, Chapter 1).

Here I harnessed the power of next-generation sequencing to explore the contributions and potential collaborations of the termite host and its hindgut consortium. My aims for this project were two-fold: 1) to describe the composition of the gut holotranscriptome and 2) to identify potential mechanisms of bacterial-derived anti-fungal defense to the fungal pathogen, *Beauveria bassiana*. Using a ribo-depletive library preparation strategy, I captured a transcriptome of the holobiont that included the often-overlooked prokaryotic symbiota. My findings highlight the potential for extensive collaboration between symbiota and the host termite including an abundance of transcripts encoding bacterial nutrient and metabolite transporters, amino acid synthetic enzymes, and carbohydrate metabolism. Additionally, I identify a candidate mechanism for bacterially-mediated anti-fungal activity in the termite gut. Overall, this study provides a snapshot of the potential functions of bacteria in *R. flavipes* guts and begins to shed light on the extent to which collaboration between the host and its prokaryotic partners plays a role in defense against fungal entomopathogens.

4.2 Materials and Methods

4.2.1 Termites and Pathogen Rearing

R. flavipes termites used in this project were collected on the campus of Purdue University in West Lafayette, IN between May and July 2014. Colonies were reared in darkness at 22±2°C with ~40% relative humidity and were provided with pine wood shims and brown paper towels as a food source. The pathogen used in this study was *B. bassiana* isolate #5477 which was cultured in darkness on potato dextrose agar (PDA) at room temperature. To collect conidia for bioassays, 10 to 12-day old *B. bassiana* culture plates were flooded with 5mL of filter sterilized 0.5% Tween-20. Conidial concentration was determined via hemocytometer and diluted to 1.25 x 10^4 conidia/mL for termite inoculation.

4.2.2 Bioassay Setup & Dissections

Groups of 30 termite workers were either submerged in a suspension of 1.25 x 10^4 conidia/mL in 0.5% filter sterilized Tween 20 or Tween 20 alone for one minute. Termites

were transferred to 33mm plastic dishes containing 2cm^2 of Whatman filter paper wetted with 100µL of deionized water. Bioassays were held at 28±2^{o}C for 48hr in complete darkness in a growth chamber. Paired treatment groups (control and pathogen challenged) from 3 distinct termite colonies served as biological replicates. After 48hrs in bioassay, termite whole guts were dissected from 25 workers per treatment, submerged in RNAlater (ThermoFisher Scientific) and stored at -80^{o}C.

4.2.3 RNA Isolation & Library Preparation

Samples were thawed on ice and all RNAlater aspirated leaving only termite gut tissue. This tissue was then homogenized in Promega SV Total RNA Isolation Kit lysis buffer and the manufacturer's protocol followed to extract total RNA from all samples. RNA concentration and relative purity was quantified using a NanoDrop 2000 (ThermoFisher Scientific).

To reduce eukaryotic bias in library preparation, total RNA was ribodepleted rather than enriched for mRNAs. The metatranscriptome libraries were prepared from total RNA using the Ovation Complete Prokaryotic RNA-Seq DR Multiplex System 1-8 (NuGEN) with the addition of custom oligos targeting the 18S rRNA from eukaryotic species anticipated to be in the samples (termite, protists, and fungal pathogen; Table C.1). Total RNA (250ng) was used as starting material for the Ovation kit and cDNA was synthesized following manufacturer instructions. After second strand synthesis, cDNA samples were frozen overnight at -20^{o}C.

After thawing, samples were sonicated using a Covaris E210 in Covaris #520045 6x16mm microtubes using the parameters specified in the Ovation protocol (Intensity = 5 not 5%) and transferred to fresh 200uL microtubes and stored overnight at -20^{o}C.

cDNA purification, end repair, barcode ligation, first strand selection, and first strand purification were performed per manufacturer's protocol. At the strand selection II step, 1µL of the 100µM custom oligo mix was added to 16µL of Solution SS5 to deplete eukaryotic rRNAs from samples. Adapter cleavage was performed as specified in the kit manual. Library amplification master mix was made fresh per protocol instructions, but the thermocycler program was modified from the manufacturer's protocol as follows:

95°C for 2 min, 20 cycles of 95°C for 30s, 60°C for 90s, and 65°C for 5 min. Bead purification of the amplified library was done with a multi-channel pipet to minimize incubation bias of the libraries.

4.2.4 Metatranscriptome Sequencing, Assembly, Annotation, and Analysis

A workflow summarizing the major steps in the metatranscriptome analysis is shown in Figure 4.1. Purified, indexed libraries were submitted to the Purdue University Genomics Core (PUGC) facility for quality control and sequenced in 1 lane on the Illumina HiSeq2500 2x100 paired-end reads. Contigs of one control library containing the least rRNA reads were assembled *de novo* using Trinity 2.1.1 by PUGC, all other libraries were mapped to this assembly to produce a read count table. Any contigs with less than 10 reads across samples or identified by homology search as rRNA were filtered out.

In an effort to annotate the contigs in a taxon specific way, a custom termite consortium database was built from existing data in the National Center for Biotechnology Information (NCBI) refseq protein database (bacteria, archaea, select protists, Table 4.1) and the *Zootermopsis nevadensis* official gene set (OGS), as this is the only termite genome available at present time (Terrapon *et al.* 2015). Contigs that were reciprocal best hits (RBH) with entries in this database at an e-value 1e-5, or less, were carried through for additional analysis. This ensured a conservative annotation of contigs in this assembly, though definitely limited detection of novel and redundant genes within the consortium.

To associate contigs with gene ontology (GO) terms, the Genbank identifiers from the list of RBH for each taxon group (bacteria, archaea, protists, and termite) were analyzed using the Uniprot retrieve/ID mapping function available online (www.uniprot.org/uploadlists/). Using the Bioconductor package for the R statistical software, edgeR differential expression analyses were done on read counts for all contigs to detect responses to the fungal pathogen (α and FDR = 0.05). To determine if any biological processes or molecular functions were enriched in a taxon group, lists of GO terms from each taxon group (bacteria, archaea, protists, or termite) were compared to

all GO terms in the gene set and enrichment was determined with a Fisher's exact test in the topGO function in the Bioconductor package.

4.2.5 Metatranscriptome Validation

Quantitative real-time polymerase chain reaction (qPCR) was used as an independent validation of read count values used to generate the contigs for differential expression analysis. Contigs representing termite, bacteria, protist, up-regulated, down-regulated, and no change groups were selected for qPCR validation (Table C.2). Using the cDNA samples generated as described previously, 1µL of cDNA, 1µL each of contig-specific forward/reverse primers, 7µL nuclease-free water, and 10µL of SensiFast SYBR no ROX master mix (Bioline) were combined for qPCR. After an initial denaturation step (10 min. at 95^{o}C), 45 cycles of denaturing (30 sec. at 95^{o}C), annealing (30 sec. at 50^{o}C), and extension (30 sec. at 72^{o}C) were performed with a real-time scan of fluorescence taken after each cycle. The log ratio CT values were regressed against log ratio of counts per million values as a measure of congruency. Regression data were analyzed by the Spearman correlation method.

4.2.6 Post-hoc Reactive Oxygen Species and Glutathione S-Transferase Activity Assays

To validate findings in the metatranscriptome, additional assays were performed test the potential for oxidative stress and increased antioxidant enzyme activity in the termite gut following *B. bassiana* challenge. Reactive oxygen species (ROS) were detected using a modified FOX1A assay (Deiana *et al.* 1999). Bioassays were repeated and dissected as described above. A mixture of 100µL of termite gut homogenate containing 10 termite gut equivalents in 100mM sodium phosphate buffer was combined with 100µL nanopure water and 100µL of FOX1A reagent was incubated for 40 minutes in the dark. Endpoint absorbance was measured at 580nm and compared to a hydrogen peroxide standard curve to estimate reactive oxygen species concentration. This was repeated in triplicate for control and pathogen-challenged groups and all biological replicates.

Glutathione S-transferase (GST) activity was measured kinetically using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. Freshly prepared 1mM CDNB in 100mM sodium phosphate buffer (pH 7.0) was combined with 10µl of gut homogenate with or

without 5mM reduced L-glutathione for a total reaction volume of 235µl. Absorbance was read kinetically for 10 minutes and mean velocity for all samples were calculated. The mean velocity of glutathione-plus samples was blank-corrected with their corresponding glutathione-minus controls. Specific activity was calculated using the extinction coefficient of CDNB of 9.5 mM^{-1} cm^{-1}.

Both ROS and GST assays measurements were normalized per milligram of protein in each sample. Protein concentration of samples was estimated using the Pierce Coomassie Plus Bradford Assay Kit (Thermo-Fisher Scientific).

4.3 Results

4.3.1 Ribodepletion effectively removes rRNA from the hyper-diverse termite gut

In total, 2,107,824 contigs were assembled from a control termite gut holotranscriptome *de novo* (Table 4.2B). Sequences analyzed contained 1.2% rRNA reads and the average rRNA contamination across all samples was estimated at 12.33% (Table 4.2A). Of the assembled contigs 258,251 had an N_{50} length of 652 bases and average length of 704 bases (Table 4.2B). After filtering out rRNA and contigs with <10 reads across samples 1,511,386 contigs remained. Additionally, a cluster dendrogram based on a Pearson distribution and a multiple dimension scaling plot agree that samples cluster together based on treatment type (control vs. *B. bassiana* challenge) rather than colony (colonies nos. 18, 21, or 22) (Figure 4.2A&C).

4.3.2 Summary of the Holotranscriptome

In order to assign annotations to potential genes of interest, all assembled, filtered contigs were reciprocally BLASTed to determine putative function and taxonomic assignment (Figure 4.1). A total of 31,156 contigs had RBH with entries in the custom termite consortium database. Each of these annotations was associated with a taxonomic group: termite, protist, bacteria, or archaea (Figure 4.3). Of these, 21,269 contigs had hits in the Uniprot ID matching database. It should be noted that the protist and archaeal annotations are more incomplete due to a lack of information available in the NCBI RefSeq database regarding termite symbionts groups (archaea, bacteria, and specific protists). The bacterial and termite contigs, however, seem considerably more complete with

annotations in many critical biosynthetic, catabolic, transport, and stress response processes (Table 4.3). Additionally, some of these categories, like amino acid biosynthesis for example, appear to have signatures of complementation between the host termite and bacterial symbionts (Table 4.3).

4.3.3 Differential gene expression analysis

Using the edgeR analysis package, a total of 563 genes exhibited significant differential expression in response to fungal pathogen challenge (FDR $p < 0.05$). The majority of these contigs were annotated as host-origin, but some symbiont contigs were also impacted (Table 4.4). In total, 162 contigs were up-regulated and 401 were down-regulated (Table 4.4). Of the differentially expressed contigs, only 223 contained Uniprot ID matches and 225 had annotated GO terms. Relative expression observed in the metatranscriptome was validated using qPCR. Log CT ratios were correlated to Log CPM ratios between treatment and control samples (Figure 4.2D). Spearman's correlation coefficient rho ($\rho = -0.69$) shows a strong negative correlation ($p = 0.008$), as would be anticipated.

In general, the termite contigs up-regulated in response to *B. bassiana* challenge reveal the hallmarks of oxidative stress (Table 4.5). Thirty-one ribosomal proteins were up-regulated which has been associated with slowed or inhibited protein translation. Additionally, a mitochondrial peroxiredoxin and a GST were up-regulated 2.8-fold and 5.1-fold respectively. Stress and immune-associated 10kDa heat shock protein and ferritin were up-regulated as well. Calcium (Ca^{2+}), iron (Fe^{3+}), zinc (Zn^{2+}), and other generic metal ion binding GO terms were abundant in the pathogen up-regulated, termite contigs. Strikingly, several components of the oxidative phosphorylation (OXPHOS) pathway were up-regulated (subunits of complex I, complex III, and complex IV and cytochrome c), however; ATP synthase contigs were not differentially expressed.

Up-regulated protist contigs also have annotations associated with responses to stress; specifically oxidative stress. Of note, two protist contigs annotated as 3'-5' exonucleases/DNA Polymerase I (DNA Pol I), which is associated with oxidative stress-linked DNA repair. The signal cascade initiators Ca^{2+}/calmodulin dependent kinase II (CAMK II) and mitogen-activated protein kinase 1 (MAPK1) were also up-regulated, and

may be involved in response to oxidative stress. The only bacterial contig with a named annotation in the up-regulated category is an amidohydrolase family 2. These enzymes have a wide-variety of catalytic capabilities, including activity against fungal metabolites.

4.3.4 *Beauveria bassiana* challenge results in oxidative stress

In addition to the up-regulation of contigs related to oxidative stress response, following 48-hour challenge with *B. bassiana*, termite guts had increased ROS present (Figure 4.4). Additionally, GST activity is significantly higher by 1.15X in pathogen challenged guts than control guts (Figure 4.5). While the origin of ROS and antioxidant/detoxification activity cannot be identified using this method, it can still be concluded that the termite gut is under oxidative stress 48-hours after inoculation with *B. bassiana*.

4.4 Discussion

4.4.1 Ribo-depletion produces a quality metatranscriptome assembly

One of the goals of this project was to identify candidate genes facilitating symbiont-mediated fungal pathogen defense in *R. flavipes*. To do this, a unique transcriptome preparation and analysis approach allowed for the ribodepletion of rRNA from total RNA rather than enriching (and potentially biasing) for mRNAs. A commercially available library preparation kit was modified to efficiently deplete all total RNA samples of anticipated prokaryotic and eukaryotic rRNAs. This strategy resulted in low rRNA content in the sequenced libraries and yielded a robust assembly of over 30,000 contigs. The contig annotation pipeline took a conservative approach to identifying termite, bacterial, protist, and archaeal sequences from a custom termite consortium database built from publically available information (Table 4.1). Though this pipeline likely misses novel transcripts and underestimates redundancies, this conservative approach produced contig annotations with reasonable confidence. Additionally, identifying 9,730 best reciprocal hits, out of the 14,610 annotated genes in the *Z. nevadensis* OGS (Terrapon *et al.* 2014), from gut tissue of worker termites in a different taxonomic family of Isoptera speaks to the quality of the assembly.

Contrastingly, the reference sequences for protist and archaeal symbionts proved to be inadequate for the purposes of this study. With only 248 and 174 RBH respectively,

undoubtedly important protist and archaeal contributions to this system have been under sampled as a whole and in reference to anti-fungal defense specifically. For instance, the absence of candidate protist-derived β-1, 3-glucanases, described to play a role in *Metarhizium anisopliae* neutralization (Rosengaus *et al.* 2014, Chapter 3), is one example of the consequence of this conservative strategy. However, the termite and bacterial libraries are more complete. From these annotated contigs I can ascertain a more complete picture of bacterial contributions to their termite host and the gut consortium as a whole. As described previously, bacterial symbionts appear to possess a complete amino acid biosynthetic toolkit (Table 4.3) (Mauldin *et al.* 1978, Nazarczuk *et al.* 1981, Husseneder *et al.* 2010, Tokuda *et al.* 2014). Additionally, the presence of nitrogen metabolism genes like nitrogenases, nitroreductases, and ureases corroborates the importance of bacterial symbionts for nitrogen recycling and fixation in the lower termite gut (Yamada *et al.* 2007, Hongoh *et al.* 2008a, Hongoh *et al.* 2008b, Hongoh *et al.* 2011, Wertz *et al.* 2012, Inoue *et al.* 2015). As with other recent studies in lower termites, this study corroborates that bacteria in *R. flavipes* guts possess a diversity of carbohydrate metabolism transcripts (Tartar *et al.* 2009*,* Do *et al.* 2014, Tokuda *et al.* 2014, Yuki *et al.* 2015). These annotations include cellulases and hemicellulases, glycosyl transferases, carboxyesterases, and polysaccharide lyases and appear to be both complementary and redundant to those encoded by the host termite (Table 4.3). Once again, this suggests that bacteria of lower termites like *R. flavipes* play an active role in wood digestion (Do *et al.* 2014, Tokuda *et al.* 2014, Peterson *et al.* 2015 [Chapter 2], Yuki *et al.* 2015) and fails to support the idea that carbohydrate metabolism is completely restricted to flagellate digestive vacuoles precluding bacterial catabolism (Brune and Dietrich 2015).

In addition to anabolic and catabolic potential, the termite gut metatranscriptome is rich in bacterial transporters. These transporters, particularly ATP-binding cassette (ABC) transporters, shed light on a practical aspect of the termite gut symbiosis. With a total of 787 annotated bacteria-derived transporters, including those responsible for all types of organic molecules and metal ions, the idea of a hypercollaborative *R. flavipes* gut environment with influx and efflux of all types of compounds between members of the

consortium is supported. ABC transporters have been noted as playing important roles in other insect-microbe relationships, particularly where metabolic partitioning is involved (Zientz *et al.* 2004, Wu *et al.* 2006, Snyder *et al.* 2010, Oakeson *et al.* 2014). Taken together, the complementary nature of the bacterial and termite contigs found in the gut metatranscriptome provides the foundation for a more complete view of this tripartite symbiosis.

4.4.2 *B. bassiana* Challenge Results in Oxidative Stress

Findings of this study indicate that, forty-eight hours post inoculation with *B. bassiana* conidia, the termite gut is experiences general oxidative stress. ROS estimation indicates a significant increase in pathogen challenged guts (Figure 4.4). Additionally, up-regulation in host antioxidant enzyme-coding genes for peroxiredoxin and GST, are corroborated at the protein-level with increases in GST enzyme activity (Table 4.5, Figure 4.5). One question this result raises is, *what is the origin of the observed ROS?* Presumably, any or all members of the consortium or the pathogen could produce free radicals. I propose, however, that the up-regulation of OXPHOS complexes I, III, and IV without corresponding up-regulation of ATP synthase may result in uncoupling-related proton leakage resulting in increased ROS (Kadenbach 2003). Production of ROS in response to pathogen challenge is a common defense strategy in eukaryotes, and coupled with more traditional immune-associated pathways (West *et al.* 2011, Buchon *et al.* 2013), may serve as a mechanism for endogenous termite anti-fungal defense.

In line with the increased oxidative stress that was observed, many of the up-regulated symbiont contigs are also associated with response to this type of damage. Two protist transcripts encoding protist signaling kinases, CAMKII and MAPK1, are up-regulated and may be involved in triggering cascades responsible for coordinating stress responses like oxidative stress and pathogen challenge (Chen *et al.* 2009, West *et al.* 2011). Additionally, two 3’-5’exonuclease genes are also up-regulated. These genes encode enzymes like DNA Pol I, which is responsible for DNA repair and has been shown to be responsive to oxidative stress (Imlay 2013).

4.4.3 Amidohydrolase 2, a candidate for Symbiont-Mediated Anti-Fungal Response

The primary aim of this project was to identify new candidate mechanisms of symbiont-mediated anti-fungal defense. The specific hypothesis tested was that bacteria collaborate with the rest of the holobiont to combat invaders. An ideal candidate gene should be up-regulated in response to pathogen presence and its product possess putative functions which might contribute to defense. Following these criteria, our dataset contained just one candidate: *amidohydrolase 2*. This amidohydrolase is a bacterial gene up-regulated 3.4X 48-hours post-inoculation with *B. bassiana.* Amidohydrolases are a large family of diverse enzymes which are catalytically promiscuous (Siebert and Raushel 2005). These activities include hydrolysis, isomerization, and decarboxylation of diverse substrates (Siebert and Raushel 2005). Amidohydrolases are found across domains of life and are particularly of note in bacteria due to their role in antibiotic resistance (Siebert and Raushel 2005). Beta-lactamases, enzyme class 3.5.2.6, catalyze the deactivation of beta-lactam antibiotics like penicillin (Bush 1989). While *B. bassiana* does not produce penicillins, it is known to produce ooconidiain which it uses to evade insect anti-fungal defenses like prophenoloxidase (PPO) and antifungal peptides (Feng *et al.* 2015). If this amidohydrolase is capable of hydrolyzing the quinone ooconidiain, this could contribute significantly to defense against *B. bassiana* in the termite gut.

In addition to amidohydrolase, there are 15 symbiont (6 protist and 9 bacterial) contigs that exhibit significant fold-change in response to *B. bassiana* pathogen challenge. While these genes have no known function, the possibility that they possess important anti-fungal propertiescannot be excluded. Unforunatley, this possibility cannot be ascertained at the present time given the current information available in public repositories like NBCI's RefSeq and Uniprot's ID mapping databases.

Finally, there remains the possibility that key symbiont-derived enzymes associated with anti-fungal defense are expressed earlier or later in the infection timeline. I chose the 48-hour time-point based on a previous study that showed fungal pathogen-induced changes to gene expression (Sen *et al.* 2015). However, that study was done with

a different fungus using vastly different microarray technology, and as such 48-hours post-inoculation may not be the optimum time-point for such observations.

4.4.4 Conclusion

In sum, this study represents the most complete metatranscriptome from *R. flavipes* to date, especially in regard to bacterial contributions. My findings shed light on the physiological collaboration in the termite gut consortium with regard to biosynthesis, catabolism, and transport of major organic molecules and ions. Notably, my data corroborate previous findings that bacteria possess the potential to play direct roles in nitrogen fixation, amino acid biosynthesis, and lignocellulose digestion.

Additionally, I propose a novel mechanism for bacterial-mediated anti-fungal defense by means of an amidohydrolase 2. The transcript for this gene is up-regulated 3.4-fold 48-hours post-inoculation with *B. bassiana* and, based on the diversity in this enzyme class, may catabolize fungal metabolites which inhibit insect immune responses and have antibiotic activity. Coupled with previous findings of protist-derived anti-fungal defenses, this proposed model of antifungal defense highlights the collaborative nature of termite holobiont immune physiology (Figure 4.6).

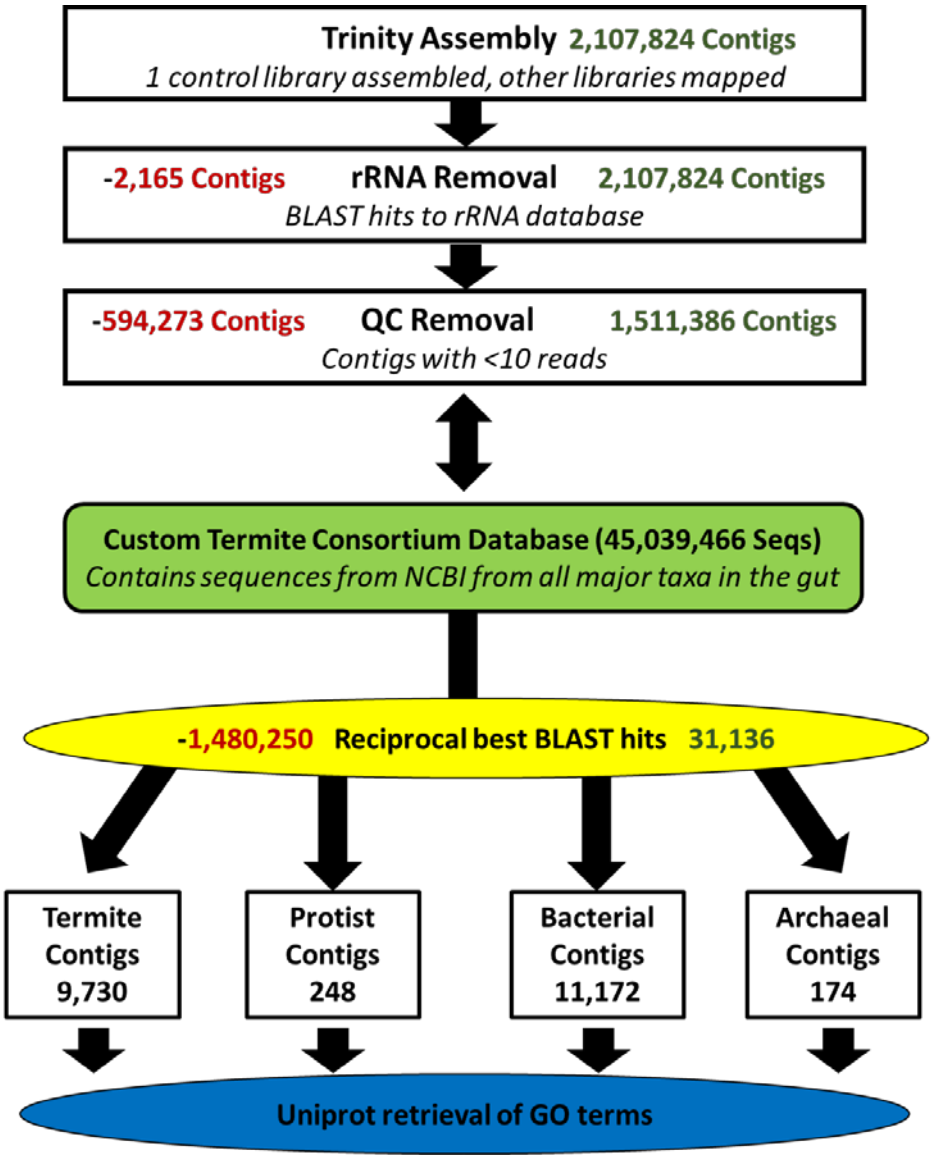

Figure 4.1 Workflow of metatranscriptome annotation.

Red numbers on the left of a given shape represent the number of contigs removed during that step and green numbers on the right of a given shape represent the number of contigs exiting the step.

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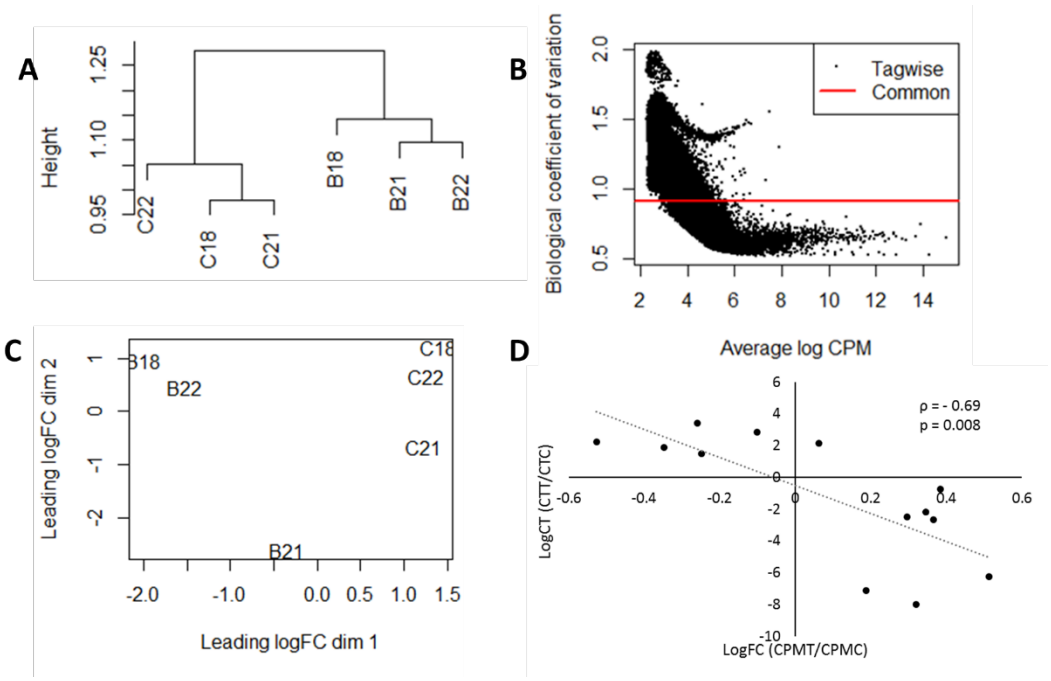

Figure 4.2 Quality control analyses of metatranscriptome.

A) Cluster dendrogram based on a Pearson distribution of all contigs following normalization. Samples are labelled with a letter indicating their treatment (C = control, B = *Beauveria)* and colony number. **B**) Plot of Biological coefficient of variation vs. average logCPM each spot represents an individual contig. **C**) Multiple dimension scaling plot showing distances in gene expression profiles across biological replicates and treatment groups. **D**) The correlation between logCT (CT of treatment/CT of control) vs. logFC (counts per million of treatment/counts per million of control), Spearman's correlation coefficient rho (ρ) reported shows a strong negative correlation (p = 0.008).

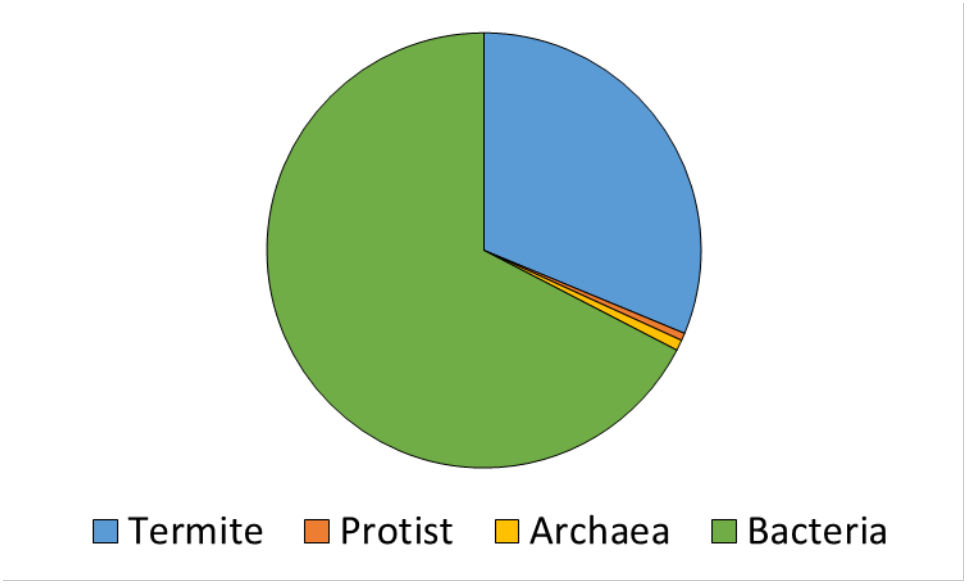

Figure 4.3 Pie chart demonstrating taxon distribution in annotated contigs.

Total proportions of the contigs from metatranscriptome annotated as belonging to each taxonomic group. Only those contigs which were RBH are included.

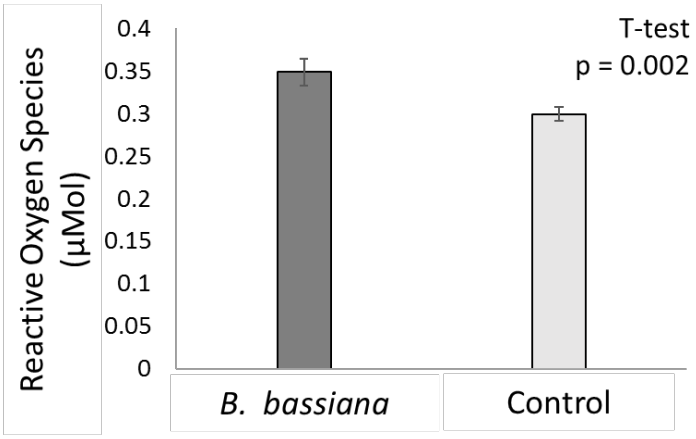

Figure 4.4 Detection of reactive oxygen species (ROS) following *B. bassiana* challenge.

Bars represent measured reactive oxygen species in *B. bassiana* (dark bar) and no treatment control (light bar) termite worker guts. Error bars represent SEM.

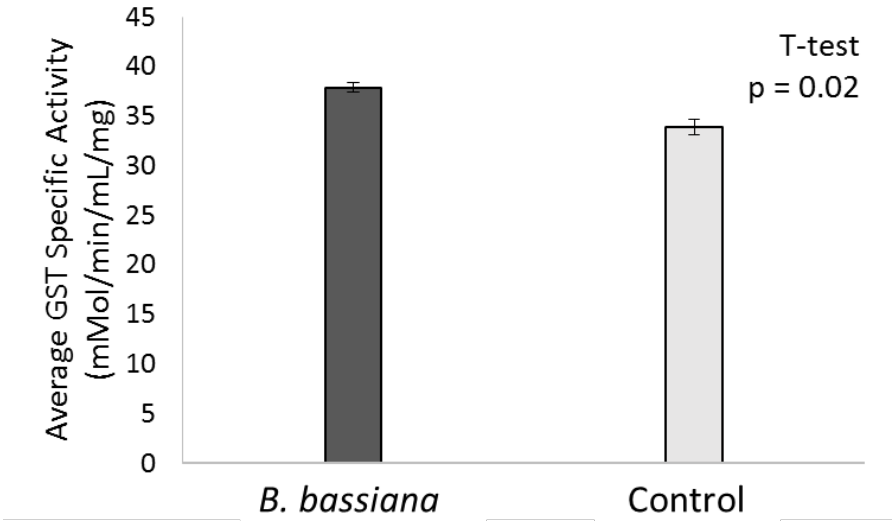

Figure 4.5 Detection of glutathione S-transferase (GST) activity following *B. bassiana* challenge.

Bars represent measured GST specific activity in *B. bassiana* (dark bar) and no treatment control (light bar) termite worker guts normalized to no-reduced glutathione blanks. Error bars represent SEM.

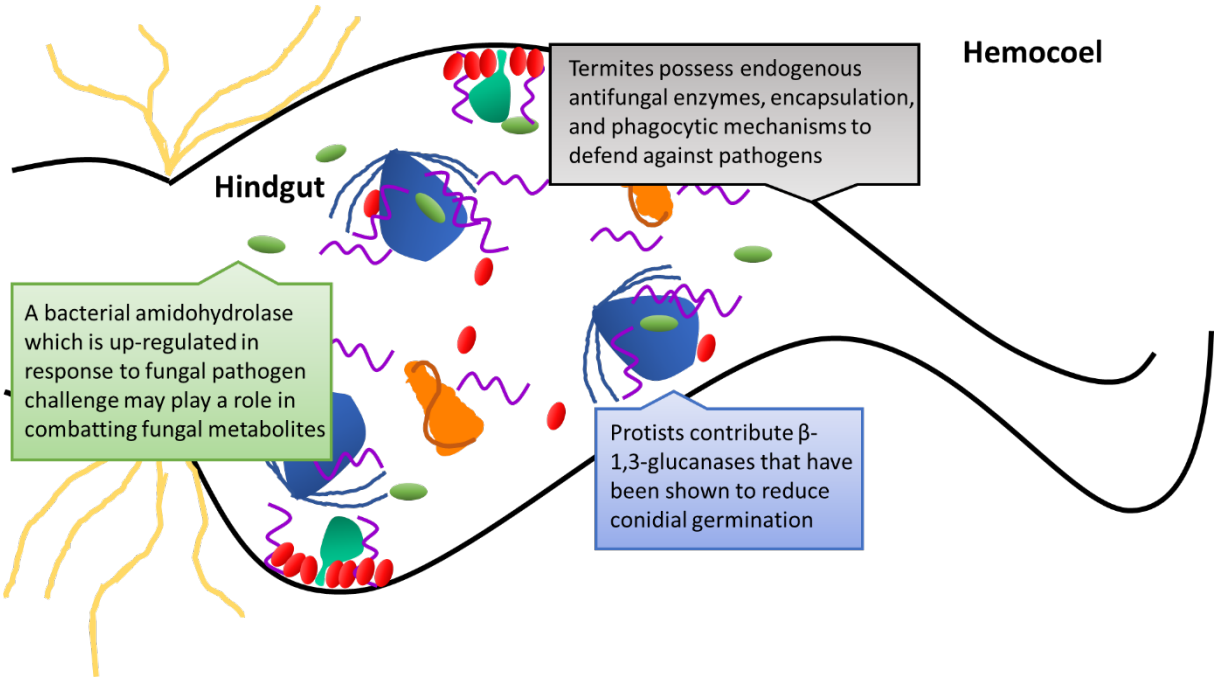

Figure 4.6 A proposed model of collaborative immune physiology.

Protists, bacteria, and the termite host all contribute to neutralizing fungal invaders within the termite hindgut.

Table 4.1 Summary table of custom termite consortium database.

Database was constructed to annotate the metatranscriptome in a taxon specific manner. Archaeal, bacterial, and protist sequences were obtained from the NCBI RefSeq database and termite sequences were obtained from the *Zootermopsis nevadensis* official gene set (OGS).

Composition of Custom Termite Consortium Database

Group	Source	No. of Seqs.
Archaea	NCBI Archaea RefSeq	851,375
Bacteria	NCBI Bacteria RefSeq	44,100,533
Protist	NCBI RefSeq for Parabasalia, Oxymonadida, Diplomonadida, and Gregarinasina	72,948
Termite	OGS for *Zootermoposis nevadensis*	14,610
	Total Sequences	**45,039,466**

Table 4.2 Summary of sequencing and assembly statistics.

A) Summary of sequencing statistics. * Indicates the library used for Trinity assembly which was selected because of low rRNA contamination. **B**) Summary of *de novo* Trinity assembly. Samples are labelled with a letter indicating their treatment (C = control, B = *Beauveria)* and colony number

A

Sequencing Statistics		
Sample	# Reads	rRNA
C18	97,357,292	3%
C21	96,954,778	6.30%
*C22	95,291,086	1.20%
B18	75,834,616	21.50%
B21	83,141,808	14.90%
B22	58,954,982	27.30%

B

Assembly Statistics		
All	No. Contigs	2,107,824
	N50	356
	Average Length	361
>500 bases	No. Contigs	258,251
	N50	652
	Average Length	704

Table 4.3 Summary of select putative bacterial and termite contig functions.

Bacterial and termite contig putative functions based on reciprocal best hits and GO molecular function. * Some candidates possess multi-functional annotations.

Functional Annotations of Contigs			
Category		Bacterial	Termite
Biosynthesis			
	Amino Acid	**143*	*11*
	Alanine	2	0
	Arginine	15	0
	Asparagine	3	1
	Cysteine	3	0
	Glutamine	2	1
	Glycine	2	1
	Histidine	28	0
	Isoleucine	7	0
	Leucine	6	0
	Lysine	16	0
	Methionine	18	3
	Phenylalanine	2	0
	Proline	9	1
	Pyrrolysine	1	0
	Serine	5	1
	Threonine	5	0
	Tryptophan	6	0
	Tyrosine	3	0
	Valine	5	0
	Other	22	3
	Vitamin	*29*	*0*
	Thiamine	27	0
	B6	2	0
	Fatty Acid	*33*	*12*
	Lipid	*5*	*2*
	Cellular Structure	*50*	*8*
	Phospholipid	16	8
	Peptidoglycan	34	0
Metabolism			
	Carbohydrate	*276*	*88*
	Glycosyl Hydrolases	204	34
	Glycosyl Transferase	67	39
	Polysaccharide Lyase	3	7

Table 4.3 Continued

	Carbohydrate Esterase	2	8
	Chitin	*2*	*33*
	Nitrogen	*32*	*7*
	Amidohydrolase	14	3
	Nitrogenase	6	0
	Nitroreductase	9	0
	Urease	9	0
	Other	8	4
	Protein	**207*	**228*
	Aminopeptidases	15	9
	Aspartic-type Peptidases	6	9
	Carboxypeptidases	18	30
	Cysteine-type Peptidases	12	24
	Dipeptidase	11	12
	Metallopeptidases	43	61
	Serine-type Peptidases	76	81
	Threonine-type Peptidases	1	12
	Other Peptidases	31	19
Transport			
	ABC Transporters	*355*	*2*
	Amino Acid	27	0
	Urea	4	0
	Carbohydrate	42	0
	Metal Ion	26	0
	C4-dicarboxylate	5	0
	Multidrug	13	0
	Excinuclease	6	0
	Other	232	2
	Other MFS Transporters	*37*	*11*
	Other Transporters	*163*	*191*
Stress Regulation			
	Antioxidant/Detoxification Enzymes	*69*	*71*
	Aldo/Keto Reductase	7	1
	Alkyl Hydroperoxide Reductase	8	0
	Catalase	1	1
	Cytochrome Oxidase P450s	0	31
	Desulfoferrodoxin	4	0
	Ferredoxin	27	0
	Glutaredoxin	0	3
	Glutathione Peroxidase	1	2

Table 4.3 continued

Glutathione S-Transferase	5	6
Peroxidase	0	6
Peroxidasin	0	3
Superoxide Dismutase	0	3
Thioredoxin	7	14
Other	9	1
Chaperonin	*15*	*16*
Other	*3*	*4*

Table 4.4 Summary of differentially expressed contigs.

Differentially expressed contigs from each taxon based on RBH annotations. Significantly up- and down-regulated contigs from each taxon were determined at α/FDR = 0.05.

Summary Statistics Table for Metatranscriptome RNAseq

Taxon	# ↑	No Change	# ↓
Termite	134	9,339	258
Protist	18	228	2
Bacteria	10	20,852	141
Archaea	0	174	0
Total	**162**	**30,593**	**401**

Table 4.5 Summary of up-regulated contigs.

Significantly up-regulated contigs at 48-hours post-inoculation with *B. bassiana*. Annotation and taxon based on RBH to the custom termite consortium database. Fold-change represents Log_2 CPM Treatment/CPM Control as calculated by edgeR.

Up-Regulated Contigs in Response to *B. bassiana* Challenge

Annotation	Fold-Change	Taxon
Amidohydrolase 2	3.43	Bacteria
Peroxiredoxin-mitochondrial	2.81	Termite
Glutathione S-transferase (GST)	5.10	Termite
Ferritin	2.85	Termite
10kDa Heat shock protein	3.40	Termite
Cytochrome b-c1 subunit 10	3.91	Termite
Cytochrome b-c1 subunit 7	3.04	Termite
Cytochrome b-c1 subunit 9	4.30	Termite
Cytochrome c	2.83	Termite
Cytochrome c oxidase subunit 6B	2.98	Termite
Cytochrome c oxidase subunit 6C	3.13	Termite
Cytochrome c oxidase subunit 7C	2.93	Termite
NADH dehydrogenase 1 alpha subunit	3.60	Termite
3'-5' exonuclease	2.82	Protist
3'-5' exonuclease/DNA Polymerase I	3.25	Protist
Ca2+/calmodulin dependent kinase II (CAMKII)	4.15	Protist
Mitogen-activated protein kinase 1 (MAPK1)	3.49	Protist

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CHAPTER 5. CONCLUSION

The findings presented in this dissertation represent efforts to better understand the extent and nature of collaborative physiological interactions between *Reticulitermes flavipes* workers and their gut symbionta. With some confidence I can say that this research highlights ways in which *Reticulitermes* termites and their symbionts interact that have never been previously described. Using a integrative approaches, including diverse laboratory techniques, and bioinformatic analyses, has allowed me to 1) quantify bacterial contributions *en masse* to termite lignocellulytic activity; 2) propose new gene/enzyme candidates for symbiont-mediated immunity to fungal pathogens; 3) develop a novel method to study prokaryotic and eukaryotic metatranscriptomes simultaneously, and 4) produce a bacterial transcriptome for the first time in lower termites. Combining the use of antimicrobial compounds, 16S sequencing, and traditional enzyme assays, I have shown that bacterial symbionts contribute to at least 23% of the efficiency in *R. flavipes* hindgut digestion (Chapter 2). And in light of Chapter 4, these contributions are likely both direct via cellulase and hemicellulase activity and indirect via nitrogen cycling, vitamin and amino acid biosynthesis, and shuttling of nutrients across the gut lumen (Figure D.1). Additionally, *R. flavipes* workers likely rely on their symbiota for pathogen defense based on data produced from bioassays, *in vitro* fungal germination assays (Chapter 3), and next-generation metatranscriptomics (Chapter 4). While the direct mechanisms of this protection remain to be clarified, this project puts forth two viable candidate mechanisms. These candidates, protist-derived glycosyl hydrolase family 7 enzymes and a bacterial-derived amidohydrolase 2 enzyme, contribute to a new model of termite collaborative immunity. In this model, termites contribute endogenous anti-

fungal responses like hemocytes, antimicrobial peptides, and reactive oxygen species; bacterial symbionts contribute enzymes which may be active against fungal metabolites; and protist symbionts contribute enzymes which break down fungal cell walls (Figure 4.6). Together the data presented here support the hypothesis that *R. flavipes* workers and their symbionts collaborate physiologically to accomplish both digestion and pathogen defense. These results also shed light on the complexity of these interactions, and lay a solid the foundation for future studies of the termite holobiont as a model system for collaborative physiology.

APPENDICES

APPENDIX A SUPPLEMENTAL INFORMATION FROM PETERSON *ET AL.* 2015

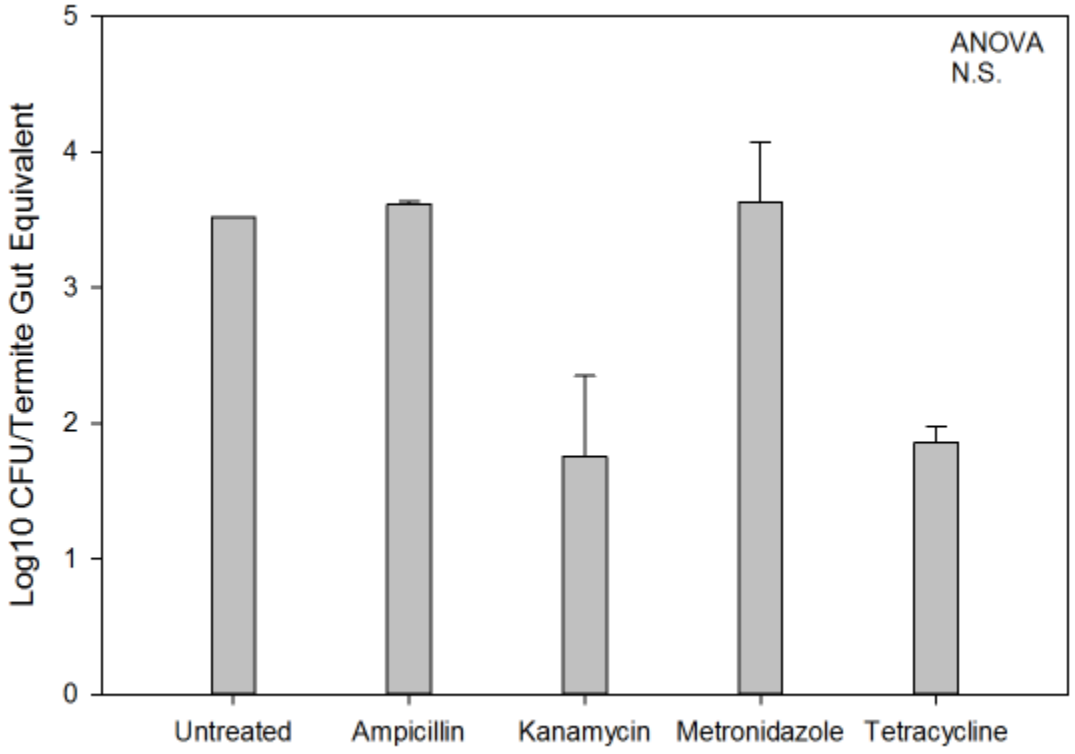


Figure A.1 Antimicrobial treatments impact the abundance of culturable bacteria per termite gut in anaerobic growth conditions. Error bars represent standard error (SEM).

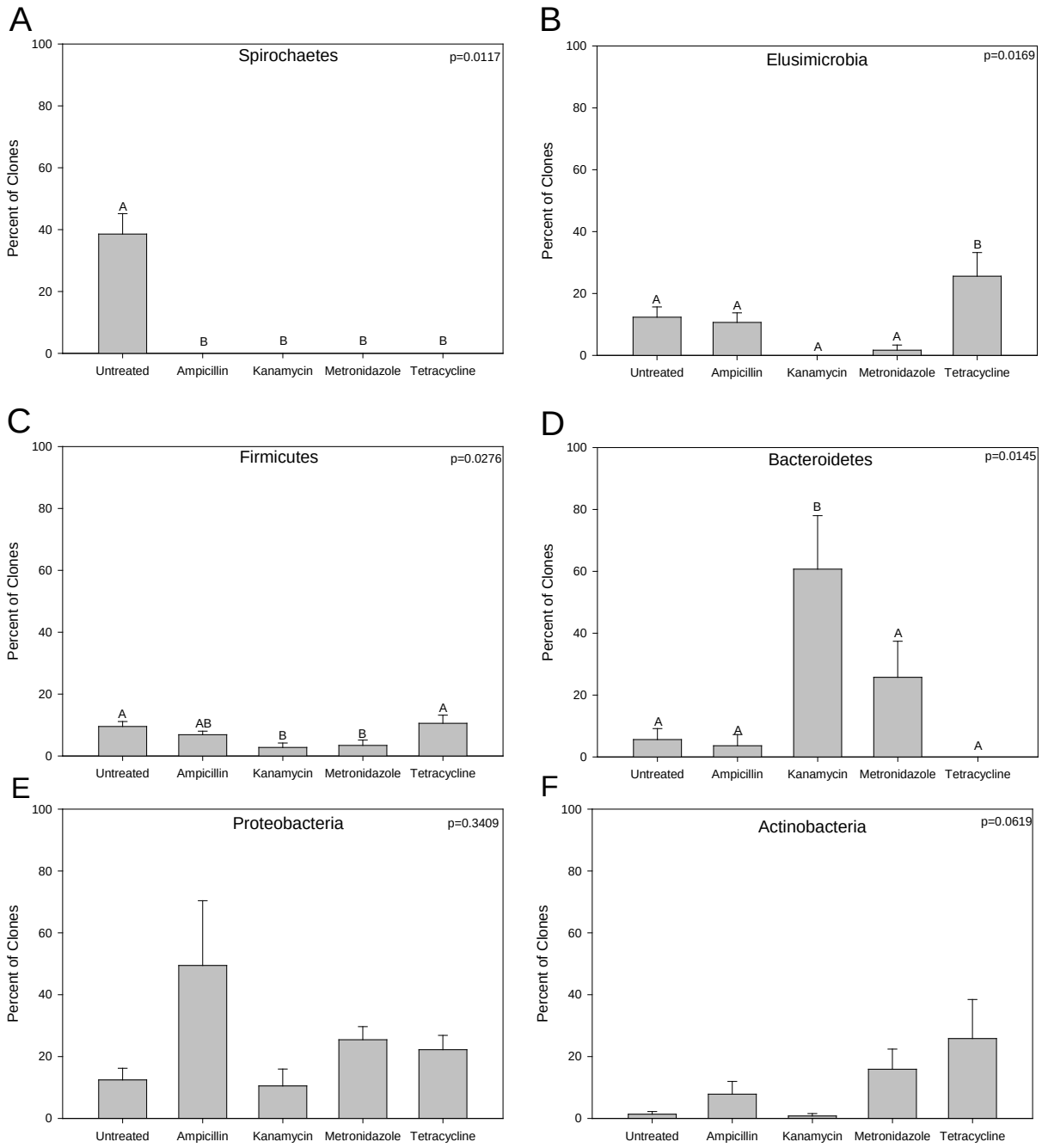
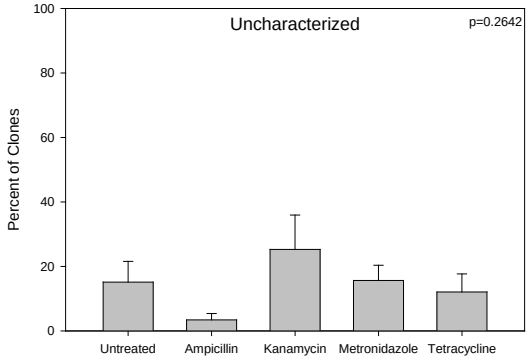


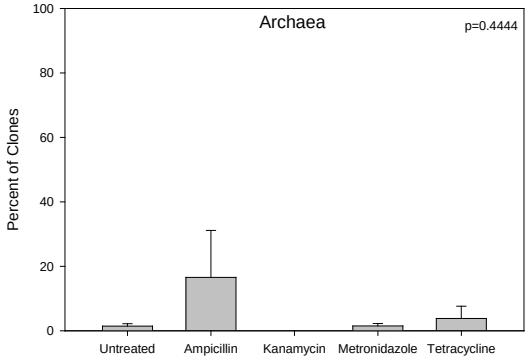
Figure A.2 A comparison of each of the dominant bacterial phyla in the termite gut (A. Spirochetes, B. Elusimicrobia, C. Firmicutes, and D. Bacteroidetes, E. Proteobacteria, F. Actinobacteria, G. Uncharacterized, and H. Archaea) which did not have significant fluctuations following antimicrobial treatment based on 16S rRNA gene clones (ANOVA; Tukey HSD). Error bars represent standard error (SEM)

Figure A.2 continued

G



H



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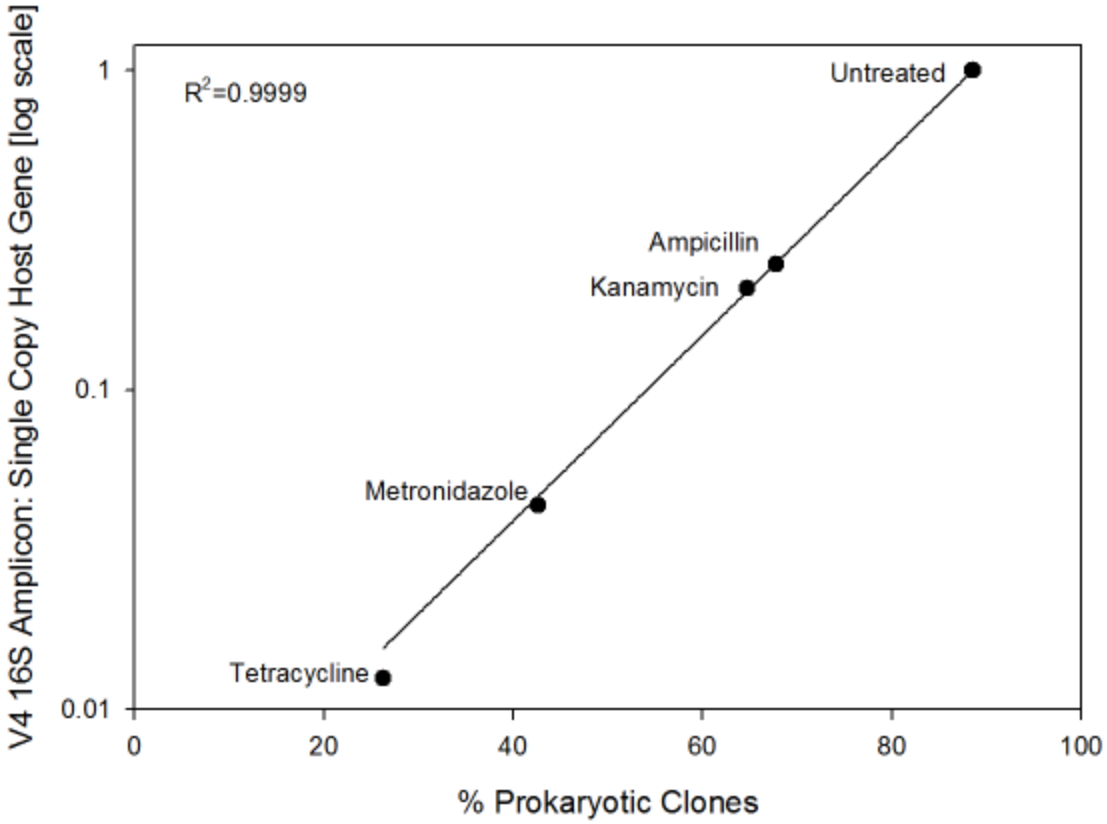

Figure A.3 A post-hoc comparison of the ratio of the 16S rDNA amplicon normalized to a host single copy gene library from each treatment group.

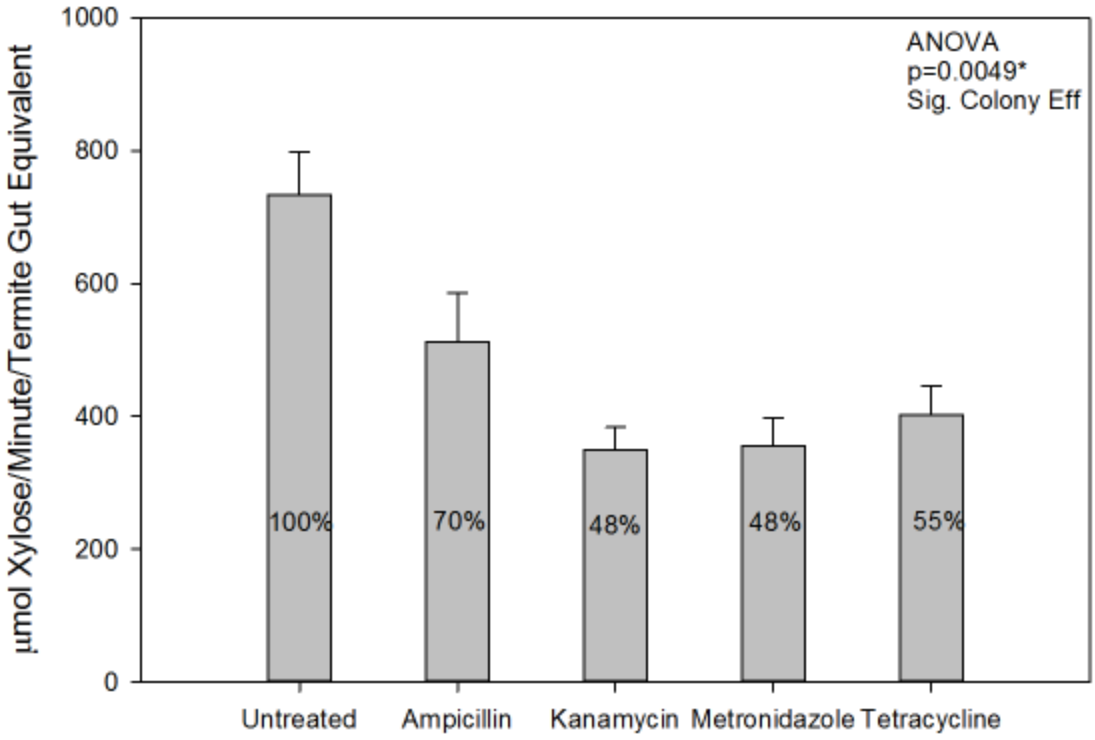

Figure A.4 Xylose liberation from pine sawdust in in vitro saccharification assays as detected by colorimetric tests. Error bars represent standard error (SEM).

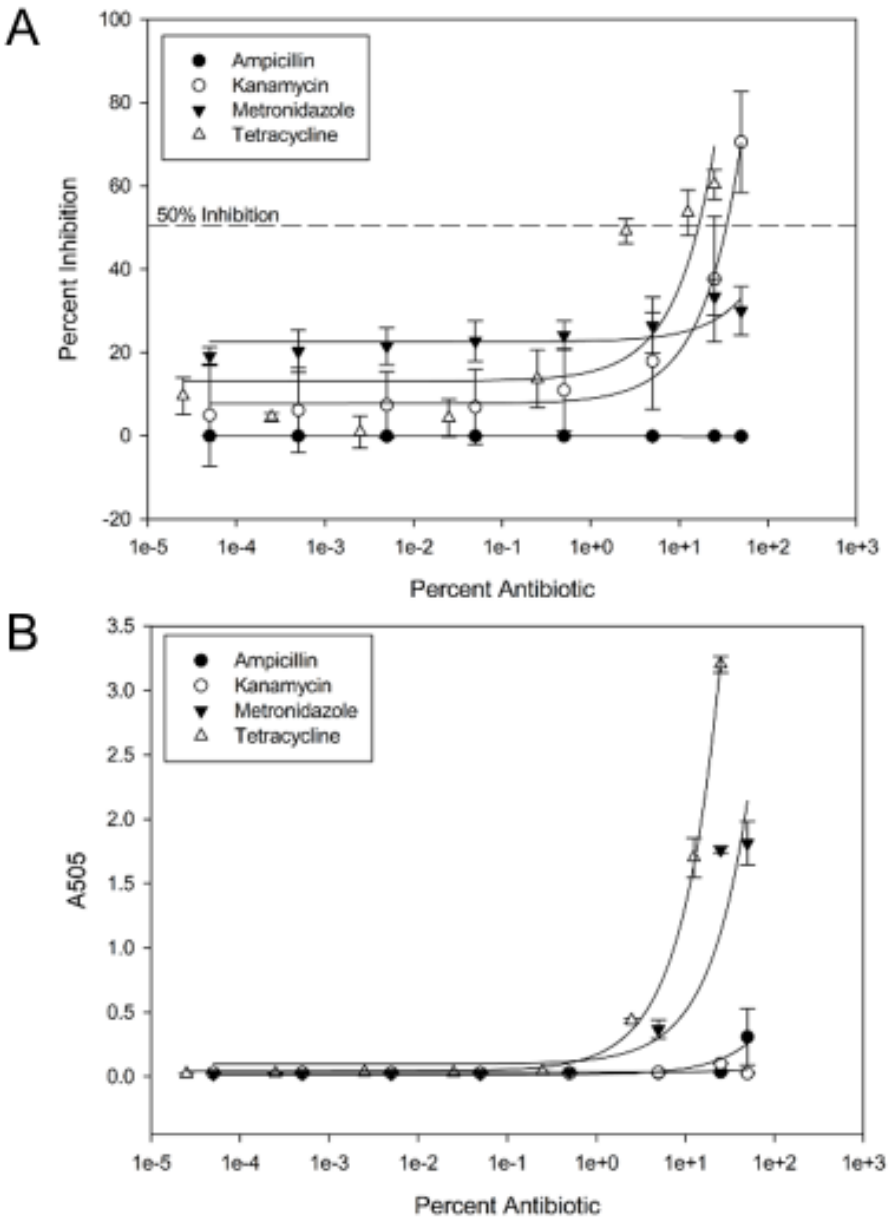

Figure A.5 A. The extent of antimicrobial inhibition of in vitro saccharification assays at various concentrations. The dashed line represents 50% inhibition. B. The extent to which antimicrobial compounds absorb at 505nm at varying concentrations. Error bars represent standard error (SEM).

Table A.1 Primer information which was utilized in this study

Target	Sequence (5'-3')	Annealing (°C)	Source	Use in This Study
Bacterial 16S U241F	CCTACGGGRSGCAGCAG	50	Wang & Qian, 2009	16S rDNA Clone Library
Bacterial 16S U519R	GWATTACCGCGGCKGCTG	50	Wang & Qian, 2009	16S rDNA Clone Library
M13 F	GTAAAACGACGGCCAG	55	Life Technologies	Clone Screening/Sequencing
M13 R	CAGGAAACAGCTATGAC	55	Life Technologies	Clone Screening/Sequencing
Bacterial 16S U515F	GTGTGCCAGCMGCCGCGGTAA	50	Rubin *et al.*, 2014	16S Amplicon qPCR
Bacterial 16S U806R	GGACTACHVGGGTWTCTAAT	50	Rubin *et al.*, 2014	16S Amplicon qPCR
Actin 5C-1_ qPCR_F	TCTGGTAGGACCACTGGTAT	50	This study	Single Copy Host qPCR
Actin 5C-1 qPCR_R	GTATCCACGCTCCGTCAAA	50	This study	Single Copy Host qPCR
Calmodulin qPCR_F	CAGCTGACTGAGGAACAGAT	50	This study	Reference Gene Expression qPCR
Calmodulin qPCR_R	CGCCATCATTGTCAGGAAT	50	This study	Reference Gene Expression qPCR
Cell-1 qPCR_F	TCACAAGCAAGCAGGCATAC	50	Zhou *et al.*, 2007	Gene Expression qPCR
Cell-1 qPCR_R	ATGAGAGCAGAATTGGCAGC	50	Zhou *et al.*, 2007	Gene Expression qPCR
GHF7-3 qPCR_F	TATGGTGGCGGATACTGTGA	50	Sethi *et al.,* 2013	Gene Expression qPCR
GHF7-3 qPCR_R	ACAGCTAATGCTGCCCTGTT	50	Sethi *et al.,* 2013	Gene Expression qPCR
β-Glu-1 qPCR_F	TGCTTCTTCATGGCTCAGAGT	50	Scharf *et al.*, 2010	Gene Expression qPCR
β-Glu-1 qPCR_R	TGGTCTCCAGGTTGTGTATCC	50	Scharf *et al.*, 2010	Gene Expression qPCR
GHF-2 qPCR_F	GCACAACCGCAGTTATGGTC	50	This study	Gene Expression qPCR
GHF-2 qPCR_R	GCCCCAGATCGTGATGAAGA	50	This study	Gene Expression qPCR
GHF-42 qPCR_F	GCTATGGGCAGACAAAGGGT	50	This study	Gene Expression qPCR
GHF-42 qPCR_R	TTGTCACTGCGAAGTCCTGG	50	This study	Gene Expression qPCR
GHF-43 qPCR_F	CGAGAGATTGTCGGGTGATT	50	Karl, 2014	Gene Expression qPCR
GHF-43 qPCR_R	CCAGAACCACTGAGCTCCTC	50	Karl, 2014	Gene Expression qPCR
GHF-26 qPCR_F	CACCATGCACCATCTGAAAC	50	Karl, 2014	Gene Expression qPCR
GHF-26 qPCR_R	TTGGTATCCGGGTGATGATT	50	Karl, 2014	Gene Expression qPCR

Table A.2 Summary of All Statistical Comparisons

Glucose				**Log10 Aerobic CFU Counts**				**GHF-2 Gene Expression**			
W=	*0.2361*			*W=*	*0.2252*			*W=*	*0.0004*		
Source	df	F ratio	p-value	Source	df	F ratio	p-value	Source	df	F ratio	p-value
Model	6	9.1067	0.0032	Model	6	14.9229	0.0006	Model	6	5.2923	0.0173
Treatment	4	13.4973	0.0012	Treatment	4	21.6798	0.0002	Treatment	4	6.4428	0.0128
Colony	2	0.3255	0.7313	Colony	2	0.1331	0.2990	Colony	2	2.9913	0.1072
Xylose				**Log10 Anaerobic CFU Counts**				**GHF-42 Gene Expression**			
W=	*0.1573*			*W=*	*0.3810*			*W=*	*0.0939*		
Source	df	F ratio	p-value	Source	df	F ratio	p-value	Source	df	F ratio	p-value
Model	6	7.9783	0.0049	Model	6	3.8282	0.0635	Model	6	6.1231	0.0113
Treatment	4	4.6287	0.0314	Treatment	4	5.3243	0.0355	Treatment	4	9.1033	0.0045
Colony	2	14.6775	0.0021	Colony	2	0.5500	0.6035	Colony	2	0.1628	0.8525
pNPC Activity				**GHF-1 Gene Expression**				**GHF-43 Gene Expression**			
W=	*0.0018*			*W=*	*0.0086*			*W=*	*0.0010*		
Source	df	F ratio	p-value	Source	df	F ratio	p-value	Source	df	F ratio	p-value
Model	6	8.2893	0.0044	Model	6	1.5620	0.2729	Model	6	4.3757	0.0296
Treatment	4	12.3531	0.0017	Treatment	4	0.5313	0.7169	Treatment	4	6.4878	0.0125
Colony	2	0.1618	0.8533	Colony	2	3.6234	0.0758	Colony	2	0.1513	0.8620
pNPG Activity				**GHF-7 Gene Expression**				**GHF-26 Gene Expression**			
W=	*0.3344*			*W=*	*0.0384*			*W<*	*0.0001*		
Source	df	F ratio	p-value	Source	df	F ratio	p-value	Source	df	F ratio	p-value
Model	6	0.8817	0.5484	Model	6	3.7241	0.0453	Model	6	7.8054	0.0053
Treatment	4	1.1150	0.4133	Treatment	4	5.3918	0.0210	Treatment	4	10.9802	0.0025
Colony	2	0.4151	0.6737	Colony	2	0.3887	0.6901	Colony	2	1.4559	0.2889
Protist Abundance				**GHF-9 Gene Expression**				**Actinobacteria 16S Clones**			
W=	*0.0041*			*W=*	*0.0441*			*W=*	*0.0015*		
Source	df	F ratio	p-value	Source	df	F ratio	p-value	Source	df	F ratio	p-value
Model	6	22.4198	0.0007	Model	6	1.9950	0.1800	Model	6	1.3761	0.3291
Treatment	4	33.5916	0.0003	Treatment	4	0.2546	0.8989	Treatment	4	2.0495	0.1800
Colony	2	1.0060	0.4200	Colony	2	5.4758	0.0318	Colony	2	0.0294	0.9711

Table A.2 Summary of All Statistical Comparisons (continued)

Archaea 16S Clones			
W<	*0.0001*		
Source	df	F ratio	p-value
Model	6	1.2959	0.3572
Treatment	4	1.1348	0.4056
Colony	2	1.6180	0.2570

Bacteroidetes 16S Clones			
W=	*0.0007*		
Source	df	F ratio	p-value
Model	6	5.5127	0.0154
Treatment	4	7.5534	0.0080
Colony	2	1.4314	0.2942

Elusimicrobia 16S Clones			
W=	*0.0152*		
Source	df	F ratio	p-value
Model	6	5.6029	0.0147
Treatment	4	7.4601	0.0083
Colony	2	1.8885	0.2129

Firmicutes 16S Clones			
W=	*0.7747*		
Source	df	F ratio	p-value
Model	6	3.8836	0.0406
Treatment	4	4.7784	0.0290
Colony	2	2.0940	0.1856

Proteobacteria 16S Clones			
W=	*0.0172*		
Source	df	F ratio	p-value
Model	6	1.3669	0.3322
Treatment	4	2.0219	0.1841
Colony	2	0.0570	0.9450

Spirochete 16S Clones			
W<	*0.0001*		
Source	df	F ratio	p-value
Model	6	23.0821	0.0001
Treatment	4	34.1231	<0.0001
Colony	2	1.0000	0.4096

Unknown 16S Clones			
W=	*0.0519*		
Source	df	F ratio	p-value
Model	6	0.8536	0.5644
Treatment	4	1.2045	0.3799
Colony	2	0.1519	0.8615

Verrucomicrobia 16S Clones			
W<	*0.0001*		
Source	df	F ratio	p-value
Model	6	1.0000	0.4852
Treatment	4	1.0000	0.4609
Colony	2	1.0000	0.4096

Table A.3 Regression Analysis of Factors Associated with Xylose Liberation Pairwise linear regressions between xylose liberation (X-variable) and other variables tested in this study (Y-Variables), with respective p-value and R2 values shown. Variables above the line show a significant correlation with xylose liberation.

X-Variable	Y-Variable	p-Value	R^2	Adjusted R^2
Xylose	Protist Abundance	0.0385	0.2895	0.2349
	Enzyme Activity-pNPC	0.0470	0.2702	0.2141
	Gene Expression-GHF-43	0.0729	0.2266	0.0671
	Firmicutes	0.0924	0.2025	0.1411
	Enzyme Activity-pNPG	0.1554	0.1489	0.0835
	Gene Expression-GHF-42	0.1705	0.1393	0.0731
	Log CFU-Aerobic	0.2365	0.1059	0.0371
	Log CFU-Anaerobic	0.2639	0.1119	0.0312
	Bacteroidetes	0.3382	0.0707	-0.0008
	Spirochetes	0.3426	0.0695	-0.0021
	Elusimicrobium	0.3576	0.0655	-0.0065
	Gene Expression-GHF-1	0.4037	0.0542	-0.0186
	Actinobacteria	0.5752	0.0248	-0.0502
	Gene Expression-GHF-26	0.6652	0.0149	-0.0609
	Gene Expression-GHF-7	0.7085	0.0111	-0.0650
	Proteobacteria	0.7381	0.0089	-0.0673
	Archaea	0.7667	0.0070	-0.0694
	Uncultured-Unknown	0.9004	0.0013	-0.0756
	Verrucomicrobia	0.9678	0.0001	-0.0768
	Gene Expression-GHF-2	0.9756	0.0001	-0.0768
	Gene Expression-GHF-9	0.9905	0.0000	-0.0769

APPENDIX B SUPPLEMENTAL INFORMATION FROM CHAPTER 3

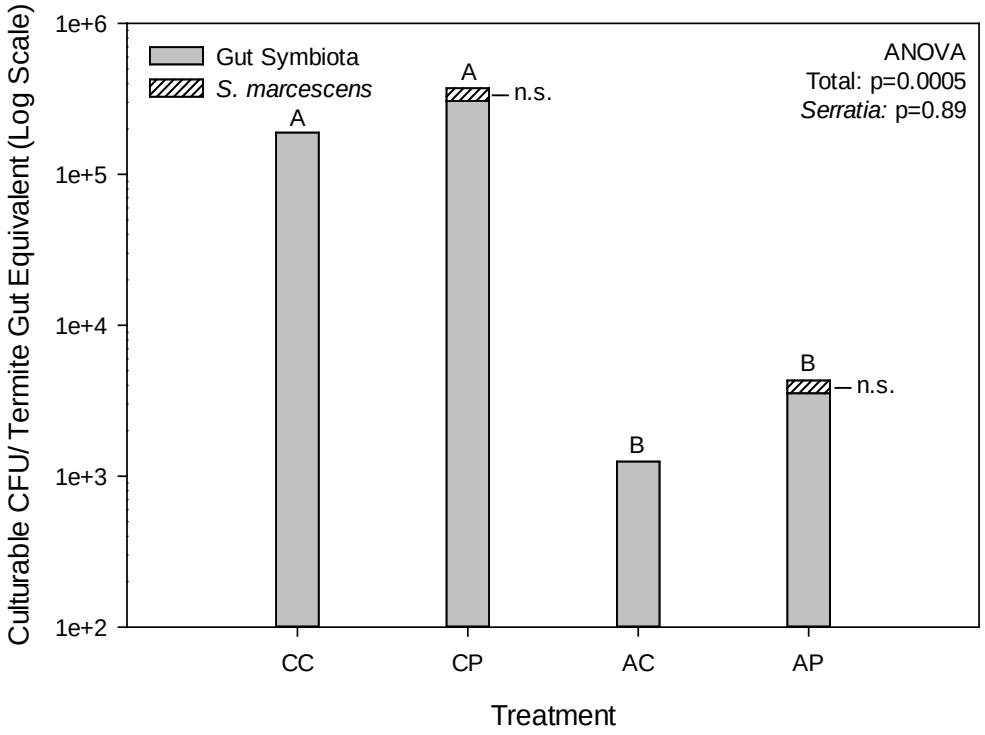

Figure B.1 Quantification of culturable bacteria from whole termite guts 48-hours post-inoculation with S. marcescens. Hashed sections of the bars represent the portion of the culturable community that was S. marcescens in the pathogen challenged groups, indicated by pink/red colored colonies. Bars with the same letter are not statistically different (α =0.05). Water treated, Blank treated (CC), Water treated, Pathogen challenged (CP), Antibiotic treated, Blank treated (AC), and Antibiotic treated, Pathogen challenged (AP).

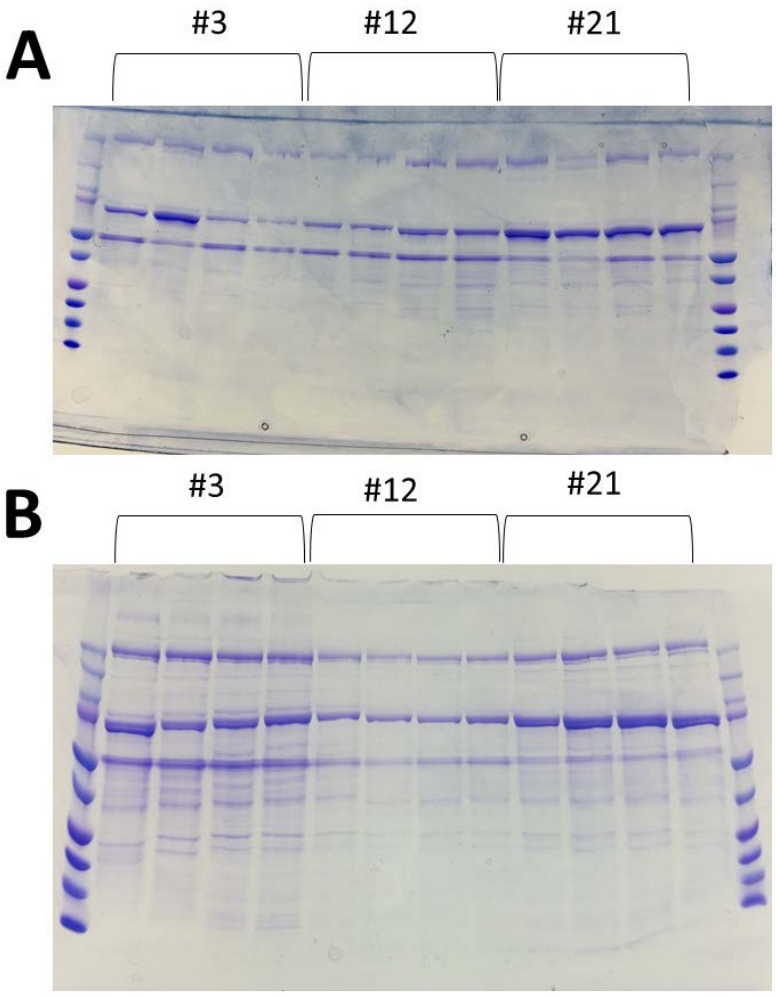

Figure B.2 Protein profiles following pathogen challenge
4-20% gradient SDS-PAGE gels of 20µg of carcass total protein per lane stained with Coomassie following **A**) *B. bassiana* and **B**) *S. marcescens.* Numbers indicate colony number of the biological rep. Within each biological rep the samples are arranged CC, CP, AC, AP.

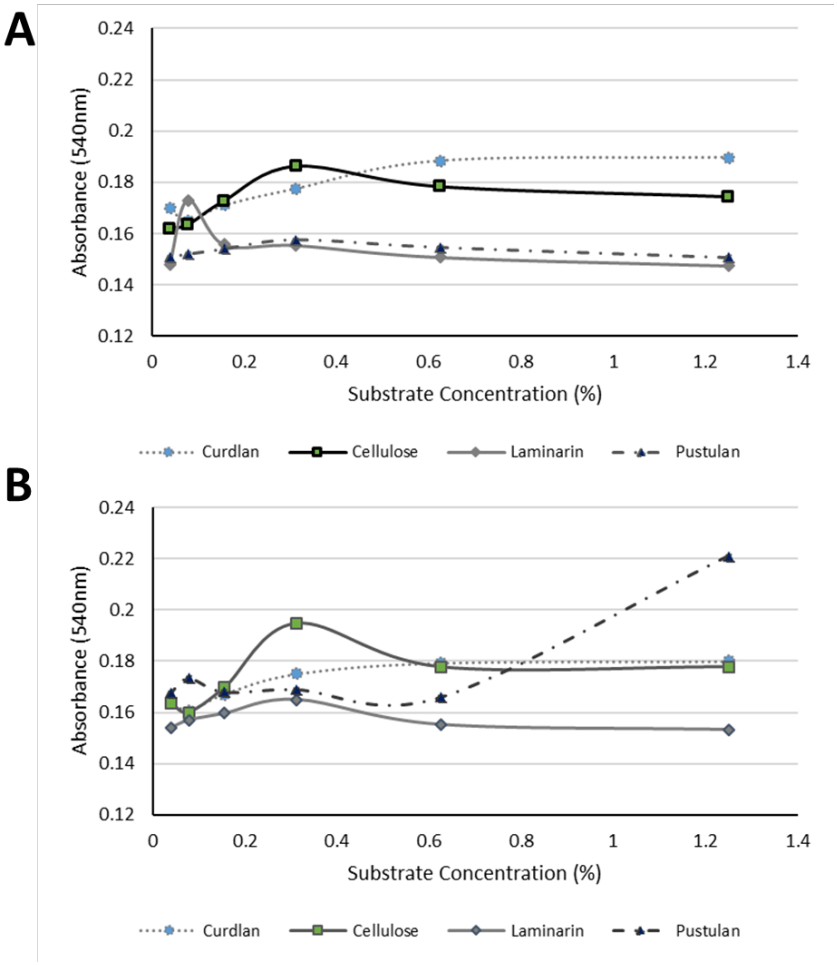

Figure B.3 **A**) GHF7-5 and **B**) GHF7-6 activity on various substrates across a concentration range from 0-1.25%. Endpoint absorbance was measured at 540nm following reaction with DNSA stop solution.

Table B.1 Primers used in Chapter 3. Many were adapted from Hussain *et al.* 2013

Primers used in Chapter 3		
Primer Name	Sequence (5'-3')	Use
14-3-3_F	GCGGCAGTTATTGAAAAGGA	qPCR
14-3-3_R	GCGATAGTAATCGGCCTTCA	qPCR
AEP_F	GTGGCCTCGTAATGGTGAAC	qPCR
AEP_R	CGGGTACTTGGACACGTCTT	qPCR
CalpainB_F	GACCGAGTGCTGACAAAACA	qPCR
CalpainB_R	CTTTTGTACGTGTGCCATCG	qPCR
CathepsinO_F	AAATCCAAAAGATGGGCAAC	qPCR
CathepsinO_R	AGAGCTCTCCTCCAGGAACA	qPCR
Ferritin2_F	TGAGGAGTGTTCGTGGTGAG	qPCR
Ferritin2_R	GGCATCAATCGCTCCATATT	qPCR
GNBP1_F	AGATCGGGCGAATAACTCCT	qPCR
GNBP1_R	TTTGCCTCCTGGATTCTCTG	qPCR
GNBP2_F	ATGCGGTCTCTTGCGATACT	qPCR
GNBP2_R	TTCCCAGTTACCACCTCCTG	qPCR
Hex2_F	ACGGAAGACGTTGGACTCAG	qPCR
Hex2_R	GAGGACCTGCTGGATCTTGT	qPCR
HSP90_F	CAATTGCTCGATCAGGAACA	qPCR
HSP90_R	TTTTCCCTGCTTCACTTTCC	qPCR
Lyso_F	AAGCGATGGACGTGAGAAAT	qPCR
Lyso_R	CGTTTATTGCGTCGGTTTTT	qPCR
Lyso-P_F	CTGTTTCTTGGTCATTGTG	qPCR
Lyso-P_R	ATATCTGTCGTTTATCTG	qPCR
MCP_F	TCAACACTCCACGCAAGAAG	qPCR
MCP_R	ACAGACCGTCCAACAGGAAG	qPCR
Termicin_F	CTGGTCGTAGTGTGCGTGTT	qPCR
Termicin_R	TGTGCGTACGGTCATCATCT	qPCR

APPENDIX C SUPPLEMENTAL INFORMATION FROM CHAPTER 4

Table C.1 Custon ribo-depletion primers developed in conjunction with NuGen to deplete the anticipated eukaryotic members of the termite holobiont and the fungal pathogen in treatment samples.

Primer Name	Primer Sequence
>1_EF363230|_0	ATGCATGTCTCAGTGCAAGC
>2_EF363230|_70	TGGTTCCTTAGATGGTGGACA
>3_EF363230|_140	GACGGAAGGGACGCTTTTAT
>4_EF363230|_210	GTTTGCCTTGGTGACTCTGAA
>5_EF363230|_280	CTGTCGATGGTAGGCTCTGC
>7_EF363230|_420	ACGGGGAGGTAGTGACGAA
>8_EF363230|_490	ATCCATTGGAGGGCAAGTCT
>9_EF363230|_560	AAGCTGTTGCGGTTAAAAAGC
>10_EF363230|_630	CCCGTCGGTGTTTAACTGG
>11_EF363230|_700	AGCGGTAACGTTCTCACACC
>12_EF363230|_770	CCGGCACGTTTACTTTGAAC
>13_EF363230|_840	TGAATACCGAGTGCATGGAA
>14_EF363230|_910	TCCCCGAGGTAATGATCAAA
>15_EF363230|_980	CGAAAGCATTTGCCAAGAAT
>16_EF363230|_1050	TTCGAAGGCGATCAGATACC
>17_EF363230|_1120	CCGAAGTTCCTCCGATGAC
>19_EF363230|_1260	CTCAACACGGGAAACCTCAC
>20_EF363230|_1330	GTGGAGCGATTTGTCTGGTT
>21_EF363230|_1400	AGTCGCATCCGGTATCCTTT
>22_EF363230|_1470	CTTCTAGCCGCACGAGATTG
>23_EF363230|_1540	CTGAAGGAATCAGCGTGTCC
>24_EF363230|_1610	TGAACCTCCTTCGTGCTAGG
>26_EF363230|_1750	CTTCGATTCCACTGGGAAGA
>28_AB032204|_0	GCCATGCAAGTGCTAGTTCA
>29_AB032204|_70	ACACGCTCAGAACCCATTTG
>30_AB032204|_140	GACATGACCTTATAGGCGTACCA
>31_AB032204|_210	TGTAGGCTATCACGGGTAACG
>33_AB032204|_350	CAAGATCGGCGGATAGGTT
>35_AB032204|_490	GCTAATCGCGTTCCAATGTT
>36_AB032204|_560	CTGAATGACTCAGCACGGTATG
>38_AB032204|_700	GGATGCGAAAGCGTTTACCT
>39_AB032204|_770	GACGGAGCGTTGTGCTATTG

>40_AB032204|_840 AGGCCTATTGGGGAACTACG
>41_AB032204|_910 TCAACGCGGAGAAACTTACC
>42_AB032204|_980 TGACTGACCGGCTAAAGACC
>43_AB032204|_1050 GGTGATTCGTGAAGCGATTT
>45_AB032204|_1190 GCTGCACGCGTTCTACAAT
>46_AB032204|_1260 CCTGAGAGGGTTTGCTACTCC
>47_AB032204|_1330 TCCCTTGTAAGCGTACGTCAA
>49_AB032205|_70 ACCCGCAAGGGTACTAAAGG
>50_AB032205|_140 GTTAAGGTGAGGACGTGACCA
>51_AB032205|_210 CCTATCAGCTTGTCCGCAGT
>52_AB032205|_280 TGAGAGACAGCGGCTATTCC
>53_AB032205|_350 CAACGAAGGAGGTGGTAACG
>54_AB032205|_420 TGTGGGGTAACCTAGGAGAGG
>55_AB032205|_490 AAGCTCGGATAGAGCGTTCA
>56_AB032205|_560 CGTGACCAAATCAGAATGCTT
>57_AB032205|_630 TCAGATCAAAGAGAGCCATCG
>58_AB032205|_700 GAAACGAATGCGAAAGCATC
>59_AB032205|_770 GGGTAGTTGCGGCCTTAAAC
>60_AB032205|_840 AGCGAAAGCTTGAGTCGTTG
>62_AB032205|_980 TGATTTTTGGTGGTGCATGG
>63_AB032205|_1050 CGTGGATTGATCTGTCATGC
>64_AB032205|_1120 GTTTAAGCAGGCGGAAGAGG
>66_AB032205|_1260 AGCGTAGTTGGGATTGACGA
>67_AB032205|_1330 CAACATTGCGCGTTGAATAC
>68_AB032206.1|_0 AAGGAAGCACACTTCGGTCA
>69_AB032206.1|_70 TGGTTTTAAATGGATAGCAGAGGT
>70_AB032206.1|_140 ATGCGATTGTTTCTCCAGAAGT
>72_AB032206.1|_280 CCATATCTACGGGTAGCAGCA
>73_AB032206.1|_350 TCGGAGGAGGTAATGACCAG
>74_AB032206.1|_420 CGTCGTGAAATCTAGCAGAGG
>75_AB032206.1|_490 AACGCCCGTAGTCTGAACTG
>76_AB032206.1|_560 TCCATTCGTTCACTGCGAAT
>77_AB032206.1|_630 TGAGAATCATCGGGGGTAGA
>79_AB032206.1|_770 GATCAAGGGCGAGAGTAGGAG
>80_AB032206.1|_840 CAAAATAGCAGTTTCGCAGGA
>81_AB032206.1|_910 TTGAAGGAATTGACGGAAGG
>83_AB032206.1|_1050 GGTTGACCTGTCTAGCGTTGA
>84_AB032206.1|_1190 TCCGTGATGTCCTTTAGATGC
>85_AB032206.1|_1260 CGACAGGGCACGCTACTCT
>86_AB032206.1|_1330 CCAGGAATCCCTTGTAAATGTG
>88_AY137594|_70 ACCGTCCATGCATGCTTTA

>90_AY137594|_210 CAGGGTTCGATACCGGAGAG
>91_AY137594|_280 ATGAGAAATGGCGACCATCA
>92_AY137594|_350 CTTCGGTTCGACAATTGGAA
>95_AY137594|_560 TCGGTTGAGGGTTGATTCAT
>98_AY137594|_770 TCGCAGCTGAACACATTAGC
>99_AY137594|_840 TGTAGTTTCCCGGCTTTGTC
>101_AY137594|_980 GACTGTCGGGGGCATTAGTAT
>104_AY137594|_1190 GCGTTAAGTTTTCGGGTTCA
>107_AY137594|_1400 TGGTGCATGGTCGTTCTTAGT
>108_AY137594|_1470 AAAGTGTTGTGGCATGGTCA
>112_AY137594|_1750 TTACGTCCCTGCCCTTTGTA
>114_AY137592|_0 GTGCGTAAAAGCCTGACTGC
>115_AY137592|_70 GGAAGGGCCGTGTTTATTAGA
>116_AY137592|_140 AATCGTCGTATCGACCTTGTG
>120_AY137592|_420 CCGCGGTAATTCCAGCTCTA
>122_AY137592|_560 CTCATTGGCGCTGAGATTG
>123_AY137592|_630 TGGTCCACTTTGGTGTGGTT
>125_AY137592|_770 GGACATCAGTGGGGTACTCG
>126_AY137592|_840 GATGGCAGCTTGTTTGGTG
>127_AY137592|_910 ATGCATCCGTTTTGTTGGTT
>130_AY137592|_1120 GGATTGGGGGTTGACCTTTA
>139_AY137592|_1750 CGCTCCTACCGATGAATGAT
>148_AY137593|_490 ATGGAGCTTTTCGTCCCTGT
>149_AY137593|_560 TTTTTCGTCGTGGGTTGATT
>153_AY137593|_840 TCTTTGTTGGGCAGGTTGTT
>160_AY137593|_1330 GTTCGGACACGGTGAGGAT
>162_AY137593|_1470 GGGGTGGTTACCGTTCCTTA
>163_AY137593|_1540 GGGGGAAGTGAGGCAATAAC
>164_AY137593|_1610 GTTTGTCCTGGCTTGGAAGA
>165_AY137593|_1680 GTGAACGCGGAATATCTCGTA
>167_AY137593|_1820 CCGCGACAAAAGAGCAGTA
>168_AY137595|_0 CGCAAACGCCCTACTATGTG
>170_AY137595|_140 TCGTATCGACTTTTGTCGATTTT
>171_AY137595|_210 CCTGACGTTAAGGTCGTGTCTT
>172_AY137595|_280 GGCCACAATTGCTAAGGTGA
>176_AY137595|_560 GGGTTTGCTGTCTGGATGTC
>180_AY137595|_840 AGCTCGAAAGGGTTAATTGGTT
>181_AY137595|_910 CGGGAGGAGAGGGGTAGAT
>183_AY137595|_1050 TGCGAAAGCATTCTTCAAGG
>185_AY137595|_1190 TTGGGGGTTGACTCAAATACA
>191_AY137595|_1610 TGAACCGCACGCGTACTACT

>192_AY137595|_1680 CTGGGGATCGGTTTTTGTAAT
>195_AF244903|_0 CCTGGTTGATCCTGCCAGT
>197_AF244903|_140 ACATGCGCAAAATCCTGACT
>198_AF244903|_210 GTCCTGCACCTTGGTGATTC
>201_AF244903|_420 CCAACACACTGGGGAGGTAG
>202_AF244903|_490 TGTAATTGGAATGGTCCGAACT
>204_AF244903|_630 CCAGGGGACGGATATTTCTC
>205_AF244903|_700 GGATTCGGAGCCTTTACTTTG
>207_AF244903|_840 GCATTTGTATTGCGTCGTCA
>210_AF244903|_1050 CAAACGACCCTTTCAGCACT
>214_AF244903|_1330 CGAGACCTCAGCCTGCTAAC
>215_AF244903|_1400 TGGTGGAAGTTTGAGGCAAT
>219_AF244903|_1680 GTGGTTTGGTGGGAAGAACA
>220_AF244903|_1750 CCGTAGGTGAACCTGCAGAA
>224_AF244904|_210 ACGCGTCCTTTGGTGATTC
>225_AF244904|_280 GGCTTGCCTTCGATGTATCA
>226_AF244904|_350 TTTTGACGGGTAACGGAGAA
>230_AF244904|_630 GGAGTTATGCTGCGGTCATT
>233_AF244904|_840 TTTTGTTGGTTTTCGAGACGA
>242_AF244904|_1470 CCGTCAACAAGCTCATCCTG
>245_AF244904|_1680 TCCGATGAAATGCTTGGACT
>249_AF244905|_140 CGGTGTAGTCGGCGCTTA
>250_AF244905|_210 CCGCAAGGTTTCAGATTTGT
>251_AF244905|_280 CTCGTATCTCGGCGATTCAT
>254_AF244905|_490 CATGGCTTGTAATTGGAATGG
>256_AF244905|_630 CGTAGTTGGATTTCGGAGGA
>257_AF244905|_700 CTGAGTGGGGGTAGGGATTT
>258_AF244905|_770 AAGCAGGCTTAACGCACCT
>259_AF244905|_840 TTGTTGGTTTATAGGATTGGTCGT
>262_AF244905|_1050 GGGATCGGAGGGTGTTAAAT
>267_AF244905|_1400 ACATGGGGGAAGTTCTAGGC
>268_AF244905|_1470 TGATGCCCTTAGATGTTCTGG
>269_AF244905|_1540 TCGTTATCGTGATGGGGATT
>272_AF244905|_1750 GGTTGTGATCGGATGAGAAGA
>273_AF244906|_0 TGACACTGCGAAAAGCTCCT
>274_AF244906|_140 CGAAAACCAGCGCTCTATGT
>275_AF244906|_210 CTCGGTCGATTTTTCATTCAA
>281_AF244906|_630 TTGATACGGGTTTGCTGGTC
>282_AF244906|_700 CAGGGATTGCTTCGTCAATTA
>283_AF244906|_770 GGTCCGGAAGGTTTACTTTGA
>285_AF244906|_910 TCCTGTCGAGTGTTGGCTATC

>286_AF244906|_980	TTGCAGGGAGACAGGAATGT
>288_AF244906|_1120	CGTTTATCATGGGCGTTTTC
>294_AF244906|_1540	CGAAAGTGTCGTGGTGTTTTT
>296_AF244906|_1680	CGCCTCAATGAGTTTGTGGT
>298_AF244906|_1820	GTCCCTGCCCTTGTACACAC
>299_AF244906|_1890	CAGGTGAAATGCTCGGATAGA
>300_AF244906|_1960	GGAAGGAGAAGTCGTAACAAGGT
>301_AF280633|_0	TACAGCGAAACTGCGAATGG
>302_AF280633|_70	TGGATAACCGTGGTAATTCTAGAGC
>303_AF280633|_140	CGACTTCGGAAGGGAGGTAT
>305_AF280633|_280	GGTATTGGCCAAACATGGTC
>306_AF280633|_350	TACATCCAAGGAAGGCAGCA
>307_AF280633|_420	AGGGCTCTTTTGGGTCTTGT
>310_AF280633|_630	CCTTTCCCTCTGTGGAACCT
>311_AF280633|_700	TGCTCGAATACATTAGCATGGA
>312_AF280633|_770	TTTCTAGGACCGCCGTAATG
>315_AF280633|_980	TCGGCACCTTACGAGAAATC
>317_AF280633|_1120	GCAGTAGCTCTGCTCCCAAA
>318_AF280633|_1190	GGCGAACCAAAGTGCTAGTCT
>319_AF280633|_1260	TTCTAAAACCAGTGTCACCAAGC
>320_AF280633|_1330	ACGGGGAAGGTTCAGAGACT
>321_AF280633|_1400	GCTCGCTCACACACTGCTTA
>322_AF280633|_1470	CGGATTGGCAAGCTCAAATA
>325_AF280633|_1680	GCCTGTATTGCTTTGGCAGT
>327_AF280633|_1820	ATCTTGTGAAACTCCGTCGTG
>328_AF280633|_1890	CAAGTCATCAGCTTGCGTTG
>329_AF280633|_1960	ACCGATTGAATGGCTCAGTG
>330_AF280633|_2030	CCGGAAAGCTCTCCAAACTC

Table C.2 Primers used in this study for qPCR validation. Primers were designed using Primer3 program (http://frodo.wi.mit.edu/) or were adapted from previous studies.

Primers used in Chapter 4		
Primer Name	Sequence (5'-3')	Use
14-3-3-F	GCGGCAGTTATTGAAAAGGA	qPCR
14-3-3-R	GCGATAGTAATCGGCCTTCA	qPCR
Actin 5C-1- F	TCTGGTAGGACCACTGGTAT	qPCR
Actin 5C-1-R	GTATCCACGCTCCGTCAAA	qPCR
Aldo-keto reductase-F	TGCTAGTCGTTCAGCATCCA	qPCR
Aldo-keto reductase-R	GAATTAAGCAGGCCCAGACA	qPCR
Amidohydrolase 2-F	GGTCGGGCTTAATTGTGAGA	qPCR
Amidohydrolase 2-R	CAGAGAACAAACACGGCAAA	qPCR
Calmodulin-F	CAGCTGACTGAGGAACAGAT	qPCR
Calmodulin-R	CGCCATCATTGTCAGGAAT	qPCR
CalpainB-F	GACCGAGTGCTGACAAAACA	qPCR
CalpainB-R	CTTTTGTACGTGTGCCATCG	qPCR
Cell-1 qPCR-F	TCACAAGCAAGCAGGCATAC	qPCR
Cell-1 qPCR-R	ATGAGAGCAGAATTGGCAGC	qPCR
Cytochrome p450 9e2-F	TGTCCACATAACGGCACAGT	qPCR
Cytochrome p450 9e2-R	TCTGTGCTTTGCCTCACATC	qPCR
Ferritin2-F	TGAGGAGTGTTCGTGGTGAG	qPCR
Ferritin2-R	GGCATCAATCGCTCCATATT	qPCR
GHF-2 qPCR-F	GCACAACCGCAGTTATGGTC	qPCR
GHF-2 qPCR-R	GCCCCAGATCGTGATGAAGA	qPCR
Hypoxia Up-regulated-F	ATGTTTTGGTGAGGCAGACC	qPCR
Hypoxia Up-regulated-R	CACTGGGCACTAAAGCAGGT	qPCR
Nitroreductase-F	GCTCTCGTTCCTGCGTTATC	qPCR
Nitroreductase-R	GGATTTAGGATTGGGCAACA	qPCR

APPENDIX D SUPPLEMENT SUMMARY MODEL

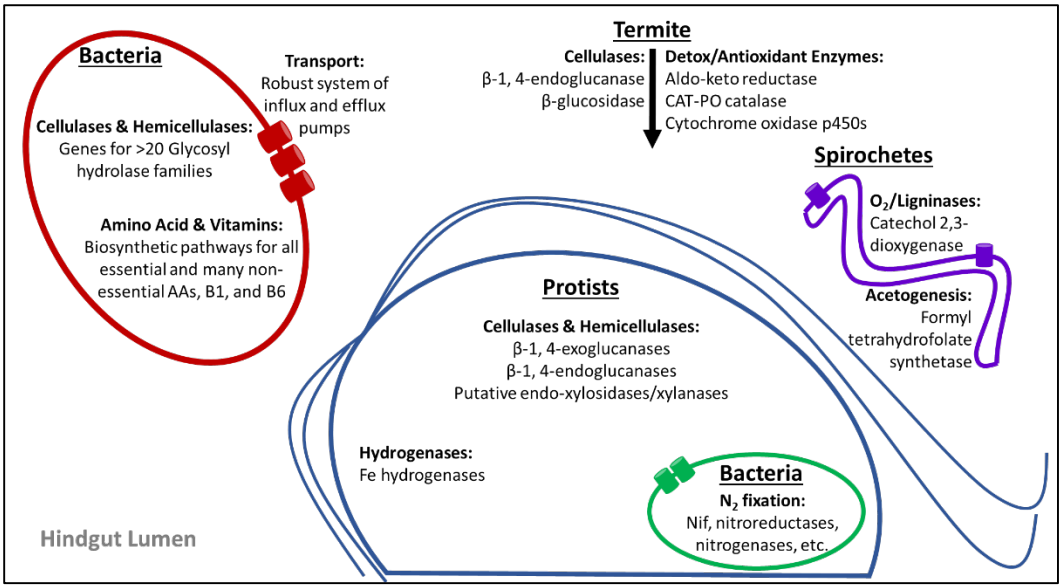

Figure D.1 Model of nutrition/digestion collaborations in the lower termite hindgut. Functions ascribed to 'protists' and/or 'bacteria' are non-specific and may be applied to any number of species within the gut, for the sake of simplicity these broad categories are used in the model.

VITA

VITA

BRITTANY F. PETERSON

Personal Information:

Date of Birth:	11 January 1987
Place of Birth:	Whiteman AFB, Missouri, USA
Parents:	(Clifford) Lee and Regina Peterson (Mascoutah, IL)
Sibling:	Zachary (brother)

Education:

Mascoutah Community High School, Mascoutah, IL (May 2005)
B.S. Microbiology, Western Illinois University (December 2008)
M.S. Biology, Western Illinois University (May 2011)
Ph.D. Entomology, Purdue University (May 2016)

Research Funding:

2014: Monsanto Research Grant		$7,320
2014: Indiana Academy of Science Senior Research Grant		$2,955
	Total:	$10,275

Travel & Professional Development Funding:

2015: Monsanto Student Travel Award	$1,250
2015: Purdue Graduate Student Government Professional Grant	$1,281
2014: PULSe Travel Grant	$300
2014: Purdue Graduate Student Government Travel Grant	$1,000
2014: Purdue Women in Science Travel Grant	$500
2013: Lillian and Alex Feir Graduate Student Travel Grant	$450
2012: PULSe Travel Grant	$200
Total:	$4,981

Honors, Scholarships, & Awards:

2015: Ohio Valley Entomological Association 2nd Place Ph.D. Student Talk
2015: PULSe Featured Student Profile
2015: William L. Brehm Memorial Scholarship

2015: Purdue University Teaching Academy Graduate Teaching Award
2015: PULSe 5-Minute Thesis Competition 1st Place Award
2015: Pest Control Magazine Scholarship
2014: Purdue College of Agriculture Graduate Ag Research Spotlight
2014: Ohio Valley Entomological Association 3rd Place Ph.D. Student Talk
2014: #Science: Effective Interdisciplinary Communication 1st Place Poster Award
2014: Annual Donald Danforth Plant Science Center Fall Symposium Best Poster Award
2014: Purdue Entomology Graduate Organization Student Spotlight
2014: Purdue University Graduate Fellowship Incentive Award
2013: Oser Family Scholarship
2012: PULSe Newly Placed Student Featured Profile
2011: WIU Biology Graduate Research Symposium 1st Place Platform Presentation
2010: Entomological Society of America President's Prize for Student Oral Presentation

Publications:

Peer-Reviewed

Peterson, **BF**, and Scharf, ME. 2016 Lower termite associations with microbes: synergy, protection, and interplay. Frontiers in Microbiol. 7, 422.

Benoit, JB, Adelman, ZN … **Peterson**, **BF** (33/80)… Schal, C, and Richards, S. 2016. Unique features of the bed bug, a global human ectoparasite, identified through genome sequencing. Nature Communications. 7, 10165.

Peterson, **BF**, Stewart, HL, and Scharf, ME, 2015. Quantifying symbiotic contributions to lower termite digestion using antimicrobial compounds. Insect Biochemistry and Molecular Biology 59, 80-88.

Sen, R, Raychoudhury, R, Cai, Y, Sun, Y, Ulrike-Lietze, V, **Peterson**, **BF**, Scharf, ME, and Boucias, DG. 2015. Molecular signatures of nicotinoid-pathogen synergy in the termite gut. PLOS ONE 10, e0123391.

Zhao, C, Escalante, LN … **Peterson**, **BF** (46/69) … Stuart, JJ, and Richards, S, 2015. A massive expansion of effector genes underlies gall-formation in the wheat pest *Mayetiola destructor*. Current Biology 25, 1-8.

Direct-Submission

Peterson, **BF**, 2014. A student's perspective on graduate training, *In* Proceedings, 14th National Conference on Urban Entomology, 18-21 May 2014, San Antonio, TX.