IRG/WP 14-10819

THE INTERNATIONAL RESEARCH GROUP ON WOOD PROTECTION

Section 1 **Biology**

Changes in bacterial gut community of *Reticulitermes flavipes* (Kollar) and *Reticulitermes tibialis* Banks after feeding on termiticidal bait material

Rachel A. Arango[1], Frederick Green III[1], Kenneth F. Raffa[2]

[1]USDA-FS, Forest Products Laboratory
One Gifford Pinchot Drive
Madison, WI, 53726-2398, USA

[2]Department of Entomology
1630 Linden Drive
345 Russell Laboratories
Madison, WI, 53706, USA

Paper prepared for the 45th Annual Meeting
Utah, USA
11-15 May 2014

Disclaimer
The opinions expressed in this document are those of the author(s) and are not necessarily the opinions or policy of the IRG Organization.
The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture or any product or service. The Forest Products Laboratory is maintained in cooperation with the University of Wisconsin. This article was written and prepared by U.S. Government employees on official time, and it is therefore in the public domain and not subject to copyright.

IRG SECRETARIAT
Box 5609
SE-114 86 Stockholm
Sweden
www.irg-wp.com

Changes in bacterial gut community of *Reticulitermes flavipes* (Kollar) and *Reticulitermes tibialis* Banks after feeding on termiticidal bait material

Rachel A. Arango[1], Frederick Green III[1], Kenneth F. Raffa[2]

[1] U.S. Forest Products Laboratory, One Gifford Pinchot Drive, Madison, WI 53726 USA;
rarango@fs.fed.us, fgreen@fs.fed.us
[2] University of Wisconsin-Madison, Department of Entomology, 1630 Linden Drive, Madison, WI 53706 USA;
raffa@entomology.wisc.edu

ABSTRACT

In this study, 454-pyrosequencing was used to evaluate the effect of two termiticidal baits, hexaflumuron and diflubenzuron, on the bacterial gut community in two *Reticulitermes flavipes* colonies and one *Reticulitermes tibialis* colony. Results showed two bacterial groups to be most abundant in the gut, the Bacteroidetes and Spirochaetes, both of which do not appear to be adversely affected by bait treatment according to analysis conducted to date. Other major bacterial lineages present included Actinobacteria, Fibrobacteres, Firmicutes, Fusobacteria, Proteobacteria, Tenericutes, TM7, Verrucomicrobia and unclassified species, which matches closely with other studies examining termite gut bacteria. Phylogenetic analysis examining similarity among treated groups versus controls showed a treatment effect in both *R. flavipes* colonies, but no effect on *R. tibialis* samples. Overall community analysis also showed treatment groups were separated by their collection location indicating a distinct bacterial community within a colony. Future analysis will focus on the types of bacteria affected by bait treatment and the role of these changes in overall termite fitness.

Keywords: *Reticulitermes flavipes* (Kollar), 454 pyrosequencing, gut bacteria, termiticidal baits

1. INTRODUCTION

Symbionts within the termite gut are responsible for aiding in a number of biological processes including, cellulose degradation, nitrogen fixation, nutrient recycling, and vitamin production among others (Potrikus and Breznak 1981; Breznak and Brune 1994; Brune and Friedrich 2000; Husseneder 2010). Additionally, symbiotic microbiota are thought to play a role in fitness, nest mate recognition (Matsuura 2001; Rosengaus et al. 2011) and are hypothesized to have had a major impact on the development of eusociality among termites (Lin and Michener 1972; Thorne 1997). While the role of protozoa in the gut of lower termites is relatively well described, the role of bacteria has only recently become a major area of study. Overall, literature to date has justified examination of the termite gut bacterial community by suggesting results from such work may provide insight into more sustainable biofuel production or lead to novel mechanisms for termite control.

The most widely used approach to assessing bacterial diversity in the termite gut to date has been cloning and sequencing of the gene encoding the 16S rRNA of the ribosomal small subunit, which has provided valuable information regarding which major taxa are present. Development of these culture-independent techniques has resulted in comprehensive bacterial community data from a number of different termite species including, *Reticulitermes speratus* (Kolbe) (Ohkuma and Kudo 1996; Hongoh et al. 2003), *Reticulitermes flavipes* (Kollar) (Fisher et al. 2007) and *Coptotermes formosanus* Shiraki (Shinzato et al. 2005) to name a few. Results from these studies suggest the presence of a wide diversity of microorganisms and highlight the fact that a large

number of gut bacteria have yet to be characterized. Despite this diversity, however, there are a number of bacterial assemblages that are almost always present including, Proteobacteria, Spirochaetes, Bacteroidetes, Actinobacteria and Endomicrobia, with Bacteroidetes and Spirochaetes being the most abundant taxa found (Brune and Friedrich 2000).

A number of researchers have examined how the loss of symbiotic gut protozoa can negatively affect termite health and a few have studied how bacterial community changes may impact termite success in terms of reproduction and survival. For example, Boucias et al. (2013) used pyrosequencing to examine gut microbiota changes after feeding *R. flavipes* lignin-rich and lignin-poor diets, finding little change in microbial composition by diet. Surprisingly few studies, however, have used these high-throughput methods in termite-microbial studies beyond evaluation of overall diversity. In the present study, 454-pyrosequencing was used to evaluate changes in the bacterial community of termite guts after feeding on either hexaflumeron or diflubenzuron termiticidal baits. Both of these bait materials are commonly used in the field for treating a colony (Su and Scheffrahn 1993). Bait materials function by being ingested by worker termites and transferred to other nestmates through trophallaxis or proctodeal feeding. We hypothesize that passage of these toxicants through the gut have potentially negative consequences on the gut microbial community that may play an additional role in overall control of termites beyond their known mode of action.

2. EXPERIMENTAL METHODS

2.1 Termite Collection and Feeding Trials

Termites were collected from three adjacent sites in each city in Muscoda, Janesville, and Hazel Green, Wisconsin along with wooden food material from these sites. Termites from Muscoda (MU) and Janesville (JV) were members of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar), while the Hazel Green (HG) termites were the arid land termite, *Reticulitermes tibialis* Banks (Arango 2014, in press). Termites were then separated into groups of 100 workers at approximately the 3rd or 4th instar and placed in plastic petri dishes with damp filter paper and either hexaflumeron (HX), diflubenzuron (DF) or food material from the initial collection site (CT). The termites and petri dishes were maintained in an incubator at 27°C and 80% RH. After 14 days, termites were killed by freezing and briefly surface washed in 70% EtOH. Guts of 50 termites from each group were removed and collected in 1.5mL vials using flame sterilized tweezers and stored at -20°C until DNA extraction and purification (~1-2 days).

2.2 DNA Extraction and Pyrosequencing

Whole genomic DNA was extracted using a chloroform-isoamyl alcohol extraction method with multiple ethanol washes. DNA was then quantified using a NanoDrop and diluted to 20 ng/µl prior to polymerase chain reaction (PCR). Universal bacterial primers 27F and 519R were chosen to amplify the V1-V3 hypervariable region of the 16S rRNA gene. Adaptors required for pyrosequencing were included in the primer sequences along with a 10 base pair tag (represented by Xs) on the reverse primer used to identify the different samples (primer sequences with adaptors underlined: **27F**: 5'- CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG AGA GTT TGA TCN TGG CTC AG -3' and **519R**: 5'- CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG XXX XXX XXX X GTN TTA CNG CGG CKG CTG -3'). PCR was performed in triplicate in a 25µl reaction containing 1µl DNA template (20 ng/µl), 5 µl 5X Herculase buffer, 0.25 µl Herculase II high fidelity polymerase (Agilent Technologies), 0.25 µl dNTPs (25mM stock conc. of each combined), 0.5 µl forward primer 27F (10 µM), 0.5 µl reverse primer 519R (10 µM), 0.5 µl DMSO and 17 µl H_2O. PCR reactions were run according to the following

conditions: 96°C for 2 minutes followed by 30 cycles each consisting of 30 seconds at 95°C, 30 seconds at 55°C and 90 seconds at 72°C. The PCR finished with a post-cycle extension of 72°C for 7 minutes. PCR products were run on a 1% low melt agarose gel (AquaPor GTAC, National Diagnostics) containing SYBR-safe, visualized on a blue light trans-illuminator (Clare Chemical Research, Dolores, CO) and the band around 600bp was excised into a 1.5mL tube using a surface sterilized metal spatula. Gel extraction was then performed using a Zymoclean™ gel DNA recovery kit and DNA was quantified using a Qubit fluorometer (Invitrogen, Grand Island, NY).

Library preparation was done by first diluting all samples to $1x10^9$ molecules/µl, pooling 10µl of each diluted sample in a tube, and diluting the pool to a final concentration of $1x10^6$ molecules/µl. Samples were then amplified via emulsion PCR (emPCR) using the GS Junior Titanium emPCR Kit (Lib-L) (Roche Applied Science) according to manufacturer's instructions. The DNA library was added so that there was a ratio of 0.95 copies per bead. After emPCR, bead recovery and sequencing was done according to manufacturer recommendations (GS Junior Titanium Series).

2.3 Data Analysis

Raw data was extracted from the GS Junior and imported into mothur (v. 1.32.1) (Schloss et al. 2009). Prior to analysis, data summary showed 90,354 sequences. Sequence analysis followed the 454 SOP (standard operating procedure) as provided online (Schloss et al. 2009; Schloss et al. 2011), which removed sequences less than 200bp long. Unique sequences were aligned using the Silva-derived reference database (Pruesse et al. 2007; Schloss 2009) and chimeras were removed using UCHIME (Edgar et al. 2011). Data was then analysed for alpha and beta diversity and phylogenetic trees were constructed (using the software package iTOL; Letunic and Bork 2006).

3. RESULTS

After removing short reads and chimeras 11,159 total sequences remained, of which, 3,412 were determined to be unique sequences. The average number of sequences per sample was approximately 413, although one sample group had only 169 sequences. Number of sequences and observed taxonomic units (OTUs) per sample location and treatment group are presented in Table 1 along with an estimate of how well the community was sampled (% coverage as determined by subsampling in mothur).

Table 1: Number of sequences, OTUs, and percent coverage for each sample site by treatment

Sample*	No. Sequences	No. OTUs	% Coverage	Sample	No. Sequences	No. OTUs	% Coverage
JV-1-HX	630	82	66	HG-3-DF	356	52	83
JV-2-HX	503	86	66	MU-1-DF	501	76	72
JV-3-HX	647	79	70	MU-2-DF	417	86	66
HG-1-HX	389	49	84	MU-3-DF	169	84	71
HG-2-HX	443	46	86	JV-1-CT	480	93	62
HG-3-HX	417	48	84	JV-2-CT	393	88	64
MU-1-HX	418	54	81	JV-3-CT	373	84	68
MU-2-HX	598	73	73	HG-1-CT	410	51	83
MU-3-HX	351	62	76	HG-2-CT	452	54	83
JV-1-DF	424	95	62	HG-3-CT	442	48	85
JV-2-DF	397	86	67	MU-1-CT	279	88	65
JV-3-DF	346	76	72	MU-2-CT	327	90	63
HG-1-DF	274	45	87	MU-3-CT	392	95	61
HG-2-DF	331	41	87				

*Location code: JV-Janesville, WI, HG-Hazel Green, WI, MU-Muscoda, WI;
Treatment Code: HX-Hexaflumeron, DF-Diflubenzuron, CT-Control

There are a number of ways to calculate diversity among the various treatment groups. In mothur, alpha diversity was measured using the inverse Simpson diversity estimate. This provided data for the variety of types of sequences in a sample (Fig. 1; larger value = higher diversity).

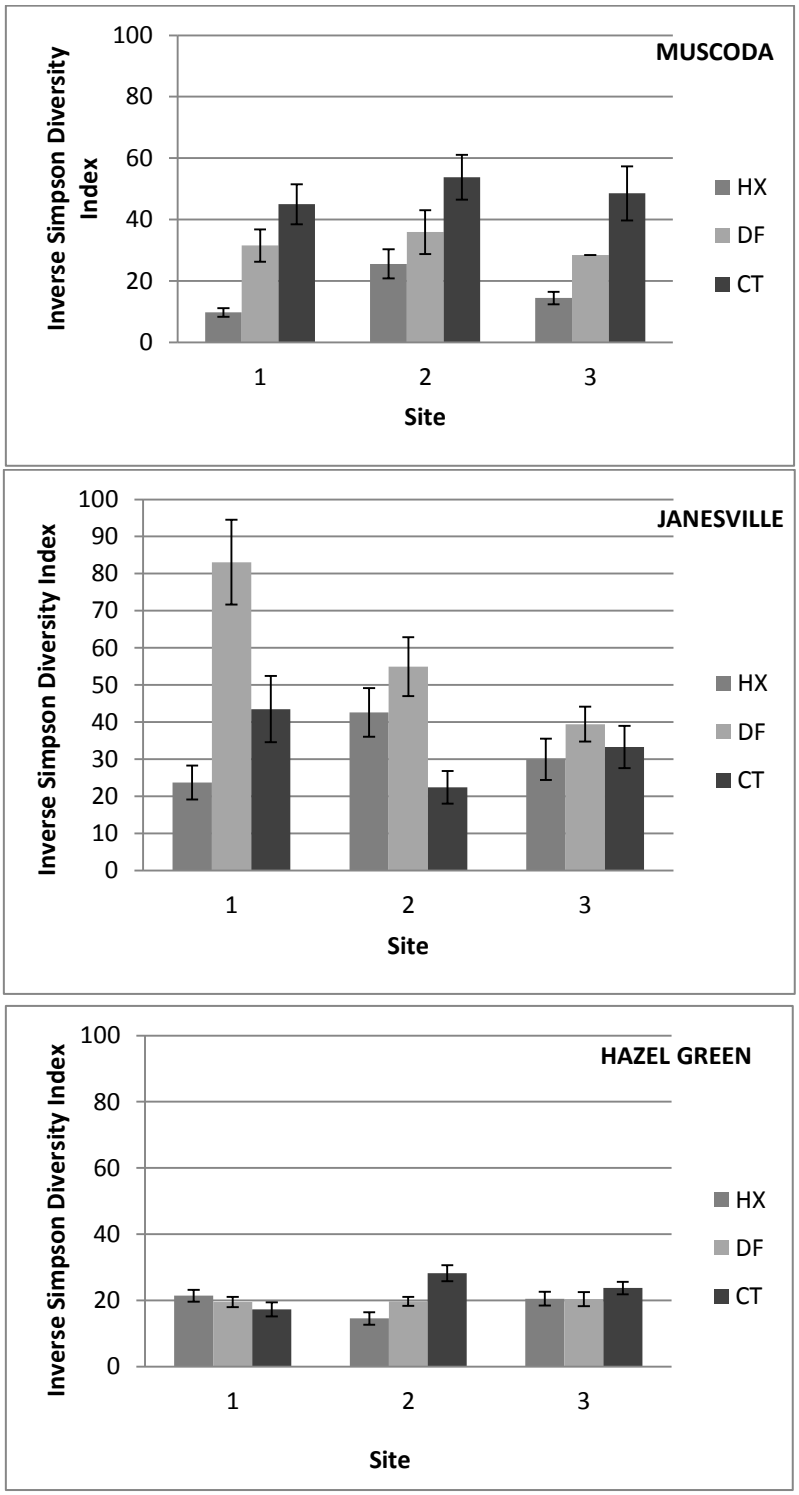

Figure 1: Inverse Simpson diversity index results separated by location (HX-Hexaflumeron, DF-Diflubenzuron, CT-Control) (Hazel Green – *R. tibialis*; Janesville & Muscoda – *R. flavipes*; error bars represent standard deviation)

The inverse Simpson diversity index showed a difference in diversity at all three Muscoda sites in termites treated either with hexaflumeron or diflubenzuron compared to the controls suggesting reduced diversity after termiticidal treatment (HX<DF<CT). Within the Janesville groups, site one showed higher diversity in the diflubenzuron treated termites compared to the others, while site two showed both treatment groups with greater diversity than the control group. For all Hazel Green termites, only site #2 showed a significant difference between control and treated groups. The other two sites showed no difference in diversity between the treated and control groups. Overall, all three Hazel Green sites showed less diversity than either Muscoda or Janesville.

Next, the three sites per location were combined and the number of shared operational taxonomic units (OTUs) among them was determined. OTUs were then broken down into major taxonomic groups which showed the presence of 11 major bacterial lineages, Bacteroidetes, Spirochaetes, unclassified, Firmicutes, Proteobacteria, Verrucomicrobia, Tenericutes, TM7, Actinobacteria, Fusobacteria and Fibrobacteres, with the dominant phyla among all samples being the Bacteroidetes and the Spirochaetes. Interestingly, the Spirochaetes tended to be more prevalent in the HG, *R. tibialis* samples compared to the dominance of Bacteoidetes among the JV and MU *R. flavipes* samples (Fig. 2). Overall, examination of the abundance of major taxonomic groups in treated versus control groups does not appear to show any major differences in analyses done thus far.

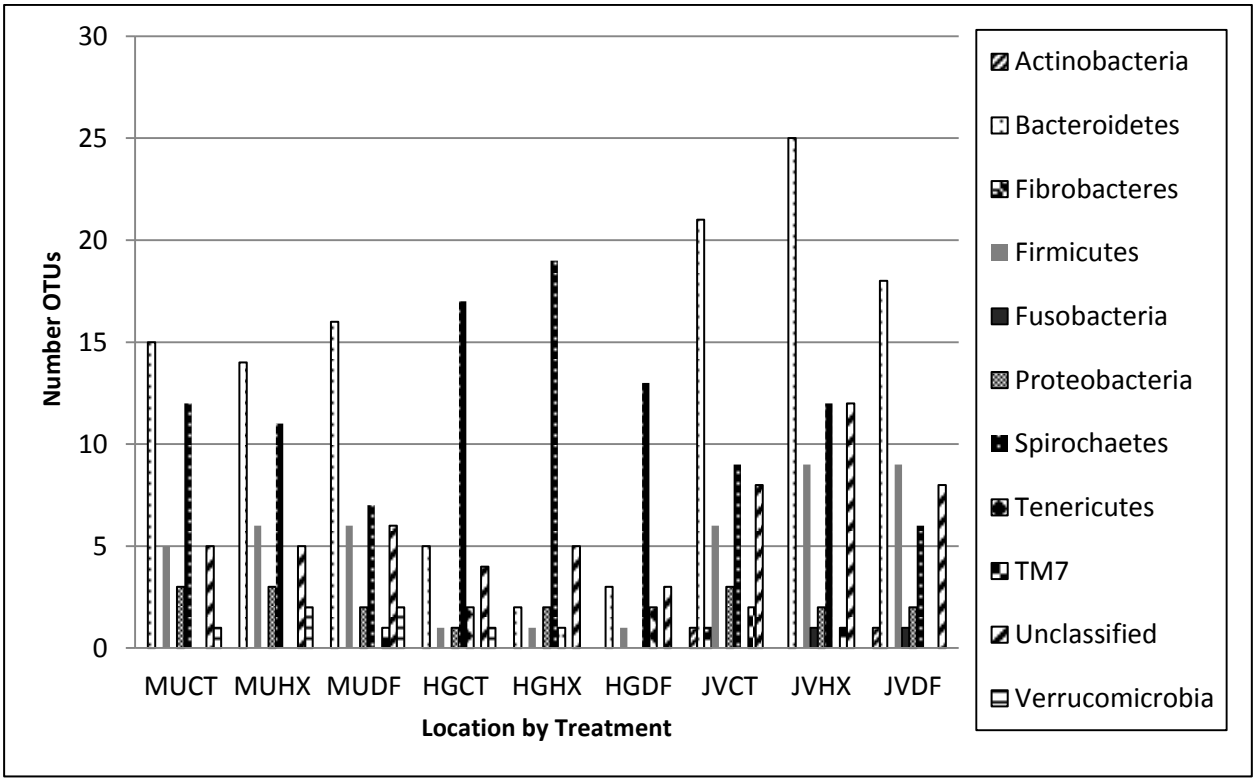

Figure 2: Presence of major bacterial phyla by treatment

To further evaluate shifts in bacterial community, both diversity and abundance of various OTUs within each experimental group was examined using two different similarity algorithms, executed in mothur. Results were used to form comparative phylogenetic trees (Letunic and Bork 2006). The Jclass phylogeny (Fig. 3) employs the traditional Jaccard similarity coefficient which is based on observed richness (presence or absence data) and the thetayc phylogeny (Fig. 4) is based on the Yue and Clayton theta similarity coefficient which takes into account OTU abundance within the sample groups.

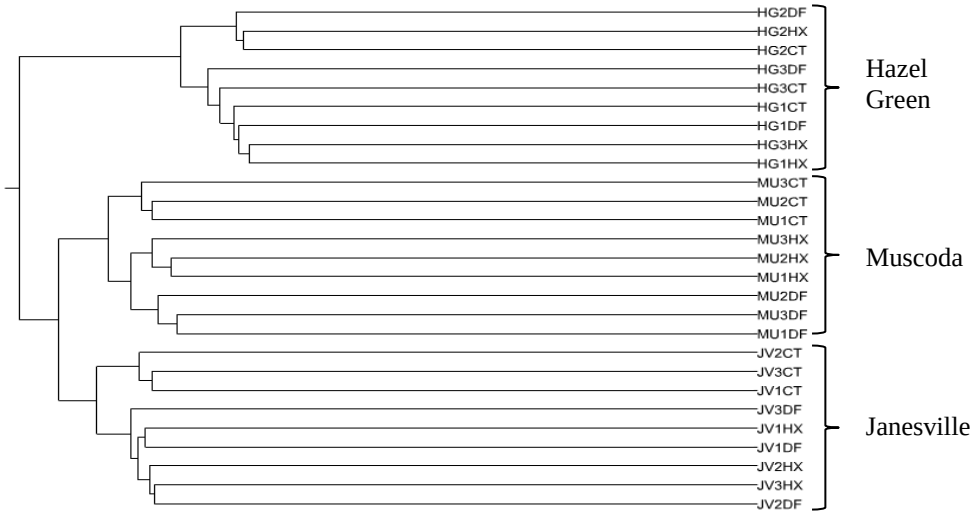

Figure 3: Bacterial community phylogeny based on Jclass dissimilarity algorithm

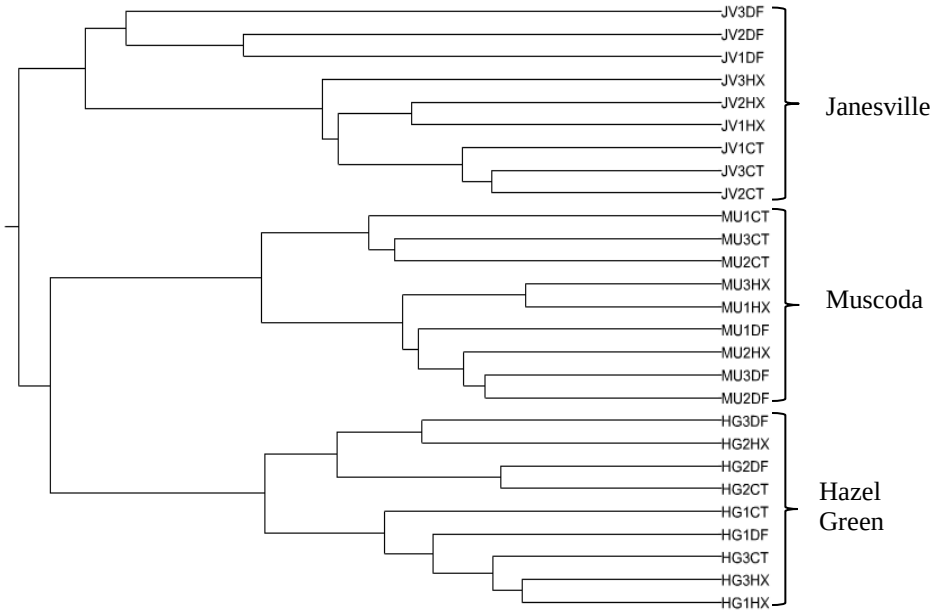

Figure 4: Bacterial community phylogeny based on thetayc dissimilarity algorithm

4. DISCUSSION

Overall, total number of sequences analysed during this study were much smaller than expected when compared to other high throughput studies (e.g. Boucias et al. 2013). In addition, estimated percent coverage of the community ranged from 61-87%, indicating that improvements should be made to the protocol to increase bacterial amplification and sequencing. Despite low sequence numbers and estimated percent coverage, however, the same major bacterial groups were present compared to other molecular studies of termites. The major bacterial phyla known from *R. flavipes* according to Fisher et al. (2007) are the Spirochaeta, Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Endomicrobia. This matches closely the diversity seen in this study as Spirochaetes and Bacteroidetes tended to make up the majority of OTUs found in *R.*

flavipes regardless of treatment. Some authors have suggested that spirochetes may account for nearly 50% of prokaryotes in termites (Brune and Friedrich 2000). Interestingly, the Bacteroidetes were found to be far less prevalent than the Spirochaetes in *R. tibialis* samples for both control and treatment groups.

Analysis of total bacterial community similarity using two different methods did show some trends with treatment. According to both the Jclass and thetayc phylogenies, individuals from the same colony were found to cluster with themselves to the exclusion of the other colonies. This suggests that termite colonies have a microbial profile that is distinct from other colonies even in the same species, while maintaining the major bacterial communities listed above. This is not surprising since *R. flavipes* acquires its gut biota via horizontal transmission through proctodeal feeding, we would expect a large degree of homogeneity in the gut fauna within a colony (Husseneder 2010). In the Jclass analysis, which differentiates groups by presence or absence of specific OTUs, *R. flavipes* groups from Muscoda separated out by treatment, indicating a difference in observed richness of OTUs in controls versus either the hexaflumuron or diflubenzuron fed termites. *Reticulitermes flavipes* from Janesville also separated controls from treated, but did not differentiate between the two treatments. There was no observed pattern by treatment in *R. tibialis* groups from Hazel Green. In the thetayc phylogeny, which evaluates OTU abundance, Janesville samples were separated out by treatment while Muscoda samples only differentiated controls versus treated, with suppressed bacterial diversity in the treated groups. Again, no observed pattern was seen in Hazel Green termite groups.

Although there does not appear to be any obvious shifts in the major bacterial phyla after termiticidal feeding, there does appear to be some shifts in diversity of the bacterial community as shown by the diversity indices and phylogenetic trees. Interestingly, these trends are apparent in *R. flavipes* but not in *R. tibialis*. In *R. flavipes*, both phylogenetic trees show the control groups separated from the treated groups. These results suggest a treatment effect of feeding on termiticidal baits on microbial community in *R. flavipes*, which was not observed in *R. tibialis*.

Since gut microbiota has been directly linked with overall termite fitness, these results could give insight into collapse of a termite colony. Changes in the bacterial community of termites could be a reduction of one assembly of bacterial species followed by increase in a more detrimental assemblage such as facultative pathogens, increasing the effectiveness of certain termiticidal products. Further analysis should be done to examine which bacterial groups may be most affected by specific bait treatments and the impact of these shifts on overall termite and colony health.

5. ACKNOLEDGEMENTS

The authors would like to thank Kelsea Jewell, Charlie Mason and Dr. Alissa Hanshew for help with sample preparation, guidance on pyrosequencing and help with use of the 454 GS Junior. We also thank Dr. Garret Suen, Dr. Dan Marschalek, Dr. Grant Kirker and Dr. Eric Matson for helpful reviews and comments on the project.

6. REFERENCES

Arango, R.A. 2014. First Record of the arid-land termite, *Reticulitermes tibialis* Banks, in Wisconsin. The Great Lakes Entomologist, In Press.

Breznak, J.A. and A. Brune. 1994. Role of microorganisms in the digestion of lignocellulose by termites. Annual Review of Entomology 39: 453-487.

Brune, A. and M. Friedrich. 2000. Microecology of the termite gut: structure and function on a microscale. Current Opinion in Microbiology 3: 263-269.

Edgar, R.C., B.J. Haas, J.C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27: 2194-2200.

Fisher, M., D. Miller, C. Brewster, C. Husseneder and A. Dickerman. 2007. Diversity of gut bacteria of *Reticulitermes flavipes* as examined by 16S rRNA gene sequencing and amplified rDNA restriction analysis. Current Microbiology 55: 254-259.

Hongoh, Y., M. Ohkuma and T. Kudo. 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiology Ecology 44: 231-242.

Husseneder, C. 2010. Symbiosis in subterranean termites: A review of insights from molecular studies. Environmental Entomology 39: 378-388.

Letunic, I. and P. Bork. 2006. Interactive Tree of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 23: 127-128.

Lin, N. and C.D. Michener. 1972. Evolution of sociality in insects. The Quarterly Review of Biology 47: 131-159.

Matsuura, K. 2001. Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. Oikos 92: 20-26. DOI: 10.1034/j.1600-0706.2001.920103.x

Ohkuma, M. and T. Kudo. 1996. Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. Applied and Environmental Microbiology 62: 461-468.

Potrikus, C.J. and J.A. Breznak. 1981. Gut bacteria recycle uric acid nitrogen in termites: a strategy for nutrient conservation. Proceedings of the National Academy of Science 78: 4601-4605.

Pruesse, E., C. Quast, K. Knittel, B.M. Fuchs, W. Ludwig, J. Peplies and F.O. Glockner. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35: 7188-7196.

Rosengaus, R.B., C.N. Zecher, K.F. Schultheis, R.M. Brucker and S. R. Bordenstein. 2011. Disruption of the termite gut microbiota and its prolonged consequence for fitness. Applied and Environmental Microbiology 77: 4303-4312.

Schloss, P.D. 2009. A high-throughput DNA sequence aligner for microbial ecology studies. PLoS ONE 4: e8230.

Schloss, P.D., S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R.A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G.G. Thallinger, D.J. Van Horn and C.F. Weber. 2009. Introducing mothur: open-source, platform independent,

community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75: 7537-7541.

Schloss, P.D., D. Gevers and S.L. Westcott. 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PloS ONE 6: e27310. Accessed January 2014.

Su, N.Y. and R.H. Scheffrahn. 1993. Laboratory evaluation of two chitin synthesis inhibitors, hexaflumuron and diflubenzuron, as bait toxicants against Formosan and Eastern subterranean termites (Isoptera: Rhinotermitidae). Journal of Economic Entomology 86: 1453-1457.

Thorne, B.L. 1997. Evolution of eusociality in termites. Annual Review of Ecology and Systematics 28: 27-54.