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Intercolony Comparisons of Gut Microbiome Composition From Lab Reared Eastern Subterranean Termites (Blattodea: Rhinotermitidae)

R. Sapkota[1,3,•] and M. E. Scharf[1,2]

[1]Department of Entomology, Purdue University, West Lafayette, IN, USA, [2]Entomology and Nematology Department, University of Florida, Gainesville, FL, USA, and [3]Corresponding author, e-mail: rajani.sapkota4801@gmail.com

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Abstract

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

Key words: termite, gut bacteria, termite colony, lab rearing

The lower termite gut possesses a unique symbiosis with different flagellates which themselves live in association with prokaryotes (Ohkuma 2008, Scharf and Tartar 2008, Brune and Ohkuma 2010). Those symbionts are mostly unique and unculturable and demonstrate a mixed mode of transfer (horizontal and vertical) within termite colonies (Hongoh 2011, Bourguignon et al. 2018). Termites can survive by feeding on dead plant materials by using the enzymes secreted by themselves and their microbial symbionts to digest complex lignocellulose (Peterson et al. 2015, Peterson and Scharf 2016a, b, Maurice and Erdei 2018). Along with cellulose/hemicellulose degradation, termite gut microbes also contribute to termite physiology through nitrogen fixation, acetogenesis, anti-fungal defense, fecundity and fitness of reproductive castes, and caste differentiation (Inoue et al. 1997, Inoue et al. 2000, Doolittle et al. 2008, Rosengaus et al. 2011, Peterson and Scharf 2016a, b, Sapkota et al. 2021).

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

Change in diet has been reported to alter the abundance and diversity of eukaryotic and prokaryotic gut symbionts in *Drosophila* (Jiménez-Padilla et al. 2020). Besides insects, host diet has also been reported to influence the gut bacterial diversity in humans (from carnivory to omnivory to herbivory), and vertical transfer has been considered as a route of transfer of symbionts in mammals (Ley et al.

2008). Similarly, termite gut bacteria have been reported to change their community structure in response to dietary changes (Auer et al. 2017, Benjamino et al. 2018). However, a 7-d lab study on a variety of diets did not result in a significant change in termite gut microbiota composition (Boucias et al. 2013). These differing results could be due to the length of restricted feeding, colony genetic differences, or rearing temperatures, e.g., high rearing temperature has been reported to reduce gut bacterial abundance and diversity (Arango et al. 2021). Colonial differences in gut microbiomes have been reported in oriental fruit flies, ants, and honeybees (Wang et al. 2011, Segers et al. 2019, Vernier et al. 2020). In addition, gut microbes in the termite are also believed to be, to a degree, colony specific (Matsuura 2001). Therefore, we planned an experiment to study the effect of identical diet and rearing temperature on the gut bacterial community from different colonies of termites with the following objectives: (1) to study the species richness, evenness, and phylogenetic relatedness of gut bacterial populations from two colonies, and (2) to compare the similarity or differences in the gut bacterial composition between colonies.

Materials and Methods

Termite Collection and Rearing

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

Sample Preparation and DNA Extraction

Whole guts from termites from both colonies were extracted in 200 μL PBS (Phosphate Buffered Saline; 0.081M monobasic sodium phosphate, 0.019 M dibasic potassium phosphate, 0.027 M potassium chloride and 1.37 M sodium chloride in high purity water) and stored at –20°C until used later for DNA extraction. The DNeasy Blood and Tissue Kit (Qiagen; Valencia, CA) was used for DNA extraction with a slight modification (overnight incubation of gut tissues) in the protocol. The quantity of DNA in the aliquots were assessed using a Nanodrop spectrophotometer (Thermo-Fisher; Waltham, MA), and the quality was assessed through the gel electrophoresis. Twenty microlitres of the extracted DNA was sent for 16S rRNA library preparation and sequencing and the rest was used to verify genetic relatedness of colonies. For this, we PCR amplified the termite mitochondrial 16S rRNA gene using the primers Forward: 5′- TTACGCTGTTATCCCTAA-3′ and Reverse: 5′- CGCCTGTTTATCAAAAACAT-3′ (Austin et al. 2005, Boucias et al. 2013). PCR was used to amplify a ~ 428 bp region, which was sequenced at the Purdue University Genomics core, IN, USA. The FASTA sequence of each colony is publicly available at GitHub repository (https://github.com/rajanisapkota/Inter-colony-comparisons-of-gut-microbiome). The resulting sequences were blasted against NCBI database to study the genetic relatedness of colonies.

Bacterial 16S rRNA Gene Sequencing

The primers used for the 16S rRNA gene sequencing were 341F- CCTACGGGAGGCAGCAG and 806R- GGACTAC VGGGTWTCTAAT, which amplified the bacterial V3-V4 region of

the 16S rRNA gene (Caporaso et al. 2011, Peterson et al. 2015). A two-step PCR was used for amplification and indexing. The indexed PCR products were normalized to 10 ng/μl, and 10 μl of each sample was pooled and concentrated to ~100 μl and cleaned using SPRI (solid reverse phase immobilization) purification. Qubit was used to quantify the cleaned library and Agilent Tapestation for fragment analysis. The library was then diluted to 2nM. The pooled samples were denatured with NaOH, diluted to 8 pM in Illumina HT1 buffer, spiked with 15% PhiX and heat denatured at 96°C for 2 minutes before loading. A MiSeq 600 (1/8th lane Stowaway) cycle v3 kit was used to sequence the sample at the University of Minnesota Genomics Center, MN, U.S. Sequences from this study are available through the NCBI Sequence Read Archive database under the BioProject accession number PRJNA782604 (SRR17005750 - SRR17005758) and Biosample id is SAMN23388497. In addition, the Qiime2 and R scripts used for statistical analysis and visualizations can be accessed from github repository- https://github.com/rajanisapkota/Inter-colony-comparisons-of-gut-microbiome.

Sequence Processing and Community Analysis

QIIME2 pipeline- moving picture tutorial (v. 2020.11, https://docs.qiime2.org/2020.11/tutorials/moving-pictures/) was used to analyze the resulting paired end illumina reads (Bolyan et al., 2019). Those sequences were filtered, trimmed to suitable depth, denoised, dereplicated, merged, and chimeras removed using the DADA2 algorithm (Divisive Amplicon Denoising Algorithm 2) with the parameters p trim-left-f 15, p trim-left--r 20, trunc-len-f 275 and trunc-len-r 215 (Callahan et al. 2016). Those parameters for DADA2 algorithm were decided from interactive quality plots. During the DADA2 process, 726,212 demultiplexed sequences were processed to obtain 525,265 high quality reads. The moving picture tutorial in qiime2 was followed to calculate diversity matrices. Alpha and beta diversity matrices were calculated using the sequences subsampled from the feature table at the subsampling depth of 46,500. The alpha diversity matrices used are- Observed features, Shannon diversity, Peilou evenness, and Faith phylogenetic diversity; while the beta diversity matrices used are phylogenetic Unifrac distances- weighted and unweighted (Lozupone et al. 2011), and nonphylogenetic distances- Jaccard and Bray Curtis. The visualization of Unifrac distances were done in R (version 4.1.1, accessed on 2021-08-10) using the packages qiime2R, dplyr, and ggplot. The reference database SILVA (version 132) was used to train the sequences after customization following the instructions on training the classifier for V3/V4 region of 16S rRNA gene. The Naïve Bayes trained Silva 132 99% OTU classifier, bounded by the 341F/806R primer set, was used as a reference read to assign taxonomy (Quast et al. 2013). Bar graphs representing the relative abundances of ASVs at the phylum and family levels were generated using the R package phyloseq from QIIME2 generated phylotype tables and taxonomy files (McMurdie and Holmes 2013). The analysis of composition of microbiomes (ANCOM) procedure was used to identify the significant differentially abundant bacterial taxa (at genus and phylum levels) using QIIME2 plugin (Mandal et al. 2015).

Results

Genetic Relatedness Among Colonies

The partial sequence of the host termite mitochondrial 16S rRNA gene was used to identify termite colony isolates. Blasting of the sequences from colonies with each other resulted in 97.95% identity match which provides evidence for colonies being different isolates of *Reticulitermes flavipes*, i.e., unique colonies. In addition, we used

the sequences from the closely related isolates of both the colonies to run Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm. MUSCLE algorithm was used to align multiple sequences and output was used to generate a phylogenetic tree (Madeira et al. 2019). The position of Whistler and Biochem colonies at different nodes of phylogenetic tree confirms that collected colonies were different isolates of *R. flavipes* (Supp. Fig. S1).

Bacterial Alpha Diversity

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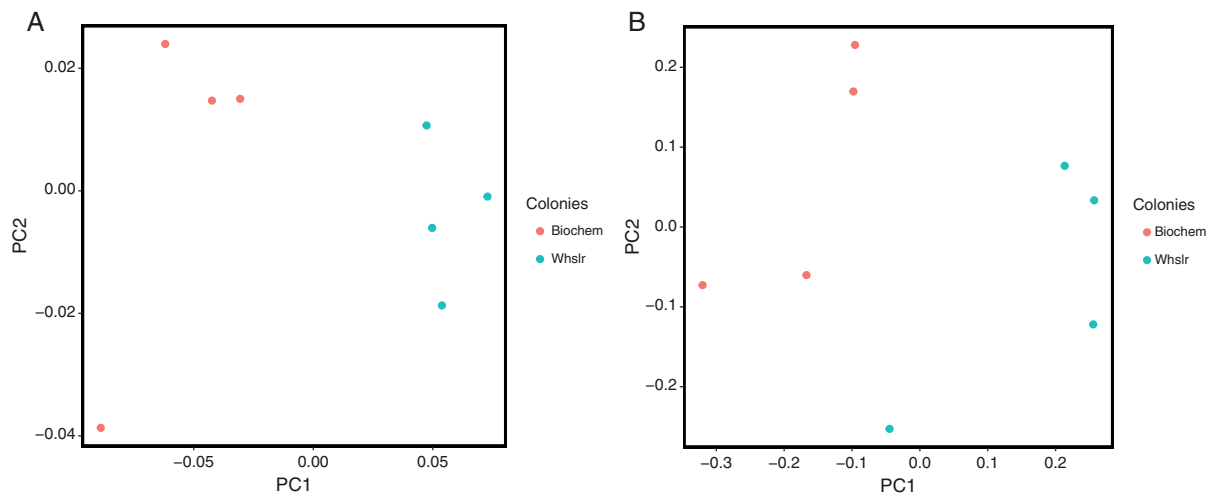
Bacterial Beta Diversity

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

Bacterial Taxonomic Composition

Based on relative abundance analysis, the taxonomic composition of dominant bacterial phyla at abundances greater than 0.01% varied slightly on their relative abundances between colonies (Fig. 3A). The major dominant phyla were Spirochaetes, Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria. Based on NCBI taxonomy, the phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are now named as Bacillota, Bacteroidota, Psudomonadota, and Actinomycetota respectively. Similarly, the taxonomic composition of bacterial families at abundances greater than 1% was computed. Based on the relative abundance graph, 16 bacterial families were present at abundances greater than 1% (Fig. 3B). On computing Analysis of Composition of Microbiomes (ANCOM) at phylum level, the phyla RsaHF231 and Verrucomicrobia were found significantly differentially abundant between colonies (Fig. 4A). In addition, at genus level, the taxa *Elizabethkingia* and *Chryseobacterium* from phylum Bacteroidetes were differentially abundant between colonies (Fig. 4B).
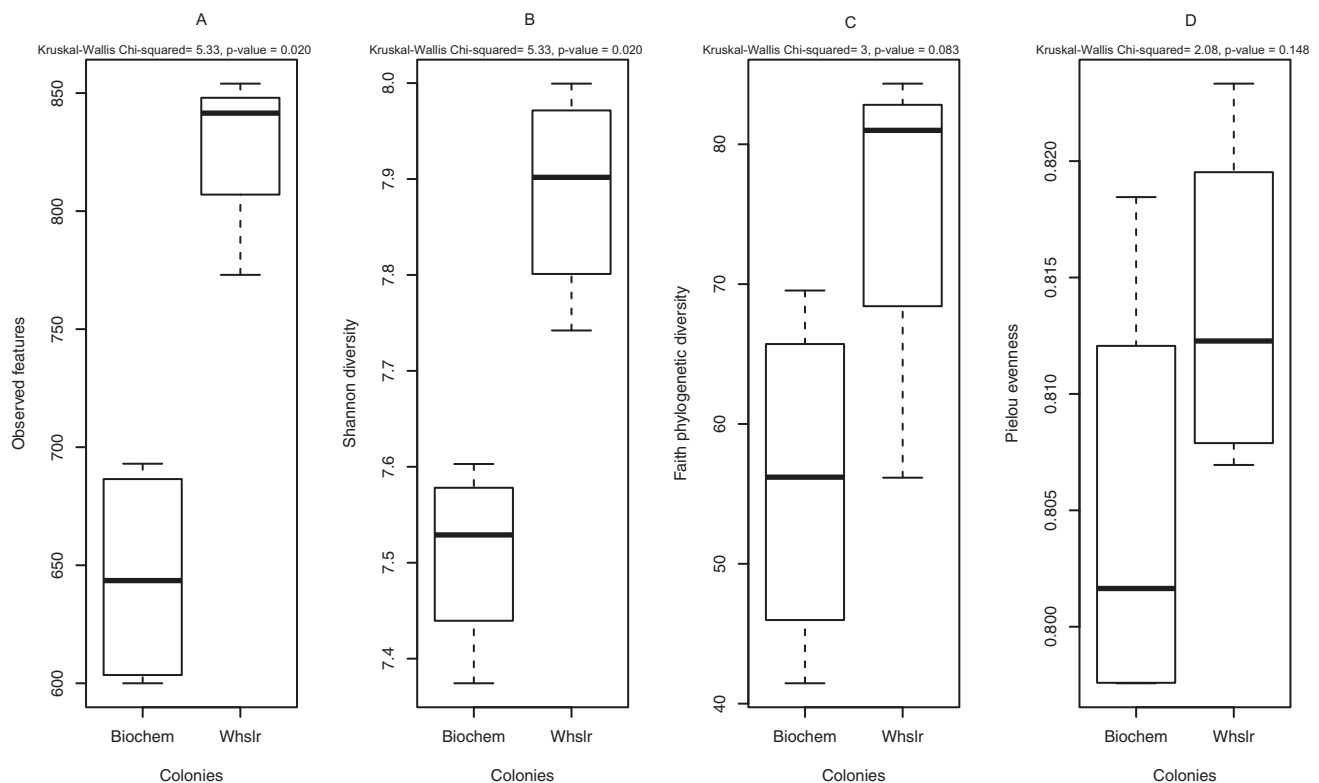
Fig. 1. Alpha diversity matrices from 16S surveys showing: Observed features (A), Shannon diversity (B), Faith phylogenetic diversity (C) and Pielou evenness (D). P-values for global Kruskal-Wallis comparisons across colonies are shown at the top of each graph.

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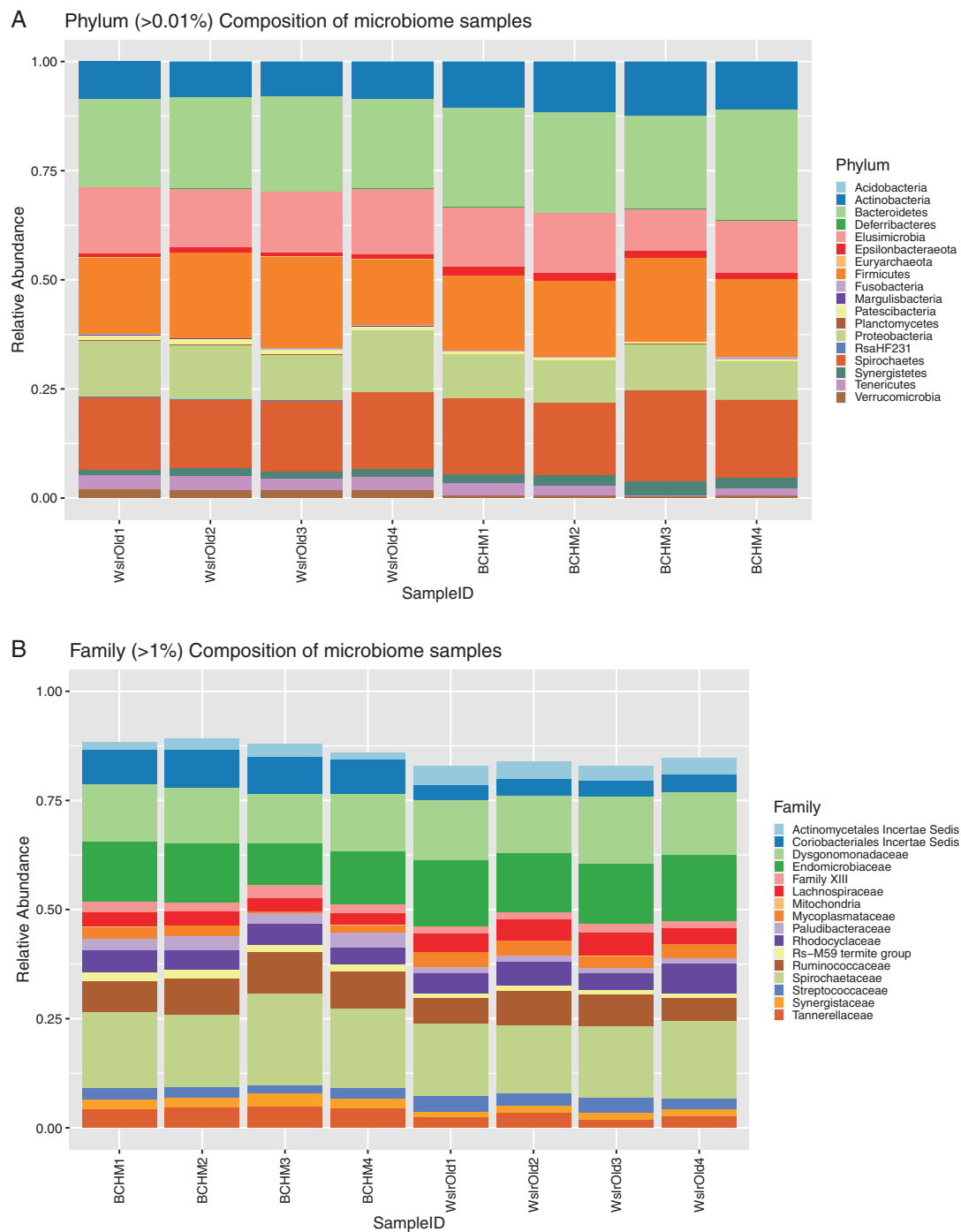
Discussion

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

The number of unique bacterial species (Observed features) and their diversities (Shannon diversity) were significantly higher in the Whslr colony, but interestingly colonies were statistically similar in terms of abundance of each bacterial species (Pielou evenness) and their phylogenetic diversities (Faith phylogenetic diversity). Alternatively, in a culture dependent study, a group of researchers found that colonies of Formosan subterranean termites resulted in a statistically similar number of culturable gut bacteria (Husseneder et al. 2009). The differences in results between studies could be due to the large number of termite gut bacteria that are unculturable and/or due to difference in rearing conditions. Based on beta diversity indices, we also found a significant signal between overall variation of bacterial communities between termite colonies. Similar to our results, distinct differences in metatranscriptome responses have been observed in termite colonies which could be tied to the gut microbial differences among them (Boucias et al. 2013, Scharf et al. 2017). Differences in gut bacteria from different colonies of oriental fruit flies and ants have been reported previously (Wang et al. 2011, Segers et al. 2019). Similarly, the composition of microbial flora in a termite gut has been believed to be colony specific (Matsuura 2001, Boucias

et al. 2013). The composition of major bacterial phyla of wood feeding termites were found stable even though variation in relative abundance occurred with their diet (Van Dexter and Boopathy 2020). Interestingly, the overall functions within a termite gut apparently were not disturbed which indicates the occurrence of metabolic overlap; an important feature in microbial ecology (Van Dexter and Boopathy 2020). Furthering to these prior studies, our study suggests that differences in gut bacterial structure among colonies are inevitable despite similar rearing conditions in the laboratory. The analysis of relative abundance of bacteria in our experiment indicates a similar composition of dominant bacterial phyla; however, a small but statistically significant difference was still prevalent suggesting that unique colonial signatures remain even after long-term feeding on identical diets. These unique signatures are likely maintained via vertical transfer of gut bacteria within colonies, leading to possible continued symbiotic associations which cannot be disrupted (Bourguignon et al. 2018).

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

A   Phylum (>0.01%) Composition of microbiome samples

B   Family (>1%) Composition of microbiome samples

Fig. 3. Relative abundance of bacteria (from abundance >0.0001) at phylum level (A) and relative abundance of bacteria (from abundance >0.01) at family level (B). Data shown are the percentage of the total bacterial abundance for each termite colony replicate.

transfer to new environments. Our findings advance current research by demonstrating a small but significant colonial variation in the gut bacterial taxa after rearing termite colonies under laboratory conditions. These findings have important implications for basic and applied termite science.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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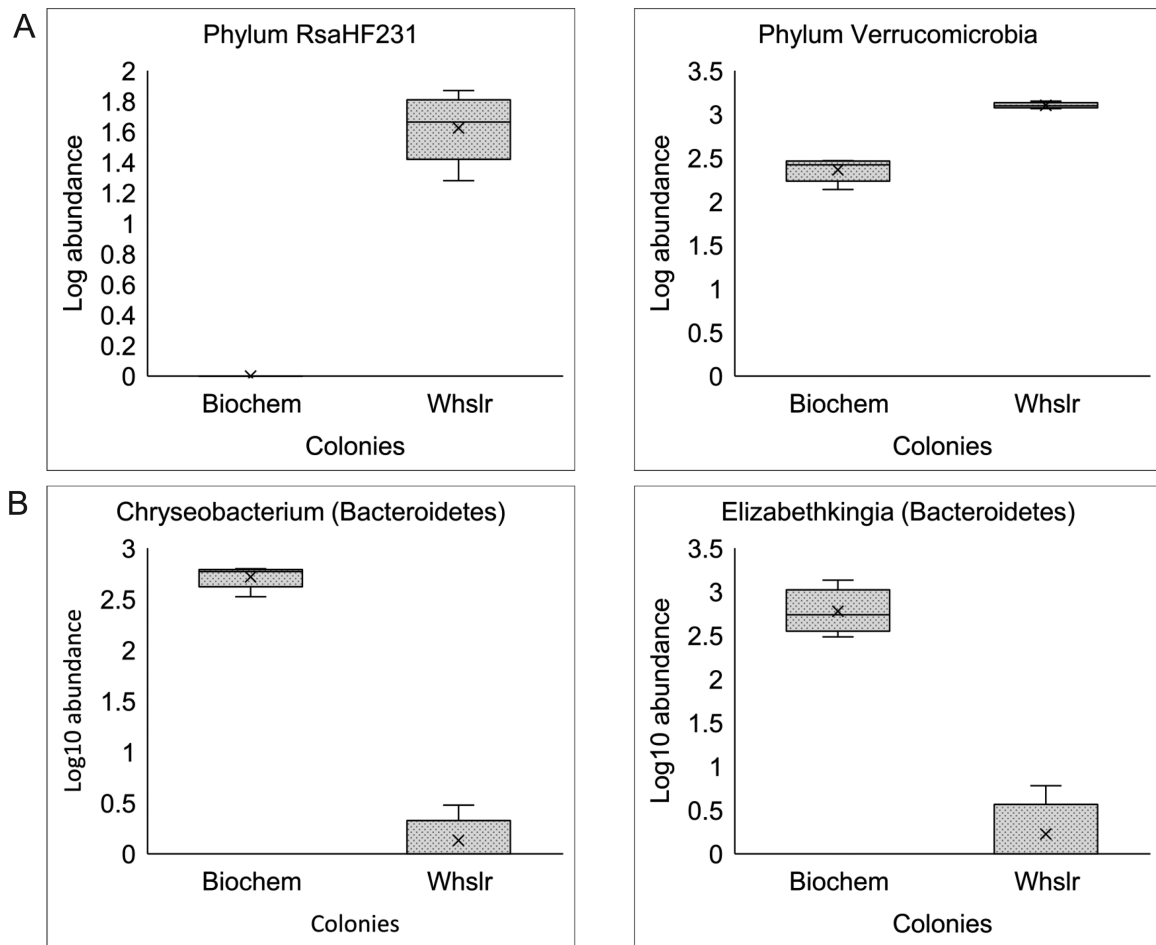
Fig. 4. Boxplots of the log abundances of bacterial phyla (A) and genera (B) (False Discovery Rate at 0.05). These phyla and genera were significantly differentially abundant as measured by ANCOM between the colonies Biochem and Whslr.

Author contribution

RS and MES: Conceptualization. RS: Data curation. RS: Formal analysis. MES: Funding acquisition. RS: Investigation. RS and MES: Methodology. MES: Resources. MES: Supervision. RS: Visualization. RS: Writing – original draft. RS and MES: Writing – review & editing.

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