Project objective: Restoring ironwood as an agroforestry species in Guam through research into the bacterial flora of ironwood trees and the guts of termites.

Name of Institution: University of Louisiana

C. Approach and Procedures

Sample collection:

Forty- five termite samples were collected in 2019-20 by the University of Guam from healthy as well as sick ironwood trees present on 14 distinct locations on the island of Guam. Geology related (location, parent material classification, site management), tree-related (tree DS, tree health, presence or absence of Ralstonia, altitude classification) and plot-related (plot average DS, plot average health, stand maturity estimate, percentage of trees with termites in the plot, percentage of dead trees in the plot) parameters were recorded (Table 1). The samples including soldiers and worker termites were partitioned into 70% ethanol (for morphological identification) and 95% ethanol (for Illumina sequencing) and were shipped to Louisiana State University.

Table 1: Description of the factors collected from ironwood tree plots by University of Guam

Factor	Description
a.) Location related	
Location	Area where the tree was located.
Parent material	The type of parent material (Lime, Tuff or Sand) at that location.
Site management	Sites were classified into three categories based on the extent of maintenance: No maintenance, Moderately managed, Highly managed
Altitude	Location of tree in relation to mean sea level (in meters), taken at ground level at the base of the tree.
Altitude classification	Altitude was classified as "low" for less than 100 meters and as "high" for greater than 100 meters.
b.) Tree related	
Presence or absence of *Ralstonia solancearum* species complex	Trees were tested for presence (+) or absence (-) of Ralstonia using the Agdia Strip Test.

Decline Severity	The level of damage to the tree due to disease determined by visual inspection based on fullness of branches and dieback (0= symptomless, 1=slight damage, 2=distinctly damaged, 3=heavily damaged, and 4=nearly dead) (Figure 1).
Health	Using tree Decline Severity, the individual trees were classified as "healthy" (DS= 0) or "sick" (DS= 1, 2, 3, or 4)
c.) Plot related	
Plot Average DS	Only live trees within the plot were counted and the average disease severity of the plot was determined.
Plot Average Health	Using Decline Severity, each tree within the plot was classified as "healthy" (DS= 0) or "sick" (DS= 1, 2, 3, or 4). An average health of the plot was determined by the percentage of sick trees to the total number of live trees in the plot.
Percentage of dead trees in plot	Percentage (%) of dead trees within the sample tree's 30m radius
Percentage of trees with termites in plot	Percentage (%) of live trees with existing or previous termite activity within a given plot.
Stand Maturity Estimate	It indicates the age/ maturity of trees in a stand. It was calculated using basal area per acre divided by number of trees per acre.

Morphological species identification of termites:

Diagnostic characters of each soldier were examined visually under a stereo microscope (Leica MZ16) using the published keys such as Chhotani 1997, Liang and Li 2016 and Su and Scheffrahn1998.

DNA extraction:

Five termites from each sample were pooled and crushed using lysis buffer (ATL) from the DNeasy Blood & Tissue kit (Qiagen, Germantown, MA). The DNA was extracted according to the manufacturer’s instructions. The concentration (>10 ng/uL) of DNA was confirmed using an Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE) with the Qubit dsDNA BR Assay Kit. Depending upon the measured quantity, DNA samples were diluted to

20ng/µl and were shipped on ice to the Hubbard Center for Genome studies at the University of New Hampshire for amplification and next-generation sequencing.

Primer selection:

One hundred thirty 16s rRNA gene full- length (1.4k) sequences of different *Ralstonia solanacearum* strains from NCBI GenBank were aligned using Geneious software and examined for variability among different V- regions (V1-V3 and V4 regions). V1-V3 region had 87.5% identical sites and V4 region had 93.5% identical sites. V1-V3 region showed more variability and was selected for next generation sequencing on Illumina NovaSeq platform (2x250). The sequencing was performed using one forward (27F) and two reverse primers (519Rmod and 519Rmodbio) to capture a broad range of biodiversity.

DNA amplification and sequencing:

Procedures for DNA amplification and sequencing were performed at University of New Hampshire Hubbard Center for Genome studies. The V1-V3 hyper variable region of the 16S rRNA gene was amplified from bacterial genomic DNA samples using the Earth Microbiome Project 16s PCR (Polymerase Chain Reaction) protocols ("https://earthmicrobiome.org/protocols-and-standards/16s/"). Successfully amplified PCR products were sequenced on the 2x250bp Illumina NovaSeq platform following Illumina Nextera Dilute library protocol (Illumina, San Diego, CA).

Bioinformatics analysis:

QIIME 2 (version 2021-4), accessible on a server provided by the Hubbard Center for Genome studies at the University of New Hampshire, was used to perform data analysis. The demultiplexed forward and reverse reads that already had barcodes and adapters removed were imported into QIIME 2 using the q2-tools plugin. The raw reads were examined for the Phred quality scores using q2-demux plugin. Visualization files (.qzv) were viewed through http://view.qiime2.org. No trimming was required as all the sequences were high quality (above Phred quality score 30). Primer sequences were removed and forward and reverse reads were truncated to 251 nucleotides. Since a considerable number of forward and reverse reads were not overlapping, paired ends were not merged, and only forward reads were subjected to denoising and chimera removal using DADA2 algorithm (Callahan et al. 2016). A table containing amplicon sequence variants (ASV) and a representative sequence file showing sequences of all the ASVs were obtained as a result of DADA2 procedure. Sequences from the same sample generated with two different reverse primer sets were merged using the group method of q2-feature-table plugin after preliminary analyses showed no significant differences in taxa composition and diversity.

The ASVs were taxonomically classified using the SILVA 132 reference database with the help of the q2-feature-classifier plugin. Different pairwise identity cutoff values (95%, 97%, 99%), i.e. the minimum percent identity match to reference sequences in the SILVA database required for a taxonomic assignment, were applied to optimize the balance between stringent classification and number of unassigned ASVs. For final taxonomic assignment sequences were classified with a 97% pairwise identity cutoff using the consensus method with the help of

BLAST algorithm. The q-2 alignment plugin was used for multiple alignment of sequences with the mafft method (Katoh et al. 2002) and the highly variable positions from the alignment were filtered out using mask command (Lane 1991). With the help of q2-fasttree plugin (FastTree 2), a midpoint-rooted phylogenetic tree was generated from aligned and masked sequences (Price et al.2010).

Taxonomy barplots showing relative abundances of taxa were generated using the 'barplot' command in the q2-taxa plugin. Unassigned ASVs were removed by filtering the ASV table. The further analysis was continued with three data sets:

1. Full data set: It is a data set that was obtained after filtering out only unassigned taxa from the raw data.

2. Only SF data set: It is a data set that contains only Spirochaetes and Fibrobacteres.

3. Without SF data set: It is a data set that was obtained after excluding Spirochaetes and Fibrobacteres from full data set.

Alpha rarefaction curves were plotted by subsampling the reads to the lowest sequencing depth of 50,227 reads per sample. In rarefaction, large samples are sub-sampled to a size at which they are equal to the smallest sample size (Chao et al. 2014). Alpha rarefaction curves represent diversity as a function of the number of resampled sequences. Along with comparing samples based on equal size, samples were also compared based on equal completeness (coverage). Sample-size and coverage-based rarefaction and extrapolation sampling curves were generated using an R package iNEXT(iNterpolation/EXTrapolation) (Hsieh et al. 2016). iNEXT package uses Hill numbers (parameterized by q) that represent the effective number of species (Chao et al. 2014, Hsieh et al. 2016). Three Hill numbers i.e., species richness ($q = 0$), Shannon diversity ($q = 1$), and Simpson diversity ($q = 2$) were used. The species richness index quantifies species based on their incidence without considering their abundance, Shannon diversity index quantifies counts species based on their abundances and Simpson diversity counts the number of dominant species. These indices estimate the effective diversity of various taxa within the samples (Chao et al. 2014, Hsieh et al. 2016). These estimates are further utilized for making statistical comparisons (Chao et al. 2014, Hsieh et al. 2016).

The ASVs obtained after denoising were classified into their respective taxa for further analysis. There are eight distinct taxonomic levels named Domain, Kingdom, Phylum, Class, Order, Family, Genus, and Species. ASVs within each phylum were plotted as the percentage of total number of ASVs. All the ASVs were categorized to their lowest SILVA assignment levels. The abundance of each ASV in different groups of all the location-, tree- and plot-related factors was also determined.

Alpha and beta diversity analyses were performed through the q2-diversity plugin. The core-metrics-phylogenetic method was applied to rarefy the sequences to a depth of 660. The alpha-diversity analysis for each sample was conducted based on incidence (Pielou's evenness, Faith's phylogenetic distance (Faith 1992)) and abundance (Number of ASVs, Shannon diversity) based indices. Correlations were assessed for factors with numerical data such as

altitude, percentage of dead trees in plot, percentage of trees with termites in the plot, stand maturity estimate, and lot average DS using Spearman rank tests(rs). Kruskal-Wallis ANOVA (H) was used to determine the group significance for factors with categorical data like location, DS, health, site management, Rs, altitude classification, parent material classification, plot DS ranking, plot health ranking, and plot average health (Table 1). The abundance-based distance metric weighted UniFrac was used to analyze beta-diversity between samples using Permutational multivariate analysis of variance (PERMANOVA). UniFrac estimates the phylogenetic distance between taxa present within a phylogenetic tree (Xia 2018). Weighted UniFrac distance was chosen because unlike unweighted UniFrac, which only evaluates the presence of a species, weighted UniFrac scales branch length based on the abundance of each species, giving more weight to more abundant lineages (Xia 2018). PERMANOVA is a non-parametric multivariate statistical test. It calculates the distance between two groups by comparing F value of data obtained after random permutation to the original F value (Anderson 2001). Principle coordinates analysis (PCoA) plots for each of the beta diversity metrics were generated using Emperor. A multifactorial PERMANOVA test known as Adonis was used in q2-diversity plugin to calculate the test statistic, significant differences and interactions. The Adonis test compares distances between the centroids of different groups with respect to the overall centroid. It calculates F-ratio and significant differences based on the framework of ANOVA and permutations of the observations. The Adonis test assumes that factors are spread homogeneously in multivariate space, i.e., the centroids of groups are equidistant from each other. To ensure that the assumption of Adonis is met, PERMDISP was performed using q2-diversity plugin. The results of Adonis test were visualized as non-metric Multi Dimensional Scaling (NMDS) plots generated using the metaMDS function in R package vegan, ggplot2 and ggordiplot (Oksanen et al. 2018) an ordination based on a distance or dissimilarity matrix.

Differential abundances in taxa responsible for differences among the groups present in tree-, plot- and location-factors were determined using the linear discriminant analysis (LDA) effect size (LEfSe) in Galaxy online workflow application (https://huttenhower.sph.harvard.edu/galaxy) (Segata et al. 2011). LEfSe performs the non-parametric factorial Kruskal-Wallis (KW) sum-rank test to identify differentially abundant ASVs between the groups, which is followed by Wilcoxon rank-sum test to access biological consistency and LDA to estimate the effect size of ASVs having significant differential abundances (Segata et al. 2011, Obanda et al. 2021). Alpha value of 0.05 was used for the factorial Kruskal-Wallis test among classes and pairwise Wilcoxon test between subclasses. Threshold on the logarithmic LDA score for discriminative features was adjusted at 3.

Pilot study for feeding experiments:

A pilot study was conducted at LSU to optimize the experimental design for feeding experiments to be conducted in the field in Guam. These preliminary experiments were performed to determine the concentration range of the bacterial solution that workers readily ate with no adverse impact on the termites.

100 ml of Casamino acid-Peptone-Glucose (CPG) broth medium was inoculated with *R. solanacearum* strain GMI1000 obtained from American Type Culture Collection (ATCC).

Ralstonia was cultured overnight (18-20 h) in a shaker-incubator at 220 rpm and 28 °C (Kelman 1954). Glycerol stocks were made for future feeding experiments by adding 500 µl of overnight culture to 500 µl of sterile glycerol (20%) in a 1.5 ml eppendorf tube followed by gentle vortexing.

Dilution series of the overnight cell culture was performed to measure optical density (OD600) in a spectrophotometer and count colony-forming units (CFUs) on agar plates. Ten sterile test tubes were taken. These tubes were labeled as 10^{-1} through 10^{-10}. Nine ml of saline (0.85%) water was added to each test tube with a sterile pipette. To the 1st test tube, 1ml of the bacterial overnight solution was added. Afterwards, 1ml solution was drawn from 1st test tube and was added into the 2nd. This was continued till the last test tube. An aliquot of 20 µl from each test tube was spread on CPG plus plates with 1.8% agar and 0.05% 2,3,5-triphenyltetrazolium chloride. After the solution was soaked into the agar, plates were kept for incubation at 28°C for 48 hours. After incubation was completed, the number of colonies on each plate was counted and CFU/ml was calculated.

Coptotermes formosanus termites collected from New Orleans, Louisiana, were used in the pilot study. Broth prepared from each of the ten dilution, along with a negative control (0.85% Saline without bacteria) was inoculated on different filter papers to observe the feeding behavior and survival of termites on different dilutions. Each dilution was replicated four times. To calculate the net consumption by termites during the feeding experiment; the dry weight of the filter papers was measured before and after termites feed on them. Dead individuals were being counted and removed from the Petri dishes daily. Fifty worker and 5 soldier termites of *C. formosanus* were placed in Petri dishes and were allowed to feed for two different time periods (two days and ten days) on filter papers inoculated with 10^{-2}, 10^{-4} and 10^{-6} bacterial dilutions along with negative control. Each treatment consisted of four replicates.

Experiments performed at University of Guam:

Feeding experiments were conducted by a LSU Graduate Student in the field on the island of Guam.

1.) Four-way choice test:

Three colonies of *N. takasagoensis* were collected from Bernard Watson's farm (13.56702, 144.87746), Mangilao Golf Couse (13.47111,144.8452) and UOG Yigo Station (13.53308, 144.87222). Parts of *N. takasagoensis* nests were transported to the laboratory and were dissected to extract the termites.

Four treatments were used:

1. Wood without *Ralstonia solanacearum* and wetwood bacteria: The wood pieces were collected from the stem of a *Ralstonia solanacearum* (Rs) negative tree that did not have any wet wood. The level of Ralstonia was detected by the Agdia test and level of wetwood was visually determined.
2. Wood with low *Ralstonia solanacearum* and low wetwood bacteria: The wood pieces were collected from a tree with low Rs having low wetwood.

3. Wood with low *Ralstonia solanacearum* and higher levels of wetwood bacteria: The wood pieces were collected from a tree with low Rs having higher levels of wetwood.
4. Wood with high *Ralstonia solanacearum* with higher levels of wetwood bacteria: The wood pieces were collected from a tree with high Rs having higher levels of wetwood.

Petri dishes (145*20mm) filled with sand at 12% moisture level were used for bioassays. Wood pieces (four treatments described above) were weighed. This was recorded as initial weight. Each of the four wood pieces were placed in a Petri plate so that they were equally distanced from each other. The experiment was designed following a Randomized Complete Block Design using 15 experimental units with five replicates from each of the three colonies.

300 workers and 60 soldiers of *Nasutitermes takasagoensis* (Thorne 1984) were released into Petri dishes. Experimental units were maintained in dark at 26 ± 2 degrees Celsius. The dead termites were removed, and their numbers were recorded every day. After three weeks, the final weight of all four wood pieces was measured to determine variation in weight before and after the bioassay.

Statistical analysis was conducted using SAS Software (SAS 9.4). The difference between mean weights of wood consumed was compared using one-way analysis of variance (ANOVA). It was followed by Tukey's Honestly-Significant Difference post-hoc test. The significance level was determined at $\alpha < 0.05$.

2.) Two-way choice test using Ralstonia overnight culture –soaked wood as treatment:

Water suspension of *Ralstonia pseudosolanacearum* bacterial culture 19-147 (from Sujan et al. 2020) was streaked on CPG plates and was incubated for 48 hours at 28 degrees Celsius (Denny 2001). Well-isolated single fluidal colonies were restreaked on several CPG plates to obtain pure cultures. Single loopfuls of bacterial growth from each CPG plate were transferred to 2 ml of sterile water in 5 ml vial. Fifteen such vials were prepared, and a cloudy suspension of bacteria was observed in water.

Overnight cultures (18-20 h) were prepared from water suspensions of Ralstonia in a shaker-incubator at 220 rpm and 28 °C. Optical density (OD600) and counts of colony-forming units (CFU/ml) was determined using dilution series of the overnight cell culture. 10-4, 10-6, and 10-8 dilutions were used for the experiments.

Three colonies of *N. takasagoensis* were collected from Bernard Watson's farm (13.56702, 144.87746), Mangilao Golf Couse (13.47111,144.8452), and UOG Yigo Station (13.53308, 144.87222). Parts of *N. takasagoensis* nests were transported to the laboratory and were dissected to extract the termites.

Wooden pieces were taken from a healthy ironwood tree, free of Ralstonia and wetwood bacteria, i.e., the pathogens associated with ironwood tree decline. The initial weight of wood pieces was recorded after drying them for 2 days at 100 degrees Celsius in a drying oven. These wood pieces were inoculated with:

- Only 0.85% saline having no Ralstonia (control)
- Different dilutions of overnight culture (10^{-4}, 10^{-6} and 10^{-8}) of *R. solanacearum*

Both treatments were kept in each Petri dish (60*20mm) filled with sand at 12% moisture level. The experiment was designed following a Randomized Complete Block Design using 45 experimental units (Three colonies * three concentrations * five replicates).

100 workers and 20 soldiers of *N. takasagoensis* (Thorne 1984) were released into Petri dishes. Experimental units were maintained in dark at 26 ± 2 degrees Celsius. The dead termites were removed and numbers were recorded every day. After three weeks, wood pieces were dried and the final weight of wood pieces was recorded.

Statistical analysis was conducted using SAS Software (SAS 9.4, 2021.1.). The difference between mean weights of wood consumed was compared using one-way analysis of variance (ANOVA). It was followed by Tukey's Honestly-Significant Difference post-hoc test. The significance level was determined at $\alpha < 0.05$.

3.) No-choice tests to measure ingestion and survival of Ralstonia in termite guts:

Three colonies of *N. takasagoensis* were collected from wooden logs brought into lab from UOG Campus (13.43020,144.80008), UOG Yigo Station (13.53356, 144.87116) and UOG Yigo Station (13.53288,144.87163). The experiment was designed following a Randomized Complete Block Design with five replicates from each of the three colonies along with a control setup in different Petri dishes. Filter paper was soaked with 10^{-4}, 10^{-6} and 10^{-8} dilution of overnight culture medium (CPG) of *Ralstonia solanacearum*. Filter paper soaked with only 0.85% saline having no Ralstonia was used as negative control. Fifty workers and 5 soldiers per colony of *N. takasagoensis* (Thorne, 1984) were released into Petri dishes (60x20 mm) to feed on filter paper with the three bacterial dilutions. *N. takasagoensis* individuals were fed on bacterial dilutions for three time periods (2 days, 4 days, and 6 days). At the end of the designated feeding period, 8 workers were removed from each Petri dish for DNA extraction and the remainders were placed in a new set of clean Petri dishes containing filter paper soaked with saline but no Ralstonia (like the control). From the remaining workers, 8 workers were removed and stored in 95% ethanol at regular intervals of 2 days.

The dry weight of the filter papers was recorded before and after the experiment. After completion of the feeding experiment, all the workers were shipped to the LSU AgCenter in vials containing 95% ethanol. The DNA was extracted by crushing whole termite workers. This DNA was sent for 16S rRNA gene Illumina sequencing to the University of New Hampshire and the sequences were analyzed for the presence and abundance of Ralstonia using QIIME2.

D. Results:

Morphological identification:

The higher termite, *N. takasagoensis* was found in 42 out of 45 (93%) samples received from Guam (Table 2). The dominant presence of *N. takasagoensis* in ironwood trees was consistent with previous findings by Park et al. (2019), that identified termites based on morphological characters and confirmed their identification via DNA barcoding. Based upon Park et al.'s (2019) study, the remaining three samples of termites attacking iron wood trees are likely to be *C. gestroi*. Because, n=3 was not enough to perform any statistical analysis, the *C. gestroi* were not included in the study.

Table 2. Data collected from ironwood tree plots by the University of Guam.

ID	Termite ID	Location	Site Management	Altitude	Altitude Classification	Parent Material Classification	Tree DS	Tree Health	Rs
19-77	Nasutitermes takasagoensis	Watson's Farm, Yigo	moderately maintained	170	high	lime	heavily damaged	sick	(+)
19-78	Nasutitermes takasagoensis	Watson's Farm, Yigo	moderately maintained	164	high	lime	nearly dead	sick	(-)
19-79	Nasutitermes takasagoensis	Watson's Farm, Yigo	moderately maintained	171	high	lime	distinctly damaged	sick	(-)
19-80	Nasutitermes takasagoensis	Watson's Farm, Yigo	moderately maintained	161	high	lime	no symptom	Healthy	(-)
19-81	Nasutitermes takasagoensis	Watson's Farm, Yigo	moderately maintained	168	high	lime	slightly damaged	sick	(-)
19-82	Nasutitermes takasagoensis	UOG Ija Station	moderately maintained	96	low	tuff	heavily damaged	sick	(+)
19-83	Nasutitermes takasagoensis	UOG Ija Station	moderately maintained	92	low	tuff	nearly dead	sick	(-)
19-84	Nasutitermes takasagoensis	Mangilao Golf Course	highly maintained	129	high	lime	no symptom	Healthy	(-)
19-85	Nasutitermes takasagoensis	Mangilao Golf Course	highly maintained	127	high	lime	nearly dead	sick	(+)
19-86	Nasutitermes takasagoensis	Windward Golf Course	highly maintained	117	high	tuff	nearly dead	sick	(-)
19-87	Nasutitermes takasagoensis	Windward Golf Course	highly maintained	126	high	tuff	slightly damaged	sick	(+)
19-88	Nasutitermes takasagoensis	Ritidian	moderately maintained	12	low	sand	slightly damaged	sick	(-)
19-89	Nasutitermes takasagoensis	Ritidian	moderately maintained	21	low	sand	no symptom	Healthy	(-)
19-90	Nasutitermes takasagoensis	Cocos Island	no maintainance	20	low	sand	distinctly damaged	sick	(-)
19-91	Nasutitermes takasagoensis	Cocos Island	no maintainance	12	low	sand	slightly damaged	sick	(-)
19-92	Nasutitermes takasagoensis	Mangilao Golf Course	moderately maintained	124	high	lime	slightly damaged	sick	(+)
19-93	Nasutitermes takasagoensis	Thousand Steps	no maintainance	17	low	lime	no symptom	Healthy	(-)
19-94	Nasutitermes takasagoensis	Ysrael Beach	no maintainance	5	low	sand	nearly dead	sick	(-)
19-95	Nasutitermes takasagoensis	Ysrael Beach	no maintainance	7	low	sand	slightly damaged	sick	(-)
19-96	Nasutitermes takasagoensis	Sagan KotturanChamoru	no maintainance	45	low	lime	slightly damaged	sick	(-)
19-98	Nasutitermes takasagoensis	AAFB	highly maintained	164	high	lime	heavily damaged	sick	(-)
19-99	Nasutitermes takasagoensis	AAFB	moderately maintained	160	high	lime	slightly damaged	sick	(-)
19-100	Nasutitermes takasagoensis	Duenas Beach	moderately maintained	11	low	sand	no symptom	Healthy	(-)
19-101	Nasutitermes takasagoensis	Duenas Beach	moderately maintained	12	low	sand	slightly damaged	sick	(-)
19-102	Nasutitermes takasagoensis	Tarague Beach	highly maintained	21	low	sand	no symptom	Healthy	(-)
19-103	Nasutitermes takasagoensis	Watson's Farm, Yigo	no maintainance	168	high	lime	slightly damaged	sick	(-)
19-104	Nasutitermes takasagoensis	Watson's Farm, Yigo	no maintainance	169	high	lime	no symptom	Healthy	(-)
19-105	Nasutitermes takasagoensis	Watson's Farm, Yigo	no maintainance	173	high	lime	slightly damaged	sick	(-)
19-106	Nasutitermes takasagoensis	Watson's Farm, Yigo	no maintainance	163	high	lime	slightly damaged	sick	(-)
19-107	Nasutitermes takasagoensis	Watson's Farm, Yigo	no maintainance	171	high	lime	no symptom	Healthy	(-)
19-108	Nasutitermes takasagoensis	UOG Yigo Station	moderately maintained	178	high	lime	no symptom	Healthy	(-)
19-109	Nasutitermes takasagoensis	UOG Yigo Station	moderately maintained	142	high	lime	slightly damaged	sick	(-)
20-123	Nasutitermes takasagoensis	Watson's Farm, Yigo	moderately maintained	163	high	lime	heavily damaged	sick	(+)
20-125	Nasutitermes takasagoensis	UOG, Mangilao	no maintainance	67	low	lime	nearly dead	sick	(+)
20-126	Nasutitermes takasagoensis	UOG, Mangilao	highly maintained	81	low	lime	heavily damaged	sick	(+)
20-129	Nasutitermes takasagoensis	Mangilao Golf Course	highly maintained	129	high	lime	slightly damaged	sick	(+)
20-130	Nasutitermes takasagoensis	UOG Yigo Station	moderately maintained	173	high	lime	nearly dead	sick	(+)
20-131	Nasutitermes takasagoensis	UOG Yigo Station	moderately maintained	173	high	lime	nearly dead	sick	(+)
20-132	Nasutitermes takasagoensis	UOG Ija Station	moderately maintained	96	low	tuff	nearly dead	sick	(+)
20-133	Nasutitermes takasagoensis	UOG Ija Station	moderately maintained	93	low	tuff	nearly dead	sick	(+)
20-134	Nasutitermes takasagoensis	UOG Ija Station	moderately maintained	82	high	tuff	nearly dead	sick	(+)
20-135	Nasutitermes takasagoensis	UOG Ija Station	moderately maintained	110	high	tuff	nearly dead	sick	(+)

ID	Termite ID	Proportion of dead trees in plot	Proportion of trees with termites in plot	Stand Maturity Estimate	Plot Average DS	Plot DS Ranking	Plot Average Health	Plot Health Average
19-77	Nasutitermes takasagoensis	0.5333	0.3333	0.53	2	distinct-damage	0.42	sick
19-78	Nasutitermes takasagoensis	0.0303	0.3939	0.03	0.47	no-symptoms	0.03	Healthy
19-79	Nasutitermes takasagoensis	0	0.5	0	1.5	distinct-damage	0.5	sick
19-80	Nasutitermes takasagoensis	0.0286	0.6571	0.03	0.91	slight-damage	0.09	sick
19-81	Nasutitermes takasagoensis	0	0.6176	0	1.29	slight-damage	0.29	sick
19-82	Nasutitermes takasagoensis	0.3913	0.4783	0.2	3	heavy-damage	1	sick
19-83	Nasutitermes takasagoensis	0.2632	0.6316	0.13	3.36	heavy-damage	1	sick
19-84	Nasutitermes takasagoensis	0.0294	0.5588	0.03	0.88	slight-damage	0.12	sick
19-85	Nasutitermes takasagoensis	0	0.4	0	1.98	distinct-damage	0.55	sick
19-86	Nasutitermes takasagoensis	0	1	0	4	nearly-dead	1	sick
19-87	Nasutitermes takasagoensis	0	1	0	1	slight-damage	0	sick
19-88	Nasutitermes takasagoensis	0	1	0	0.9	slight-damage	0.3	sick
19-89	Nasutitermes takasagoensis	0.0652	0.6304	0.02	1.07	slight-damage	0.26	sick
19-90	Nasutitermes takasagoensis	0.0761	0.2174	0.03	1.08	slight-damage	0.29	sick
19-91	Nasutitermes takasagoensis	0.0756	0.2558	0.03	0.86	slight-damage	0.14	sick
19-92	Nasutitermes takasagoensis	0.1143	0.6	0.11	2.1	distinct-damage	0.68	sick
19-93	Nasutitermes takasagoensis	0.0612	0.449	0.06	0.83	slight-damage	0.13	sick
19-94	Nasutitermes takasagoensis	0.0759	0.1646	0.03	0.33	no-symptoms	0.03	Healthy
19-95	Nasutitermes takasagoensis	0.0909	0.1697	0.03	0.59	slight-damage	0.12	sick
19-96	Nasutitermes takasagoensis	0.0857	0.5143	0.09	0.88	slight-damage	0.16	sick
19-98	Nasutitermes takasagoensis	0	0.6	0	0.8	slight-damage	0.2	sick
19-99	Nasutitermes takasagoensis	0.1429	0.7143	0.14	3	slight-damage	1	sick
19-100	Nasutitermes takasagoensis	0.1	0.6	0.03	1.06	slight-damage	0.33	sick
19-101	Nasutitermes takasagoensis	0.1294	0.3882	0.04	0.84	slight-damage	0.2	sick
19-102	Nasutitermes takasagoensis	0.0811	0.2162	0.03	0.82	slight-damage	0.21	sick
19-103	Nasutitermes takasagoensis	0.2571	0.2857	0.26	1.54	slight-damage	0.38	sick
19-104	Nasutitermes takasagoensis	0.1613	0.1935	0.16	0.92	slight-damage	0.04	sick
19-105	Nasutitermes takasagoensis	0	0.3333	0	0.92	slight-damage	0.14	sick
19-106	Nasutitermes takasagoensis	0.0278	0.3611	0.03	1.06	slight-damage	0.2	sick
19-107	Nasutitermes takasagoensis	0.0833	0.0833	0.08	1.21	slight-damage	0.55	sick
19-108	Nasutitermes takasagoensis	0.1667	0.5667	0.17	0.44	slight-damage	0.04	sick
19-109	Nasutitermes takasagoensis	0	0.5656	0	0.67	slight-damage	0.11	sick
20-123	Nasutitermes takasagoensis	0.381	0.2381	0.38	1.62	slight-damage	0.31	sick
20-125	Nasutitermes takasagoensis	0.1333	0.2667	0.13	2	slight-damage	0.77	sick
20-126	Nasutitermes takasagoensis	0	1	0	4	slight-damage	1	sick
20-129	Nasutitermes takasagoensis	0.0976	0.6585	0.1	1.65	slight-damage	0.51	sick
20-130	Nasutitermes takasagoensis	0.0909	0.3636	0.09	2.2	slight-damage	0.7	sick
20-131	Nasutitermes takasagoensis	0.0909	0.3636	0.09	2.2	slight-damage	0.7	sick
20-132	Nasutitermes takasagoensis	0.2857	0.619	0.14	3.4	slight-damage	1	sick
20-133	Nasutitermes takasagoensis	0.375	0.5417	0.19	3.07	slight-damage	1	sick
20-134	Nasutitermes takasagoensis	0.6087	0.2609	0.3	3	slight-damage	0.89	sick
20-135	Nasutitermes takasagoensis	0.5833	0.2083	0.29	2.9	slight-damage	1	sick

Evaluation of next-generation sequencing data:

A total of 11,106,360 sequences were recovered and the ASV table obtained after DADA2 quality filtering and chimera removal contained 9,902,718 sequence reads. A mean number of 119,309 reads per sample and 767 reads per ASV were observed. Further removal of the unassigned taxa resulted in 1,709,419reads, a mean number of 40,700 reads per sample and 933 reads per ASV. The contingency-based filter confirmed that each ASV was present in more than two samples.

Sequencing-depth based alpha rarefaction curves:

The alpha rarefaction curves for observed number of ASVs (richness), Faith's phylogenetic distance (Faith's PD), and Shannon diversity indices were plotted against sequencing depth for all samples. Faith's PD represents the sum of all branch lengths of the phylogenetic tree based on ASVs in each sample. Faith's PD is confounded by ASV richness, but also takes phylogenetic relationship into account. The Shannon index is a measure of the richness which scales the samples based on the evenness of the community. Most alpha rarefaction curves based on ASV richness and Faith's PD indices started to level out after a sequencing depth of 10,000 to 15,000 was reached and the curve based on Shannon diversity started leveling out at less than 5000 sequencing depth. This indicates that sufficient sequencing depth was achieved to capture most of the taxa and diversity present in the community and collection of more sequences beyond that depth of sampling is unlikely to result in the discovery of any new ASVs or increased diversity.

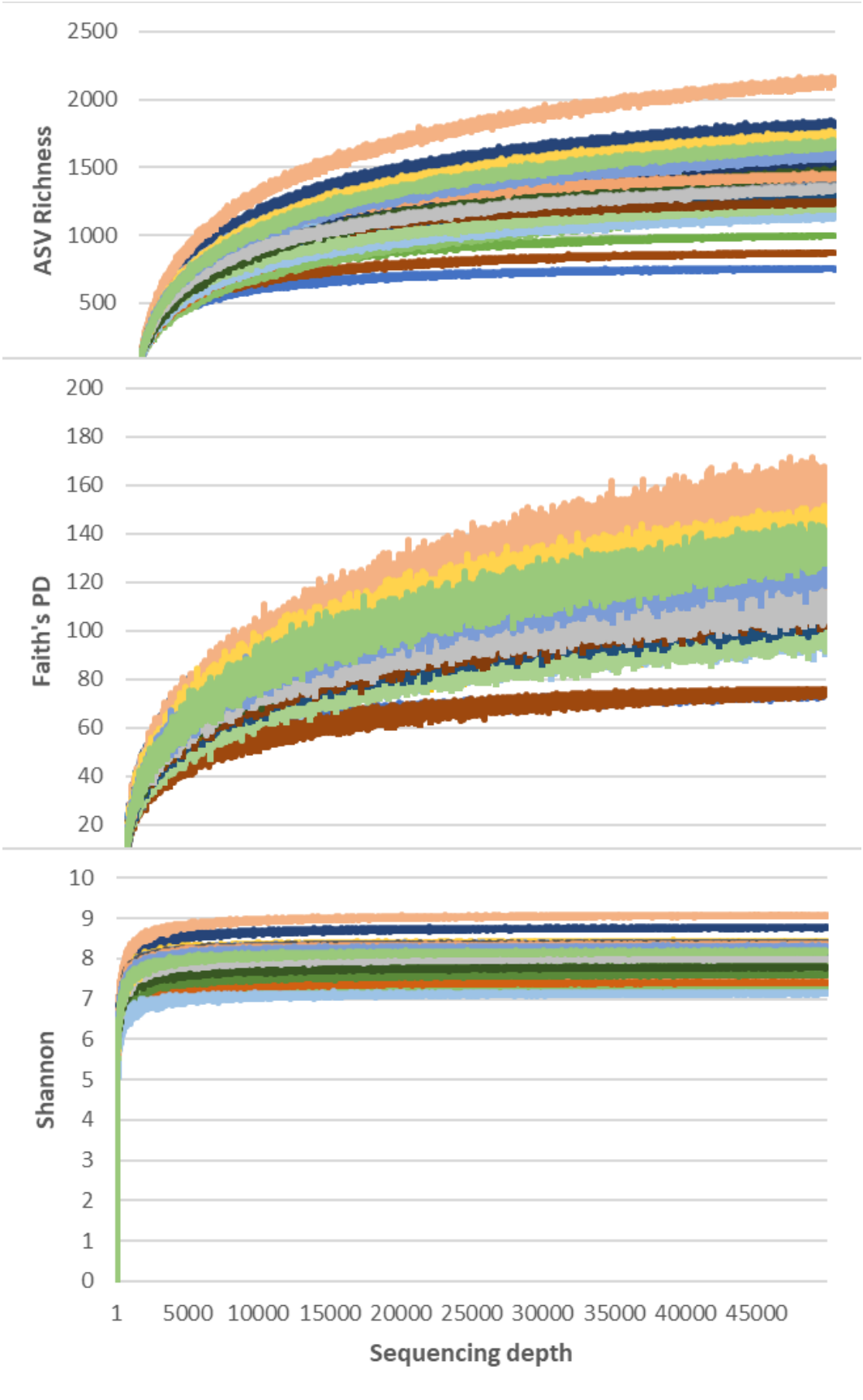
ASV Richness
2500
2000
1500
1000
500
Faith's PD
200
180
160
140
120
100
80
60
40
20
Shannon
10
9
8
7
6
5
4
3
2
1
0
1
5000
10000
15000
20000
25000
30000
35000
40000
45000
Sequencing depth

Fig 1. Alpha rarefaction curves of bacteria diversity showing the number of ASVs, Faith's phylogenetic distance and Shannon diversity indices in 43 samples of *Nasutitermes takasagoesis* workers plotted against sequencing depth.

Sample-size-based rarefaction curves:

Sample-size-based rarefaction curves depict effective bacterial diversity in relation to the number of samples collected by measuring their ASV richness, Shannon diversity and Simpson inverse indices. The input data was in the form of incidence frequency of ASVs within 42 samples. The solid lines of the plots are the interpolated portion based on the actual sample size of 42, and the dotted lines are based on an extrapolation of the effective diversity if the number of samples was doubled. The actual sample size captured the majority of effective bacteria diversity since the interpolated portions of curves for Shannon and Simpson inverse indices levelled off at an effective diversity of 150 and 100, respectively, and extrapolation did not increase the captured diversity. The curve for ASV richness started to level off but did not reach an asymptote at 42 samples and doubling the sample size would have increased the effective diversity by 200 (Fig. 2). However, added richness would largely be based on rare ASVs since the abundance (Shannon diversity), and dominance of ASVs (Simpson inverse) did not increase with added richness.

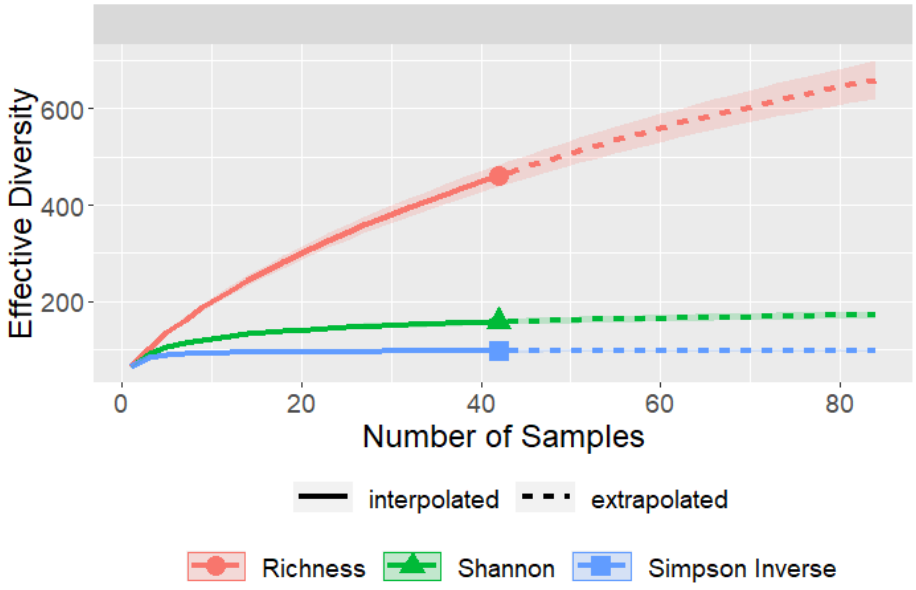

Fig 2: Sample-based rarefaction curves for the full dataset with effective bacterial diversity for different metrics plotted against the number of samples

Coverage-based rarefaction curves:

The coverage-based rarefaction curves depict effective diversity with respect to sample completeness. The interpolated portions of the coverage-based rarefaction curves reach over 90% sample coverage at an effective richness of 450, Shannon diversity of 150 and Simpson inverse of 100 (Fig. 3). The extrapolated portion of the curves extends the coverage to almost 95% which increases the richness to more than 600 but the Shannon diversity and Simpson inverse only increase incrementally (Fig. 3). In concordance with sample-based rarefaction (Fig. 2), the increase in richness upon extrapolation depicts that number of captured ASVs can be increased by doubling the sample size. However, added ASV richness by doubling the number of samples is likely based on rare ASVs since there is little impact on sample completeness measured by Shannon diversity and Simpson inverse.

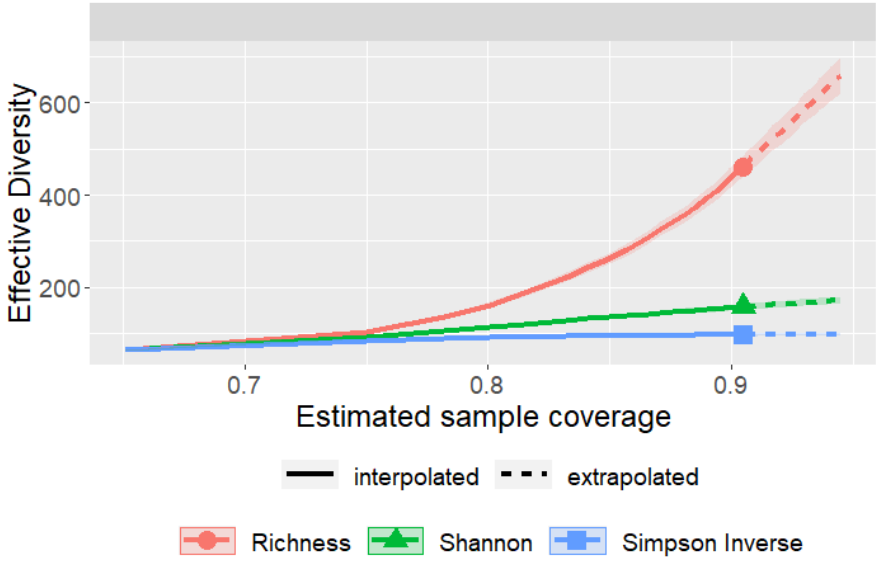

Fig. 3: Coverage-based rarefaction curves for the entire dataset with effective diversity plotted against estimated sample coverage.

Taxa Composition:

At taxonomic level two in SILVA (Phylum level), Spirochaetes (48.22 %) and Fibrobacteres (41.43 %) were found to be the most dominant phyla followed by Bacteroidetes (3.61%), Proteobacteria (3.35%), Margulisbacteria (0.84%), Acidobacteria (0.77%), Planctomycetes (0.65%), and others (1.61%) (Fig. 4).

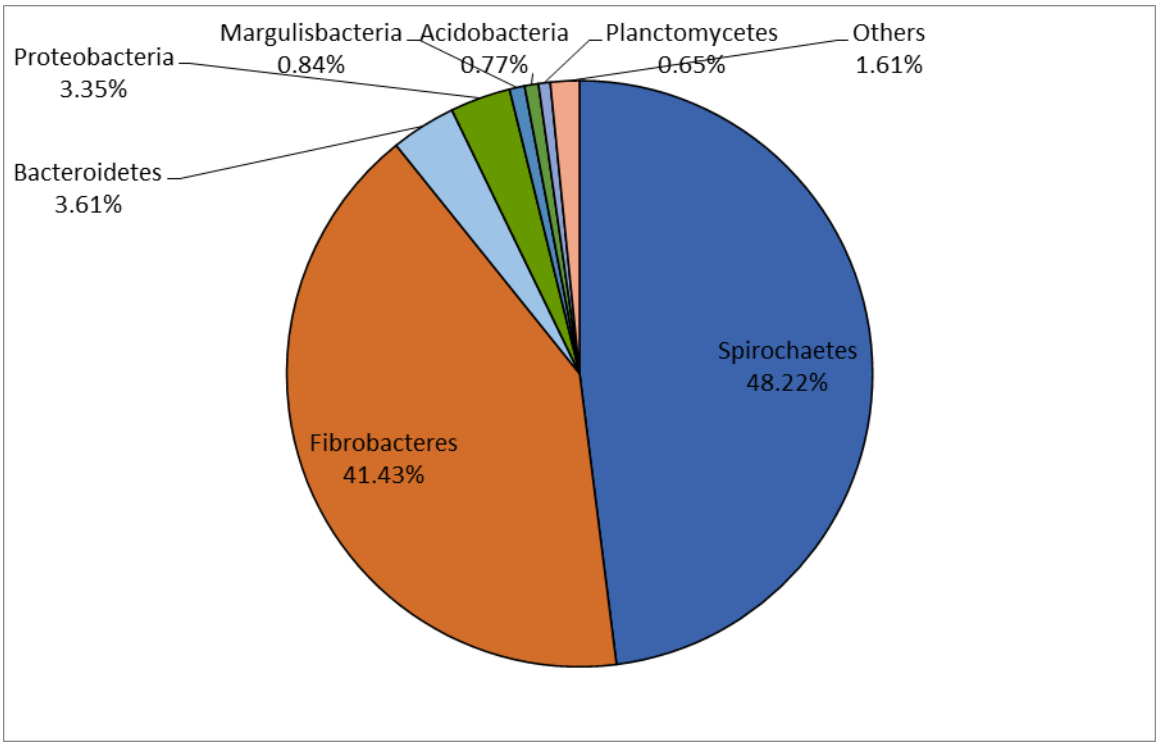

Fig. 4: Relative abundance of bacterial phyla (SILVA level 2) in *N. takasagoensis* workers based on the full dataset across all samples

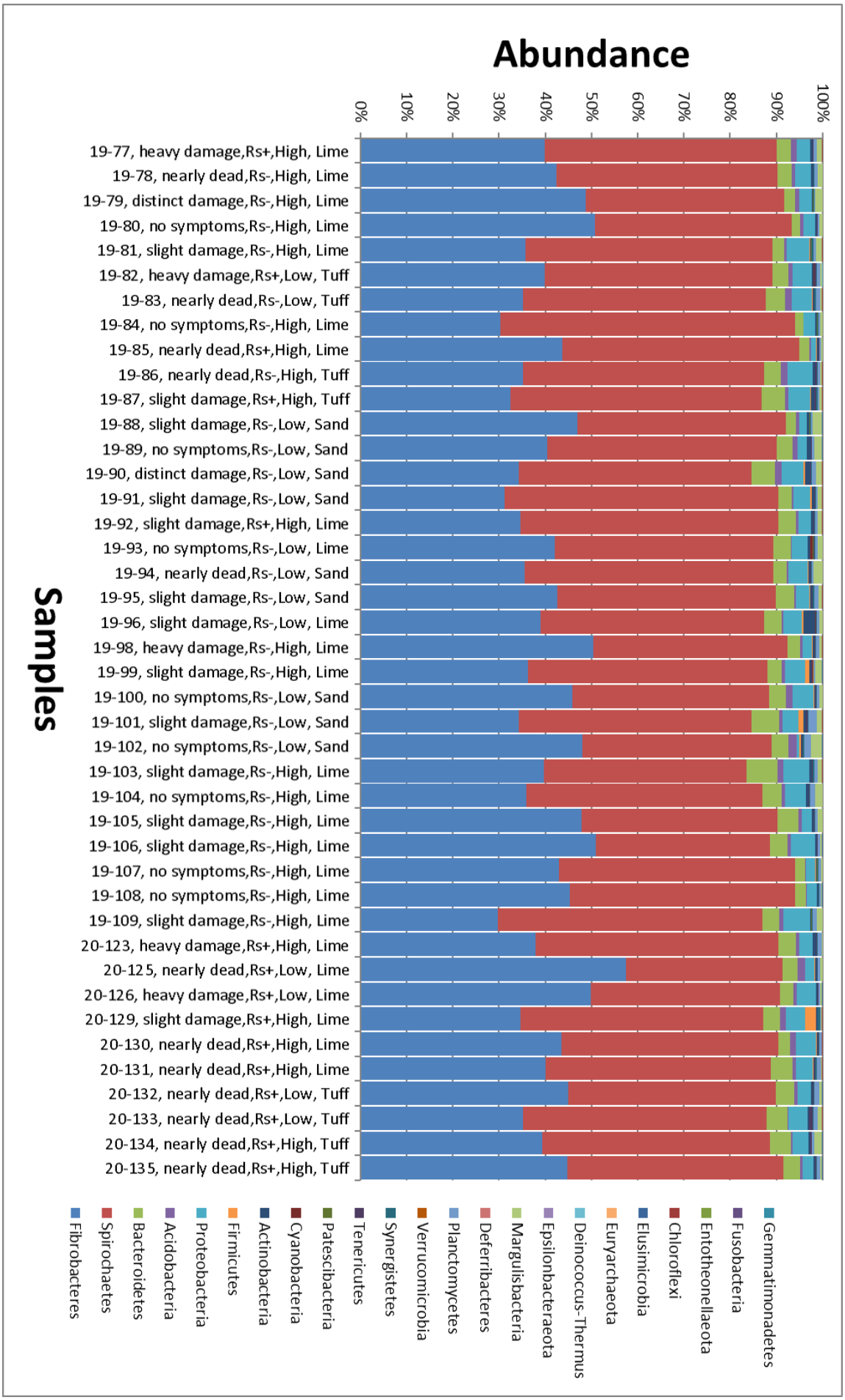
Abundance
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Samples
19-77, heavy damage,Rs+,High, Lime
19-78, nearly dead,Rs-,High, Lime
19-79, distinct damage,Rs-,High, Lime
19-80, no symptoms,Rs-,High, Lime
19-81, slight damage,Rs-,High, Lime
19-82, heavy damage,Rs+,Low, Tuff
19-83, nearly dead,Rs-,Low, Tuff
19-84, no symptoms,Rs-,High, Lime
19-85, nearly dead,Rs+,High, Lime
19-86, nearly dead,Rs-,High, Tuff
19-87, slight damage,Rs+,High, Tuff
19-88, slight damage,Rs-,Low, Sand
19-89, no symptoms,Rs-,Low, Sand
19-90, distinct damage,Rs-,Low, Sand
19-91, slight damage,Rs-,Low, Sand
19-92, slight damage,Rs+,High, Lime
19-93, no symptoms,Rs-,Low, Lime
19-94, nearly dead,Rs-,Low, Sand
19-95, slight damage,Rs-,Low, Sand
19-96, slight damage,Rs-,Low, Lime
19-98, heavy damage,Rs-,High, Lime
19-99, slight damage,Rs-,High, Lime
19-100, no symptoms,Rs-,Low, Sand
19-101, slight damage,Rs-,Low, Sand
19-102, no symptoms,Rs-,Low, Sand
19-103, slight damage,Rs-,High, Lime
19-104, no symptoms,Rs-,High, Lime
19-105, slight damage,Rs-,High, Lime
19-106, slight damage,Rs-,High, Lime
19-107, no symptoms,Rs-,High, Lime
19-108, no symptoms,Rs-,High, Lime
19-109, slight damage,Rs-,High, Lime
20-123, heavy damage,Rs+,High, Lime
20-125, nearly dead,Rs+,Low, Lime
20-126, heavy damage,Rs+,Low, Lime
20-129, slight damage,Rs+,High, Lime
20-130, nearly dead,Rs+,High, Lime
20-131, nearly dead,Rs+,High, Lime
20-132, nearly dead,Rs+,Low, Tuff
20-133, nearly dead,Rs+,Low, Tuff
20-134, nearly dead,Rs+,High, Tuff
20-135, nearly dead,Rs+,High, Tuff
Fibrobacteres
Spirochaetes
Bacteroidetes
Acidobacteria
Proteobacteria
Firmicutes
Actinobacteria
Cyanobacteria
Patescibacteria
Tenericutes
Synergistetes
Verrucomicrobia
Planctomycetes
Deferribacteres
Margulisbacteria
Epsilonbacteraeota
Deinococcus-Thermus
Euryarchaeota
Elusimicrobia
Chloroflexi
Entotheonellaeota
Fusobacteria
Gemmatimonadetes

Fig. 5: Relative abundance of bacterial phyla associated with *N. takasagoensis* workers collected from ironwood trees.

Table 3: Incidence (number of reads) of 40 most abundant ASVs associated with *N. takasagoensis* samples

Phylum	Lowest SILVA assignment	No symptoms	Slight damage	Distinct damage	Heavy damage	Nearly dead	Sick	Healthy	Moderate	High	None	Positive	Negative	High	Low	Lime	Tuff	Sand	No Symptoms Plot	Slightly Damaged Plot	Distinctly damaged Plot	Heavily Damaged Plot	Nearly Dead Plot	Healthy Plot	Sick Plot
Spirochaetes	uncultured Treponema sp.	88415	147055	20265	51443	162050	253050	216178	213629	116592	139007	191731	277497	295313	173915	284295	91532	93401	39487	206481	128592	69194	25474	264587	204641
Fibrobacteres	uncultured Fibrobacteres bacterium	84885	126474	17799	70636	163068	267139	195723	210970	107164	144728	205469	257393	290424	172438	306131	82240	74491	33877	196441	139559	69910	23075	258962	203900
Spirochaetes	uncultured Treponema sp.	27505	44767	7915	21483	48602	79514	70758	72001	28564	49707	61588	88684	88670	61602	88043	30632	31597	10947	66021	43204	21402	8698	91722	58550
Fibrobacteres	uncultured Fibrobacteres bacterium	33129	37934	8369	16815	36487	62611	70123	66869	31884	33981	44146	88588	74642	58092	71749	25403	35582	6723	66061	30621	18121	11208	76100	56634
Spirochaetes	Termite Treponema cluster	33647	31098	5496	18635	40856	67580	62152	70830	31755	27147	51510	78222	80899	48833	74630	28006	27096	5555	58780	34342	23363	7692	72611	57121
Fibrobacteres	uncultured Chitinivibrionia bacterium	21626	21716	5751	6216	31550	43764	43095	40801	18754	27304	31368	55491	47328	39531	48261	16779	21819	4763	37759	27118	10948	6271	47252	39607
Spirochaetes	Treponema	12553	21833	3681	6970	22613	35651	31999	35796	13803	18051	30100	37550	41446	26204	38279	16168	13203	3200	28213	20356	11708	4173	36540	31110
Proteobacteria	uncultured delta proteobacterium	7332	15657	1989	5493	14514	23957	21028	21253	9963	13769	18305	26680	29623	15362	27256	10629	7100	2685	18634	12837	6731	4098	25640	19345
Margulisbacteria	uncultured candidate division ZB3 bacterium	3661	4416	1081	1264	3987	6433	7976	7065	2510	4834	3961	10448	7448	6961	7140	2376	4893	1283	7751	2697	2318	360	9951	4458
Bacteroidetes	Candidatus Symbiothrix	2572	4790	782	1714	4380	7226	7012	6357	3655	4226	5398	8840	7874	6364	7045	3465	3728	834	7180	3172	2227	825	8922	5316
Bacteroidetes	uncultured Bacteroidetes bacterium	2377	4949	508	1280	4992	7178	6928	6911	2039	5156	6561	7545	9194	4912	9183	2862	2061	573	5191	5438	2365	539	7792	6314
Fibrobacteres	uncultured Fibrobacterales bacterium	3465	2163	86	852	6245	7237	5574	4370	3863	4578	5374	7437	7764	5047	9220	1781	1810	1858	4931	3486	2218	318	6667	6144
Fibrobacteres	uncultured Chitinivibrionia bacterium	3397	3888	694	1311	3082	5388	6984	5877	2748	3747	3565	8807	6503	5869	7070	1826	3476	952	6905	2576	1527	412	8683	3689
Acidobacteria	Holophagaceae	2108	2903	652	1137	3439	5796	4443	4565	2774	2900	4126	6113	5357	4882	5625	1782	2832	370	4432	3407	1186	844	5618	4621
Planctomycetes	Pirellulaceae	2080	2972	484	1357	3155	5153	4895	5044	2066	2938	3645	6403	5410	4638	5316	2077	2655	524	5068	2345	1654	457	6324	3724
Proteobacteria	Rhodospirillales	1707	2525	578	523	3218	4328	4223	3640	1286	3625	2829	5722	3841	4710	4514	1622	2415	1009	4266	1287	1621	368	5276	3275
Bacteroidetes	COB P4-1 termite group	1466	2903	537	1033	2598	4582	3955	3819	1959	2759	3347	5190	4845	3692	4574	1812	2151	468	3895	2306	1373	495	5089	3448
Bacteroidetes	Paludibacteraceae	1479	2226	386	711	2446	3835	3413	3259	1468	2521	2829	4419	4130	3118	4025	1641	1582	372	3369	1720	1332	455	4162	3086
Bacteroidetes	Tannerellaceae	843	1654	279	636	1608	2611	2409	2563	1059	1398	2020	3000	2841	2179	2564	1356	1100	344	2275	1088	990	323	3052	1968
Spirochaetes	Spirochaetaceae	830	1099	80	352	976	1458	1879	1431	691	1215	1010	2327	2167	1170	1867	863	607	129	1756	557	737	158	2048	1289
Tenericutes	Acholeplasmataceae	456	794	156	269	1336	1803	1208	1556	614	841	1428	1583	1625	1386	1314	1067	630	182	1204	589	822	214	1504	1507
Actinobacteria	Coriobacteriales Incertae Sedis	472	960	155	352	914	1468	1385	1318	638	897	1210	1643	1778	1075	1643	617	593	173	1354	796	376	154	1779	1074
Acidobacteria	Holophagaceae	408	718	277	587	610	1521	1079	1372	532	696	836	1764	1859	741	1749	413	438	155	1081	973	141	250	1772	828
Bacteroidia	Rikenellaceae	581	788	91	193	943	1275	1321	1165	573	858	1025	1571	1559	1037	1559	556	481	140	1149	700	463	144	1454	1142
Synergistetes	Synergistaceae	487	966	87	290	735	1287	1278	1140	778	647	1073	1492	1590	975	1448	533	584	128	1154	786	352	145	1487	1078
Bacteroidetes	Candidatus Armantifilum	341	763	73	256	1021	1453	1001	1094	598	762	1254	1200	1341	1113	1132	809	513	169	956	520	602	207	1138	1316
Actinobacteria	Propionibacteriaceae	287	1182	153	161	408	756	1435	485	272	1434	523	1668	541	1650	1475	178	538	109	1531	381	84	86	1696	495
Proteobacteria	Burkholderiaceae	294	666	428	188	518	1203	891	1023	416	655	462	1632	923	1171	755	441	898	80	1281	284	257	192	1432	662
Bacteroidetes	Bacteroidales	275	822	148	141	678	985	1079	1033	277	754	856	1208	1177	887	1235	371	458	66	1010	650	298	40	1242	822
Bacteroidetes	uncultured Chlorobi bacterium	370	607	95	233	575	944	936	899	363	618	677	1203	1150	730	1155	338	387	118	845	548	286	83	1149	731
Actinobacteria	Coriobacteriales	388	514	148	263	506	938	881	728	427	664	667	1152	1181	638	1095	355	369	104	895	478	246	96	1193	626
Spirochaetes	Treponema	11	1470	17	80	67	164	1481	120	0	1525	147	1498	1555	90	1622	0	23	0	1391	254	0	0	1561	84
Bacteroidetes	Bacteroidales	217	617	55	184	560	831	802	858	219	556	792	841	1105	528	1035	327	271	76	588	672	266	31	985	648
Chloroflexi	Dehalococcoidia	150	363	40	138	353	566	478	496	292	256	471	573	622	422	541	282	221	49	471	279	164	81	597	447
Firmicutes	Lactococcus	76	689	75	18	139	236	761	752	54	191	126	871	312	685	292	158	547	24	684	33	256	0	724	273
Bacteroidetes	Candidatus Symbiothrix	182	283	29	162	258	449	465	482	207	225	366	548	590	324	641	122	151	73	466	248	89	38	606	308
Planctomycetes	Mastotermes darwiniensis	174	320	40	87	264	391	494	507	104	274	366	519	505	380	465	196	224	41	429	222	174	19	563	322
Spirochaetes	M2PT2-76 termite group	137	315	55	71	290	437	431	452	163	253	380	488	527	341	483	224	161	35	371	249	164	49	490	378
Firmicutes	Streptococcus pneumoniae	0	705	0	0	0	705	0	0	705	0	705	0	705	0	705	0	0	0	0	705	0	0	0	705
Proteobacteria	Anaplasmataceae	132	289	19	30	182	231	421	352	32	268	163	489	459	193	616	2	34	74	413	133	0	32	384	268

One of the aims of this project was to detect the presence and abundance of Ralstonia bacteria in the termite as it is one of the most important causal organism behind IWTD in Guam. However, taxonomic level seven (Genus level) analysis did not show any *Ralstonia* sp. regardless if termites were collected from healthy iron wood trees or trees with confirmed Ralstonia infection. Since Ralstonia is a common soil bacterium and pathogen in iron wood trees (Ayin et al. 2019), this result was surprising. *Klebsiella* sp. (phylum Proteobacteria) is also considered to be associated with IWTD in Guam (Ayin et al. 2019). This wetwood bacteria, *Klebsiella* sp. was found in samples 19-93, 19-94, and 19-101 albeit only in minor abundances. The lack of Ralstonia and the low presence and abundance of wetwood bacteria suggest that termites are not a vector for these pathogens of IWTD.

Alpha diversity:

Alpha diversity analysis was conducted to determine if bacterial diversity within termite samples is significantly related to tree disease or any underlying geological or environmental factors (Table 4). Along with the full data set, it was decided to also analyze a data set containing the phyla Spirochaetes and Fibrobacteres separately because a) taxonomic composition revealed that Spirochaetes and Fibrobacteres were dominant phyla (89.65% combined relative abundance) having a fairly equal distribution across all samples (Fig. 5) and b) it is likely that these phyla contain the majority of obligate symbionts (Brune 2014; Breznak 2002). Thus, further analysis was continued with three data sets:

1. Full data set: contains all ASVs with taxonomic assignment.
2. Only SF data set: contains only the major phyla Spirochaetes and Fibrobacteres.
3. Without SF data set: contains the minor phyla after excluding Spirochaetes and Fibrobacteres from full data set.

Factor	Full Dataset				Without SF Dataset				Only SF Dataset			
	ASV Richness	Faith's PD	Shannon	Evenness	ASV Richness	Faith's PD	Shannon	Evenness	ASV Richness	Faith's PD	Shannon	Evenness
a.) Location related												
Location												
Parent material												
Site management			S	S	M							
Altitude										S		
Altitude classification						M			M	S		
b.) Tree related												
Presence or absence of Ralstonia						M			S		S	
Decline Severity						M						
Health		S			S	S						
c.) Plot related												
Plot Average DS				S								
Plot Average Health				S				S				
Proportion of dead trees in plot			S			S						
Proportion of trees with termites in plot				S			M					
Stand Maturity Estimate						S						

Table 4: Alpha diversity of location-, tree- and plot-related factors in Full data set, Without SF (Spirochaetes and Fibrobacteres) dataset and Only SF data set

Out of all the factors (Table 1), location and parent material had no significant influence on alpha diversity in any of the three data sets (Full, Only SF and without SF) while the rest of the factors, i.e., site management, altitude classification, presence or absence of Ralstonia, tree DS, tree health, plot average DS, plot average health, stand maturity estimate, percentage of trees with termites, percentage of dead trees in plot in plot showed significant results in at least one of the datasets.

a.) Tree related factors:

1. Ralstonia: The presence or absence of Ralstonia in trees from which termites were collected showed no significant effect on the total bacteria community (Full dataset). However, Ralstonia presence had a significant influence on ASV richness and Shannon diversity of the Spirochaetes and Fibrobacteres (Only SF) community and marginal influence on environmental bacteria (Without SF). Spirochete and Fibrobacteres communities of termites (Only SF) from trees with Ralstonia infestation (n=15) showed significantly higher richness (ASV Richness, p=0.02, H=5.21, Kruskal-Wallis ANOVA) compared to those from trees without Ralstonia (n=27) (Fig. 6). Phylogenetic distance in the Only SF dataset was not significantly influenced by Ralstonia infestation and only marginally significant in the Without SF dataset (Faith's PD, p=0.08, H= 2.95, Kruskal-Wallis ANOVA). The presence of Ralstonia also was associated with a significant increase in Shannon diversity (p=0.03, H=4.35, Kruskal-Wallis ANOVA) of the Spirochaetes and Fibrobacteres community (Fig. 23), but not evenness (p=0.26, H=1.24, Kruskal-Wallis ANOVA).

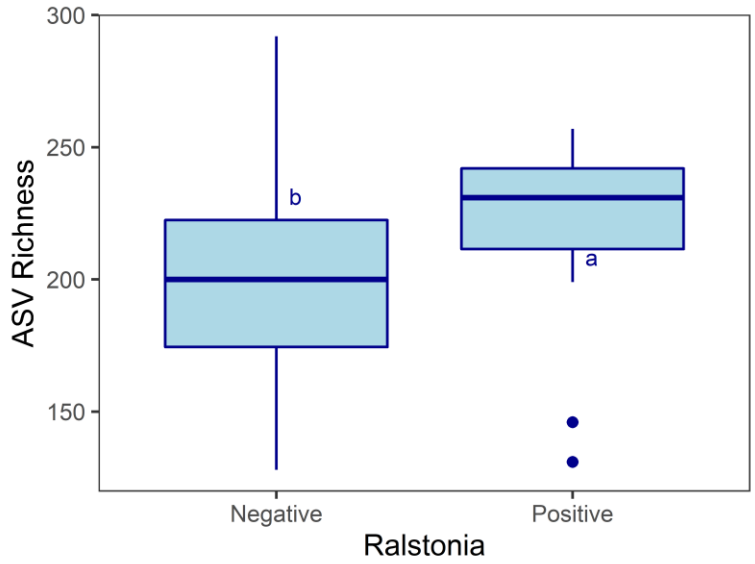

Fig. 6: ASV richness of Spirochete and Fibrobacteres communities of termites collected from trees showing presence or absence of Ralstonia (Only SF dataset). Different letters indicate significant difference.

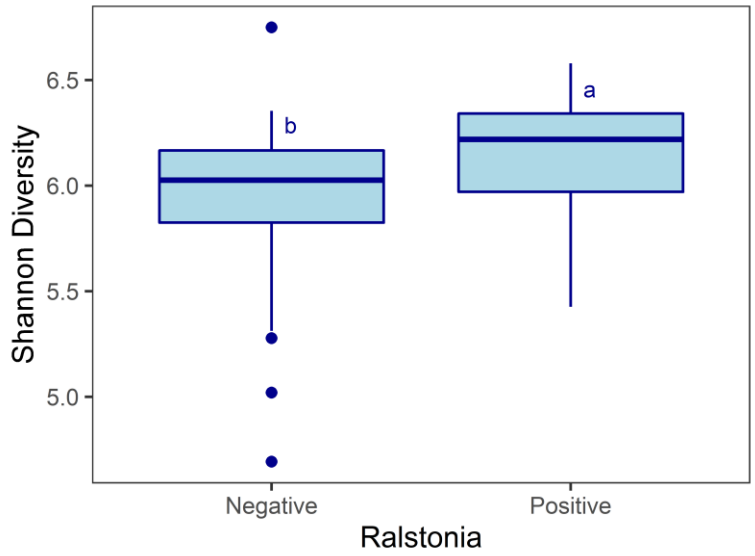

Fig. 7: Shannon diversity of Spirochete and Fibrobacteres communities of termites collected from trees showing presence or absence of Ralstonia (Only SF dataset). Different letters indicate significant difference.

2. Tree DS: Decline severity of the tree marginally influenced phylogenetic diversity (Faith's PD, p=0.067, H= 8.76, Kruskal-Wallis ANOVA) of the termites' bacteria community in the Without SF dataset. Pairwise tests revealed that the overall marginal

difference in phylogenetic distance was caused by significantly higher Faith's PD values (p=0.005, H= 7.68, Kruskal-Wallis ANOVA) among minor bacteria taxa present in termites collected from nearly dead (n=12) trees as compared to trees with no symptom (n=9). Other pairings (slight damage, distinct damage, high damage) did not show significant differences with respect to one another. Moreover, Pielou's evenness, ASVs richness and Shannon indices also showed no significant influence of the decline severity of trees on bacteria communities of termites attacking those trees in any of the three data sets (Full, Only SF and Without SF).

3. Tree health: When DS categories were reduced to sick (DS 1-4) vs healthy trees (DS 0), significant results were obtained within the Full data set and the Without SF data set. The bacterial communities of termites collected from sick trees (n=33) showed significantly higher richness (ASV Richness, p=0.008, H=5.21, Kruskal-Wallis ANOVA) compared to those from healthy trees (n=9) in the Without SF data set (Fig.8). The phylogenetic distances were also greater between the bacterial communities of termites collected from sick trees than those of healthy trees in both Without SF data set (Faith's PD, p=0.019, H= 5.49, Kruskal-Wallis ANOVA) (Fig. 9) and Full data set (Faith's PD, p=0.024, H= 5.07, Kruskal-Wallis ANOVA) (Fig. 10). There were no significant differences in Pielou's evenness and Shannon diversity between samples collected from healthy and sick trees in any of the data sets.

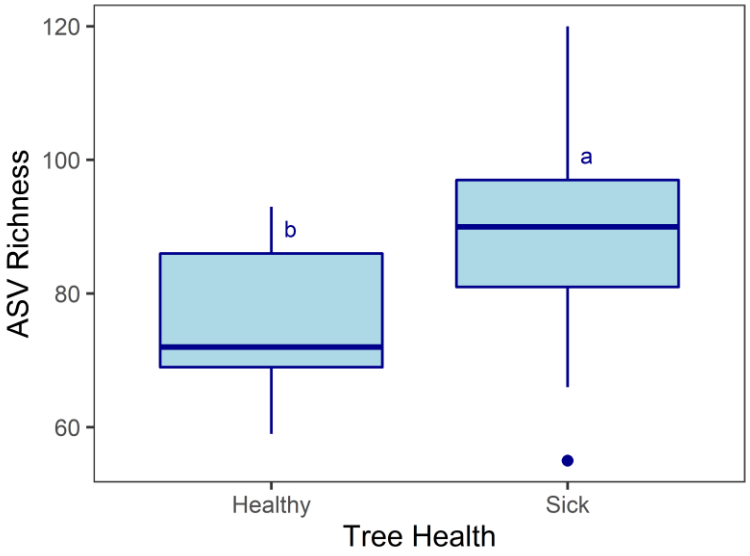

Fig. 8: ASV richness of bacteria communities of termites collected from healthy and sick trees (Without SF data set). Different letters indicate significant difference.

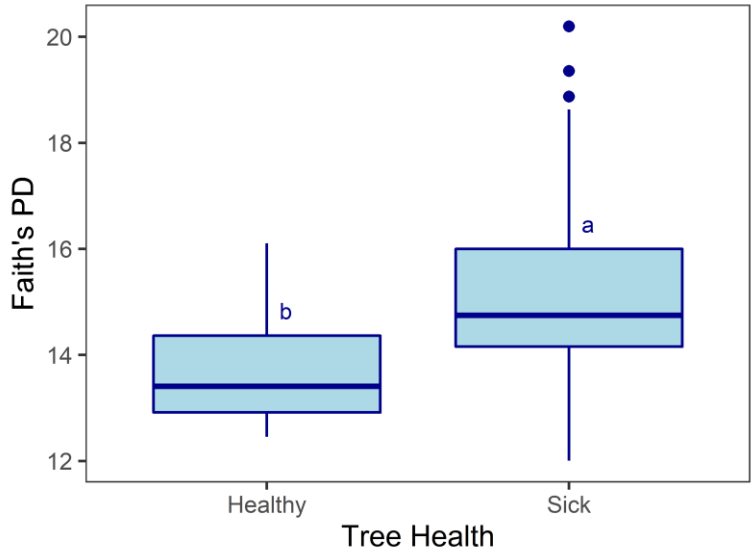

Fig. 9: Faith's PD of bacteria communities of termites collected from healthy and sick trees (Without SF data set). Different letters indicate significant difference.

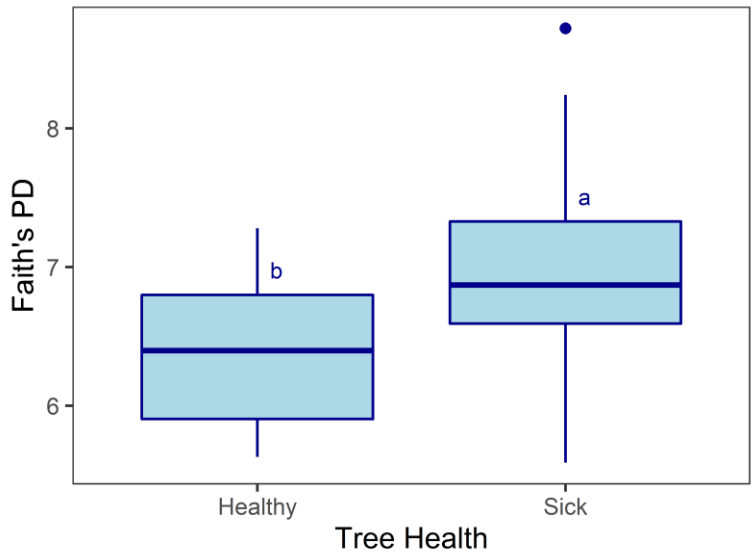

Fig. 10: Faith's PD of bacteria communities of termites collected from healthy and sick trees (Full data set). Different letters indicate significant difference.

b.) Plot related factors:

1. Plot Condition (plot Average DS, plot average health and proportion of dead trees in plot): The average decline severity (Plot Average DS) of the plots influenced some aspects of the bacterial diversity in the termites. The bacterial composition within termite samples (Full data set) was more even (Pielou's evenness p=0.008, r_s= -0.3094, Spearman's Rank test) in less damaged tree plots.

   When Plot Average Health (percentage of sick trees with DS 1-4) was considered, the same pattern was observed. Significant results were obtained for increasing evenness with decreasing percentage of sick trees in the Full data set (Pielou's evenness, p=0.014, r_s = -0.37, Spearman's Rank test) and Without SF data set (Pielou's evenness, p=0.04, r_s = -0.31, Spearman's Rank test). No significant differences for plot average DS and plot average health were obtained in ASV richness, Faith's PD and Shannon diversity in any data set.

   When plot condition was measured as the percentage of dead trees in plots, phylogenetic distance among the environmental bacterial communities (Without SF data set) within termite samples rose significantly with higher proportions of dead trees in the plot (Faith's PD, p=0.019, r_s=0.549, Spearman's Rank test). Pielou's evenness, ASV richness and Shannon diversity did not show significance for percentage of dead trees in plots in any of the data set.

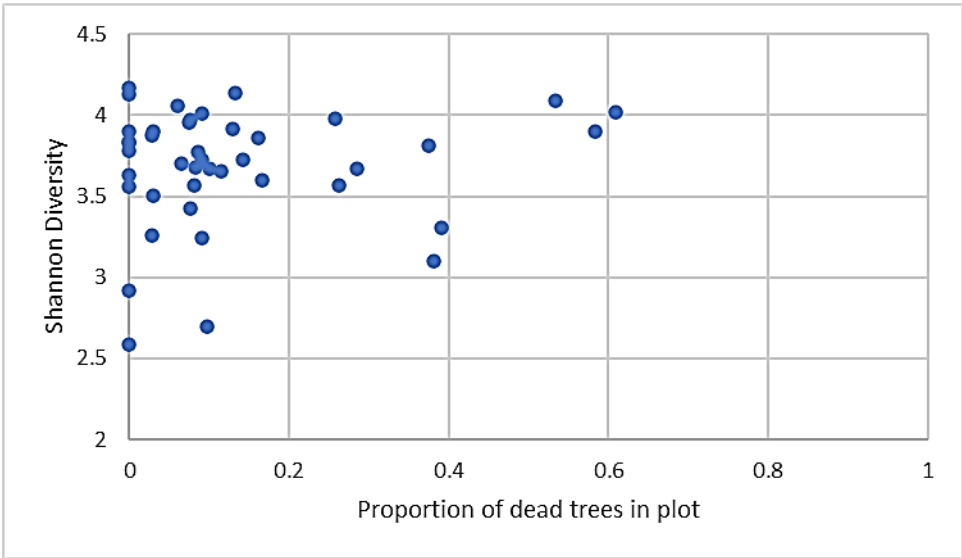

Fig. 11: Correlation between Shannon diversity of bacterial communities of termites collected from ironwood trees and proportion of dead ironwood trees in plots (Full dataset).

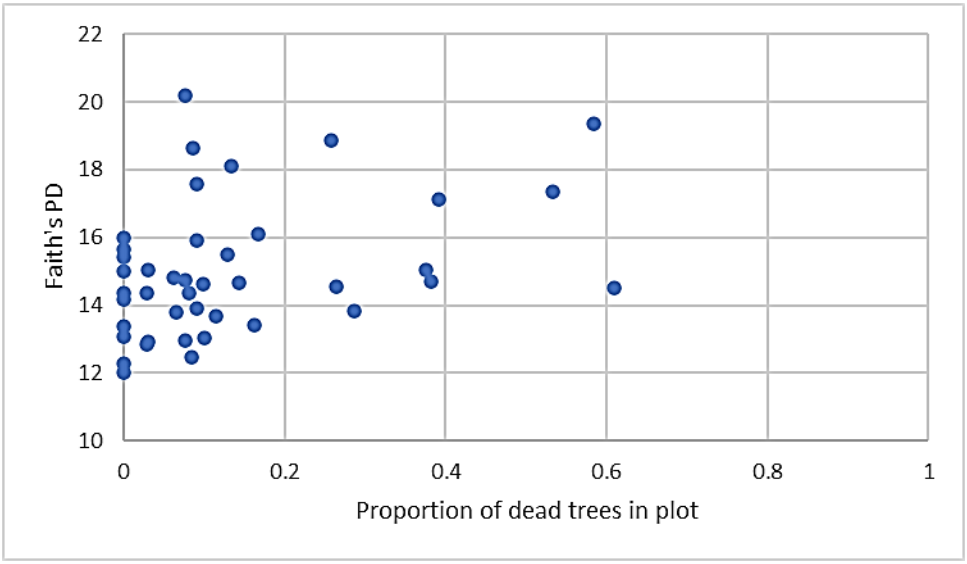

Fig. 12: Correlation between Faith's PD of bacterial communities of termites collected from ironwood trees in a plot and proportion of dead ironwood trees plot (Without SF dataset).

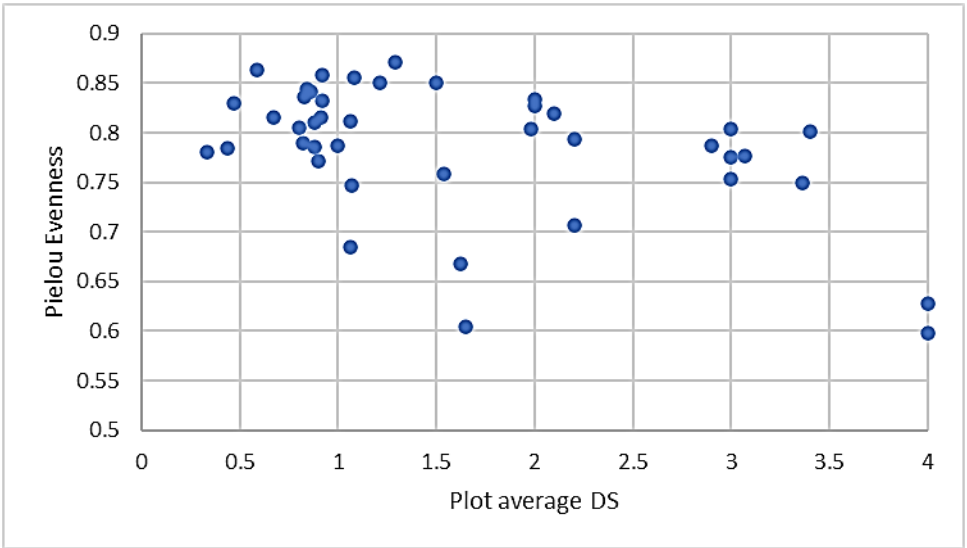

Fig. 13: Correlation between Pielou's Evenness of bacterial communities of termites collected from trees having various levels of decline severity (0-4) and average disease severity of tree plots (Full dataset).

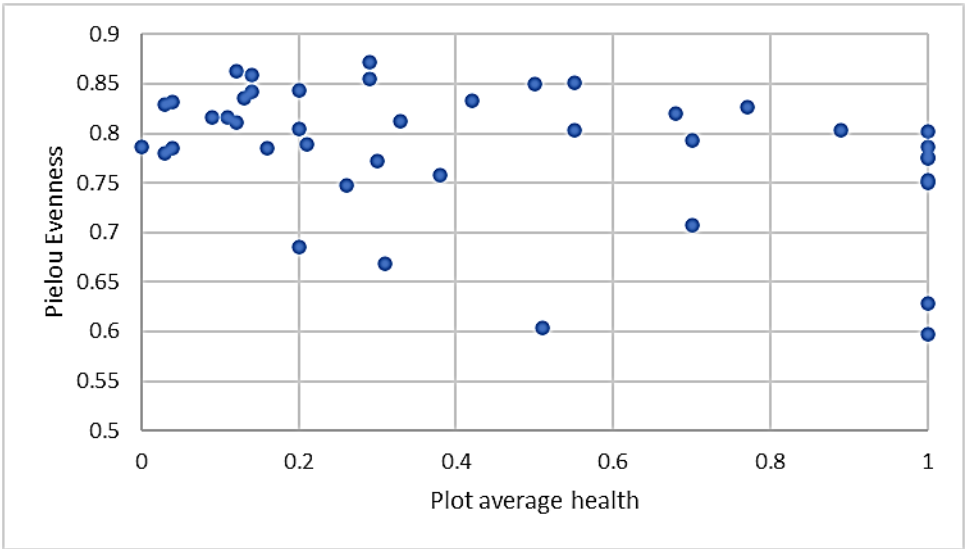

Fig. 14: Correlation between Pielou's Evenness of bacteria communities of termites collected from healthy and sick plots and average health of plot (Full dataset).

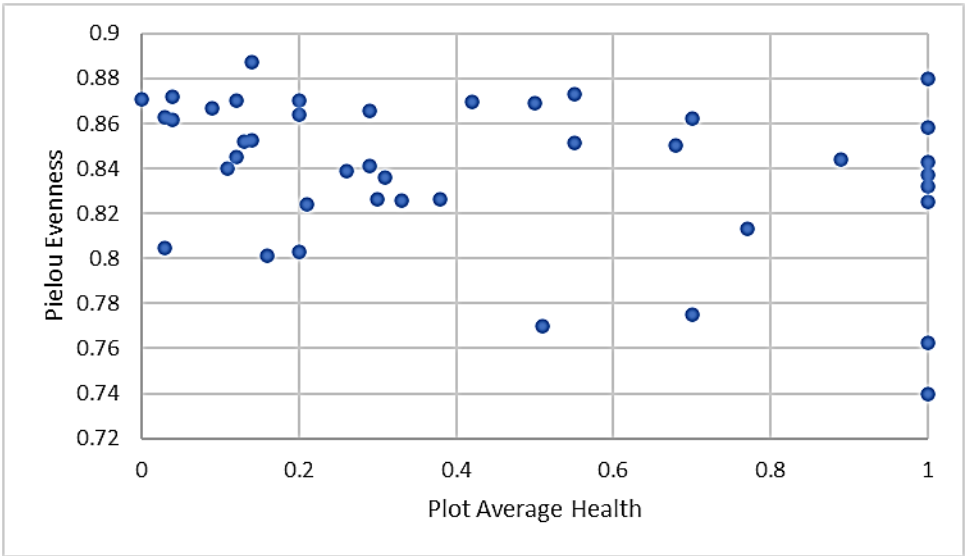

Fig. 15: Correlation between Pielou's Evenness of bacteria communities of termites collected from healthy and sick plots and average health of plot (Without SF dataset).

2. Percentage of trees with termites in plot: Increasing number of termite infested trees in a plot was significantly associated with less diversity in terms of evenness (Pielou's evenness, $p=0.011$, $r_s = -0.38$, Spearman's Rank test) and evenness relative to richness

(Shannon index, p=0.012, r_s = -0.38, Spearman's Rank test) of the total bacterial community of termites feeding on those Pielou's evenness, ASV richness and Faith's PD did not show any significance for percentage of dead trees in plots in any of the data set.

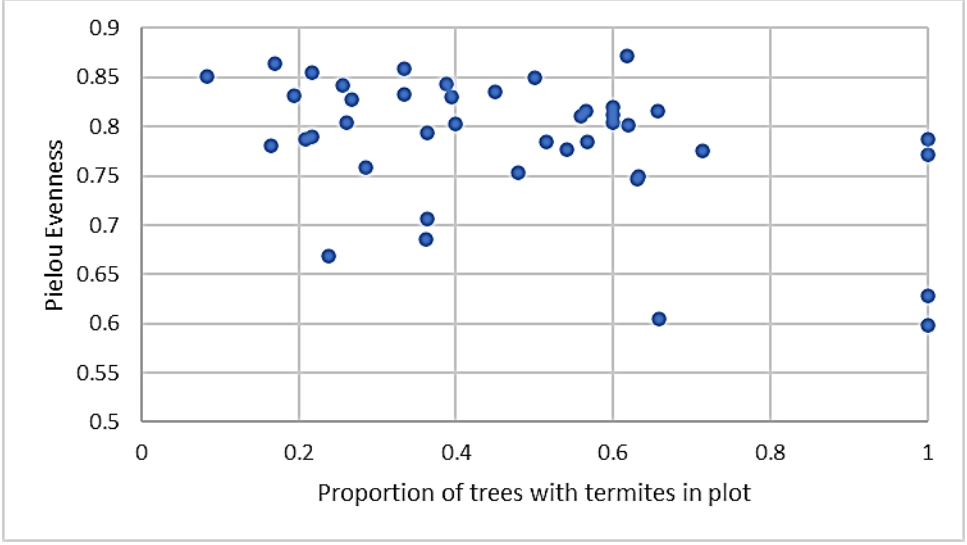

Fig. 16: Correlation between Faith's PD of bacterial communities of termites collected from termite infested trees in a plot and proportion of trees with termites in plot (Full dataset).

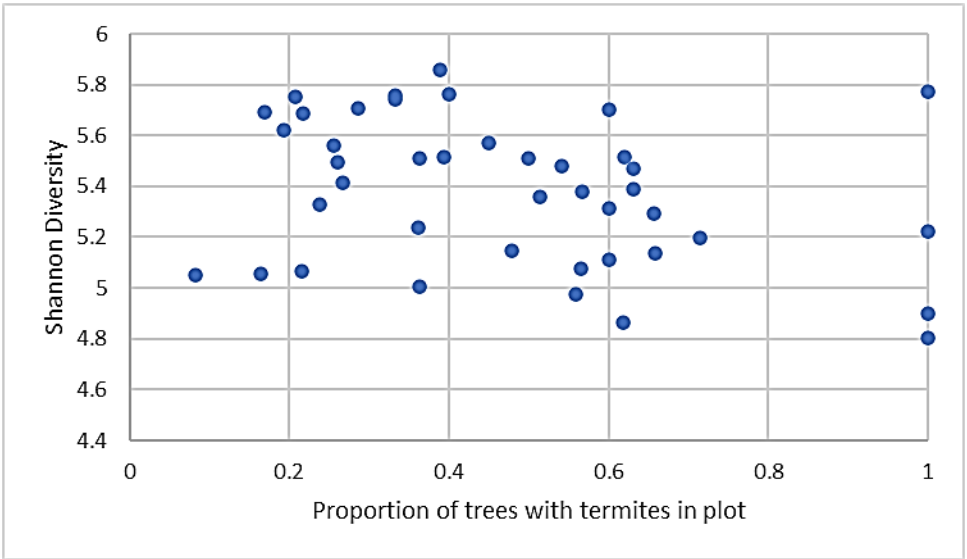

Fig. 17: Correlation between Shannon diversity of bacterial communities of termites collected from termite infested trees in a plot and proportion of trees with termites in plot (Without SF dataset).

3. Stand Maturity Estimate: The age of the tree stand showed a marginal correlation (Faith's PD, p=0.09, r_s=0.26) to the phylogenetic diversity of the bacteria in the Full dataset and significant correlation (Faith's PD, p=0.01, r_s=0.36, Spearman's Rank test) in Without SF data set. Termites collected from mature trees had greater phylogenetic distances among the bacterial taxa, than those collected from young trees. Pielou's evenness, ASVs richness and Shannon indices did not have significant influence on stand maturity in any of the three data sets.

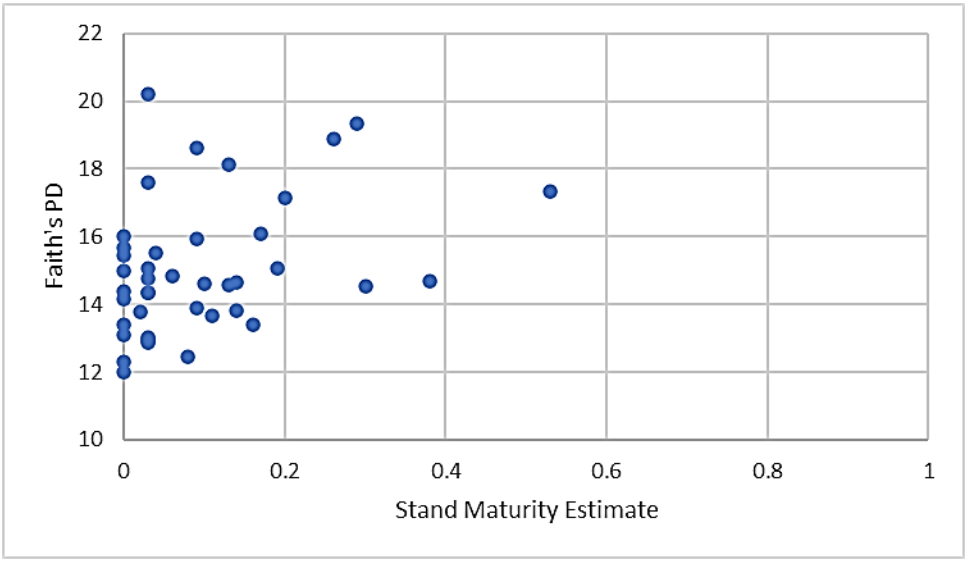

Fig. 18: Correlation between Faith's PD of bacterial communities of termites collected from different maturity levels and Stand Maturity Estimate (Without SF dataset).

c.) Location-related factors:

1. Location: Location had no significant influence on alpha diversity in any of the three data sets (Full, Only SF and without SF).
2. Site management (low, moderate, high): The evenness and Shannon diversity of the bacterial community associated with termites (Full data set) was significantly influenced by the level of site management (Fig. 19, Fig. 20). It was found that intense site management lead to less evenness (Pielou's evenness, p= 0.045, H =6.17, Kruskal-Wallis ANOVA) and lower Shannon diversity (p=0.024, H=7.41, Kruskal-Wallis ANOVA) of the bacterial community of the termites. However, site management did not have

significant influence on ASVs richness and Faith's PD in any of the three data sets (Full, Only SF and without SF).

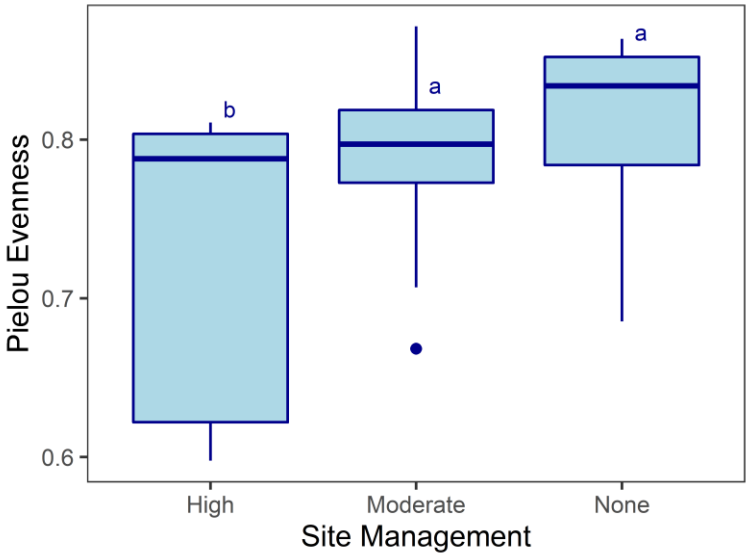

Fig. 19: Pielou's evenness of bacterial communities of termites collected from highly, moderately or non-managed sites (Full data set). Different letters indicate significant difference.

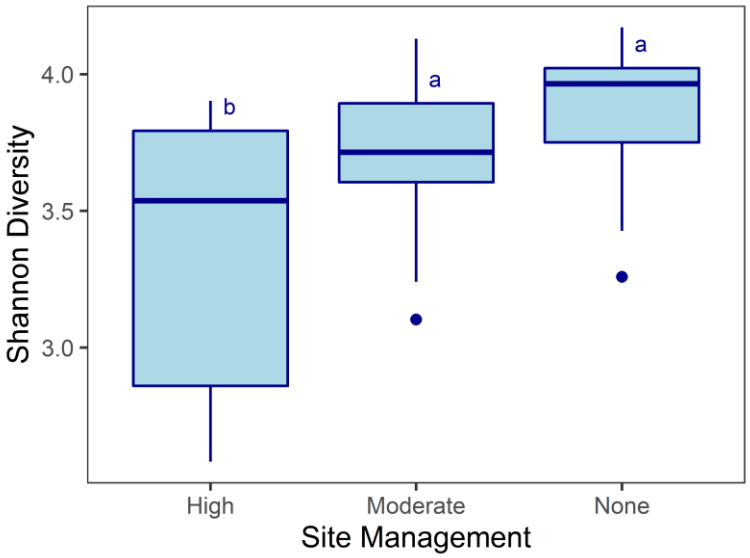

Fig. 20: Shannon diversity index of bacterial communities of termites collected from highly, moderately or non-managed sites (Full data set). Different letters indicate significant difference.

3. Altitude: The altitude of the tree location showed a weak but significant correlation (Faith's PD, p=0.0462 r_s=0.0462, Spearman's Rank test) to the phylogenetic diversity of the bacteria in the Only SF dataset. Termites collected from trees at high altitude had greater phylogenetic distances among the bacterial taxa, than those collected from trees at low altitude. Pielou's evenness, ASVs richness and Shannon indices did not have significant influence on stand maturity in any of the three data sets.

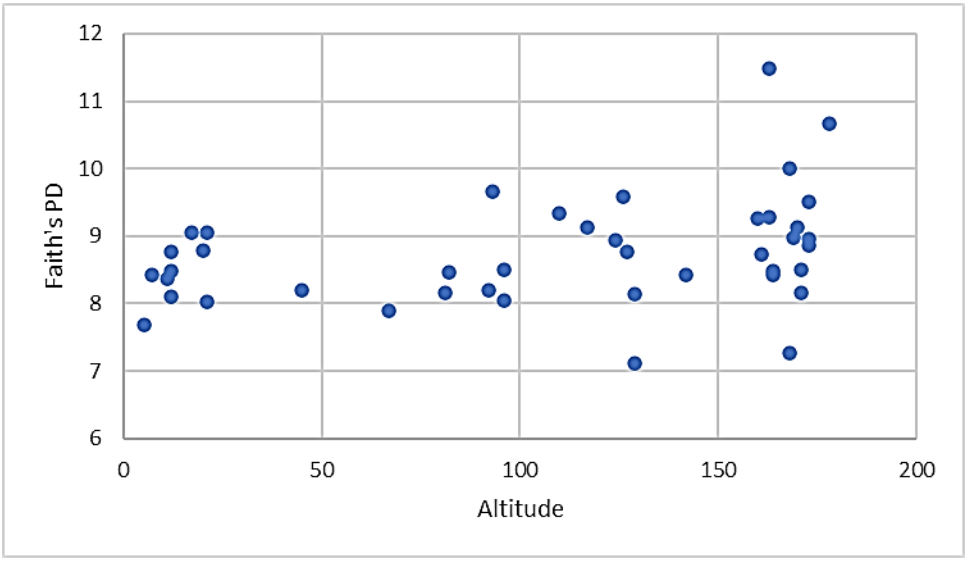

Fig. 21: Correlation between Faith's PD of bacterial communities of termites collected from trees at different altitude levels and altitude of the tree location (Only SF dataset).

4. Altitude Classification (High> 100 m vs Low< 100m): The bacterial communities in the phyla Spirochaetes and Fibrobacteres (Only SF dataset) of termites collected from ironwood trees at high altitudes (n=25) showed greater phylogenetic distance (Faith's PD, p=0.017, H= 5.61, Kruskal-Wallis ANOVA) within samples than those of termites from trees located at low (n=17) altitude. The phylogenetic distance in the Without SF dataset was also marginally greater (Faith's PD, p= 0.074, H =3.17, Kruskal-Wallis ANOVA) in termite samples from trees at high altitude. The full dataset did not show a significant effect of Altitude classification on Faith's PD. Also, Pielou's evenness, ASVs richness and Shannon indices did not have significant influence on altitude classification in any of the three data sets (Full, Only SF and without SF).

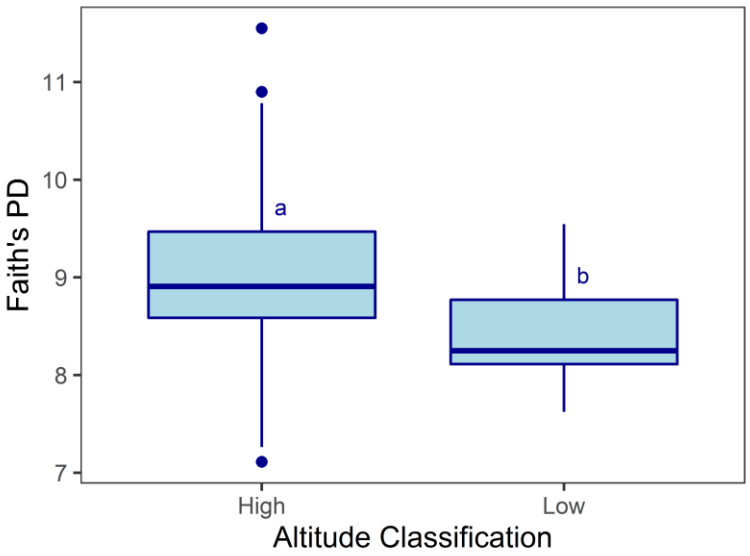

Fig. 22: Faith's PD of bacterial communities of termites collected from trees at high and low altitudes (Only SF dataset). Different letters indicate significant difference.

5. Parent Material Classification: Parent material had no significant influence on alpha diversity in any of the three data sets (Full, Only SF and without SF).

Beta diversity:

PERMANOVA identified three factors that had significant effects on beta diversity of the bacterial community, i.e. Ralstonia presence (p=0.011, pseudo-F= 3.28), Altitude (p=0.042, pseudo-F= 2.84) and Parent Material (p= 0.004, pseudo-F= 3.65). Bacteria diversity was significantly different between termite samples taken from trees growing on Lime and Sand (p= 0.003, pseudo-F = 5.577) as well as Sand and Tuff (p= 0.011, pseudo-F = 4.284), while samples from Lime and Tuff did not show significant differences (p = 0.425, pseudo-F= 0.916) (Table 5).

Table 5: PERMANOVA test results. Asterisks indicate significant effect.

Factor		Sample size	Permutations	pseudo-F	p-value
Ralstonia					
Negative	Positive	42	999	3.282	0.016*
Altitude Classification					
High	Low	42	999	2.843	0.029*

Parent Material					
Lime	Sand	34	999	5.577	0.003*
Lime	Tuff	33	999	0.916	0.425
Sand	Tuff	17	999	4.284	0.011*

PERMDISP showed no significant difference in dispersion for the three factors. Since, assumption of homogeneous spread was not violated, the Adonis test was performed. The Adonis test results showed that presence or absence of Ralstonia (Pr(>F) = 0.024) in the ironwood trees the termites were collected from and Altitude (Pr(>F) =0.04) of the tree location had significant effects on bacteria community similarity of termite samples. However, Parent Material (Pr(>F) = 0.166) did not show significant results (Table 5). The R^2 values for Ralstonia (R^2 = 0.0758) and Altitude (R^2=0.0557) were low, indicating that these factors are only explaining a low percentage regarding the variation in the data. The interaction among Ralstonia and Parent Material showed marginal significance with a low R^2 value (Pr(>F) = 0.09, R^2 = 0.0506) while no significant interaction was observed in other pairwise combinations.

Table 6: Adonis test results. Asterisks indicate significant effect.

Factor	Df	Sums Of Sqs	Mean Sqs	F.Model	R^2	Pr(>F)
Rs	1	0.181	0.181	3.4610	0.0758	0.024*
Altitude	1	0.133	0.133	2.5445	0.0557	0.04*
Parent Material	2	0.1523	0.0762	1.4571	0.0638	0.166
Rs: Altitude	1	0.0248	0.0248	0.4742	0.0104	0.746
Rs: Parent Material	1	0.1207	0.1207	2.3096	0.0506	0.09
Altitude: Parent Material	1	0.0379	0.0379	0.7253	0.0159	0.541
Rs: Altitude: Parent Material	1	0.0115	0.0115	0.2192	0.0048	0.917
Residuals	33	1.725	0.0523		0.722921	
Total	41	2.387			1	

The NMDS plots revealed that centroids of Ralstonia positive and Ralstonia negative groups as well as high and low altitude groups were positioned close to one another, indicating that the composition of these groups were similar (Fig. 23, Fig. 24). With the exception of an outlier sample collected from a tree negative for Ralstonia and located at low altitude, the groups

of both the factors showed similar dispersion in concordance with the non-significant PERMDISP results.

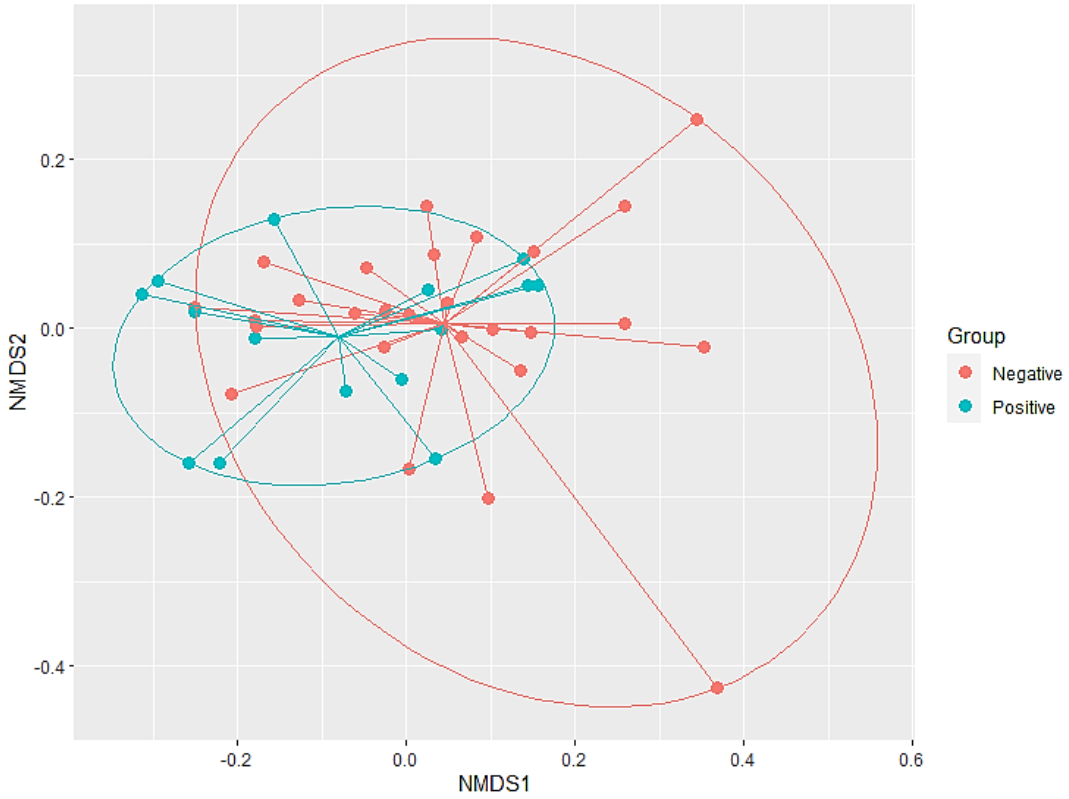

Fig. 23: Plot of the first two dimensions of the NMDS ordination of the weighted Unifrac distance matrix showing the similarity of bacteria communities of termite samples within and between the Ralstonia negative and Ralstonia positive groups.

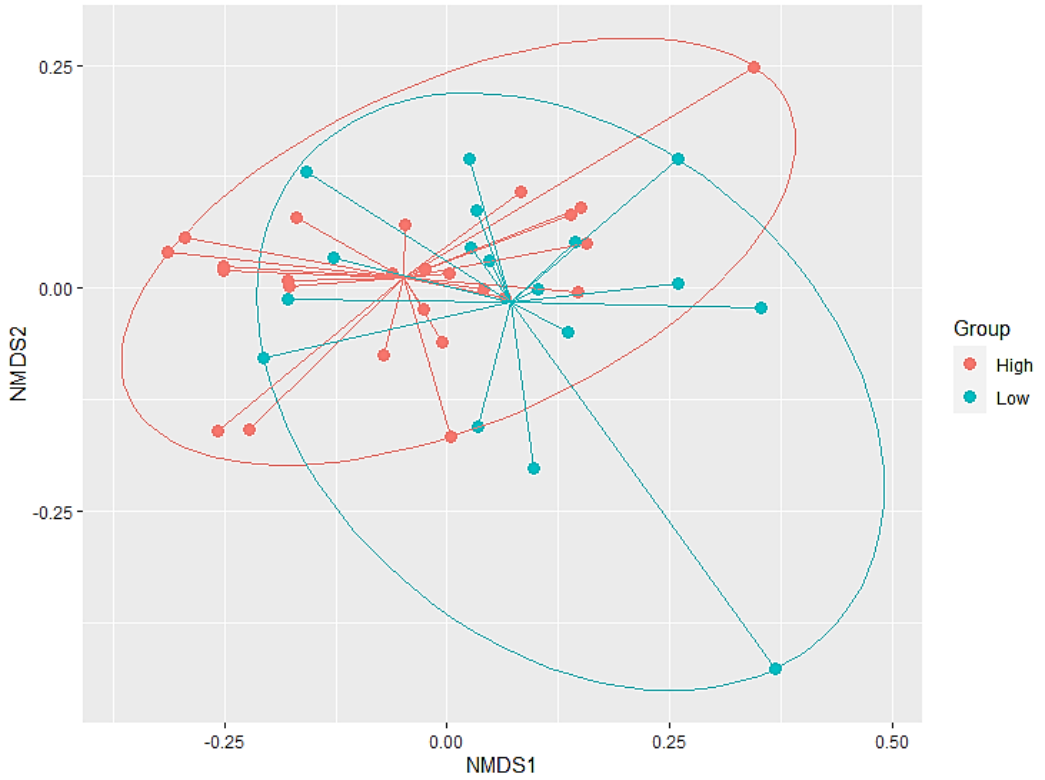

Fig. 24: Plot of the first two dimensions of the NMDS ordination of the weighted Unifrac distance matrix showing the similarity of bacteria communities of termite samples within and between the high altitude and low altitude groups.

Differential Abundance:

At effect size threshold of 3 on the LDA score for discriminative features and significance level of 0.05, LEfSe analysis showed that Margulisbacteria (Uncultured candidate division ZB3 bacterium) had significantly higher abundance in termites collected from Ralstonia negative trees and Dysgonomonadaceae as well as Rhodospirillales had significantly higher abundance in termites collected from trees at low altitude.

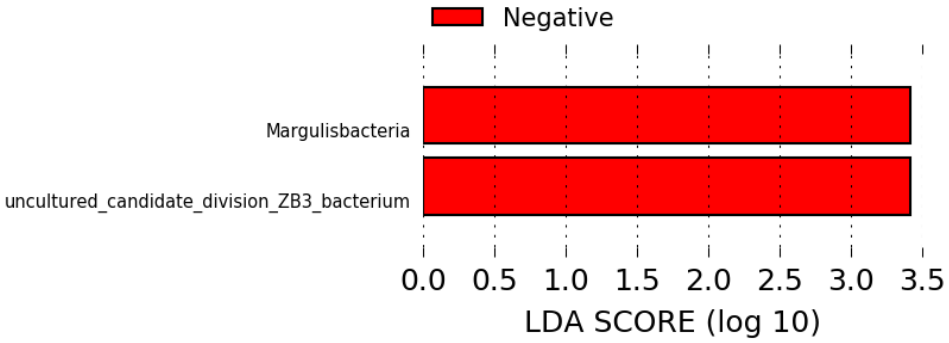

Fig 25: Differential abundance of bacteria ASVs with respect to Ralstonia presence in ironwood trees (LEfSe differentials). At alpha level of 0.05 and an effect size threshold of three on the LDA score for discriminative features, two ASV were enriched in termite samples collected from Ralstonia negative trees as compared to Ralstonia positive trees.

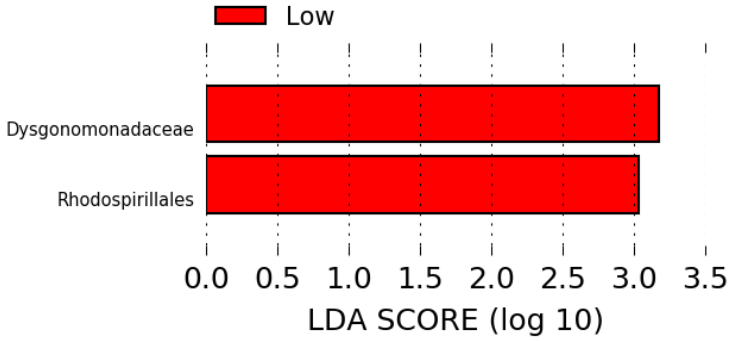

Fig 26: Differential abundance of bacteria ASVs with respect to Altitude of ironwood tree from mean sea level (LEfSe differentials). At alpha level of 0.05 and an effect size threshold of three on the LDA score for discriminative features, two ASV were enriched in termite samples collected from trees at low altitude as compared to trees at high altitude.

<u>Feeding experiment at LSU with *C. formosanus* to determine optimum concentration of bacterial dilution:</u>

The results for ANOVA showed significant differences at 2 days (p=0.01, F = 20.76) and 10 days (p=0.01, F= 21. 68). For both 2 days and 10 days, 10^{-2} had lowest consumption and control had highest consumption. No significant differences were found between consumption of control

and 10^{-6} at both 2 days and 10 days. After 2 days, the consumption of 10^{-4} was significantly lower than control, however, after 10 days, the difference of consumption between 10^{-4} and control became insignificant.

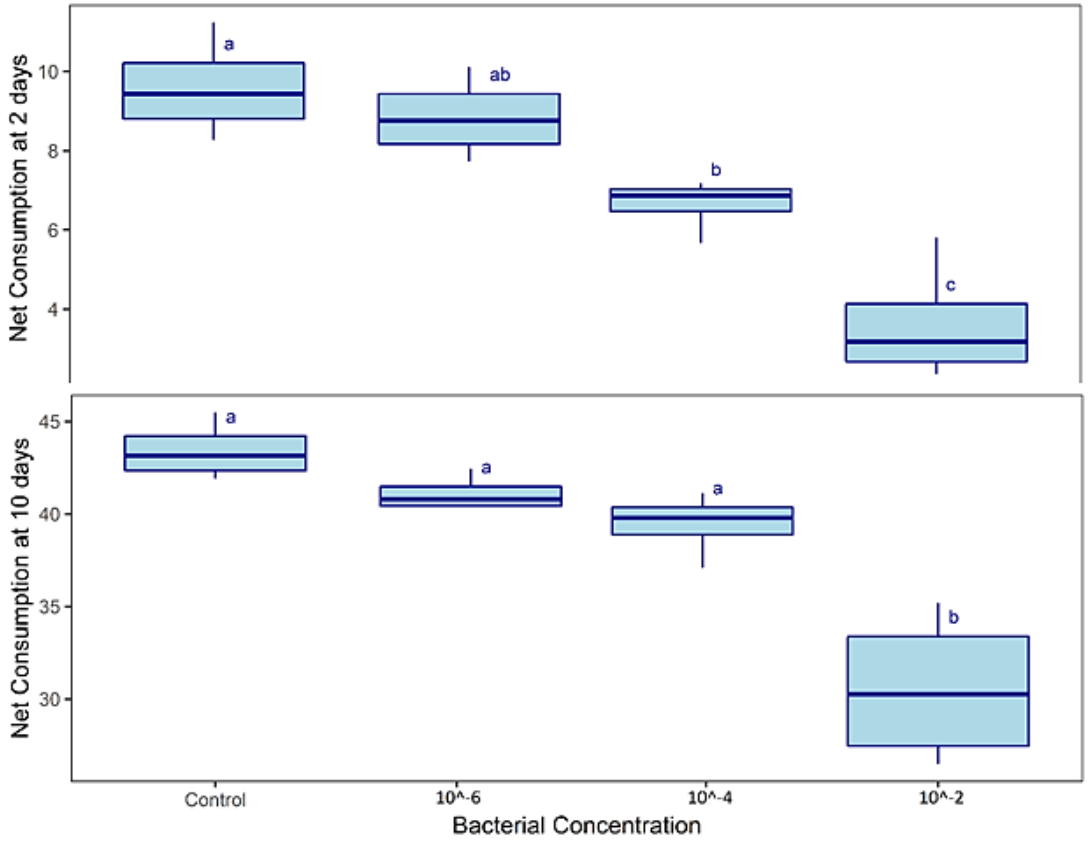

Fig. 27: Net Consumption (mg) of filter paper by *C. formosanus* workers at bacterial dilutions of 10^{-2}, 10^{-4}, 10^{-6} and control, One-way ANOVA and Tukey's Studentized Range (HSD) Test at 2 days and 10 days. Different letters indicate significant difference at the same time point.

<u>Difference between the consumption of wood pieces with different levels of Ralstonia and wet wood bacteria by *N. takasagoensis* workers:</u>

For all the bioassays, the average mortality rate of 32.9 ± 5.69% was observed. One-Way Analysis of Variance showed a significant effect ($p = 0.0007$, $R2 = 0.2604$) of the food source on net consumption by *N. takasogoensis* workers. Figure 45 shows that "High Rs and High WW" had the lowest consumption and "No Rs and No WW" had the highest consumption.

Tukey's Studentized Range (HSD) Test showed that net consumption of "High Rs and High WW" wood by *N. takasagoensis* workers is significantly different from "No Rs and No WW" wood ($p = 0.0004$) and "Low Rs and High WW" wood ($p = 0.0180$) (Table 7). However, net consumption of "No Rs and No WW" wood, "Low Rs and High WW" wood and "Low Rs and Low WW" wood by *N. takasagoensis* workers was not significantly different; and the net

consumption of “Low Rs and Low WW” wood and “High Rs and High WW” was also not significantly different (Table 7). These results indicate that termites tend to prefer wood without bacteria over wood with high concentrations of bacteria on it.

Table 7: Pairwise differences in consumption (above the diagonal) between four treatments with different wood pieces (along the diagonal) by *N. takasagoensis* workers and Tukey's Studentized Range (HSD) Test (below the diagonal) for the four-choice bioassay. Food source is along the diagonal, difference in consumption is above the diagonal and p value is below the diagonal. Difference in consumption is presented with the minuend to the left of the subtrahend along the diagonal.

Difference in consumption of food source (g)

No Rs and No WW	0.0608	0.0409	0.1387
0.2425	Low Rs and Low WW	-0.0110	0.0780
0.5837	0.9250	Low Rs and High WW	0.0979
0.0004	0.0836	0.0180	High Rs and High WW

p value

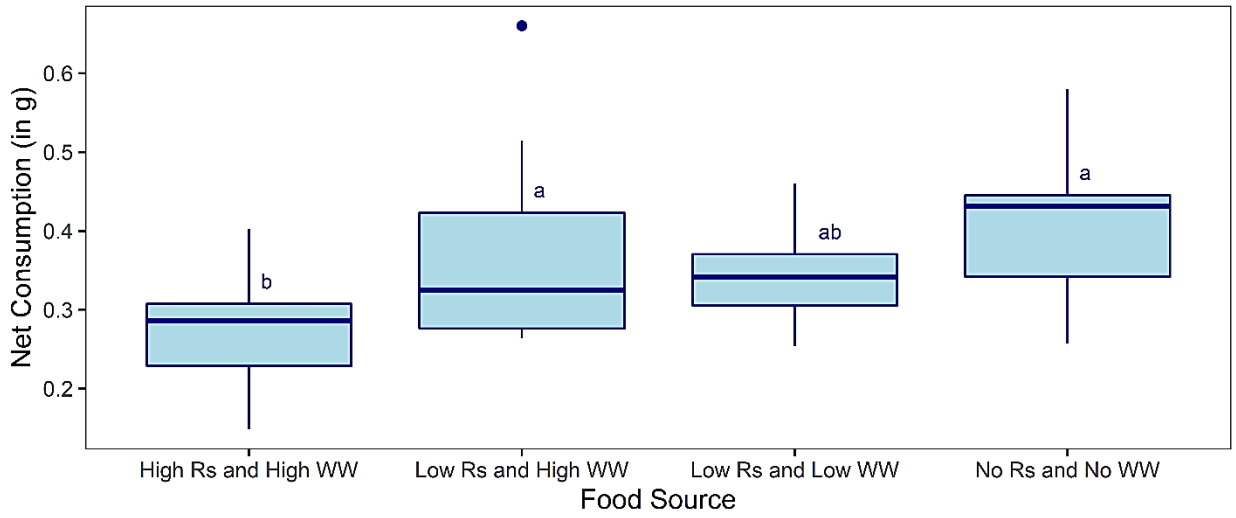

Fig. 28: Net consumption (g) of wood pieces with different levels of Ralstonia (Rs) and wetwood (WW) bacteria by *N. takasagoensis* workers. Different letters indicate significant differences determined by One-way ANOVA and Tukey's Studentized Range (HSD) Test for four-choice bioassay.

Difference between the consumption of Ralstonia inoculated wood and healthy wood by *N. takasagoensis* workers:

The bacterial culture that was used to make dilutions for inoculating wood pieces had optical density (OD_{600}) of 2.5 and 9.251E+9 colony forming units per ml (CFU/ml). Marginal differences were observed between consumption of 10^{-4} and Control ($p=0.0925$, $R^2 = 0.0810$) as well as between 10^{-6} ($p=0.0846$, $R^2= 0.0959$) and control. No significant differences were observed between the consumption of 10^{-8} and Control ($p=0.1176$, $R^2= 0.1180$) (Fig. 29).

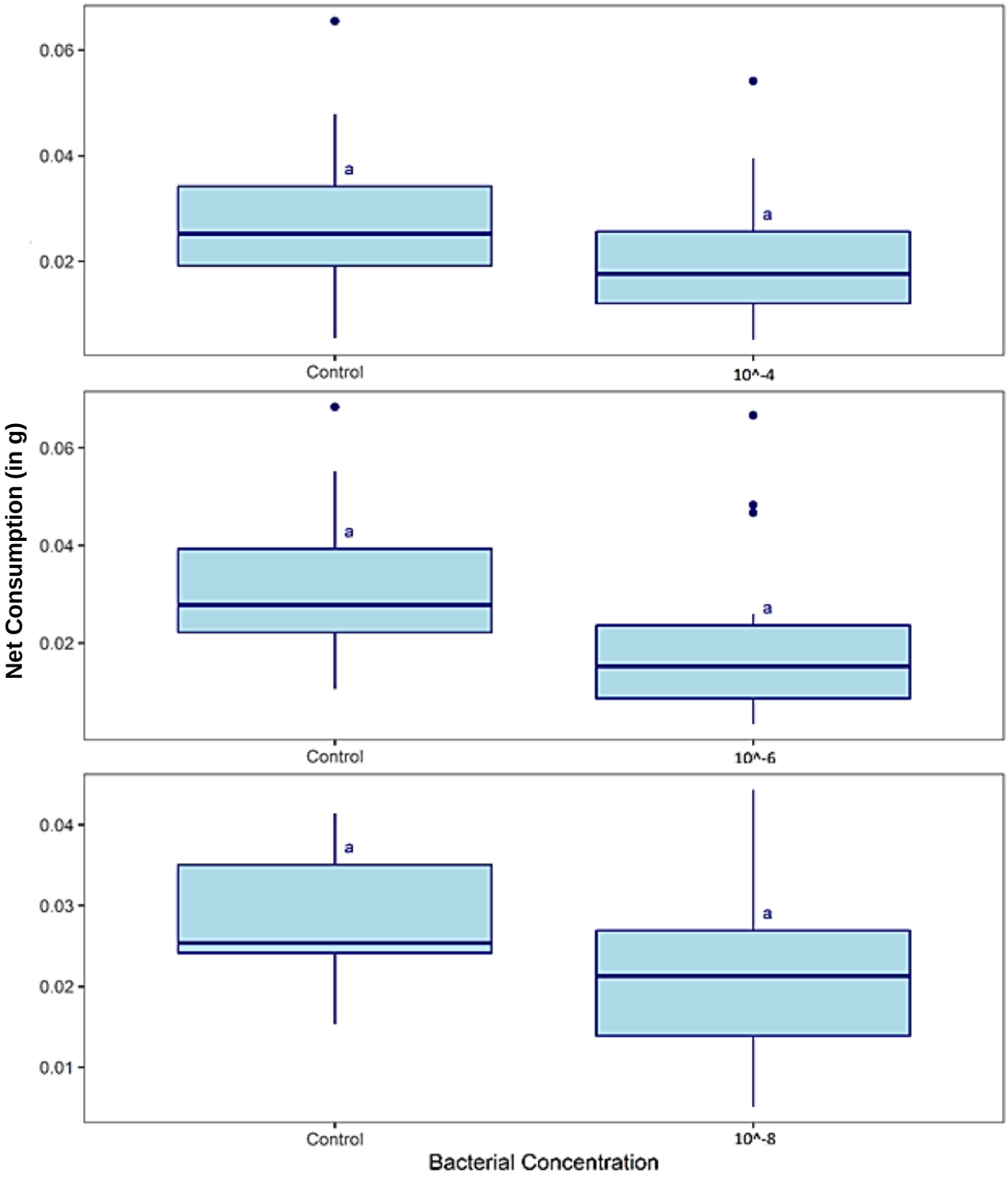

Fig. 29: Net Consumption (g) of wood pieces inoculated with 10^{-4}, 10^{-6} and 10^{-8} bacterial concentration by *N. takasagoensis* workers. Same letters indicate lack of significant differences determined by One-way ANOVA and Tukey's Studentized Range (HSD) Test for two-choice bioassay.

References:

Anderson, Marti J. 2001. “A New Method for Non-Parametric Multivariate Analysis of Variance.” *Austral Ecology* 26 (1): 32–46. https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x.

Ayin C.M., Alvarez A.M., Awana C., Schleinzer F.M., Marx B.D., Schlub R.L. 2019. *Ralstonia solanacearum*, *Ganoderma australe*, and bacterial wetwood as predictors of ironwood tree (*Casuarina equisetifolia*) decline in Guam. Australasian Plant Pathol. 48, 625–636 doi:10.1007/s13313-019-00666-8

Breznak, J. A. (2002). Phylogenetic Diversity and Physiology of Termite Gut Spirochetes. *Integrative & Comparative Biology*, *42*(2), 313. https://doi.org/10.1093/icb/42.2.313

Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. *Nature Reviews Microbiology*, *12*(3), 168–180. https://doi-org.libezp.lib.lsu.edu/10.1038/nrmicro3182

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016 Jul;13(7):581-3. doi: 10.1038/nmeth.3869. Epub 2016 May 23. PMID: 27214047; PMCID: PMC4927377.

Chao, A., Gotelli, N.J., Hsieh, T.C., Sander, E.L., Ma, K.H., Colwell, R.K. and Ellison, A.M. (2014), Rarefaction and extrapolation with Hill numbers: a framework for sampling and estimation in species diversity studies. Ecological Monographs, 84: 45-67. https://doi.org/10.1890/13-0133.1

Chhotani, O. B. 1997. The fauna of India and the adjacent countries. Isoptera (Termites): (family Termitidae), Vol. 2. Zoological Survey of India, Calcutta, India.

Denny, TP; Hayward, AC. 2001. Gram-negative bacteria: Ralstonia. Pages 151-174 in: Laboratory guide for identification of plant pathogenic bacteria, 3rd ed. Schaad, NW; Jones, JB; Chun, W, eds. APS Press, St. Paul, M. N.

Faith, D. P. 1992. Conservation evaluation and phylogenetic diversity. Biol. Conserv. 61, 1–10.

Hsieh, T. C. & Ma, K. & Chao, Anne. (2016). iNEXT: An R package for rarefaction and extrapolation of species diversity (Hill numbers). Methods in Ecology and Evolution. 7. 10.1111/2041-210X.12613.

Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002 Jul 15;30(14):3059-66. doi: 10.1093/nar/gkf436. PMID: 12136088; PMCID: PMC135756.

Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

Lane, D. J. (1991). 16S/23S rRNA sequencing, p. 115-175. In E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.

Liang, W. R., and H. F. Li. 2016. Redescriptions of three *Nasutitermes* species (Isoptera: Termitidae: Nasutitermitinae) occurring in Taiwan. Ann. Entomol. Soc. Am. 109: 779–795.

Obanda, D. N., Keenan, M. J., Page, R., Raggio, A. M., Taylor, C. M., Marx, B. D., Stout, R. W., Guice, J., Luo, M., Welsh, D. A., Coulon, D., & Husseneder, C. (2021). Gut Microbiota Composition and Predicted Microbial Metabolic Pathways of Obesity Prone and Obesity Resistant Outbred Sprague-Dawley CD Rats May Account for Differences in Their Phenotype. *Frontiers in nutrition*, *8*, 746515. https://doi.org/10.3389/fnut.2021.746515

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D,Minchin RR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH,Szoecs E, Wagner H (2018) Vegan: community ecology package. Rpackage version 2.5–2. https://CRAN.R-project.org/package=vegan

Price MN, Dehal PS, Arkin AP FastTree 2-approximately maximum-likelihood trees for large alignments. *PLoS One.* 2010;5(3):e9490. doi: 10.1371/journal.pone.0009490.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome biology*, *12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Su, N.-Y., and R. H. Scheffrahn. 1998. *Coptotermes vastator* Light (Isoptera: Rhinotermitidae) in Guam. Pro. Hawaii. Entomol. Soc. 33: 13–18.

Thorne BL. 1984. Polygyny in the Neotropical termite *Nasutitermes corniger*: Life history consequences of queen mutualism. Behavioral Ecology and Sociobiology 14: 117-136. https://doi.org/10.1007/BF00291903

Xia Y., Sun, J., & Chen, D.-G. (2018). *Statistical analysis of microbiome data with R*. Springer.