1-24-2023

INVESTIGATING THE POTENTIAL ROLE OF TERMITES AS PATHOGEN VECTORS IN THE DECLINE OF IRONWOOD TREES (CASUARINA EQUISETIFOLIA) IN GUAM

Garima Setia
Louisiana State University and Agricultural and Mechanical College

INVESTIGATING THE POTENTIAL ROLE OF TERMITES AS PATHOGEN VECTORS IN THE DECLINE OF IRONWOOD TREES (CASUARINA EQUISETIFOLIA) IN GUAM

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Entomology

by
Garima Setia
B. S., Punjab Agricultural University, 2020
May 2023

Acknowledgments

I am ineffably grateful to my major advisor Dr. Claudia Husseneder for her constant guidance throughout the research. Her way of thinking, passion towards research, and immense knowledge have always fascinated and inspired me. I was fortunate to have her as my mentor as I have learned immensely from her during these past two years. I am certain that these learnings will help me grow professionally and personally throughout my life.

I would like to acknowledge Dr. Robert Schlub for his invaluable inputs during the entire project. I will always be thankful to Dr. Schlub, Julia, Elizabeth, Alex, and Ethan from the University of Guam for helping me tremendously with the research during my stay in Guam. I would extend my thanks to my committee members Dr. Michael Stout and Dr. Qian "Karen" Sun for reviewing my thesis. I would like to express my deep gratitude towards Dr. Jin Tao, Junyan Chen, and Dr. Ilgoo Kang for helping me with molecular work for my project. I am extremely grateful to my parents and my brother for their profound encouragement and patience throughout the duration of this research. Special thanks to my friends Suman, Bijaylaxmi, Sandeep, Jyoti, Sindhu and Rajat for their love and support during my stay at Louisiana.

This thesis is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2018-38640-28418 through the Western Sustainable Agriculture Research and Education program under project number SW19-906. USDA is an equal opportunity employer and service provider. Any opinions, findings, conclusions, or recommendations expressed in this thesis do not necessarily reflect the view of the U.S. Department of Agriculture.

Table of Contents

Abstract

Termites have been found associated with the decline of *Casuarina equisetifolia* (ironwood), an important agro-forestry species in Guam. While the ironwood tree decline (IWTD) was first reported in 2002, its association with termites as well as with bacteria *Ralstonia solanacearum* and *Klebsiella* species (*K. oxytoca* and *K. variicola*) was determined in 2010 and 2012 respectively. In 2019, the termite species attacking the ironwood trees were identified as *Nasutitermes takasagoensis*, *Coptotermes gestroi,* and *Microcerotermes crassus*. However, the role of termites in IWTD is not known. Since termites are repositories of bacteria and consume wood, termite foragers might feed on ironwood trees infested with IWTD-associated pathogens and spread these pathogens from infested to healthy trees. We hypothesized that termite workers are vectors for the pathogens associated with IWTD. The bacterial communities of worker samples collected from sick and healthy ironwood trees were analyzed through 16S rRNA gene sequencing. Relation of the bacterial community of worker samples with tree-, plot-and location-related factors was also analyzed. Feeding experiments were performed to investigate if wood consumption by termite workers was influenced by the bacterial load of ironwood. The ability of workers to ingest and sustain *R. solanacearum* bacteria was determined through feeding experiments. *Ralstonia* spp. were not detected in any of the *N. takasagoensis* samples regardless if they were collected from trees with confirmed Ralstonia infection or healthy trees. One *C. gestroi* sample and none of the *M. crassus* samples showed Ralstonia. Four *N. takasagoensis* samples and one *C. gestroi* sample showed *Klebsiella* species albeit in low amounts. Tree Health and Site Management impacted the bacterial community of both *N. takasagoensis* and *C. gestroi* workers. The bacterial community of *N. takasagoensis* workers was also impacted by Presence of Ralstonia, Altitude, Plot Average Decline Severity, Plot Average Decline, Proportion of Dead

Tree in the Plot, and Proportion of Trees with Termite Damage in the Plot. The feeding experiments showed that termite workers can ingest *R. solanacearum* bacteria, however, the bacteria are not able to thrive in the body of healthy workers. We concluded that termite workers are not vectors for the potential pathogens associated with IWTD.

Introduction

Casuarina equisetifolia (Ironwood), a tree species with remarkable utilities and significance, has been in decline in Guam, which is a Micronesian island (Athens and Ward 2004, Stone 1970, Fosberg et al. 1979, Elevitch and Wilkinson 2000). In 2002, Bernard Watson noticed that 20 of his 10-year old ironwood windbreak trees on this farm were in poor health (Mersha et al. 2009, Mersha et al. 2010). The trees exhibited two sets of symptoms. Grouped together were five trees with wilt symptoms, which consisted of a rapid upward progression of yellowing and death of foliage. These trees were dead within 6 months, while retaining much of their dead foliage. Scattered among healthy trees, in at least two locations were 15 trees that exhibited foliage thinning, and branch die-back. Over time, the severity of symptoms would increase and often end in tree death within 5 years. This condition would eventually be referred to as ironwood tree decline (IWTD). From an informal survey of tree sites across Guam, it become apparent that tree wilt was rare, whereas IWTD was widespread and likely impacting the health of Guam's ironwood population. In addition to the external symptoms of IWTD are internal symptoms of wood staining and ooze formation (Schlub 2013). The ooze contained various bacteria including *Ralstonia*, *Klebsiella*, *Kosakonia*, *Enterobacter*, *Pantoea*, *Erwinia* and *Citrobacter*. Among these bacteria, *Ralstonia solanacearum* and *Klebsiella* species (*K. oxytoca* and *K. variicola*) were frequently isolated and studied (Schlub 2013, Ayin et al. 2019, Schlub et al. 2020).

The mechanism behind the transmission of IWTD associated pathogens from trees in decline to healthy trees is still unclear. Various potential abiotic and biotics factors have been studied to understand their role in association with IWTD. Many have been ruled out as IWTD contributors

including the wood-rotting fungi (*Phellinus* spp.), beetles (*Protaetia pryeri* and *Protaetia orientalis*), and the gall-inducing wasp *Selitrichodes casuarinae* Fisher & La Salle (Campora 2005, Schlub 2010, Mersha et al. 2011, Schlub 2013). However, the level of human management at an ironwood tree site, the presence of the wood-rotting fungus and termites on a tree were shown to be significantly associated with IWTD (Schlub 2010). In 2019 it was determined that the presence of the wood-rotting fungus *Ganoderma australe* on the roots or base of a tree was a significant predictor of IWTD. Additionally, it was determined that the presence of *R. solanacearum* was a predictor, but not the presence of *Klebsiella* spp. or *K. oxytoca*. However, wetwood bacteria (*Klebsiella* spp) were significantly related tothe rate of ooze formation in a cross-section and the percent cross-sectional area with bacterial wetwood. Although previous studies have identified a strong association between IWTD and termite infestation, the role of termites as a potential vector of IWTD associated pathogens has not been studied.

Our study aimed at understanding the role of termites as a potential vector in IWTD. We performed deep sequencing of the bacterial communities present in three different termite species named *Nasutitermes takasagoensis*, *Coptotermes gestroi* and *Microcerotermes crassus*, that infest ironwood trees in Guam to detect the presence of pathogens associated with IWTD (Park et al. 2019). The association of bacterial communities of these termites with respect to various tree-, location-, and plot-related factors was analyzed. We performed feeding experiments with *N. takasagoensis* and *C. gestroi* workers to understand if termites show any difference in consumption among ironwood with different pathogen loads. Termites were fed with *Ralstonia solanacearum*, a pathogen associated with IWTD, to determine the ability of termites to ingest and sustain this pathogen inside their bodies. Recognizing the role of termites in IWTD can give a new direction

to the decades long ongoing research to preserve ironwood trees in Guam, for example, by designing an effective Integrated Pest Management plan for combating the decline in ironwood trees.

Chapter 1. Literature Review

1.1. Guam

Guam is the largest and southernmost of the Mariana islands present in Micronesia (Lazaro et al. 2020, Donnegan and Butler 2004, Young 1988, Safford 1905, Tracey et al. 1964). It is a territory of the United States (Rottman 2004). Guam is located at latitude 13°28' N and longitude 144°45' E (Tracey et al. 1964). The island is 48 kilometers in length, 4 to 18 kilometers in width, 160 kilometers in circumference with an area of 549 square kilometers and population density of 2,007 per square kilometers (Young 1988, Tracey et al. 1964). The highest mountain on the island is Mount Lamlam with an altitude of around 406 meters, which make Guam the third highest of the southern Mariana Islands (Young 1988).

The island originated about 70 million years ago and is comprised of two distinct parts (Donnegan and Butler 2004, Young 1988, Tracey et al. 1964). The northern half of the island is a smooth limestone plateau bordered by cliffs. On the plateau are three hills, Mount Santa Rosa and Mataguac Hill are volcanic outcroppings and Barrigada Hill is a limestone outcropping (Young 1988, Tracey et al. 1964). The southern half of the island is a result of volcanic eruptions. It is irregular and consists largely of igneous rock. Due to the porous limestone, the Northern part does not contain any streams (Young 1988, Tracey et al. 1964). However, numerous streams flow on the impermeable volcanic rocks of the southern half (Ward and Brookhart 1962). The soil of Guam is made up of three different parent materials, coralline limestone, residuum derived from tuff and tuff breccia, and water deposited coral sand (https://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx). Guam consists of two barrier

reefs named Cocos, and Luminao due to which the shoreline of the island has many beaches (Tracey et al. 1964).

The island of Guam experiences a tropical marine climate, which is warm and humid moderated by seasonal trade winds and a wet and dry season (Young 1988). Being a tropical island, Guam possesses a rich biodiversity that includes a variety of flora and fauna (Lazaro et al. 2020, Donnegan and Butler 2004, Safford 1905, Stone 1970). However, the biodiversity of Guam is under threat. Native species of Guam are being negatively impacted by invasive species such as cycad scale (*Aulacaspis yasumatsui*), Rhinoceros beetle (*Oryctes rhinoceros*), *Leucaena leucocephala*, *Vitex parviflora*, Brown Tree Snake *(Boiga irregularis)*, African tulip tree (*Spathodea campanulata*), among others (Lazaro et al. 2020, Moore and Smith 2008). Diseases have also caused threats to various plant species of Guam. Betal nut tree population were drastically reduced for a few years due to bud rot disease (Drenth 2004). The number of coconut trees in Guam has been depressed for decades due to Coconut Tinanga Viroid (Wall and Randles 2003). Since 2002, ironwood trees in Guam are also experiencing a sudden decline and the reasons and mechanisms behind this decline is still being investigated (Schlub 2013, Schlub et al. 2020).

1.2. Ironwood tree

Casuarina equisetifolia, also called ironwood, she-oak, horsetail, beefwood, Australian pine and 'gago', is spread across the island of Guam (Stone 1970, Fosberg et al. 1979, Diouf et al. 2009). Ironwood trees are known to be indigenous to Australia, Malaysia, Southeast Asia, and the Melanesian and Polynesian regions of the Pacific (Safford 1905, Swearing 1997). Ironwood trees frequently grow on disturbed sites such as beaches, land-fills, wetlands, roadsides, cleared land,

and vacant lots (Morton 1980, Elfers 1998). Hammock communities and swamps are described as its natural habitat (Binggeli 1997). Ironwood trees have been continually propagated in Guam for hundreds of years as confirmed by the records of soil pollen samples (Athens and Ward 2004). However, these trees do not compete with native tree species in the limestone forests (Moore 1973).

Ironwood trees belong to the Casuarinaceae family (Diouf et al. 2008, Chonglu et al. 2010). These trees are evergreen angiosperms (Diouf et al. 2008). The bark is brittle with reddish brown to grey color. Ironwood trees have thin needle-like, jointed, greyish green branchlets bearing the anatomical minute tooth-shaped leaves (Chonglu et al. 2010). The flower type of Guam's population is 80% monoecious, and 13% dioecious trees of which 3% are male (Schlub et al. 2011). The fruits have small and winged seed formed in brown cone-like woody clusters (Swearingen 2005). Healthy ironwood trees in Guam have an estimated life span of 45 to 90 years and may grow up to 24 m in height with a diameter at breast height of 92 cm (Schlub et al. 2020).

Ironwood trees are fast-growing trees that can grow at a rate of 1.5-3 meters per year (Swearingen and Plant Conservation Alliance 2005). The favorable annual temperature range for the tree is 22℃ to 27 ℃ and average rainfall requirement varies from 700 to 2000 millimeters (NRC US Advisory Committee on Technology Innovation 1980, Duke 1983, Snyder 1992). Since ironwood trees are salt-tolerant trees and can withstand partial water logging, they are found growing on marginal lands (Pinyopusarerk and House 1994). The trees are also tolerant of typhoon damage, due to which they can survive on the Mariana Islands where typhoons are frequent (Pinyopusarerk and House 1994). Ironwood trees also have an ability to fix atmospheric nitrogen by forming root

nodules with microbial associations (Diouf et al. 2008).

Ironwood trees are one of the most significant agro-forestry species of Guam (Elevitch and Wilkinson 2000). The trees are tightly integrated into the environment of the island because of their cultural, ecological and environmental importance. In Guam, the trees are commonly used as fuelwood, windbreak, shelterbelt and mulch. Ironwood trees are also used as ornamental plants along the roadside in urban areas. In other areas, ironwood trees have been found to be highly effective in controlling erosion, stabilizing sand dunes, reclaiming marshy soils and combating desertification (Vietmeyer 1986, Snyder 1992). In Guam, ironwood trees also serve as a perch for predatory birds (Chonglu et al. 2010, Schlub et al. 2020). Ironwood trees are an important perching tree for the white-collared Kingfisher (*Todiramphus chloris*) and Mariana Fruit Dove, *Ptilinopus roseicapilla* (Marshall 1949).

1.3. Ironwood tree decline

A forestry inventory in 2002 surveyed over 115,000 ironwood trees larger than 12.7 centimeters in diameter at breast height and reported that ironwood trees are among the healthiest tree species on the island (Donnegan and Butler 2004). The same report claimed the ironwood tree to be one of the eight species of tree growing larger than 28 cm in diameter. During the same year, a local farmer, Mr. Bernard Watson observed that his ironwood trees were dying prematurely (Schlub 2013). His trees were less than 10 years old and were planted in windbreaks. The trees exhibited two distinct kinds of symptoms. Within a group of 10 trees, 5 trees showed wilting symptoms with a rapid yellowing of foliage, tip burn of two lower branchlets and tree death within 6 months. The dead foliage often remained on the tree giving the tree a singed appearance (Mersha et al. 2009,

Mersha et al. 2010). The other symptom observed on the farm was a gradual and uniform thinning of foliage leading to lethal progressive dieback of branches. By the time the Natural Resources Personnel with Commander Navy Region Marianas became aware of trees dying in large numbers at the Naval station in 2004, approximately one-third of the trees at that place were dead. By 2005, the decline was widespread in Guam; hundreds of trees were dead, and thousands of trees exhibited symptoms of decline (Schlub 2013). The condition of foliage thinning and dieback on ironwood was referred to as Ironwood Tree Decline (IWTD) (Mersha et al. 2009, Mersha et al. 2010).

A five-day IWTD conference was held in January 2009. The participants visited fourteen sites to collect samples, reviewed literature regarding the tree and concluded that Guam's ironwood trees are not only dying from natural causes but are also in a state of the decline attributed to various biotic and abiotic factors. A survey of 1,427 ironwood trees from 44 sites was conducted to study the factors that could possibly have an impact on the tree (Schlub 2010). Factors such as disease severity, number of branches, circumference at breast height, density, fire damage, typhoon damage, presence of conk, presence of termites, latitude, longitude, altitude, and human management were studied (Schlub 2010). A statistical modeling technique known as logic model was applied to analyze the data. The trees were visually categorized based on branch fullness and extent of dieback symptoms. The decline severity (DS) was classified into discrete values from 0 to 4. A perfectly healthy tree with full foliage will have a decline severity value of 0. With a decrease in foliage on its branches the value of decline severity will increase; 0= symptomless, 1= slightly damaged, 2= distinctly damage, 3= heavily damaged, 4= nearly dead (Schlub 2010). Presence of termites, presence of conks, and level of human management were found significantly associated ($p < 0.01$) with decline severity of the tree (Schlub 2010). A tree having termites, conks,

or higher level of human management is more likely to have IWTD as compared to the tree without termites, without conks and less human management (Schlub 2010).

The bacterial pathogen *Ralstonia solanacearum* was discovered in the ooze and tissue samples of declining ironwood trees by Melody Putman using immunostrips (Agdia, Inc.) (Schlub et al. 2011). An additional survey conducted in 2012 confirmed the presence of *R. solanacearum* and *Klebsiella* spp. *(K. oxytoca, K. variicola)* in the ooze of diseased trees through Loop-mediated isothermal amplification (LAMP) primers developed by Kubota et al. (2011). Ayin et al. (2015) showed that strain of *R. solanacearum* isolated from ironwood trees in Guam is closest to *R. solanacearum* strain GMI1000.

Ralstonia solanacearum is an aerobic, motile, soil-borne and xylem-residing bacterium found on six continents which causes bacterial wilt disease of many crops (Yabuuchi et al. 1995). Over 50 dicot and monocot families are hosts of *R. solanacearum* (Yabuuchi et al. 1995, Hayward 1991, Denny 2007). The pathogen forms a heterogenous species complex that contains thousands of distinct strains. *Ralstonia solanacearum* can persist in soil or water reservoirs for long periods (Alvarez et al. 2008), and when in contact with a suitable host, it can enter the plant through wounds or lateral root emergence points (Denny 2007). Thereafter it colonizes the water-transporting plant xylem vessels and thrives there (Denny 2007, Xue et al. 2020). Massive production of exopolymeric substances likely contributes to the clogging of the xylem channels, leading to blockage of water transport, followed by symptoms like wilting leaves, stunted growth, stem discoloration, and death (Denny 2007, Xue et al. 2020).

The two species of *Klebsiella* isolated from ironwood trees, *K. oxytoca* and *K. variicola*, are

facultative anaerobic and non-motile bacteria (Brisse et al. 2021). *Klebsiella* species are among the bacteria that cause wetwood symptoms in the tree resulting in brown discoloration of the wood (Hartley et al. 1961, Jeremic et al. 2004). The central core of the tree is usually impacted when the tree is attacked by *Klebsiella* species (Schlub 2013). The bacteria are translocated by the xylem vessels of *C. equisetifolia* seedlings following wound inoculation (Schlub 2013). Along with *R. solanacearum* and *Klebsiella* spp. *(K. oxytoca, K. variicola)*, various other bacteria from genera such as *Pantoea*, *Enterobacter*, *Citrobacter*, *Erwinia*, and *Kosakonia*, have been isolated from the ooze of ironwood trees (Ayin et al. 2019). However, *R. solanacearum*, *Klebsiella oxytoca* and *K. variicola*, are consistently present in declining trees (Schlub 2013).

Hollowing of trunks, which is a symptom of termite attack was observed in ironwood trees under decline in Guam (Schlub 2013). Although termites were rarely observed on young trees and not all diseased ironwood trees showed termite damage, it was important to study their association with this problem since there is a possibility that termite attacks were accelerating the decline by causing feeding damage (Schlub 2013).

Initially, the termite species attacking ironwood trees of Guam were not identified (Schlub 2010?). In 1946, Light mentioned the presence of three termite species in Guam, *Cryptotermes domesticus* (Haviland), *Neotermes connexus* Snyder, and *Prorhinotermes inopinatus* Silvestri. In addition, previous reports of termite infestation in Guam considered the termites attacking structures in Guam as *Coptotermes formosanus* (Hromada 1970, Lai 1977, Su and Tamashiro 1987). Su and Scheffrahn (1998) conducted a termite survey in Guam and reported the presence of six species through morphological identification. These six termite species were *Coptotermes vastator* Light,

Cryptotermes dudieyi Bank, *Microcerotermes* sp., *Nasutitermes* sp., *Cryptotermes* sp., and *Prorhinotermes* sp. However, they did not find any *C. formosanus* specimens. A recent phylogenetic analysis has shown that the *C. vastator* specimens found in Guam by Su and Scheffrahn in 1998 are synonymous to *C. gestroi* termites (Yeap et al. 2007). In a recent study by Park et al. (2019), termites were collected from ironwood trees in Guam. Using morphology and DNA barcoding technique, termites were identified based on the closest match to GenBank sequence references as *Nasutitermes takasagoensis*, *Coptotermes gestroi*, *Microcerotermes crassus* and *Microcerotermes* species (Park et al. 2019).

1.4. Termites

1.4.1. General biology and evolution

Termites are social and cryptic insects and are considered as most efficient organism for degradation of lignocellulose. Termites belong to the order Blattodea, superfamily Blattoidea and epifamily Termitoidea (Inward et al. 2007, Zhang 2011). Termites are most abundant in tropical and subtropical climatic zones (Brune 2014, Arora et al. 2022). Termites prefer moist warm environments due to their soft cuticles (Bignell 2011). Nine families and more than 3000 different species of termites have been found around the world (Arora et al. 2022, Krishna et al. 2013). The termite colony consists of several morphologically and functionally distinct castes (Bignell 2011). Workers are wingless, usually blind, and sterile (except for family Hodotermitidae), helping the colony in constructing nests, in foraging for food and water, in digesting plant material with the aid of symbiotic gut microbes and feeding other members of the colony (Bignell 2011). Soldiers are also blind, sterile and are unable to feed themselves; however, they protect the colony from predators using the mandibular defenses or by chemical squirts (Bignell 2011). The reproductive

forms consist of the queens and kings as well as the nymphs and alates, from which they originate (Bignell 2011). In some termite species, if the primary reproductives die, they are replaced by neotenic reproductives. These neotenic reproductives can be nymphoids or ergatoids. The nymphoids or secondary supplementary reproductives are developed from nymphs and the ergatoids or tertiary supplementary reproductives are developed from workers (Bignell 2011, Yashiro and Matsuura 2014).

Around 150 million years ago, in the Late Jurassic period, some omnivorous cockroaches acquired flagellated protists, also called as flagellates, within their hindgut (Bignell et al. 2011, Brune 2014, Bourguignon et al. 2015). The presence of flagellates enabled that group of cockroaches to feed on wood (Bignell et al. 2011, Brune 2014, Bourguignon et al. 2015). These were the sub-social cockroaches from the Genus Cryptocercus (Brune and Dietrich 2015). Termites are the sister group of Genus Cryptocercus (Brune and Dietrich 2015, Arora et al. 2022, Lo et al. 2000). Descendance of termites from cockroaches has been confirmed by both morphological and molecular assessment (Inward et al. 2007a, Inward et al. 2007b). Like cockroaches, termites are hemimetabolous and have styli at the rear end of the abdomen (Harris 1957). Some wood-boring species of cockroaches consume decaying wood and require intestinal protozoa to digest the cellulose (Lo and Eggleton 2011). There are considerable similarities between the protozoa present in these cockroaches and the ones present in primitive termites (Krishna and Weesner 1969). Moreover, the ancestors of termites as well as wood feeding cockroaches form their oothecae internally and share the behavior of intraspecific coprophagy along with gregariousness (Inward et al. 2007). The nitrogen-recycling behavior of the termite is also inherited from its cockroach ancestors which consumes exuviae and performs cannibalism to obtain nitrogen (Nalepa 1994,

Raina et al. 2008, Chouvenc and Su 2012, Kakkar et al. 2016a, Moore 1969, Mullins and Su 2018). The termites that retained close similarities to wood-feeding cockroaches including cellulolytic flagellates are called “lower” termites (families Archotermopsidae, Mastotermitidae, Stolotermitidae, Kalotermitidae, Hodotermitidae, Stylotermitidae, Rhinotermitidae and Serritermitidae). About 60 million years ago, in the Eocene period, a termite lineage lost flagellates and the role of flagellates was replaced by fungi and bacteria (Ni and Tokuda 2013, Bignell et al. 2011, Arora et al. 2022, Otani et al. 2014, Aanen et al. 2002). These termites are called “higher” termites (family Termitidae). These “higher” termites are highly successful in survival as more than 80 percent of the current termite species belong to eight subfamilies (Apicotermitinae, Cubitermitinae, Foraminitermitinae, Macrotermitinae, Nasutitermitinae, Sphaerotermitinae, Syntermitinae and Termitinae) within the family Termitidae (Brune 2014).

1.4.2. Feeding preference of wood-feeding termites

All termite species consume lignocellulose that is present in cell walls of plants (Arora et al. 2022). Lignocellulose is the major compound in wood and most abundant biomolecule on earth (Arora et al. 2022, Brune 2014, Breznak and Brune 1994). Lower termites feed only on wood while higher termites have diversified their diets to include fungi, dung, and humus, along with wood (Brune 2014, Donovan et al. 2001, Eggleton and Tayasu 2001, Wood and Johnson 1986). Although wood-feeding termites are capable of eating and digesting any wood, it has been observed that termites can prefer to feed on one type of wood over the other. Various feeding preference experiments have been conducted to determine the preference or aversion of termites for a particular wood, when two or more than two types of woods are offered. Kofoid and Bowe (1934) suggested species of termite, type of wood, the moisture content of the wood, type and amount of extractives in

wood, difference between heartwood and sapwood, hardness of the wood and the extent of any previous attack by fungi or other insect, as factors determining preference of wood by termites. A review by Thorne (1998) also identified temperature, caste ratio of the colony, mortality rate and size of food source as the factors affecting wood preference of termites. Wolcott (1946, 1953) showed that termites tend to resist feeding on wood with high resin as well as high lignin content. Marchán (1964) depicted the impact of lignin, ash, and protein content of the wood on the termite preferences. *Reticulitermes flavipes* workers avoided wood with high hardness in a study performed by Behr et al. (1972). Many studies have shown the impact of fungi on the food preference of termites. Smythe et al. (1971) and Getty and Haverty (1998) showed preference of *C. formosanus* and *R. flavipes* workers for fungal decayed wood over non decayed wood. Studies have also shown that amount and stage of fungal decay also impact wood preference (Getty and Haverty 1998, Lenz et al. 1991).

1.4.3. Dual digestion system of termites and symbionts

Termites perform a complex process for the degradation of lignocellulose that they consume. Lignocellulose is difficult to digest. The lignocellulose microfibrils have glycosidic bonds and are connected by a network of hemicelluloses (Brune 2014). The cellulases access glycosidic bonds from chain ends or amorphous regions as the access of cellulases to the crystalline core is hindered by hemicelluloses. Presence of lignin hinders the digestion process (Brune 2014, Watanabe et al. 2010). For digesting the lignocellulose, the digestive enzymes produced by the termites on their own are not sufficient. Therefore, termites make associations with various microbes that assist termites in digesting the lignocellulose. These microbes can either be exosymbionts or endosymbionts. Exosymbionts help some termites in digesting the wood outside the body of these

termites (Makonde et al. 2013). Fungi act as exosymbiont for digestion in fungus-feeding termites (Poulsen et al. 2014, Otani et al. 2014). Endosymbionts are present exclusively in the termite hindgut and help termite workers in completing wood digestion after initial breakdown by the workers' own wood digesting enzymes in fore- and midgut (Nakashima et al. 2002a, Breznak and Brune 1994, Sethi et al. 2014, Peterson et al. 2015, Brune 2014). Since both the termites and the symbionts cannot survive without each other, these symbionts are called mutualistic obligate symbionts (Cleveland 1926, Brune 2014). The endosymbionts consist of bacteria, archaea and flagellates (Makonde et al. 2013, Ohkuma and Brune 2010, Arora et al. 2022). All termites possess bacteria and archaea while only lower termites have flagellates (Brune 2014). Cleveland proved the importance of flagellate symbionts for the survival of lower termites as absence of flagellates in the gut of termite caused death of termites due to starvation (Cleveland 1926, Breznak and Brune 1994, Brune 2014).

The wood ingested by termites is initially crushed into small pieces by mandibles and gizzard in foregut, thereby increasing the surface area of wood (Watanabe and Tokuda 2010, Brune 2014). In the midgut, endoglucanases begin the hydrolysis of cellulose to produce oligosaccharides. The endoglucanases are secreted by salivary glands in lower termites and by midgut epithelium in higher termites. The resulting oligosaccharides are cleaved to glucose by β-glucosidases. The digestion processes in foregut and midgut only achieve partial degradation of lignocellulose. The digestion of crystalline cellulose and hemicelluloses is accomplished by mutualistic symbionts present in hindgut with the help of glycosidic hydrolases such as exoglucanases, endoglucanases and β-glucosidases. The lignocelluloses are converted into polysaccharides, hydrogen and acetate. These products are eventually converted into short-chain fatty acids to be resorbed by the termite

(Watanabe and Tokuda 2010, Brune 2014, Brune and Dietrich 2015).

Lower termites have flagellates in hindgut that possess glycosidic hydrolases (exoglucanases and endoglucanases) and hemicellulases (xylanases, arabinosides, mannosidases) for digestion of lignocellulose to produce acetate, hydrogen and carbon dioxide (Bignell et al. 2011). The lactate and formate produced by flagellates within the hindgut during the digestion process is converted into acetate and carbon dioxide by bacteria. So far, around 500 different flagellates species have been identified (Song et al. 2021, Jasso-Selles et al. 2020, Inagaki et al. 2020). These flagellates belong to phylum Parabalasia and Class Oxymonadea of phylum Preaxostyla comprising 60-90 percent weight of termite worker's hindgut (Brune 2014). These cellulolytic flagellates also help termites in recycling and accessing nitrogen as well as in hydrogen cycling (Inoue et al. 2005, 2007, Cleveland 1926, Katzin and Kirby 1939, Ohkuma 2003, Sethi et al. 2014). The flagellates interact with bacteria present within the gut (Husseneder 2010). Habitat, feeding habits and caste of termites also determine the diversity of flagellates within them (Embley and Martin 2006, Bourguignon et al. 2015, Yamin 1979, Kitade and Matsumoto 1998).

In lower termites approximately twenty-nine bacterial phyla have been identified in termites, out of which around eleven are core phyla (Arora et al. 2022). For instance, *Reticulitermes flavipes* workers have Spirochaetes, Elusimicrobia, Firmicutes, Bacteroidetes, Proteobacteria and Fibrobacteres as major phyla and Verrumicrobia, Actinobacteria and Tenericutes as minor phyla. In *Coptotermes formosanus* workers, Bacteroidetes are the most dominant phylum, followed by Firmicutes, Spirochaetes, Actinobacteria, Proteobacteria and Tenericutes (Husseneder et al. 2010).

Higher termites lack flagellates and therefore cellulolytic bacteria in the hindgut secrete glycoside

hydrolases that help in lignocellulose degradation (Brune 2014). Wood-feeding higher termites have Spirochaetes as their dominant phylum followed by Fibrobacteres, Bacteroidetes and Firmicutes (Hongoh et al. 2006, Vikram et al. 2021). Fungus-feeding higher termites (family Termitidae, subfamily Macrotermitinae) live in symbiosis with fungi from phylum Basidiomycota (dominant in gut) and Ascomycota (dominant in nest) (Makonde et al. 2013, Moriya et al. 2005, Visser et al. 2009, Zhou et al. 2019). The digestion takes place in two cycles. In the first cycle, workers ingest plant litter along with fungal spores. The undigested feces are deposited on fungus gardens present in the nest. In the second cycle these termites consume the preprocessed plant fiber and nutrients are absorbed in the body (Brune 2014, Wood and Thomas 1989, Radek 1999, Poulsen et al. 2014, Otani et al. 2014). Phyla such as Bacteroidetes and Firmicutes are dominant in fungus-feeding termites. Other phyla such as Spirochaetes, Proteobacteria and Margulisbacteria are also present within these termites (Hongoh et al. 2005). Dung-feeding higher termites feed on lignocellulose which is partially humified. The humified lignocellulose has lower cellulose content and more nitrogenous products than wood (Brune 2014). Because of presence of high amounts of peptides in humus, these termites have highest abundance of phylum Firmicutes. Spirochaetes is the second dominant phylum within these termites, followed by phylum Bacteroidetes and Proteobacteria (Brune 2014, Thongaram et al. 2005).

Phylum Spirochaetes helps in reductive acetogenesis, fermentation, nitrogen fixation and acts as endosymbiont of flagellates (Warnecke et al. 2007, Breznak and Leadbetter 2006, Dröge et al. 2008). Bacteroidetes play an important role in lignocellulose degradation, nitrogen fixation and recycling (Ohkuma et al. 1996, Noda et al. 2006). Phylum Firmicutes is involved in lignocellulose digestion, nitrogen fixation, chitin and sugar degradation and acetogenesis (Poulsen et al. 2014,

Husseneder 2010). Phylum Margulisbacteria exploit phylum Spirochaetes as their hydrogen sinks (Utami et al. 2018).

Archaea are responsible for anaerobic methanogenesis in both lower and higher termites (Brune 2010, Hongoh and Ohkuma 2010, Leadbetter et al. 1998, Leadbetter and Breznak 1996, Ohkuma and Brune 2010, Paul et al. 2012, Shi et al. 2015). Sixteen families of Archaea have been identified in termite guts (Arora et al. 2022). Archaea produce methane from excess hydrogen generated during fermentation of polysaccharides (Purdy 2007). The utilization of excess hydrogen also helps flagellates in increasing efficiency of cellulose digestion. The diversity of archaea is higher in higher termites as compared to lower termites (Brune 2014, Paul et al. 2012). The methane produced during this process make termites a useful source for biofuel production (Sun and Chen 2010).

1.4.4. Methods for identification of symbionts

A plethora of microorganisms present within the termites are still uncultured and unknown. In order to identify these microorganisms, a wide range of techniques have been employed by the researchers. Methods such as microscopy, biochemical tests, immunological assays, among others, have been conventionally used to identify these microorganisms (Houpikian and Raoult 2002, Järvinen et al. 2009). Most of the conventional identification methods require culturing of these microorganisms on standard media under laboratory conditions, which rely on factors such as temperature, pH, and nutrition, as a result making it almost impossible to culture all the microbes present in a sample (Kamble et al. 2020, Stewart 2012). Only less than 1% of microorganisms present in nature can be cultured, while the rest of them are still unculturable (Solden et al. 2016,

Santos et al. 2020, Rashid and Stingl 2015). This drawback of conventional microbiological techniques poses major constraints to the study of microbial diversity.

Initially, Sanger sequencing was used to sequence the microbial communities (Sanger et al. 1977). But this sequencing technique required cloning of 16S rRNA gene after the amplification and could only allow the analysis of individual sequences, making the evaluation of the microbial communities in the environmental sample, a tedious and costly process. The introduction of Next Generation Sequencing technologies, within the last decade, revolutionized microbial diversity studies, as it has reduced the cost and increased the efficiency of DNA sequencing (Kircher and Kelso 2010, Goodwin and McCombie 2016, Mardis 2008). Different approaches can be utilized for the taxonomic and phylogenetic studies of microbiota in termite samples. In whole genome sequencing, the entire DNA sequence within an individual can be analyzed, including both coding and non-coding regions of DNA (Morris et al. 2021). Metagenomics involves analysis of the microbial DNA from environmental communities by sequencing and assembling the whole genomes of all the microbes present within the sample (Handelsman et al. 1998). Whole genome sequencing provides the broadest view of an individual's genome. It is used for detecting and analyzing variations within the individual, and for identifying the organism at a resolution lower than the species level (strain level identification). When single or multiple genes within the microbial genomes of the sample are targeted using specific primers, it is called amplicon sequencing or metataxanomics (Suenaga and Suenaga 2012). Amplicon sequencing is synonymous with DNA barcoding, which involves sequencing a standard region of DNA with variable regions called "barcodes" for taxonomic identification of the species (Arnot et al. 1993, Herbert et al. 2003). The process in which DNA barcoding is used to simultaneously identify

multiple species within an environmental DNA sample with the help of universal or group-specific primers is known as DNA metabarcoding (Creer et al. 2016). Sequencing in which a particular region of the genome is targeted provides higher coverage and throughput, takes lesser time and is less laborious than whole genome sequencing because of the small sequence read length. Targeted genome sequencing is mostly used for taxonomic profiling. Whole and targeted genome sequencing techniques do not provide sufficient information about the functional profile of the individual; for which transcriptomics, i,e., RNA sequencing to measure levels of gene expression, is used. Metatranscriptomics is a study of genes that are expressed by the microbial community within a given sample (Aguiar-Pulido et al. 2016).

Dubnau et al. (1965), and Woese (1987) had reported the significance of the 16S rRNA gene for studying prokaryotic taxonomy and phylogeny. Approximately 1500 nucleotides long 16S rRNA is present in 30S small ribosomal subunit of a prokaryotic cell along with a set of 21 proteins (Böttger 1989, Harmsen and Karch 2004). The 16S rRNA gene has a critical role in the initiation of protein synthesis in prokaryotic cells (Böttger 1989, Harmsen and Karch 2004). The unique characteristics of this gene makes it the most common genetic marker used for the taxonomic profiling of prokaryotes (bacteria and archaea) (Santos et al. 2020, Janda et al. 2007, Patel 2001). The 16S rRNA gene sequence is ubiquitous, highly conserved in all prokaryotic organisms, and contains multiple hypervariable regions (Amit 2014, Woese and Fox 1977). The presence of conserved regions makes this gene suitable for designing universal primers, while the variable regions (V1-V9) help in taxonomic identifications (Gutell et al. 1993, Clarridge 2004, Gray et al. 1984, Relman 1999, Baker et al. 2003). Because of the presence of variable regions within the gene, it is not mandatory to sequence the full-length16S rRNA gene; as depending on the purpose

of the study and the organism being studied, an individual or combination of variable regions can be chosen (Suenaga and Suenaga 2012).

The different sequencing platforms produce reads of different base pair lengths and can be used for different purposes based on the requirements of the research. Short read sequencing platforms such as SOLiD (Valouev 2008), 454 pyrosequencing Roche (Margulies 2005), Ion Torrent's PGM (Thermo Fisher) (Rothberg 2011), Illumina (Bennett 2004), as well as long read sequencing platforms Pacific Biosciences (PacBio) (Eid 2009), and Nanopore (Kono and Arakawa 2019), produce millions of reads in a single sequencing run. Illumina is the most popular high throughput sequencing technology for short length reads (Goodwin and McCombie 2016, Escobar-Zepeda et al. 2015, Kumar et al. 2019, Sandmann et al. 2017). However, output of 50 bp to 300 bp sequence length by Illumina does not allow whole 16S rRNA gene sequencing; due to which the variable regions within the gene are mostly utilized for 16S gene analysis with Illumina (Wommack et al. 2008).

However, the sequence output from next generation sequencing techniques could contain some erroneous reads, significant variation among the quality scores of sequencing reads, and deviation from desirable library fragment sizes (Leasi et al. 2018, Zinger et al. 2019, Weiss et al. 2017). These issues can lower the quality of the raw data for downstream analyses. To overcome these issues, various types of analysis tools can be used. These tools can filter out reads with lower quality scores and remove less abundant and spurious sequences. The sequences of 16S rRNA gene regions generated after metabarcoding can be analyzed using open-source pipelines such as MOTHUR (Schloss et al. 2009), MG-RAST (Keegan et al. 2016), and QIIME2 (Caporaso et al.

2010), which involve steps such as demultiplexing, denoising, taxonomic analysis, phylogenetic analysis, and diversity analysis.

QIIME2 ('Quantitative Insights Into Microbial Ecology') is a next-generation microbiome bioinformatics platform that is used for analyzing and interpreting the raw sequencing reads (Caporaso et al. 2010). This pipeline is developed to enhance the reproducibility of the results obtained during a study. QIIME2 deciphers the results of sequencing data by performing quality-filtering, sequence alignment, phylogenetic tree formation and taxon-based diversity analysis (Bolyen et al. 2019). Since QIIME2 is based on plugin architecture, it incorporates other programs such as DADA2 (Callahan et al. 2016), BLAST (Camacho et al. 2008), MAFFT (Katoh et al. 2002), FastTree 2 (Price et al. 2010), among others, to contribute to the assessment. The interactive visualization tools (QIIME 2 View) provided by this pipeline allows interpretation of data. Output of QIIME2 is presented in the form of amplicon sequence variants (ASVs) in which 100% identical sequence reads are combined into unique representative sequences (Edgar 2016).

Chapter 2. Taxonomic Profiling and Diversity Analysis of Bacterial Communities of *Nasutitermes takasagoensis* Workers Associated with Ironwood Trees (*Casuarina equisetifolia*) in Guam

2.1. Introduction

Casuarina equisetifolia, the ironwood tree, has been a part of the natural ecosystem of Guam for thousands of years (Athens and Ward 2004, Stone 1970, Fosberg et al. 1979, Elevitch and Wilkinson 2000). The tree can be found all around the island including forests areas, beaches, and road shoulders (Mersha et al. 2009). The ironwood tree has remarkable properties and utilities ranging from being an excellent wind breaker, mulch, fuel wood and construction wood to contributing to land reclamation and soil fertility improvement by reducing soil erosion and fixing atmospheric nitrogen (Pinyopusarerk and House 1993, Touati et al. 2016, Conglu et al. 2010, Diouf et al. 2009, Mersha et al. 2009, Schlub 2013). This evergreen angiosperm grows very fast and has an ability to grow to as large as 92 cm in diameter (2002 forest resources report, Donnegan et al. 2004). All these qualities make the ironwood tree one of the most significant agroforestry species of Guam.

However, in 2002, a sudden death of ironwood trees in the field of a local farmer in Guam was reported (Mersha et al. 2009). The trees that were dying showed symptoms such as yellowing and thinning of foliage, dieback, and droplets of ooze and areas of wetwood in the cross-section. The ironwood trees exhibiting these symptoms are unable to recover and gradually die over a course of several years. This condition has been referred to as Ironwood Tree Decline (IWTD) in Guam (Mersha et al. 2009, Mersha et al. 2010, Schlub et al. 2011, Schlub 2013). Over the past two decades, the number of locations in Guam where IWTD occurs appears to have changed little since

the peak of IWTD 15 years ago (Schlub et al. 2020). Tree death continues at these IWTD hotspots, but at a slower rate than in years past (Schlub et al. 2020).

Ralstonia solanacearum, a pathogen known to cause bacterial wilt (Yabuuchi et al. 1995, Smith 1896), was detected in the wood drill shavings and ooze from ironwood trees under decline using immunodiagnostic strips (Agdia, Inc.) (Mersha et al. 2010, Schlub et al. 2011, Schlub 2013). A strain of *R. solanacearum* was isolated from cultures obtained from bacterial ooze of infected ironwood trees and was shown to cause wilting symptoms in healthy ironwood seedlings (Ayin et al. 2013, 2015, Paudel 2020). Two species from genus *Klebsiella* (*K. oxytoca* and *K. variicola*), that cause wetwood symptoms in plants (Hartley et al. 1961, Jeremic et al. 2004), were also isolated from bacterial ooze of infected ironwood trees and were regarded as likely opportunistic pathogens for IWTD (Ayin et al. 2015, Ayin et al. 2019). *Ralstonia solanacearum* and wetwood bacterial species *Klebsiella* spp. were consistently isolated from bacterial ooze of infected ironwood trees (Schlub 2013).

The *Ralstonia* and *Klebsiella* spp. penetrate the tree through root wounds. Once inside, they travel through the roots water-transport xylem vessels into the tree's stem (Denny 2007, Schlub 2013). A major difference between the wilt pathogen Ralstonia and the wetwood bacteria such as Klebsiella is the fact that Ralstonia colonizes a tree's sapwood and young xylem vessels (roots, stem, and branches) which ultimately leads to clogging the vessels and wilting of the host, whereas Klebsiella growth is limited in young xylem vessels and does not appear to influence the transport of water (Schlub 2013, Schlub et al. 2020). The highest quantity of wetwood bacteria are found in the tree's heartwood where xylem cells are compacted, filled with resins, and no longer transport

water (Schlub 2013, Schlub et al. 2020). Along with *Ralstonia* and *Klebsiella*, bacterial isolates from infested ironwood trees in Guam included also bacteria from the genera *Kosakonia*, *Enterobacter*, *Pantoea*, *Erwinia* and *Citrobacter* (Ayin et al. 2019).

One of the principal modes of transmission of plant diseases caused by pathogens are insect vectors (Agrios 2008, Harris and Maramorosch 1980, Heck 2018, Cilia et al. 2011, Wielkopolan et al. 2021, Perilla-Henao and Casteel 2016, Ghosh et al. 2017). Plant pathogens can either be dependent on an insect host for their survival or an insect can be just a medium for the spread of that pathogen from one plant to another. Insects can transmit plant pathogens externally, e.g., when pathogens stick to the mouthpart or body of the insect, as well as internally by carrying them inside their digestive tract where the plant pathogen may or may not multiply (Agrios 2008, Gedling et al. 2018). All plant pathogenic viruses, phytoplasmas, and some fungi, bacteria, protozoa, as well as nematodes are internally transmitted by insects while some fungi and bacteria can also be transmitted externally on the insect bodies (Agrios 2008, Perilla-Henao and Casteel 2016).

Various insects including beetles, gall wasp and termites have been found attacking ironwood trees in Guam (Mersha et al. 2009). The presence of beetles (*Protaetia pryeri* (Janson) and *Protaetia orientalis* (Gory and Percheron) on ironwood trees was found not to be associated with IWTD (Campora 2005). The gall wasp (genus *Selitrichodes*) that damages the tip of the branchlets on ironwood trees was also found unlikely to be associated with IWTD as the symptoms of IWTD initiate from the roots (Schlub 2013). A survey of 1,427 ironwood trees across the island determined presence of termites on the ironwood trees as a significant factor associated with IWTD (Schlub 2010, Schlub et al. 2011). Heavy infestations of termites on declining ironwood trees have

been observed (Mersha 2009, Schlub 2013). Symptoms of termite attack such as tunnelling, hollowing of trunk, and presence of conspicuous nests have been commonly found on ironwood trees in Guam. The termites attacking ironwood trees in Guam were identified as *Nasutitermes takasagoensis* (Nawa) (Blattodea: Termitidae), *Coptotermes gestroi* (Wasmann) (Blattodea: Rhinotermitidae), *Microcerotermes crassus* Snyder (Blattodea: Termitidae) and another *Microcerotermes* species by the closest match of their DNA barcodes to reference sequences in NCBI GenBank (Park et al. 2019). Over 90% of ironwood trees in Guam that had an infestation with live termites were attacked by a species from the *Nasutitermes takasagoensis* species complex (Park et al. 2019).

As a member of so-called "higher" termites, *Nasutitermes takasagoensis* (Family Termitidae) workers harbor a mutualistic hindgut microbiome constituted by prokaryotes (Inward et al. 2007, Brune 2014, Köhler et al. 2012). The core bacterial phyla present in *Nasutitermes* spp. include Spirochaetaes, Fibrobacteres, Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Margulisbacteria (TG3 phylum) (Rossmassler et al. 2015, Vikram et al. 2021, Köhler et al. 2012, Hongoh et al. 2006, Miyata et al. 2007, Warnecke et al. 2007). Most of the bacteria present within the termites are obligate symbionts because they are important for the termite colonies' survival and reproduction (Hongoh et al. 2008, Brune 2014). Meanwhile termite bodies can also harbor transient bacteria from the environment that are not always present in their gut. These environmental bacteria can enter the termite gut with consumed plant matter or attach to the surface of the body integument whereby they can be ingested through grooming. Survival of environmental bacteria in the gut of termites depends on whether these bacteria are adapted to the conditions in the termite gut determined, for example, by host species, diet, pH, and O_2 and H_2

gradient (Mikaelyan et al. 2017, Husseneder et al. 2009, Rahman et al. 2015, Tai et al. 2015). If termites come in contact or ingest wood infested with the pathogens associated with IWTD, these pathogens could stick to the body surface or enter the termite bodies. which might cause termites to spread these pathogens to other trees while foraging. Thus, it was hypothesized that members of *N. takasagoensis*, which represents the major termite species associated with IWTD, are vectors for pathogenic bacteria causing IWTD. To test this hypothesis termite samples were collected from healthy and sick ironwood trees that were negative or positive for Ralstonia infestation in Guam in order to (1.) describe the bacteria taxa associated with workers of *N. takasagoensis* attacking ironwood trees in Guam to test if termites carry plant pathogens, (2.) test for relations between the tree-, plot-, and location-related factors associated with ironwood trees attacked by *N. takasagoensis* workers and microbial diversity of those *N. takasagoensis* worker samples, (3.) determine if *N. takasagoensis* termites prefer feeding on parts of ironwood trees with low pathogen content compared to high pathogen content, and (4.) determine whether *R. solanacearum* bacteria are ingested and survive in the termite gut.

2.2. Materials and methods

2.2.1. Samples and metadata

2.2.1.1. Termite samples

Forty-two *N. takasagoensis* termite samples were collected from ironwood trees in 2019-20 on the island of Guam by the team of the University of Guam (Figure 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. Diagnostic characters of each soldier were examined visually under a stereo microscope (Leica MZ16) using

the published keys such as Chhotani (1997) and Liang and Li (2016) for morphological species identification. Tree data was recorded for each termite sample. This included information on the tree-related factors (Table 1), on the plot-related factors (Table 2), and on the location-related factors (Table 3).

2.2.1.2. Tree-related factors

Presence of Ralstonia: Each tree from which termite samples were collected, was assayed by lab members at the University of Guam for *R. solanacearum using R. solanacearum*-specific immunodiagnostic test kits manufactured by Agdia, Inc. of Elkhart Indiana. U.S.A. (Ayin et al. 2015). This kit screens plant/bacterial culture samples for *R. solanacearum* using an antigen-antibody based test. The kit contains ImmunoStrips® and sample bags containing BEB1 buffer. Upon successful completion of the reaction, two lines consisting of a control line and a test line can be seen. Presence of both the lines within 30 minutes at any intensity, was considered a positive indication of the presence of Ralstonia in the sample. However, this test does not measure concentration of Ralstonia.

Decline Severity (DS): The ironwood trees in Guam were categorized into five categories based on the level of damage and fullness of branches at the time termites were sampled. This categorization was performed based on visual inspection described by Schlub et al. (2010). Trees with no disease symptoms (symptomless) were given a DS value of 0, trees that were showing a few symptoms (slightly damaged) were given a DS value of 1, trees with visible disease symptoms (distinctly damaged), were given a DS value of 2, trees that were showing severe disease symptoms (heavily damaged) were given a DS value of 3 and trees that had shed almost all their leaves and

were about to die (nearly dead) were given a DS value of 4 (Schlub 2010).

Tree Health: Assessment of ironwood trees decline was further simplified by categorizing trees as healthy or sick based on their DS values. Symptomless trees (DS = 0) were categorized as healthy while those with symptoms (DS = 1 through 4) were categorized as sick.

2.2.1.3. Plot-related factors

Plot Average DS: A "plot" was described as the circular area with a radius of ≈30m, with a tagged termite sample tree at its center. The Plot Average DS was determined by, first, adding the DS values of all the live trees within the plot and dividing the sum by the number of live trees within that plot. Average DS = $(DS_1 + DS_2 + \cdots DS_n) / n$ (where "n" = total number of live trees within plot).

Plot Average Decline: The average decline of the plot was determined by the percentage of sick trees relative to the total number of live trees in the plot.

Proportion of Dead Trees in Plot was determined by dividing the number of dead trees by the total number of trees within a 30m plot radius.

Proportion of Trees with Termites in Plot was determined by dividing the number of live trees with termite activity within a given plot by the number of live trees. The termite activity was determined based on the presence of termite nests or tunnels on the tree.

2.2.1.4. Location-related factors

Geographical location was the name of the area (village in Guam) in which the ironwood tree was

located from which a termite sample was taken.

Altitude was measured from the base of the tree with respect to mean sea level (in meters).

Altitude Classification: An altitude less than 100 meters was classified as “low” and altitude greater than 100 meters was classified as “high”.

Parent Material refers to unconsolidated, relatively unweathered minerals or organic matter from which soil at the tree location developed. The information regarding parent material was retrieved using Soils section of Natural Resources Conservation Service by United States Department of Agriculture (USDA) (https://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx). The parent material coralline limestone is referred to as “lime”, residuum derived from tuff and tuff breccia is referred to as “tuff” and water deposited coral sand is referred to as “sand”.

Site Management describes the condition of the site in terms of the level of human management. If a site was not managed at all, for example an abandoned lot, it was categorized as not managed. Sites such as farm wind-rows were categorized as moderately managed and well-managed sites, such as golf courses; were categorized as highly managed.

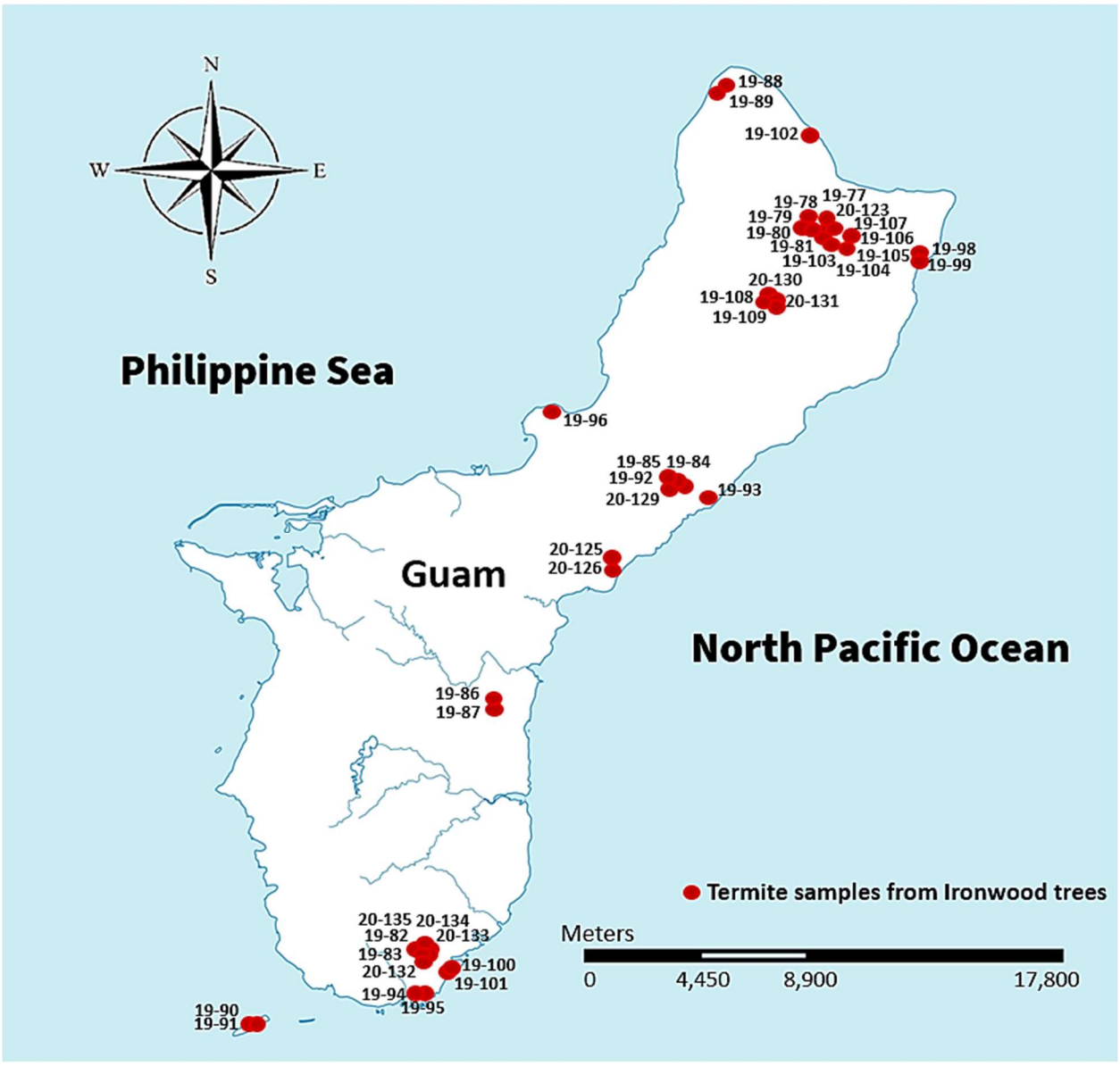

Figure 1. Map of ironwood tree sites where 42 samples of *Nasutitermes takasagoensis* termites were collected. Tree metadata for each termite sample can be found in Table 1, 2, and 3.

Table 1. Tree-related metadata for *Nasutitermes takasagoensis* termite samples.

Termite sample ID	Presence of Ralstonia	Tree Decline Severity	Tree Health
19-77	Positive	heavily damaged	Sick
19-78	Negative	nearly dead	Sick
19-79	Negative	distinctly damaged	Sick
19-80	Negative	symptomless	Healthy
19-81	Negative	slightly damaged	Sick
19-82	Positive	heavily damaged	Sick
19-83	Negative	nearly dead	Sick
19-84	Negative	symptomless	Healthy
19-85	Positive	nearly dead	Sick
19-86	Negative	nearly dead	Sick
19-87	Positive	slightly damaged	Sick
19-88	Negative	slightly damaged	Sick
19-89	Negative	symptomless	Healthy
19-90	Negative	distinctly damaged	Sick
19-91	Negative	slightly damaged	Sick
19-92	Positive	slightly damaged	Sick
19-93	Negative	symptomless	Healthy
19-94	Negative	nearly dead	Sick
19-95	Negative	slightly damaged	Sick
19-96	Negative	slightly damaged	Sick
19-98	Negative	heavily damaged	Sick
19-99	Negative	slightly damaged	Sick
19-100	Negative	symptomless	Healthy
19-101	Negative	slightly damaged	Sick
19-102	Negative	symptomless	Healthy
19-103	Negative	slightly damaged	Sick
19-104	Negative	symptomless	Healthy
19-105	Negative	slightly damaged	Sick
19-106	Negative	slightly damaged	Sick
19-107	Negative	symptomless	Healthy
19-108	Negative	symptomless	Healthy
19-109	Negative	slightly damaged	Sick
20-123	Positive	heavily damaged	Sick
20-125	Positive	nearly dead	Sick
20-126	Positive	heavily damaged	Sick
20-129	Positive	slightly damaged	Sick
20-130	Positive	nearly dead	Sick
20-131	Positive	nearly dead	Sick
20-132	Positive	nearly dead	Sick
20-133	Positive	nearly dead	Sick
20-134	Positive	nearly dead	Sick
20-135	Positive	nearly dead	Sick

Table 2. Plot-related metadata for *Nasutitermes takasagoensis* termite samples.

Termite sample ID	Plot Average DS	Plot Average Decline	Proportion of Dead Trees in Plot	Proportion of Trees with Termites in Plot
19-77	2	0.42	0.5	0.33
19-78	0.47	0.03	0.03	0.39
19-79	1.5	0.5	0	0.5
19-80	0.91	0.09	0.03	0.66
19-81	1.29	0.29	0	0.62
19-82	3	1	0.39	0.48
19-83	3.36	1	0.26	0.63
19-84	0.88	0.12	0.03	0.56
19-85	1.98	0.55	0	0.4
19-86	4	1	0	1
19-87	1	0	0	1
19-88	0.9	0.3	0	1
19-89	1.07	0.26	0.07	0.63
19-90	1.08	0.29	0.08	0.22
19-91	0.86	0.14	0.08	0.26
19-92	2.1	0.68	0.11	0.6
19-93	0.83	0.13	0.06	0.45
19-94	0.33	0.03	0.08	0.16
19-95	0.59	0.12	0.09	0.17
19-96	0.88	0.16	0.09	0.51
19-98	0.8	0.2	0	0.6
19-99	3	1	0.14	0.71
19-100	1.06	0.33	0.1	0.6
19-101	0.84	0.2	0.13	0.39
19-102	0.82	0.21	0.08	0.22
19-103	1.54	0.38	0.26	0.29
19-104	0.92	0.04	0.16	0.19
19-105	0.92	0.14	0	0.33
19-106	1.06	0.2	0.03	0.36
19-107	1.21	0.55	0.08	0.08
19-108	0.44	0.04	0.17	0.57
19-109	0.67	0.11	0	0.57
20-123	1.62	0.31	0.38	0.24
20-125	2	0.77	0.13	0.27
20-126	4	1	0	1
20-129	1.65	0.51	0.1	0.66
20-130	2.2	0.7	0.09	0.36
20-131	2.2	0.7	0.09	0.36
20-132	3.4	1	0.29	0.62
20-133	3.07	1	0.38	0.54
20-134	3	0.89	0.61	0.26
20-135	2.9	1	0.58	0.21

Table 3. Location-related metadata for *Nasutitermes takasagoensis* termite samples.

Termite sample ID	Geographical Location	Altitude (m)	Altitude Classification	Parent Material Classification	Site Management
19-77	Watsons Farm Yigo	170	High	Lime	Moderate
19-78	Watsons Farm Yigo	164	High	Lime	Moderate
19-79	Watsons Farm Yigo	171	High	Lime	Moderate
19-80	Watsons Farm Yigo	161	High	Lime	Moderate
19-81	Watsons Farm Yigo	168	High	Lime	Moderate
19-82	UOG Ija Station	96	Low	Tuff	Moderate
19-83	UOG Ija Station	92	Low	Tuff	Moderate
19-84	Mangilao Golf Course	129	High	Lime	High
19-85	Mangilao Golf Course	127	High	Lime	High
19-86	Mangilao Golf Course	117	High	Tuff	High
19-87	Mangilao Golf Course	126	High	Tuff	High
19-88	Ritidian	12	Low	Sand	Moderate
19-89	Ritidian	21	Low	Sand	Moderate
19-90	Cocos Island	20	Low	Sand	None
19-91	Cocos Island	12	Low	Sand	None
19-92	Mangilao Golf Course	124	High	Lime	Moderate
19-93	Thousand Steps	17	Low	Lime	None
19-94	Ysrael Beach	5	Low	Sand	None
19-95	Ysrael Beach	7	Low	Sand	None
19-96	Sagan Kotturan Chamoru	45	Low	Lime	None
19-98	AAFB	164	High	Lime	High
19-99	AAFB	160	High	Lime	Moderate
19-100	Duenas Beach	11	Low	Sand	Moderate
19-101	Duenas Beach	12	Low	Sand	Moderate
19-102	Tarague Beach	21	Low	Sand	High
19-103	Watsons Farm Yigo	168	High	Lime	None
19-104	Watsons Farm Yigo	169	High	Lime	None
19-105	Watsons Farm Yigo	173	High	Lime	None
19-106	Watsons Farm Yigo	163	High	Lime	None
19-107	Watsons Farm Yigo	171	High	Lime	None
19-108	UOG Yigo Station	178	High	Lime	Moderate
19-109	UOG Yigo Station	142	High	Lime	Moderate
20-123	Watsons Farm Yigo	163	High	Lime	Moderate
20-125	UOG Mangilao	67	Low	Lime	None
20-126	UOG Mangilao	81	Low	Lime	High
20-129	Mangilao Golf Course	129	High	Lime	High
20-130	UOG Yigo Station	173	High	Lime	Moderate
20-131	UOG Yigo Station	173	High	Lime	Moderate
20-132	UOG Ija Station	96	Low	Tuff	Moderate
20-133	UOG Ija Station	93	Low	Tuff	Moderate
20-134	UOG Ija Station	82	High	Tuff	Moderate
20-135	UOG Ija Station	110	High	Tuff	Moderate

2.2.2. DNA extraction, primer selection and Illumina sequencing

Total DNA was extracted from the termite workers received from Guam in 95% EtOH using the DNeasy Blood & Tissue kit (Qiagen, Germantown, MA). Five workers per sample were pooled and homogenized in lysis buffer (Buffer AL, DNeasy Blood & Tissue kit) using a sterile pestle (Thermo Fisher Scientific, Wilmington, DE). The concentration of DNA was confirmed using an Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE) with the Qubit dsDNA BR Assay Kit (Invitrogen™, Life Technologies™) and 2.5 µl/ng DNA per sample was shipped to the University of New Hampshire for next-generation sequencing.

To select the most informative region for detecting and separating Ralstonia strains by their DNA sequence, 130 16S rRNA gene full-length (1.4k) sequences of different *Ralstonia solanacearum* strains were retrieved from NCBI GenBank, aligned using Geneious Prime (Version 2022.2.1) software and examined for variability among different V-regions (V1-V3 and V4 regions). The V1-V3 region had 87.5% identical sites and V4 region had 93.5% identical sites. The V1-V3 region showed more variability and was selected for next generation sequencing.

Library preparation and Illumina sequencing were performed at the University of New Hampshire Hubbard Center for Genome Studies. The V1-V3 hyper variable region of the bacterial 16S rRNA gene was amplified from the genomic DNA samples using one forward (27F) and two reverse primers (519Rmod and 519Rmodbio) to capture a broad range of biodiversity (Thomas et al. 2019, Miya et al. 2015). Successfully amplified PCR products were sequenced on the 2x250bp Illumina NovaSeq platform following Illumina Nextera Dilute library protocol (Illumina, San Diego, CA).

2.2.3. Bioinformatics and statistical analysis

The Quantitative Insights into Microbial Ecology (QIIME2) pipeline (Caporaso et al. 2010, Estaki et al. 2020) version 2021-4, accessible on a server of the Hubbard Center for Genome Studies at the University of New Hampshire, was used to perform sequence data analysis. Demultiplexed sequencing reads were obtained from University of New Hampshire after Illumina sequencing in FASTQ format. Demultiplexing involves sorting the reads to the samples to which they belong using barcode sequences. These barcodes are sequences of base pairs that are attached uniquely to each sample before combining the samples for sequencing during library preparation. The downstream analysis was started with visualizing the demultiplexed reads using the demux plugin in QIIME2. The DADA2 plugin in QIIME2 was used for denoising of single-end and paired-end reads (Callahan et al. 2016). All demultiplexed reads had Phred quality scores >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 sufficiently overlapping, paired ends were not merged, and only forward reads were subjected to denoising and chimera removal using the DADA2 algorithm. A table containing amplicon sequence variants (ASVs), i.e., unique sequences or "bacterial strains" and a representative sequence file showing sequences of all the ASVs were obtained as a result of the DADA2 procedure. Sequences from the same sample generated with two different reverse primer sets were merged. All the sequences used in this study were submitted to GenBank (BioProject ID PRJNA883256).

Rarefaction is a process in which samples are sub-sampled to equal sample size to allow comparison independent of sample size. The sequencing process typically results in different total sequencing depth (number of reads) among samples. Therefore, rarefaction was employed to

subsample sequence reads without replacement to the common sequencing depth that was equal to the sample with the lowest sequencing depth (50,277) so that samples can be compared at the same sequencing depth for diversity analysis. Alpha rarefaction curves showing alpha diversity as a function of the sequencing depth (number of resampled sequences) were plotted using alpha-rarefaction method of the qiime diversity plugin. Three different alpha diversity indices were measured. The ASV richness index measures number of ASVs. Phylogenetic distance between the ASVs was computed using Faith's Phylogenetic Diversity (PD) index (Faith 1992). The Shannon diversity index scales the richness of ASVs based on their evenness (Shannon and Weaver 1949). The leveling of the alpha rarefaction curves signifies that sequencing depth used for the analysis was enough to capture most of the diversity present within the samples. Along with sequencing depth, samples were also compared based on equal sampling size and 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). The iNEXT package uses Hill numbers to represent the effective diversity present in a sample (Chao et al. 2014, Hsieh et al. 2016). The effective diversity was described as "the number of equally abundant species that would be needed to give the same value of a diversity measure" (Chao et al. 2014). Hill numbers are a class of diversity measures (parameterized by q) that quantify effective diversity by integrating both relative abundance and richness (MacArthur 1965, Chao et al. 2014, Hill 1973). In general, according to Chao et al. (2014) species richness is measured at q=0, which quantifies number of species, Shannon diversity (q=1) measures the effective number of species by counting species in proportion to their abundance and Simpson diversity (q=2) measures the effective number of dominant species. In this study, Hill numbers i.e., ASV richness (q = 0),

Shannon diversity (q = 1), and Simpson diversity (q = 2) were used to estimate the effective diversity of bacteria strains (ASVs) within the samples (Chao et al. 2014, Hsieh et al. 2016). To obtain sample-based rarefaction curves, effective diversity was plotted against the number of samples and to obtain coverage-based rarefaction curves, effective diversity was plotted against estimated coverage. Both sample-, and coverage-based rarefaction curves were also extrapolated to compute the effective diversity when sample size was doubled.

The SILVA reference database was used for taxonomic assignment of ASVs. SILVA (http://www.arb-silva.de) is a public database that contains aligned ribosomal RNA sequence data from Bacteria, Archaea and Eukaryota which has been quality checked (Quast et al. 2013). The BLAST algorithm (Camacho et al. 2009) was used to taxonomically classify ASVs obtained after denoising based on the sequences in SILVA 132 reference database (Quast et al. 2013). The pairwise identity cutoff of 97% was used for the purpose of taxonomic assignment of the reads. All the ASVs that showed less than 97% sequence identity to the reference sequences in the SILVA database remained unassigned and were filtered from the ASV table using filter-table method in qiime taxa plugin. From the filtered ASV table, bar plots showing relative abundances of taxa within each sample were generated using barplot method in qiime taxa plugin. Using the BLAST+ algorithm, the top 20 ASVs according to total number of reads across all the samples were assigned to the top BLAST hit in the NCBI GenBank database (Benson et al. 2015) to obtain more detailed taxonomic assignment as GenBank contains more sequence reads albeit less carefully curated than SILVA database. The MAFFT method (Katoh et al. 2002) was used for multiple alignment of sequences and the highly variable positions from the alignment were filtered out using mask command (Lane 1991). From these aligned and masked sequences, a midpoint-rooted phylogenetic

tree was generated (Price et al. 2010).

Alpha diversity represents the taxa and their abundances within samples while beta diversity represents the difference in diversity among groups of samples. Incidence-based (ASV richness, Faith's PD) and abundance-based (Pielou's evenness, Shannon diversity) alpha diversity indices were calculated using core-metrics-phylogenetic, alpha-correlation, and alpha-group-significance method of qiime diversity plugin in QIIME2 at the lowest sequencing depth of 15,951 common to all samples obtained after filtering out the unassigned ASVs. ASV richness measures number of ASVs present within a sample (DeSantis 2006), Faith's PD measures length of phylogenetic tree branches of all ASVs present within a sample (Faith 1992), Pielou's evenness measures relative evenness of ASVs present within a sample (Pielou 1966), and Shannon diversity calculates diversity based on richness and evenness of ASVs present within a sample (Shannon and Weaver 1949). Kruskal-Wallis ANOVA (H) (Kruskal and Wallis 1952), followed by false discovery rate correction using Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) was used to determine the group significance for factors with categorical data, i.e., Presence of Ralstonia, Tree DS, Tree Health, Location, Altitude Classification, Parent Material, and Site Management (Table 1, and 2). Spearman rank (rs) tests were used to assess correlations for factors with numerical data, i.e., Altitude, Plot Average DS, Plot Average Decline, Proportion of Dead Trees in Plot, and Proportion of Trees with Termites in Plot (Table 1, 2, and 3).

Beta diversity, i.e., the differentiation of the microbial composition between termite samples grouped by factors, was analyzed at 999 permutations using Permutational Multivariate Analysis of Variance (PERMANOVA) based on the weighted Unifrac distance metric which takes

abundance and phylogenetic distance into account (Anderson 2001, Lozupone and Knight 2005). PERMANOVA is a non-parametric multivariate statistical test. It calculates the distance between two groups by comparing the F value of data obtained after random permutation to the original F value (Anderson 2001). The PERMDISP test at 1000 permutations was performed to ensure the homogeneity of variance of the bacteria composition among groups of termite samples determined by multivariate spread. It compares dispersion within groups using F test. Homogeneity of variance is an important assumption of PERMANOVA-based tests because being robust tests, they do not take dispersion of the samples into consideration while calculating differences in beta diversity. After testing the significance of each factor for beta diversity using one-factorial PERMANOVA tests, the multifactorial PERMANOVA test, also known as ADONIS (Anderson 2001) was used for the factors that were found significant, to confirm their significance and determine the interactions between these factors. The beta diversity was calculated using core-metrics-phylogenetic, beta-correlation, beta-group-significance, and ADONIS method of qiime diversity plugin in QIIME2 at the lowest sequencing depth of 15,951 common to all samples obtained after filtering out the unassigned ASVs. The results of ADONIS test were visualized as non-metric Multi Dimensional Scaling (NMDS) plots. The NMDS ordination based on weighted Unifrac distance matrix was generated using the metaMDS function in R package vegan, ggplot2, and ggordiplot (Oksanen et al. 2018).

Differential abundances in taxa responsible for differences among the groups within factors significant for beta diversity, were determined using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) in QIIME2 (Lin and Peddada 2020). This method provides p-values for individual taxa that are differentially abundant and corrects the false discovery rate of

the samples using Benjamini-Hochberg procedure (Lin and Peddada 2020, Benjamini and Hochberg 1995).

2.2.4. Feeding experiments to assess consumption of *R. solanacearum* by *N. takasagoensis* workers

2.2.4.1. Termite samples for feeding experiments

Three different feeding experiments were conducted during a four-week stay at the University of Guam to assess feeding preference of *N. takasagoensis* workers (1) depending on the presence of *R. solanacearum* and wetwood bacteria in natural ironwood pieces (four-choice tests), (2) between *R. solanacearum* inoculated *C. equisetifolia* wood and control with no *R. solanacearum* inoculation (two-choice tests) and (3) to test whether *R. solanacearum* is ingested and survives in the termite gut by force-feeding *N. takasagoensis* workers on *R. solanacearum* inoculated filter paper (no-choice tests) (Figure 2). For four- and two-choice tests, three colonies of *N. takasagoensis* were collected from Bernard Watson's farm (GPS coordinates: 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 immediately after being collected from the field. All the experimental units were set up on the same day. No-choice tests were conducted with *N. takasagoensis* termites collected from ironwood tree logs brought into the lab from UOG Campus (13°43020',144°80008'), UOG Yigo Station (13°53356', 144°87116') and UOG Yigo Station (13°53288',144°87163'). For all bioassays, the experimental units were maintained in the dark at 26 ± 2 °C. Dead termites were removed daily, and the numbers of dead termites recorded.

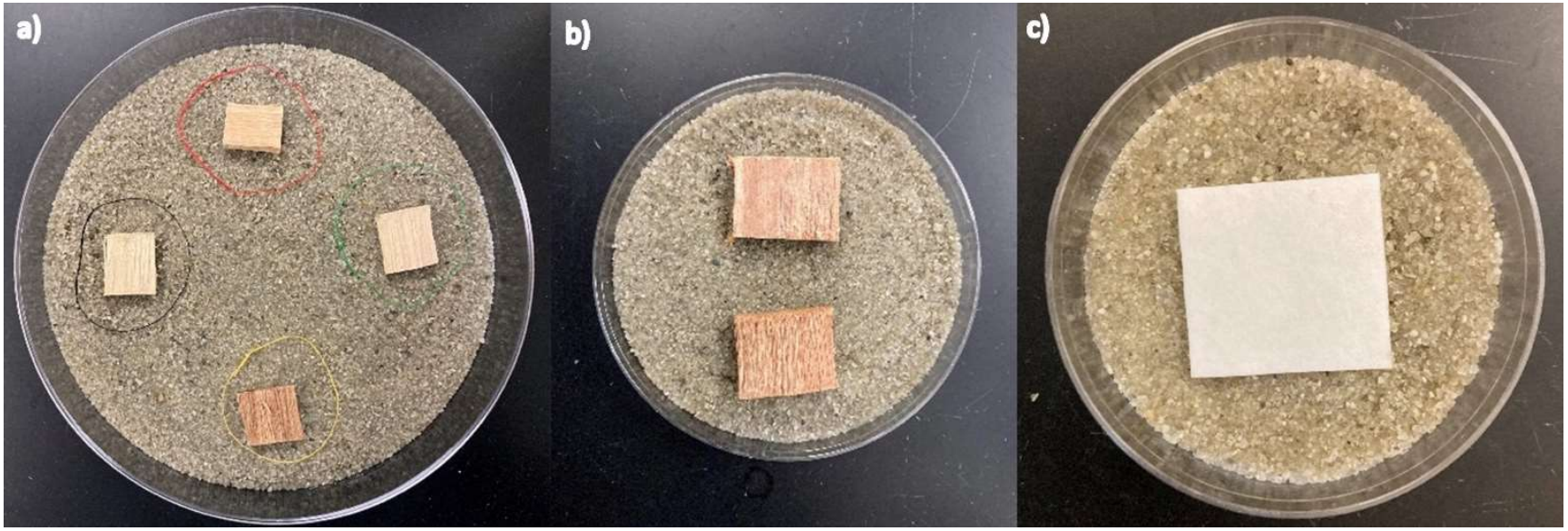

Figure 2. a) Four-choice tests were set up in Petri dishes containing four *C. equisetifolia* wood pieces: 1 (red) was negative for *R. solanacearum* and contained low amounts of wetwood bacteria, 2 (green) was positive for *R. solanacearum* and contained low amounts of wetwood bacteria, 3 (yellow) was negative for *R. solanacearum* and contained high amounts of wetwood bacteria, and 4 (black) was positive for *R. solanacearum* and contained high amounts of wetwood bacteria, b) Two-choice tests consisted of Petri dishes with two *C. equisetifolia* wood pieces: *R. solanacearum* inoculated wood and control with no *R. solanacearum* inoculation c) No-choice tests used Petri dishes with *R. solanacearum* inoculated filter paper. Images were taken by Garima Setia.

2.2.4.2. Four-choice tests between four *C. equisetifolia* wood pieces having different amounts of *R. solanacearum* and wetwood bacteria

For the treatments in the four-choice test, four *C. equisetifolia* trees with positive or negative test results for *R. solanacearum* and low or high amounts of wetwood bacteria were cut through the trunk to obtain the wood pieces for the test (Figure 3). To eliminate cross contamination, the circular saw was surface sterilized with 10% Clorox solution between trees. Log pieces were sliced in roughly 2 cm thick disks using a circular saw. Then, wood pieces (approximately 0.5x2x1.5 cm^3) were cut from the central portion of the disks with a chisel and hammer; thereby, maximizing the recovery of wetwood tissue. Trees without wetwood stains in their center were assumed to contain low amounts of wetwood bacteria (Figure 3 a and b) while trees with visible dark stains in the center were assumed to contain high amounts of wetwood bacteria (Figure 3 c and d). In addition, tests for *R. solanacearum* were performed (using immunodiagnostic strips (Agdia, Inc.) to select four treatments: (1) *R. solanacearum* negative and low wetwood bacteria (Tree location: Yigo Experiment Station (13°533523', 144°871081')) (2) *R. solanacearum* positive and low wetwood bacteria (Tree location: Yigo Experiment Station (13°533523', 144°871130')) (3) *R. solanacearum* negative and high wetwood bacteria (Tree location: Bernard Watson's Farm (13°34.026', 144°52.584')) (4) *R. solanacearum* positive and high wetwood bacteria (Tree location: Bernard Watson's Farm (13°34.028', 144°52.597')).

Figure 3. Image showing cross-sections of ironwood tree trunks being (a) *R. solanacearum* negative with low amounts of wetwood bacteria (b) *R. solanacearum* positive with low amounts of wetwood bacteria (c) *R. solanacearum* negative with high amounts of wetwood bacteria and (d) *R. solanacearum* positive with high amounts of wetwood bacteria. Images were taken by Garima Setia.

These wood pieces were weighed, and their initial weight was recorded. Each of the four wood pieces was placed with equal distance in a Petri dish (145x20mm) filled with sand at 12% moisture level. The experiment was designed following a randomized complete block design using 15 experimental units with five replicates from each of the three colonies. For each replicate 300 workers and 60 soldiers of *N. takasagoensis* were released into the Petri dishes. After three weeks, the final weight of all four wood pieces was measured to determine the difference 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) followed by Tukey's Studentized Range post-hoc test. The significance level was determined at $\alpha < 0.05$.

2.2.4.3. Validation of the Ralstonia strain through pathogenicity tests and sequencing

For two-choice test and no-choice test, instead of natural wood, *N. takasagoensis* workers were given a food source inoculated with a known amount of *R. solanacearum*. For these tests, the *R. solanacearum* bacterial isolate 19-147 was used (Paudel 2020). This 19-147 isolate was a subculture of the original isolate obtained from the bacterial ooze from a root section of a heavily damaged (DS=3) ironwood tree from Guam and successfully purified at the University of Hawaii in 2019 (Paudel 2020). To confirm the pathogenicity of this isolate 19-147, pathogenicity experiments were performed with the help of Dr. Robert Schlub and lab members at the University of Guam.

The 19-147 isolate present in the form of water cultures was streaked onto Casamino Acid-Peptone-Glucose (CPG) Agar to obtain subcultures. The CPG agar was prepared by adding 1 g

Casamino acid (casein hydrolysate), 10 g Peptone, 5 g Glucose and 17 g Agar in 1-liter distilled water followed by sterilization in an autoclave at 121˚C for 20 minutes (Kelman 1954). The streaked CPG agar plates were kept in an incubator for 48 hours at 28 ˚C to allow the bacteria to grow. After 48 hours, well-isolated single fluidal colonies were re-streaked on several CPG plates to obtain pure cultures. The re-streaked CPG agar plates were again kept for incubation at 28˚C for 48 hours. After bacterial growth was obtained in re-streaked CPG plates, a loopful of bacteria was transferred to 5 ml vials containing 2ml of sterile distilled water. Fifteen such vials containing cloudy suspensions of bacteria in water were prepared. Overnight cultures (18-20 h) of water suspensions of Ralstonia were prepared using CPG broth medium in a shaker-incubator at 220 rpm and 28°C (Kelman 1954). The inoculum for the pathogenicity test was prepared by adding 1 ml of overnight culture into 750 ml of CPG broth (Kelman 1954) and placing it in a shaker incubator at 220 rpm and 28°C for 24 hours. Serial dilutions from 10^{-1} to 10^{-10} of the overnight culture were performed to count colony-forming units (CFU/ml) on CPG agar plates. From each dilution, a 20µL aliquot was plated on CPG agar, and the number of CFU was determined from countable plates. A CFU per ml of 8.834E+9 was obtained. The 750 ml of bacterial overnight culture was transferred into an approximate 20 liters plastic bucket and 18 liters of water were added to the bucket.

Indicator plants (Two ironwood sprouts per cone (approximately 5 weeks old), one tomato seedling per cone (approximately 3 weeks) and one ironwood seedling (approximately 2 months)) were planted in tree-seedling cones (2.5in D x10in L) containing potting soil. Six tomato varieties were used: Baby Roma, Black Krim, Brandywine, Cherokee Purple, Beefsteak, and Garden Leader Monster. Ironwood (*C. equisetifolia*) seeds were first generation 1992-1994 international

provenance seeds collected on Guam: provenance country and CSIRO Seedlot no.: India (18015), Kenya (18144), Papua New Guinea (18153), Vietnam (18152), Egypt (18126), Malaysia (18348), and Australia (18008). Seeds were also collected from Guam's indigenous ironwood trees; these seeds are comparable to provenance seeds 18121.

On December 31st 2021, three replicates of each tomato seedlings, ironwood sprouts and ironwood seedlings were wound inoculated, and one replicate of each treatment was wounded but not inoculated (control). Wounding was the result of thrusting each plant root 10 times with a narrow, thin, u-shaped, metal probe, with a serrated edge. Wounding was followed by submerging the plant tubes either in a bucket containing inoculum or a bucket with clean water (control). Plants were dipped a second time on the following day. After the third day, clean water was applied to each plant as needed. The seedlings were monitored daily for any disease symptoms. In March (7-11) above ground tissue samples were collected from each plant and tested for Ralstonia with the immunodiagnostic strips (Agdia, Inc.).

The experiment resulted in all the tomato plants and 50% of the ironwood sprouts and seedlings developing symptoms. Plant death was 100% for the inoculated tomatoes and 50% for the inoculated sprouts. None of the ironwood seedlings died. All controls survived to the end of the experiment with the exception of the Malaysia tree. All the inoculated tomatoes and ironwood sprouts and 33% of the ironwood seedlings tested positive for *R. solanacearum*.

The bacterial isolate 19-147 was also validated by sequencing. Bacteria from water suspensions of isolate 19-147 were pelleted down by centrifugation and were shipped from University of Guam to LSU AgCenter in 95% ethanol. The DNA was extracted from the bacteria pellet using the

DNeasy Blood & Tissue kit (Qiagen, Germantown, MA) and included with the other samples for sequencing on the Illumina-Novaseq platform of the University of New Hampshire (see 2.2.3). The results obtained after performing BLAST against SILVA 132 database were as expected. The bacteria were identified as *Ralstonia solanacearum* according to both SILVA 132 database and top GenBank match (Accession number: MT598218.1, Percent identity: 100%, Query Coverage: 100%, E value: 3e-115).

2.2.4.4. Two-choice test comparing consumption of wood soaked with Ralstonia overnight culture to a saline control

Two-choice tests were performed to assess the consumption of *R. solanacearum* inoculated *C. equisetifolia* wood by *N. takasagoensis* workers compared to *C. equisetifolia* wood not inoculated by *R. solanacearum*. Overnight cultures were prepared using CPG broth medium in a shaker-incubator at 220 rpm and 28°C and serial dilutions from 10^{-1} to 10^{-10} were performed using 0.85% saline. For control, only 0.85% saline without *R. solanacearum* was used. The optical density (OD_{600}) was measured using a spectrophotometer (SPECTRONIC™ 200, Thermo Scientific) and the number of colony-forming units (CFU/ml) was determined using CPG agar plates. The bacterial culture that was used to make dilutions for inoculating wood pieces had an optical density (OD_{600}) of 2.5 and 9.251E+9 colony forming units per ml (CFU/ml). Dilutions of 10^{-4}, 10^{-6} and 10^{-8} were used for the two-choice tests, because bacteria at these dilutions did not repel workers or impact their survival as determined based on the results of a pilot study performed on *C. formosanus* at LSU using *R. solanacearum* strain GMI1000 obtained from American Type Culture Collection (ATCC).

Wood pieces (around 0.5x2x1.5 cm^3) were cut with a circular saw from the center of the trunk of

a healthy *R. solanacearum* negative ironwood tree determined via immunodiagnostic strips (Agdia, Inc.) with no evidence of wetwood staining. The initial weight of the wood pieces was recorded after drying them for 2 days at 100 °C in a drying oven. Wood pieces were inoculated with 200 µL of different dilutions of overnight culture (10^{-4}, 10^{-6} and 10^{-8}) of *R. solanacearum* and saline control. Treatments were kept in each Petri dish (60x20mm) filled with sand at 12% moisture level (Figure 2). The experiment was designed following a randomized complete block design using 45 experimental units (three colonies x three concentrations x five replicates). One hundred workers and 20 soldiers of *N. takasagoensis* were released into each replicate. After three weeks, wood pieces were dried, and the final weight of each wood piece was recorded. Statistical analysis was conducted as described in for the four-choice test (2.2.4.1).

2.2.4.5. No-choice tests to measure ingestion and survival of Ralstonia in termite guts

The experiment was designed following a randomized complete block design with five replicates from each of the three colonies (Section 2.2.4) setup in different Petri dishes along with controls for each of them. Filter paper was soaked with 100 µL of 10^{-4}, 10^{-6} or 10^{-8} dilutions of overnight culture of *R. solanacearum* made in 0.85% saline. Filter paper soaked with only 0.85% saline having no Ralstonia was used as a negative control. Fifty workers and 5 soldiers per colony of *N. takasagoensis* were released into Petri dishes (60x20 mm) to feed on the different bacterial dilutions (10^{-4}, 10^{-6}, 10^{-8} or control) for three time periods (2 days, 4 days, and 6 days) using separate sets of Petri dishes. These first six days of the experiment were referred to as Phase 1. The termites feeding for the longest time (6 days) on different Ralstonia concentrations (10^{-4}, 10^{-6}, 10^{-8} and no Ralstonia) in Phase 1 were transferred to filter paper with no Ralstonia for additional two days in Phase 2 of the experiment to test if ingested Ralstonia survives. Thus, the total duration

of both phases of the experiment combined was 8 days. After every two days during the experiment, i.e., at 2, 4 and 6 days of Phase 1 and at 8 days, i.e., 6 days (Phase 1) plus 2 days into Phase 2, eight workers were removed from each Petri dish and were stored in 95% ethanol to measure presence of Ralstonia over time in the termites. 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 pooling five termite workers from each sample using the DNeasy Blood & Tissue kit (Qiagen, Germantown, MA) following manufacturer's instructions. The V4 region of the 16S rRNA gene of the bacterial DNA was amplified using 515F and 926R primers (Caporaso et al. 2011, 2012). Successfully amplified PCR products were sequenced on the 2x250bp Illumina NovaSeq platform following Illumina Nextera Dilute library protocol (Illumina, San Diego, CA) at the University of New Hampshire Hubbard Center for Genome Studies. The sequence analysis was performed using QIIME2 as described above (2.2.3) to determine the presence and abundance of Ralstonia as well as change in the termites' bacterial communities during and after feeding on Ralstonia.

2.3. Results

2.3.1. Number of sequence reads and ASVs

A total of 11,106,360 raw sequences were recovered across the 42 *N. takasagoensis* samples and the ASV table obtained after DADA2 quality filtering and chimera removal contained 9,902,718 sequence reads and 12,903 ASVs. Further removal of the unassigned taxa with < 97% similarity to references in the SILVA database resulted in 1,709,419 reads, a mean number of 40,700 reads per sample and a total of 462 ASVs across all samples. The final number of ASVs with taxonomic assignment at 97% identity cutoff represented only 3.5% of the total ASVs. The minimum

sequencing depth common to all samples was reduced from 50,410 to 15,951 after filtering out the unassigned ASVs.

2.3.2. Sequence depth-, sample- and coverage-based rarefaction

The sequence-depth based rarefaction curves of most samples employing 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 a depth of less than 5,000 sequences (Figure 4a). This indicates that sufficient sequencing depth was achieved to capture most of the taxa and diversity present in each sample and collection of more sequences beyond that sequencing depth is unlikely to result in a considerable increase in diversity.

The sample-based rarefaction (Figure 4b) across all samples showed that the interpolated portions of curves for Shannon and Simpson inverse indices levelled off at an effective diversity of 150 and 100, respectively, and extrapolation to twice the sample-size 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 number of ASVs from 462 to more than 600 (Figure 4b). However, added richness would largely be based on rare ASVs since the Shannon diversity, and Simpson inverse index did not increase with added richness.

The coverage-based rarefaction (Figure 4c) depicts effective diversity with respect to sample completeness. The interpolated portions of the coverage-based rarefaction curves reached over 90% sample coverage at an ASV richness of 462, Shannon diversity of around 150 and Simpson inverse of around 100 (Figure 4c). 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 (Figure 4c).

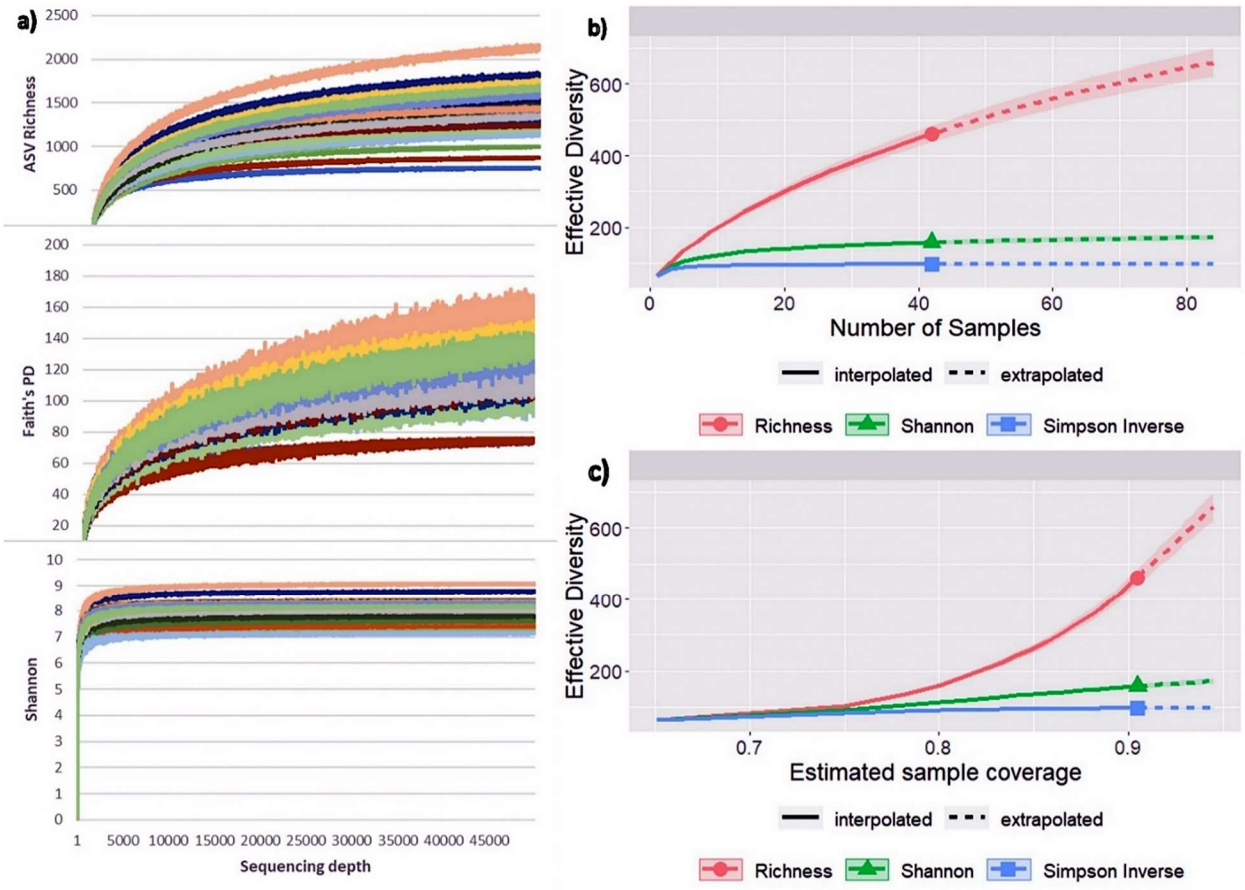

Figure 4. a) Sequence-based rarefaction curves of bacteria diversity showing the number of ASVs, Faith's phylogenetic distance and Shannon diversity indices in 42 samples of *Nasutitermes takasagoesis* workers plotted against sequencing depth. b) Sample-based rarefaction curves with effective bacterial diversity for different metrics plotted against the number of samples. c) Coverage-based rarefaction curves with effective diversity plotted against estimated sample coverage. Solid lines indicate intrapolation up to the actual sample size; dashed lines represent extrapolation to twice the sample size. Rarefaction was performed over the total bacteria diversity (with and without taxonomical assignment).

2.3.3. Taxa composition

Twenty-two bacterial phyla were identified in the 42 *N. takasagoensis* samples collected from ironwood trees in Guam (Table 4). Spirochaetes (48.16 %) and Fibrobacteres (41.38 %) were found to be the most dominant phyla followed by Bacteroidetes (3.61%), Proteobacteria (3.38%), Margulisbacteria (0.84%), Acidobacteria (0.77%), Planctomycetes (0.65%), Actinobacteria (0.47%), Synergistetes (0.19%), Firmicutes (0.19%), Tenericutes (0.18%), and others (0.16%) (Table 4, Figure 5).

The majority of the top 20 ASVs according to total number of reads were present in all 42 samples (Table 5). The most commonly detected ASVs, found in all the samples, were assigned to the dominant phyla Spirochaetes and Fibrobacteres. Treponema from phylum Spirochaetes was identified as the most dominant genus associated with *N. takasagoensis* workers. An uncultured Fibrobacteres bacterium from phylum Fibrobacteres was the second most frequent taxon. An uncultured *Treponema* sp. (phylum Spirochaetes) was the third most dominant taxon, followed by an uncultured Fibrobacteres bacterium (phylum Fibrobacteres), an uncultured *Treponema* sp. (phylum Spirochaetes), an uncultured Chitinivibrionia bacterium (phylum Fibrobacteres), an uncultured bacterium of the Phylum Spirochaetes, and an uncultured delta proteobacterium (phylum Proteobacteria), which all averaged over 1,000 reads per sample. Top 20 ASVs present in most *N. takasagoensis* samples but with reads averaging less than 1,000 per samples were an uncultured candidate division ZB3 bacterium (phylum Margulisbacteria), two uncultured Bacteroidetes bacteria (phylum Bacteroidetes), an uncultured Chitinivibrionia bacterium (phylum Fibrobacteres), uncultured Acidobacteria (phylum Acidobacteria), uncultured planctomycete

(phylum Planctomycete), an uncultured Alphaproteobacteria bacterium (phylum Proteobacteria), three uncultured Bacteroidetes bacterium (phylum Bacteroidetes) and an uncultured Spirochaetes bacterium (phylum Spirochaetes).

One of the aims of this project was to test whether bacteria associated with IWTD were present in termites collected from ironwood trees in Guam. The taxonomic analysis did not show any *Ralstonia* spp. regardless if termites were collected from healthy ironwood trees or trees with confirmed Ralstonia infection. However, various other genera, such as Comamonas, Duganella, Hydrogenophaga, Lautropia, Massilia, Ottowia, Parapusillimonas and Xenophilus from the same family (Burkholderiaceae) as the Genus Ralstonia were present in the termite samples. An unidentified species from the genus *Klebsiella* (phylum Proteobacteria), i.e., the genus which is believed to be associated with wetwood symptomatic tissue and IWTD in Guam, was found in a few samples albeit in minor abundances (19-86: 2 sequence reads, 19-93: 1 read, 19-94: 1 read, and 19-101: 5 reads). The lack of Ralstonia and the low presence and abundance of wetwood bacteria suggest that *N. takasagoensis* workers are not a vector for these putative IWTD pathogens.

Table 4. Number of reads across all samples, relative abundance of all the phyla associated with *N. takasagoensis* samples and the number of samples these phyla were observed in.

Phylum	Number of reads across all samples	Relative abundance (%)	Number of samples
Spirochaetes	823,678	48.16	42
Fibrobacteres	707,688	41.38	42
Bacteroidetes	61,718	3.61	42
Proteobacteria	57,851	3.38	42
Margulisbacteria	14,451	0.84	42
Acidobacteria	13,221	0.77	42
Planctomycetes	11,174	0.65	42
Actinobacteria	8,005	0.47	42
Synergistetes	3,216	0.19	42
Firmicutes	3,179	0.19	42
Tenericutes	3,055	0.18	42
Chloroflexi	1,102	0.06	42
Cyanobacteria	638	0.04	20
Elusimicrobia	531	0.03	26
Patescibacteria	523	0.03	25
Deinococcus-Thermus	46	<0.01	3
Gemmatimonadetes	45	<0.01	1
Verrucomicrobia	45	<0.01	2
Entotheonellaeota	44	<0.01	1
Epsilonbacteraeota	44	<0.01	1
Deferribacteres	43	<0.01	1
Fusobacteria	43	<0.01	1

Table 5. The most abundant 20 ASVs associated with *N. takasagoensis* samples according to total number of reads with their assignments in SILVA and NCBI GenBank, their total number of reads along with the number of samples the ASVs were observed in and the average number of reads and standard deviation per sample.

Phylum	Order	Lowest SILVA assignment	Top GenBank match	Percent identity to top match	Accession number in GenBank	Number of reads	Number of samples	Average reads per sample	Standard deviation
Spirochaetes	Spirochaetales	uncultured Treponema sp.	uncultured Treponema sp.	97.40%	AB255887.1	469,228	42	11,172	4,444.25
Fibrobacteres	Fibrobacterales	uncultured Fibrobacteres bacterium	uncultured Fibrobacteres bacterium	98.27%	AB255945.1	462,862	42	11,021	5,630.75
Spirochaetes	Spirochaetales	uncultured Treponema sp.	uncultured Treponema sp.	97.83%	EF453947.2	150,272	42	3,578	1,508 .33
Fibrobacteres	Fibrobacterales	uncultured Fibrobacteres bacterium	uncultured Fibrobacteres bacterium	99.57%	EF455000.2	132,734	42	3,160	2,036.78
Spirochaetes	Spirochaetales	termite gut metagenome	uncultured Treponema sp.	100.00%	EF454958.2	129,732	42	3,089	1,514.09
Fibrobacteres	Fibrobacterales	uncultured Chitinivibrionia bacterium	uncultured Chitinivibrionia bacterium	98.27%	AB255928.1	86,859	42	2,068	1,913.11
Spirochaetes	Spirochaetales	uncultured bacterium	uncultured bacterium	97.41%	KM023955.1	67,650	42	1,611	722.46
Proteobacteria	Rs-K70 termite group	uncultured delta proteobacterium	uncultured delta proteobacterium	98.26%	EF454740.2	44,985	42	1,071	569.36
Margulisbacteria	uncultured candidate division ZB3 bacterium	uncultured candidate division ZB3 bacterium	uncultured Candidatus Marinamargulisbacteria bacterium	99.57%	EF454953.2	14,409	42	343	243.45
Bacteroidetes	Bacteroidales	uncultured bacterium	uncultured bacterium	99.57%	KM024025.1	14,238	42	339	187.88
Bacteroidetes	Bacteroidales	uncultured Bacteroidetes bacterium	uncultured Bacteroidetes bacterium	99.13%	AB255906.1	14,106	42	336	263.9
Fibrobacteres	Fibrobacterales	uncultured Fibrobacterales bacterium	uncultured Fibrobacterales bacterium	97.36%	EF454859.2	12,811	37	305	496.87
Fibrobacteres	Chitinivibrionia Incertae Sedis	uncultured Chitinivibrionia bacterium	uncultured Chitinivibrionia bacterium	99.57%	AB255930.1	12,372	42	295	187.12
Acidobacteria	Holophagales	Holophagaceae	uncultured bacterium	100.00%	KM024015.1	10,239	42	244	198.44
Planctomycetes	Pirellulales	uncultured planctomycete	uncultured planctomycete	100.00%	KM651184.1	10,048	42	239	136.78
Proteobacteria	Rhodospirillales	uncultured alpha proteobacterium	uncultured Alphaproteobacteria bacterium	99.13%	AB192062.1	8,551	42	204	174.73
Bacteroidetes	Bacteroidales	uncultured Bacteroidetes bacterium	uncultured Bacteroidetes bacterium	100.00%	AB255911.1	8,537	42	203	103.96
Bacteroidetes	Bacteroidales	uncultured Bacteroidales bacterium	uncultured Bacteroidales bacterium	98.70%	AB255907.1	7,248	42	173	92.68
Bacteroidetes	Bacteroidales	Tannerellaceae	uncultured bacterium	99.13%	KM024037.1	5,020	42	120	53.8
Spirochaetes	Spirochaetales	uncultured Spirochaetes bacterium	uncultured Spirochaetes bacterium	97.39%	KM651173.1	3,337	39	79	82.78

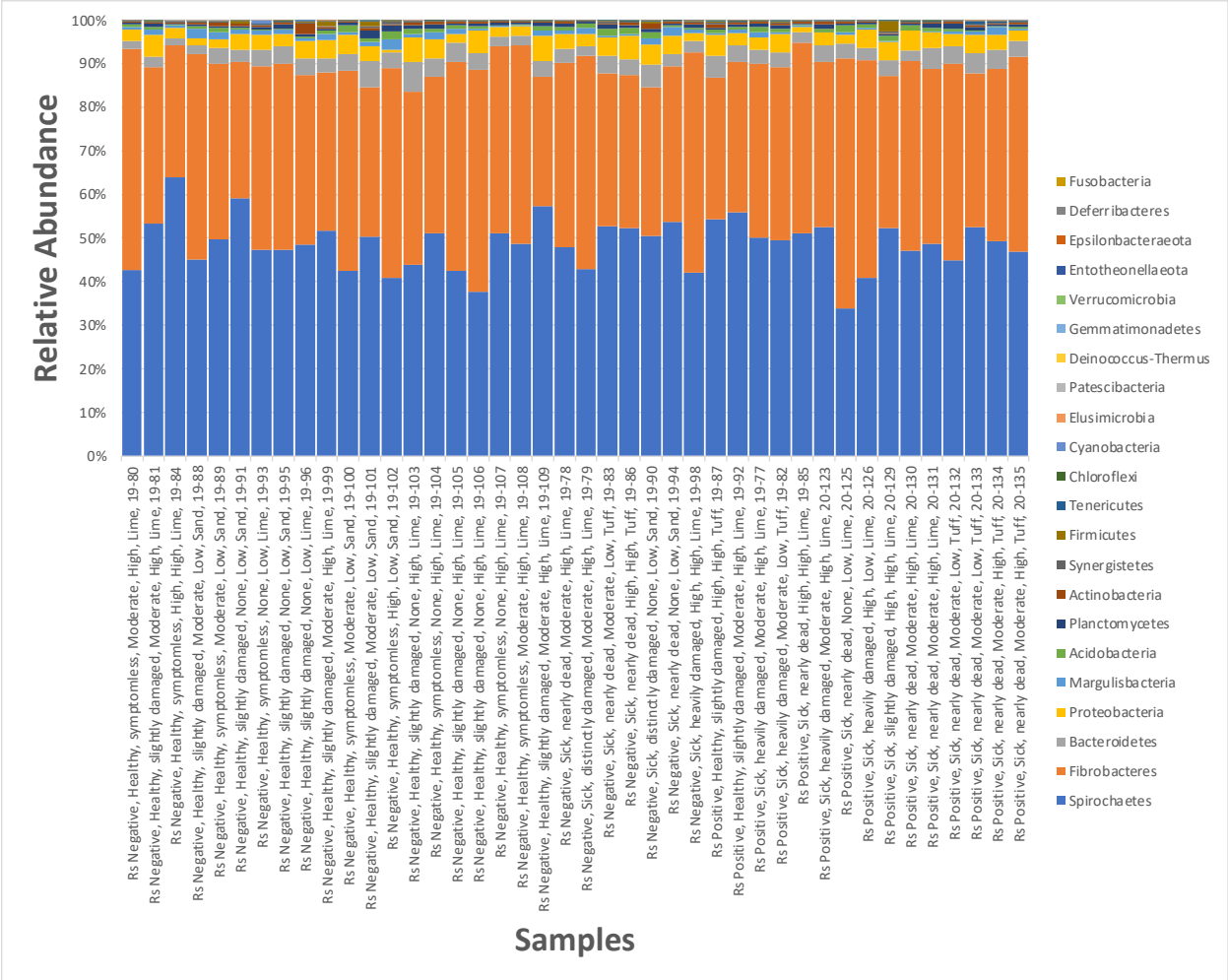

Figure 5. Taxa bar plots showing the relative abundance of bacterial phyla associated with 42 samples of *N. takasagoensis* workers collected from ironwood trees in Guam. Phyla are shown in decreasing abundance from bottom to top. The sample name on the x-axis encodes the following seven factors: (1) presence (Positive) or absence (Negative) of Ralstonia (Rs), (2) Tree Health (Healthy or Sick), (3) Tree DS (symptomless (DS=0), slightly damaged (DS=1), distinctly damaged (DS=2), heavily damaged (DS=3), and nearly dead (DS=4)), (5) level of Site Management (None, Moderate, High), (6) Altitude Classification (High, Low) and (7) Parent Material (Sand, Lime, Tuff).

2.3.4. Alpha diversity

No significant effects on any of the alpha diversity indices of termite bacterial communities were observed for presence or absence of Ralstonia, Tree DS, Location, Altitude as well as Parent Material of the tree from which termites were collected. While no significant differences in alpha diversity were detected across the five DS stages, there was a significant effect when decline severity categories were reduced to sick (DS 1-4) vs healthy trees (DS 0). The phylogenetic distances of bacteria communities were significantly greater (Faith's PD, $p=0.02$, $H= 5.07$, Kruskal-Wallis ANOVA, Figure 6a) in termites collected from sick trees ($n=33$) than those of healthy trees ($n=9$). There were no significant differences in ASV Richness, Pielou's evenness and Shannon diversity between termite samples collected from healthy and sick trees.

The evenness and Shannon diversity of the bacterial community associated with termites was significantly influenced by the level of Site Management. It was found that intense Site Management led to less evenness (Pielou's evenness, $p= 0.04$, $H =6.17$, Kruskal-Wallis ANOVA, Figure 6b) and lower Shannon diversity ($p=0.02$, $H=7.41$, Kruskal-Wallis ANOVA, Fig. 3c) of the bacterial community of the termites compared to moderate and high management levels. However, Site Management did not have significant influence on ASVs richness and Faith's PD.

For Plot Average DS, Plot Average Decline, Proportion of Dead Trees in Plots and Proportion of Trees with Termites in Plot, increase in decline was negatively associated with some aspects of the bacterial diversity in the termites. Evenness of the bacteria community within termite samples was negatively correlated to Plot Average DS (Pielou's evenness, $p=0.01$, $r_s= -0.3094$, Spearman's Rank test, $n=42$, Figure 6d), i.e., evenness was higher in less damaged tree plots than in plots with

severe damage. When Plot Average Decline (percentage of sick trees with DS 1-4) was considered, a similar negative correlation of increasing evenness with decreasing percentage of sick trees was observed (Pielou's evenness, p=0.014, r_s = -0.37, Spearman's Rank test, n=42, Figure 6e). When plot condition was measured as the Proportion of Dead Trees in Plots, Shannon diversity of bacterial communities within termite samples was inverse correlated to higher Proportion of Dead Trees in Plots (Shannon diversity, p= 0.03, r_s= -0.32, Spearman's Rank test, n=42, Figure 6f). Pielou's evenness, ASV richness and Faith's PD did not show significant correlation to the Proportion of Dead Trees in Plots. Increasing number of Trees with Termites in Plot was significantly associated with less diversity in terms of evenness of the bacterial community of termites feeding on those trees (Pielou's evenness, p=0.01, r_s = -0.38, n=42, Spearman's Rank test, Figure 6g). Proportion of Dead Trees did not significantly influence ASV richness, Shannon diversity and Faith's PD.

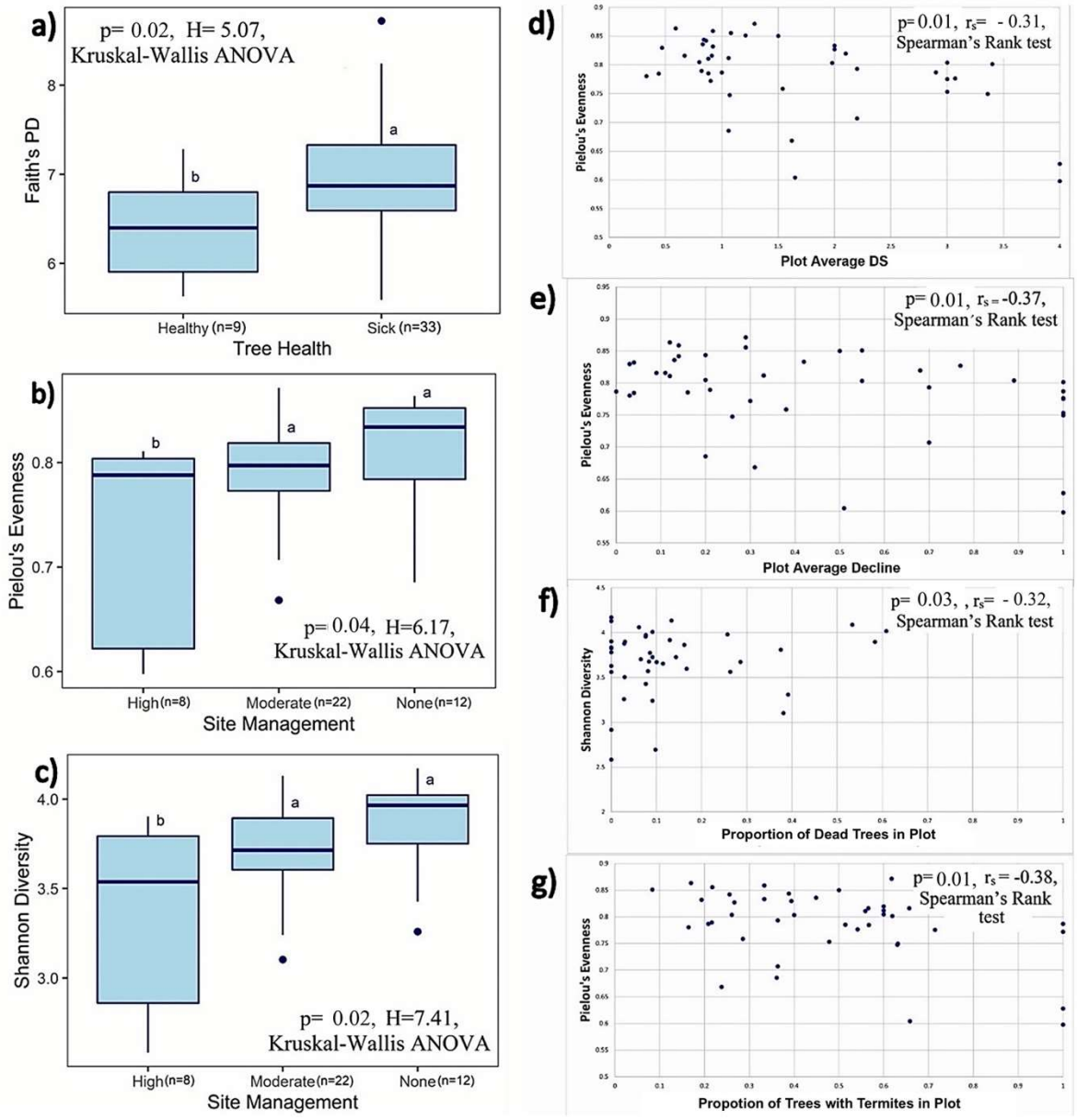

Figure 6. Tree-, plot and location-related factors with significant effects on different aspects of alpha diversity of termite bacteria communities. Different letters indicate significant difference. a) Faith's PD of bacteria communities of termites collected from healthy and sick ironwood trees. b) Pielou's evenness and c) Shannon diversity index of bacterial communities of termites collected from highly, moderately or non-managed sites. d) Correlation between Pielou's evenness of termite bacterial communities and Plot Average DS. e) Correlation between Pielou's evenness of termite bacteria communities and Plot Average Decline. f) Correlation between Shannon diversity of bacterial communities of termites and Proportion of Dead Trees in Plots. g) Correlation between Faith's PD of bacterial communities of termites and Proportion of Trees with Termites in Plot.

2.3.5. Beta diversity

Ralstonia presence (p=0.02, pseudo-F= 3.28, n=42), Altitude (p=0.03, pseudo-F= 2.84, n=42) and Parent Material (p= 0.01, pseudo-F= 3.65, n=42) showed significant effects on the beta diversity of the bacterial community in termites using single factor PERMANOVA. Bacteria composition was significantly different between termite samples taken from trees growing on Lime and Sand (p= 0.01, pseudo-F = 5.58, n=34) as well as Sand and Tuff (p= 0.01, pseudo-F = 4.28, n=17), while samples from Lime and Tuff did not show significant differences (p = 0.43, pseudo-F= 0.92, n=33). Homogeneity for multivariate variances was confirmed using PERMDISP that showed no significant difference in dispersion for the three factors (Table 6). Since the assumption of homogeneity for multivariate variances was fulfilled, a multifactorial ADONIS test was performed.

Table 6. Pairwise comparison of groups within the factors Presence of Ralstonia, Altitude Classification and Parent Material using PERMANOVA (999 permutations) and PERMDISP (1,000 permutations). Asterisks indicate significant effect.

			PERMANOVA		PERMDISP	
Factor		Sample size	pseudo-F	p-value	F-value	p-value
Presence of Ralstonia						
Negative	Positive	42	3.28	0.02*	0.24	0.67
Altitude Classification						
High	Low	42	2.84	0.03*	1	0.32
Parent Material						
Lime	Sand	34	5.58	0.01*	1.4	0.25
Lime	Tuff	33	0.92	0.43	0.06	0.83
Sand	Tuff	17	4.28	0.01*	1.27	0.27

Presence of Ralstonia (Pr(>F) = 0.02) in the ironwood trees from which the termites were collected and Altitude Classification (Pr(>F) =0.04) of the tree location showed significant effects on bacteria community similarity of termite samples based on the ADONIS test results (Table 7). In contrast to the single factor PERMANOVA results, Parent Material (Pr(>F) = 0.17) showed only marginally significant results. Ralstonia explained the highest percentage of variation in the dataset at 8% (R^2 = 0.08) followed by Altitude Classification at 6% (R^2=0.06). The interaction among Ralstonia and Parent Material showed marginal significance with a low R^2 value (Pr(>F) = 0.09, R^2 = 0.05) while no significant interaction was observed in other pairwise combinations. The separation of the centroids of Ralstonia positive and Ralstonia negative groups as well as high and low altitude groups in the NMDS plots confirm the differentiation (Figure 7). However, since the factors explained only a small fraction of the variability (Table 7) there was some overlap in the bacteria composition between the groups (Figure 7). Except for 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.

Table 7. Adonis test results showing factors affecting the differentiation of bacteria composition among groups of termite samples and their interactions. Asterisks indicate significant effects.

Factor	Df	Sums Of Squares	Mean Squares	F.Model	R^2	Pr(>F)
Presence of Ralstonia (Positive, Negative)	1	0.18	0.18	3.46	0.08	0.02*
Altitude Classification (Low, High)	1	0.13	0.13	2.54	0.06	0.04*
Parent Material (Lime, Sand, Tuff)	2	0.15	0.08	1.46	0.06	0.17
Presence of Ralstonia: Altitude Classification	1	0.02	0.02	0.47	0.01	0.75
Presence of Ralstonia: Parent Material	1	0.12	0.12	2.31	0.05	0.09
Altitude Classification: Parent Material	1	0.04	0.04	0.73	0.02	0.54
Presence of Ralstonia: Altitude Classification: Parent Material	1	0.01	0.01	0.22	0.01	0.92
Residuals	33	1.73	0.052		0.72	
Total	41	2.39			1	

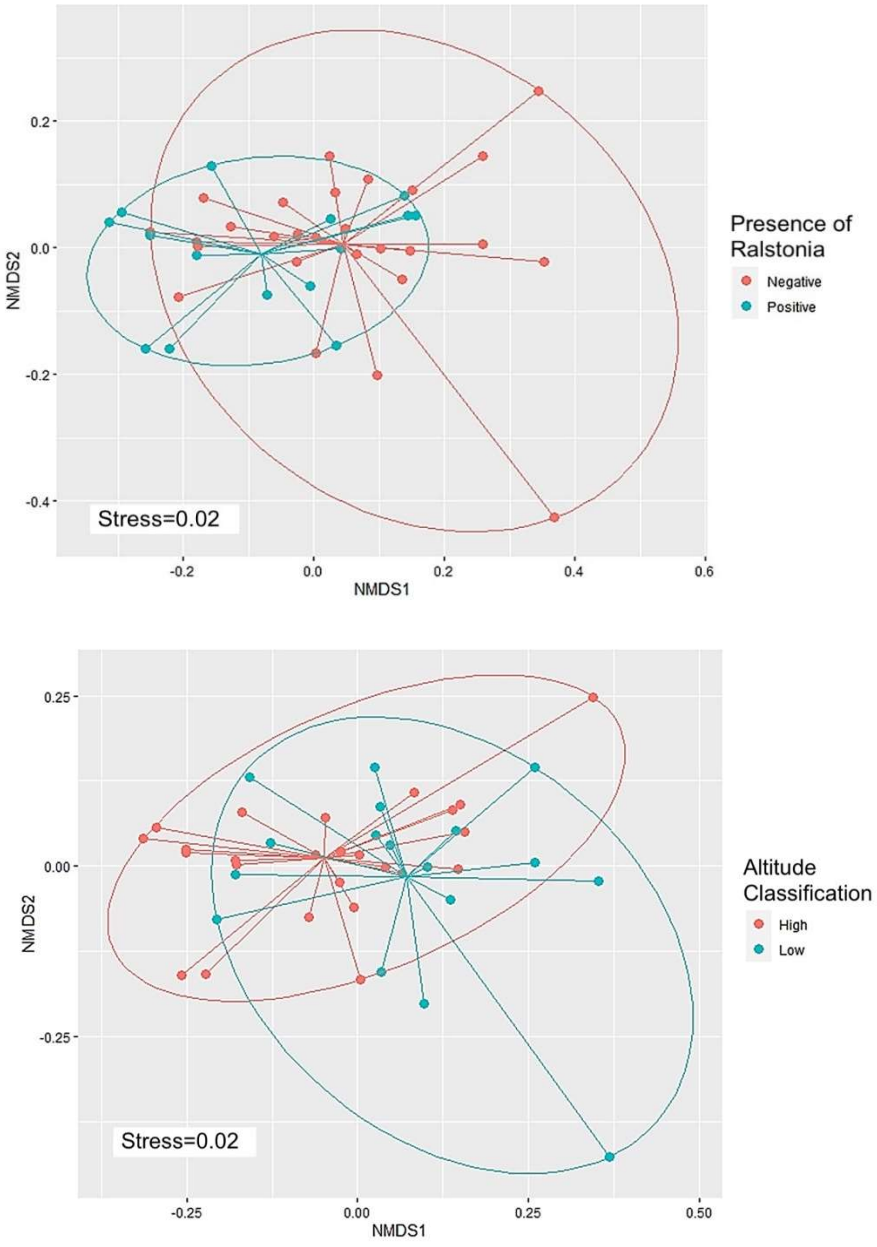

Figure 7. Plot of the first two dimensions of the NMDS ordination of the weighted Unifrac distance matrix showing the similarity of bacteria communities among termite samples from Ralstonia negative and Ralstonia positive trees and, termite samples collected from trees at high altitude and low altitude. Individual samples (circles) are connected (lines) to the centroid of their group showing the overall dispersion (ellipses) within the group. Stress denotes the goodness of fit of the predicted ordination distances against the original distance matrix.

2.3.6. Differential Abundance

Analysis of differentially abundant taxa among the sample groups using ANCOM-BC showed that an ASV within phylum Bacteroidetes (adjusted p value=0.01) and an ASV belonging to Genus Treponema within phylum Spirochaetes (adjusted p value=0.03) had a significantly higher abundance in termites collected from Ralstonia negative trees as compared to those collected from Ralstonia positive trees (Table 8). The bacterial communities of termites collected from trees growing at high altitude were significantly enriched in two ASVs from Genus Treponema within phylum Spirochaetes (adjusted p value= 0.01 and 0.04), respectively (Table 8). Seven ASVs were differentially abundant between the termite samples collected from sand as compared to lime (all adjusted p value<0.05) while 24 ASVs were differentially abundant between the termite samples collected from tuff as compared to lime (all adjusted p value<0.05) (Table 8).

Table 8. ASVs with significant differential abundance in termites collected from *R. solanacearum* (Ralstonia) positive versus negative trees, low versus high altitude, sand versus lime, and tuff versus lime parent material. Significantly differential abundance was determined by ANCOM-BC at q value (adjusted p value) less than 0.05. W is the test statistic for determining differential abundance. Positive values for W depict that the ASV is significantly enriched in termites collected from trees that are a) Ralstonia positive compared to negative, or b) at low versus high altitude, or c) on sand or tuff parent material compared to lime and vice versa for negative W values. The enrichment of each ASV for a particular group is marked in blue.

Phylum	Family	Lowest SILVA assignment	Differential abundance		
a) Presence of Ralstonia (Positive versus Negative)					
			W (Positive)	Positive	Negative
Bacteroidetes	uncultured bacterium	uncultured bacterium	-4.77		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-4.02		
b) Altitude Classification (Low versus High)					
			W (Low)	Low	High
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-5.08		
c) Parent Material (Sand versus Lime)					
			W (Sand)	Sand	Lime
Fibrobacteres	uncultured bacterium	uncultured Chitinivibrionia bacterium	5.32		
Fibrobacteres	uncultured bacterium	uncultured Fibrobacteres bacterium	4.48		
Margulisbacteria	uncultured candidate division ZB3 bacterium	uncultured candidate division ZB3 bacterium	4.25		
Spirochaetes	Spirochaetaceae	uncultured bacterium	-7.15		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-4.69		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-4.42		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-4.21		

(table cont'd.)

Phylum	Family	Lowest SILVA assignment	Differential abundance		
c) Parent Material (Tuff versus Lime)					
			W (Tuff)	Tuff	Lime
Actinobacteria	uncultured bacterium	uncultured bacterium	3.95		
Bacteroidetes	Tannerellaceae	uncultured bacterium	7.61		
Bacteroidetes	Dysgonomonadaceae	uncultured bacterium	5.72		
Bacteroidetes	Dysgonomonadaceae	uncultured bacterium	4.37		
Bacteroidetes	Tannerellaceae	uncultured bacterium	4.14		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacteres bacterium	6.97		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacteres bacterium	4.90		
Margulisbacteria	uncultured candidate division ZB3 bacterium	uncultured candidate division ZB3 bacterium	-3.96		
Planctomycetes	Pirellulaceae	uncultured planctomycete	4.25		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	6.66		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	6.38		
Spirochaetes	Spirochaetaceae	uncultured bacterium	5.84		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.65		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.58		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-5.20		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-5.18		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.04		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.01		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.79		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.54		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.15		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.12		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.07		
Tenericutes	Acholeplasmataceae	uncultured Mollicutes bacterium	4.26		

2.3.7. Consumption of wood pieces with different levels of Ralstonia and wetwood bacteria by *N. takasagoensis* workers

When IWTD pathogens were not detected in considerable amounts in *N. takasagoensis* termites during bacterial diversity analysis it was evaluated whether termites would prefer wood with naturally low pathogen load over wood with Ralstonia present and high amounts of wetwood bacteria. A significant effect of food source on net consumption by *N. takasogoensis* workers was detected in four-way choice bioassays between food sources consisting of natural wood pieces with different amounts of Ralstonia and wetwood bacteria ($p = 0.01$, $R^2 = 0.26$, One-Way ANOVA, Figure 8). Net consumption of "Ralstonia positive and high amounts of wetwood bacteria" food source by *N. takasagoensis* workers was significantly lower than of "Ralstonia negative and low amounts of wetwood bacteria" food source ($p = 0.01$,) and "Ralstonia negative and high amounts of wetwood bacteria" food source ($p = 0.02$, Tukey's Studentized Range Test, Table 9). However, net consumption of "Ralstonia negative and low amounts of wetwood bacteria", "Ralstonia negative and high amounts of wetwood bacteria" and "Ralstonia positive and low wetwood" food source by *N. takasagoensis* workers was not significantly different; and the net consumption of wood pieces designated as "Ralstonia positive and low amounts of wetwood bacteria" and "Ralstonia negative and high amounts of wetwood bacteria" was also not significantly different (Table 9, Figure 8). These results indicate that termites tend to prefer wood without or low amounts of IWTD pathogenic bacteria over wood containing high amounts of IWTD pathogenic bacteria. The mortality rate of *N. takasagoensis* workers was high in the feeding experiments (32.9 ± 5.69%). However, the average mortality rate was constant among the treatments.

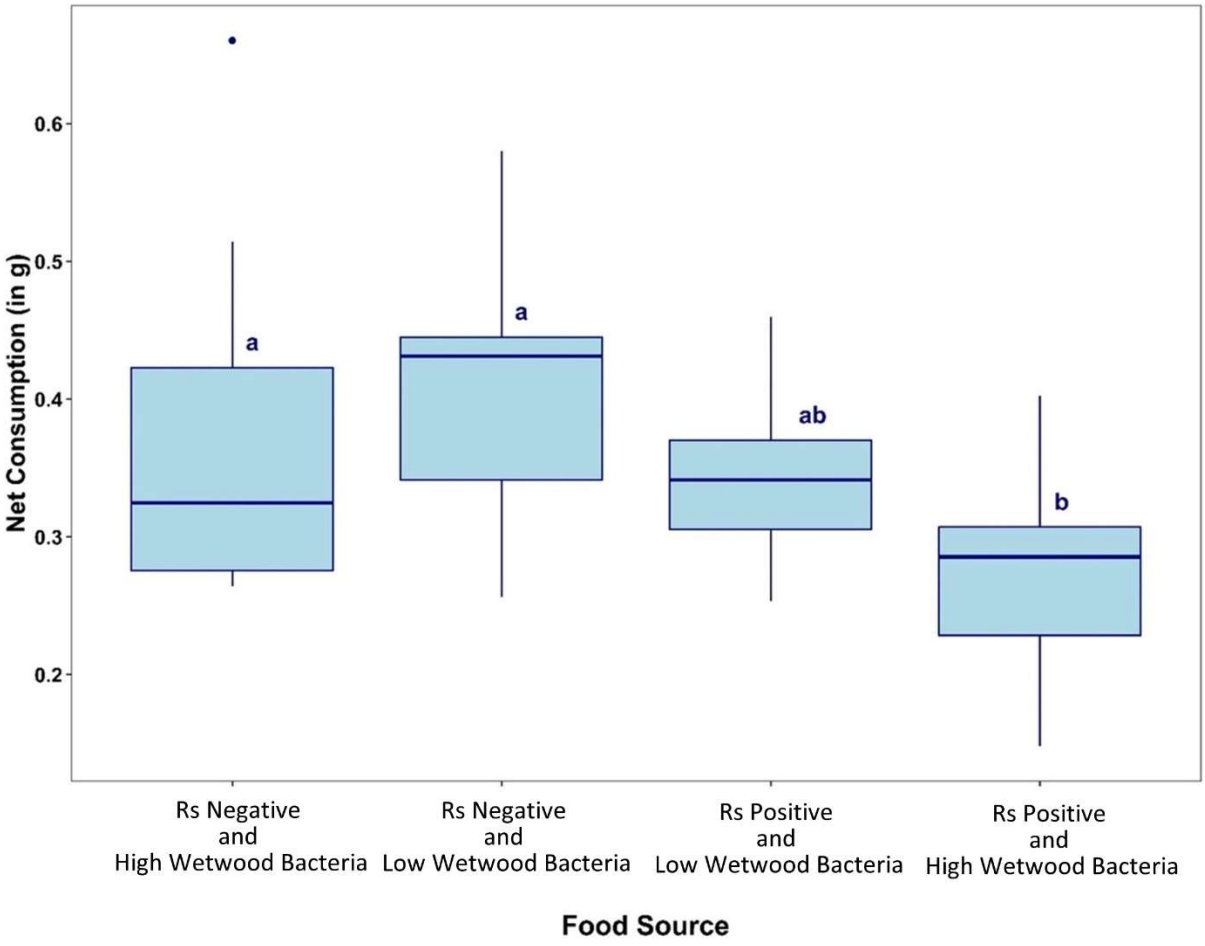

Figure 8. Net consumption (g) of wood pieces which tested positive or negative for *R. solanacearum* (Rs) and contained low or high amounts of wetwood bacteria by *N. takasagoensis* workers. Different letters indicate significant differences determined by Tukey's Studentized Range Test for four-choice bioassay (Table 9).

Table 9. Pairwise differences in consumption (above the diagonal) between four treatments with different natural wood pieces (along the diagonal) by *N. takasagoensis* workers and Tukey's Studentized Range Test results (below the diagonal) for the four-choice bioassay.

Difference in consumption of food source (g)			
Rs Negative and Low Wetwood Bacteria	0.06	0.04	0.14
0.24	Rs Positive and Low Wetwood Bacteria	-0.01	0.08
0.58	0.93	Rs Negative and High Wetwood Bacteria	0.1
0.01	0.08	0.02	Rs Positive and High Wetwood Bacteria
p value			

2.3.8. Termite consumption of wood pieces inoculated with different concentrations of Ralstonia versus saline control

When *N. takasagoensis* workers had the choice to feed on symptomless wood inoculated with a known concentration of *R. solanacearum* and a saline control (symptomless wood without Ralstonia), consumption in controls was marginally higher than consumption of wood inoculated with a 10^{-4} ($p=0.09$, $R^2 = 0.08$, n=3 colonies x 5 replicates, ANOVA) as well as a 10^{-6} dilution of Ralstonia ($p=0.08$, $R^2= 0.09$, n=3 colonies x 5 replicates, ANOVA), Figure 9). At lowest dilution (10^{-8}) no differences in consumption remained ($p= 0.11$, $R^2 = 0.11$, n=3 colonies x 5 replicates, ANOVA). The average mortality rate (30.94 ± 19.22%) was constant among the treatments.

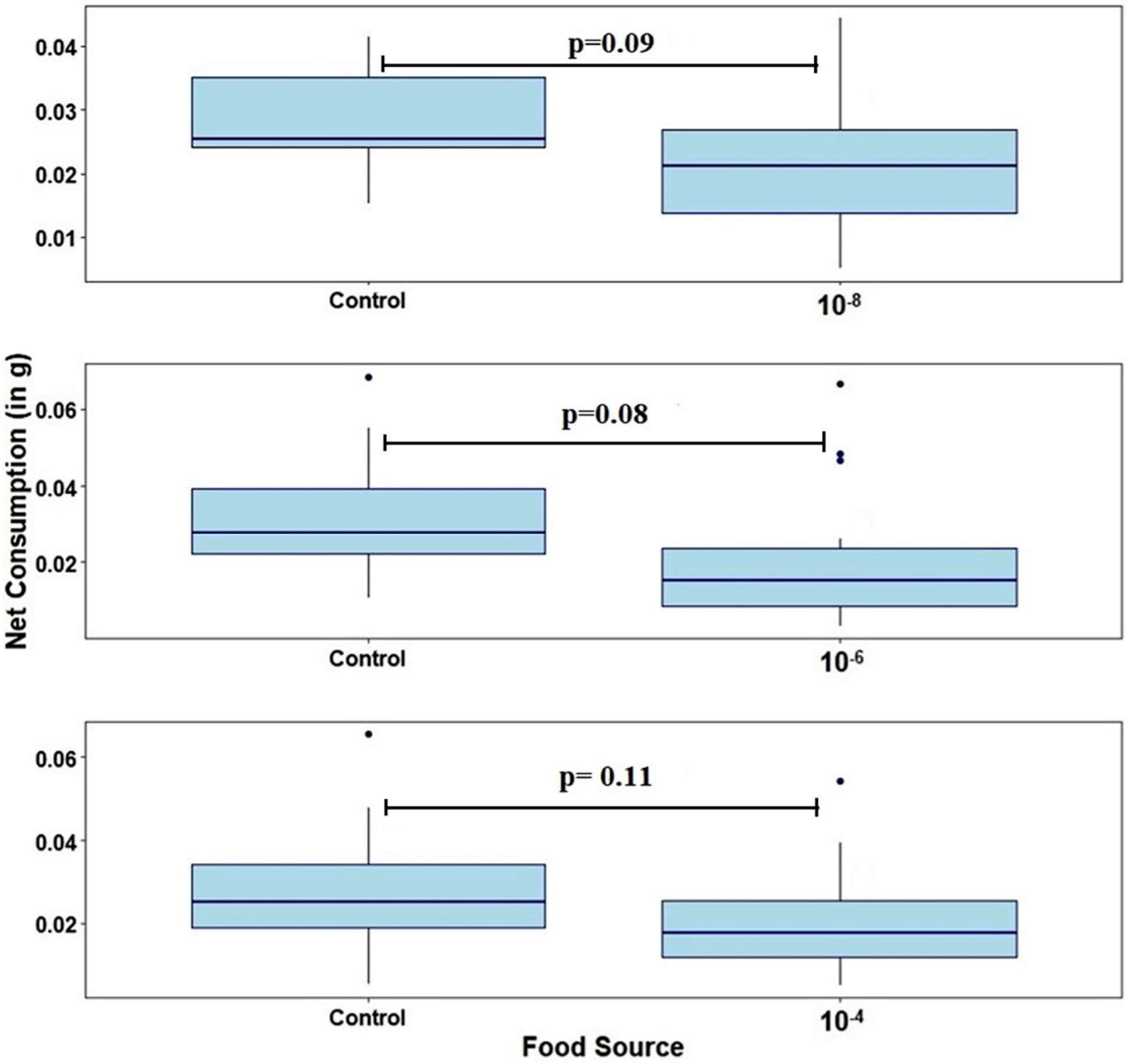

Figure 9. Net Consumption (g) of wood pieces inoculated with 10^{-4}, 10^{-6} and 10^{-8} bacterial dilution versus control by *N. takasagoensis* workers.

2.3.9. Termite consumption of filter paper inoculated with different concentrations of Ralstonia without any choice

2.3.9.1. Qualitative confirmation of consumption

During the first half of the experiment or Phase 1, the treatment used for feeding the termites was filter paper with different Ralstonia concentrations (10^{-4}, 10^{-6}, 10^{-8} dilutions along with no Ralstonia as control) to test if termites ingest Ralstonia. After Phase 1 of the experiment was completed, the remaining termites that were fed the longest (6 days) on Ralstonia and filter paper were transferred for 2 days to filter paper without Ralstonia to test if Ralstonia would persist in the gut without continued inoculation in Phase 2 of the experiment. Consumption was observed in controls and treatments based on holes in the filter paper, but since the termites deposited feces on the filter paper, weight change of filter paper was not measured. Sequencing of the16S rRNA gene was performed for the termite samples from Phase 1 and 2 to determine if *R. solanacearum* is ingested and capable of surviving in workers.

2.3.9.2. Number of sequence reads and ASVs

Across the 240 samples used in no-choice tests, DADA2 quality filtering resulted in a total of 15,124,113 sequence reads and 9,852 ASVs. After filtering out the unassigned ASVs below the percent identity of 97% to SILVA database references, the number of reads was reduced to 10,427,594 sequences and the remaining number of ASVs with taxonomical assignment was 617. The minimum sequencing depth common to all samples was reduced from 5,423 to 4,268 after filtering out the unassigned ASVs.

2.3.9.3. Sequence depth-, sample- and coverage-based rarefaction

The sequence-depth based rarefaction curves based on ASV richness, Faith's PD and Shannon diversity for most termite samples started to level out at a sequencing depth of 2,000, 1,000 and 500 respectively (Figure 10a). These rarefaction curves showed that the sequencing depth for the samples used in this study was sufficient to capture most of the diversity present within the samples. The sample-based rarefaction curves across 240 samples used in the no-choice test for Shannon diversity and Simpson inverse diversity started to level off at an effective diversity of around 150 and 100, respectively, while for ASV richness the curve did not level out (Figure 10b). When the curves were extrapolated to twice the sample size, Shannon diversity and Simpson inverse index remained the same but the number of ASV increased approximately by 25%. Leveling out of Shannon diversity and Simpson inverse indices indicated that the added richness would be based on rare ASVs. The coverage-based rarefaction curves (Figure 10c) showed that a high coverage of around 98% was obtained. On extrapolation of the curve, by doubling the sample size, the coverage was increased to 100% (Figure 10c).

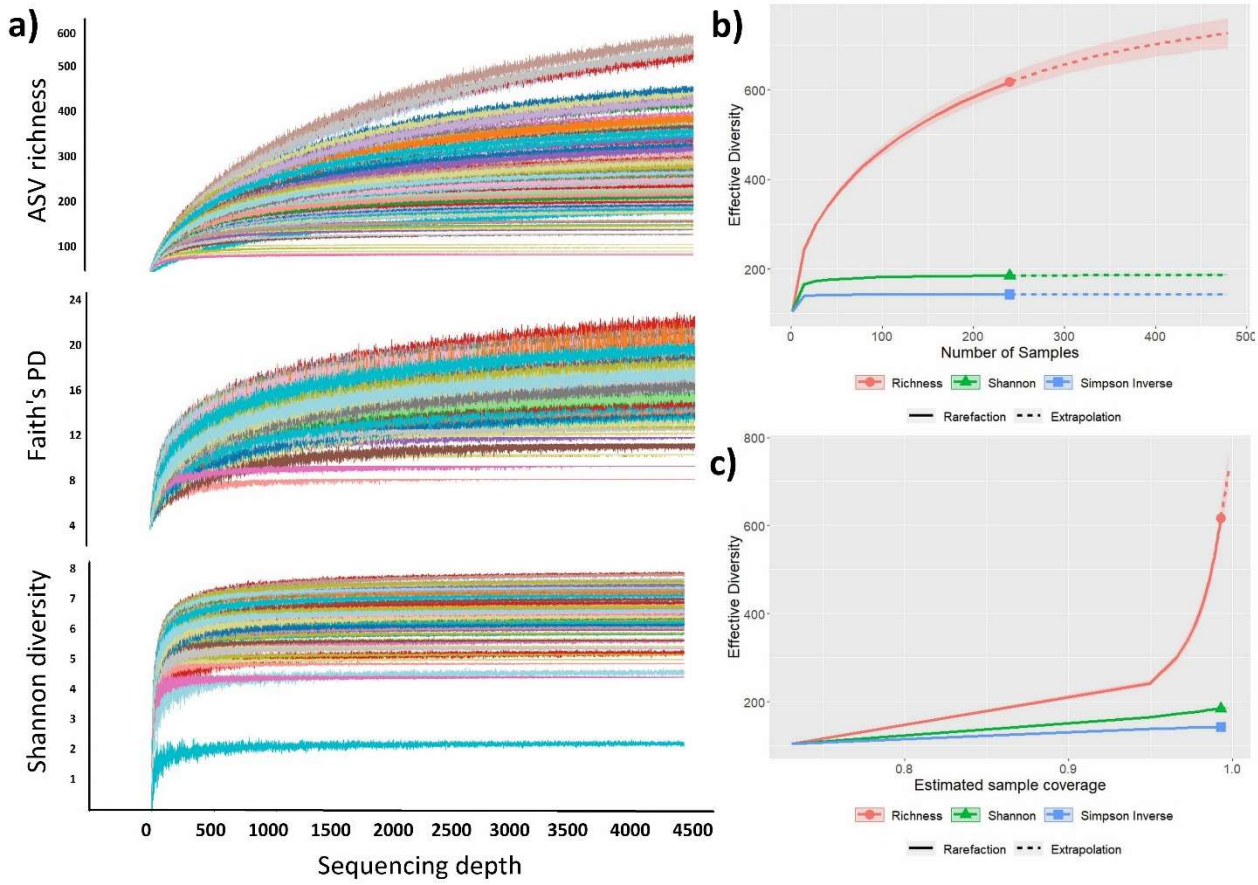

Figure 10. a) Sequence-based rarefaction curves of bacteria diversity showing the number of ASVs (ASV richness), Faith's phylogenetic distance and Shannon diversity indices for each of the 240 samples of *Nasutitermes takasagoesis* workers used in no-choice test plotted against sequencing depth. b) Sample-based rarefaction curves across all 240 samples with effective bacterial diversity for different metrics plotted against the number of samples. c) Coverage-based rarefaction curves across all 240 samples with effective diversity plotted against estimated sample coverage. Solid lines indicate intrapolation up to the actual sample size; dashed lines represent extrapolation to twice the sample size. Rarefaction was performed over the total bacteria diversity (with and without taxonomical assignment).

2.3.9.4. Bacteria composition of termites used in no-choice tests

Illumina sequencing did not detect any Ralstonia in the workers while they were feeding on filter paper inoculated with Ralstonia at any of the Ralstonia concentrations or time points. However, after six days of Ralstonia feeding (Phase 1) and 2 days of feeding on filter paper only (Phase 2),

Ralstonia was observed in 6 samples, but not in the controls. Sample HN3M6DR4 had 32,535 reads of Ralstonia, HN1H6DR3 and HN2H6DR2, both, had 27,472 reads, HN2L6DR3 had 13,798 reads, HN3L6DR1 had 8,137 reads, and HN2L6DR4 had 5,820 reads. However, the number of samples in which Ralstonia was found across all concentration ranges and feeding durations (6 of 240) and the relative abundance of Ralstonia as compared to other bacteria in these samples (<0.01%) was low.

Although Ralstonia was not detected in most of the samples despite the force-feeding, a difference in the ranking in relative abundance of some phyla was observed in all samples between day 6 of Phase 1 and Phase 2 of the experiment (Figure 11). The average relative abundance of Fibrobacteres decreased by half from 12% to 6%, which caused the Fibrobacteres to drop from the second most dominant phylum in Phase 1 to the fourth ranked in Phase 2. All other phyla retained their relative ranking but changed in relative abundance: Spirochaetes also decreased but to a lesser extent than Fibrobacteres from 66% to 57% while the relative abundance of Bacteroidetes increased 8% to 12%. Firmicutes also increased from 6% to 9%, Proteobacteria increased from 4% to 9% and Planctomycetes increased slightly from 2% to 3%. The diversity analysis showed that alpha diversity (Pielou's evenness, Faith's PD, ASV richness, Shannon diversity) and beta diversity of microbiota was significantly different (all $p<0.009$, Kruskal-Wallis ANOVA for alpha diversity and $p=0.001$, PERMANOVA for beta diversity, Table 10) between day 6 of Phase 1 and Phase 2 of the experiment. Furthermore, to test whether this shift in microbiota was caused by Ralstonia feeding or not, the effects of Ralstonia concentration, as well as duration of feeding on Ralstonia and filter paper on the phyla composition and alpha- and beta diversity were determined. A total of 107 ASVs from five different Phyla Fibrobacteres, Spirochetes, Bacteroidetes,

Firmicutes, Proteobacteria, Planctomycetes, Acidobacteria, and Synergistetes were differentially abundant ($q<0.05$) between Phase 1 and Phase 2 (Table 11).

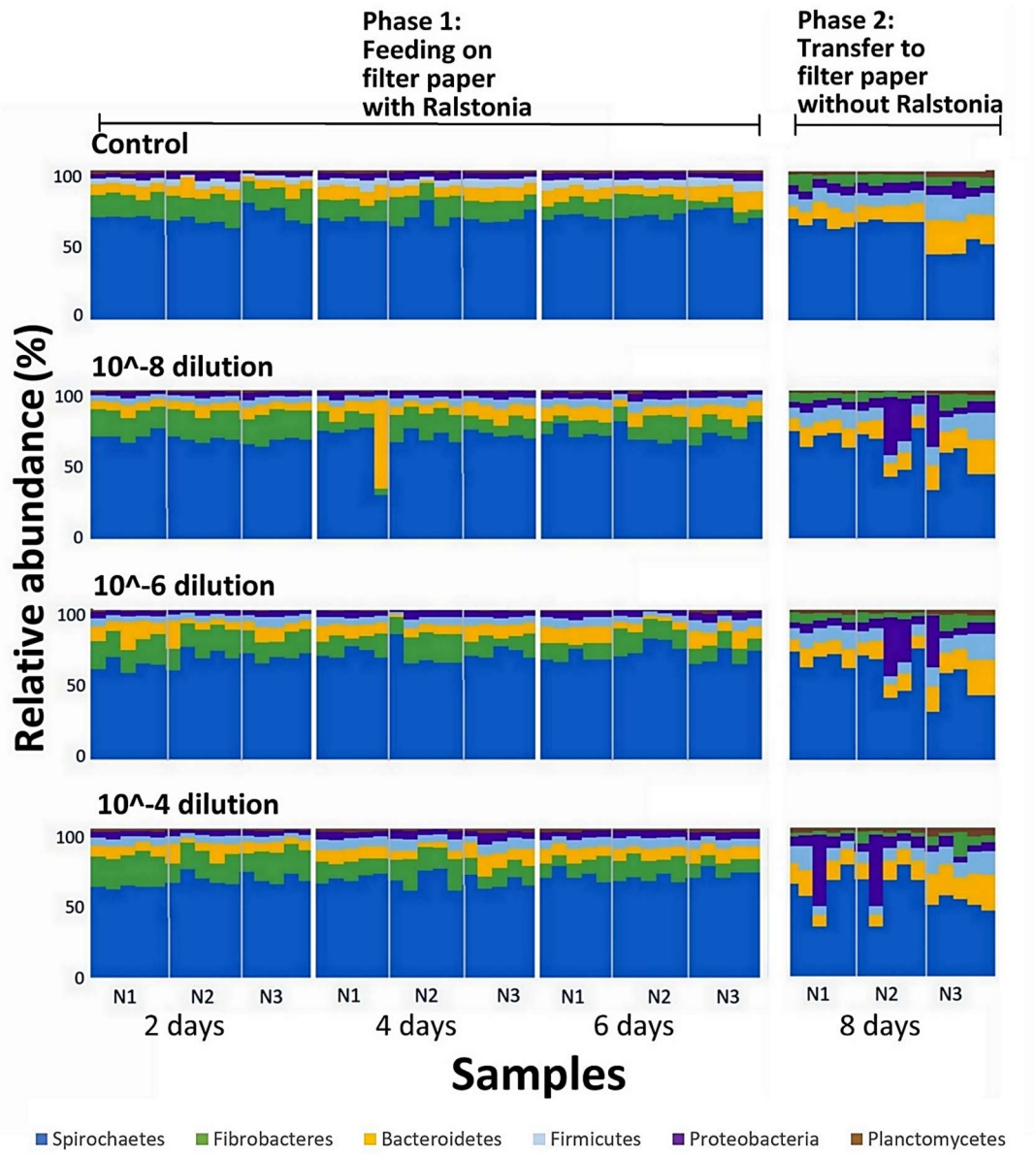

Figure 11. Taxa barplots showing the shift in relative abundance of bacterial phyla of termites between the two phases of the experiment and indicating the similarities among different Ralstonia concentrations (No Ralstonia control, 10^{-8}, 10^{-6}, and 10^{-4}), differences among different durations of feeding on Ralstonia inoculated filter paper (Phase 1: 2,4, and 6 days) and between Phase 1 and Phase 2, and different colonies (N1, N2, N3). There were five replicates for each Ralstonia concentration, time point and colony. Phase 1: Termites were fed for 2, 4, and 6 days with different concentrations of Ralstonia. Phase 2: Termites fed for 6 days with Ralstonia were fed for two additional days on filter paper only for a total duration of the experiment of 8 days.

Table 10. Significant differences in alpha diversity (Pielou's evenness, Faith's PD, ASV richness, Shannon diversity) and beta diversity (Weighted Unifrac distance) between the bacteria communities of termites on day 6 of Phase 1 (feeding on filter paper with Ralstonia) and Phase 2 (2 days after transfer to filter paper without Ralstonia). Asterisks indicate the significant differences.

Metric		Kruskal-Wallis ANOVA	p value
Alpha diversity	Pielou's evenness	33.64	6.64E-09*
	Faith's PD	10.72	0.001*
	ASV richness	6.81	0.009*
	Shannon diversity	29	7.24E-08*
		PERMANOVA	
Beta diversity	Weighted unifrac	pseudo-F	p value
		38.38	0.001*

Table 11. Differentially abundant ASVs between the two phases of the experiment (Phase 1: After 6 days of feeding on filter paper with Ralstonia and Phase 2: After transfer to filter paper without Ralstonia for 2 days). Significance of differential abundance was determined by ANCOM-BC at q value less than 0.05. W is the test statistic for determining differential abundance. Positive values for W depict that the ASV is significantly enriched in Phase 1 (6 days) and negative values for W depict significant enrichment in Phase 2 (8 days). The phase in which an ASV is enriched is marked in blue for easy interpretation.

Taxon			**Differential abundance**		
Phylum	Family	Lowest SILVA assignment	W (Phase 1 (6 days))	Phase 1 (6 days)	Phase 2 (8 days)
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	7.27		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	5.95		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	5.81		
Fibrobacteres	uncultured bacterium	uncultured Chitinivibrionia bacterium	5.79		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	5.53		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	5.38		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	5.08		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	4.72		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	4.69		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	4.65		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	4.3		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	4.3		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	3.97		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	3.93		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	8.6		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-8.51		
Spirochaetes	Spirochaetaceae	uncultured Spirochaetes bacterium	-6.9		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-6.77		
Spirochaetes	Leptospiraceae	uncultured bacterium	-6.74		

(table cont'd.)

Taxon			Differential abundance		
Phylum	Family	Lowest SILVA assignment	W (Phase 1 (6 days))	Phase 1 (6 days)	Phase 2 (8 days)
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-6.5		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	6.17		
Spirochaetes	Spirochaetaceae	uncultured Spirochaetes bacterium	5.96		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.93		
Spirochaetes	Spirochaetaceae	uncultured Spirochaetaceae bacterium	-5.86		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.86		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.77		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-5.7		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-5.57		
Spirochaetes	Spirochaetaceae	uncultured Spirochaetes bacterium	5.52		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-5.44		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-5.32		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.17		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.13		
Spirochaetes	Leptospiraceae	uncultured spirochete	5.1		
Spirochaetes	Spirochaetaceae	Termite Treponema cluster	5.06		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-5.03		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5.02		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	5		
Spirochaetes	Spirochaetaceae	uncultured Spirochaetes bacterium	4.96		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.92		

(table cont'd.)

Taxon			Differential abundance		
Phylum	Family	Lowest SILVA assignment	W (Phase 1 (6 days))	Phase 1 (6 days)	Phase 2 (8 days)
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.89		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.83		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.69		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-4.65		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.64		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.56		
Spirochaetes	Spirochaetaceae	Termite Treponema cluster	-4.54		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.51		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.50		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.48		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-4.46		
Spirochaetes	Spirochaetaceae	uncultured Spirochaetes bacterium	4.34		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.32		
Spirochaetes	Spirochaetaceae	Termite Treponema cluster	-4.21		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.20		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-4.18		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.15		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.14		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	4.07		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	3.99		

(table cont'd.)

Taxon			Differential abundance		
Phylum	Family	Lowest SILVA assignment	W (Phase 1 (6 days))	Phase 1 (6 days)	Phase 2 (8 days)
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-3.95		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	3.94		
Spirochaetes	Spirochaetaceae	uncultured *Treponema* sp.	-3.92		
Bacteroidetes	uncultured bacterium	uncultured Bacteroidetes bacterium	-7.29		
Bacteroidetes	uncultured bacterium	uncultured Bacteroidales bacterium	6.04		
Bacteroidetes	Dysgonomonadaceae	uncultured bacterium	-5.3		
Bacteroidetes	Dysgonomonadaceae	uncultured Bacteroidales bacterium	-4.82		
Bacteroidetes	Rikenellaceae	uncultured Bacteroidales bacterium	4.60		
Bacteroidetes	Tannerellaceae	uncultured bacterium	-4.37		
Bacteroidetes	Dysgonomonadaceae	uncultured Bacteroidales bacterium	-4.27		
Bacteroidetes	Tannerellaceae	uncultured bacterium	-3.95		
Firmicutes	Peptococcaceae	uncultured Firmicutes bacterium	-7.77		
Firmicutes	Enterococcaceae	Enterococcus raffinosus cftri2200	-7.74		
Firmicutes	Ruminococcaceae	uncultured Firmicutes bacterium	-7.41		
Firmicutes	Ruminococcaceae	uncultured Lachnospiraceae bacterium	7.15		
Firmicutes	Ruminococcaceae	uncultured Firmicutes bacterium	-6.11		
Firmicutes	Christensenellaceae	uncultured Clostridiales bacterium	-6.11		
Firmicutes	Ruminococcaceae	uncultured Lachnospiraceae bacterium	-5.75		
Firmicutes	Ruminococcaceae	uncultured Clostridiaceae bacterium	-5.66		
Firmicutes	Lachnospiraceae	uncultured bacterium	5.57		
Firmicutes	Peptococcaceae	uncultured Veillonellaceae bacterium	5.53		
Firmicutes	Christensenellaceae	uncultured bacterium	-5.02		
Firmicutes	Peptococcaceae	uncultured Firmicutes bacterium	-4.76		

(table cont'd.)

Taxon			Differential abundance		
Phylum	Family	Lowest SILVA assignment	W (Phase 1 (6 days))	Phase 1 (6 days)	Phase2 (8 days)
Firmicutes	Christensenellaceae	uncultured bacterium	-4.73		
Firmicutes	Christensenellaceae	uncultured rumen bacterium	-4.57		
Firmicutes	Christensenellaceae	uncultured bacterium	-4.55		
Firmicutes	uncultured bacterium	uncultured Clostridiales bacterium	4.47		
Firmicutes	Peptococcaceae	uncultured Firmicutes bacterium	-4.27		
Firmicutes	Ruminococcaceae	uncultured Lachnospiraceae bacterium	4.19		
Firmicutes	Ruminococcaceae	uncultured Firmicutes bacterium	-4.18		
Firmicutes	uncultured bacterium	uncultured Firmicutes bacterium	-4.08		
Firmicutes	Christensenellaceae	uncultured bacterium	-4.04		
Planctomycetes	Pirellulaceae	uncultured Planctomycetales bacterium	-7.04		
Planctomycetes	Pirellulaceae	uncultured planctomycete	-4.99		
Planctomycetes	Pirellulaceae	uncultured planctomycete	-4.07		
Planctomycetes	uncultured bacterium	uncultured alpha proteobacterium	6.49		
Planctomycetes	Rhodocyclaceae	uncultured bacterium	-5.42		
Planctomycetes	Desulfovibrionaceae	uncultured bacterium	-4.63		
Planctomycetes	Desulfovibrionaceae	uncultured bacterium	-4.06		
Acidobacteria	Propionibacteriaceae	uncultured actinobacterium	-4.57		
Acidobacteria	Propionibacteriaceae	uncultured actinobacterium	-4.42		
Acidobacteria	uncultured bacterium	uncultured actinobacterium	-4.17		
Acidobacteria	Holophagaceae	uncultured Acidobacteriales bacterium	-4.12		
Acidobacteria	uncultured bacterium	uncultured bacterium	-4.10		
Acidobacteria	Holophagaceae	uncultured Acidobacteriales bacterium	-3.96		
Acidobacteria	uncultured bacterium	uncultured bacterium	-3.94		
Synergistetes	Synergistaceae	uncultured bacterium	-4.56		

2.3.9.5. Feeding on different Ralstonia concentrations did not impact termite microbiome

Initial feeding with different Ralstonia concentrations (10^{-4}, 10^{-6}, 10^{-8} and no Ralstonia) in Phase 1 showed no significant effect on bacterial communities of termites; neither in Phase 1 while termites were feeding on Ralstonia nor in Phase 2 after termites were transferred to Ralstonia free filter paper (Table 12). The microbiome of termites during Phase 1 was similar across Ralstonia concentrations as alpha diversity (Pielou's evenness, Faith's PD, ASV richness, Shannon diversity) and beta diversity (PERMANOVA) of the microbiome did not show any significant difference (all $p \geq 0.41$, Kruskal-Wallis ANOVA for alpha diversity and $p=0.27$, PERMANOVA for beta diversity). Similarly, there was no difference in Phase 2 among the microbiomes of termites initially fed on different Ralstonia concentrations during Phase 1 (all $p \geq 0.16$, Kruskal-Wallis ANOVA for alpha diversity and $p=0.83$, PERMANOVA for beta diversity) (Figure 11, Table 12). Furthermore, the shift in microbiome composition between Phase 1 and Phase 2 also occurred in the No Ralstonia controls. This indicates that the presence or concentration of Ralstonia was not causing the shift in the microbiome observed during the experiment (Figure 11).

Table 12. Lack of significant differences in alpha diversity (Pielou's evenness, Faith's PD, ASV richness, Shannon diversity) and beta diversity of the bacteria community of termites in both phases of the no-choice experiment with respect to the initial feeding with different Ralstonia concentrations (No Ralstonia, 10^{-8}, 10^{-6}, and 10^{-4}). Phase 1: Termites were fed for 2, 4, and 6 days with different concentrations of Ralstonia. Phase 2: Termites fed for 6 days with Ralstonia were fed for two additional days on filter paper only.

Metric		Phase 1: Feeding on filter paper with different Ralstonia concentrations			Phase 2: Transfer to filter paper without Ralstonia	
		Kruskal-Wallis ANOVA	p value		Kruskal-Wallis ANOVA	p value
Alpha diversity	Pielou's evenness	2.87	0.41		4.15	0.24
	Faith's PD	1.81	0.61		3.23	0.35
	ASV richness	0.61	0.89		5.21	0.16
	Shannon diversity	0.05	0.99		4.65	0.20
		PERMANOVA			PERMANOVA	
Beta diversity	Weighted unifrac	pseudo-F	p value		pseudo-F	p value
		1.14	0.27		0.6	0.83

2.3.9.6. Impact of duration of feeding on termite microbiome

Since presence of Ralstonia was not impacting the microbiome in termites, we analyzed if the duration of the experiment, i.e. the duration termites were fed on filter paper under lab conditions separated from their colony, was impacting the bacterial communities of termites. It was found that the duration of feeding on filter paper (regardless of Ralstonia concentrations) had impact on most of the alpha diversity indices in both phases of the experiment (Figure 12, Table 13). The alpha diversity of the bacterial community (measured by ASV richness, Faith's PD, Pielou's evenness, Shannon diversity) significantly increased (all $p \leq 0.04$, Kruskal-Wallis ANOVA) during Phase 1, i.e., from 2 days to 6 days of filter paper feeding with Ralstonia (Figure 12, Table 13). Furthermore, all alpha diversity indices significantly increased from the beginning of Phase 1 to

Phase 2 (Figure 12). The pairwise comparisons between bacterial communities of termites at 2 days, 4 days, and 6 days of Phase 1 as well as Phase 2 (at 8 days) were significantly different (all $p \leq 0.01$, PERMANOVA) (Table 13). A total of 18 ASVs from six different phyla Fibrobacteres, Spirochetes, Bacteroidetes, Firmicutes, Proteobacteria and Patescibacteria were differentially abundant ($q < 0.05$) between the termite samples collected at 2 days and 4 days while a total of 31 ASVs from the same six phyla were differentially abundant between 2 days and 6 days (Table 14).

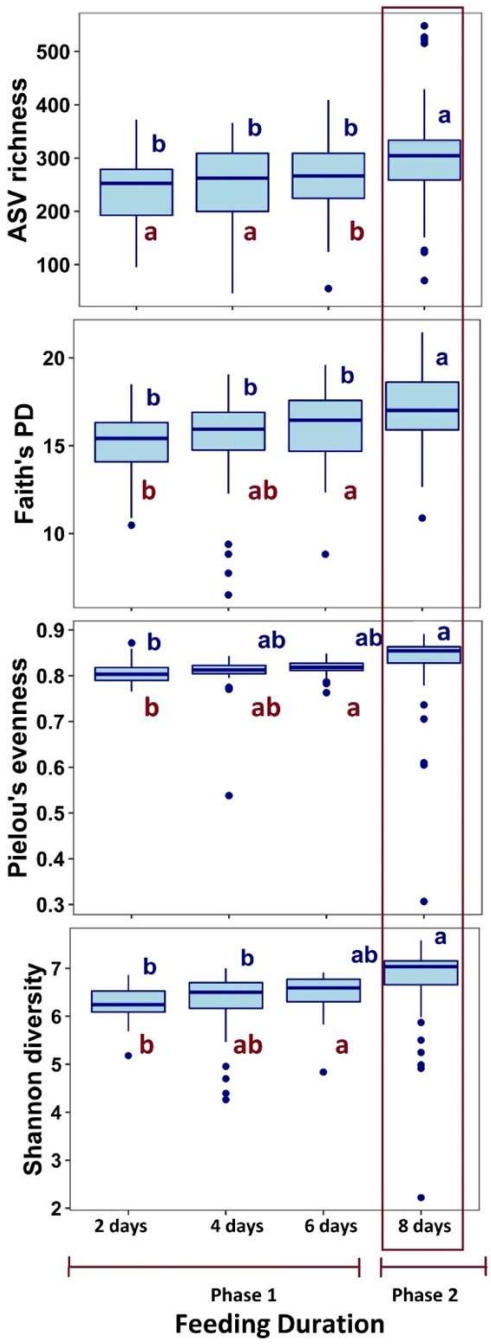

Figure 12. Differences in alpha diversity (ASV richness, Faith's PD, Pielou's evenness, Shannon diversity) of bacterial communities in termites during Phase 1 (represented by red letters below the boxes) and for the overall duration of feeding including Phase 1 and Phase 2 (represented by blue letters above the boxes). The red box in the figure is separating the Phase 2 from Phase 1.

Table 13. Alpha diversity (Pielou's evenness, Faith's PD, ASV richness, Shannon diversity) and beta diversity of bacteria community of termites with respect to duration of feeding in both phases of the no-choice experiment. Phase 1: Termites were fed for 2, 4, and 6 days with different concentrations of Ralstonia. Phase 2: Termites fed for 6 days with Ralstonia were fed for two additional days on filter paper only.

Metrics		Phase 1 (2, 4, 6 days)			Phase 1 (6 days) versus Phase 2 (8 days)	
		Kruskal-Wallis ANOVA	p value		Kruskal-Wallis ANOVA	p value
Alpha diversity	Pielou's evenness	16.9	0.0002*		67.25	1.65E-Q4*
	Faith's PD	9.98	0.006*		34.66	1.43E-07*
	ASV richness	6.27	0.04*		25.22	0.00001*
	Shannon diversity	16.09	0.0003*		10.22	3.39E-13*
		PERMANOVA			PERMANOVA	
Beta diversity	Weighted unifrac	pseudo-F	p value		pseudo-F	p value
		14.59	0.0009*		40.55	0.0010*
		Pairwise PERMANOVA			Pairwise PERMANOVA	
		pseudo-F	q value		pseudo-F	q value
	2 days to 4 days	13.28	0.001*		12.74	0.0012*
	4 days to 6 days	2.52	0.010*		2.66	0.0010*
	2 days to 6 days	34.16	0.010*		34.12	0.0012*
	2 days to Phase2	-	-		91.85	0.0012*
	4 days to Phase2	-	-		41.72	0.0012*
	6 days to Phase2	-			37.72	0.0012*

Table 14. Differentially abundant ASVs in Phase 1 (between 2 days and 4 days, and between 2 days and 6 days) of the experiment. Significance of differential abundance was determined by ANCOM-BC at q value less than 0.05. W is the test statistic for determining differential abundance. Positive values for W depict that the ASV is significantly enriched at 2 days compared to 4 days or 6 days and vice versa for negative W values. The significant enrichment of each ASV for a particular time point is marked in blue.

Taxon			Differential abundance		
2 days vs 4 days					
Phylum	Family	Lowest SILVA assignment	W (4 days)	2 days	4 days
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.49		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.35		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.19		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.57		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-3.92		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.40		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-3.46		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-3.24		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	4.02		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	-4.12		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	-3.70		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	-3.59		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	-4.09		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	4.27		
Bacteroidetes	Rikenellaceae	uncultured Bacteroidales bacterium	-4.21		
Firmicutes	Ruminococcaceae	uncultured Firmicutes bacterium	-3.67		
Patescibacteria	Patescibacteria	uncultured bacterium	4.09		
Proteobacteria	Moraxellaceae	Acinetobacter sp. SJ-15	3.79		

(table cont'd.)

Taxon			Differential abundance		
2 days vs 6 days					
Phylum	Family	Lowest SILVA assignment	W (6 days)	2 days	6 days
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-5.94		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.68		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.38		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-7.46		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-5.58		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-5.37		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-5.08		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.64		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.47		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.26		
Fibrobacteres	Fibrobacteraceae	uncultured Fibrobacterales bacterium	-4.21		
Spirochaetes	Spirochaetaceae	uncultured Spirochaetaceae bacterium	3.93		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	-5.24		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	5.11		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	-4.78		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	-4.55		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	-4.25		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	-4.10		

(table cont'd.)

Taxon			Differential abundance		
2 days vs 6 days					
Phylum	Family	Lowest SILVA assignment	W (6 days)	2 days	6 days
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	4.51		
Spirochaetes	Spirochaetaceae	uncultured Treponema sp.	4.09		
Bacteroidetes	Dysgonomonadaceae	uncultured Bacteroidales bacterium	4.59		
Bacteroidetes	uncultured	uncultured Bacteroidetes bacterium	4.18		
Bacteroidetes	Rikenellaceae	uncultured Bacteroidales bacterium	-4.74		
Bacteroidetes	Rikenellaceae	uncultured Bacteroidales bacterium	4.11		
Bacteroidetes	Rikenellaceae	uncultured Bacteroidales bacterium	4.17		
Firmicutes	Ruminococcaceae	uncultured Firmicutes bacterium	-4.41		
Firmicutes	uncultured	uncultured Clostridiales bacterium	4.08		
Patescibacteria	Patescibacteria	uncultured bacterium	3.80		
Proteobacteria	uncultured	uncultured alpha proteobacterium	-4.51		
Proteobacteria	uncultured	uncultured rumen bacterium	4.40		
Proteobacteria	Moraxellaceae	Acinetobacter sp. SJ-15	5.03		

2.4. Discussion

The goal of this research was to determine if the termite *N. takasagoensis* plays a role as a pathogen vector in the decline of ironwood trees of Guam. We hypothesized that termite workers infesting diseased ironwood trees would carry IWTD pathogens, because they would have ingested bacteria by consuming diseased wood (Agrios 2008, Perilla-Henao and Casteel 2016, Brune 2014, Rossmassler et al. 2015, Vikram et al. 2021). If true, termites would be possible vectors for disease transmission to healthy trees while foraging. Our results based on 16S rRNA gene amplicon sequencing of the bacterial population present in *N. takasagoensis* worker samples collected from IWTD pathogen infested and healthy ironwood trees in Guam suggest that *N. takasagoensis* workers are not a vector for pathogens causing IWTD as we did not find any of the pathogens associated with IWTD in considerable amounts in these termite samples. *Ralstonia solanacearum* was absent, *Klebsiella* species were present in only a few samples, and other potential pathogens previously isolated from diseased ironwood trees like *Kosakonia*, *Enterobacter*, *Pantoea*, *Erwinia* and *Citrobacter* were also not detected in the termite samples (Ayin et al. 2015, Ayin et al. 2019).

It is unlikely that the absence of *R. solanacearum* in the bacterial microbiota of the termite samples collected from ironwood trees in Guam was a technical artifact caused by sequencing failure, lack of reference sequences, or insufficient sequencing- or sampling-depth. The primers used in this study to amplify Ralstonia were verified by performing BLAST against NCBI GenBank database for their ability to detect *R. solanacearum*. Moreover, pure cultures of *R. solanacearum* isolated from IWTD that were used in feeding experiments were positively identified using the same methods. Also, other species within the same family as the genus Ralstonia were detected while

analyzing bacterial communities of these termite samples (2.3.3). Although Ralstonia was not detected in freshly collected *N. takasagoensis* workers, it was present after the no-choice feeding experiment caused microbiota changes in lab reared termites. The target bacteria, *R. solanacearum* and related strains of the Ralstonia complex, are present in the database (SILVA 132) used for taxonomic assignment of ASVs obtained after sequencing of bacterial communities in this study. In addition, rarefaction analyses showed that sequencing-depth and sample effort was sufficient to capture the vast majority of bacteria associated with the termite samples.

Despite the absence of *R. solanacearum*, all the expected core bacteria were found in the *N. takasagoensis* worker samples collected from ironwood trees in Guam. The core bacterial phyla present in *N. takasagoensis* include Spirochaetes, Fibrobacteres, Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Margulisbacteria (TG3 phylum) (Hongoh et al. 2006, Warnecke et al. 2007, Miyata et al. 2007, Köhler et al. 2012, Marynowska et al. 2017, Calusinska et al. 2020). In our study, Spirochaetes and Fibrobacteres were the most dominant phyla which was similar to the most recent studies based on 16S rRNA gene amplicon high-throughput sequencing of the gut microbiome of higher termites including *Nasutitermes* species performed on the Illumina MiSeq platform (Marynowska et al. 2017, Calusinska et al. 2020). Furthermore, studies performing taxonomic analysis of bacterial populations specifically of *N. takasagoensis*, always identified the Spirochaetes as the most dominant phylum even when different sequencing methods were used (Hongoh et al. 2006, Miyata et al. 2007, Köhler et al. 2012). However, the abundance of Fibrobacteres varied among all the previous literature on *N. takasagoensis* (Hongoh et al. 2006, Warnecke et al. 2007, Miyata et al. 2007, Köhler et al. 2012). Hongoh et al. (2006) and Miyata et al. (2007) used clone-based methods and ranked Fibrobacteres as the second and fourth

most abundant phylum, respectively, while Köhler et al. (2012) found Fibrobacteres only in minor abundance (0.04%) in the termite gut using Pyrotag sequencing. Unlike our study, none of the previous study found Fibrobacteres (41.38%) in a comparable abundance to Spirochaetes (48.16%) (Table 5, Figure 5). This difference in Fibrobacteres abundance might be due to different primers, sequencing methods, and analysis methods used in these studies; alternatively, it is possible that abundance of Fibrobacteres is impacted based on the geographical region, environmental conditions, or type of diet. Overall, the taxonomic profiling performed in our study was able to detect all the core phyla that were observed in previous studies of *N. takasagoensis*, moreover, we also detected some phyla such as Tenericutes, Patescibacteria, Deinococcus-Thermus, Gemmatimonadetes, Entotheonellaeota, and Epsilonbacteraeota that were not detected in previous studies.

The probability of *R. solanacearum* presence in termite bodies under natural conditions is less than 1 in 9 million bacteria, since no *R. solanacearum* sequences were identified among the 9 million sequencing reads from 42 *N. takasagoensis* worker samples freshly collected from ironwood trees in Guam. The reason behind absence of *R. solanacearum* and other IWTD pathogens in these *N. takasagonesis* worker samples could be that these termites either do not feed on infected wood or, if they feed and ingest bacteria from infected trees, the pathogens do not survive in the termites. The feeding experiments giving termites the choice between wood from healthy ironwood trees and wood pieces containing pathogens indicate that termites prefer consuming healthy wood. In a four-choice test between healthy ironwood and wood with naturally different amounts of Ralstonia and wetwood bacteria, termites consumed more of the healthy wood than wood containing pathogens. Similarly, when ironwood pieces were artificially inoculated with Ralstonia, termites

showed a marginal tendency to consume more of the negative control than of Ralstonia inoculated wood. Thus, our results indicate that given a choice, termites are less likely to feed on wood that contains IWTD pathogens.

However, even with a preference for healthy wood, it is unlikely that termites can avoid all exposure to Ralstonia and other pathogens while feeding and nesting on trees with Ralstonia infection. Therefore, it was hypothesized that Ralstonia might not be able to establish itself in a healthy termite. This was confirmed by no-choice feeding experiments in the lab. Even when *N. takasagoensis* workers were forced to come into contact and consume high concentrations of Ralstonia on filter paper, no Ralstonia could be detected in the first 6 days. Typically, termites ingest bacteria within a day and transfer them rapidly to colony members via trophallaxis and grooming as shown in previous studies with genetically engineered fluorescent bacteria (Husseneder et al. 2005, Husseneder and Grace 2005). This is strong evidence that a healthy Nasutitermes microbiota is refractory to invasion by Ralstonia. This could be due to conditions in the gut preventing Ralstonia from growing as *N. takasagoensis* being a higher termite has an alkaline gut pH (6-10) and the favorable conditions for *R. solanacearum* to thrive includes a low pH (pH 4.5–5.5) (Brune 2014, Li et al. 2017). In addition, it has been reported that termites have an innate and adaptive immunity that provides them protection against the invasion by foreign microbes present in feeding sites and food sources (Rosengaus et al. 1999, Traniello et al. 2002, Cremer et al. 2007, Bulmer et al. 2009, Cremer et al. 2018). The indigenous gut symbionts of termites prevent foreign microbes from colonizing the termite guts (Veivers et al. 1982, Dillon and Dillon 2004, Sen et al. 2015, Peterson and Scharf 2016, Oberpaul et al. 2020). Moreover, specialized microbial communities and antimicrobial proteins have been detected in termite nests,

different from the surrounding soil, that add another level of protection to the termite colony (Bulmer et al. 2009, Visser et al. 2012, Chouvenc et al. 2013, Oberpaul et al. 2020, Soukup et al. 2021 and Witasari et al. 2022).

Interestingly, some *N. takasagoensis* worker samples did show considerable numbers of Ralstonia after 6 days of feeding on Ralstonia inoculated filter paper plus two additional days of feeding on filter paper only. This rise of Ralstonia in some samples coincided with a shift in the microbiota in all samples marked by a reduction of Fibrobacteres, an increase in bacteria diversity and a decline in termite health and is most likely explained as dysbiosis, i.e., an imbalance in the microbiota (Messer and Chang 2018). It is possible that the dysbiosis facilitated invasion by foreign bacteria because protection by indigenous gut symbionts was weakened (Brown et al. 2012, Toor et al. 2019, Pan et al. 2020). As a result, Ralstonia was able to thrive at least in some termite laboratory colonies, although it was not detected in the field samples. However, this dysbiosis was not caused by Ralstonia feeding as Ralstonia concentrations had no effect on microbiota and termites not fed with Ralstonia showed the same shift in relative abundance of phyla but without Ralstonia invasion (Figure 11, Table 12).

This dysbiosis might have occurred due to removal of the termite workers from their natural environment and lab rearing them on an artificial diet, i.e., processed filter paper, since in contrast to Ralstonia concentration, the duration of the experiment showed significant effects on the microbiota (Figure 11). This shift in microbiota observed after more than one week (8 days) of feeding on filter paper was in accordance with results from previous studies which suggested that a shift in microbiome might take place between one and two weeks when termites are fed on an

artificial diet (Tanaka et al. 2006, Miyata et al. 2007, Su et al. 2017). Boucias et al. (2013) fed termite workers (*Reticulitermes flavipes*, Rhinotermitidae) on lignin-rich and lignin-poor cellulose diet for one week but did not observe any significant changes in microbiota. However, Su et al. (2017) observed a significant shift in gut microbiota after feeding workers of a different rhinotermitid species (*Tsaitermes ampliceps*, Rhinotermitidae) for two weeks on filter paper. The relative abundance of Spirochaetes was decreased, while the relative abundance of Proteobacteria, Bacteroidetes and Firmicutes was increased in termites feeding on filter paper as compared to those feeding on wood after two weeks (Su et al. 2017). Miyata et al. (2007), also observed a significant increase in diversity and richness of microbiota in *N. takasagoensis* workers feeding for three weeks on artificial diets as compared to those feeding on wood (Miyata et al. 2007). The relative abundance of Spirochaetes and Fibrobacteres was decreased and relative abundance of Bacteroidetes and Firmicutes was increased in termites feeding on artificial diets as compared to those feeding on wood (Miyata et al. 2007). The shift in microbiota including the reduction in Fibrobacteres abundance observed in Miyata et al.'s (2007) study on *N. takasagoensis* workers after 3 weeks of feeding on filter paper was in accordance with the microbiota shift observed in our study after 8 days of filter paper diet for the same species. Furthermore, Tanaka et al. (2006) fed *C. formosanus* workers for four weeks and observed an increase in protozoa diversity in termites fed with artificial diet as compared to those fed with wood. A change in protozoa diversity likely influences bacteria diversity as many bacteria species are known to be associated with protozoa as ecto-and endo-symbionts (Brune and Dietrich 2015).

Along with the highly distinct shift that occurred between Phase 1 and Phase 2 (i.e., after 8 days), a gradual shift in bacterial diversity was observed within Phase 1 as well (Figure 12, Table 13),

right after the termites started feeding on the artificial diet. The increase in microbiome diversity in Phase 1 might be the result of a population increase in previously rare bacteria species that were preadapted to the new conditions along with new exposure to bacteria in the laboratory experiment. Bacteria that were not able to thrive on filter paper diet or the bacteria that were not required in large amounts for digesting the processed cellulose in filter paper, experienced a population reduction. This is indicated by a decrease in abundance of Fibrobacteres as the majority of bacterial species in this phylum are obligate symbionts adapted to hydrolyzing complex lignocellulose from wood (Warnecke et al. 2007, Ransom-Jones et al. 2012). We did not examine the effects of filter paper diet on the microbiota for longer than 8 days, however, based on the results of previous studies in different termite species, it can be assumed that the microbiome will most likely stabilize after the dysbiosis if the conditions stay constant (Boucias et al. 2006, Su et al. 2017, Miyata et al. 2007, Tanaka et al. 2006).

Although *R. solanacearum,* was not found in termites samples collected from ironwood trees in decline and bacterial diversity within termite samples (alpha diversity) was not impacted by Ralstonia presence in trees, bacterial communities differed between the group of termite samples collected from trees that were infested with *R. solanacearum* as compared to termites collected from *R. solanacearum* free trees. This difference in beta diversity was at least partially driven by differential abundance of a *Treponema* sp. from phylum Spirochaetes and an uncultured bacterium from phylum Bacteroidetes as both of these bacteria species were enriched in termites attacking trees that tested negative for Ralstonia. Our knowledge regarding these uncultured bacterial species found within termite samples is limited, however, we can still make inferences from described relatives of these bacteria. Most bacterial species within phyla Spirochaetes and Bacteroidetes are

obligate symbionts of the termites (Tokuda et al. 2018, Zhang et al. 2012). *Treponema* species in termite guts are known to contribute to reductive acetogenesis and nitrogen fixation and some bacteria within phylum Bacteroidetes are known to play a significant role in fermentation of sugars and uric acid degradation (Breznak and Leadbetter 2006, Tokuda et al. 2018, Ohkuma 1996, Zhang et al. 2012).

The mechanism by which Ralstonia infection of trees might alter termite microbiota composition is not known. It is unlikely that consumption of Ralstonia alone would alter the termite microbiota since the feeding experiments showed that Ralstonia alone is not the reason for microbial shifts and Ralstonia does not establish itself easily in a healthy termite. It is, however, possible that other factors confounded by or in interaction with Ralstonia infestation influence the beta diversity. One of such factors that was found impacting beta diversity of the bacterial composition of termites in this study was altitude of the ironwood tree location with respect to mean sea level, as bacterial communities within termite samples collected from high altitude trees were enriched in *Treponema* sp. as compared to the samples collected from low altitude trees. However, the factors and their interactions investigated in this study explained only a small percentage (0.14%) of the variance in beta diversity of microbiota, suggesting that there are other abiotic or biotic factors influencing the microbial composition that need to be researched in future studies.

While there was differentiation of termite microbiota between termite samples from Ralstonia positive and negative trees (beta diversity), the bacterial diversity within the individual termite samples (alpha diversity) was not affected by Ralstonia presence. Since Ralstonia is one of the main agents involved in IWTD one might have expected that other factors measuring tree- and

plot-health would show similar results. However, none of the health-related factors impacted beta diversity, but several affected alpha diversity of termite microbiota. This discrepancy might be explained by the fact that not all the trees that were categorized as "sick" based on visual symptoms of decline tested positive for Ralstonia. Possible reasons could be that symptoms of decline were appearing because of reasons other than *Ralstonia* infection such as aging or presence of wetwood. In addition, the immunodiagnostic method of detecting Ralstonia might cause false negatives when Ralstonia amounts are low.

In termites that were feeding on trees categorized as "sick" based on visual symptoms of decline, higher phylogenetic diversity of bacteria was observed than in "healthy" trees. With increase in tree stress due to higher level of site management, increase of decline in plot, increase of sick trees in plot, and increase of termite infestation in plot, bacterial communities of termites feeding on those trees showed less evenness. A higher proportion of dead trees in the plot was associated with decreased Shannon diversity of bacterial communities of termites feeding on the trees. Antimicrobial defense mechanisms in the wood of living trees, protect the tree from pathogen invasion (Pearce 1995). The microbiome present in these plants acts as an additional layer of defense against pathogenic organisms (Teixeira et al. 2019). However, a previous study observed a shift in microbiome composition of trees that have diseases as compared to asymptomatic trees (Koskella et al. 2017). Koskella et al. (2017) found a number of opportunistic pathogens that were highly abundant in diseased trees as compared to healthy trees and suggested that when tree health is impacted due to disease, the opportunity for pathogenic bacteria to colonialize the tree tissues is increased. Thus, it is possible that in our study as well, the sickness of the tree, increase in tree stress or death of the tree might change wood chemistry and, subsequently, the microbiome of the

tree. As a result of which the defenses of ironwood trees against opportunistic bacteria might have been weakened and more bacteria were able to attack the trees in decline as compared to healthy trees. A change in tree microbiome might have direct or indirect effects on termite microbiota diversity. However, the overall bacterial richness within the termite samples was not impacted by tree stress or disease. A change of evenness, phylogenetic distances and Shannon diversity, without change in ASV richness shows that the number of species in the gut ecosystem are in a stable equilibrium limited by the number of ecological and functional niches available. To better understand stress related microbiota changes, ironwood tree microbiomes should be studied in association with tree-, plot- and location related factors. Along with this, relationships between the tree microbiota, bacteria in the surrounding soil and in the termites that attack the tree can also be studied in the future to determine the reasons behind causation and transmission of IWTD with the goal to develop an integrated management plan for controlling the spread of this decline to other Micronesian islands.

In conclusion, we can rule out members of *N. takasagoensis* as vectors of IWTD pathogens. However, there are still various biotic and abiotic factors that might influence the spread of IWTD pathogens, including other species of termites. It is possible that other termite species found on the ironwood trees of Guam (Park et al. 2019), that have not been covered in this chapter, might play some role in transmission of these pathogens. For instance, members of *Coptotermes gestroi*, which is a lower termite and is the second most dominant termite species, have slightly acidic to neutral gut pH unlike *N. takasagoensis* members that have alkaline gut pH (Brune et al. 1995, Brune 1996). So, *C. gestroi* workers can possibly provide a favorable environment for Ralstonia to survive. Moreover, *R. solanacearum* was found in the guts of *C. formosanus* alates, a closely

related species to *C. gestroi* (Chen et al. unpublished) indicating the potential that Ralstonia could be part of the microbiota of *C. gestroi* workers. The microbiota of other termite species, *C. gestroi* and *M. crassus* found on ironwood trees in Guam will be investigated in the next chapters of the thesis (Chapter 3, 4).

Chapter 3. Taxonomic Profiling and Diversity Analysis of Bacterial Communities of *Coptotermes gestroi* Workers Associated with Ironwood Trees (*Casuarina equisetifolia*) in Guam

3.1. Introduction

Ironwood (*Casuarina equisetifolia*), a tree widespread across Guam, is one of the most significant agro-forestry species on the island (Elevitch and Wilkinson 2000, Stone 1970, Fosberg et al. 1979). It has been continually propagated in Guam for hundreds of years as confirmed by the records of soil pollen samples (Athens and Ward 2004). The tree has tolerance to salt and partial waterlogging, resistance to pests and diseases, and the ability to fix atmospheric nitrogen (Touati et al. 2016, Conglu et al. 2010, Diouf et al. 2008). The tree is commonly used in Guam as fuelwood, windbreak, shelterbelt, and mulch (Chonglu et al. 2010). It is highly effective in controlling soil erosion, stabilizing sand dunes, reclaiming marshy soils, and combating desertification (Touati et al. 2016, Conglu et al. 2010).

In 2002, a local farmer in Guam reported a sudden decline of ironwood trees on his land (Mersha et al. 2009, Mersha et al. 2010). It was found that the ironwood trees in Guam are being infected with a disease that leads to gradual thinning of their foliage, progressive dieback of branches and eventually death of the tree (Mersha et al. 2009, Mersha et al. 2010). The dead foliage often remains on the tree giving the tree a singed appearance (Mersha et al. 2009, Mersha et al. 2010). The condition of foliage thinning and dieback on ironwood was referred to as Ironwood Tree Decline (IWTD) (Mersha et al. 2009, Mersha et al. 2010). Although the causal organisms behind this decline are still not confirmed, bacterial pathogens like *Ralstonia solanacearum* and *Klebsiella* species (*K. oxytoca* and *K. variicola)* have been frequently isolated from the ooze of ironwood

trees in decline and therefore these bacteria have been considered associated with IWTD (Mersha et al. 2010, Schlub et al. 2011, Schlub et al. 2012, Ayin et al. 2015, Ayin et al. 2019, Schlub et al. 2020). The bacterium *Ralstonia solanacearum* causes bacterial wilt and *Klebsiella* species cause wetwood symptoms and are translocated through xylem vessels of the tree following wound inoculation (Schlub 2013). Pathogens such as *Kosakonia*, *Enterobacter*, *Pantoea*, *Erwinia* and *Citrobacter* were also detected during the phylogenetic analysis of bacterial isolates from declining ironwood trees collected from Guam (Ayin et al. 2019).

A study conducted by Schlub (2010) determined various biotic and abiotic factors associated with IWTD. This study showed significant results (p-value <0.01) for the association of termites with ironwood trees under decline (Schlub 2010). To determine the role of termites in IWTD, termites attacking ironwood trees in Guam were identified using morphological and molecular means (Park et al. 2019). The termite *Coptotermes gestroi* (Wasmann) (Blattodea: Rhinotermitidae) was found to be one of the termite species attacking ironwood trees in Guam (Park et al. 2019).

Coptotermes gestroi is a subterranean termite, native to Southeastern Asia (Wasmann 1896, Kirton and Brown 2003). This termite can feed on timber, wood, paper, cardboard, leather, plastic, rubber, clothes as well as living trees and as a result causes a lot of economic damage (Bignell and Lo 2011, Su and Scheffrahn 1990, Kirton and Brown 2003). This termite is frequently found associated with dead or dying trees (Chouvenc et al. 2018). Foraging tunnels made by *C. gestroi* termites are thin and highly branched, with the overall pattern appearing like a mosaic or jigsaw puzzle (Hapukotuwa et al. 2010, Scheffrahn et al. 1993). While feeding on live trees, these termites hollow out portions of large trees to build a colony (Greaves 1962, Becker 1975). Attack by *C.*

gestroi colonies can led to tree decline and death (Chouvenc et al. 2018). *Coptotermes gestroi* termites are known to attack plants with significantly damaged root system (Fage 1987).

Unlike higher termites (Family: Termitidae) that have diversified diet including wood, fungus and humus, lower termites (Families Archotermopsidae, Mastotermitidae, Stolotermitidae, Kalotermitidae, Hodotermitidae, Stylotermitidae, Rhinotermitidae and Serritermitidae) rely only on wood as a source of cellulose. To digest the ingested wood, the microbial composition of lower termite workers includes both cellulolytic flagellates as well as bacteria in their guts in contrast to higher termite workers in which only bacteria are present (Brune 2014). While protists perform the majority of wood digestion, the bacteria present in the lower termite workers perform lignin and chitin degradation, acetogenesis, recycling of nitrogen in form of uric acid present in the faeces and atmospheric nitrogen fixation (Brune 2013, Brune and Friedrich 2000, Ohkuma 2003, Hongoh et al. 2008, Arora et al. 2022). Along with obligate symbionts, that aid in wood digestion and are vital for the survival of termites, the microbial community of termites can also contain environmental bacteria that are ingested by foraging workers along with the wood that termites are feeding on (Keast and Walsh 1979, Hänel 1982, Brune 2014, Diouf et al. 2018, Fröhlich et al. 2007, Vikram et al. 2021). Plant pathogens such as *Erwinia*, *Pantoea*, *Pseudomonas*, *Burkholderia*, *Acidovorax*, *Xanthomonas*, *Clavibacter*, *Streptomyces,* as well as *Ralstonia* have been previously found inside the bodies of *Coptotermes* termites and their nests (Oberpaul et al. 2020). If the chemical composition and pH are favorable for a particular pathogen, it is possible, that termites could harbor that pathogen in their bodies and transmit it while foraging. Based on this, we hypothesized that members of *C. gestroi* that are found attacking ironwood trees in Guam are vectors for pathogenic bacteria causing IWTD. In this study, the samples of *C. gestroi* workers

were collected from various *R. solanacearum* positive and negative ironwood trees with the objectives of (1.) performing taxonomic profiling of the bacterial composition *C. gestroi* workers that attack ironwood trees in Guam to investigate the incidence and abundance of pathogens associated with IWTD, (2.) determining the impact of tree- and location-related factors associated with ironwood trees attacked by *C. gestroi* workers on the bacterial communities of *C. gestroi* worker samples, and (3.) determine if *C. gestroi* workers prefer feeding on parts of ironwood trees with low pathogen content compared to high pathogen content.

3.2. Materials and methods

3.2.1. Sample collection

Twenty-seven *C. gestroi* termite samples were collected in 2021 by staff members from the University of Guam from ironwood trees at 15 different locations in Guam (Figure 1, Table 1). These termite samples were shipped to LSU AgCenter in 95% ethanol for DNA sequencing. Along with termite samples, information regarding the tree- related factors (Presence of *Ralstonia*, Tree decline severity (DS) and Tree Health), and location-related factors (Site Management, Parent Material, Altitude and Altitude Classification) was also collected from each tree (2.2.1.1). Presence of *Ralstonia solanacearum* in ironwood trees from which termite samples were collected was determined with Immunodiagnostic strips (Agdia, Inc.). A detailed description of these factors is provided in the Materials and methods section of chapter 2 (2.2.1.1).

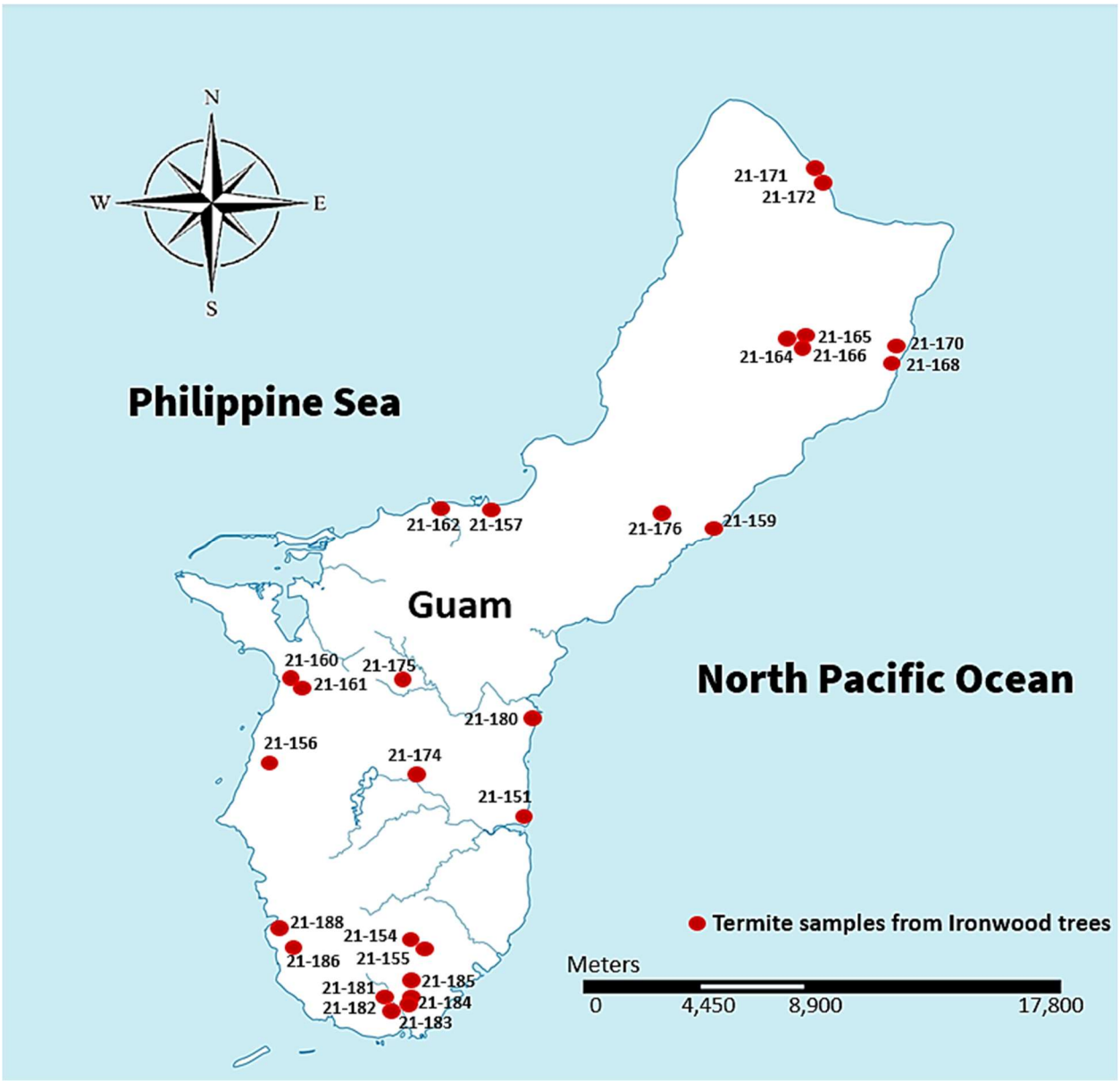

Figure 1. Map of ironwood tree sites where 27 samples of *C. gestroi* termites were collected. Tree metadata for each termite sample can be found in Table 1.

Table1. Location- and tree-related data recorded for *C. gestroi* samples. This data was recorded by the staff members from the University of Guam. Data that was not available is marked as "n/a".

Termite Sample ID	Location	Tree DS	Tree Health	Site Management	Presence of Ralstonia	Altitude (m)	Altitude Classification	Parent Material
21-151	UOG Inarajan Station	n/a	n/a	n/a	n/a	16	Low	Lime
21-154	UOG Ija Station	n/a	n/a	Moderately Managed	n/a	89	Low	Tuff
21-155	UOG Ija Station	nearly dead	Sick	Moderately Managed	Positive	93	Low	Tuff
21-156	Nimitz Park	symptomless	Healthy	Highly Managed	Negative	4	Low	Sand
21-157	Paseo Park Hagatna	symptomless	Healthy	Highly Managed	Negative	6	Low	Lime
21-159	Mangilao Golf Course	n/a	n/a	Moderately Managed	n/a	128	High	Lime
21-160	Apaca Point Agat	symptomless	Healthy	Moderately Managed	Negative	6	Low	Tuff
21-161	Apaca Point Agat	symptomless	Healthy	Moderately Managed	Negative	6	Low	Tuff
21-162	Governor's Complex Aniquia	symptomless	Healthy	Highly Managed	Negative	6	Low	Tuff
21-164	UOG Yigo Station	nearly dead	Sick	Highly Managed	Positive	144	High	Lime
21-165	Watson's Farm Yigo	nearly dead	Sick	Moderately Managed	Positive	159	High	Lime
21-166	Watson's Farm Yigo	heavily damaged	Sick	Moderately Managed	Positive	159	High	Lime
21-168	AAFB	nearly dead	Sick	Highly Managed	Negative	155	High	Lime
21-170	AAFB	slight damage	Healthy	Moderately Managed	Negative	155	High	Lime
21-171	AAFB tarague beach	nearly dead	Sick	Highly Managed	Positive	9	Low	Sand
21-172	AAFB tarague beach	heavily damaged	Sick	Moderately Managed	Negative	9	Low	Sand
21-174	Windward hills golf course	nearly dead	Sick	Highly Managed	Positive	115	High	Tuff
21-175	UOG Inarajan Station	n/a	n/a	n/a	n/a	115	High	Lime
21-176	Mangilao Golf Course	n/a	n/a	Moderately Managed	n/a	128	High	Lime
21-180	Country Club of the Pacific	heavily damaged	Sick	Highly Managed	Negative	26	Low	Tuff
21-181	UOG Ija Station	n/a	n/a	n/a	n/a	75	Low	Tuff
21-182	UOG Ija Station	heavily damaged	Sick	Moderately Managed	Positive	86	Low	Tuff
21-183	UOG Ija Station	n/a	Sick	Highly Managed	n/a	86	Low	Tuff
21-184	UOG Ija Station	n/a	Sick	Highly Managed	n/a	86	Low	Tuff
21-185	UOG Ija Station	n/a	Sick	Highly Managed	n/a	86	Low	Tuff
21-186	Merizo Cemetary	heavily damaged	Sick	No maintenance	Positive	25	Low	Tuff
21-188	Fort Soledad	heavily damaged	Sick	Highly Managed	Negative	42	Low	Tuff

3.2.2. DNA extraction

Total DNA from *C. gestroi* worker samples received from Guam in 95% ethanol was extracted using the DNeasy Blood & Tissue kit (Qiagen, Germantown, MA). Total DNA was extracted by pooling and homogenizing five *C. gestroi* workers per sample using lysis buffer (Buffer AL, DNeasy Blood & Tissue kit) with a sterile pestle (Thermo Fisher Scientific, Wilmington, DE). Quantity of the extracted DNA was tested using the Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE) with the Qubit dsDNA BR Assay Kit). After extraction, 2.5 µl/ng of DNA per sample was sent to University of New Hampshire Hubbard Center for Genome Studies for next generation sequencing. The V4 region of the bacterial 16S rRNA gene present in termite worker samples was amplified using 515F and 926R primers (Caporaso et al. 2010, 2012). Sequencing of DNA was performed using Illumina Nextera Dilute library protocol (Illumina, San Diego, CA). The sequences used in this study were deposited in NCBI GenBank as BioProject ID PRJNA883256.

3.2.3. Bioinformatics and statistical analysis

Bioinformatic analysis was performed using Quantitative Insights Into Microbial Ecology (QIIME2) pipeline (Caporaso et al. 2010, Estaki et al. 2020). The demultiplexed reads were obtained after sequencing from the University of New Hampshire (2.2.3). The Phred quality score of the demultiplexed sequence reads was found to be above 30, therefore no trimming was performed for forward and reverse sequence reads. Using DADA2 denoising, primers were trimmed off. The length of the reads was too short and forward and reverse sequences did not overlap; thus, only forward reads were used for analysis.

Sequence-depth, sample size- as well as coverage-based rarefaction curves were generated to evaluate if sequencing depth, number of samples and coverage used for the analysis were sufficient to capture most of the bacterial diversity present within the samples (2.2.3). Alpha rarefaction curves were plotted using QIIME2 by subsampling the sequence reads without replacement to the common sequencing depth that was equal to the sample with the lowest sequencing depth (4,982). Sample size- and coverage-based curves were plotted using R package iNEXT (iNterpolation/ EXTrapolation) (Hsieh et al. 2016) (2.2.3.).

For taxonomic assignment of sequence reads, i.e., Amplicon Sequence Variants (ASVs, 2.2.3), SILVA 132 reference database (http://www.arb-silva.de) was used (Quast et al. 2013). All the ASVs were taxonomically classified using consensus method in BLAST algorithm (Camacho et al. 2009) at 97% pairwise identity cutoff. The sequences were aligned using MAFFT method and the alignments were masked to remove the variable positions (Lane 1991). Finally, the ASVs that could not be taxonomically assigned, were filtered out and taxa bar plots representing relative abundances of assigned taxa were made. The taxonomic assignment of the top 20 ASVs with the highest number of reads across all samples was confirmed by performing BLAST for these sequences against NCBI GenBank which contains more sequences than SILVA 132, but the latter is better curated.

The diversity of bacteria within the termite worker samples was assessed through alpha diversity indices such as Pielou's evenness, Faith's phylogenetic distance, Number of ASVs and Shannon diversity (2.2.3). Group significance for factors with categorical data such as Presence of Ralstonia, Tree DS, Tree Health, Location, Parent Material, Site Management and Altitude

Classification (2.2.1.2, 2.2.1.3) was determined with Kruskal-Wallis ANOVA (H) test statistic (Kruskal and Wallis 1952), followed by false discovery rate correction using Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). To determine the correlations for the factor with numerical data, i.e., Altitude, Spearman rank test (rs) was used. The differentiation of the bacterial composition between termite samples grouped by factors (beta-diversity), was analyzed at 999 permutations using Permutational multivariate analysis of variance (PERMANOVA). Weighted Unifrac (Lozupone and Knight 2005) was used as the distance metric as it computes the phylogenetic distances between the bacterial taxa based on their abundances (2.2.3).

3.2.4. Pilot study for feeding experiments

Preliminary feeding experiments with colonies of *Coptotermes formosanus*, a congeneric species closely related to *C. gestroi*, were performed at Louisiana State University to determine the concentration range of the bacterial solution that workers readily ate with no adverse impact on their survival. Casamino acid-Peptone-Glucose (CPG) broth was prepared by adding 1 g Casamino acid (casein hydrolysate), 10 g Peptone, 5 g Glucose and sterilizing it in autoclave at 121˚C for 20 minutes (Kelman 1954). A hundred microlitres of CPG broth medium was inoculated with *R. solanacearum* strain GMI1000 obtained from American Type Culture Collection (ATCC). *Ralstonia solanacearum* was cultured overnight (18-20 h) in a shaker-incubator at 220 rpm and 28 °C (Kelman 1954). The overnight culture had an optical density (OD_{600}) of 2.5. The reading for OD_{600} was obtained using a spectrophotometer (SPECTRONIC™ 200, Thermo Scientific). 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.

Serial dilutions from 10^{-1} to 10^{-10} of the overnight cell culture were performed in 0.85% saline. An aliquot of 20 µl from each test tube was spread on CPG agar plates. 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 for each microliter, 8.731E+10 CFUs were obtained.

Fresh *C. formosanus* termites collected from a colony in Brechtel park at New Orleans, Louisiana, were used in the pilot study. A hundred microliters from each of the ten dilutions prepared from overnight culture of *R. solanacearum*, along with a negative control (0.85% saline without bacteria) was pipetted on different filter papers to observe the feeding behavior and survival of termites on different dilutions. Around 2X2 cm^2 filter paper was dry weighed and placed into a Petri dish. Fifty worker and 5 soldier termites of *C. formosanus* were placed in each Petri dish 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 the negative control. Each treatment consisted of four replicates. Net consumption by termites during the feeding experiment was calculated by subtracting the dry weight of the filter papers at the end of the feeding period from the initial weight. Dead individuals were being counted and removed from the Petri dishes daily.

Significant effects of Ralstonia dilutions on consumption were detected for both feeding durations at 2 days ($p=0.01$, $F = 20.76$) and 10 days ($p=0.01$, $F= 21.68$, One-way ANOVA). For both 2 days and 10 days, the highest concentration had lowest consumption and control had highest consumption, showing that high pathogen loads reduce feeding. 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. There was no significant difference between the mortality rate at any of the concentrations (all $p>0.05$, One-way ANOVA). Based on this pilot study the 10^{-4} dilution was selected for choice-tests performed on *C. gestroi* workers in Guam because this dilution showed reduction in feeding in the short-term compared to controls without impacting worker health. In addition, lower dilutions (10^{-6}, 10^{-8}) were added to determine the minimum threshold for effects of pathogen loads on consumption.

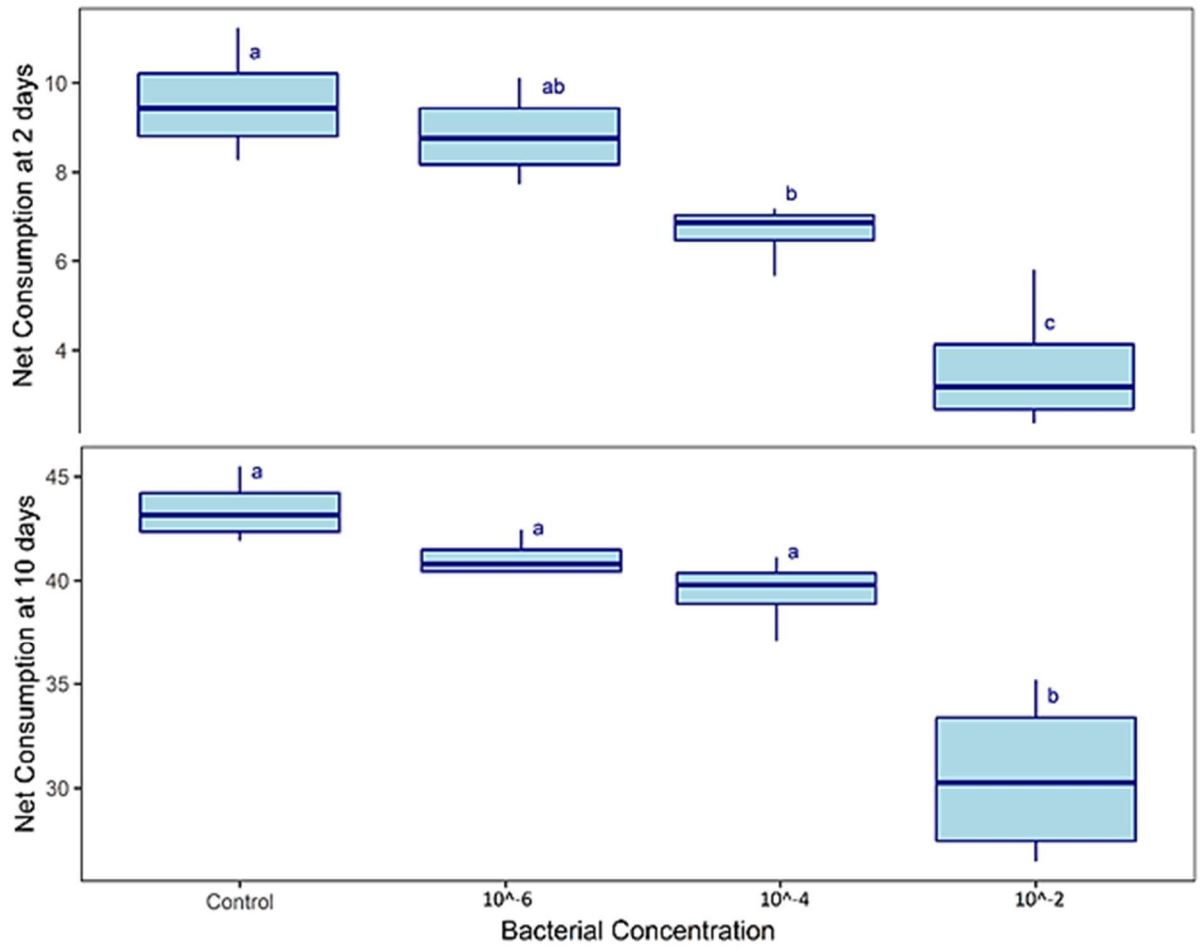

Figure 2. Net Consumption (mg) of filter paper by *C. formosanus* workers at 10^{-2}, 10^{-4}, 10^{-6} dilutions prepared from overnight culture of *R. solanacearum* and control, One-way ANOVA and Tukey's Studentized Range (HSD) Test at 2 days and 10 days. Different letters indicate significant difference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

3.2.5. Feeding experiments in Guam to assess the consumption of *R. solanacearum* by *C. gestroi* workers

Three colonies of *C. gestroi* were collected from Apaca Point, Agat (13.40239, 144.66307), Bernard Watson's farm (13.56553, 144.87749), and UOG Ija Experiment Station (13.26523, 144.71623). The *C. gestroi* termites were collected using plastic milk crate traps. Plastic milk crates (33.2x33.2x28.1 cm) containing a wooden lattice structure of 44 pieces of softwood lumber; arranged horizontally (3.5 x 3.5 x 29.5 cm) and vertically (3.5 x 3.5 x 27 cm) were buried in the ground and were covered with 3-5 cm of soil (Gautam and Henderson 2011b). These crates were brought back to the laboratory to collect the workers and soldiers. For each Petri dish, 300 workers and 30 soldiers were used in four-choice tests (see 2.2.4.1, Figure 2a), 100 workers and 10 soldiers for two-choice tests (see 2.2.4.2, 2.2.4.3, Figure 2b) and 50 workers and 5 soldiers per Petri dish were used for no-choice test (see 2.2.4.4, Figure 2b). The procedure for four-choice, two-choice and no-choice feeding experiments was the same as described in Materials and methods of chapter 2 except no DNA sequencing was performed after no choice test and preference of termites between *R. solanacearum* inoculated and non-inoculated filter paper was determined based on difference in consumption.

3.3. Results

3.3.1. Number of sequence reads and ASVs

A total of 5,599,951 raw sequences were obtained from 27 samples of *C. gestroi* collected from *R. solanacearum* positive and negative ironwood trees in Guam. After removal of low-quality reads and chimeras, a total of 4,333,087 sequence reads and 8,976 different ASVs were obtained. When the unassigned ASVs with < 97% similarity to references in the SILVA database were removed

from the samples, a total of 2,168,762 sequence reads and 747 ASVs remained. The removal of unassigned ASVs reduced the lowest sequencing depth common to all samples from 16,513 to 4,982.

3.3.2. Sequence depth-, sample- and coverage-based rarefaction

The sequence-depth based rarefaction curves (Figure 3a) of most samples started to level off at a sequencing depth around 2,000 for ASV richness, around 8,000 for Faith's PD, and less than 2,000 for Shannon diversity. The leveling of these curves indicates that the sequencing depth was sufficient to capture most of the bacterial diversity present in each of the samples. The sample-based rarefaction (Figure 3b) across 27 samples showed an ASV richness of 747, Shannon diversity of less than 450 and Simpson inverse of around 300. The extrapolation of curves by doubling the sample size increased ASV richness from 747 to more than 900 while Shannon diversity and Simpson inverse index did not increase considerably. The coverage-based rarefaction (Figure 3c) shows that sequence reads of 27 termite samples were able to achieve 90% coverage. Increasing the coverage to around 95% by extrapolation to twice the sample size would have increased the ASV richness to more than 900; however, Shannon diversity and Simpson inverse index would have remained the same. This suggests that the additional ASV richness would most likely be due to rare ASVs.

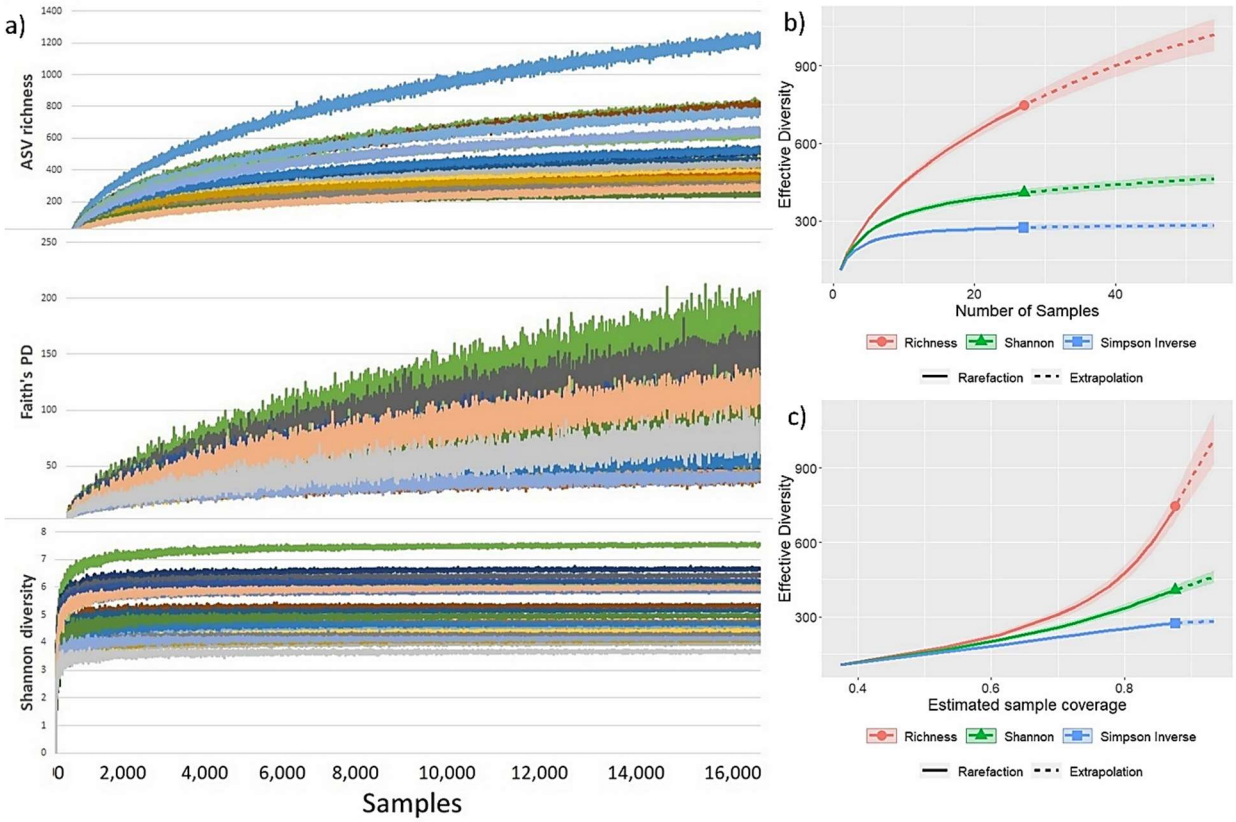

Figure 3. a) Sequence-based rarefaction curves of bacteria diversity showing the number of ASVs, Faith's phylogenetic distance and Shannon diversity indices in 27 samples of *C. gestroi* workers plotted against sequencing depth. b) Sample-based rarefaction curves with effective bacterial diversity for different metrics plotted against the number of samples. c) Coverage-based rarefaction curves with effective diversity plotted against estimated sample coverage. Solid lines indicate intrapolation up to the actual sample size; dashed lines represent extrapolation to twice the sample size. Rarefaction was performed over the total bacteria diversity (with and without taxonomical assignment).

3.3.3. Taxa composition

In 27 samples of *C. gestroi* collected from ironwood trees in Guam, 28 different phyla were observed (Table 2). The most dominant phyla were Spirochaetes (45.46%), Bacteroidetes (23.41%) and Fibrobacteres (15.1%). The minor phyla obtained in *C. gestroi* workers were Proteobacteria (6.14%), Firmicutes (4.42%), Planctomycetes (1.7%), Synergistetes (0.86%), Cloacimonetes (0.77%), Acidobacteria (0.74%), Actinobacteria (0.72%), Elusimicrobia (0.19%), Margulisbacteria (0.18%) and others (0.3%) (Table 2, Figure 4).

Only one of the top 20 ASVs according to total number of reads was present in all 27 samples; this ASV was an uncultured *Treponema* sp. from Phylum Spirochaetes (Table 3). Another uncultured *Treponema* sp., was the most dominant ASV but it was present in only 15 out of 27 samples. An uncultured Spirochaetes bacterium (Phylum: Spirochaetes), a *Treponema* sp. (Phylum: Spirochaetes), an uncultured delta proteobacterium (Phylum Proteobacteria) and an uncultured Eubacteriaceae bacterium (Phylum Firmicutes) were present in more than 20 samples. The remaining 14 ASVs of the top 20 ASVs were present in less than 20 samples. The ASV belonging to *Candidatus Azobacteroidetes* bacteria (Phylum Bacteroidetes) had the fifth highest number of reads, but it was found only in one sample (Table 3).

Six ASVs that were not in the top 20 ASVs were assigned to taxa that contain pathogenic bacteria putatively associated with IWTD as these bacteria were identified during the phylogenetic analysis of ooze from ironwood trees in decline (Table 4). The *Ralstonia* sp. ECPB06, *Enterobacter* sp., *Klebsiella oxytoca*, *Pantoea* sp., and *Citrobacter* sp. were each detected in only one sample with 9 sequencing reads (Sample: 21-166), 2 sequencing reads (21-183), 13 sequencing reads (21-157),

7 sequencing reads (21-172) and 303 sequencing reads (21-182), respectively. *Citrobacter amalonaticus* was detected in 5 samples (21-155, 21-161, 21-166, 21-168, 21-170) with a total of 98 sequencing reads.

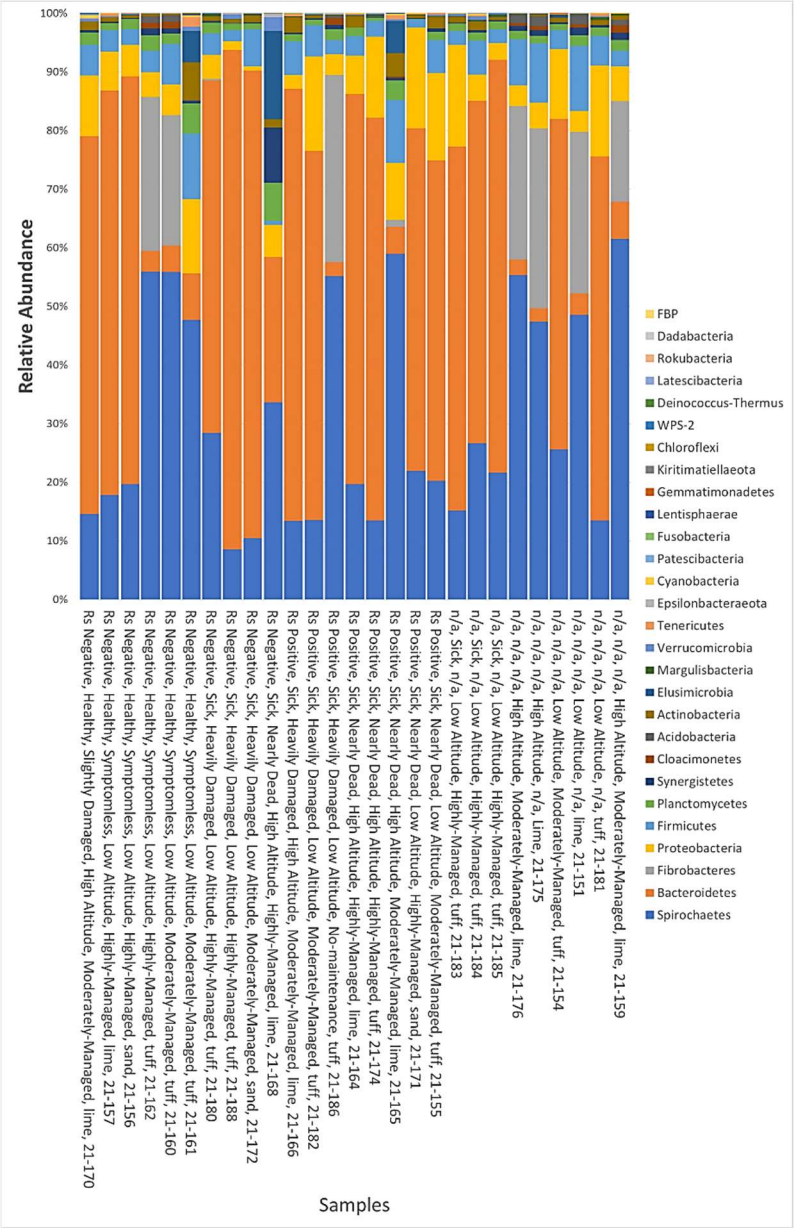

Figure 4. Taxa bar plots showing the relative abundance of bacterial phyla associated with 27 samples of *C. gestroi* workers collected from ironwood trees in Guam. Phyla are shown in decreasing abundance from bottom to top. The sample name on the x-axis encodes the following factors: presence (Positive) or absence (Negative) of Ralstonia (Rs), Tree Health (Healthy or Sick), Tree DS (symptomless (DS=0), slightly damaged (DS=1), distinctly damaged (DS=2), heavily damaged (DS=3), and nearly dead (DS=4)), Altitude (High, Low), level of site management (not managed (None), moderately managed (Moderate), highly managed (High)), and Parent Material (Sand, Lime, Tuff).

Table 2. Number of reads across all samples and relative abundance of all phyla associated with *C. gestroi* samples and the number of samples these phyla were observed in.

Phylum	Number of reads	Relative abundance	Number of samples
Spirochaetes	984,367	45.46	27
Bacteroidetes	507,021	23.41	27
Fibrobacteres	327,011	15.1	13
Proteobacteria	132,943	6.14	27
Firmicutes	95,656	4.42	27
Planctomycetes	36,808	1.7	27
Synergistetes	18,661	0.86	26
Cloacimonetes	16,637	0.77	9
Acidobacteria	16,094	0.74	19
Actinobacteria	15,565	0.72	27
Elusimicrobia	4,069	0.19	14
Margulisbacteria	3,844	0.18	24
Verrucomicrobia	2,154	0.1	24
Tenericutes	1,815	0.08	20
Epsilonbacteraeota	1,462	0.07	18
Cyanobacteria	803	0.04	17
Patescibacteria	207	0.01	16
Fusobacteria	92	<0.01	5
Lentisphaerae	85	<0.01	2
Gemmatimonadetes	56	<0.01	4
Kiritimatiellaeota	29	<0.01	4
Chloroflexi	17	<0.01	5
Deinococcus-Thermus	15	<0.01	3
Candidate phylum WPS-2	15	<0.01	2
Candidate phylum Rokubacteria	4	<0.01	1
Candidate phylum Latescibacteria	4	<0.01	1
Candidate phylum Dadabacteria	3	<0.01	1
Candidate phylum FBP	2	<0.01	1

Table 3. The most abundant 20 ASVs associated with *C. gestroi* samples according to the total number of reads with their assignments in SILVA and NCBI GenBank, their total number of reads along with the number of samples the ASVs were observed in and the average number of reads and standard deviation per sample.

Phylum	Order	Lowest SILVA assignment	Scientific name	Percent identity to top match	Accession number in GenBank	Number of Reads	Number of Samples	Average Reads per sample	Standard deviation
Spirochaetes	Spirochaetales	uncultured Treponema sp.	uncultured *Treponema sp.*	100.00%	AB243261.1	641,173	15	23,747	68,853
Bacteroidetes	Bacteroidales	*Candidatus Azobacteroides*	uncultured bacterium	96.10%	KP690848.1	345,819	20	12,808	16,306
Fibrobacteres	Fibrobacterales	uncultured Chitinivibrionia bacterium	uncultured Chitinivibrionia bacterium	99.57%	AB255963.1	268,988	13	9,963	23,368
Spirochaetes	Spirochaetales	uncultured Treponema sp.	uncultured *Treponema sp.*	99.57%	AB191798.1	203,210	27	7,526	20,837
Bacteroidetes	Bacteroidales	*Candidatus Azobacteroides*	uncultured Bacteroidetes bacterium	100.00%	GQ502497.1	59,656	1	2,209	11,481
Spirochaetes	Spirochaetales	uncultured Spirochaetes bacterium	uncultured Spirochaetes bacterium	97.84%	GQ502630.1	56,587	21	2,096	2,491
Fibrobacteres	Fibrobacterales	uncultured Fibrobacteres bacterium	uncultured Fibrobacteres bacterium	99.57%	AB192077.2	55,196	8	2,044	6,967
Proteobacteria	Rs-K70 termite gr	uncultured delta proteobacterium	uncultured delta proteobacterium	99.57%	EF454984.2	49,756	24	1,843	5,408
Spirochaetes	Spirochaetales	Treponema	uncultured Spirochaetes bacterium	98.70%	GQ502639.1	23,806	20	882	1,011
Proteobacteria	Enterobacteriales	uncultured bacterium	*Trabulsiella odontotermitis*	100.00%	MH542345.1	21,900	13	811	1,764
Firmicutes	Clostridiales	uncultured Clostridiaceae bacterium	uncultured Clostridiaceae bacterium	99.57%	AB192211.1	21,289	18	788	2,383
Proteobacteria	Rickettsiales	Wolbachia	Wolbachia	100.00%	DQ837199.1	21,000	17	778	1,186
Planctomycetes	Pirellulales	uncultured planctomycete	uncultured planctomycete	99.13%	KM651184.1	20,882	9	773	2,084
Firmicutes	Clostridiales	uncultured Eubacteriaceae bacterium	uncultured Eubacteriaceae bacterium	99.13%	AB243287.1	16,888	23	625	1,259
Cloacimonetes	Cloacimonadales	uncultured bacterium	uncultured bacterium	97.40%	AB191981.1	16,637	9	616	1,934
Bacteroidetes	SJA-28	uncultured Chlorobi bacterium	uncultured Chlorobi bacterium	98.70%	AB192127.1	14,292	9	529	1,777
Bacteroidetes	Bacteroidales	uncultured Bacteroidales bacterium	uncultured Bacteroidales bacterium	99.57%	AB191992.1	13,772	8	510	1,903
Spirochaetes	Spirochaetales	Termite Treponema cluster	uncultured Spirochaetes bacterium	99.13%	GQ502643.1	12,907	19	478	598
Bacteroidetes	Bacteroidales	uncultured Bacteroidales bacterium	uncultured Bacteroidales bacterium	98.27%	AB192005.1	12,265	9	454	1,311
Acidobacteria	Holophagales	uncultured Acidobacteria bacterium	uncultured Acidobacteria bacterium	99.57%	AB192122.1	12,000	9	444	1,168

Table 4. Six ASVs that were assigned to putative pathogenic bacteria from *C. gestroi* workers collected from ironwood trees in Guam

Phylum	Order	Genus	Species	Total number of	Number of samples
Proteobacteria	Betaproteobacteriales	Ralstonia	Ralstonia sp. ECPB06	9	1
Proteobacteria	Enterobacteriales	Enterobacter	Ambiguous taxa	2	1
Proteobacteria	Enterobacteriales	Enterobacter	*Klebsiella oxytoca*	13	1
Proteobacteria	Enterobacteriales	Pantoea	uncultured prokaryote	7	1
Proteobacteria	Enterobacteriales	Citrobacter	*Citrobacter amalonaticus*	98	5
Proteobacteria	Enterobacteriales	Citrobacter	unidentified bacteria	303	1

3.3.4. Diversity analysis

There was no significant impact of Altitude, Altitude Classification, Presence of Ralstonia, Tree DS, and Parent Material on alpha diversity of the bacterial communities of *C. gestroi* workers collected from ironwood trees in Guam. Tree Health and Site Management had a significant impact on the alpha diversity of bacterial communities in these termites (Figure 5). Bacterial communities of termites collected from healthy trees (n=6) had significantly higher richness (ASV Richness, p=0.0496, H=6.0075, Kruskal-Wallis ANOVA) compared to those collected from sick trees (n=15). The phylogenetic distances of bacteria were greater in termites collected from moderately managed sites (n=11) than those collected from highly managed sites (n=12) (Faith's PD, p= 0.0067, H= 7.3334, Kruskal-Wallis ANOVA) (Figure 6). There was no significant difference in beta diversity of bacterial communities between termite samples collected at high and low altitude (p=0.245, pseudo-F = 1.4807, PERMANOVA), from trees with presence or absence of Ralstonia (p=0.679, pseudo-F = 0.4699, PERMANOVA), or from healthy or sick trees (p= 0.07, pseudo-F = 2.6804, PERMANOVA). Bacterial beta diversity was also not significant among termite samples collected from trees with different decline severity, i.e., symptomless, slightly damaged, distinctly damaged, heavily damaged or nearly dead trees (p=0.463, pseudo-F = 0.8779, PERMANOVA), from sites that were highly, moderately or not managed (p=0.07, pseudo-F = 2.6804, PERMANOVA), and from trees growing on sand, lime or tuff parent material (p=0.209, pseudo-F = 1.6301, PERMANOVA).

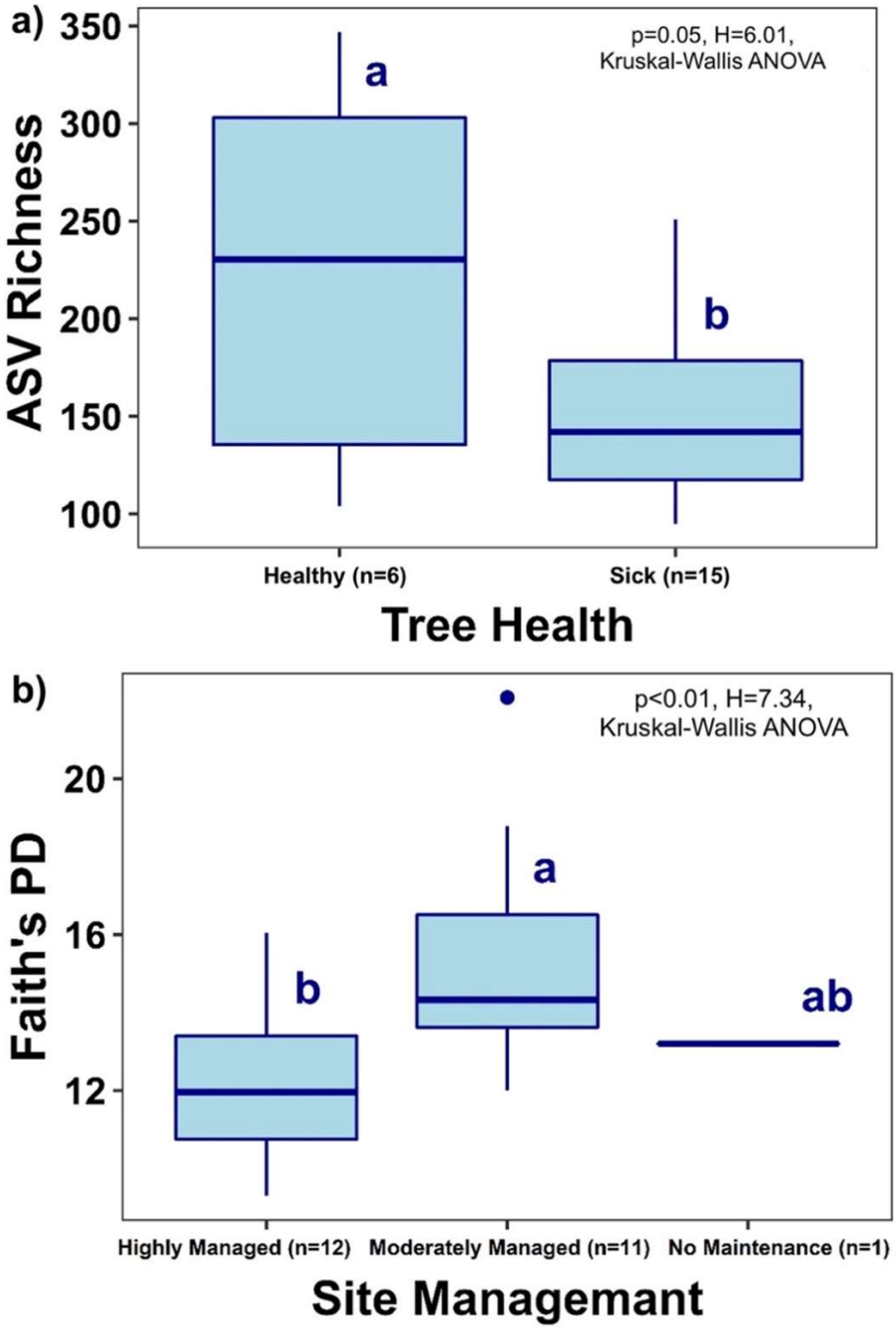

Figure 5. Ironwood tree-related factors with significant effects on different aspects of alpha-diversity of termite bacteria communities. Different letters indicate significant difference. a) ASV Richness of bacteria communities of termites collected from Healthy and Sick trees. b) Faith's PD of bacterial communities of termites collected from highly, moderately or non-managed sites (Highly-Managed, Moderately-Managed and No-Maintenance).

3.3.5. Different Ralstonia and wetwood bacteria amounts did not impact feeding behavior of *C. gestroi* workers

Since IWTD pathogens were only detected in small amounts and few *C. gestroi* samples collected from ironwood trees of Guam, feeding experiments were designed to understand the feeding behavior of termites with respect to food source containing pathogens associated with IWTD. In four-choice tests, where termites were fed with four different natural wood sources from Ralstonia positive or negative ironwood trees with high or low amounts of wetwood bacteria, no significant difference in net consumption of any of the four food sources was detected ($p=0.418$, One-Way ANOVA followed by HSD test). (Figure 6). Two-choice tests for determining the difference between the consumption of ironwood pieces inoculated with different dilutions of *R. solanacearum* versus wood not inoculated with *R. solanacearum* did not show any significant differences between the consumption of wood with 10^{-4} dilution ($p= 0.141$), 10^{-6} dilution ($p= 0.823$), or 10^{-8} dilution ($p=0.251$) ($n=15$) of Ralstonia and wood with no Ralstonia ($n=15$, One-Way ANOVA followed by HSD test, Figure 7). Also, no significant difference was observed in no-choice tests between consumption, of filter paper inoculated with *R. solanacearum* at different concentrations (10^{-4}, 10^{-6}, 10^{-8}) and the control, both, after feeding for one week ($p=0.249$) and two weeks ($p=0.876$, One-Way ANOVA followed by HSD test, Figure 8). The average mortality rate ($15.3 \pm 8.12\%$) was constant among the treatments. The results from all three feeding experiments suggest that *C. gestroi* workers are not deterred by food sources infected with Ralstonia.

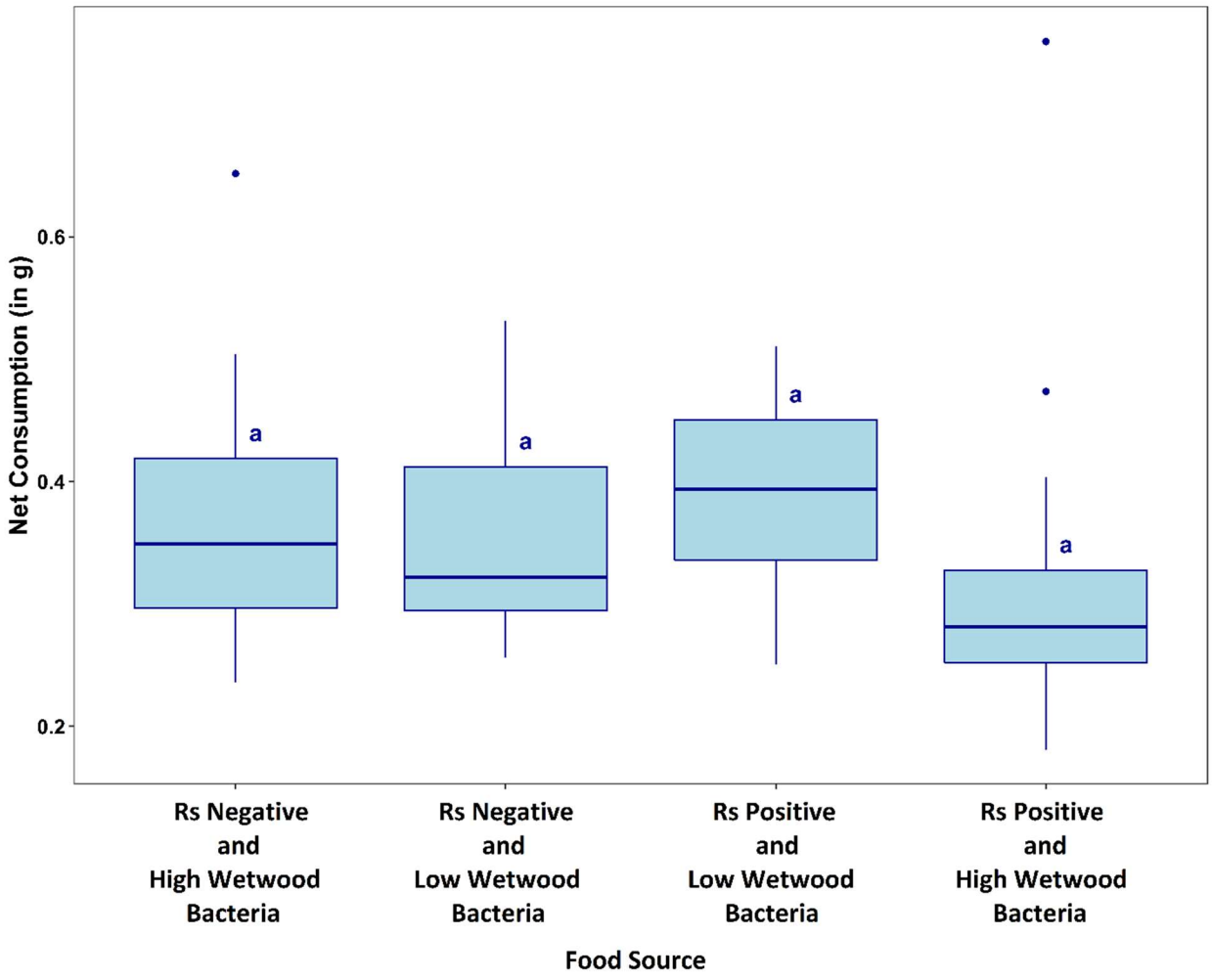

Figure 6. Four-choice test measuring net consumption (g) by *C. gestroi* workers of four food sources consisting of natural wood pieces: *R. solanacearum* negative and low amounts of wetwood bacteria, *R. solanacearum* positive and low amounts of wetwood bacteria, *R. solanacearum* negative and high amounts of wetwood bacteria, and *R. solanacearum* positive and high amounts of wetwood bacteria). Same letters indicate lack of significant differences determined by One-way ANOVA and Tukey's Studentized Range (HSD) Test.

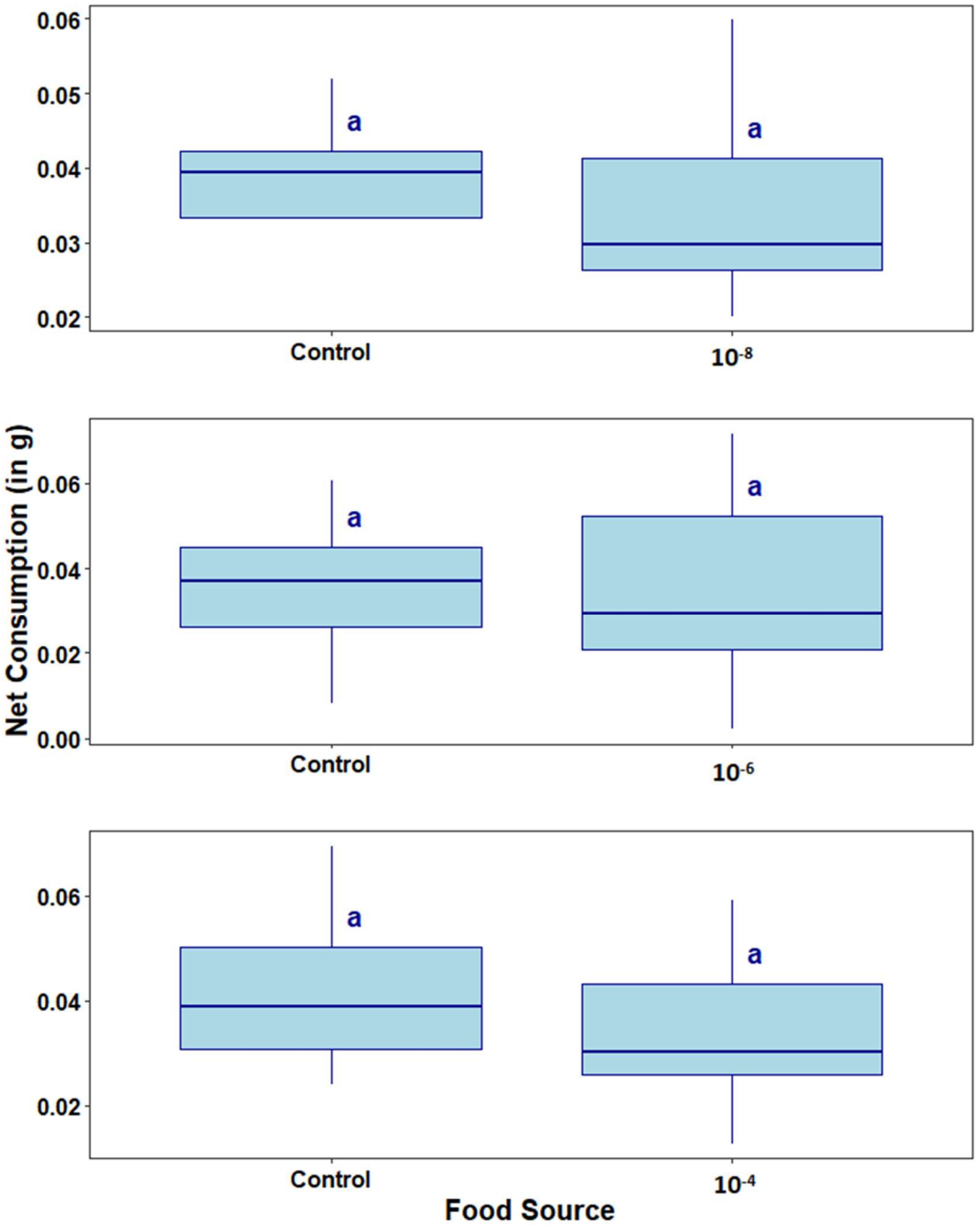

Figure 7: Two-choice test measuring net consumption (g) of wood pieces inoculated with 10^{-4}, 10^{-6} and 10^{-8} Ralstonia dilution by *C. gestroi* workers in comparison to a control with no Ralstonia. Same letters indicate lack of significant differences determined by One-way ANOVA and Tukey's Studentized Range (HSD) Test.

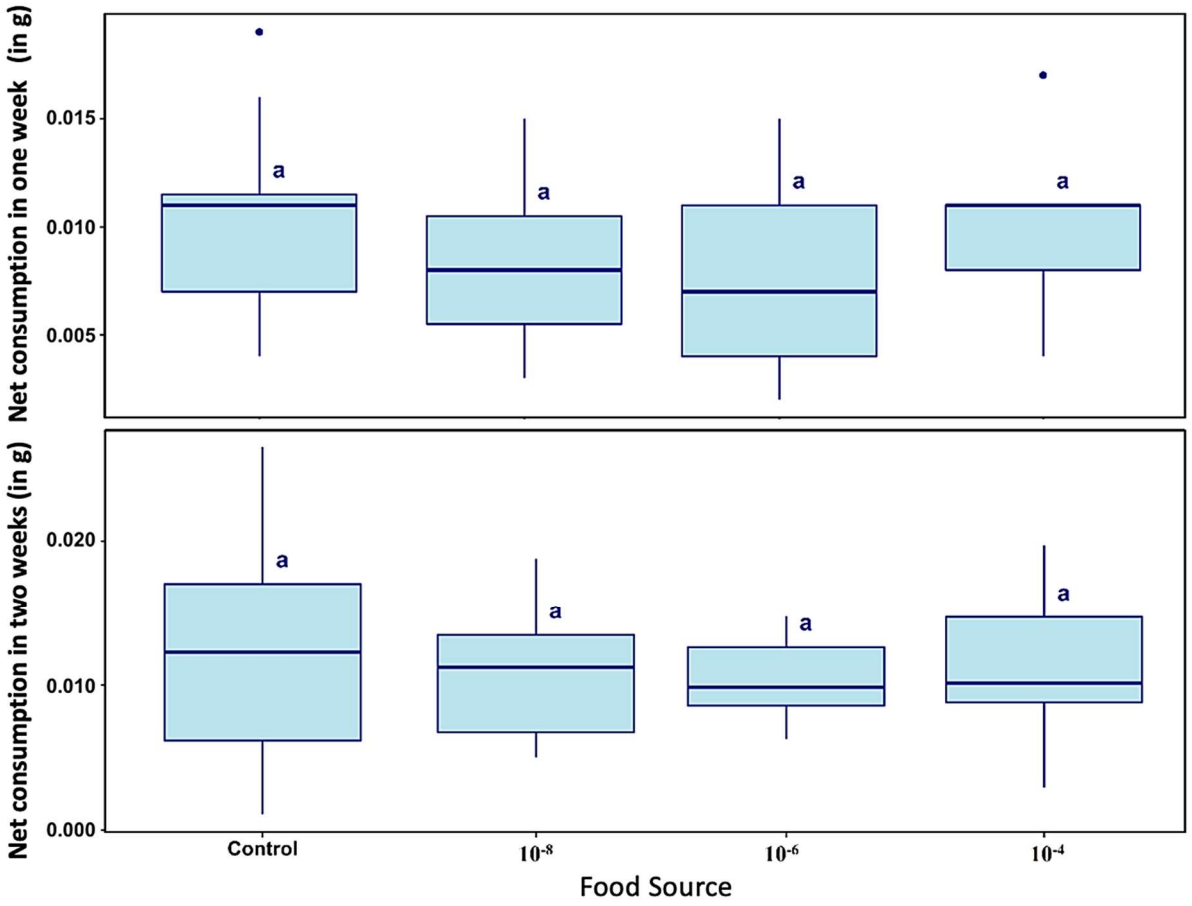

Figure 8. No-choice test measuring net consumption (g) of filter papers inoculated with 10^{-4}, 10^{-6} and 10^{-8} bacterial concentration by *C. gestroi* workers. Same letters indicate lack of significant differences determined by One-way ANOVA and Tukey's Studentized Range (HSD) Test.

3.4. Discussion

Coptotermes gestroi was the termite species with second highest infestation of ironwood trees (*Casuarina equisetifolia*), after *Nasutitermes takasagoensis* termites (Park et al. 2019). The earlier records considered the *Coptotermes* species found in Guam as *Coptotermes formosanus* (Hromada 1970, Lai 1977, Su and Tamashiro 1987). Later a survey conducted by Su and Scheffrahn (1998) identified the *Coptotermes* species in Guam as *Coptotermes vastator*. However, a phylogenetic study based on DNA sequence comparisons of mitochondrial genes by Yeap et al. (2007) showed *C. vastator* to be synonymous to *C. gestroi*. A study by Evans et al. (2013), reported the presence of *C. gestroi* on the island of Guam. The *Coptotermes* species collected from ironwood trees in Guam was also identified as *C. gestroi* using morphological characters and DNA barcoding (Park et al. 2019).

The bacterial composition of freshly collected whole bodies of *C. gestroi* workers was described with high-throughput sequencing of the V4 region of the bacterial 16SrRNA gene to investigate the role of *C. gestroi* workers as pathogen vectors in decline of ironwood trees in Guam. Spirochaetes (45.46% relative abundance), Bacteroidetes (23.41%), Fibrobacteres (15.1%), Proteobacteria (6.14%), and Firmicutes (4.42%) were found to be the dominant phyla in *C. gestroi* workers from Guam. All of these dominant phyla except the for Fibrobacteres were also found in two previous deep sequencing studies in *C. gestroi* workers (Do et al. 2014, Oberpaul et al. 2020) and are, therefore, core phyla. However, the relative ranking according to the abundance of each phylum differed among these studies, which might be due to geographical population differences and/or differences in sample collection, rearing and sequencing. Do et al. (2014) performed

metagenomic sequencing of bacterial genomes in *C. gestroi* worker guts from Vietnam. In contrast to our study, which used field collected termites, the termites in Do et al. (2014)'s study were kept in the lab and fed on pinewood. Moreover, Do et al. (2014) targeted the free-living gut microbiota. Thus, bacteria that are endosymbionts of the protists (more than 70% of bacteria in the hindgut) (Noda et al. 2005) and bacteria that are attached to the gut walls (3-20% of bacteria in the hindgut) (Nakajima et al. 2006) were underrepresented in Do et al.'s (2014) study. The dominant phyla (>4% relative abundance) reported by this metagenomic sequencing study included Firmicutes (22.48%), Proteobacteria (17.84%), Spirochaetes (17.40%), Bacteroidetes (11.60%) and Synergistetes (4.27%), but Fibrobacteres were not among the major phyla. Oberpaul et al. (2020) performed high-throughput sequencing of the V3-V4 region of the bacterial 16S rRNA gene from whole bodies of workers from three *Coptotermes* species, including *C. gestroi,* similar to our study. However, the termites in Oberpaul et al.'s study were reared for decades in lab colonies fed on birchwood, which might explain why their phyla rankings also differed from our study. Instead of Spirochetes, followed by Bacteroidetes, Oberpaul et al. (2020) reported Bacteroidetes (52%), Alphaproteobacteria (12%), Spirochetes (11%), Firmicutes (7%), and Actinobacteria (6%) as the dominant phyla in descending order across the three *Coptotermes* species with Fibrobacteres representing only a minor fraction.

The high abundance of Fibrobacteres set our study apart from the previous *C. gestroi* bacteria inventories (15.1% in our study versus <1% in Oberpaul et al. 2020 and no mention in Do et al. 2014). Unlike Spirochaetes, Bacteroidetes, Proteobacteria, and Firmicutes that were present in all 27 samples, the Fibrobacteres were found in only 13 samples and, thus, are not among the core phyla. Fibrobacteres is a small phylum (Garrity and Holt 2001) with only one formal genus

(Fibrobacter) and two cultured species (*Fibrobacter succinogenes* and *Fibrobacter intestinalis*), which are involved in degradation of lignocellulosic material (Ransom-Jones et al. 2012). Fibrobacteres is usually present as a dominant phylum in higher termites as members of this phylum are important contributors to lignocellulose digestion in higher termites. Since lower termites have cellulolytic flagellates, a high abundance of Fibrobacteres is usually not observed (Brune 2014). Horizontal transfer of microbiota does occur among termite species, either through aggressive encounters or shared feeding substrates (Bourguignon et al. 2018). Therefore, it is noteworthy that *C. gestroi* and *N. takasagoensis* colonies sometimes forage and nest in close proximity on the same ironwood tree (Park et al. 2019, G. Setia personal observation). As a higher termite *N. takasagoensis* harbors Fibrobacteres among its dominant core bacteria (Chapter 2). Although there is no direct evidence of inter-species transfer of bacteria in this study, it is possible that *C. gestroi* workers acquired additional Fibrobacteres through interaction. As a termite symbiont, Fibrobacteres are well adapted to thrive in termite guts and might complement lignocellulose digestion of *C. gestroi* workers and their flagellates, especially if the population of flagellates is low.

The majority of the top 20 most frequent bacteria across all samples was uncultured. However, some important bacteria such as *Candidatus Azobacteroides*, *Trabulsiella odontotermitis* and Wolbachia were identified among the frequent bacteria. *Candidatus Azobacteroides* from phylum Bacteroidetes and order Bacteroidales is the intracellular endosymbiont of cellulase digesting protozoa from the genus *Pseudotrichonympha* and has coevolved with its host (Noda et al. 2005, Noda et al. 2007, Chen et al. 2022). *Candidatus Azobacteroides* bacteria are involved in nitrogen and carbohydrate metabolism in the rhinotermitid genera *Coptotermes* and *Heterotermes* (Hongoh

et al. 2008, Arora et al. 2022). This protozoa endosymbiont was first sequenced in Pseudotrichonympha of *C. formosanus* (Hongoh et al. 2008). *Candidatus Azobacteroides* bacteria was also detected in previous high-throughput study in *C. gestroi* workers (Oberpaul et al. 2020). *Trabulsiella odontotermitis* found in *C. gestroi* workers in our study belongs to phylum Proteobacteria and order Enterobacteriales. This species is usually found in various fungus growing termites and was first isolated from a fungus growing termite named *Odontotermes formosanus* (Sapountzis et al. 2015, Chou et al. 2007). *Trabulsiella odontotermitis* is considered as a facultative symbiont possibly responsible for carbohydrate metabolism and aflatoxin degradation (Sapountzis et al. 2015). Moreover, *T. odontotermitis* was cultured from *C. formosanus* and has been used as genetically engineered symbiont in paratransgenesis based termite control studies (Tikhe et al. 2016). Wolbachia from phylum Proteobacteria and order Rickettsiales was also found in our study. Wolbachia is one of the most common endoparasite of various insects and nematodes (Taylor et al. 2018, Duron et al. 2008). Wolbachia has been detected in the Coconut rhinoceros beetle (*Oryctes rhinoceros*) (Coleoptera: Scarabaeidae) on the islands of Guam, Palau, and Taiwan (Grasela and Moore 2022).

Bacterial genera that were previously identified in ironwood trees afflicted by IWTD, i.e., *Ralstonia*, *Enterobacter*, *Klebsiella*, *Pantoea*, and *Citrobacter,* were detected only in a few *C. gestroi* workers samples collected from ironwood trees of Guam (Ayin et al. 2019) (3.3.3). However, of all these plant pathogens, only *R. solanacearum*, which had been previously isolated from ooze of sick ironwood trees in Guam, was able to cause wilting symptoms on healthy ironwood trees (Paudel 2020). The isolates of *Klebsiella* sp. from ironwood trees in Guam were unable to elicit symptoms of wetwood in healthy trees and the remaining putative plant pathogens

identified from these ironwood trees are still not tested for their pathogenicity in ironwood trees (Ayin et al. 2015, 2019).

Therefore, *R. solanacearum* is the only pathogen associated with IWTD that has been confirmed to cause symptoms in ironwood trees through isolation and reinoculation. We found *Ralstonia* sp. in one of the *C. gestroi* samples analyzed in this study. This termite sample was collected from a Ralstonia positive tree that showed severe symptoms of IWTD (Highly damaged DS= 3). However, the abundance of sequence reads assigned to *Ralstonia* sp. was less than 0.01%. The failure to detect Ralstonia in the majority of samples was not likely due to insufficient sequencing or sampling effort. It was ascertained through alpha rarefaction analysis that sequencing depth, number of samples and coverage used in this study was sufficient to capture most of the bacterial diversity.

One possible explanation for the low detection of Ralstonia could have been avoidance of consumption of wood infested with pathogens by *C. gestroi* workers. However, the choice tests that were conducted between natural wood from healthy ironwood trees and wood from ironwood tree with high pathogen load; or between wood inoculated with Ralstonia and wood from a healthy ironwood tree, showed that termites consumed the wood equally regardless of IWTD bacteria load. These results suggested that *C. gestroi* workers do not avoid pathogen infested wood at least in the concentration range (10^{-4}, 10^{-6}, 10^{-8} dilutions) tested (3.3.5). However, the pilot study with *C. formosanus* workers showed that higher concentrations (10^{-2} dilution) of Ralstonia than those employed in this study did lead to reduced consumption (3.2.4). In contrast to *C. gestroi* workers, *N. takasagoensis* workers did show preference for healthy wood over pathogen infested wood even

at low pathogen loads (Chapter 2). The lack of deterrence by pathogens of *C. gestroi* compared to *N. takasagoensis* workers might be explained by different feeding behaviors of these termite species. Unlike *N. takasagoensis*, *Coptotermes* termites are known for their voracious and opportunistic feeding behavior as they are capable of feeding on anything that contains cellulose; indicated by the huge damage that they termites cause to structures and trees (Cowie et al. 1989, Tho and Kirton 1990, Dhanarajan 1969, Tho 1974, Kirton 1995).

The results from microbiome analysis and feeding experiments indicate that *C. gestroi* workers likely ingest Ralstonia as (1) we did find Ralstonia in worker bodies, albeit in low incidences and (2) feeding experiments showed that Ralstonia did not act as feeding deterrent. However, Ralstonia could not establish itself in large quantities in the termite bodies for various reasons. Termites exhibit individual and social immunity against the invasion of foreign bacteria (Rosengaus et al. 1999, Traniello et al. 2002, Cremer et al. 2007, Bulmer et al. 2009, Cremer et al. 2018). Being social insects termites exhibit immune responses against foreign bacteria such as alarm behaviors, allo-grooming, avoidance, burial of dead bodies, and cannibalism (Kramm et al. 1982, Rosengaus et al. 1998, Liu et al. 2015, Yanagawa and Shimizu 2007, Chouvenc and Su 2010, He et al. 2018). The native microbiota as well as antimicrobial compounds present in the termite bodies and termite nests also prevent the invasion and further colonization by foreign bacteria (Veivers et al. 1982, Dillon and Dillon 2004, Sen et al. 2015, Peterson and Scharf 2016, Oberpaul et al. 2020, Rosengaus et al. 2011, Bulmer et al. 2009, Visser et al. 2012, Chouvenc et al. 2013, Soukup et al. 2021 and Witasari et al. 2022, Mevers et al. 2017). Moreover, the optimum pH for the growth of *R. solanacearum* is 4.5–5.5 (Li et al. 2017), while *C. gestroi* workers have slightly acidic to neutral pH. The pH range of *C. gestroi* worker guts is closer to the optimum pH range for *R. solanacearum*

as compared to the pH range of *N. takasagoensis* worker guts (Chapter 2), but the gut environment is still not ideal for the growth of *R. solanacearum*.

However, Ralstonia was a major component of microbiota in some *Coptotermes formosanus* alates (Chen at al., unpublished). *Coptotermes formosanus* is a close relative of *C. gestroi*. Alates are the reproductive castes of the termites and harbor fewer numbers of symbionts in their gut as compared to workers (Shimada et al. 2013). Alates carry the initial inoculum of symbionts when they swarm to found new colonies, but their hind guts are not specialized like those of workers to house a diverse climax community of symbionts in a stable equilibrium. Alates would be a more efficient vector for Ralstonia than workers as (1) they can spread Ralstonia from disease infested trees to healthy trees while swarming and forming new colonies, and (2) their gut microbiota has not reached optimum symbiont community and, thus, might have niches for temporary colonization by environmental bacteria. Therefore, future studies are necessary to confirm if *C. gestroi* alates do carry Ralstonia in their bodies although most workers do not.

Feeding on sick ironwood trees impacted the bacterial composition of *C. gestroi* workers by decreasing bacteria richness (number of ASVs) compared to richness of the bacteria community of termites feeding on healthy trees. In case of *N. takasagoensis* workers (Chapter 2), the phylogenetic distances of bacteria communities were greater in workers feeding on sick trees than those of healthy trees. Previous research on bleeding canker disease (*Pseudomonas syringae pv aesculi*) showed the impact of disease on the alpha diversity of the tree microbiome. There was a decrease in alpha diversity in the symptomatic trees as compared to asymptomatic trees (Koskella et al. 2017). The authors claimed that a change in alpha diversity parameters due to presence of a

disease weakens the defense mechanism of a plant against the opportunistic pathogens and therefore they can enter the plant tissue (Pearce 1995, Koskella et al. 2017). Since presence of tree disease seems to impact microbiome of both tree (Koskella et al. 2017) and the insect feeding on that tree (our study), it is possible that there is a correlation between tree microbiome and the insect microbiome that needs to be further investigated.

The level of site management where the ironwood tree is located impacts the severity of the IWTD (Schlub 2010). A tree growing on a site with high level of human management is more likely to have IWTD than a tree on a site with lower level of management (Schlub 2010). In this study, it was observed that the management of the site also impacted the bacterial communities of termites feeding on the trees present at that site. A high level of site management led to decrease in phylogenetic diversity of the bacteria present in termites feeding on those trees. Similarly, the bacterial community of workers of *N. takasagoensis* showed less evenness and Shannon diversity with increased site management (Chapter 2). A site managed by removing unwanted plants, would reduce the overall plant diversity of the ecosystem. A reduced plant diversity can reduce the microbial diversity of the soil and subsequently the microbial diversity of the tree (Lavelle et al. 1995, Wardle et al. 2004). A decrease in microbiota in the soil that subterranean termites like *C. gestroi* workers tunnel through and of the wood they feed on could impact the microbiota of termites. However, more studies on the association of site management with soil-, tree- and termite microbiomes are needed to understand their combined contribution to IWTD.

In conclusion, this study shows that *C. gestroi* workers are unlikely to be a vector of pathogens associated with IWTD in Guam because the termites' pathogen load is low; however, the wounds

caused by the termite infestation could still be a cause for entry of plant pathogens and lead to acceleration of the decline (Agrios 2008). Moreover, termites are well-known as the predominant decomposers of organic matter (Sugimoto et al. 2000). Therefore, there is also the possibility that termites were statistically associated with IWTD not because they are actively causing tree decline but as an opportunistic feeder on weakened trees under decline or dead trees. Thus, further research is required to determine whether other biotic or abiotic factors might be responsible for onset, acceleration, and the spread of IWTD.

Chapter 4. Taxonomic Profiling of Bacterial Communities of *Microcerotermes crassus* Workers Associated with Ironwood Trees (*Casuarina equisetifolia*) in Guam

4.1. Introduction

Guam is the southernmost Mariana Island and contains a rich diversity of flora and fauna. Ironwood trees are an integral part of the island's flora. Ironwood trees have an ability to fix atmospheric nitrogen and have an innate resistance to various pests and diseases (Diouf et al. 2008, Touati et al. 2016, Conglu et al. 2010). High salt tolerance makes ironwood one of the few trees that can survive along the coastlines. Since ironwood trees act as windbreak and prevent soil erosion, they play an important role in protecting the island from typhoons (Chonglu et al. 2010, Schlub 2013).

The ironwood trees in Guam are dying in large numbers due to a sudden decline known as ironwood tree decline (IWTD). This decline was first reported in 2002 (Mersha et al. 2009, Schlub 2011). Due to IWTD, thousands of trees were dead in a short span of time (Mersha et al. 2009, Schlub 2011). The trees under decline show symptoms such as thinning of foliage and dieback of tree branches from tip to downwards (Mersha et al. 2009, Schlub 2011). The crossection of the tree under decline shows areas of wet wood and ooze. Plant pathogenic bacteria such as *Ralstonia solanacearum* and *Klebsiella* species (*K. oxytoca* and *K. variicola*) were identified when the ooze from infested trees was analyzed (Ayin et al. 2013, 2015). Association of IWTD was studied with different biotic and abiotic factors such as presence of conks, presence of termites, tree damage due to typhoon, tree density, level of human management, longitude and latitude (Schlub et al. 2020). The study showed that the presence of termites was associated significantly ($p<0.01$) with

IWTD (Schlub 2010). Symptoms of termite attack including hollowing of trees and tunnels are often observed on ironwood trees in Guam (Schlub 2013). Termite species attacking ironwood trees in Guam were identified as *Nasutitermes takasagoensis* (Nawa), *Coptotermes gestroi* (Wasmann), *Microcerotermes crassus* Snyder (Blattodea: Termitidae) and *Microcerotermes* species (Blattodea: Termitidae) through DNA barcoding techniques and morphological characters (Park et al. 2019). The *Nasutitermes takasagoensis* and *Coptotermes gestroi* termites are more frequent on ironwood trees as compared to the *Microcerotermes crassus* termites (Park et al. 2019). The association of *Nasutitermes takasagoensis* and *Coptotermes gestroi* termites with IWTD was studied in Chapter 2 and Chapter 3 and it was shown that neither of these species can be considered a vector for IWTD pathogens. To complete the assessment of potential association of termites with IWTD, it is important to study the vector potential of *Microcerotermes crassus* termites.

The genus *Microcerotermes* is the largest among the subfamily Termitinae (Scheffrahn & Huchet 2010) and is known to be cosmotropical. It is found commonly distributed among Southeastern regions of Asia (Wong and Lee 2010, Tho 1992). There are around 148 species within this genus. Most of the species within this genus build arboreal nests with carton material. However, some species also build mounds or even underground nests (Takematsu et al. 2003). Most of the species from this genus feed on dead wood but some species can damage the living trees as well (Park et al. 2019).

The species *M. crassus* is a wood-feeding higher termite of the family Termitidae. The gut of the Termitidae is highly compartmentalized and has a high pH (Brune 2014). Wood-feeding

Termitidae depend upon the bacteria present inside the gut for the digestion of wood to complement the termites' limited cellulolytic capability (Brune 2014). The bacterial community present within these termites plays important roles performing nitrogen fixation, reductive acetogenesis, chitin and lignin digestion, among others (Arora et al. 2022, Brune 2014). A wide diversity of bacterial species present in these termites can include both obligate symbionts, which are essential for the survival of termite and cannot live outside the termite guts, as well as bacteria from the environment that the termites may have ingested while feeding and were able to survive in the termite gut environment (Hongoh et al. 2010, Brune 2014, Mikaelyan et al. 2017, Husseneder et al. 2009, Rahman et al. 2015, Tai et al. 2015).

Since termites are the "hotspots" for bacteria diversity and the colonies of *M. crassus* have been found attacking ironwood trees in Guam (Park et al. 2019), the goal of this study was to test whether *M. crassus* workers harbor IWTD pathogens and might, thus, act as vectors for IWTD. For this purpose, we used 16S rRNA gene amplicon sequencing for taxonomic analysis of bacterial communities of *M. crassus* workers attacking ironwood trees.

4.2. Materials and methods

4.2.1. Termite collection and DNA sequencing

Six *M. crassus* termite samples containing workers and soldiers were collected from ironwood trees at different locations in Guam by the team of University of Guam in 2021 (Figure 1). The collected *M. crassus* termite samples were shipped to LSU AgCenter in 95% ethanol. The DNeasy Blood & Tissue kit (Qiagen, Germantown, MA) was used to extract DNA from pools of five workers per sample following manufacturer's instructions. The homogenization of termite samples

was performed using a sterile pestle (Thermo Fisher Scientific, Wilmington, DE) in lysis buffer (Buffer AL, DNeasy Blood & Tissue kit). The concentration of extracted DNA was quantified with the Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE) and the Qubit dsDNA BR Assay Kit (Invitrogen™, Life Technologies™). DNA sequencing was performed at University of New Hampshire Hubbard Center for Genome studies. The 515F and 926R primers from the V4 region of the 16S rRNA gene of the bacterial DNA were used for DNA amplification (Parada et al. 2016). Sequencing was performed on the Illumina NovaSeq (2x250bp) platform using Illumina Nextera Dilute library protocol (Illumina, San Diego, CA). All the sequences used in this study were uploaded to NCBI GenBank under BioProject ID PRJNA883256.

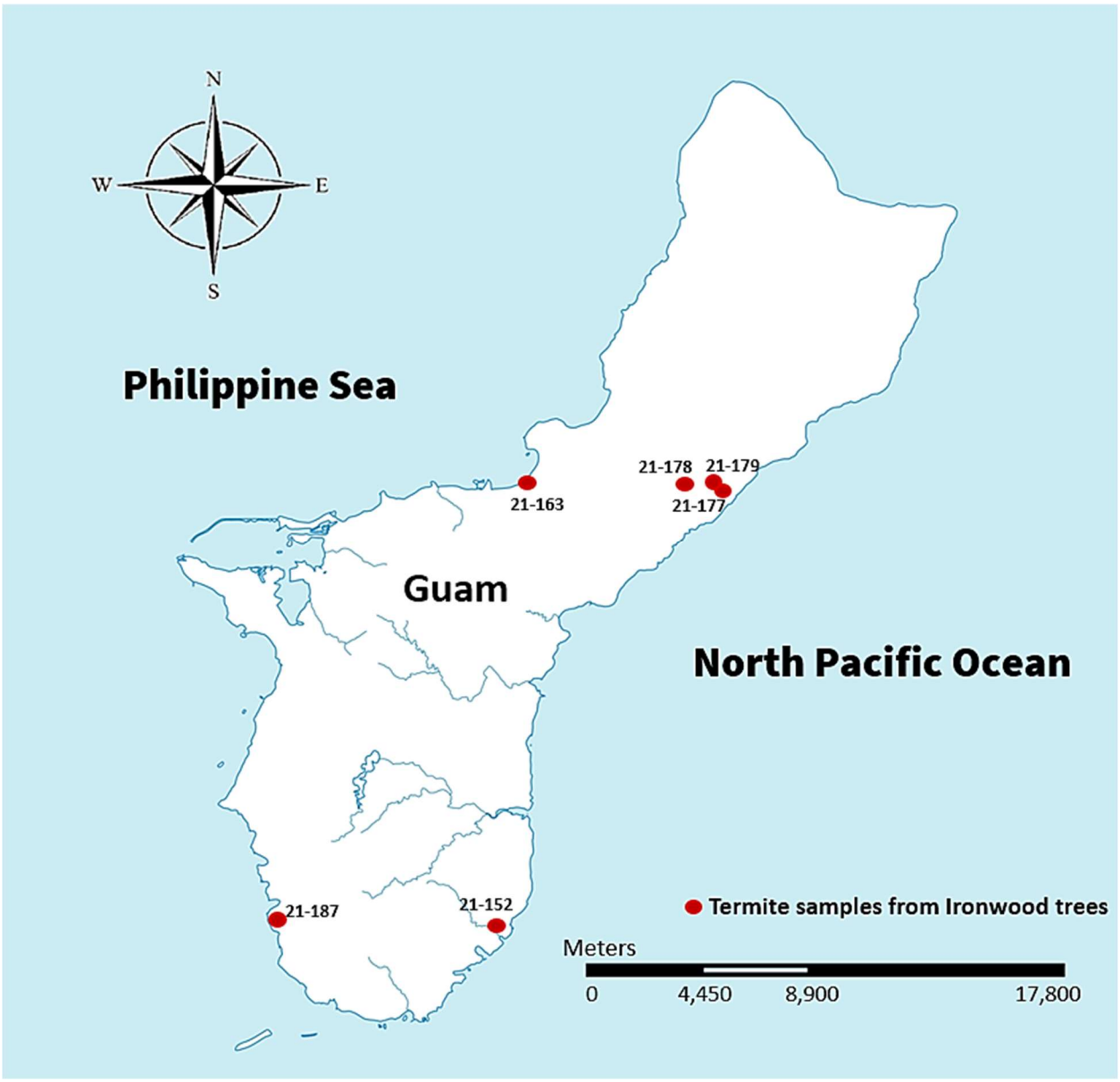

Figure 1. Map of ironwood tree sites on Guam where 6 samples of *M. crassus* termites were collected.

4.2.2. Bioinformatics and statistical analysis

The Quantitative Insights into Microbial Ecology (QIIME2) pipeline was used to perform bioinformatic analysis of demultiplexed sequence reads received from University of New Hampshire Hubbard Center for Genome studies in FASTQ format (Caporaso et al. 2010, Estaki et al. 2020). The Phred quality score of the demultiplexed reads was analyzed using DADA2 (Callahan et al. 2016). The Phred quality scores of all the sequence reads were above 30, therefore no trimming was required. The primers and chimera sequences were removed, and 251 nucleotide long sequence reads were obtained. Forward and reverse reads were not overlapping due to the short length of reads. Because of this, only forward reads were used for downstream analysis. Sequence depth based-, sample based- and coverage based- rarefaction curves were plotted to evaluate if sufficient bacterial diversity has been captured. QIIME2 was used to plot alpha rarefaction curves for Amplicon Sequence Variant (ASV) richness, Faith's Phylogenetic Distance (Faith's PD) and Shannon diversity of each sample against sequencing depth after subsampling the sequence reads to the number of sequences in the sample with the lowest sequencing depth of 4,118. The unique nucleotide sequences are called as ASVs Number of ASVs present within a sample is measured through ASV richness index. Faith's PD index measure the phylogenetic distance between the ASVs and Shannon diversity measures ASVs richness based on the evenness. Sample size- and coverage-based curves were plotted using R package iNEXT (iNterpolation/ EXTrapolation) as described in chapter 2 (2.2.4, Hsieh et al. 2016). The reference database SILVA 132 was used for taxonomic assignment of ASVs (Quast et al. 2013). Sequence reads were taxonomically classified using consensus method in BLAST at 97% pairwise identity cutoff. The sequences were aligned using MAFFT method and the alignments were masked to remove the

variable positions (Lane 1991). Finally, the reads that remained unassigned were filtered out and taxa bar plots representing relative abundances of assigned taxa were made. The taxonomic identity of the top 20 ASVs according to their number of reads across all samples was also confirmed using references in NCBI GenBank database by performing BLAST (Benson et al. 2016).

4.3. Results

4.3.1. Number of sequence reads and ASVs

The number of raw sequence reads was 652,571 across the six *M. crassus* samples collected from ironwood trees in Guam. A total of 2,165 ASVs in 378,976 sequence reads were observed after quality filtering using DADA2 with a range of 4,118 to 236,400 sequence reads per sample. A total of 831 ASVs remained with a total of 231,967 sequence reads after removal of ASVs with no taxonomical assignment at 97% identity to references in the SILVA database.

4.3.2. Sequence depth-, sample- and coverage-based rarefaction

The sequence-depth based rarefaction curve for ASV richness and Faith's PD (Figure 2a) started to level out at a sequencing depth of 1,500. For Shannon diversity, the curves for all the samples levelled out at sequencing depth of 500. This indicates that sequencing depth of the samples used in this study was enough for capturing most of the bacteria strains and their diversity within each sample.

The sample-based rarefaction curves (Figure 2b) for Shannon diversity and Simpson inverse started to level out at the effective diversity of around 150 and 120, respectively and the extrapolation of the curves to twice the sample size did not increase the effective diversity

considerably. However, the sample-based rarefaction curve did not level out at the observed ASV richness of 194. This indicates that the number of samples used in this study were sufficient to capture most of the diversity based on richness and evenness (Shannon diversity) and the diversity of the dominant bacterial species (Simpson inverse) but doubling the number of samples would have increased the ASV richness from 194 to around 250. However, this increase in ASV numbers would be based on rare ASVs as the extrapolation did not show much increase in Shannon diversity and Simpson inverse indices.

The coverage-based rarefaction curves (Figure 2c) showed that the six *M. crassus* samples collected in the study were able to achieve almost 80% coverage. The extrapolation of the curves by doubling the sample size increased the coverage to 95% while increasing the ASV richness from 194 to more than 250 without considerably impacting the Shannon diversity and Simpson inverse.

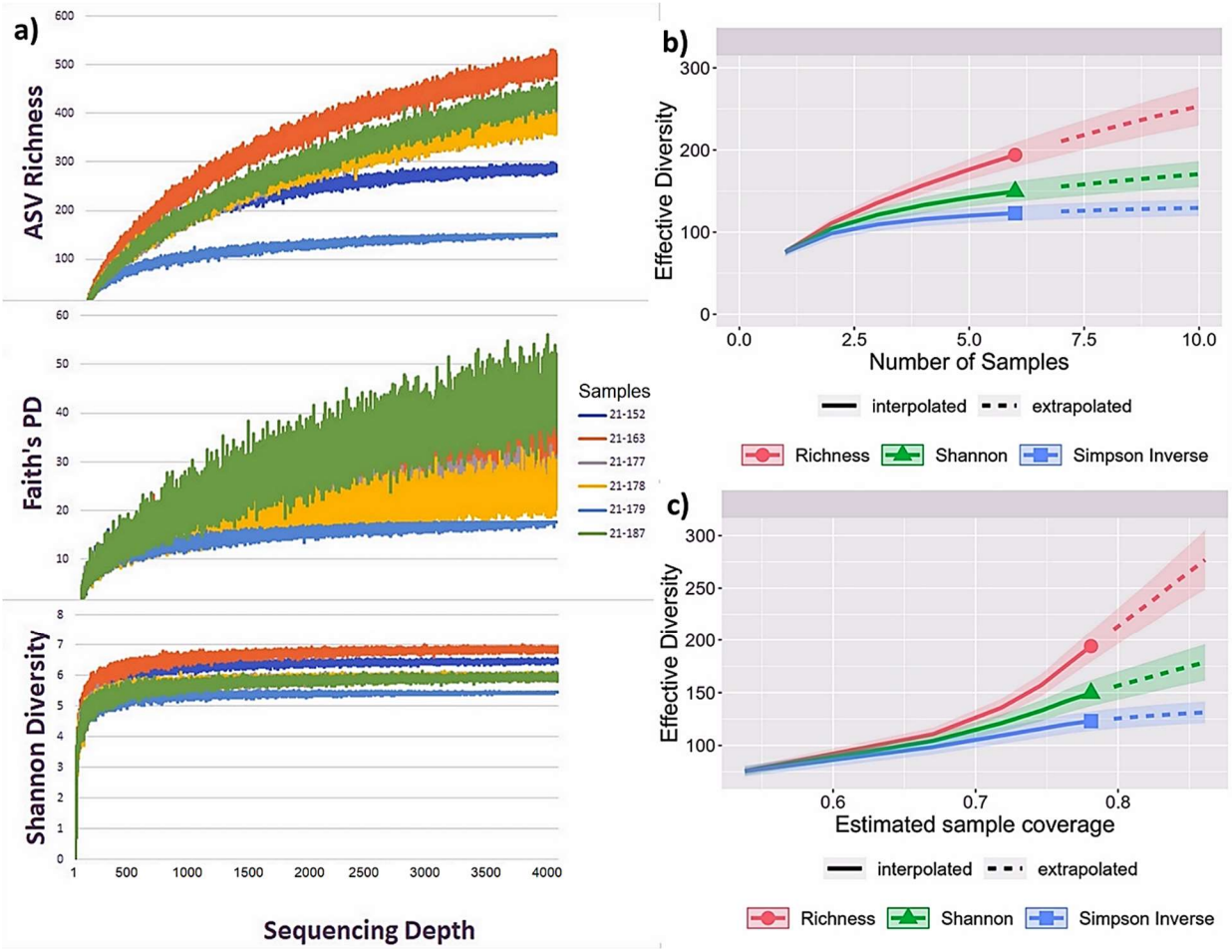

Figure 2. a) Sequence-based rarefaction curves of bacteria diversity showing the number of ASVs, Faith's phylogenetic distance and Shannon diversity indices in six *M. crassus* worker samples plotted against sequencing depth. b) Sample-based rarefaction curves with effective bacterial diversity for different metrics plotted against the number of samples. c) Coverage-based rarefaction curves with effective diversity plotted against estimated sample coverage. Solid lines indicate intrapolation up to the actual sample size; dashed lines represent extrapolation to twice the sample size. Rarefaction was performed over the total bacteria diversity (with and without taxonomical assignment).

4.3.3. Taxa composition

Twenty different phyla were observed in *M. crassus* workers collected from ironwood trees in Guam. Spirochaetes, Firmicutes, Proteobacteria, Fibrobacteres, Acidobacteria, Cloacimonetes, Epsilonbacteraeota, Synergistetes, Margulisbacteria, Planctomycetes and Bacteroidetes were present in all six samples and are, thus, likely core phyla. Actinobacteria was present in five samples and other phyla such as Cyanobacteria, Chloroflexi, Patescibacteria, Elusimicrobia, Tenericutes, Fusobacteria, Rokubacteria and Verrucomicrobia were present in less than three samples.

Spirochaetes was the most dominant phylum with a relative abundance of 53.78% and 13 ASVs across all samples. Twelve Spirochaete ASVs belonged to order Spirochaetales and family Spirochaetaceae and one was assigned to the order Leptospirales and family Leptospiraceae. Only two Spirochete genera were identified, i.e., Spirochaeta and Treponema, both from the family Spirochaetaceae. The second most dominant phylum observed in *M. crassus* samples was Fibrobacteres with two classes (Fibrobacteria and Chitinivibrionia) and four different ASVs at a combined relative abundance of 28.31%. Only one genus named Fibrobacter was identified within order Fibrobacterales, family Fibrobacteraceae and class Fibrobacteria, and all other genera remained unidentified.

While Spirochaetes and Fibrobacteres were the most dominant phyla, Firmicutes, Proteobacteria and Bacteroidetes were the most diverse phyla observed within the *M. crassus* workers. The phylum Firmicutes had the largest number of ASVs (57) with a relative abundance of 4.66%. The phylum Proteobacteria had 43 ASVs with a relative abundance of 4.21% and the phylum

Bacteroidetes had 30 ASVs at a relative abundance of 3.48%.

The top 20 ASVs with the highest number of reads across the samples of *M. crassus* workers belong to bacteria from the phyla Spirochaetes, Fibrobacteres, Proteobacteria, Planctomycetes, Firmicutes, Cloacimonetes, Bacteroidetes, Synergistetes, and Acidobacteria (Table 2). An uncultured *Treponema* sp. from the phylum Spirochaetes was the most dominant bacteria species, followed by uncultured Chitinivibrionia bacterium from the phylum Fibrobacteres as the second most abundant. However, the pathogenic bacteria causing IWTD, i.e., *Ralstonia* spp. and *Klebsiella* spp. were absent in the *M. crassus* samples collected from ironwood trees in Guam.

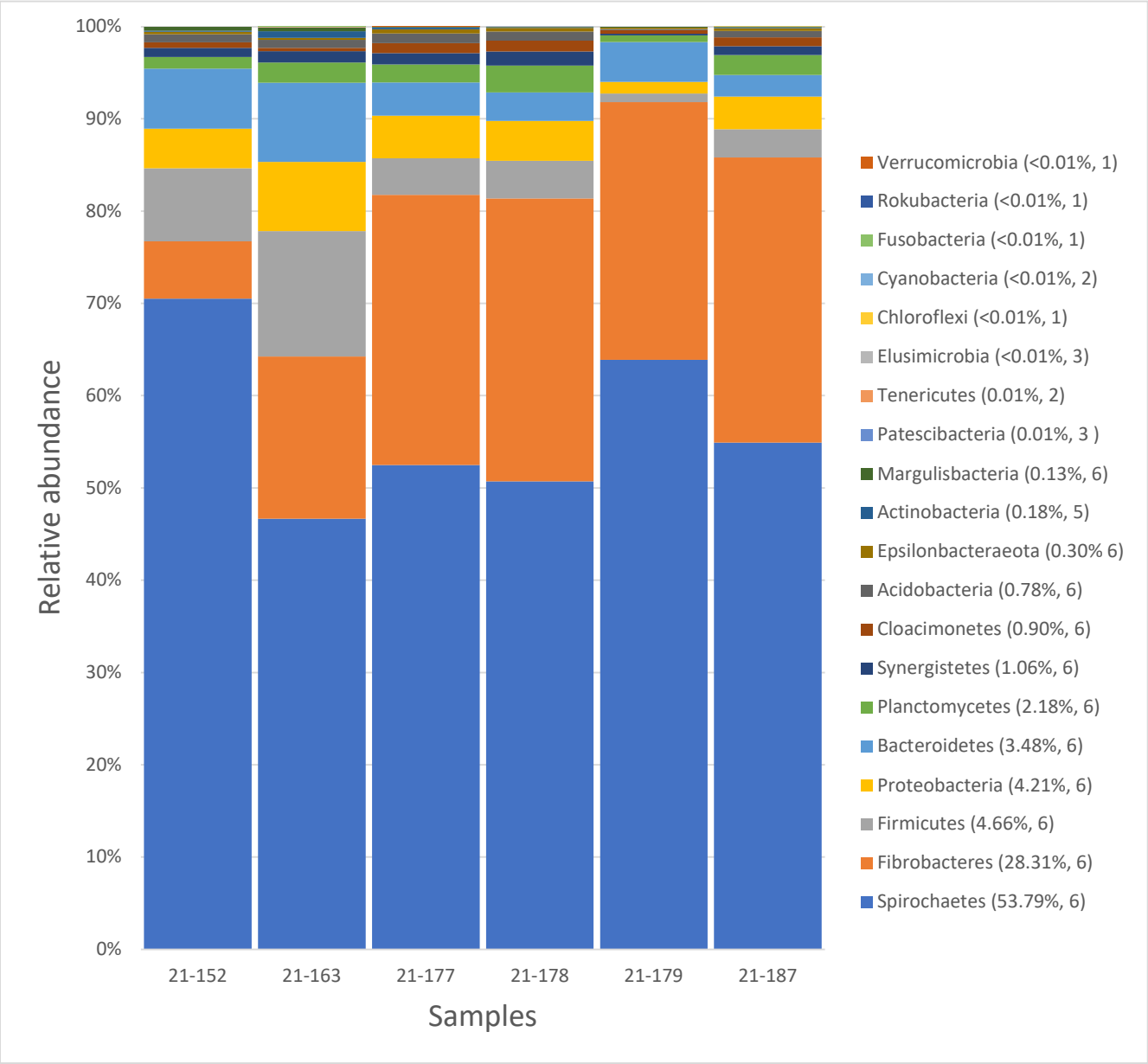

Figure 3. Taxa bar plots showing the relative abundance of bacterial phyla associated with six samples of *M. crassus* workers collected from ironwood trees in Guam. Legend depicts name of the phylum with its relative abundance (in %) and number of samples in which that phylum was found.

Table 2. The 20 ASVs with highest number of reads across the *M. crassus* samples classified according to the SILVA 132 and GenBank database.

Phylum	Order	Lowest SILVA assignment	Scientific name	Percent identity to top match	Accession number in GenBank	Number of Reads	Number of Samples	Average Reads per sample	Standard deviation
Spirochaetes	Spirochaetales	uncultured Treponema sp.	uncultured Treponema sp.	99.13%	AB191906.1	98,787	6	16,465	23,843
Fibrobacteres	Fibrobacterales	uncultured Chitinivibrionia bacterium	uncultured Chitinivibrionia bacterium	98.27%	AB255975.1	63,462	6	10,577	16,764
Spirochaetes	Spirochaetales	uncultured Treponema sp.	uncultured Treponema sp.	98.70%	AB191972.1	24,063	6	4,011	5,111
Proteobacteria	Rs-K70 termite gr	uncultured delta proteobacterium	uncultured delta proteobacterium	100.00%	AB255924.1	7,250	6	1,208	1,538
Planctomycetes	Pirellulales	uncultured planctomycete	uncultured planctomycete	99.13%	KM651184.1	4,707	6	785	1,129
Firmicutes	Clostridiales	uncultured Eubacteriaceae bacterium	uncultured bacterium	99.13%	AB277904.1	4,334	6	722	907
Firmicutes	Clostridiales	uncultured Clostridiaceae bacterium	uncultured Clostridiaceae bacterium	100.00%	AB192029.1	2,324	5	387	840
Cloacimonetes	Cloacimonadales	uncultured bacterium	uncultured bacterium	97.40%	AB191981.1	2,097	6	350	525
Bacteroidetes	SJA-28	uncultured Chlorobi bacterium	uncultured Chlorobi bacterium	98.70%	AB192128.1	1,903	6	317	330
Fibrobacteres	Fibrobacterales	uncultured Fibrobacteres bacterium	uncultured Fibrobacteres bacterium	99.57%	AB192087.1	1,703	6	284	336
Bacteroidetes	Bacteroidales	uncultured Bacteroidales bacterium	uncultured Bacteroidales bacterium	98.27%	AB191992.1	1,528	5	255	261
Bacteroidetes	Bacteroidales	uncultured Bacteroidales bacterium	uncultured Bacteroidales bacterium	98.27%	AB191995.1	1,430	6	238	309
Firmicutes	Clostridiales	uncultured Clostridiaceae bacterium	uncultured Clostridiaceae bacterium	99.13%	AB192023.1	1,418	6	236	263
Synergistetes	Synergistales	uncultured bacterium	uncultured bacterium	98.70%	KM023943.1	971	5	162	186
Synergistetes	Synergistales	uncultured bacterium	uncultured bacterium	99.13%	AB243291.1	969	6	162	202
Acidobacteria	Holophagales	uncultured Acidobacteria bacterium	uncultured Acidobacteria bacterium	98.27%	AB192122.1	957	5	160	183
Acidobacteria	uncultured Acidob	uncultured Acidobacteria bacterium	uncultured Acidobacteria bacterium	97.40%	AB192123.1	848	6	141	184
Bacteroidetes	Bacteroidales	uncultured Bacteroidetes bacterium	uncultured Bacteroidetes bacterium	99.57%	AB192003.1	763	5	127	157
Proteobacteria	Rhodospirillales	uncultured alpha proteobacterium	uncultured Alphaproteobacteria bacteriu	100.00%	AB192062.1	743	5	124	163
Bacteroidetes	Bacteroidales	uncultured Bacteroidales bacterium	uncultured Bacteroidetes bacterium	98.27%	KM650310.1	723	5	121	147

4.4. Discussion

The termite *Microcerotermes crassus*, is one of the four termite species attacking ironwood trees, that are significantly associated with IWTD on the island of Guam (Park et al. 2019, Schlub 2010). Since termites harbor large numbers of symbiotic and environmental bacteria in their guts and on their body surface, we hypothesized that termites could be vectors for IWTD pathogens. Previous studies of the bacterial taxonomy and diversity of *N. takasagoensis* and *C. gestroi* (Chapter 2 and 3) rejected this hypothesis for both species. To complete the assessment concerning association of termites with IWTD we analyzed the bacteria composition of *M. crassus* workers collected from Guam's ironwood trees to test if they harbor plant pathogens associated with IWTD. However, similar to the results of *N. takasagoensis* and *C. gestroi* workers, the taxonomic profiling of bacterial communities in *M. crassus* workers did not show any pathogens associated with IWTD as well. This indicates that none of the termite (*N. takasagoensis*, *C. gestroi* and *M. crassus*) workers are a vector of plant pathogens associated with the IWTD.

The alpha rarefaction plots indicated that increase in number of samples would have increased the number of bacterial species (ASV richness). However, increase in richness would have been caused by rare ASVs as Shannon diversity and Simpson inverse indices did not increase when the number of samples was virtually doubled by extrapolation. This indicates that even if the plant pathogenic bacteria would have been present among those rare ASVs acquired after increasing the sample size, the amount of the pathogens would not be sufficient for the termites to be considered as vectors. None of the 652,571 total reads obtained after sequencing was identified as *R. solanacearum*, or as *Klebsiella* spp. underscoring the low (< 1/652,571) probability of presence of

IWTD pathogens in *M. crassus* workers.

The failure of detection of IWTD pathogens was not due to primer bias or lack of reference sequences since (1) the ability of the primers used in this study to identify DNA sequences of *R. solanacearum* was ascertained by performing BLAST searches against NCBI GenBank database, (2) reference sequences for *Ralstonia* and *Klebsiella* species were present in the SILVA 132 database (Chapter 2, Chapter 3), (3) both *Ralstonia* and *Klebsiella* species were detected in *C. gestroi* samples collected from sick and healthy ironwood trees (Chapter 3, 3.3.3), (4) Ralstonia was detected in the *N. takasagoensis* samples from force feeding test using the same primers (Chapter 2, 2.3.9.4) and (5) the primers successfully confirmed *R. solanacearum* bacterial isolate (19-147) from the declining ironwood trees (Paudel 2020) (Chapter 2, 2.2.4.3).

The absence of pathogens associated with IWTD in termites might be because termites do not prefer to feed on wood infested with pathogens as shown in the case of *N. takasagoensis*, which are also members of the Termitidae as *M. crassus* (Chapter 2). Although the concentration of *R. solanacearum* in ironwood trees is not known, the results from a pilot study in *Coptotermes formosanus* (Chapter 3) indicate that if the concentration of *R. solanacearum* is high (10^{-2} dilution was used in pilot study) in ironwood trees, the termites might tend to avoid that wood.

For the environmental bacteria to survive in body of termites, they must adapt to the conditions in termite gut such as pH, O_2 and H_2 gradient. As aerobic bacteria species *R. solanacearum* bacteria should be able to survive in the microoxic zones of termite guts (Brune 2014). However, *R. solanacearum* bacteria flourishes well in an environment with low pH (pH 4.5–5.5) (Li et al. 2017). The alkaline milieu of midgut and hindgut region of higher termites (e.g., pH ~ 6-10 in

Microcerotermes parvus workers, Brune et al. 1995) is outside of the pH optimum of *R. solanacearum* and might, thus, not be favorable for survival of *R. solanacearum*.

The individual and social immunity of termites against the invasion of foreign bacteria can also be the reason behind the absence of pathogens in the termite bodies (Cremer et al. 2007, Cremer et al. 2018). Termites are capable of secreting antimicrobial compounds (Rosengaus et al. 2011, Bulmer et al. 2019). The antimicrobial compounds are also present in the nest of termites resulting in an additional protection from invasion of pathogens (Witasari et al. 2022). The microbial community present in termite guts and nests aids the termite's immunity by preventing foreign bacteria from colonizing the termite gut (Rosengaus et al. 1999, Traniello et al. 2002, Cremer et al. 2007, Bulmer et al. 2009, Cremer et al. 2018, Veivers et al. 1982, Dillon and Dillon 2004, Sen et al. 2015, Peterson and Scharf 2016, Oberpaul et al. 2020). Moreover, termites exhibit hygienic behavior including allo-grooming, avoidance, cannibalism, and burial of nest-mates that are exposed to pathogens (Kramm et al. 1982, Rosengaus et al. 1998, Liu et al. 2015, Yanagawa and Shimizu 2007). Similar resilience to change in gut microbiota was observed in *N. takasagoensis* workers as *R. solanacearum* bacteria were not able to establish themselves in healthy *N. takasagoensis* workers despite force-feeding in high concentration (Chapter 2, 2.3.9). Ralstonia was only detected after artificial feeding conditions induced dysbiosis, i.e., disrupted the balance in the termite guts, and, thus, weakened the gut health of the termites and the microbial defenses (Chapter 2, 2.3.9).

Despite the absence of *R. solanacearum*, all the core bacterial phyla that have been observed in previous studies of the *Microcerotermes* genus and other higher termites were found in *M. crassus*

worker samples collected from ironwood trees in Guam. The core bacterial phyla such as Spirochaetes, Fibrobacteres, Firmicutes, Proteobacteria, Bacteroidetes, and Margulisbacteria have been observed in previous studies of bacterial taxonomic profiling in the *Microcerotermes* genus and other higher termites (Auer et al. 2017, Diouf et al. 2015, Hongoh et al. 2006, Miyata et al. 2007, Köhler et al. 2012, Dietrich et al. 2014, Mikaelyan et al. 2015, Wang et al. 2016, Su et al. 2016). Spirochaetes and Fibrobacteres were the dominant phyla (>5% relative abundance) found in *M. crassus* workers by Illumina sequencing of the V4 region of 16S rRNA gene in our study. Studies that performed Illumina sequencing of the V3-V4 region of 16S rRNA gene (*Microcerotermes parvus* (Mikaelyan et al. 2015, Auer et al. 2017), *Nasutitermes corniger* (Mikaelyan et al. 2015), *Nasutitermes takasagoensis* (Mikaelyan et al. 2015), *Nasutitermes ephratae* (Auer et al. 2017)) and a clone-based study (*Nasutitermes takasagoensis* and *Microcerotermes* species workers (Hongoh et al. 2006)) also showed the dominance of Spirochaetes and Fibrobacteres similar to our study. However, studies that performed Pyrosequencing (*Mironasutitermes shangchengensis* (Wang et al. 2016), *Mironasutitermes shangchengensis* (Su et al. 2016), *Nasutitermes* spp. (Köhler et al. 2012), *Nasutitermes arborum* (Diouf et al. 2015) and another clone-based study (*Nasutitermes takasagoensis* (Miyata et al. 2007)) found Spirochaetes, Firmicutes and Bacteroidetes in higher abundance than Fibrobacteres. The difference in sequencing method seems to influence the relative abundance of bacterial phyla. The difference in abundance of phyla obtained in this study as compared to other studies in Termitidae might also be because of different termite species or populations from different geographical regions, different primers, or different analysis methods were used in these studies as compared to our study.

In summary, the lack of plant pathogens among the bacterial inventory of *M. crassus* workers, as well as *N. takasagoensis* and *C. gestroi* workers (Chapter 2 and Chapter 3) showed that none of the termite species attacking ironwood trees serve as vectors for IWTD pathogens. However, there is still a possibility that mechanical tree damage caused by termite infestation could weaken the tree and act as entry point for pathogens present in the surrounding soil. As of to date, the modes and patterns of the rapid spread of IWTD and whether there are vectors involved, remain unknown.

Chapter 5. Conclusion and future directions

This thesis showed that none of the termite species (*N. takasagonesis*, *C. gestroi* and *M. crassus*) that attack ironwood trees in Guam were vectors for IWTD pathogens. The IWTD associated pathogens were either absent or were scarcely detected in the termite worker samples collected from sick and healthy ironwood trees in Guam. Only *Klebsiella* sp. was detected in the worker samples of *N. takasagonesis*. Putative pathogens from genera *Ralstonia*, *Klebsiella*, *Enterobacter*, *Pantoea*, and *Citrobacter* were detected in low amounts (0.02% relative abundance) in the worker samples of *C. gestroi*. No pathogenic bacteria species associated with IWTD were detected in the worker samples of *M. crassus*.

The feeding experiments presented possible explanations for the failure to detect IWTD pathogens in termites. *Nasutitermes takasagonesis* workers prefer to feed on wood with low pathogenic load over wood with high pathogenic load. The feeding experiments in *N. takasagonesis* workers showed that termite workers can ingest *R. solanacearum* bacteria, however, the bacteria were not able to establish themselves in the body of healthy workers; validating the results of previous studies that termites and their native microbiota defend against the colonialization by foreign bacteria. Although workers of *C. gestroi* did not show any preference at the concentration range tested for Ralstonia (10^{-4}, 10^{-6} and 10^{-8} dilutions), the results from a pilot study performed using *C. formosanus* workers showed that consumption was reduced when termites were fed with a high Ralstonia concentration (10^{-2} dilution). This suggests that *C. gestroi* workers might show similar avoidance as *N. takasagonesis* workers but at a higher concentration of Ralstonia.

Bacterial communities of *N. takasagonesis* workers were found to be impacted by Presence of

Ralstonia, Tree Health, Plot Average DS, Plot Average Decline, Proportion of Dead Trees in the Plot, Proportion of Trees with Termites in the Plot, Altitude, Parent Material and Site Management while those of *C. gestroi* workers were impacted by Tree Health and Site Management. The number of *M. crassus* samples was too limited to investigate the effects of those factors on the bacterial community.

Termites are associated with IWTD (Schlub 2010); however, it is still unknown whether termite infestation is one of the causes of IWTD or it is a consequence. It is possible that other castes of termites such as alates (winged reproductives) might be responsible for carrying the pathogens, as Ralstonia has been detected in alate populations of *Coptotermes formosanus*, a close relative to *Coptotermes gestroi* (Chen et al. unpublished). Alternatively, the association between termites and IWTD might also be indirect. The mechanical tree damage due to feeding of termites might be serving as a point of entry for the IWTD associated pathogens. To understand the indirect association of termites with IWTD, the pattern of termite infestation at the different stages of IWTD severity needs to be studied. This would elucidate if association of termites are causal to IWTD, or these termites simply serve as opportunistic feeders.

There is a need to study the microbiome of the healthy and diseased ironwood trees and analyze the relationship of tree microbiome in relationship with different tree-, plot- and location related factors. Furthermore, the study of microbiota of the soil surrounding the trees and its association with tree-, plot- and location related factors could also be helpful to determine the reason behind IWTD. It is also essential to determine which of the putative plant pathogens isolated from ooze cause IWTD. For this purpose, the pathogenicity of all the bacteria isolated from the ooze of

ironwood trees in decline should be examined. Determining the factors behind the cause and transmission of IWTD would help in developing an integrated disease management plan to prevent the spread of this decline to healthy ironwood trees within the island of Guam and the neighboring islands.

References

Aanen, D. K., Eggleton, P., Rouland-Lefevre, C., Guldberg-Froslev, T., Rosendahl, S., & Boomsma, J. J. (2002). The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(23), 14887–14892. https://doi.org/10.1073/pnas.222313099

Agrios, G. N. (2008). "Transmission of plant diseases by insects," in *Encyclopedia of Entomology.* ed. J. L. Capinera (Dordrecht: Springer Netherlands), 3853–3885

Aguiar-Pulido, V., Huang, W., Suarez-Ulloa, V., Cickovski, T., Mathee, K., & Narasimhan, G. (2016). Metagenomics, Metatranscriptomics, and Metabolomics Approaches for Microbiome Analysis. *Evolutionary Bioinformatics*, *12*, 5–16. https://doi.org/10.4137/ebo.s36436

Alvarez, B., Vasse, J., Le-Courtois, V., Trigalet-Démery, D., López, M. M., & Trigalet, A. (2008). Comparative behavior of Ralstonia solanacearum biovar 2 in diverse plant species. *Phytopathology*, *98*(1), 59–68. https://doi.org/10.1094/PHYTO-98-1-0059

Amalendu Ghosh, Ghosh, A., Dey, D., Timmanna, Basavaraj, Bikash Mandal, Mandal, B., Mandal, B., & Jain, R. K. (2017). *Thrips as the Vectors of Tospoviruses in Indian Agriculture*. 537–561. https://doi.org/10.1007/978-981-10-5672-7_24

Anderson, M. 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

Arnot, D. E., Roper, C., & Bayoumi, R. A. (1993). Digital codes from hypervariable tandemly repeated DNA sequences in the Plasmodium falciparum circumsporozoite gene can genetically barcode isolates. *Molecular and biochemical parasitology*, *61*(1), 15–24. https://doi.org/10.1016/0166-6851(93)90154-p

Arora, J., Kinjo, Y., Šobotník, J., Buček, A., Clitheroe, C., Stiblik, P., Roisin, Y., Žifčáková, L., Park, Y. C., Kim, K. Y., Sillam-Dussès, D., Hervé, V., Lo, N., Tokuda, G., Brune, A., & Bourguignon, T. (2022). The functional evolution of termite gut microbiota. *Microbiome*, *10*(1), 78. https://doi.org/10.1186/s40168-022-01258-3

Athens, J. S., & Ard, J. V. W. (2004). Holocene vegetation, savanna origins and human settlement of Guam. In A Pacific Odyssey: Archaeology and Anthropology in the Western Pacific. Papers in Honour of Jim Specht. *Records of The Australian Museum, Supplement*, *29*, 15–30. https://doi.org/10.3853/j.0812-7387.29.2004.1398

Auer, L., Lazuka, A., Sillam-Dussès, D., Miambi, E., O'Donohue, M., & Hernandez-Raquet, G. (2017). Uncovering the Potential of Termite Gut Microbiome for Lignocellulose Bioconversion in Anaerobic Batch Bioreactors. *Frontiers in microbiology*, *8*, 2623. https://doi.org/10.3389/fmicb.2017.02623

Ayin, C. M., Alvarez, A. M., Awana, C., Schleinzer, F. M., Marx, B. D., B. D. Marx, & Schlub, R. L. (2019). Ralstonia solanacearum, *Ganoderma australe*, and bacterial wetwood as predictors of ironwood tree (*Casuarina equisetifolia*) decline in Guam. *Australasian Plant Pathology*, *48*(6), 625–636. https://doi.org/10.1007/s13313-019-00666-8

Ayin, C.M., Schlub R.L., Alvarez A.M. (2013) Identification of bacteria associated with decline of ironwood trees (*Casuarina equisetifolia*) in Guam. Phytopathology 103:S2.10

Ayin, C.M., Schlub, R.L., Yasuhara-Bell, J. *et al.* Identification and characterization of bacteria associated with decline of ironwood (C*asuarina equisetifolia*) in Guam. *Australasian Plant Pathol.* **44,** 225–234 (2015). https://doi.org/10.1007/s13313-014-0341-4

Baker, G. C., Smith, J. J., & Cowan, D. A. (2003). Review and re-analysis of domain-specific 16S primers. *Journal of microbiological methods*, *55*(3), 541–555. https://doi.org/10.1016/j.mimet.2003.08.009

Behr, E. A., Behr, C. T., & Wilson, L. F. (1972). Influence of Wood Hardness on Feeding by the Eastern Subterranean Termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitadae). *Annals of The Entomological Society of America*, *65*(2), 457–460. https://doi.org/10.1093/aesa/65.2.457

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B-Methodological*, *57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bennett, S. (2004). Solexa Ltd. *Pharmacogenomics*, *5*(4), 433–438. https://doi.org/10.1517/14622416.5.4.433

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2017). GenBank. *Nucleic Acids Research*, *45*(D1), D37–D42. https://doi-org.libezp.lib.lsu.edu/10.1093/nar/gkw1070

Bignell, D. E., Roisin, Y., & Lo, N. (2011). *Biology of Termites: A Modern Synthesis*. https://doi.org/10.1007/978-90-481-3977-4

Binggeli, P. (1997). *Casuarina equisetifolia* L. (Casuarinaceae), Woody Plant Ecology.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., Cope, E. K., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Böttger, E. C. (1989). Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. *Fems Microbiology Letters*, *65*, 171–176.

https://doi.org/10.1111/j.1574-6968.1989.tb03617.x

Boucias, D. G., Cai, Y., Sun, Y., Lietze, V. U., Sen, R., Raychoudhury, R., & Scharf, M. E. (2013). The hindgut lumen prokaryotic microbiota of the termite Reticulitermes flavipes and its responses to dietary lignocellulose composition. *Molecular ecology*, *22*(7), 1836–1853. https://doi.org/10.1111/mec.12230

Bourguignon, T., Lo, N., Cameron, S. L., Šobotník, J., Hayashi, Y., Shigenobu, S., Watanabe, D., Roisin, Y., Miura, T., & Evans, T. A. (2015). The evolutionary history of termites as inferred from 66 mitochondrial genomes. *Molecular biology and evolution*, *32*(2), 406–421. https://doi.org/10.1093/molbev/msu308

Bourguignon, T., Lo, N., Dietrich, C., Šobotník, J., Sidek, S., Roisin, Y., Brune, A., & Evans, T. A. (2018). Rampant Host Switching Shaped the Termite Gut Microbiome. *Current Biology*, *28*(4), 649. https://doi.org/10.1016/j.cub.2018.01.035

Breznak, J. A., & Brune, A. (1994). Role of Microorganisms in the Digestion of Lignocellulose by Termites. *Annual Review of Entomology*, *39*(1), 453–487. https://doi.org/10.1146/annurev.en.39.010194.002321

Breznak, J. A., & Leadbetter, J. R. (2006). *Termite Gut Spirochetes*. https://doi.org/10.1007/springerreference_3861

Brisse, S., Grimont, F., Grimont, P.A.D. (2006). The Genus *Klebsiella*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, KH., Stackebrandt, E. (eds) The Prokaryotes. Springer, New York, NY. https://doi.org/10.1007/0-387-30746-X_8

Brown, K., DeCoffe, D., Molcan, E., & Gibson, D. L. (2012). Diet-Induced Dysbiosis of the Intestinal Microbiota and the Effects on Immunity and Disease. *Nutrients*, *4*(8), 1095–1119. https://doi.org/10.3390/nu4081095

Brune, A. (2006). *Symbiotic Associations Between Termites and Prokaryotes*. 439–474. https://doi.org/10.1007/978-3-642-30194-0_20

Brune, A. (2010). *Methanogens in the Digestive Tract of Termites*. 81–100. https://doi.org/10.1007/978-3-642-13615-3_6

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

Brune, A., & Dietrich, C. (2015). The Gut Microbiota of Termites: Digesting the Diversity in the Light of Ecology and Evolution. *Annual Review of Microbiology*, *69*(1), 145–166. https://doi.org/10.1146/annurev-micro-092412-155715

Brune, A., & Friedrich, M. W. (2000). Microecology of the termite gut: Structure and function on a

microscale. *Current Opinion in Microbiology*, *3*(3), 263–269. https://doi.org/10.1016/s1369-5274(00)00087-4

Brune, A., & Kühl, M. (1996). pH profiles of the extremely alkaline hindguts of soil-feeding termites (Isoptera: Termitidae) determined with microelectrodes. *Journal of Insect Physiology*, *42*, 1121–1127. https://doi.org/10.1016/s0022-1910(96)00036-4

Brune, A., Emerson, D. R., Emerson, D., Breznak, J. A., & Breznak, J. A. (1995). The Termite Gut Microflora as an Oxygen Sink: Microelectrode Determination of Oxygen and pH Gradients in Guts of Lower and Higher Termites. *Applied and Environmental Microbiology*, *61*(7), 2681–2687. https://doi.org/10.1128/aem.61.7.2681-2687.1995

Bulmer, M. S., Bachelet, I., Raman, R., Rosengaus, R. B., & Sasisekharan, R. (2009). Targeting an antimicrobial effector function in insect immunity as a pest control strategy. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(31), 12652–12657. https://doi.org/10.1073/pnas.0904063106

Bulmer, M. S., Franco, B. A., & Fields, E. G. (2019). Subterranean Termite Social Alarm and Hygienic Responses to Fungal Pathogens. *Insects*, *10*(8), 240. https://doi.org/10.3390/insects10080240

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., Johnson, A., & Holmes, S. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Calusinska, M., Marynowska, M., Bertucci, M., Untereiner, B., Klimek, D., Goux, X., Sillam-Dussès, D., Gawron, P., Halder, R., Wilmes, P., Ferrer, P., Gerin, P. A., Roisin, Y., Philippe Delfosse, Delfosse, P., & Delfosse, P. (2020). Integrative omics analysis of the termite gut system adaptation to Miscanthus diet identifies lignocellulose degradation enzymes. *Communications Biology*, *3*(1), 1–12. https://doi.org/10.1038/s42003-020-1004-3

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J. S., Bealer, K., & Madden, T. L. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics*, *10*(1), 421–421. https://doi.org/10.1186/1471-2105-10-421

Campora, C. (2005). Potential relationship between the decline of mature *Casuarina equisitifolia* L. on Guam Naval Station and the presence of two invasive beetles, *Protaetia pryeri* (Janson) and *Protaetia orientalis* (Gory and Percheron) (Coleoptera: Scarabaediae): Preliminary report, 13 pp.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Jeremy E. Koenig, Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M. J., Bauer, M., Gormley, N. A., Gilbert, J. A., Smith, G., & Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*, *6*(8), 1621–1624. https://doi.org/10.1038/ismej.2012.8

Chao, A., Gotelli, N. J., Hsieh, T. C., Sander, E. L., H., K., Colwell, R. K., & Ellison, A. M. (2014). Rarefaction and extrapolation with Hill numbers: A framework for sampling and estimation in species diversity studies. *Ecological Monographs*, *84*(1), 45–67. https://doi.org/10.1890/13-0133.1

Chen, J., Setia, G., Sun, Q., & Husseneder, C. (2022). Dynamics of Gut Symbionts in Termite Kings and Queens During Early Colony Development. Unpublished.

Chhotani, O. B. (1997). *The Fauna of India and the Adjacent Countries: Isoptera (Termites) Volume II. Family Termitidae*.

ChongLu, Z., Yong, Z., Yu, C., Zhen, C., Qingbin, J., Pinyopusarerk, K., & Franche, C. (2010). *Potential Casuarina species and suitable techniques for the GGW*. 163–170. https://doi.org/10.4000/books.irdeditions.2123

Chou, J. H., Chen, W. M., Arun, A. B., & Young, C. C. (2007). *Trabulsiella odontotermitis* sp. nov., isolated from the gut of the termite *Odontotermes formosanus* Shiraki. *International journal of systematic and evolutionary microbiology*, *57*(Pt 4), 696–700. https://doi.org/10.1099/ijs.0.64632-0

Chouvenc, T., & Foley, J. R. (2018). Coptotermes gestroi (Wasmann) (Blattodea [Isoptera]: Rhinotermitidae), a threat to the southeastern Florida urban tree canopy. *The Florida Entomologist*, *101*(1), 79–90.

Chouvenc, T., & Su, N. Y. (2012). When subterranean termites challenge the rules of fungal epizootics. *PloS one*, *7*(3), e34484. https://doi.org/10.1371/journal.pone.0034484

Cilia, M., Tamborindeguy, C., Fish, T., Howe, K., Thannhauser, T. W., & Gray, S. (2011). Genetics coupled to quantitative intact proteomics links heritable aphid and endosymbiont protein expression to circulative polerovirus transmission. *Journal of virology*, *85*(5), 2148–2166. https://doi.org/10.1128/JVI.01504-10

Clarridge J. E., 3rd (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical microbiology reviews*, *17*(4), 840–862. https://doi.org/10.1128/CMR.17.4.840-862.2004

Cleveland, L. R. (1926). Symbiosis Among Animals with Special Reference to Termites and Their Intestinal Flagellates. *The Quarterly Review of Biology*, *1*(1), 51–60. https://doi.org/10.1086/394236

Cowie, R. H., Logan, J. W. M., & Wood, T. G. (1989). Termite (Isoptera) damage and control in tropical forestry with special reference to Africa and Indo-Malaysia: A review. *Bulletin of Entomological Research*, *79*(2), 173–184. https://doi.org/10.1017/s0007485300018150

Creer, S., Deiner, K., Frey, S. D., Porazinska, D. L., Taberlet, P., Thomas, W. K., Potter, C., & Bik, H. M. (2016). The ecologist's field guide to sequence-based identification of biodiversity. *Methods in Ecology and Evolution*, *7*(9), 1008–1018. https://doi.org/10.1111/2041-210x.12574

Cremer, S., Pull, C. D., & Fürst, M. A. (2018). Social Immunity: Emergence and Evolution of Colony-Level Disease Protection. *Annual Review of Entomology*, *63*(1), 105–123. https://doi.org/10.1146/annurev-ento-020117-043110

Cremer, Sylvia, Armitage, S. A. O., & Schmid-Hempel, P. (2007). Social Immunity. *Current Biology*, *17*(16), R693–R702. https://doi.org/10.1016/j.cub.2007.06.008

Denny, T. P. (2007). *Plant pathogenic Ralstonia species*. 573–644. https://doi.org/10.1007/978-1-4020-4538-7_16

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., & Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and environmental microbiology, 72(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Dhanarajan, G. (1969). *The termite fauna of Malaya and its economic significance*. *July 1969*, *32 (3)*(3), 274–278.

Dietrich, C., Köhler, T., & Brune, A. (2014). The Cockroach Origin of the Termite Gut Microbiota: Patterns in Bacterial Community Structure Reflect Major Evolutionary Events. *Applied and Environmental Microbiology*, *80*(7), 2261–2269. https://doi.org/10.1128/aem.04206-13

Dillon, R. J., & Dillon, V. M. (2004). The gut bacteria of insects: Nonpathogenic interactions. *Annual Review of Entomology*, *49*(1), 71–92. https://doi.org/10.1146/annurev.ento.49.061802.123416

Diouf, D., Sy, M.O., Gherbi, H., Bogusz, D., Franche, C. (2008) Casuarinaceae. In: Kole CR, Scorza R, Hall TC (eds) Compendium of transgenic crop plants: transgenic forest tree species, vol 9. Blackwell Publishing, Oxford, pp. 279–292

Diouf, M., Hervé, V., Mora, P., Robert, A., Robert, A., Frechault, S., Rouland-Lefèvre, C., & Miambi, E. (2018). Evidence from the gut microbiota of swarming alates of a vertical transmission of the bacterial symbionts in *Nasutitermes arborum* (Termitidae, Nasutitermitinae). *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, *111*(4), 573–587. https://doi.org/10.1007/s10482-017-0978-4

Diouf, M., Roy, V., Mora, P., Frechault, S., Lefebvre, T., Hervé, V., Rouland-Lefèvre, C., & Miambi, E. (2015). Profiling the Succession of Bacterial Communities throughout the Life Stages of a

Higher Termite *Nasutitermes arborum* (Termitidae, Nasutitermitinae) Using 16S rRNA Gene Pyrosequencing. *PLOS ONE*, *10*(10). https://doi.org/10.1371/journal.pone.0140014

Donovan, S. E., Eggleton, P., & Bignell, D. E. (2001). Gut content analysis and a new feeding group classification of termites. *Ecological Entomology*, *26*(4), 356–366. https://doi.org/10.1046/j.1365-2311.2001.00342.x

Drenth, A. (2004). Phytophthora Bud Rot in Areca Nut on Guam and the Northern Martianas. *CRC for Tropical Plant Protection, Indooroopilly Research Centre, Brisbane*. 27 pp.

Dröge, S., Rachel, R., Radek, R., & König, H. (2008). *Treponema isoptericolens* sp. Nov., a novel spirochaete from the hindgut of the termite *Incisitermes tabogae*. *International Journal of Systematic and Evolutionary Microbiology*, *58*(5), 1079–1083. https://doi.org/10.1099/ijs.0.64699-0

Dubnau, D., Smith, I., Smith, I., Morell, P., P Morell, & Marmur, J. (1965). Gene conservation in *Bacillus* species. I. Conserved genetic and nucleic acid base sequence homologies. *Proceedings of the National Academy of Sciences of the United States of America*, *54*(2), 491–498. https://doi.org/10.1073/pnas.54.2.491

Duke J. A., (1983). *Casuarina equisetifolia J.R. & G. Forst.,* Center for New Crops & Plant Products, Purdue University.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J., & Hurst, G. D. (2008). The diversity of reproductive parasites among arthropods: Wolbachia do not walk alone. *BMC biology*, *6*, 27. https://doi.org/10.1186/1741-7007-6-27

Edgar, R. C. (2016). UNOISE2: Improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv*, 081257. https://doi.org/10.1101/081257

Eggleton, P., & Tayasu, I. (2001). Feeding groups, lifetypes and the global ecology of termites. *Ecological Research*, *16*(5), 941–960. https://doi.org/10.1046/j.1440-1703.2001.00444.x

Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., Otto, G., Peluso, P., Rank, D. R., Baybayan, P., Bettman, B., Bibillo, A., Arkadiusz Bibillo, Bjornson, K., Chaudhuri, B., Frederick Christians, Christians, F. C., Cicero, R. L., Clark, S., … Turner, S. (2009). Real-Time DNA Sequencing from Single Polymerase Molecules. *Science*, *323*(5910), 133–138. https://doi.org/10.1126/science.1162986

Elevitch, C. R., & Wilkinson, K. M. (2000). *Agroforestry guides for Pacific Islands / Craig R. Elevitch and Kim M. Wilkinson, (editors)*. vii. https://doi-org.libezp.lib.lsu.edu/https://www.agroforestry.net/afg/book.html

Elfers, S.C. (1998). ELEMENT STEWARDSHIP ABSTRACT for Casuarina equisetifolia Australian Pine To the User: Element Stewardship Abstracts (ESAs) are prepared to provide The Nature

Conservancy's Stewardship staff and other land managers with current management-related information.

Embley, T. M., & Martin, W. (2006). Eukaryotic evolution, changes and challenges. *Nature*, *440*(7084), 623–630. https://doi.org/10.1038/nature04546

Escobar-Zepeda, A., de León, A. V.P., & Sanchez-Flores, A. (2015). The Road to Metagenomics: From Microbiology to DNA Sequencing Technologies and Bioinformatics. *Frontiers in Genetics*, *6*, 348–348. https://doi.org/10.3389/fgene.2015.00348

Estaki, M., Mehrbod Estaki, Jiang, L., Bokulich, N. A., McDonald, D., Gonzalez, A., Kosciolek, T., Martino, C., Zhu, Q., Birmingham, A., Vázquez-Baeza, Y., Dillon, M. R., Bolyen, E., Caporaso, J. G., & Knight, R. (2020). QIIME 2 Enables Comprehensive End-to-End Analysis of Diverse Microbiome Data and Comparative Studies with Publicly Available Data. *Current Protocols in Human Genetics*, *70*(1). https://doi.org/10.1002/cpbi.100

Evans, T. A., Forschler, B. T., & Grace, J. K. (2013). Biology of Invasive Termites: A Worldwide Review. *Annual Review of Entomology*, *58*(1), 455–474. https://doi.org/10.1146/annurev-ento-120811-153554

Faith, D. P., & Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation*, *61*(1), 1–10. https://doi.org/10.1016/0006-3207(92)91201-3

Fosberg, F. R., Sachet, M. H., & Oliver, R. (1979). A geographical checklist of the Micronesian dicotyledonae. *Micronesica; Journal of the University of Guam*, *15*(1/2), 41–295.

Fröhlich, J., Koustiane, C., Kämpfer, P., Rosselló-Móra, R., Valens, M., Berchtold, M., Kuhnigk, T., Hertel, H., Maheshwari, D. K., & König, H. (2007). Occurrence of rhizobia in the gut of the higher termite *Nasutitermes nigriceps*. *Systematic and Applied Microbiology*, *30*(1), 68–74. https://doi.org/10.1016/j.syapm.2006.03.001

Garrity, G. M., & Holt, J. G. (2015). The Road Map to the Manual. *Bergey's Manual of Systematics of Archaea and Bacteria*, 119–166. https://doi.org/10.1007/978-0-387-21609-6_15

Gedling, C. R., Smith, C. M., LeMoine, C. M. R., & Cassone, B. J. (2018). The Mexican bean beetle (*Epilachna varivestis*) regurgitome and insights into beetle-borne virus specificity. *PLOS ONE*, *13*(1). https://doi.org/10.1371/journal.pone.0192003

Getty, G. M., & Haverty, M. I. (1998). Consumption of sound and decayed ponderosa pine and Douglas-fir by Reticulitermes spp. (Isoptera: Rhinotermitidae) from northern California. *Journal of Economic Entomology*, *91*(3), 650–654. https://doi.org/10.1093/jee/91.3.650

Goodwin, S., Mcpherson, J. D., & McCombie, W. R. (2016). Coming of age: Ten years of next-generation sequencing technologies. *Nature Reviews Genetics*, *17*(6), 333–351. https://doi.org/10.1038/nrg.2016.49

Grasela, James J., & Moore, Aubrey. (2022). Preliminary detection of Wolbachia in the coconut rhinoceros beetle *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) from Guam, Palau, and Taiwan. Zenodo. https://doi.org/10.5281/zenodo.6672841

Gray, M. W., Sankoff, D., & Cedergren, R. (1984). On the evolutionary descent of organisms and organelles: A global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. *Nucleic Acids Research*, *12*(14), 5837–5852. https://doi.org/10.1093/nar/12.14.5837

Gutell, R. R., Gray, M. W., & Schnare, M. N. (1993). A compilation of large subunit (23S and 23S-like ribosomal RNA structures: 1993. *Nucleic Acids Research*, *21*(13), 3055–3074.

Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J., & Goodman, R. M. (1998). Molecular biological access to the chemistry of unknown soil microbes: A new frontier for natural products. *Chemistry & Biology*, *5*(10). https://doi.org/10.1016/s1074-5521(98)90108-9

Hänel, H. (1982). The life cycle of the insect pathogenic fungus *Metarhizium anisopliae* in the termite *Nasutitermes exitiosus*. *Mycopathologia*, *80*(3), 137–145. https://doi.org/10.1007/bf00437576

Harmsen, D. & Karch, H. (2004). 16S rDNA for diagnosing pathogens: A living tree. *ASM news*. 70. 19-24.

Harris, K. F., Maramorosch, K., & Maramorosch, K. (1980). *Vectors of Plant Pathogens*.

Harris, W. V. (1957). *An Introduction to Malayan Termites*. https://books.google.com/books?id=4ldizgEACAAJ

Hartley, C., Davidson, R. W., & Crandall, B. S. (1961). *Wetwood, bacteria, and increased pH in trees*. 2215, 1–34.

Hayward, A. C. (1991). Biology and Epidemiology of Bacterial Wilt Caused by *Pseudomonas Solanacearum*. *Annual Review of Phytopathology*, *29*(1), 65–87. https://doi.org/10.1146/annurev.py.29.090191.000433

He, S., Johnston, P. R., Kuropka, B., Lokatis, S., Weise, C., Plarre, R., Kunte, H.J., & McMahon, D. P. (2018). Termite soldiers contribute to social immunity by synthesizing potent oral secretions. *Insect Molecular Biology*, *27*(5), 564–576. https://doi.org/10.1111/imb.12499

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of The Royal Society B: Biological Sciences*, *270*(1512), 313–321. https://doi.org/10.1098/rspb.2002.2218

Heck, M. (2018). Insect Transmission of Plant Pathogens: A Systems Biology Perspective. *MSystems*, *3*(2). https://doi.org/10.1128/msystems.00168-17

Hongoh, Y., & Ohkuma, M. (2010). *Termite Gut Flagellates and Their Methanogenic and Eubacterial Symbionts*. 55–79. https://doi.org/10.1007/978-3-642-13615-3_5

Hongoh, Y., Deevong, P., Hattori, S., Inoue, T., Noda, S., Noparatnaraporn, N., Kudo, T., & Ohkuma, M. (2006). Phylogenetic Diversity, Localization, and Cell Morphologies of Members of the Candidate Phylum TG3 and a Subphylum in the Phylum Fibrobacteres, Recently Discovered Bacterial Groups Dominant in Termite Guts. *Applied and Environmental Microbiology*, *72*(10), 6780–6788. https://doi.org/10.1128/aem.00891-06

Hongoh, Y., Ekpornprasit, L., Inoue, T., Moriya, S., Trakulnaleamsai, S., Ohkuma, M., Noparatnaraporn, N., & Kudo, T. (2006). Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. *Molecular ecology*, *15*(2), 505–516. https://doi.org/10.1111/j.1365-294X.2005.02795.x

Hongoh, Y., Sharma, V. K., Prakash, T., Noda, S., Toh, H., Taylor, T. D., Kudo, T., Sakaki, Y., Sakaki, Y., Toyoda, A., Hattori, M., & Ohkuma, M. (2008). Genome of an Endosymbiont Coupling N2 Fixation to Cellulolysis Within Protist Cells in Termite Gut. *Science*, *322*(5904), 1108–1109. https://doi.org/10.1126/science.1165578

Houpikian, P., & Raoult, D. (2002). Traditional and molecular techniques for the study of emerging bacterial diseases: One laboratory's perspective. *Emerging Infectious Diseases*, *8*(2), 122–131. https://doi.org/10.3201/eid0802.010141

Hromada, C. J. (1970). Formosan termites on Guam. A massive control job for Terminix. *Pest Control* 38: 10–25.

Hsieh, T. C., H., K., & Chao, A. (2016). INEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). *Methods in Ecology and Evolution*, *7*(12), 1451–1456. https://doi.org/10.1111/2041-210x.12613

Hunt, J. H., & Nalepa, C. A. (Eds.). (1994). *Nourishment and evolution in insect societies*. Westview Press, Inc.

Husseneder, C. (2010). Symbiosis in Subterranean Termites: A Review of Insights From Molecular Studies. *Environmental Entomology*, *39*(2), 378–388. https://doi.org/10.1603/en09006

Husseneder, C., & Grace, J. K. (2005). Genetically engineered termite gut bacteria (*Enterobacter cloacae*) deliver and spread foreign genes in termite colonies. *Applied Microbiology and Biotechnology*, *68*(3), 360–367. https://doi.org/10.1007/s00253-005-1914-5

Husseneder, C., Berestecky, J. M., J. K. Grace, Grace, J. K., & J. K. Grace. (2009). Changes in Composition of Culturable Bacteria Community in the Gut of the Formosan Subterranean Termite Depending on Rearing Conditions of the Host. *Annals of The Entomological Society of America*, *102*(3), 498–507. https://doi.org/10.1603/008.102.0321

Husseneder, C., Ho, H. Y., & Blackwell, M. (2010). Comparison of the bacterial symbiont composition of the formosan subterranean termite from its native and introduced range. *The open microbiology journal*, *4*, 53–66. https://doi.org/10.2174/1874285801004010053

Husseneder, C., Wise, B. R., & Higashiguchi, D. T. (2005). Microbial diversity in the termite gut: a complementary approach combining culture and culture-independent techniques. *Fifth International Conference on Urban Pests, Singapore, 11-13 July 2005*, 189–195.

Huyen, T., Nguyen, T. T., Nguyen, T. N., Le, Q. G., Nguyen, C., Nguyen, C. T., Kimura, K., & Truong, N. H. (2014). Mining biomass-degrading genes through Illumina-based de novo sequencing and metagenomic analysis of free-living bacteria in the gut of the lower termite *Coptotermes gestroi* harvested in Vietnam. *Journal of Bioscience and Bioengineering*, *118*(6), 665–671. https://doi.org/10.1016/j.jbiosc.2014.05.010

Inagaki, T., Yanagihara, S., Fuchikawa, T., Taro Fuchikawa, Taro Fuchikawa, & Matsuura, K. (2020). Gut microbial pulse provides nutrition for parental provisioning in incipient termite colonies. *Behavioral Ecology and Sociobiology*, *74*(6), 1–11. https://doi.org/10.1007/s00265-020-02843-y

Inoue, J., Saita, K., Kudo, T., Sadaharu Ui, Ui, S., & Ohkuma, M. (2007). Hydrogen production by termite gut protists: Characterization of iron hydrogenases of Parabasalian symbionts of the termite *Coptotermes formosanus*. *Eukaryotic Cell*, *6*(10), 1925–1932. https://doi.org/10.1128/ec.00251-07

Inoue, T., Moriya, S., Ohkuma, M., & Kudo, T. (2005). Molecular cloning and characterization of a cellulase gene from a symbiotic protist of the lower termite, *Coptotermes formosanus*. *Gene*, *349*, 67–75. https://doi.org/10.1016/j.gene.2004.11.048

Inward, D. J. G., Beccaloni, G. W., & Eggleton, P. (2007a). Death of an order: A comprehensive molecular phylogenetic study confirms that termites are eusocial cockroaches. *Biology Letters*, *3*(3), 331–335. https://doi.org/10.1098/rsbl.2007.0102

Inward, D. J. G., Vogler, A. P., & Eggleton, P. (2007b). A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. *Molecular Phylogenetics and Evolution*, *44*(3), 953–967. https://doi.org/10.1016/j.ympev.2007.05.014

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology*, *45*(9), 2761–2764. https://doi.org/10.1128/JCM.01228-07

Järvinen, A. K., Laakso, S., Piiparinen, P., Aittakorpi, A., Lindfors, M., Huopaniemi, L., Piiparinen, H., & Mäki, M. (2009). Rapid identification of bacterial pathogens using a PCR- and microarray-based assay. *BMC microbiology*, *9*, 161. https://doi.org/10.1186/1471-2180-9-161

Jasso-Selles, D. E., De Martini, F., Velenovsky, J. F., Joseph F. Velenovsky, Mee, E. D., Montoya, S.

J., Hileman, J. T., Garcia, M. D., Su, N.-Y., Chouvenc, T., & Gile, G. H. (2020). The Complete Protist Symbiont Communities of *Coptotermes formosanus* and *Coptotermes gestroi*: Morphological and Molecular Characterization of Five New Species. *Journal of Eukaryotic Microbiology*, *67*(6), 626–641. https://doi.org/10.1111/jeu.12815

Jeremic, D., Cooper, P. A., & Srinivasan, U. (2004). Comparative analysis of balsam fir wetwood, heartwood, and sapwood properties. *Canadian Journal of Forest Research*, *34*(6), 1241–1250. https://doi.org/10.1139/x03-287

Kakkar, G., Chouvenc, T., Osbrink, W. L. A., & Su, N.Y. (2016). Temporal Assessment of Molting in Workers of Formosan Subterranean Termites (Isoptera: Rhinotermitidae). *Journal of Economic Entomology*, *109*(5), 2175–2181. https://doi.org/10.1093/jee/tow180

Kamble, A., Sawant, S., & Singh, H. (2020). *16S ribosomal RNA gene-based metagenomics: A review*. *7*(1), 5. https://doi.org/10.4103/bmrj.bmrj_4_20

Katoh, K., Kazuharu Misawa, Misawa, K., Kuma, K., Miyata, T., Miyata, T., & Takashi Miyata. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, *30*(14), 3059–3066. https://doi.org/10.1093/nar/gkf436

Katzin, L. I., & Kirby, H. (1939). The Relative Weights of Termites and Their Protozoa. *Journal of Parasitology*, *25*(5), 444. https://doi.org/10.2307/3272319

Keast, D., Leonie G. Walsh, & Walsh, L. G. (1979). Passage and Survival of Chlamydospores of *Phytophthora cinnamomi* Rands, the Causal Agent of Forest Dieback Disease, Through the Gastrointestinal Tracts of Termites and Wild Birds. *Applied and Environmental Microbiology*, *37*(3), 661–664. https://doi.org/10.1128/aem.37.3.661-664.1979

Keegan, K. P., Glass, E. M., & Meyer, F. (2016). MG-RAST, a Metagenomics Service for Analysis of Microbial Community Structure and Function. *Methods of Molecular Biology*, *1399*, 207–233. https://doi.org/10.1007/978-1-4939-3369-3_13

Kelman, A. (1954). The relationship of pathogenicity of Pseudomonas solanacearum to colony appearance in a tetrazolium medium. *Phytopathology*, *44*(12).

King, J. H. P., Mahadi, N. M., Bong, C. F. J., Ong, K. H., & Hassan, O. (2014). Bacterial microbiome of *Coptotermes curvignathus* (Isoptera: Rhinotermitidae) reflects the coevolution of species and dietary pattern. *Insect Science*, *21*(5), 584–596. https://doi.org/10.1111/1744-7917.12061

Kircher, M., & Kelso, J. (2010). High-throughput DNA sequencing – concepts and limitations. *BioEssays*, *32*(6), 524–536. https://doi.org/10.1002/bies.200900181

Kirton, L. G. (1995). *Habitat and Host Associations of Coptotermes (Isoptera: Rhinotermitidae) in Peninsular Malaysia*. University of London. https://books.google.com/books?id=1vjbtgAACAAJ

Kirton, L. G., & Brown, V. K. (2003). The taxonomic status of pest species of Coptotermes in Southeast Asia: Resolving the paradox in the pest status of the termites, *Coptotermes gestroi*, *C. havilandi* and *C. travians* (Isoptera: Rhinotermitidae). *Sociobiology*, *42*(1), 43–63.

Kitade, O., & Matsumoto, T. (1998). Characteristics of the symbiotic flagellate composition within the termite family Rhinotermitidae (Isoptera). *Symbiosis*, *25*, 271–278.

Kofoid, C.A. and Bowe, E.E. (1934). A standard biological method of testing the termite resistivity of cellulose-containing materials. pp. 517-545. In C.A. Kofoid [ed.], Termites and termite control. University of California Press, Berkeley.

Köhler, T., Dietrich, C., Scheffrahn, R. H., & Brune, A. (2012). High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites (*Nasutitermes* spp.). *Applied and Environmental Microbiology*, *78*(13), 4691–4701. https://doi.org/10.1128/aem.00683-12

Kono, N., & Arakawa, K. (2019). Nanopore sequencing: Review of potential applications in functional genomics. *Development Growth & Differentiation*, *61*(5), 316–326. https://doi.org/10.1111/dgd.12608

Koskella, B., Meaden, S., Crowther, W. J., Leimu, R., Roche, B., & Metcalf, C. J. E. (2017). A signature of tree health? Shifts in the microbiome and the ecological drivers of horse chestnut bleeding canker disease. *New Phytologist*, *215*(2), 737–746. https://doi.org/10.1111/nph.14560

Kramm, K. R., West, D. F., & Rockenbach, P. G. (1982). Termite pathogens: transfer of the entomopathogen *Metarhizium anisopliae* between Reticulitermes sp. termites. *Journal of Invertebrate Pathology*, *40*(1), 1-6.

Krishna, K., & Weesner, F. M. (1969). *Biology of termites, Vol. I.*

Krishna, K., Grimaldi, D. A., Krishna, V., & Engel, M. S. (2013). Treatise on the Isoptera of the World. *Bulletin of the American Museum of Natural History*, *377*(377), 1–2704. https://doi.org/10.1206/377.6

Kruskal, W., & Wallis, W. A. (1952). Use of Ranks in One-Criterion Variance Analysis. *Journal of the American Statistical Association*, *47*(260), 583–621. https://doi.org/10.1080/01621459.1952.10483441

Kubota, R., Schell, M.A., Peckham, G.D. *et al. In silico* genomic subtraction guides development of highly accurate, DNA-based diagnostics for *Ralstonia solanacearum* race 3 biovar 2 and blood disease bacterium. *J Gen Plant Pathol* **77**, 182–193 (2011). https://doi.org/10.1007/s10327-011-0305-2

Kumar, K. R., Cowley, M. J., & Davis, R. L. (2019). Next-Generation Sequencing and Emerging Technologies. *Seminars in thrombosis and hemostasis*, *45*(7), 661–673.

https://doi.org/10.1055/s-0039-1688446

La Fage, J. P. (1987). Practical considerations of the Formosan subterranean termite in Louisiana: a 30-year-old problem. *Research Extension Series - College of Tropical Agriculture and Human Resources, University of Hawaii, Cooperative Extension Service*, *Oct 1987. (083)*(083), 37–42.

Lai, P. Y. (1977). *Biology and ecology of the Formosan subterranean termite, Coptotermes formosanus, and its susceptibility to the entomogenous fungi, Beauveria bassiana and Metarrhizium anisopliae.* [Doctoral dissertation, University of Hawaii].

Lane, D. J. (1991). *16S/23S rRNA sequencing*. 115–175.

Lavelle, P., Lattaud, C., Trigo, D., D. Trigo, & Barois, I. (1995). Mutualism and biodiversity in soils. *Plant and Soil*, *170*(1), 23–33. https://doi.org/10.1007/978-94-011-0479-1_2

Leadbetter, J. R., & Breznak, J. A. (1996). Physiological ecology of *Methanobrevibacter cuticularis* sp. Nov. And *Methanobrevibacter curvatus* sp. Nov., isolated from the hindgut of the termite *Reticulitermes flavipes*. *Applied and Environmental Microbiology*, *62*(10), 3620–3631. https://doi.org/10.1128/aem.62.10.3620-3631.1996

Leadbetter, J. R., Crosby, L. D., & Breznak, J. A. (1998). *Methanobrevibacter filiformis* sp. Nov., a filamentous methanogen from termite hindguts. *Archives of Microbiology*, *169*(4), 287–292. https://doi.org/10.1007/s002030050574

Leasi, F., Sevigny, J. L., Laflamme, E. M., Artois, T., Casu, M., Curini-Galletti, M., Alberto de Jesus Navarrete, Alberto de Jesus Navarrete, de Jesús Navarrete, A., Di Domenico, M., Goetz, F. E., Hall, J. A., Hochberg, R., Jörger, K. M., Jondelius, U., Todaro, M. A., Wirshing, H. H., Norenburg, J. L., & Thomas, W. K. (2018). Biodiversity estimates and ecological interpretations of meiofaunal communities are biased by the taxonomic approach. *Communications Biology*, *1*(1), 112–112. https://doi.org/10.1038/s42003-018-0119-2

Len, M., T.L. Amburgey, D. Zi-Rong, J.K. Mauldin, A.F. Preston, D. Rudolph, and E.R. Williams. (1991). Interlaboratory Studies on Termite-Wood Decay Fungi Associations .2. Response of Termites to Gloeophyllum-Trabeum Grown on Different Species of Wood (Isoptera, Mastotermitidae, Termopsidae, Rhinotermitidae, Termitidae). *Sociobiology*, *18*(2), 203–254.

Li, S., Liu, Y., Wang, J., Yang, L., Zhang, S., Xu, C., & Ding, W. (2017). Soil Acidification Aggravates the Occurrence of Bacterial Wilt in South China. *Frontiers in Microbiology*, *8*, 703–703. https://doi.org/10.3389/fmicb.2017.00703

Li, S., Xu, C., Wang, J. *et al.* Cinnamic, myristic and fumaric acids in tobacco root exudates induce the infection of plants by *Ralstonia solanacearum* . *Plant Soil* **412**, 381–395 (2017). https://doi.org/10.1007/s11104-016-3060-5

Light, S. F. (1946). *Insects of Guam*. Bernice P. Bishop Museum.

Lijuan, S., Yang, L., Huang, S., Su, X., Li, Y., Yan Li, Li, Y., Li, Y., Wang, F., Entao Wang, Wang, E. T., Kang, N., Jian Xu, Jian Xu, Xu, J., Andong Song, Song, A., Andong Song, & Andong Song. (2016). Comparative Gut Microbiomes of Four Species Representing the Higher and the Lower Termites. *Journal of Insect Science*, *16*(1), 97. https://doi.org/10.1093/jisesa/iew081

Lin, H., & Peddada, S. D. (2020). Analysis of compositions of microbiomes with bias correction. *Nature Communications*, *11*(1), 3514. https://doi.org/10.1038/s41467-020-17041-7

Liu, L., Li, G., Sun, P., Lei, C., & Huang, Q. (2015). Experimental verification and molecular basis of active immunization against fungal pathogens in termites. *Scientific reports*, *5*(1), 1-12.

Lo, N., and Eggleton, P. (2011). Termite phylogenetics and cocladogenesis with symbionts. In: Biology of termites: a modern synthesis (eds) Bignell, D.E., Roisin, Y., and Lo, N. Springer, Dorderecht. pp. 27-50.

Lo, N., Tokuda, G., Watanabe, H., Rose, H. A., Slaytor, M., Maekawa, K., Bandi, C., & Noda, H. (2000). Evidence from multiple gene sequences indicates that termites evolved from wood-feeding cockroaches. *Current Biology*, *10*(13), 801–804. https://doi.org/10.1016/s0960-9822(00)00561-3

Lozupone, C. A., & Knight, R. (2005). UniFrac: A New Phylogenetic Method for Comparing Microbial Communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/aem.71.12.8228-8235.2005

Makonde, H. M., Boga, H. I., Osiemo, Z., Mwirichia, R., Stielow, J. B., Göker, M., & Klenk, H.-P. (2013). Diversity of Termitomyces associated with fungus-farming termites assessed by cultural and culture-independent methods. *PLOS ONE*, *8*(2). https://doi.org/10.1371/journal.pone.0056464

Marchán, F. J. (1964). The lignin, ash, and protein content of some neo-tropical woods. *Caribbean Forester* 7: 121-35.

Mardis, E. R. (2008). Next-Generation DNA Sequencing Methods. *Annual Review of Genomics and Human Genetics*, *9*(1), 387–402. https://doi.org/10.1146/annurev.genom.9.081307.164359

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., Berka, J., Jan Berka, Michael S. Braverman, Braverman, M. S., Braverman, M. S., Chen, Y.-J., Chen, Z., Dewell, S., Du, L., Fierro, J. M., Gomes, X. V., Godwin, B. C., He, W., … Rothberg, J. M. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature*, *437*(7057), 376–380. https://doi.org/10.1038/nature03959

Marshall, , Joe T. (1949). The Endemic Avifauna of Saipan, Tinian, Guam and Palau. *The Condor*, *51*(5), 200–221. https://doi-org.libezp.lib.lsu.edu/10.2307/1364563

Marynowska, M., Goux, X., Sillam-Dussès, D., Rouland-Lefèvre, C., Roisin, Y., Delfosse, P.,

Calusinska, M., & Magdalena Calusinska. (2017). Optimization of a metatranscriptomic approach to study the lignocellulolytic potential of the higher termite gut microbiome. *BMC Genomics*, *18*(1), 1–14. https://doi.org/10.1186/s12864-017-4076-9

Mersha, Z,. Schlub, R.L., Moore, A. (2009). The state of ironwood (*Casuarina equisetifolia* subsp. *equisetifolia*) decline on the pacific island of Guam. Phytopathology 99:S85

Mersha, Z., Schlub, R.L., Spaine, P., Smith, J., Nelson, S., Moore, A., McConnell, J., Pinyopusarerk, K., Nandwani, D., Badilles, A. (2010). In Fungal associations and factors in *Casuarina equisetifolia* decline, 9th International Mycological Congress, Edinburgh, UK:IMC9:P3.268

Mersha, Z., Schlub, R.L., Spaine, P.O., Smith, J.A., Nelson, S.C. (2010) Visual and quantitative characterization of ironwood tree (*Casuarina equisetifolia*) decline on Guam. *Phytopathology* 100:S82

Messer, J. S., & Chang, E. B. (2018). *Microbial Physiology of the Digestive Tract and Its Role in Inflammatory Bowel Diseases*. 795–810. https://doi.org/10.1016/b978-0-12-809954-4.00036-0

Mevers, E., Chouvenc, T., Su, N.Y., & Clardy, J. (2017). Chemical Interaction among Termite-Associated Microbes. *Journal of Chemical Ecology*, *43*(11), 1078–1085. https://doi.org/10.1007/s10886-017-0900-6

Mikaelyan, A., Dietrich, C., Köhler, T., Poulsen, M., Sillam-Dussès, D., & Brune, A. (2015). Diet is the primary determinant of bacterial community structure in the guts of higher termites. *Molecular Ecology*, *24*(20), 5284–5295. https://doi.org/10.1111/mec.13376

Mikaelyan, A., Meuser, K., & Brune, A. (2017). Microenvironmental heterogeneity of gut compartments drives bacterial community structure in wood- and humus-feeding higher termites. *FEMS Microbiology Ecology*, *93*(1). https://doi.org/10.1093/femsec/fiw210

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., J. Y. Poulsen, Poulsen, J. Y., Sato, K., Keiichi Sato, Minamoto, T., Yamamoto, S., Yamanaka, H., Yamanaka, H., Araki, H., Kondoh, M., & Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. *Royal Society Open Science*, *2*(7), 150088–150088. https://doi.org/10.1098/rsos.150088

Miyata, R., Noda, N., Tamaki, H., Kinjyo, K., Aoyagi, H., Uchiyama, H., & Tanaka, H. (2007). Influence of Feed Components on Symbiotic Bacterial Community Structure in the Gut of the Wood-Feeding Higher Termite *Nasutitermes takasagoensis*. *Bioscience, Biotechnology, and Biochemistry*, *71*(5), 1244–1251. https://doi.org/10.1271/bbb.60672

Moore, B. P. (1969). *13 – Biochemical Studies in Termites*. 407–432. https://doi.org/10.1016/b978-0-12-395529-6.50017-9

Moore, P. H. (1973). Composition of a limestone forest community on Guam. *Micronesica*, *July*

1973, *9 (1)*(1), 45–58.

Moriya, S., Inoue, T., Ohkuma, M., Yaovapa, T., Johjima, T., Suwanarit, P., Sangwanit, U., Vongkaluang, C., Noparatnaraporn, N., & Kudo, T. (2005). Fungal Community Analysis of Fungus Gardens in Termite Nests. *Microbes and Environments*, *20*(4), 243–252. https://doi.org/10.1264/jsme2.20.243

Morris, H. R., Houlden, H., & Polke, J. M. (2021). Whole-genome sequencing. *Practical Neurology*, *21*(4), 322–327. https://doi.org/10.1136/practneurol-2020-002561

Morton, J. F., & Collectanea, M. (1980). *The Australian Pine Or Beefwood (Casuarina equisetifolia L), An Invasive "Weed" Tree In Florida*.

Nakashima, K., Watanabe, H., Saitoh, H., Tokuda, G., & Azuma, J. I. (2002). Dual cellulose-digesting system of the wood-feeding termite, Coptotermes formosanus Shiraki. *Insect biochemistry and molecular biology*, *32*(7), 777–784. https://doi.org/10.1016/s0965-1748(01)00160-6

Ni, J., & Tokuda, G. (2013). Lignocellulose-degrading enzymes from termites and their symbiotic microbiota. *Biotechnology Advances*, *31*(6), 838–850. https://doi.org/10.1016/j.biotechadv.2013.04.005

Noda, S., Iida, T., Kitade, O., Nakajima, H., Kudo, T., & Ohkuma, M. (2005). Endosymbiotic Bacteroidales bacteria of the flagellated protist *Pseudotrichonympha grassii* in the gut of the termite *Coptotermes formosanus*. *Applied and Environmental Microbiology*, *71*(12), 8811–8817. https://doi.org/10.1128/aem.71.12.8811-8817.2005

Noda, S., Inoue, T., Hongoh, Y., Kawai, M., Nalepa, C. A., Vongkaluang, C., Kudo, T., & Ohkuma, M. (2006). Identification and characterization of ectosymbionts of distinct lineages in Bacteroidales attached to flagellated protists in the gut of termites and a wood-feeding cockroach. *Environmental Microbiology*, *8*(1), 11–20. https://doi.org/10.1111/j.1462-2920.2005.00860.x

Noda, S., Kitade, O., Inoue, T., Inoue, T., Kawai, M., Kawai, M., Kanuka, M., Hiroshima, K., Hongoh, Y., Constantino, R., Uys, V., Zhong, J., Kudo, T., & Ohkuma, M. (2007). Cospeciation in the triplex symbiosis of termite gut protists *(Pseudotrichonympha* spp.), their hosts, and their bacterial endosymbionts. *Molecular Ecology*, *16*(6), 1257–1266. https://doi.org/10.1111/j.1365-294x.2006.03219.x

Oberpaul, M., Zumkeller, C. M., Culver, T., Spohn, M., Spohn, M., Mihajlovic, S., Leis, B., Glaeser, S. P., Plarre, R., McMahon, D. P., Hammann, P., Schäberle, T. F., Glaeser, J., & Vilcinskas, A. (2020). High-Throughput Cultivation for the Selective Isolation of Acidobacteria From Termite Nests. *Frontiers in Microbiology*, *11*, 597628–597628. https://doi.org/10.3389/fmicb.2020.597628

Ohkuma, M. (2003). Termite symbiotic systems: Efficient bio-recycling of lignocellulose. *Applied Microbiology and Biotechnology*, *61*(1), 1–9. https://doi.org/10.1007/s00253-002-1189-z

Ohkuma, M., & Brune, A. (2010). *Diversity, Structure, and Evolution of the Termite Gut Microbial Community*. 413–438. https://doi.org/10.1007/978-90-481-3977-4_15

Ohkuma, M., Noda, S., Usami, R., Horikoshi, K., & Kudo, T. (1996). Diversity of Nitrogen Fixation Genes in the Symbiotic Intestinal Microflora of the Termite Reticulitermes speratus. *Applied and Environmental Microbiology*, *62*(8), 2747–2752. https://doi.org/10.1128/aem.62.8.2747-2752.1996

Oksanen, J., & Oksanen, J. (2007). *Vegan: Community Ecology Package. R package version 1.8-5*.

Otani, S., Mikaelyan, A., Nobre, T., Hansen, L. H., Koné, N. A., Sørensen, S. J., Duur K. Aanen, Duur K. Aanen, Aanen, D. K., Boomsma, J. J., Brune, A., & Poulsen, M. (2014). Identifying the core microbial community in the gut of fungus-growing termites. *Molecular Ecology*, *23*(18), 4631–4644. https://doi.org/10.1111/mec.12874

Pan, F., Pan, F., Pan, F., Xu, X., Zhang, L.-L., Luo, H.-J., Chen, Y., Long, L., Lin Long, Wang, X., Zhuang, P.-T., Li, E.-M., Li, E.-M., Xu, L., Xu, L.-Y., & Xu, L.-Y. (2020). Dietary riboflavin deficiency induces genomic instability of esophageal squamous cells that is associated with gut microbiota dysbiosis in rats. *Food & Function*, *11*(12), 10979–10979. https://doi.org/10.1039/d0fo01944e

Parada, A. E., David M. Needham, Needham, D. M., Needham, D., & Fuhrman, J. A. (2016). Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology*, *18*(5), 1403–1414. https://doi.org/10.1111/1462-2920.13023

Park, J.S., Husseneder, C., Robert L Schlub, & Schlub, R. L. (2019). Morphological and Molecular Species Identification of Termites Attacking Ironwood Trees, *Casuarina equisetifolia* (Fagales: Casuarinaceae), in Guam. *Journal of Economic Entomology*, *112*(4), 1902–1911. https://doi.org/10.1093/jee/toz097

Patel, J. B. (2001). 16s rRNA Gene Sequencing for Bacterial Pathogen Identification in The Clinical Laboratory. *Molecular Diagnosis*, *6*(4), 313–321. https://doi.org/10.1054/modi.2001.29158

Patwardhan, A., Ray, S., & Roy, A. (2014). Molecular markers in phylogenetic studies-a review. *Journal of Phylogenetics & Evolutionary Biology*, *2014*.

Paudel, Sujan (2020). *Evolutionary relationships and molecular diagnostics of Ralstonia solanacearum species complex associated with declining ironwood trees in Guam* [Master's thesis, University]. http://hdl.handle.net/10125/70339

Paul, K., Nonoh, J., Mikulski, L., & Brune, A. (2012). "Methanoplasmatales," Thermoplasmatales-

related archaea in termite guts and other environments, are the seventh order of methanogens. *Applied and Environmental Microbiology*, *78*(23), 8245–8253. https://doi.org/10.1128/aem.02193-12

Pearce, R. B. (1996). Antimicrobial defences in the wood of living trees. *New Phytologist*, *132*(2), 203–233. https://doi.org/10.1111/j.1469-8137.1996.tb01842.x

Perilla-Henao, L. M., & Casteel, C. L. (2016). Vector-Borne Bacterial Plant Pathogens: Interactions with Hemipteran Insects and Plants. *Frontiers in Plant Science*, *7*, 1163–1163. https://doi.org/10.3389/fpls.2016.01163

Peterson, B. F., & Scharf, M. E. (2016). Metatranscriptome analysis reveals bacterial symbiont contributions to lower termite physiology and potential immune functions. *BMC Genomics*, *17*(1), 772–772. https://doi.org/10.1186/s12864-016-3126-z

Peterson, B. F., Stewart, H. L., & Scharf, M. E. (2015). Quantification of symbiotic contributions to lower termite lignocellulose digestion using antimicrobial treatments. *Insect Biochemistry and Molecular Biology*, *59*, 80–88. https://doi.org/10.1016/j.ibmb.2015.02.009

Pielou, E. C. (1966). The measurement of diversity in different types of biological collections. *Journal of Theoretical Biology*, *13*, 131–144. https://doi-org.libezp.lib.lsu.edu/10.1016/0022-5193(66)90013-0

Pinyopusarerk, K., & House, A. P. N. (1993). *Casuarina: An annotated bibliography of C. equisetifolia, C. junghuhniana and C. oligodon*.

Poulsen, M., Hu, H., Li, C., Chen, Z., Xu, L., Otani, S., Nygaard, S., Nobre, T., Klaubauf, S., Schindler, P. M., Hauser, F., Pan, H., Yang, Z., Sonnenberg, A. S. M., de Beer, Z. W., Zhang, Y., Wingfield, M. J., Grimmelikhuijzen, C. J. P., de Vries, R. P., … Zhang, G. (2014). Complementary symbiont contributions to plant decomposition in a fungus-farming termite. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(40), 14500–14505. https://doi.org/10.1073/pnas.1319718111

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2—Approximately maximum-likelihood trees for large alignments. *PLOS ONE*, *5*(3). https://doi.org/10.1371/journal.pone.0009490

Purdy, K. J. (2007). The distribution and diversity of Euryarchaeota in termite guts. *Advances in Applied Microbiology*, *62*, 63–80. https://doi.org/10.1016/s0065-2164(07)62003-6

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Gloeckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596.

Quast, C., Pruesse, E., Yilmaz, P., Jan Gerken, Gerken, J. E., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: Improved data

processing and web-based tools. *Nucleic Acids Research*, *41*, 590–596. https://doi.org/10.1093/nar/gks1219

Radek, R. (1999). Flagellates, bacteria, and fungi associated with termites: diversity and function in nutrition–a review. *Ecotropica*, *5*(1), 183-196.

Rahman, N. A., Parks, D. H., Willner, D., Engelbrektson, A., Goffredi, S. K., Warnecke, F., Scheffrahn, R. H., & Hugenholtz, P. (2015). A molecular survey of Australian and North American termite genera indicates that vertical inheritance is the primary force shaping termite gut microbiomes. *Microbiome*, *3*(1), 5–5. https://doi.org/10.1186/s40168-015-0067-8

Raina, A. K., Park, Y. I., & Gelman, D. B. (2008). Molting in workers of the Formosan subterranean termite *Coptotermes formosanus*. *Journal of Insect Physiology*, *54*(1), 155–161. https://doi.org/10.1016/j.jinsphys.2007.08.015

Ransom-Jones, E., Jones, D. R., Jones, D. L., McCarthy, A. J., & McDonald, J. E. (2012). The Fibrobacteres: An Important Phylum of Cellulose-Degrading Bacteria. *Microbial Ecology*, *63*(2), 267–281. https://doi.org/10.1007/s00248-011-9998-1

Rashid, M. U., Mamoon Rashid, Rashid, M., & Stingl, U. (2015). Contemporary molecular tools in microbial ecology and their application to advancing biotechnology. *Biotechnology Advances*, *33*(8), 1755–1773. https://doi.org/10.1016/j.biotechadv.2015.09.005

Relman, D. A. (1999). The Search for Unrecognized Pathogens. *Science*, *284*(5418), 1308–1310. https://doi.org/10.1126/science.284.5418.1308

Rosengaus, R. B., Traniello, J. F. A., Chen, T., Brown, J. J., & Karp, R. D. (1999). Immunity in a Social Insect. *Naturwissenschaften*, *86*(12), 588–591. https://doi.org/10.1007/s001140050679

Rosengaus, R. B., Traniello, J. F., & Bulmer, M. S. (2010). Ecology, behavior and evolution of disease resistance in termites. In *Biology of termites: a modern synthesis* (pp. 165-191). Springer, Dordrecht.

Rossmassler, K., Dietrich, C., Thompson, C. L., Mikaelyan, A., Nonoh, J., Scheffrahn, R. H., Sillam-Dussès, D., & Brune, A. (2015). Metagenomic analysis of the microbiota in the highly compartmented hindguts of six wood- or soil-feeding higher termites. *Microbiome*, *3*(1), 56–56. https://doi.org/10.1186/s40168-015-0118-1

Rothberg, J. M., Jonathan M. Rothberg, Jonathan M. Rothberg, Hinz, W., Rearick, T., Schultz, J., Mileski, W. J., Mel Davey, Davey, M., Leamon, J. H., Johnson, K. L., Milgrew, M. J., Mark James Milgrew, Edwards, M. D., Hoon, J., Jeremy Hoon, Jan F. Simons, Simons, J. F., Marran, D., … Bustillo, J. (2011). An integrated semiconductor device enabling non-optical genome sequencing. *Nature*, *475*(7356), 348–352. https://doi.org/10.1038/nature10242

Rottman, G. L., & Gerrard, H. (2004). *Guam 1941 & 1944: Loss and Reconquest*.

Safford, W. E. (1905). The useful plants of the island of guam. Govt. Print. Off.

Sandmann, S., de Graaf, A. O., van der Reijden, B. A., Jansen, J. H., & Dugas, M. (2017). GLM-based optimization of NGS data analysis: A case study of Roche 454, Ion Torrent PGM and Illumina NextSeq sequencing data. *PloS one*, *12*(2), e0171983. https://doi.org/10.1371/journal.pone.0171983

Sanger, F., Nicklen, S., & Coulson, A. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(12), 5463–5467. https://doi.org/10.1073/pnas.74.12.5463

Santos, A., van Aerle, R., Barrientos, L., & Martinez-Urtaza, J. (2020). Computational methods for 16S metabarcoding studies using Nanopore sequencing data. *Computational and structural biotechnology journal*, *18*, 296–305. https://doi.org/10.1016/j.csbj.2020.01.005

Sapountzis, P., Gruntjes, T., Otani, S., Estevez, J., da Costa, R. R., Plunkett, G., Perna, N. T., & Poulsen, M. (2015). The enterobacterium *Trabulsiella odontotermitis* presents novel adaptations related to its association with fungus-growing termites. *Applied and Environmental Microbiology*, *81*(19), 6577–6588. https://doi.org/10.1128/aem.01844-15

Scheffrahn, R. H., & Huchet, J.B. (2010). A new termite species (Isoptera: Termitidae: Termitinae: Amitermes) and first record of a Subterranean Termite from the Coastal Desert of South America. *Zootaxa*, *2328*(1), 65–68. https://doi.org/10.11646/zootaxa.2328.1.3

Scheffrahn, R. H., Robbins, W. P., Busey, P., Su, N.Y., & Mueller, R. K. (1993). Evaluation of a novel, hand-held, acoustic emissions detector to monitor termites (Isoptera: Kalotermitidae, Rhinotermitidae) in wood. *Journal of Economic Entomology*, *86*(6), 1720–1729. https://doi.org/10.1093/jee/86.6.1720

Scheffrahn, R. H., Su, N.Y., & Diehl, B. (1990). Native, Introduced, and Structure-Infesting Termites of the Turks and Caicos Islands, B.W.I. (Isoptera: Kalotermitidae, Rhinotermitidae, Termitidae). *The Florida Entomologist*, *73*(4), 622–627. https://doi.org/10.2307/3495276

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Stres, B., Thallinger, G. G., David J. Van Horn, Van Horn, D. J., Carolyn F. Weber, Carolyn F. Weber, Weber, C., & Weber, C. F. (2009). Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541. https://doi.org/10.1128/aem.01541-09

Schlub, K. A. (2010). *Investigating The Ironwood Tree (Casuarina equisetifolia) Decline on Guam Using Applied Multinomial Modeling*. https://doi.org/10.31390/gradschool_theses.2676

Schlub, R. L., Ayin, C. M., Alvarez, A. M., Paudel, S., Arif, M., Marx, B. D., Husseneder, C., Schlub, K. A., Quintanilla, M., Klopfenstein, N. B., Kennaway, L. F., Zhang, Y., Zhong, C., &

Nicodemus, A. (2020). *Ecology of Guam's Casuarina equisetifolia and research into its decline* In Sixth International Casuarina Workshop Krabi, Thailand, 21-25 October 2019 (Eds Haruthaithanasan M, Pinyopusarerk K, Nichodemus A, Bush D, and Thomson L.), pp.237-245

Schlub, R. L., Mersha, Z., Aime, C.M., Badilles, A., Cannon, P.G., Marx, B.D., McConnell, J., Moore, A., Nandwani, D., Nelson, S.C., et al. (2011) Guam ironwood (*Casuarina equisetifolia*) tree decline conference and follow-up, 4th International Casuarina Workshop, March 21-25, 210 Haikou, China. (Eds Zhong, C., Pinyopusarerk, K., Kalinganire, A., Franche, C.), pp 239-246

Schlub, R. L., Schlub, K. A., Alvarez, A. M., Aime, C. M., Cannon, P. G., & Persad, A. (2012). Integrated perspective on tree decline of ironwood (*Casuarina equisetifolia*) on Guam. In *Proceedings of the 60th Annual Western International Forest Disease Work Conference: October* (pp. 8-12).

Schlub, R. L. (2013). Gago, Guam ironwood tree, *Casuarina equisetifolia*: past, present, future. Guam Cooperative Extension Service Publication. (http://cnas-re.uog.edu/wp-content/uploads/2016/01/finalreducedwsareironwood-manual-03-25.pdf).

Sen, R., Raychoudhury, R., Cai, Y., Sun, Y., Lietze, V.-U., Peterson, B. F., Scharf, M. E., & Boucias, D. G. (2015). Molecular signatures of nicotinoid-pathogen synergy in the termite gut. *PLOS ONE*, *10*(4). https://doi.org/10.1371/journal.pone.0123391

Sethi, A., Delatte, J., Foil, L. D., & Husseneder, C. (2014). Protozoacidal Trojan-Horse: Use of a ligand-lytic peptide for selective destruction of symbiotic protozoa within termite guts. *PLOS ONE*, *9*(9). https://doi.org/10.1371/journal.pone.0106199

Shannon, C. E., & Weaver, W. (1949). *The mathematical theory of communication.* University of Illinois Press.

Shi, Y., Huang, Z., Han, S., Fan, S., & Yang, H. (2015). Phylogenetic diversity of Archaea in the intestinal tract of termites from different lineages. *Journal of Basic Microbiology*, *55*(8), 1021–1028. https://doi.org/10.1002/jobm.201400678

Shimada, K., Lo, N., Kitade, O., Wakui, A., & Maekawa, K. (2013). Cellulolytic protist numbers rise and fall dramatically in termite queens and kings during colony foundation. *Eukaryotic Cell*, *12*(4), 545–550. https://doi.org/10.1128/ec.00286-12

Shinzato, N., Muramatsu, M., Matsui, T., & Watanabe, Y. (2005). Molecular phylogenetic diversity of the bacterial community in the gut of the termite *Coptotermes formosanus*. *Bioscience, Biotechnology, and Biochemistry*, *69*(6), 1145–1155. https://doi.org/10.1271/bbb.69.1145

Smith, E. F. (1896). *A bacterial disease of the tomato, eggplant, and Irish potato (Bacillus solanacearum n. sp.)*. G.P.O.

Smith, S. L., & Moore, A. (2008). *Early detection pest risk assessment: Coconut rhinoceros beetle*.

Smythe, R. V., Carter, F. L., & Baxter, C. C. (1971). *Influence of wood decay on feeding and survival of the eastern subterranean termite, Reticulitermes flavipes (Isoptera: Rhinotermitidae)*. *Jan 15*, *1971*, *64 (1)*(1), 59–62.

Snyder, S. A. (1992). *Casuarina* spp. In: Fire Effects Information System, [Online]. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory (Producer).

Solden, L. M., Lloyd, K. G., & Wrighton, K. C. (2016). The bright side of microbial dark matter: Lessons learned from the uncultivated majority. *Current Opinion in Microbiology*, *31*, 217–226. https://doi.org/10.1016/j.mib.2016.04.020

Song, Y., Hervé, V., Radek, R., Pfeiffer, F., Zheng, H., & Brune, A. (2021). Characterization and phylogenomic analysis of *Breznakiella homolactica* gen. Nov. Sp. Nov. Indicate that termite gut treponemes evolved from non-acetogenic spirochetes in cockroaches. *Environmental Microbiology*, *23*(8), 4228–4245. https://doi.org/10.1111/1462-2920.15600

Soukup, P., Větrovský, T., Větrovský, T., Stiblik, P., Votýpková, K., Chakraborty, A., David Sillam-Dussès, Sillam-Dussès, D., Kolařík, M., Odriozola, I., Lo, N., Nathan C. Lo, Baldrian, P., Šobotník, J., & Bourguignon, T. (2020). Termites Are Associated with External Species-Specific Bacterial Communities. *Applied and Environmental Microbiology*, *87*(2). https://doi.org/10.1128/aem.02042-20

Stewart, E. J. (2012). Growing Unculturable Bacteria. *Journal of Bacteriology*, *194*(16), 4151–4160. https://doi.org/10.1128/jb.00345-12

Stone (1970) The flora of Guam. Micronesica 6:1–659

Su, N. Y., & Scheffrahn, R. H. (1998). *Coptotermes vastator Light (Isoptera: Rhinotermitidae) in Guam*. *33*, 13–18.

Su, N.Y., & Scheffrahn, R. H. (1990). Potential of insect growth regulators as termiticides: A review. *Sociobiology*, *17*(2), 313–328.

Su, N.Y., and M. Tamashiro. (1987). An overview of the Formosan subterranean termite in the world, p. 3–15. In M. Tamashiro and N.Y. Su (eds.), Biology and control of the Formosan subterranean termite. College of Tropical Agriculture and Human Resources, University of Hawaii, Honolulu, HI.

Suenaga, H. (2012). Targeted metagenomics: a high-resolution metagenomics approach for specific gene clusters in complex microbial communities. *Environmental microbiology*, *14*(1), 13–22. https://doi.org/10.1111/j.1462-2920.2011.02438.x

Sugimoto, A., Bignell, D. E., & Macdonald, J. A. (2000). *Global Impact of Termites on the Carbon Cycle and Atmospheric Trace Gases*. 409–435. https://doi.org/10.1007/978-94-017-3223-9_19

Sun, J., & Chen, C. (2010). Cellulolytic insects and their potentials for viable biofuels: a new frontier discipline in entomology and bioengineering. *Chinese Bulletin of Entomology*, *47*(6), 1033-1042.

Swearingen, J. M. (2005). *Paperbark Tree*. Plant Conservation Alliance, Alien Plant Working Group. https://books.google.com/books?id=W18KzQEACAAJ

Tai, V., James, E. R., Nalepa, C. A., Scheffrahn, R. H., Perlman, S. J., & Keeling, P. J. (2015). The role of host phylogeny varies in shaping microbial diversity in the hindguts of lower termites. *Applied and Environmental Microbiology*, *81*(3), 1059–1070. https://doi.org/10.1128/aem.02945-14

Taylor, M. J., Bordenstein, S. R., & Slatko, B. E. (2018). Microbe Profile: Wolbachia: A sex selector, a viral protector and a target to treat filarial nematodes. *Microbiology*, *164*(11), 1345–1347. https://doi.org/10.1099/mic.0.000724

Teixeira, P. J. P., Colaianni, N. R., Fitzpatrick, C. R., & Dangl, J. L. (2019). Beyond pathogens: Microbiota interactions with the plant immune system. *Current Opinion in Microbiology*, *49*, 7–17. https://doi.org/10.1016/j.mib.2019.08.003

Tho, Y. P. (1974). The termite problem in plantation forestry in peninsular Malaysia. *Malaysian Forester* , *37*(4), 278–283.

Tho, Y. P., & Kirton, L. G. (1992). *Termites of peninsular Malaysia.*

Thomas, F., Dittami, S. M., Brunet, M., Le Duff, N., Tanguy, G., Leblanc, C., Leblanc, C., & Gobet, A. (2020). Evaluation of a new primer combination to minimize plastid contamination in 16S rDNA metabarcoding analyses of alga-associated bacterial communities. *Environmental Microbiology Reports*, *12*(1), 30–37. https://doi.org/10.1111/1758-2229.12806

Thongaram, T., Hongoh, Y., Kosono, S., Ohkuma, M., Trakulnaleamsai, S., Noparatnaraporn, N., & Kudo, T. (2005). Comparison of bacterial communities in the alkaline gut segment among various species of higher termites. *Extremophiles*, *9*(3), 229–238. https://doi.org/10.1007/s00792-005-0440-9

Thorne, B. L. (1998). Biology of subterranean termites of the genus Reticulitermes, pp. 1-30. In NPCA Research Report on Subterranean Termites. National Pest Control Association, Dunn Loring, VI.

Tikhe, C. V., Martin, T. M., Howells, A., Delatte, J., & Husseneder, C. (2016). Assessment of genetically engineered *Trabulsiella odontotermitis* as a 'Trojan Horse' for paratransgenesis in termites. *BMC Microbiology*, *16*(1), 202–202. https://doi.org/10.1186/s12866-016-0822-4

Tokuda, G., Mikaelyan, A., Fukui, C., Matsuura, Y., Watanabe, H., Fujishima, M., & Brune, A. (2018). Fiber-associated spirochetes are major agents of hemicellulose degradation in the hindgut of wood-feeding higher termites. *Proceedings of the National Academy of Sciences of the United*

States of America, *115*(51), 201810550. https://doi.org/10.1073/pnas.1810550115

Toor, D., Wsson, M. K., Kumar, P., Kumar, P., Kumar, P., Karthikeyan, G., Karthikeyan, G., Naveen Kumar Kaushik, Kaushik, N. K., Chhavi Goel, Goel, C., Singh, S., Anil Kumar, Kumar, A., & Prakash, H. (2019). Dysbiosis Disrupts Gut Immune Homeostasis and Promotes Gastric Diseases. *International Journal of Molecular Sciences*, *20*(10), 2432. https://doi.org/10.3390/ijms20102432

Touati, J., Chliyeh, M., Ouazzani Touhami, A., Benkirane, R., Douira, A. (2016). Effect of mycorrhizae on growth and root development of *Casuarina* spp. under greenhouse conditions. *International Journal of Advances in Pharmacy, Biology and Chemistry*. 5(3): 2277–4688.

Tracey, Jr., JI, SO Schlanger, JT Stark, DB Doan, and HG May. (1964). General Geology of Guam: Geology and Hydrology of Guam, Mariana Islands. USGS Professional Paper no. 403-A, pp. A1 – A103.

Traniello, J. F. A., Rosengaus, R. B., & Savoie, K. (2002). The development of immunity in a social insect: Evidence for the group facilitation of disease resistance. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(10), 6838–6842. https://doi.org/10.1073/pnas.102176599

Utami, Y. D., Kuwahara, H., Igai, K., Murakami, T., Sugaya, K., Morikawa, T., Nagura, Y., Yuki, M., Deevong, P., Inoue, T., Kihara, K., Lo, N., Yamada, A., Ohkuma, M., & Hongoh, Y. (2019). Genome analyses of uncultured TG2/ZB3 bacteria in "Margulisbacteria" specifically attached to ectosymbiotic spirochetes of protists in the termite gut. *The ISME Journal*, *13*(2), 455–467. https://doi.org/10.1038/s41396-018-0297-4

Valouev, A., Ichikawa, J., Tonthat, T., Stuart, J. R., Ranade, S., Peckham, H. E., Zeng, K., Malek, J. A., Gina Costa, Costa, G., McKernan, K., Sidow, A., Fire, A., & Johnson, S. M. (2008). A high-resolution, nucleosome position map of *C. elegans* reveals a lack of universal sequence-dictated positioning. *Genome Research*, *18*(7), 1051–1063. https://doi.org/10.1101/gr.076463.108

Veivers, P. C., O'Brien, R. W., & Slaytor, M. (1982). Role of bacteria in maintaining the redox potential in the hindgut of termites and preventing entry of foreign bacteria. *Journal of Insect Physiology*, *28*(11), 947–951. https://doi.org/10.1016/0022-1910(82)90111-1

Vietmeyer, N. (1986). Casuarina: weed or windfall? *American Forests* :22-25.

Vikram, S., Arneodo, J. D., Joel D. Arneodo, Joel D. Arneodo, Calcagno, J. A., Ortiz, M., Mon, M. L., Etcheverry, C., Cowan, D. A., & Talia, P. (2021). Diversity structure of the microbial communities in the guts of four neotropical termite species. *PeerJ*, *9*. https://doi.org/10.7717/peerj.10959

Visser, A. A., Ros, V. I., De Beer, Z. W., Debets, A. J., Hartog, E., Kuyper, T. W., Laessøe, T., Slippers, B., & Aanen, D. K. (2009). Levels of specificity of Xylaria species associated with

fungus-growing termites: a phylogenetic approach. *Molecular ecology*, *18*(3), 553–567. https://doi.org/10.1111/j.1365-294X.2008.04036.x

Wall, G. C. and Randles, J. W. 2003. Coconut Tinangaja Viroid. Chapter 36 in: Hadidi, A. et al. (Eds.) (2003). CSIRO Publishing, Australia, or Science Publishers, Enfield, NH. Pp. 242-245.

Wang, Y., Lijuan, S., Huang, S., Bo, C., Sen, Y., Li, Y., Li, Y., Yan Li, Li, Y., Wang, F., Xie, H., Jian Xu, Xu, J., & Song, A. (2016). Diversity and resilience of the wood-feeding higher termite *Mironasutitermes shangchengensis* gut microbiota in response to temporal and diet variations. *Ecology and Evolution*, *6*(22), 8235–8242. https://doi.org/10.1002/ece3.2497

Ward, P. E., & Brookhart, J. W. (1962). *Military Geology of Guam, Mariana Islands. Water Resources Supplement*. https://doi.org/10.21236/ada519596

Wardle, D. A., Bardgett, R. D., Klironomos, J. N., Setälä, H., van der Putten, W. H., & Wall, D. H. (2004). Ecological linkages between aboveground and belowground biota. *Science*, *304*(5677), 1629–1633. https://doi.org/10.1126/science.1094875

Warnecke, F., Luginbühl, P., Ivanova, N., Ghassemian, M., Richardson, T. H., Stege, J. T., Cayouette, M., McHardy, A. C., Djordjevic, G., Aboushadi, N., Sorek, R., Tringe, S. G., Podar, M., Martin, H. G., Kunin, V., Dalevi, D., Madejska, J., Kirton, E., Platt, D., Szeto, E., … Leadbetter, J. R. (2007). Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature*, *450*(7169), 560–565. https://doi.org/10.1038/nature06269

Watanabe, H., Watanabe, H., & Tokuda, G. (2010). Cellulolytic Systems in Insects. *Annual Review of Entomology*, *55*(1), 609–632. https://doi.org/10.1146/annurev-ento-112408-085319

Weiss, S., Xu, Z. Z., Peddada, S. D., Amir, A., Bittinger, K., Gonzalez, A., Lozupone, C. A., Zaneveld, J. R., Vázquez-Baeza, Y., Birmingham, A., Hyde, E. R., & Knight, R. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*, *5*(1), 27–27. https://doi.org/10.1186/s40168-017-0237-y

Wielkopolan, B., Jakubowska, M., & Obrępalska-Stęplowska, A. (2021). Beetles as Plant Pathogen Vectors. *Frontiers in plant science*, *12*, 748093. https://doi.org/10.3389/fpls.2021.748093

Witasari, L. D., Wahyu, K. W., Anugrahani, B. J., Kurniawan, D. C., Haryanto, A., Nandika, D., Karlinasari, L., Arinana, A., Batubara, I., Santoso, D., Rachmayanti, Y., Firmansyah, D., Sudiana, I. K., & Hertanto, D. M. (2022). Antimicrobial activities of fungus comb extracts isolated from Indomalayan termite (*Macrotermes gilvus* Hagen) mound. *AMB Express*, *12*(1), 14. https://doi.org/10.1186/s13568-022-01359-0

Woese, C. R. (1987). Bacterial evolution. *Microbiological Reviews*, *51*(2), 221–271. https://doi.org/10.1128/mr.51.2.221-271.1987

Woese, C. R., & Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: The primary

kingdoms. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(11), 5088–5090. https://doi.org/10.1073/pnas.74.11.5088

Wolcott, G. N. (1953). Termite-repellent wood extractives. *The Journal of Agriculture of the University of Puerto Rico*, *37*(3), 224–227. https://doi.org/10.46429/jaupr.v37i3.12751

Wolcott, G.N. (1946) Factors in the natural resistance of wood to termite attack. Caribbean Forester 7: 121-35.

Wommack, K. E., Bhavsar, J., & Ravel, J. (2008). Metagenomics: Read Length Matters. *Applied and Environmental Microbiology*, *74*(5), 1453–1463. https://doi.org/10.1128/aem.02181-07

Wong, N., & Lee, C.Y. (2010). Intra- and interspecific agonistic behavior of the subterranean termite *Microcerotermes crassus* (Isoptera: Termitidae). *Journal of Economic Entomology*, *103*(5), 1754–1760. https://doi.org/10.1603/ec10060

Wood, T. G. & Johnson, R. A. (1986) The biology, physiology and ecology of termites. In: *Economic impact and control of social insects* (eds S. B. Vinson) pp. 1–67. Praeger, Greenwood Press, Westport, CT.

Wood, T. G., & Thomas, R. J. (1989). *The mutualistic association between Macrotermitinae and Termitomyces*. *14*, 69–92. https://doi.org/10.1016/b978-0-12-751800-8.50009-4

Xue, H., Lozano-Durán, R., & Macho, A. P. (2020). Insights into the Root Invasion by the Plant Pathogenic Bacterium *Ralstonia solanacearum*. *Plants (Basel, Switzerland)*, *9*(4), 516. https://doi.org/10.3390/plants9040516

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., & Nishiuchi, Y. (1995). Transfer of two Burkholderia and an Alcaligenes species to Ralstonia gen. Nov.: Proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. Nov., *Ralstonia solanacearum* (Smith 1896) comb. Nov. and *Ralstonia eutropha* (Davis 1969) comb. Nov. *Microbiology and immunology*, *39*(11), 897–904. https://doi.org/10.1111/j.1348-0421.1995.tb03275.x

Yamin, M. A. (1979). Scanning Electron Microscopy of Some Symbiotic Flagellates from the Termite Zootermopsis. *Transactions of the American Microscopical Society*, *98*(2), 276. https://doi.org/10.2307/3226032

Yanagawa, A., & Shimizu, S. (2007). Resistance of the termite, *Coptotermes formosanus* Shiraki to *Metarhizium anisopliae* due to grooming. *BioControl*, *52*(1), 75-85.

Yashiro, T., & Matsuura, K. (2014). Termite queens close the sperm gates of eggs to switch from sexual to asexual reproduction. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(48), 17212–17217. https://doi.org/10.1073/pnas.1412481111

Yeap, B.K., Othman, A. S., Lee, V. S., & Lee, C.Y. (2007). Genetic relationship between

Coptotermes gestroi and *Coptotermes vastator* (Isoptera: Rhinotermitidae). *Journal of Economic Entomology*, *100*(2), 467–474. https://doi.org/10.1603/0022-0493(2007)100[467:grbcga]2.0.co;2

Young, F. J. (Fred J. 1952, University of Guam., Guam. Department of Commerce., & United States. Soil Conservation Service. (1988). *Soil survey of territory of Guam*. The Service.

Yow-Pong, T., & Kirton, L. G. (1990). *The economic significance of Coptotermes termites in Malaysian forestry*.

Zhang, D., de Souza, R. F., Anantharaman, V., Iyer, L. M., Iyer, L. M., & Aravind, L. (2012). Polymorphic toxin systems: Comprehensive characterization of trafficking modes, processing, mechanisms of action, immunity and ecology using comparative genomics. *Biology Direct*, *7*(1), 18–18. https://doi.org/10.1186/1745-6150-7-18

Zhang, X., Matson, E. G., & Leadbetter, J. R. (2011). Genes for selenium dependent and independent formate dehydrogenase in the gut microbial communities of three lower, wood-feeding termites and a wood-feeding roach. *Environmental Microbiology*, *13*(2), 307–323. https://doi.org/10.1111/j.1462-2920.2010.02330.x

Zhou, J., Duan, J., Gao, M., Wang, Y., Wang, X., & Zhao, K. (2019). Diversity, Roles, and Biotechnological Applications of Symbiotic Microorganisms in the Gut of Termite. *Current microbiology*, *76*(6), 755–761. https://doi.org/10.1007/s00284-018-1502-4

Zinger, L., Bonin, A., Alsos, I. G., Bálint, M., Bik, H., Boyer, F., Chariton, A. A., Creer, S., Coissac, E., Deagle, B. E., De Barba, M., Dickie, I. A., Dumbrell, A. J., Ficetola, G. F., Fierer, N., Fumagalli, L., Gilbert, M. T. P., Jarman, S., Jumpponen, A., Kauserud, H., … Taberlet, P. (2019). DNA metabarcoding-Need for robust experimental designs to draw sound ecological conclusions. *Molecular ecology*, *28*(8), 1857–1862. https://doi.org/10.1111/mec.15060

Vita

Garima Setia, born in Punjab, India, received a Bachelor of Science (honors) degree in the discipline of Agriculture from Punjab Agricultural University in August 2020. In January 2021, she started her Master of Science program in Entomology at Louisiana State University in Dr. Claudia Husseneder's lab. She anticipates graduating in May 2023.