Tree diversity and functional leaf traits drive herbivore-associated microbiomes in subtropical China

Runing title: Drivers of herbivore-associated microbes

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Abstract

Herbivore insects acquire microorganisms from host plants or soil, but it remains unclear how the diversity and functional composition of host plants contribute to structuring herbivore microbiomes. Within a controlled tree-diversity setting, we used DNA metabarcoding of 16S rRNA to assess the contribution of Lepidoptera species and their local environment (particularly, tree diversity, host tree species, and leaf traits) to the composition of associated bacterial communities. In total, we obtained 7,909 bacterial OTUs from 634 caterpillar individuals comprising 146 species. Tree diversity was found to drive the diversity of caterpillar-associated bacteria both directly, and indirectly via effects on caterpillar communities, and tree diversity was a stronger predictor of bacterial diversity than diversity of caterpillars. Leaf toughness and dry matter content were important traits of the host plant determining bacterial species composition, while leaf calcium and potassium concentration influenced bacterial richness. Our study reveals previously unknown linkages between trees and their characteristics, herbivore insects, and their associated microbes, which contributes to developing a more nuanced understanding of functional dependencies between herbivores and their environment, and has implications for the consequences of plant diversity loss for trophic interactions.

Key words

BEF-China; Lepidoptera; herbivore-associated microbiome; 16S rRNA gene; Phyllosphere; leaf characteristics

1 | Introduction

Herbivore insects acquire microorganisms that occur in and on host plants or in their surroundings, such as the soil in which the host plant grows (Sugio, Dubreuil, Giron &

53 Simon, 2015). In recent years, evidence has emerged suggesting that herbivore symbionts
54 contribute to herbivore-plant interactions (Frago, Dicke & Godfray, 2012). Insect symbionts
55 comprise a community of bacteria, fungi and viruses, and persist both in the insects and on
56 the cuticle of their exoskeleton as well as on the plants on which they reside, while engaging
57 in a variety of interactions (Klepzig & Six, 2004). Many symbionts found in the gut of insects
58 are allied to microorganisms of the immediate environment, and thus it is likely that diverse
59 environments are encountered at different life stages of some of these microbial species
60 (Frago, Dicke & Godfray, 2012). In addition to gut symbionts, herbivorous insects are known
61 to acquire intra- and extra-cellular microbes also from host plants (Caspi, Inbar, Mozes,
62 Katzir & Zchori, 2011; Li, Ahmed, Lv, Shi & Qiu, 2016).
63 Lepidoptera posses extraordinary species richness (Scoble, 1992) yet are ecologically
64 homogeneous in that 99% of the species are herbivorous (Strong, Lawton & Southwood,
65 1984), and thus they are an ideal model taxon for studying bacterial functionality in herbivory
66 processes. Some recent studies have suggested that caterpillars lack a resident gut
67 microbiome (Hamme, Janzen, Hallwachs, Jaffe & Fierer, 2017; Hammer, Sanders & Fierer,
68 2019). Moreover, most of the microbes found in the Lepidoptera gut were found to be shared
69 with the surrounding leaf surface or with the soil in which the host plant grew, and the
70 bacteria seem to have no effects on growth and survival of the caterpillar (Hamme, Janzen,
71 Hallwachs, Jaffe & Fierer, 2017; Whitaker, Shayla, Jon, Martin & Pierce, 2016). If indeed
72 lacking a persistent microbiome, then any microbial-driven processes in caterpillars might be
73 more environmentally dependent than would otherwise be.
74 From the perspective of a herbivore, plants are a heterogeneous resource from which
75 species or individuals are selected. The amount of herbivory experienced by a plant is to a
76 large part determined by chemical and physical traits of its leaves (Pérez et al., 2003;
77 Carmona, Lajeunesse & Johnson, 2011; Loranger et al., 2012). Leaf traits of species describe

how they interact with their surrounding environments, which includes the local plant community. Thus, some leaf traits have been shown to respond to the local level of plant diversity, which occurs via biotic interactions such as competition and facilitation. In consequence, ecosystem functions are affected, such as nutrient uptake and light acquisition which are reflected in trait values (e.g. specific leaf area, leaf toughness and C: N ratio; Walter et al., 2012; Abbas et al., 2013; Kostenko, Mulder, Courbois & Bezemer, 2017). Moreover, research has shown that variation in leaf traits along a plant diversity gradient contributes to associational resistance to herbivory, as traits respond to both changes in the light environment and conspecific interactions of the herbivores (Muiruri et al., 2018).

Tissue damage from herbivory triggers plant defences which alter the susceptibility to further attacks by insects (Agrawal, 1998), and also microbes (Bressan et al., 2009; Thaler, Humphrey & Whiteman, 2012). Thus, it is likely that herbivore-associated microbes are involved in such herbivory regulation processes. In addition, some recent studies have demonstrated the interlinkages between tree species, ecosystem functions and the cycling of elements (Cardinale et al., 2012; Tilman, Isbell & Cowles, 2014), and the mutual connectedness between tree diversity and the abundance and richness of higher trophic levels (e.g. herbivores and predators) through food webs (Scherber et al., 2010; Haddad, Crutsinger, Gross, Haarstad & Tilman, 2011; Gossner et al., 2016; Giling et al., 2019; Schuldt et al., 2019). However, the extent to which tree species diversity corresponds to the composition of the herbivore microbiome and the direction of any relationships between them and insect herbivory, are unknown (Badri, Zolla, Bakker, Manter & Vivanco, 2013).

Here, we analyze the relationships between the diversity and composition of trees and herbivores, microbes, and herbivory. This is conducted in the “BEF-China” experiment, a large-scale forest biodiversity experiment incorporating random extinction scenarios of tree species, and used to estimate the ecological effects of biodiversity loss (Bruelheide et al.,

2014). We target microbial symbionts on and in the caterpillar body, using 16S rRNA gene sequencing (Figure 1). We hypothesized that the community composition of herbivore-associated microbes is driven by specific aspects of their surroundings (leaf traits, host tree species identity and diversity of tree species in the host tree community) and by the composition and diversity of the host herbivore species. Our aim was to assess the relative importance of direct host-mediated effects versus environmentally-mediated effects on the herbivore microbiome. Moreover, we hypothesized that herbivore-associated microbial composition affects insect herbivory, which would indicate that the herbivore microbiome plays an important role in mediating interactions between host plant diversity and herbivores.

2 | Materials and Methods

2.1 | Experimental design for study sites

The study was conducted in the BEF-China forest biodiversity experiment, which was established in the south-east of China (Xingangshan, Jiangxi Province, 29°08′–29°11′N, 117°90′–117°93′E) in 2009. The study area has a subtropical monsoon climate with an annual mean temperature of 16.7°C and precipitation of 1,821 mm (Yang et al., 2013). The 38.4 ha study area consists of two sites including a total of 566 plots (25.8 *25.8 m/plot, 271 plots in site ‘A’ and 295 plots in site ‘B’). In each plot, 400 trees were planted in 20 rows and 20 columns. The species pool includes 40 species of trees. Species were selected for each plot was according to a random broken stick design for extinction scenarios of 24, 16, 8, 4, 2 mixtures and monocultures (the 24-species mixtures are an additional treatment on top of this design; Bruelheide et al., 2014).

2.2 | Sample collection of individual caterpillars

We focus on the larval stage, being the primary feeding stage of Lepidoptera and typically the focus for studies of herbivory. The collection of lepidopteran larvae used herein has been previously reported, with focus on tree diversity effects on herbivores themselves in Wang et al. (2019; 2020); thus, here we focus on the microbiomes associated with these herbivores. In October 2018, we selected individuals from the caterpillar samples for extraction of bacterial DNA. To ensure the comparability of caterpillar-associated bacteria across the tree diversity levels, we selected the individual caterpillars randomly from different plots based on the BEF-China design (118 caterpillars came from monocultures, and 110, 104, 97, 98, 107 came from the mixtures of 2, 4, 8, 16 and 24 species, respectively; details see from Table S1). Those Lepidoptera species that had the highest abundances in each plot were selected as representative for the plot. As the number of caterpillars among plots and that of different Lepidoptera species varied greatly, we selected the caterpillars to sample as many tree different species per plot as possible. Because of tree mortality, the whole sample included 37 tree species from 54 plots (Table S1). For each plot, all bacterial sequences obtained from the selected caterpillars served as the bacterial community of the Lepidoptera larvae from the given plot. Totally, this resulted in the selection of 444 trees covering the full tree diversity gradient and a total of 634 caterpillar individuals.

As the current strategy for sampling caterpillar-associated bacteria at the plot level has some limitations (particularly, the bacterial composition of some rare Lepidoptera species was not taken into account), we tested whether the results were affected by sample size. To this end, we used additional linear models to check the relationships between bacterial and tree species richness at the tree richness level (Figure S1). We found that the different Lepidoptera species feeding on the same tree species showed similar bacterial communities (relative abundance of bacterial phyla; Figure S2), and there was no significant difference in bacterial diversity (results not shown).

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153 **2.3 | DNA extraction, amplification, quantitation and sequencing**

154 Total DNA was extracted from the individual caterpillars using Qiagen DNeasy Tissue Kit
155 (QIAGEN GmbH, Hilden, Germany), following the manufacturer's protocol. Samples were
156 processed using sterile tools and conditions. The DNA extracts were quantified using the
157 Qubit 4.0 Fluorometer and stored at -20°C for further processing.

158 The V3 and V4 regions of the 16S rRNA gene, a fragment 468 bp in length, was
159 targeted as it has amongst the highest taxonomic coverage in bacteria (Klindworth, Pruesse,
160 Schweer, Jörg & Frank, 2012). V3 and V4 were amplified using the 16S forward (5′ -
161 ACTCC TACGG GAGGC AGCAG -3′) and reverse (5′ - GGACT ACNVG GGTWT
162 CTAAT - 3′; Zeng et al., 2011) primers. The reaction system involved 4 μL of 5×FastPfu
163 Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of forward primer, 0.8 μL of reverse primer, 0.4 μL
164 of FastPfu Polymerase, 0.2 μL of BSA, 9.5 μL of water and 10 ng of template DNA. The
165 conditions of the PCR were 3 mins template denaturation at 95 °C, followed by 30 cycles at
166 95°C for 30 s per cycle, 30 s annealing at 53 °C, elongation at 72 °C for 45 s, and 10 mins
167 extension at 72 °C finally. The resulting PCR products were extracted from 2% agarose gel
168 and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union
169 City, CA, USA), and quantified using QuantiFluor™-ST (Promega, USA) according to the
170 manufacturer's protocol.

171 Caterpillar-derived amplicons were purified and barcoded (Wang et al., 2019). Purified
172 amplicons were quantified, pooled in equimolar, and paired-end sequenced using the V2
173 Illumina chemistry (2x300 bp) on an Illumina MiSeq platform (Illumina, San Diego, USA;
174 Illumina, Inc. 2015) according to the standard protocols by Majorbio Bio-Pharm Technology
175 Co. Ltd. (Shanghai, China).

176

177 **2.4 | Bioinformatics analyses**

178 We used both VSEARCH v 2.8.1 (Rognes et al., 2016) and USEARCH v 11 (Edgar, 2010) to
179 process raw sequences. Firstly, the read pairs were merged, primers were trimmed, and
180 quality filtering excluded short and low-quality reads (below 25), using VSEARCH. Then we
181 dereplicated the data (retaining abundance information), denoised, and generated OTUs using
182 the unoise3 command of USEARCH. Finally, the OTUs were assigned taxonomic
183 information using the Silva 138 SSU database by using VSEARCH, after which non-bacteria
184 OTUs were removed (Pruesse et al., 2007). Bacteria were classified at the level of phylum,
185 class, order, family, genus and species.

186 Phylogenetic diversity (PD) of bacteria was incorporated as response variables. To
187 construct the bacterial phylogeny, we used MAFFT v 7.0 (Misawa, Katoh, Kuma & Miyata,
188 2002) to align the sequences, trimmed the alignment with MEGA v 7.0 (Kumar, Stecher &
189 Tamura, 2016) and inferred the phylogeny using the ML software IQ-TREE v 1 (Nguyen,
190 Schmidt, von Haeseler & Minh, 2015). The abundance of the bacterial OTUs was defined as
191 the number of reads per sample.

192

193 **2.5 | Leaf traits, herbivory and plot covariables**

194 We selected a suite of 11 morphological and chemical leaf traits which we considered prime
195 candidates for determining leaf quality for herbivore insects, and to characterize plot
196 conditions in accordance to nutritional quality and potential defense traits of the trees. We
197 used leaf area, specific area, dry matter content and toughness as the main morphological
198 traits, and leaf potassium, calcium, magnesium, sodium, phosphorus, carbon, and nitrogen
199 content, and C: N ratio, as the chemical leaf traits (see Table S2 for abbreviations that will be
200 used below). Leaf traits were measured on sun-exposed, fully expanded, undamaged leaves

from five to seven individuals per tree species according to standard protocols (Kröber, Böhnke, Welk, Wirth & Bruelheide, 2012).

Herbivory was measured visually according to damage levels at the end of the growing season (site A: September 2014; site B: September 2015; Schuldt et al., 2017). For each tree, we inspected seven leaves on three randomly selected branches from different positions of the tree canopy (21 leaves per tree). Herbivory was quantified as the overall leaf damage per leaf by chewing, mining, galling and sucking insects, and assigned a percentage class (0, < 5%, < 25%, < 50%, < 75%, > 75%); as standard (e.g. Scherber et al., 2010; Schuldt et al., 2010; Schuldt et al, 2012; Ness JH, Rollinson & Whitney, 2011).

Other plot variables retrived and included in modelling were means of elevation, slope, eastness (sine-transformed radian values of aspect) and northness (cosine-transformed radian values of aspect), which were generated from a 5-meter digital elevation model based on differential GPS measurements (Scholten et al., 2017).

2.6 | Community-weighted mean trait values, functional and phylogenetic diversity

We used the community-weighted mean (CWM) of each trait as well as the functional diversity of selected traits for each tree species, which is the mean value of each species' trait weighted by the species contribution to the plot wood volume. The CWM values of each trait in each plot were calculated by the following equation: $\mathrm{CW}M_{tp} = \sum_{i=1}^{S} V_{ip} \times t_i$, where V_{ip} is the relative tree wood volume of species *i* in plot p and t_i is the mean trait value of species *i* (Garnier et al., 2004). Tree wood volume was estimated from basal area and tree height measured on trees in the center of each plot according to Fichtner et al. (2017). We also applied the CWM of tree volume as an additional predictor in our models. We used species-mean trait values as previous studies in BEF-China demonstrated that variability in trait–

environment relationships were much more pronounced at the interspecific than the intraspecific level (Schuldt et al., 2012).

To characterize the plot conditions of the study sites the following metrics were calculated at the plot level. The functional diversity of trees was calculated by the mean pairwise distance of trait values among tree species, weighted by relative wood volume, and expressed as Rao's Q (Ricotta & Moretti, 2011). We also depicted PD of tree communities by wood volume-weighted phylogenetic mean pairwise distance (MPD), which in the abundance-weighted case is equivalent to Rao's Q (Tucker, Cadotte, Carvalho, Davies & Mazel, 2016). In addition, we calculated the mean nearest taxon distance (MNTD), a measure of the phylogenetic distance to the nearest taxon, which for each taxon quantifies the extent of terminal clustering (Webb, 2000; Webb, 2002). Phylogenetic indices were calculated on a maximum likelihood phylogenetic tree of all woody species recorded in all plots (Purschke, Michalski, Bruelheide & Durka, 2017).

In addition to plants, indices of Lepidoptera diversity were included. We incorporated Faith's PD, abundance-weighted phylogenetic MPD and MNTD of the lepidopteran communities sampled per study plot (Wang et al., 2019) as predictors in our models. The phylogenetic data were obtained from a maximum likelihood phylogenetic tree based on all lepidopteran samples we collected from 2017 and 2018 (Wang et al., 2020).

2.7 | Statistical analyses

Statistical analyses were conducted using the packages picante (Kembel et al., 2010), vegan (Oksanen, 2008), ape (Paradis & Schliep, 2019), edgeR (Robinson, McCarthy & Smyth, 2010), phyloseq (McMurdie & Holmes, 2013), lavaan (Rosseel, 2012) and lulu (Frøslev et al., 2017) in R v3.5.2 (http://www.R-project.org). Firstly, to eliminate the impacts on differing read numbers across samples, the number of sequences of all samples was rarefied

to the lowest number by using the "rarefy" function of the vegan package. The bacterial S_{obs} (observed bacterial richness), Chao1 (nonparametric estimator for bacterial richness), Shannon diversity, and Pielou's evenness were calculated for each plot from bacterial abundance using the "diversity" function of the vegan package. To improve normality and variance in homogeneity of the model residuals, tree species richness, Lepidoptera richness, abundance, and bacterial S_{obs} were log-transformed, and Chao1, Shannon diversity, and Pielou's evenness were square-root transformed. All continuous predictors were standardized before the analyses.

To avoid multicollinearity affecting our statistical analyses, we tested correlations among all predictors through the Pearson's correlation coefficients ($r > 0.7$ interpreted as a strong correlation) and examined variance inflation factors (VIF) in statistical models. Single regression analyses were first used to assess the relationships between species richness, herbivory, phylogenetic metrics of the diversity of trees/lepidopteran larvae (PD, MPD & MNTD) and alpha diversity of the bacterial community. Then, we used linear models to test the potential effects of tree species richness, Lepidoptera richness, leaf traits and plot covariables on caterpillar-associated bacteria. We used bacterial richness (S_{obs} and Chao1), bacterial PD, Shannon diversity, and Pielou's evenness as response variables. For predictors, we included tree species richness, MPD, MNTD, tree functional diversity, CWMs of the selected leaf traits and woody volume, Lepidoptera richness, abundance, MPD and MNTD. We did not include Lepidoptera richness and phylogenetic diversity in the same models because of their strong collinearity (Pearson's $r > 0.9$, $p < 0.001$). The same applied to tree species richness and MPD (Pearson's $r > 0.7$, $p < 0.001$). In addition, we used the interaction between site and tree species richness/tree functional diversity in our models. The linear models were simplified in a stepwise procedure until we obtained the model with the lowest AICc.

Path analyses were conducted to explore the potential causal relationships among tree species richness, Lepidoptera richness, leaf traits, plot covariables, and richness of caterpillar-associated bacteria. Based on prior and theoretical knowledge, we hypothesized that species richness of trees and Lepidoptera might directly influence bacterial communities. In addition, tree species richness could also indirectly influence bacterial communities via Lepidoptera richness. Concurrently, leaf traits may have direct or indirect effects on bacterial communities. Non-significant pathways were gradually removed if their removal improved model fit (Scherber et al., 2010). The model fit was assessed by comparative p-value, fit index value (CFI), Akaike Information Criteria (AIC) and root mean square errors of approximation (RMSEA). Adequate model fits are indicated by high CFI, low AIC, and low RMSEA.

To quantify the homogeneity of dissimilarity variances (bacterial composition dissimilarity among plots) within each tree richness level, the variances of dissimilarities were compared using the "betadisper" function in vegan (Anderson, 2006). This test is similar to Levene's test for homogeneity of variances. We applied analysis of similarities (ANOSIM) to test significance on beta diversity between tree species richness (Warton, Wright & Wang, 2012), with a p value obtained from 9,999 permutations. Moreover, we used a Mantel test to check whether bacterial composition was influenced by spatial location. Differentially abundant bacterial OTUs were detected using edgeR's generalized linear model (GLM) approach. This method allows for testing differential bacterial abundance between different levels of factors by employing a design matrix to account for complex experimental designs. We fitted a generalized linear model with a negative binomial distribution to the normalized values for each of the bacterial OTUs. Differential abundance was tested using a likelihood ratio test, using an adjusted P value cutoff of 0.01. The bacterial OTUs that were enriched in mixtures were compared with bacterial counts from the

300 monoculture plots as reference, and in addition, for higher species richness, also with the
301 bacterial counts from 2, 4 and 8 species mixtures.
302 Distance-based redundancy analysis (db-RDA analysis) based on Bray-Curtis
303 distances was performed using the function “capscale” from the R Package vegan. To
304 determine whether plot covariables and CWM of leaf traits contribute to explaining the
305 microbial community structure of lepidopteran samples, we applied variance partitioning,
306 based on the db-RDA analysis.
307
308 **3 | Results**
309 After merging reads and filtering for quality, we obtained 16,490,248 reads (out of
310 20,466,051 raw reads) in total from 634 caterpillar individuals, delineated to 7,909 bacterial
311 OTUs. There were minor shifts in bacterial phyla among tree genera of plots (Figure 2 &
312 Table S3). The three most abundant bacterial phyla were Proteobacteria, Firmicutes and
313 Actinobacteria. Among classes, Alpha- and Gamma-proteobacteria classes predominated.
314 We found six core bacterial OTUs, present in > 90% of the samples, belonging to four phyla
315 (Proteobacteria, Actinobacteria, Firmicutes, Deinococcus-Thermus). We chose the four most
316 abundant Lepidoptera species (> 24 individuals and observed at all tree diversity levels) to
317 examine distribution patterns of their bacterial phyla (Figure S3). We found that the bacteria
318 of a given Lepidoptera species varied greatly in composition among tree diversity levels, and
319 the bacteria of different Lepidoptera species often varied substantially within a given tree
320 diversity level. Further, based on the assigned taxonomic information, we inferred that only
321 two of the core species are likely to have been derived from the gut of caterpillars, the
322 remainder likely from the leaf surface or elsewhere in the environment.
323 There was no significant correlation between herbivory and bacterial richness (Pearson’s
324 $r = -0.19$, $p = 0.29$) or diversity (Pearson’s $r = -0.16$, $p = 0.38$) at the plot level. Bacterial

richness (S_{obs} and Chao1) and Shannon diversity differed across the study plots, and both were significantly correlated with tree species richness (Figure 3, Table1). These results were confirmed when analyzing the relationship between tree species richness and bacterial diversity at the tree richness level (i.e. based on similar numbers of caterpillars in each richness level; Figure S1). Besides, bacterial richness at the plot level (S_{obs} and Chao1) was also affected by Lepidoptera abundance and richness, although this effect differed somewhat in magnitude at the two study sites (Table1). Tree species richness also corresponded to the caterpillar microbiomes when using Pielou's evenness. Moreover, bacterial richness was positively correlated with elevation (observed bacterial richness), slope and CWMs of several leaf traits of tree communities, especially LDMC, LA, and K content, for which the linear models were similar for S_{obs} and Chao1. Shannon diversity was correlated with tree species richness and the CWMs of SLA, Ca content and K content (Table 1). Pielou's evenness was positively correlated with tree species richness and the CWMs of LT, SLA, Ca content and Mg content.

The path analyses (Figure 6; Table S4) showed that tree species richness directly influenced Lepidoptera richness, which in turn affected the bacterial richness. At the same time, bacterial richness was driven by tree species richness both independently and directly, and this influence was far greater than that of Lepidoptera richness on bacterial richness. Moreover, lead traits also negatively affected bacterial richness, and had an indirect influence through effects on Lepidoptera richness. The CWM of LDMC had a positive effect on bacterial richness both directly and indirectly, through Lepidoptera richness.

Analysis of homogeneity (betadisper) showed that bacterial composition within each tree species richness level varied along the tree diversity gradient, as evidenced by the mean distance to the centroid of each group (Figure 4). The dispersion of the variance of dissimilarities along the diversity gradient demonstrated differences in homogeneity among

tree richness levels ($F = 5.99$, $p < 0.01$), indicating that the difference in bacterial composition between plots within lower tree diversity levels was more pronounced, and that between plots of higher diversity was less so. Analysis of similarity (ANOSIM) revealed that bacteria species composition was not significantly different among tree species richness levels (ANOSIM statistic R: 0.02, P = 0.38). Mantel test indicated that there was no significant relationship between bacterial composition and spatial location (r= 0.07, P=0.14).

db-RDA analysis was performed to determine whether plot covariables and CWM of leaf traits affected the bacterial community structure of lepidopteran samples. The bacterial community structure differed across the study plots but was not significantly affected by species richness of trees or Lepidoptera. However, it was influenced by CWM LDMC (db-RDA pseudo-F = 2.51, Padj < 0.01), CWM LT (db-RDA pseudo-F = 1.88, Padj < 0.01) and tree richness (db-RDA pseudo-F = 1.41, Padj < 0.05; Figure 7).

The analyses of differentially abundant bacterial OTUs between tree richness levels were conducted by fitting a generalized linear model with a negative binomial distribution to normalized values for each of the 7,909 bacterial, and testing for differential abundance using a likelihood ratio test. We first used bacterial counts from monoculture plots as a control and an adjusted P value cutoff of 0.01, and compared it with the bacterial counts from 2, 4, 8, 16 and 24 species mixed plots separately. As shown in Figure 5, the enriched bacterial counts were always greater than depleted counts in comparison to the control. Then, we used the counts from 2, 4, 8 and 16 tree species mixtures as a control and compared successively with higher diversity mixtures. We found that the counts of the enriched species were higher than that of depleted species with increasing diversity when using monocultures and 2 species mixtures as controls. Although when using the 4-species mixtures as a control, the counts of depleted bacteria exceeded the enriched, and the counts of both reduced significantly. In

addition, Figure S4 shows the numbers of differentially enriched and depleted bacterial OTUs between each tree richness level compared with different controls.

Discussion

This study highlights the impacts of tree diversity on the diversity and community composition of caterpillar-associated bacteria, and shows they are influenced by tree diversity and characteristics of the leaf, in what is both a direct and indirect interaction. The direct effect of tree diversity on bacterial diversity was found to predominate, whereas the composition of bacterial communities were to a large part determined by tree diversity and leaf functional traits, especially LDMC and LT, but also chemical leaf traits such as calcium and potassium concentrations. Considering that there is an increasing number of studies reporting that the larva of Lepidoptera recruit microbes from the environment, and that they lack a persistent gut microbiome (Hammer TJ, Janzen DH, Hallwachs, Jaffe & Fierer, 2017; Hammer, Sanders & Fierer, 2019), these are key findings that help to better understand which environmental factors determine these microbial communities and how such environmental effects may influence herbivore functioning. In contrast, we did not find a relationship of microbial diversity and the degree of herbivory, pointing to limiting effects of caterpillar-associated bacteria on this important ecosystem process.

Tree diversity affected the diversity of caterpillar-associated bacteria through influencing the abundance and diversity of lepidopteran larvae. Wang et al. (2019) reported that the impact of tree diversity on herbivore diversity is generally indirect, as tree diversity had strong effects on herbivore abundances, which in turn can affect herbivore diversity. The increase in bacterial diversity that follows increasing Lepidoptera diversity at the plot level is consistent with the expectation that more Lepidoptera individuals and species provide more niche opportunities for bacteria (Akiko, Dubreuil, David & Jean-Christophe, 2015). Thus,

399 diversity at one trophic level begets biodiversity at other trophic levels. Moreover, tree
400 diversity was found to also directly influence the diversity of caterpillar-associated bacteria.
401 The most common bacterial groups of the phyllosphere are Acidobacteria, Actinomycetes,
402 Bacteroidetes, Firmicutes and Proteobacteria (Bodenhausen, Horton & Bergelson, 2013;
403 Bulgarelli, Schlaeppi, Spaepen, van Themaat & SchulzeLefert, 2013), the latter both the most
404 abundant taxonomic group observed in our study as well as generally associated with the
405 phyllosphere reported by others (Humphrey, Nguyen, Villalobos & Whiteman, 2014). This
406 suggests that the phyllosphere is one of the main sources of the herbivore microbiome
407 (Hammer, Janzen, Hallwachs, Jaffe & Fierer, 2017; Whitaker, Shayla, Jon, Martin & Pierce,
408 2016). Kembel et al. (2014) reported that phyllosphere microbial composition differs
409 according to position and height of tree leaves, and physiological and biochemical features
410 such as water content, leaf mass, nitrogen and phosphorus concentrations, leaf surface
411 structure and thickness. Moreover, both bacterial and fungal communities of the phyllosphere
412 are seasonally dynamic (Jumpponen & Jones, 2010; Rastogi et al., 2012). We suspect that
413 diversity in environmental drivers is one of the main reasons for the very high bacterial
414 compositional dissimilarity observed between caterpillars in this study site; with merely 6
415 bacteria OTU of 7,909 that were commonly observed. Further, taxonomic analysis showed
416 that only two of these core species originate from the caterpillar gut; the remainder
417 presumably from the leaf surface or elsewhere in the environment. This result is generally
418 consistent with the finding reported by Hammer et al. (2017; 2019), that resident microbial
419 symbionts are generally absent, or present in low numbers, in the caterpillar gut. Our result
420 implies a correspondence between herbivore associated microbes and their host plants.
421 Another question which remains to be tested is the long term stability of phyllosphere to
422 herbivore microbial interaction, and to what degree they are altered upon herbivores
423 encountering new host plants through movement or feeding.

It is important to note the effect of leaf traits on the diversity and distribution of caterpillar-associated bacteria. As mentioned above, the phyllosphere appears to be a key source for herbivore microbiomes, and is modereated by tree characterisitcs such as leaf structure (also, LDMC is directly affected by leaf thickness, structure, and specific leaf area and reflects the ability of plants to obtain resources). LDMC and LT are usually expected to negatively associate with herbivory because structurally robust leaves are relatively difficult to consume (Pérez et al., 2003). However, both the results herein and some previous reports suggest a positive relationship between LDMC and leaf herbivory (Lepidoptera richness in this study; Schuldt et al., 2012), probably because there are herbivores specifically adapted to tough leaves (Pérez et al., 2003) and herbivores have to consume more of less nutritious foliage to gain the same nitrogen accumulation rates (Scriber & Slansky 1981). Herbivore insects not only damage the plant tissues but also induce plant defenses, which in turn affects the susceptibility of plants to insect (Agrawal, 1998) and microbial attacks (Bressan et al., 2009; Thaler, Humphrey & Whiteman, 2012). Humphrey et al. (2019) reported that insect herbivory modifies bacterial diversity patterns of leaves via induction of plant defenses. However, our finding that herbivory was not related to bacterial richness or diversity at the plot level does not support the idea that the insect-associated microbes on the leaves promote or inhibit insect herbivory. However, the exploration of whether herbivory is affected by bacterial composition or by particular groups of bacteria might provide further insights into this question.

In addition to the two leaf traits (LDMC and LT) mentioned above, we also found that leaf potassium (K) content and calcium (Ca) content can affect bacterial richness, Shannon diversity or Pielou's evenness of the bacteria community, thus there are potential links between leaf traits, herbivores and their associated microbes. Compared to other leaf traits, leaf potassium (K) and calcium (Ca) content have received little attention with respect to

herbivory, but some studies have shown that they can have either positive or negative impacts on herbivore insects (e.g. fecundity; Awmack & Leather, 2002). There is, however, considerable variation in mineral requirements of herbivore insects.

Tree species richness was found to be an important factor that affected caterpillar-associated bacteria community composition. A remarkable result was that certain bacterial OTUs were more abundant in tree species mixtures compared to monoculture plots. However, the accumulation rate of bacterial taxa in more species rich mixtures gradually decreased. That the increase of tree diversity might have a certain stabilizing effect on the herbivore-associated bacterial community was also supported by our finding that the bacterial species composition became more homogenous with tree species richness. From this we would conclude that more tree species-rich forests might have richer but more stable and homogeneous bacterial communities.

We conclude that tree diversity and leaf traits of the tree community are strong drivers of the caterpillar-associated bacteria communities in our subtropical forest. Our study revealed the linkages between tree (leaves), herbivore insects and herbivore-associated microbes, which contributes to develop a more comprehensive understanding of relationship between herbivores and their environment. Moreover, the driving and stabilizing effects of tree diversity on herbivore-associated bacteria suggests that future research should take effects of plants on herbivore-associated microbes into consideration, when studying the relationships between plants and herbivores.

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Author contributions

CDZ conceived the idea for the manuscript; YL designed research; KPM and HB set up the BEF-China experiment; YL, DC, MQW, TW, AS, PFG, JTC, PA, NLZ, QSZ, CSW and KPM collected and/or contributed data and advice; YL conducted the bioinformatic analyses and the statistical analyses and wrote the manuscript, with input by AS, HB, DC, and all coauthors.

Data accessibility statement

The data is available via BEF-China project database at https://data.botanik.uni-halle.de/bef-china/datasets. DNA sequence data can be accessed on Genbank.

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720 TABLE 1 | Summary results of linear models for observed bacterial richness, estimated
721 bacterial richness (Chao1 estimator), Shannon diversity and Pielou's evenness of bacterial
722 communities across a tree species richness gradient. Standardized parameter estimates (with
723 standard errors, t and p values) are shown for the variables retained in the minimal models.

	Observed bacterial richness			
	Est.	SE	*t*	*P*
(Intercept)	7.047	0.062	112.293	< .001
Lepidoptera abundance (log)	0.278	0.165	1.678	.101
Lepidoptera richness (log)	-0.302	0.162	-1.860	.070
Tree richness (log)	0.243	0.054	4.491	< .001
Tree FD (Rao's Q)	0.026	0.051	0.519	.607
SiteB	-0.050	0.120	-0.421	.675
Elevation	-0.106	0.046	-2.291	.027
Slope	0.110	0.033	3.278	.002
CWM LDMC	0.136	0.050	2.670	.010
CWM LA	0.086	0.039	2.195	.034
CWM K	-0.150	0.036	-4.118	< .001
Lepidoptera abundance: SiteB	0.413	0.197	-2.101	.041
Lepidoptera richness: SiteB	0.525	0.196	2.678	.010
Tree FD (Rao's Q): SiteB	-0.170	0.083	-2.045	.047
	Estimated bacterial richness			
	Est.	SE	*t*	*P*
(Intercept)	7.348	0.061	119.556	< .001
Lepidoptera abundance (log)	0.029	0.193	1.520	.136
Lepidoptera richness (log)	-0.252	0.184	-1.371	.178
Tree richness (log)	0.293	0.043	6.767	< .001
SiteB	0.153	0.115	1.340	.187

Slope	0.082	0.036	2.294	.027
CWM LDMC	0.168	0.058	2.880	.006
CWM LA	0.117	0.047	2.476	.017
CWM K	-0.117	0.040	-2.916	.006
CWM Tree volume	0.091	0.042	2.161	.036
Lepidoptera abundance: SiteB	-0.470	0.232	-2.028	.048
Lepidoptera richness: SiteB	0.488	0.216	2.258	.029
	Shannon diversity			
	Est.	SE	*t*	*P*
(Intercept)	1.550	0.035	43.790	< .001
Tree richness (log)	0.083	0.024	3.407	< .005
SiteB	0.123	0.558	2.206	.032
CWM SLA	0.059	0.026	2.276	.027
CWM K	-0.015	0.032	-4.680	< .001
CWM Ca	-0.097	0.032	3.021	< .005
	Pielou's evenness			
	Est.	SE	*t*	*P*
(Intercept)	-0.337	0.021	-16.031	< .001
Tree richness (log)	0.054	0.022	2.495	.016
CWM SLA	0.066	0.028	2.351	< .001
CWM Ca	0.165	0.049	3.347	< .005
CWM Mg	-0.115	0.045	-.2.575	.013
CWM LT	-0.068	0.031	-2.205	.032

724 Note: CWM Ca, Community-weighted mean value of leaf Calcium concentration; CWM K,
725 Community-weighted mean value of leaf potassium concentration; CWM Mg, Community-
726 weighted mean value of leaf magnesium concentration; CWM LDMC, Community-weighted
727 mean value of leaf dry matter content; CWM LT, Community-weighted mean value of leaf

toughness; CWM SLA, Community-weighted mean value of specific leaf area; CWM LA, Community-weighted mean value of leaf area; FD, Functional diversity.

FIGURE 1 | Overview of the study; (a) location of the study site; Xin-Gang mountain, Jiangxi Province (29° 08′ – 29° 11′ N, 117° 90′ – 117° 93′ E), southeast China, with a typical subtropical climate; (b) two example plots in the study site, with tree species richness of 2 & 4; (c) presence / absence of three Lepidoptera species in the two plots; (d) the relationships between lepidopteran samples and their associated bacterial OTUs in two plots.

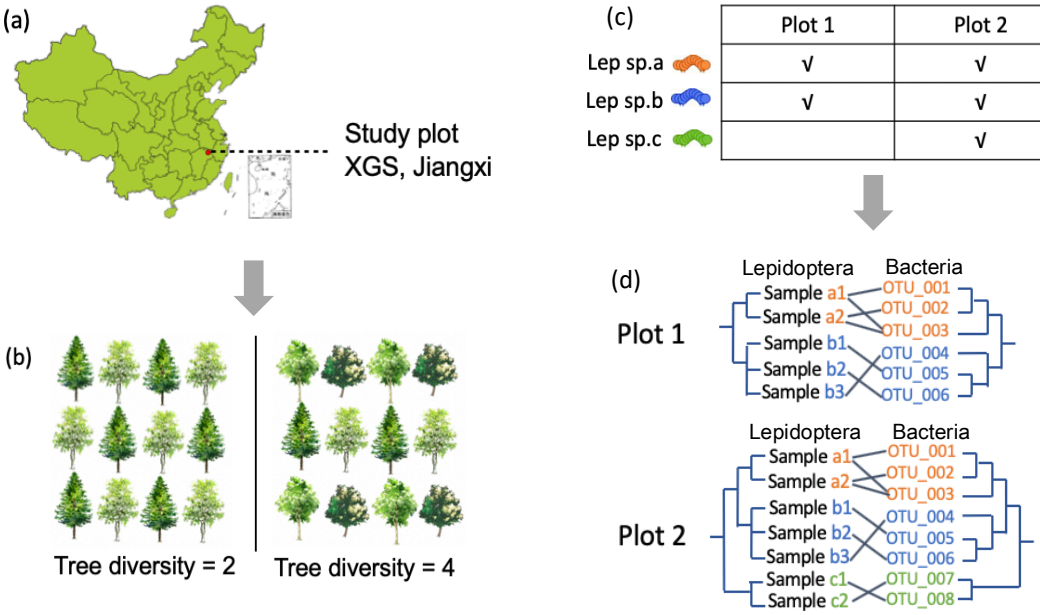

FIGURE 2 | Relative distribution of the bacterial phyla in relation to tree genera. Each stacked bar also represents the relative abundance of the bacterial phyla obtained in each tree genus. The bacterial phyla are listed in the legend. Analysis is limited to phyla with site relative abundance >= 0.1%.

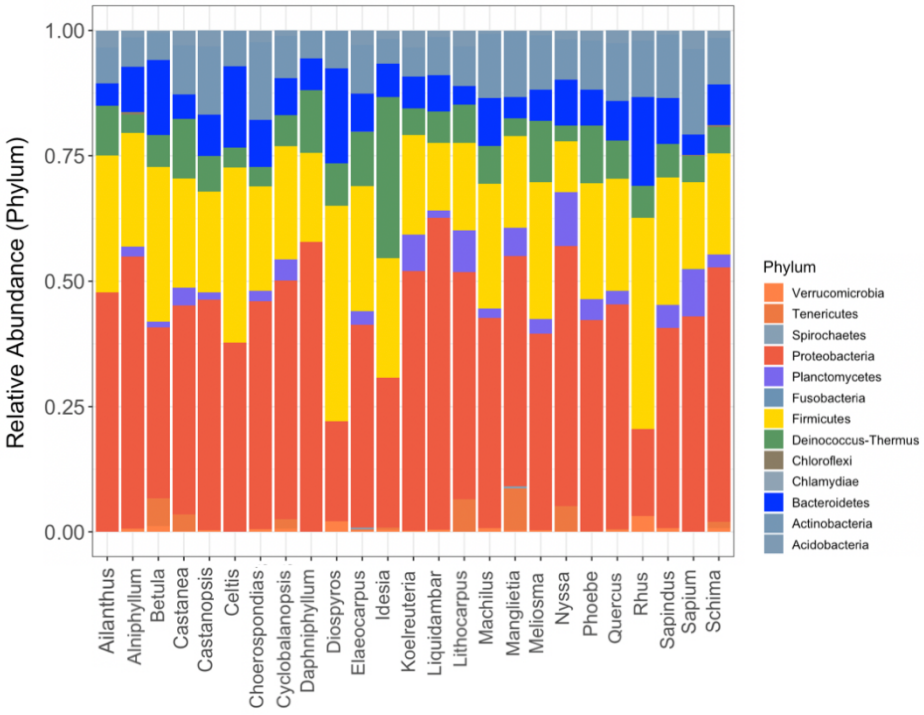

FIGURE 3 | Relationships between (a) tree species richness and bacterial richness, (b) tree species richness and bacterial Shannon diversity, (c) tree species richness and bacterial Pielou's evenness, (d) Lepidoptera richness and bacterial richness. Regression lines (with 95% confidence bands) show significant ($p \leqslant .05$) relationships. The axis values are on a log-scale for tree species richness, Lepidoptera richness and richness of bacteria.

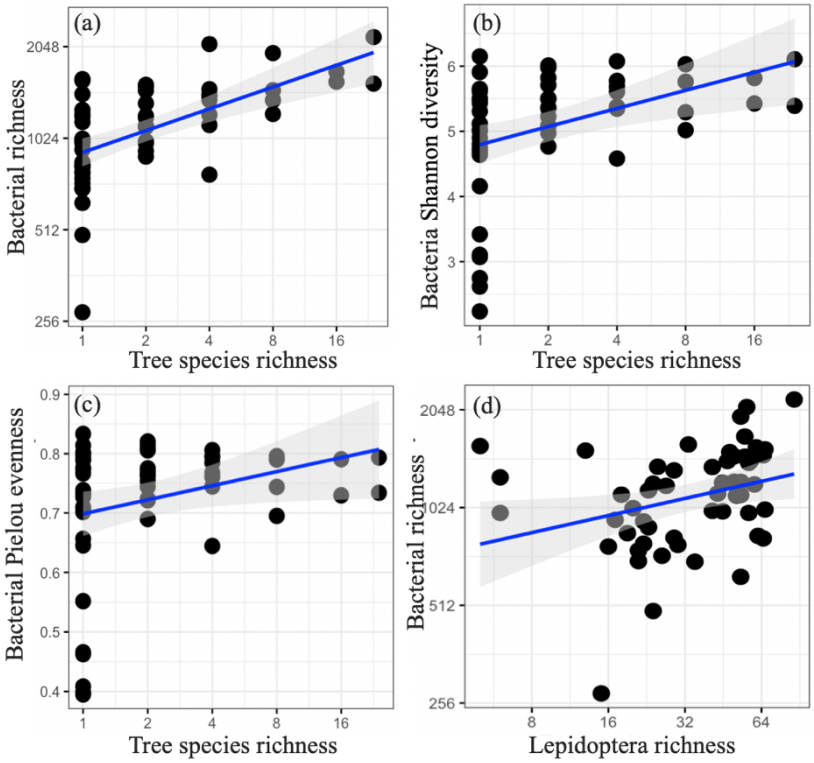

FIGURE 4 | Analysis of homogeneity (betadisper) showing that the dissimilarities of bacterial composition among plots within each tree richness level declined along tree richness levels, as evidenced by the mean distance to the centroid of each group.

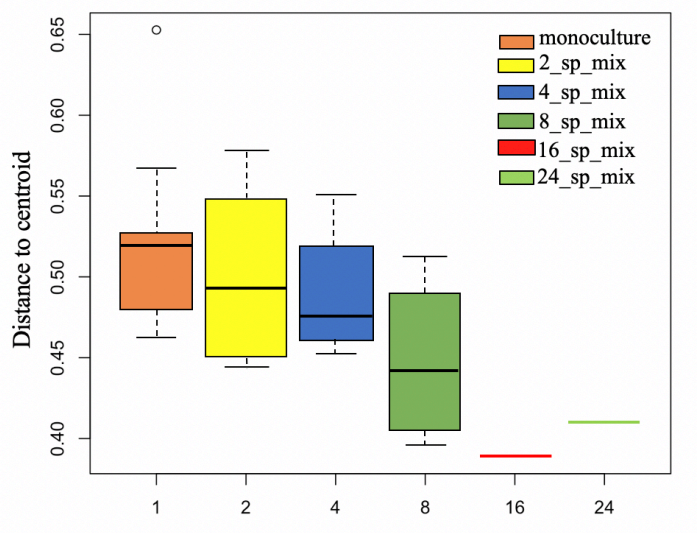

FIGURE 5 | Differentially enriched bacterial OTUs across tree species richness levels. We firstly used bacterial counts from monocultural plots as a control and compared it with the bacterial counts from 2, 4, 8, 16 and 24 species mixed plots successively. Part (a) to (d) represent the results of using the monocultures to 8 species mixtures as a control separately. Each point represents an individual species, and the position along the y axis represents the abundance fold change compared with the control.

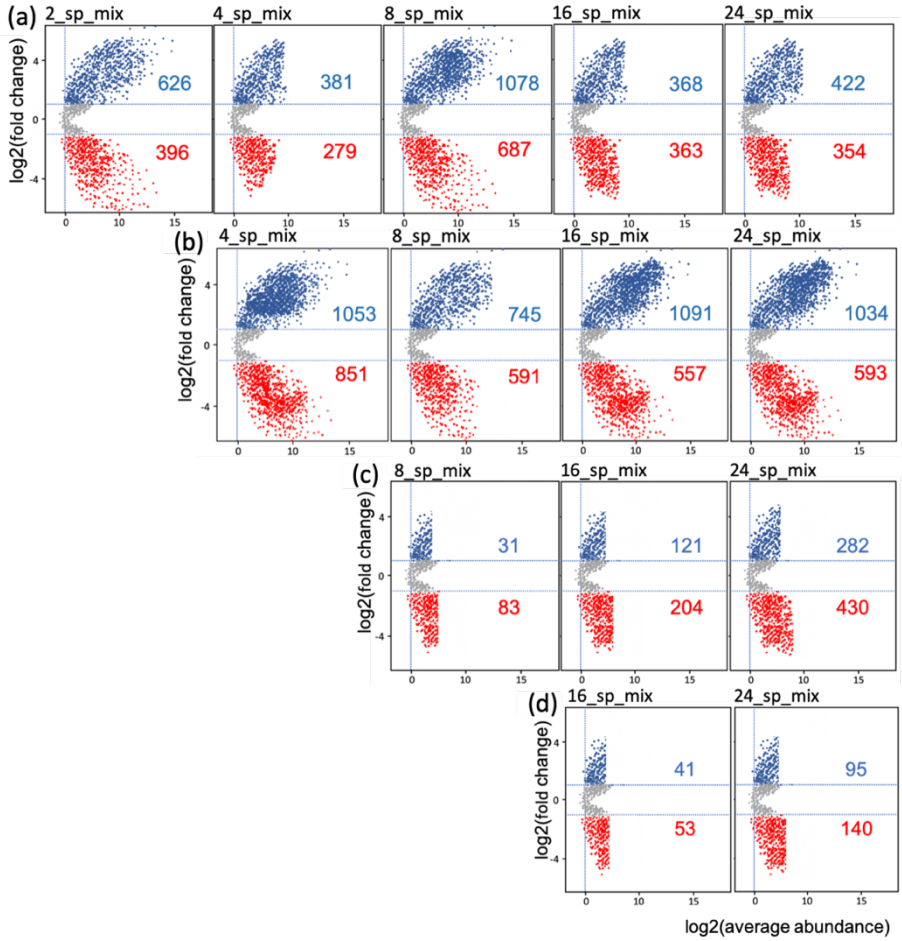

FIGURE 6 | Path model of the effects of tree species richness (direct effect and indirect effects through Lepidoptera abundance and Lepidoptera richness), Lepidoptera richness, CWM LT (direct effect and indirect effect through Lepidoptera richness), CWM LDMC (indirect effect through Lepidoptera richness) on richness of bacterial community. The path coefficients next to the arrows represent the strength of the positive or negative effects of one variable on another (** < 0.001; * < 0.05). See Table S2 & S4 for abbreviations and statistical values.

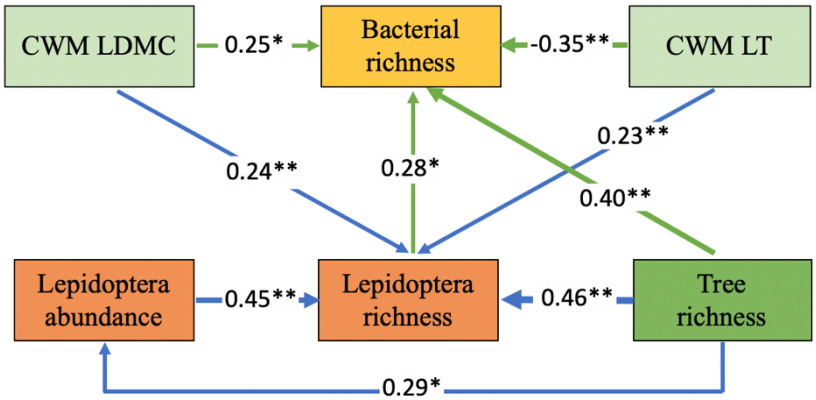

FIGURE 7 | Distance-based redundancy analysis plot showing the relationships of CWM LDMC, CWM LT and tree richness to the bacterial community structure. The plot represents db-RDA analysis based on Bray–Curtis distance with all of the plot covariables and CWM of leaf traits as explanatory variables. CWM LDMC, CWM LT and tree richness were the only significant explanatory variables ($p < 0.05$).

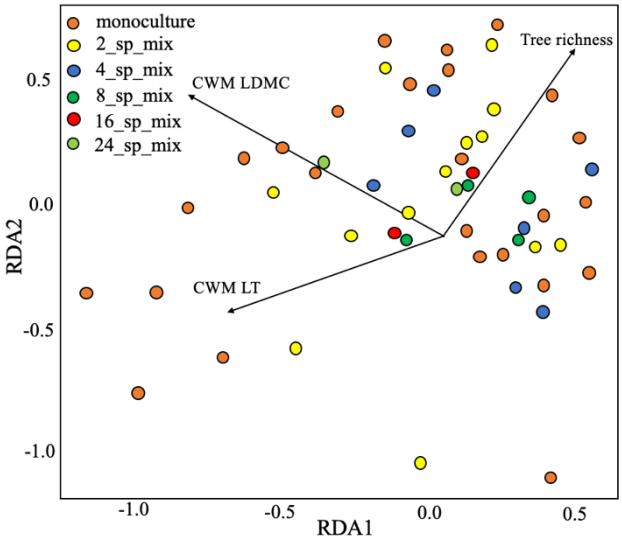

Figure Legends for this manuscript:

Figure.1 Overview (pipeline) of the study.

Figure.2 Relative distribution of the bacterial phyla in relation to tree genera.

Figure.3 Relationships between tree diversity and bacterial richness, diversity, and Pielou's evenness. Also relationship between Lepidoptera richness and bacterial richness.

Figure.4 Analysis of homogeneity (betadisper) between 6 tree richness levels.

Figure.5 Differentially enriched bacterial OTUs across tree species richness levels.

Figure.6 Results of the path model analysis.

Figure.7 Results of the distance-based redundancy analysis.

Figures and tables in the supplementary materials include the following:

Table. S1 Tree species richness, composition and caterpillar sample size of the study plots.

Table. S2 The list of leaf traits and corresponding abbreviations.

Table. S3 Relative abundance of bacterial phyla as correlated with tree genus.

Table. S4 Summary of the path model results.

Figure. S1 Relationships between tree species richness and bacterial richness & Shannon diversity at richness level.

Figure. S2 Relative abundance of individual phyla that correlated with Lepidoptera species in this study.

Figure.S3 Relative abundance of individual phyla that correlated with 4 most abundant Lepidoptera species in this study.

Figure. S4 Numbers of differentially enriched and depleted bacterial OTUs between each tree richness level compared with different controls.