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The effects of host age and spatial location on bacterial community composition in the English Oak tree (*Quercus robur*)

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Summary

Drivers of bacterial community assemblages associated with plants are diverse and include biotic factors, such as competitors and host traits, and abiotic factors, including environmental conditions and dispersal mechanisms. We examine the roles of spatial distribution and host size, as an approximation for age, in shaping the microbiome associated with *Quercus robur* woody tissue using culture-independent 16S rRNA gene amplicon sequencing. In addition to providing a baseline survey of the *Q. robur* microbiome, we screened for the pathogen of acute oak decline. Our results suggest that age is a predictor of bacterial community composition, demonstrating a surprising negative correlation between tree age and alpha diversity. We find no signature of dispersal limitation within the Wytham Woods plot sampled. Together, these results provide evidence for niche-based hypotheses of community assembly and the importance of tree age in bacterial community structure, as well as highlighting that caution must be applied when diagnosing dysbiosis in a long-lived plant host.

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Introduction

Many lines of evidence suggest that microbes are crucial for plant health and function (Kim *et al.*, 2011; Berendsen *et al.*, 2012), and yet we have a relatively poor understanding of which mechanisms shape the plant-associated microbial community or how this might in-turn influence host traits. Furthermore, although plant microbiome research has primarily focused on the below ground portion of the plant (the rhizosphere), knowledge of the phylloplane (the microbial composition of leaves) is increasing (Lindow and Brandl, 2003; Vorholt, 2012), demonstrating an equally important role in shaping plant phenotype. Still less is known regarding the microbial composition of other organs, with distinct communities reported across tissues within the host, often playing a more important role than biogeography (Ottesen *et al.*, 2013; Leff *et al.*, 2014; Coleman-Derr *et al.*, 2016). For tree species in particular, the dermosphere (bark associated microbial community, (Lambais *et al.*, 2014) may be particularly important given that bacterial pathogens often invade the host through wounds in the bark (Tattar, 2012; Misas-Villamil *et al.*, 2013). This variation among tissues mirrors what is observed in other long-lived hosts, including humans, where data is most abundant; distinct bacterial communities have been isolated from different skin sites (Grice *et al.*, 2009) and these differences appear stable over time (Costello *et al.*, 2009). Such variation is also likely to exist across individual plant microbiomes given that they can be heritable (Peiffer *et al.*, 2013), shaped by host genetics (Bodenhausen *et al.*, 2014; Beckers *et al.*, 2016), and play functional roles that include sensitizing the plant immune system (Pieterse *et al.*, 2014).

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The root-associated microbiomes of healthy *Arabidopsis* plants are arguably the best understood plant microbiome (Lundberg *et al.*, 2012) with the mechanisms behind host regulation recently coming to light (Lebeis *et al.*, 2015). However, many more non-model plant species have had their microbiomes characterized. For example, a number of studies have explored the nature of tree microbiomes, providing baseline taxonomic surveys and assessing the drivers of community composition, typically contrasting host traits with climatic or geographic variables. Many of these studies find a strong effect of host phylogeny on the bacterial community, with a greater effect of tree species than geographic distance, even across continents (Redford *et al.*, 2010; Lambais *et al.*, 2014). Similarly, in a tropical environment in Malaysia, Kim et al. (Kim *et al.*, 2012) found a strong signal of host phylogeny on bacterial community composition. Functional host traits such as growth rate and leaf mass have also been demonstrated as key drivers of composition, alongside phylogeny (Kembel *et al.*, 2014). In contrast, Finkel et al. (Finkel *et al.*, 2012) found trees of the same species in a different desert locations host distinct microbial communities. Given these conflicting results across the scales examined, it is unclear whether phylloplane microbiomes are subject to niche-based or neutral models of community assembly.

Specifically, the roles of dispersal and immigration, in combination with ecological selection and drift (Vellend, 2010), have been the focus of a number of theoretical models of community assembly, many of which are applicable to microbes (Sloan *et al.*, 2006; Nemergut *et al.*, 2013). The niche

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assembly model states that the dispersal of bacteria is unhindered by physical constraints, and all organisms can be found anywhere but it is the environment which selects for their persistence (de Wit and Bouvier, 2006). Conversely, the dispersal assembly hypothesis states that the biodiversity we observe can largely be explained by stochastic local extinctions and dispersal-limitation, typified by the idea of island biogeography (Hubbell, 2001; Volkov *et al.*, 2003). Whilst this is essentially the "niche vs. neutral" debate, Fierer (Fierer, 2008) provides the nuances of the microbial context including the much higher species richness and evenness, and the rapidity of species turnover typical of most bacterial communities.

The English oak tree, *Quercus robur*, study system provides an opportunity to test these competing hypotheses. If microbial community assembly is purely a dispersal-driven process, we would predict a positive relationship between tree age and diversity, as older organisms will have experienced more colonization events. Such a positive relationship has been demonstrated for trees and their plant epiphytes and lichens (Flores-Palacios and Garcia-Franco, 2006; Johansson *et al.*, 2007), but has not been shown before in tree-associated bacterial communities. Alternatively, if the process is strictly niche-driven, older trees could represent an alternative environment to smaller trees, favoring proliferation of particular species but not necessarily harboring a greater diversity.

As well as dispersal, host traits are likely to govern the microbes present. Among the host factors known to influence microbial diversity, host age is

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131 often a key predictor. Data from humans suggests diversity consistently
132 increases with age from birth across populations (Yatsunenko *et al.*, 2012). In
133 insects, honey bee queens undergo massive compositional shifts in their
134 microbiome as they age (Tarpy *et al.*, 2015) and in a wild bird, *Rissa*
135 *tridactyla*, chicks harbor a greater diversity of bacteria than adults (van
136 Dongen *et al.*, 2013). In plants, bacterial diversity can be highest on younger
137 leaves in lettuces (Dees *et al.*, 2015), however the evidence is mixed as tree-
138 associated bacterial communities can be strongly influenced by season
139 (Peñuelas *et al.*, 2012).
140
141 In this study we describe and explore the bacterial composition of *Q.robur* tree
142 cores in a well-studied UK forest, Wytham Woods, in order to answer three
143 key questions: firstly, what are the typical bacterial taxa associated with this
144 Woodland site; secondly, does geographic distance affect dispersal, such that
145 there is a spatial pattern of community composition and distance between
146 trees; and thirdly, is *Q. robur* host age or location important in structuring
147 bacterial communities. To answer these questions, we first describe the tree-
148 associated microbiota using amplicon sequencing of the 16S rDNA gene of 64
149 trees. Using a long-term woodland census we then assess correlations
150 between alpha and beta diversity and factors such as age and spatial
151 location. Additionally, we use these data to compare the predicted metabolic
152 functionality and screen our dataset for the pathogenic clade *Brenneria*, the
153 causative agent of acute oak decline, from which the UK *Q. robur* population
154 is currently experiencing an epidemic (Denman *et al.*, 2012). This survey
155 presents a unique opportunity to assess the practicality of high throughput

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sequencing in environmental monitoring. Given the critical importance of detecting and preventing the emergence of tree diseases before large-scale spread, a better understanding of tree microbiomes offers additional value in surveillance.

Experimental Procedures

Study System

Wytham Woods is one of the most intensively studied tree populations in Europe and undergoes extensive surveys every 2 years. As such, it provides a practical system for correlating a vast number of ecological variables and demographic traits and has been the source of numerous important papers (Hunter *et al.*, 1997; Morecroft *et al.*, 2003; Butt *et al.*, 2009). The UK *Q. robur* population is suffering a number of infectious diseases, collectively known as oak decline. This comprises chronic oak decline, sudden oak decline and acute oak decline (AOD) (Denman and Webber, 2009). Symptoms, such as stem bleeding, are strikingly similar, which makes misdiagnosis with Phytophthora or bupestrid beetles possible. A number of bacterial species from the *Brenneria* genus have been isolated from *Q. robur* trees suffering from AOD and it is likely that this species is the causal agent (Denman *et al.*, 2012). The disease has not yet been reported in Wytham Woods (Kirby *et al.*, 2014) so the absence of *Brenneria* species on healthy trees would buttress the existing evidence that *Brenneria* is the primary pathogen.

Site sampling

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We sampled 64 *Q. robur* trees in a single hectare, collecting 192 samples in over 3 days in September, 2013. Tree diameter at breast height (DBH) was recorded as a proxy for tree age. This method is endorsed by the UK forestry commission as a non-destructive mode of estimating tree age (Commission, 1998). Whilst comparisons among trees at different sites due to crowding may be inaccurate, comparisons of the same species at the same site provides reliable estimates of tree age. Further, using trees from our dataset that had known planting dates, we observe a linear relationship between diameter and age, reinforcing the view that DBH is a good proxy for age (SI, Figure 1).

Core tissue samples were obtained using the Trephor tool (Rossi *et al.*, 2006), allowing for three small (approximately 3 cm) microcore samples to be taken at breast height at three separate sites (North, Southwest, and Southeast). The tool was sterilized and wiped thoroughly using 70% ethanol in between each sample extraction. Samples were flash frozen in the field for transportation back to the laboratory. Upon return to the laboratory, samples were homogenized using a Fast-Prep 24 instrument (MP Biomedicals) for five minutes with the addition of two 0.5cm steel beads. Total DNA was then extracted from the resulting homogenate using a Qiagen DNeasy Plant Mini Kit, following the protocol provided. For amplification of the V4 region of the 16S rDNA gene, the universal primer set GTGCCAGCMGCCGCGGTAA (5' --- 3') and GGACTACHVGGGTWTCTAAT (5′ --- 3′) was used.

Brenneria amplification

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To demonstrate that the primers used in our study could amplify *Brenneria goodwinii* we cultured strain 931-23 (provided by R. Jackson, University of Reading) and chose a random subset of 5 of the primers used to amplify the V4 region to test for amplification in these positive controls.

Bioinformatic analysis

Illumina MiSeq 250bp paired-end reads were demultiplexed and de-barcoded at the sequencing centre (Source Biosciences, Oxford). Sequences have been deposited in the NCBI short-read archive (accession PRJNA 298668). Quality filtering of reads was conducted using the Qiime (1.9.0) pipeline (Caporaso *et al.*, 2010). Reads were joined and filtered with the default settings (Bokulich *et al.*, 2013). Briefly, a maximum of 3 consecutive low quality base calls was allowed before truncating the read, phred-score threshold was set at 30 (which provides a 99.9% accuracy of base call), 75% of the read was required to consist of high-quality, consecutive base call and all reads with N character base calls were dropped. Open reference OTU picking was conducted using the Uclust algorithm and the Silva 111 16S rDNA database at the 97% identity level (Pruesse *et al.*, 2007; Edgar, 2010). Chimera removal was performed with Chimera Slayer (Haas *et al.*, 2011); OTUs present at abundances less than 0.005% of the dataset were removed as were OTUs observed in only a single instance, as both are known to inflate diversity estimates (Bokulich *et al.*, 2013). Mitochondrial and chloroplast sequences were also removed. This left a remaining dataset with a total of 1013881 sequences spread across 115 samples, containing a median count of 1830 sequences per sample (mean 8816, length: 251.8 bp). Using the

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same trimmed sequence files, closed-reference OTU picking was performed against the GreenGenes (1.5) (DeSantis *et al.*, 2006) database (as required by Picrust) using the uclust algorithm implemented in Qiime (1.9). Functional predictions of observed taxa was made using the Picrust program (Langille *et al.*, 2013) using the Kegg orthology database (Kanehisa *et al.*, 2014).

Statistical analysis

Rarefaction was performed for diversity analyses to a depth of 500 sequences per sample. Whilst this is relatively low for microbiome studies, we aimed to maintain high levels of biological replication at the cost of sampling depth within individual samples. Pseudo R^2 values were calculated using the residual and null deviance from model outputs as described in Faraway (2006). UniFrac scores were generated in Qiime and statistical analyses were performed in R (R Core Team, 2015) using the packages 'vegan' (Oksanen *et al.,* 2016) and 'cluster' (Maechler *et al.*, 2015).

Results

Baseline survey of the Quercus robur *microbiome*

The most abundant bacterial class observed within our samples was the alphaproteobacteria, with a mean relative abundance of 26% (+/- 12 S.D.), followed by the thermoleophilia with 22% (+/- 15 S.D.), and the betaproteobacteria, contributing a mean of 13% (+/- 15 S.D.). Overall, acidobacteria, actinobacteria and proteobacteria were the three most abundant phyla, making up over 80% of OTUs (Figure 1).

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256

257 *Age related decline in microbial diversity*

258 We identified a weak negative correlation between tree size and species
259 richness (using observed OTUs) when controlling for uneven sampling of
260 individual trees (GLM, $F_{1,87}$=4.13, p=0.0453, pseudo R^2=0.048) (Figure 2.).
261 Observed OTU count was used as the measure of species richness, however
262 the result was non-significant when Faith's phylogenetic distance or Chao 1
263 estimator (Chao *et al.*, 2004) was used (p=0.12 and 0.16 respectively). There
264 was no effect of sample orientation (cardinal direction), and this factor was
265 therefore excluded from the model during stepwise model simplification.
266 Interestingly, these correlations strengthened when sample size was
267 increased to 110 samples by using a lower rarefaction depth (100 sequences,
268 data not shown). A similar result was mirrored by beta-diversity, where tree
269 size was a significant predictor of microbial community composition using both
270 abundance weighted UniFrac scores (PERMANOVA, $F_{1,87}$=4.63, p=0.0036,
271 R^2=0.052, permutations=9999) and unweighted UniFrac scores
272 (PERMANOVA, $F_{1,87}$=2.93, p=0.027, R^2=0.033, permutations=9999) when
273 tree ID was controlled for.

274

275 *Taxa correlations*

276 To investigate changes in composition further we performed Spearman rank
277 correlations against tree size for each OTU in the dataset, and found no
278 significant associations following correction for multiple testing. To further
279 assess whether there were higher taxonomic level associations between
280 specific bacterial clades and tree size we selected the three most abundant

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phyla. Collectively, the Proteobacteria, Actinobacteria and Acidobacteria made up over 80% of our sequences. We found a significant decrease in the relative abundance of Proteobacteria with tree size (Kendall's rank correlation, τ=-0.22, z=-3.39, p=0.0007), a significant increase in the relative abundance of Actinobacteria (τ=0.19, z=3.04, p=0.0023) and a non-significant decrease in Acidobacteria (τ=-0.14, z=-2.13, p=0.033) following Bonferroni correction (Figure 3).

Functional predictions

In order to predict how the function of communities associated with our *Q. robur* trees changed as they aged we created a predicted metagenome using the Picrust program (Langille *et al.*, 2013). However, we found no correlation between any of the predicted individual genes or functional pathways associated with our observed microbiome and tree size, perhaps indicating high functional redundancy of the more diverse microbiota of smaller trees.

Assessing spatial patterns

Finally, to look for patterns of biogeography, or dispersal limitation, we performed Mantel correlations between a spatial matrix from the Euclidean distances between trees and the UniFrac scores that measure bacterial community composition. A correlation would be indicative that the spatial distribution of trees does indeed affect the bacterial composition of the community. There was no effect of abundance for either weighted (Mantel r = 0.0009, p=0.47, permutations=9999) or unweighted UniFrac scores (r=0.002, p=0.46, permutations=9999), suggesting an absence of dispersal limitation.

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306

307 *Brenneria*

308 Reassuringly, we found no sequences identified as Brenneria in our dataset
309 (prior to rarefaction), despite confirming that all our tested primers could
310 successfully amplify this species following culture *in vitro*.

311

312 **Discussion**

313 Our study of the bacterial microbiomes of 64 English oak trees (*Quercus*
314 *robur*) in a single woodland provides a number of insights into the drivers of
315 bacterial community structure and dispersal. Firstly, our census of the
316 microbiome of *Q. robur* tissue is consistent with a previous report that found
317 the same 3 most dominant phyla in the roots of oak trees: Actinobacter,
318 Proteobacteria and Acidobacter (Uroz *et al.*, 2010). The high abundance of
319 Acidobacter is also consistent with other culture-independent studies of the
320 phyllospheric microbiota from tropical trees (Kim *et al.*, 2012).

321

322 By comparing tree size with species richness, we found no sign of an increase
323 in bacterial diversity as trees age. This is of particular interest as it suggests
324 factors other than dispersal affect microbiome structure, as would be
325 expected by an increase in microbial diversity with growth as a result of
326 species accumulation. When observed OTUs was used as the measure of
327 alpha diversity we found a weak but significant decline in species richness
328 with tree age. Furthermore, negative correlations between tree age and
329 species richness were significant when the sample size was increased by
330 reducing rarefaction depth (and therefore excluding fewer samples). Detecting
331 subtle changes in species diversity require maximal statistical power, and

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there is clearly a trade-off between sampling depth and statistical power. Exploring this trade-off in regard to microbial community sampling clearly warrants further study as alternative approaches have yet to be widely adopted (McMurdie and Holmes, 2014). Moreover, quantifying the shape of the age-diversity relationship through the tree lifetime requires longitudinal studies to build on cross-sectional studies like the data presented here. One suggestion for observed age-related differences is variation in the chemical and physiological state of the host tissue (van Dongen *et al.*, 2013) and this could be the case between younger and older *Q.robur* tree tissues.

A flat or negative correlation between tree age and bacterial alpha diversity contrasts the positive association found between epiphytic plants and lichens and tree host age (Flores-Palacios and Garcia-Franco, 2006; Johansson *et al.*, 2007) perhaps suggesting that bacteria are less dispersal-limited than other tree-associated organisms. To explore these ideas further, and based on the conflicting niche assembly and dispersal assembly hypotheses (Hubbell, 2001; de Wit and Bouvier, 2006), we predicted that if microbiome structure is purely a function of dispersal, such that communities are assembled by stochastic dispersal events and local extinctions, we would find a correlation between spatial distance among trees and community dissimilarity scores (beta diversity). Conversely, if microbes have unlimited dispersal within the forest, as is often assumed, one would expect no correlation with beta diversity. Our results suggest that latter models are most informative, whereby we find no signature of dispersal limitation (i.e. the

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community composition of our samples are not influenced by the proximity of others). There is the potential for microbes to disperse at global scales (Morris *et al.*, 2008), however evidence for true cosmopolitan distribution has been mixed to date (Caporaso *et al.*, 2012; Finkel *et al.*, 2012; Sul *et al.*, 2013) and, as demonstrated by Bell (Bell, 2010) also in Wytham Woods, microbial dispersal limitation may be more important over short time scales.

We also found an increase in the relative abundance of Actinobacter and a decrease in Proteobacter and Acidobacter (although the latter was only nearing significance) with tree size. Mechanistically, it is hard to ascribe functions to whole phyla as they encompass a range of morphologies, metabolic diversity and pathogenicity (Dworkin *et al.*, 2006). The Acidobacter are, however, reported to be slow growing with low metabolic rates (Ward *et al.*, 2009), sometimes referred to as k-selection strategists due to their higher abundances in soils with lower resource availability (Fierer *et al.*, 2008). Carbon mineralization rate can also be a good predictor of Acidobacter soil abundance, but how well these finding translates to an alternative niche, such as tree cores, remains unknown (Fierer *et al.*, 2008). If this were the case in our system we would expect Acidobacter and Proteobacteria to be inversely correlated; but we find the opposite. Maignien et al. (Maignien *et al.*, 2014) have also suggested that phyllosphere communities are first colonized by r-strategists (such as Acinetobacter and Pseudomonas). Moreover, when multiple OTUs of the same species are present in the source community, for example rainfall, only one becomes established in the phyllosphere

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community, indicative of niche competition (Maignien *et al.*, 2014). Given that the Acidobacter are consistently isolated at high relative abundances from soil it seems likely that the soil is the major contributing source for the interior microbiota of oak trees. Acidobacter have also been detected at high relative abundances in the trunk of *Gingko bilbao* trees but not in the leaves of the same trees, again suggesting soil derived rather than phyllospheric dispersal (Leff *et al.*, 2014). Whether this is through transport of microbes through the phloem or a function of early, seedling colonization remains undetermined. Interestingly, and as a word of caution, we identified the presence of Ralstonia in our negative sequencing controls, which has been identified by Salter et al. (Salter *et al.*, 2014) as a common kit contaminant. However this group also includes many plant pathogens and wouldn't be unexpected in our environmental samples, highlighting the difficulty in identifying contaminant sequences from environmental samples and the need for negative controls.

Whilst we have described a shift in bacterial community structure with age, the correlations between specific taxa and age are only present at the phylum level and not at the OTU level. The variability in genomic content, even among closely related bacteria (Perna *et al.*, 2001; Guidot *et al.*, 2007), is often used to justify a lack of ecological or metabolic similarity among hosts. However there is evidence for functional convergence at higher taxonomic ranks (Philippot *et al.*, 2010), including trophic and biogeographic differences (Fierer *et al.*, 2008; Philippot *et al.*, 2009). One mechanism for our observation of size-based differences could be that the age of a plant is the most important factor in determining its induced defenses (Quintero and Bowers,

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2011). Indeed, the complex interactions between host immune systems and commensal bacteria are coming to light in different systems (Brestoff and Artis, 2013; Franzenburg *et al.*, 2013). For example, the presence of commensal microbes is non-random in a tropical tree host and has been demonstrated to prevent pathogen success, particularly in fungal endophytes (Arnold *et al.*, 2003).

Despite being present at low numbers, many species could collectively play a role in microbial community function. To explore this idea further we used metagenomic predictions based on our 16S sequences to assess functional diversity. Given that we found a significant shift in the microbial composition (at the Phylum level) with tree age, we expected to find a similar effect of functional traits. We found no such trend, as no individual genes or functional pathways were over or under represented in older tree samples. This lack of functional correlation, despite a taxonomic correlation implies a level of redundancy in gene pathways among bacterial phyla, or lack of sensitivity in the methods used to predict a metagenome. If the latter is true, and the limitation is the quality of annotation in metagenomic databases then ultimately, more metagenomic sequencing may not yield more insight into community function.

A focus on *Q. robur* allows us to answer some important applied questions: A reassuring outcome of this analysis was that we failed to identify a single sequence from *Brenneria* species. The UK oak population is undergoing an epidemic of acute oak decline (AOD) and the *Brenneria* clade of bacteria have

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been isolated from oaks experiencing the disease (Denman *et al.*, 2012). Koch's postulates have also been reported in the Spanish oak (*Quercus ilex*) (Poza-Carrión *et al.*, 2008). However acute oak decline was not found to be present in Wytham in 2014 (Kirby *et al.*, 2014) and our data supports that conclusion. This further strengthens the inference that *Brenneria* is a causative agent of the disease, as suggested by Denman et al. (Denman *et al.*, 2012). Our census provides a baseline of healthy microbial flora in UK *Q. robur* and comparison with trees in diseased states is a crucial area for further study. Additionally, the observed differences in microbiome among differently aged trees provides a caution for defining tree microbiome health. The healthy microbiome of a young tree may well appear similar as that of a dysbiotic microbiome of an old tree. As such, when using microbiome studies in the context of plant health, fair comparisons among plant demographics must be made in order to make useful diagnoses.

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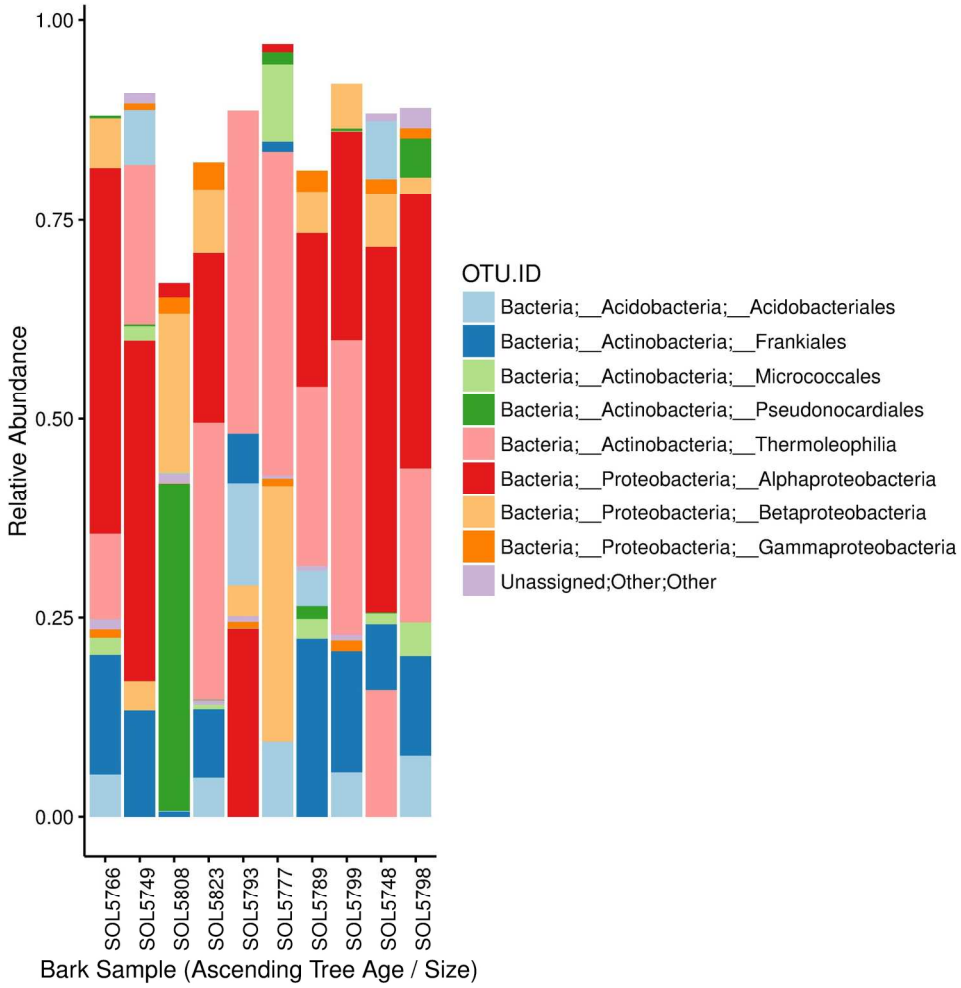
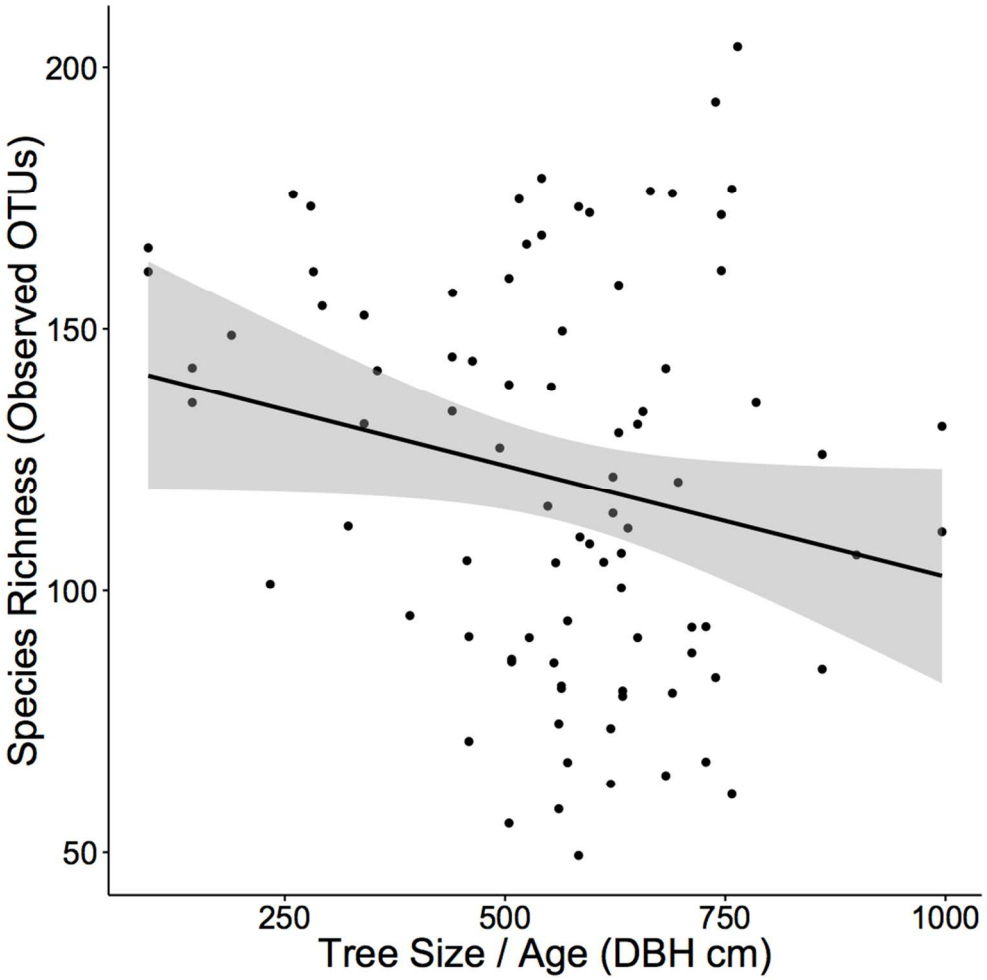
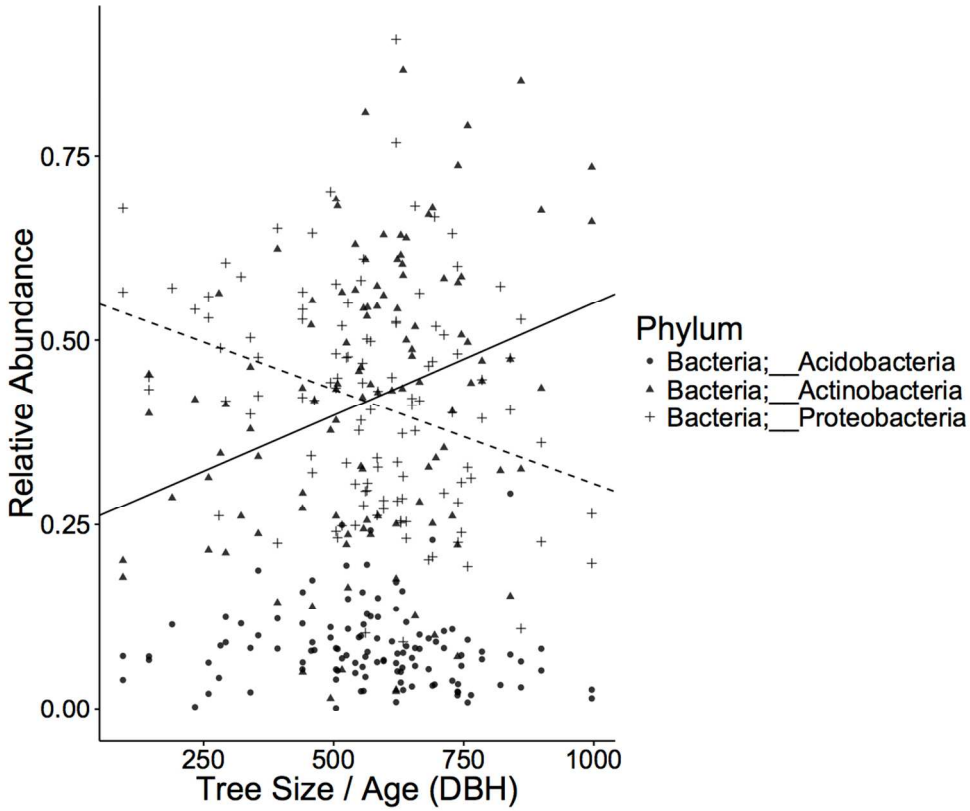


Randomly selected barplots showing the 9 most common bacterial taxa ordered by ascending tree age (from left to right). Alpha diversity decreases in older trees and there is much variation in beta-diversity. Only those taxa with a mean relative abundance greater than 2.5% across the entire dataset were retained for the figure for clarity.
177x177mm (300 x 300 DPI)

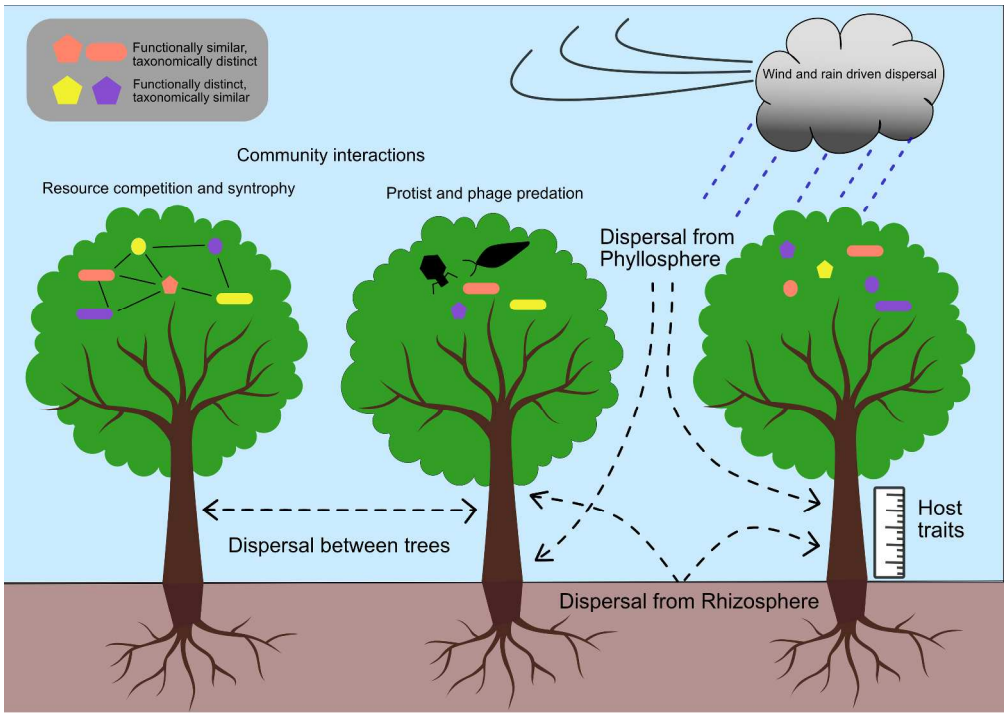


Age based decline in species richness based on species richness, as measured by observed OTUs, following rarefaction to a depth of 500 sequences per sample. GLM, $F_{1,87}=4.13$, $p=0.0453$. Intercept =142, slope = -0.045.
180x177mm (150 x 150 DPI)



Taxa-specific correlations with Oak Tree age. The relative abundance of Actinobacteria increases with tree age (triangles, solid line, intercept = 0.25, slope = 0.00030) (Kendall's rank correlation, τ=0.19, z=3.04, p=0.0023), whilst the relative abundance of Proteobacteria declines (crosses, dashed line, intercept = 0.56, slope = -0.00026) (τ=-0.22, z=-3.39, p=0.0007). The relative abundance of Acidobacteria also declines however this is non-significant after Bonferroni correction (τ=-0.14, z=-2.13, p=0.033).
217x177mm (150 x 150 DPI)

Only



Conceptual diagram of potential drivers of bacterial community composition in our Oak tree system. Communities may be seeded from wind and rain driven dispersal, or colonize the plant directly from the soil during growth. Following initial colonization, the microbes must survive, and potentially thrive, in the observed niche. The niche is likely to be dictated by, among others, competition for host resources, predation and environmental conditions.

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