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## RESEARCH ARTICLE

# Complex associations between cross-kingdom microbial endophytes and host genotype in ash dieback disease dynamics

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## Abstract

1. Tree pathogens are a major threat to forest ecosystems. Conservation management strategies can exploit natural mechanisms of resistance, such as tree genotype and host-associated microbial communities. However, fungal and bacterial communities are rarely looked at in the same framework, particularly in conjunction with host genotype. Here, we explore these relationships and their influence on ash dieback disease, caused by the pathogen *Hymenoscyphus fraxineus*, in European common ash trees.
2. We collected leaves from UK ash trees and used microsatellite markers to genotype trees, quantitative PCR to quantify *H. fraxineus* infection load and ITS and 16S rRNA amplicon sequencing to identify fungal and bacterial communities, respectively.
3. There was a significant association between *H. fraxineus* infection intensity and ash leaf fungal and bacterial community composition. Higher infection levels were positively correlated with fungal community alpha-diversity, and a number of fungal and bacterial genera were significantly associated with infection presence and intensity. Under higher infection loads, leaf microbial networks were characterized by stronger associations between fewer members than those associated with lower infection levels. Together these results suggest that *H. fraxineus* disrupts stable endophyte communities after a particular infection threshold is reached, and may enable proliferation of opportunistic microbes. We identified three microbial genera associated with an absence of infection, potentially indicating an antagonistic relationship with *H. fraxineus* that could be utilized in the development of anti-pathogen treatments.
4. Host genotype did not directly affect infection, but did significantly affect leaf fungal community composition. Thus, host genotype could have the potential to indirectly affect disease susceptibility through genotype × microbiome interactions, and should be considered when selectively breeding trees.

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5. *Synthesis.* We show that the diversity, composition and network structure of ash leaf microbial communities are associated with the severity of infection from ash dieback disease, with evidence of disease-induced dysbiosis. We also show that host genotype influences leaf fungal community composition, but does not directly influence tree infection. These findings help to elucidate relationships between host genetics, the microbiome and a tree pathogen, highlighting potential resistance mechanisms and possible co-infection concerns that could inform ash tree management.

#### KEYWORDS

amplicon sequencing, bacteria, *Fraxinus excelsior*, fungi, genetic diversity, *Hymenoscyphus fraxineus*, microbiome, tree pathogen

## 1 | INTRODUCTION

Invasive pathogens are an increasing threat to trees and forest ecosystems across the globe (Burdon, Thrall, & Ericson, 2005). This rise can be largely attributed to human activity. For example, the international trade in wood products and live plants has introduced pathogens to naive tree populations with no evolved resistance mechanisms, whilst climate change has also rendered environments more conducive to tree infection and pathogen proliferation in many areas (Anderson et al., 2004; Linnakoski, Forbes, Wingfield, Pulkkinen, & Asiagbu, 2017; Roy et al., 2014). Large-scale mortalities in tree species endanger biodiversity, natural capital and ecosystem service provision (Boyd, Freer-Smith, Gilligan, & Godfray, 2013; Freer-Smith & Webber, 2017), and are therefore a key priority area for natural resource management and conservation.

One pathogen of great concern is *Hymenoscyphus fraxineus* (Ascomycota; Leotiomycetes, Helotiales; Helotiaceae), which causes ash dieback disease in a number of ash species, including European ash (*Fraxinus excelsior*)—a highly abundant and ecologically, economically and culturally important tree species. This fungal pathogen produces the toxic compound viridiol, which damages leaves, stems and eventually, the trunk, ultimately causing xylem necrosis and canopy loss (Grad, Kowalski, & Kraj, 2009). Ash dieback has caused up to 85% mortality in plantations within 20 years of exposure (Coker et al., 2019; McKinney et al., 2014), and is driving extensive declines across mainland Europe and the UK (Coker et al., 2019; Jepson & Arakelyan, 2017; McKinney et al., 2014; Mitchell et al., 2014). The disease is likely to have been introduced by trade and is largely spread by wind and water-borne ascospores at a rate of approximately 20–30 km per year (Gross, Zaffarano, Duo, & Grünig, 2012). Due to its severity and the lack of effective treatment or control methods, the import of ash trees is currently banned in the UK.

A range of silvicultural and arboricultural management practices have been suggested for ash dieback mitigation, such as increasing local tree species diversity, removing infected tissue and/or autumn leaf fall, reducing tree density and applying fungicides (Hrabětová, Černý, Zahradník, & Havrdová, 2017; Skovsgaard et

al., 2017). However, such methods may be expensive, labour-intensive and damaging to the environment. Exploiting natural host resistance mechanisms offers a promising alternative, which may provide a more long-term solution whilst avoiding some of these disadvantages.

Ash dieback resistance has a strong host genetic component; nearly 50% of phenotypic variation in crown damage is based on host genotype (McKinney et al., 2014; McKinney, Nielsen, Hansen, & Kjær, 2011; Muñoz, Marçais, Dufour, & Dowkiw, 2016). Furthermore, progeny from low-susceptibility mother clones exhibit lower symptoms of disease, indicating a heritable basis for tolerance (Lobo, McKinney, Hansen, Kjær, & Nielsen, 2015). The specific genetic drivers of tolerance are still unclear, but may be linked to genetically induced variation in phenology (McKinney et al., 2011; Stener, 2018). In addition, a suite of 20 gene expression markers associated with low susceptibility to *H. fraxineus* have been identified (Harper et al., 2016; Sollars et al., 2017), demonstrating that coding regions of the host genome are intrinsically involved in disease resistance.

Whilst selective breeding for tolerant genotypes may be desirable for timber production purposes, there are problems associated with this approach. Given the long generation time of trees, reduced genetic diversity could leave populations vulnerable to extinction through pathogen evolution as well as other emerging threats (e.g. emerald ash borer, *Agrilus planipennis*) (Jacobs, 2007). In addition, the proportion of trees tolerant to ash dieback are currently unknown but are likely to be very low, perhaps in the range of 1%–5% (McKinney et al., 2014; McMullan et al., 2018). Furthermore, mortality occurs most rapidly at the sapling stage, meaning selection pressure is very high and the pool of genetic diversity to draw from may be low. Thus, a more holistic understanding of the mechanisms of tolerance may assist the development of management strategies to maximize the regeneration potential of trees and forests at a local and landscape level. This approach will allow managers to identify tolerant individuals in the wider landscape, which could then form the basis of tree breeding programmes.

The plant microbiome forms an important component of disease tolerance. Host-microbiome interactions encompass a range

of types from antagonistic to mutualistic, however, the overwhelming benefits of a healthy microbiome are now clear, including protection from infectious diseases (Turner, James, & Poole, 2013). In several tree species, changes in microbiome composition in response to pathogenic infection have been observed (Busby, Peay, & Newcombe, 2016; Cross et al., 2017; Koskella, Meaden, Crowther, Leimu, & Metcalf, 2017), suggesting an interaction between the host microbiome and invasive pathogens. As such, interest is growing in the potential to engineer host microbiomes to enhance or induce microbially mediated traits (Foo, Ling, Lee, & Chang, 2017; Mueller & Sachs, 2015; Quiza, St-Arnaud, Yergeau, & Rey, 2015; Sheth, Cabral, Chen, & Wang, 2016; Yergeau et al., 2015). Identifying particular leaf endophytes that limit *H. fraxineus* infection may allow us to manipulate the leaf microbiome (i.e. the phyllosphere) for tree resistance. This could be achieved through a number of mechanisms including; selection of individuals based on microbial communities associated with host tolerance (Becker et al., 2015); addition of microbial inoculants that inhibit pathogenic growth (Marcano, Díaz-Alcántara, Urbano, & González-Andrés, 2016); alteration of environmental conditions that promote a desirable microbiome (Bender, Wagg, & van der Heijden, 2016; Thijs, Sillen, Rineau, Weyens, & Vangronsveld, 2016); or genetic modification of trees that alters signalling or selection traits that determine microbial community composition and function (Beckers et al., 2016). Culturing studies have identified a number of endophytic fungi of ash trees that inhibit the growth or germination of *H. fraxineus* and thus could be used as potential micro-biocontrol agents (Haňáčková, Havrdová, Černý, Zahradník, & Koukol, 2017; Kosawang et al., 2018; Schlegel et al., 2018; Schulz, Haas, Junker, André, & Schobert, 2015).

In order to implement such strategies, we first need to characterize the phyllosphere community in response to infection. Cross et al. (2017) previously showed fungal community composition in ash leaves altered as *H. fraxineus* infection intensified over time, however it is not clear if this was driven by infection dynamics or temporal variation across the season. In addition, the role of cross-kingdom (e.g. bacterial and fungal) interactions in determining microbiome function is of growing interest (Menezes, Richardson, & Thrall, 2017). For example, cross-kingdom interactions may be important for biofilm production on leaf surfaces (Frey-Klett et al., 2011; van Overbeek & Saikkonen, 2016), and fungal communities can influence bacterial community colonization via the modulation of carbon, nitrogen and environmental pH (Hassani, Durán, & Hacquard, 2018; Johnston, Hiscox, Savoury, Boddy, & Weightman, 2018). Thus, such interactions may be important for limiting pathogen invasion, although bacterial-fungal associations are not well-characterized in this context (but see Jakuschkin et al., 2016). There are also complex interactions between host genotype and microbiome composition (Aglar et al., 2016; Bálint et al., 2013; Griffiths et al., 2018; Smith, Snowberg, Gregory Caporaso, Knight, & Bolnick, 2015; Wagner et al., 2016) that can also alter pathogen susceptibility (Koch & Schmid-Hempel, 2012; Ritpitakphong et al., 2016). Understanding genetic influences on microbial community composition may allow us to use

these two powerful determinants of disease susceptibility in combination to maximize disease tolerance across populations.

Here, we integrate these genetic and microbial factors within one framework by using microsatellite characterization of host genotype, ITS rRNA and 16S rRNA sequencing to identify fungal and bacterial communities of leaves, qPCR to quantify *H. fraxineus* infection and phenotypic scoring of tree infection levels across two sites (Manchester and Stirling, UK) to. We aimed to: (a) identify differences in fungal and bacterial communities associated with ash leaves (i.e. the phyllosphere) according to *H. fraxineus* infection (at specific time points for multiple stands); (b) identify co-occurrence patterns between fungal and bacterial communities according to *H. fraxineus* infection; and (c) identify relationships between host genotype, phyllosphere composition and *H. fraxineus* infection intensity.

## 2 | MATERIALS AND METHODS

### 2.1 | Tree scoring, leaf sampling and DNA extraction

We conducted sampling and transport of ash material under Forestry Commission licence number FCPHS2/2016. We collected leaves from ash trees in semi-natural stands during the summer months from two areas. We sampled saplings from Balquhidderoch Wood in Stirling, Scotland (25th July 2016) and mature trees from multiple sections of the off-road National Cycle Route 6 in Manchester, England (the Fallowfield Loop, River Irwell and Drinkwater Park; 19th–25th August 2017). We sampled later in the season to maximize the potential for trees to have been exposed to *H. fraxineus*, and at both sites, widespread and epidemic levels of ash dieback were evident. We selected and scored trees displaying a range of ash dieback infection signs, from visibly clear of infection (infection score = 0) to heavily infected with extensive signs of ash dieback (infection score = 5). We collected leaves that were visibly clear of infection from 25 trees in Stirling (three leaves per tree) and 63 trees in Manchester (one leaf per tree) in sterile bags and froze these immediately in the field using dry ice. We transferred samples to a  $-20^{\circ}\text{C}$  freezer within 12 hr of collection, where they remained until DNA extraction. We weighed 50mg of leaf material and disrupted samples in a TissueLyser bead beater (Qiagen) for 2 min. We extracted DNA using the Qiagen DNeasy Plant MiniKit (along with two extraction blanks) according to the manufacturer's protocol, and used this DNA for all downstream molecular analyses.

### 2.2 | *Hymenoscyphus fraxineus* quantitative PCR

To quantify *H. fraxineus* infection, we conducted quantitative PCR (qPCR) on leaves according to a modified version of loos et al. (loos, Kowalski, Husson, & Holdenrieder, 2009) and loos & Fourrier (loos & Fourrier, 2011). Based on preliminary assessments of Ct values obtained during qPCRs (Cross et al., 2017), we diluted our DNA by a factor of 10. We conducted 10  $\mu\text{l}$  reactions using 0.4  $\mu\text{l}$  each of 10  $\mu\text{M}$  forward (5'-ATTATATTGTTGCTTTAGCAGGTC-3') and reverse (5'-TCCTCTAGCAGGCACAGTC-3') primers, 0.25  $\mu\text{l}$  of

8  $\mu\text{M}$  dual-labelled probe (5'-FAM-CTCTGGGCGTCGGCCTCG-MGBNFQ-3'), 5  $\mu\text{l}$  of QuantiNova PCR probe kit (Qiagen), 1.95  $\mu\text{l}$  of molecular grade water and 2  $\mu\text{l}$  of template DNA (~2ng). We used the following thermocycler conditions; initial denaturation of 95°C for 2 min followed by 50 cycles of 95°C for 10 s and 65°C for 30 s, using the green channel on a RotorGene Q real-time PCR machine (Qiagen). We included *H. fraxineus* standards ranging from 0.1 to 100 ng. We ran samples, standards and extraction blanks in duplicate and used the mean average of these for subsequent analyses. We multiplied the concentrations obtained from the qPCRs by the dilution factor of 10, and normalized the data for further analyses by calculating log concentrations using  $\log(H. fraxineus \text{ infection})+1$  (henceforth 'log *H. fraxineus* infection'). Additionally, based on the distribution of *H. fraxineus* qPCR data (Figure S1a-c), we assigned samples with infection categories of 'absent' for samples with 0 ng/ $\mu\text{l}$ ; 'low' for <200ng/ $\mu\text{l}$ ; 'medium' for 200 < 2000 ng/ $\mu\text{l}$  and 'high' for >2000 ng/ $\mu\text{l}$ .

### 2.3 | Host genotype characterization

To characterize tree genotype, we used 10 previously developed *F. excelsior* microsatellite markers (Brachet, Jubier, Richard, Jung-Muller, & Frascaria-Lacoste, 1998; Lefort, Brachet, Frascaria-Lacoste, Edwards, & Douglas, 1999; Harbourne, Douglas, Waldren & Hodkinson, 2005) (Table S1). We used a three-primer approach to fluorescently label PCR products (Neilan, Wilton, & Jacobs, 1997) using universal primers (Blacket, Robin, Good, Lee, & Miller, 2012; Culley et al., 2013) tagged with the fluorophores FAM, NED and PET (Table S1). We carried out PCRs in 10  $\mu\text{l}$  singleplex reactions using 5  $\mu\text{l}$  MyTaq Red Mix (Bioline), 1–10 ng DNA, 1  $\mu\text{M}$  of the 5' modified forward primer and 4  $\mu\text{M}$  each of the reverse primer and universal primer. PCR cycling conditions varied in annealing temperature among loci (Table S1), but otherwise consisted of an initial denaturation of 95°C for 3 min, 30 cycles of 95°C for 15 s, 46–60°C for 15 s and 72°C for 15 s, followed by a final extension step at 72°C for 5 min. PCR products for certain loci were then multiplexed for automated capillary electrophoresis, and the remaining loci were analysed separately (Table S1). Capillary electrophoresis was carried out at the University of Manchester Genomic Technologies Core Facility using a 3,730 DNA Sequencer (Thermo Fisher Scientific) with GeneScan 500 LIZ (Thermo Fisher Scientific).

We scored and binned alleles using GeneMapper v3.7 (ThermoFisher Scientific) and MsatAllele v1.05 (Alberto, 2009). One locus, CPFRA6, was monomorphic and was therefore removed from subsequent analyses. We estimated null allele frequency using the Expectation Maximization algorithm (Dempster, Laird, & Rubin, 1977) as implemented in FreeNA (Chapuis & Estoup, 2007). We removed loci with null allele frequencies above 20% for each site for individual-level heterozygosity analyses to reduce bias associated with false homozygotes. We also removed locus CPFRA5 from the Manchester data file as this was monomorphic. This made datasets of five loci and eight loci for Manchester and Stirling respectively. Five measures of individual-level heterozygosity (proportion of heterozygous loci, observed heterozygosity, expected heterozygosity,

internal relatedness and homozygosity by locus) were calculated using the *genhet* function (Coulon, 2010) in RStudio (v1.2.1335) (RStudio Team, 2016) for R (v3.4.1) (R Core Team, 2017).

Pairwise Euclidean genetic distances between trees were calculated for each site separately, and again together, using GenoDive v2.0b23 (Meirmans & Van Tienderen, 2004). As missing data can skew genetic distance calculations, we used GenoDive to impute missing data based on overall site allele frequencies prior to calculations. To investigate the presence of genetic differentiation in trees between sites, we estimated  $F_{ST}$  corrected for null alleles using ENA correction (Chapuis & Estoup, 2007) in FreeNA, and conducted an Analysis of Molecular Variance (AMOVA) in GenoDive using the least squares method. We also carried out a principle coordinates analysis (PCoA) in GenAlEx v6.503 (Peakall & Smouse, 2012) based on Euclidean distances using the standardized covariance method to visualize the variation in host genotype according to site.

### 2.4 | ITS 1F-2 and 16S V4 rRNA amplicon sequencing

To identify leaf fungal communities, we amplified DNA for the ITS 1F-2 rRNA gene (White, Bruns, Lee, & Taylor, 1990) using single indexed reverse primers and a modified protocol of Smith and Peay (2014) and Nguyen et al. (2014). Briefly, we ran PCRs in duplicate using Solis BioDyne 5x HOT FIREPol® Blend Master Mix, 2  $\mu\text{M}$  primers and 1.5  $\mu\text{l}$  of sample DNA. Thermocycling conditions used an initial denaturation at 95°C for 10 min, with 30 cycles of 95°C for 30 s, 52°C for 20 s and 72°C for 30 s, and a final extension at 72°C for 8 min. We combined PCR replicates into a single PCR plate and cleaned products using HighPrep™ PCR clean up beads (MagBio) according to the manufacturer's instructions. We quality checked the PCR products using an Agilent TapeStation 2200. To quantify the number of sequencing reads per sample, we constructed a library pool using 1  $\mu\text{l}$  of each sample. We ran a titration sequencing run with this pool using an Illumina v2 nano cartridge (paired-end reads; 2  $\times$  150 bp) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) on an Illumina MiSeq at the University of Salford. Based on the percentage of reads sequenced per library, we calculated the volume required for the full sequencing run and pooled these accordingly. Full ITS rRNA amplicon sequencing was conducted using paired-end reads with an Illumina v3 (2  $\times$  300 bp) cartridge on an Illumina MiSeq. We also included negative (extraction blanks) and positive (fungal mock community and *H. fraxineus*) controls.

To identify bacterial communities in leaves, we amplified DNA for the 16S rRNA V4 region using dual indexed forward and reverse primers according to Kozich et al. (Kozich et al., 2013) and Griffiths et al. (Griffiths et al., 2018). We ran PCRs in duplicate as described above, using thermocycling conditions of 95°C for 15 min, followed by 28 cycles of 95°C for 20 s, 50°C for 60 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. To quantify individual libraries, we again pooled 1  $\mu\text{l}$  of each library and sequenced this using

an Illumina v2 nano cartridge as described above, then pooled samples according to read coverage and conducted a full paired-end sequencing run using Illumina v2 (2 × 250 bp) chemistry. We included extraction blanks and a mock bacterial community as negative and positive controls respectively.

## 2.5 | Pre-processing of amplicon sequencing data

We trimmed remaining adapters and primers for ITS rRNA sequencing data using cutadapt (Martin, 2011). This step was not required for 16S rRNA sequencing data. Unless otherwise stated, we conducted all subsequent data processing and analysis in RStudio (see supplementary files for full code).

A total of 6,346,506 raw sequence reads from 139 samples were generated during ITS sequencing. We conducted ITS rRNA gene amplicon sequence processing in DADA2 v1.5.0 (Callahan et al., 2016). Modal contig length was 181 bp (range 75–315 bp) once paired-end reads were merged. We did not conduct additional trimming based on sequence length as the ITS region is highly variable (Schoch et al., 2012). No contaminants were identified in the negative controls. We removed chimeras and assigned taxonomy using the UNITE v7.2 database (UNITE, 2017). We obtained a median of 29,043 reads per sample. We exported the final exact sequence variant (ESV) table, taxonomy table and sample metadata to the PHYLOSEQ package (McMurdie & Holmes, 2013). DADA2 identified 12 unique sequence variants in the sequenced mock community sample comprising 12 fungal isolates.

A total of 4,055,595 raw sequence reads from 139 samples were generated during 16S rRNA sequencing. As with ITS rRNA amplicon data, we conducted 16S rRNA gene amplicon sequence processing in DADA2 v1.5.0. Modal contig length was 253 bp once paired-end reads were merged. We removed ESVs with length >260 bp (78 ESVs; 0.026% of total sequences) along with chimeras and two ESVs found in the negative controls. We assigned taxonomy using the SILVA v128 database (Quast et al., 2013; Yilmaz et al., 2014). We stripped out chloroplasts and mitochondria from ash leaf samples, and removed 31 samples for which no sequence data remained, leaving a median of 2,930 reads per sample. We exported the final ESV table, taxonomy table and sample metadata to phyloseq. DADA2 identified 20 unique sequence variants in the sequenced mock community sample comprising 20 bacterial isolates.

## 2.6 | Phyllosphere composition by site and *H. fraxineus* infection

We converted the ESV abundance data of individual samples to relative abundances for fungi and bacteria separately. We produced box plots visualizing the variation in relative abundance of the top 10 most abundant classes according to site and *H. fraxineus* infection category as described above (i.e. 'absent', 'low', 'medium', or 'high'). We conducted a permutational ANOVA (PERMANOVA; adonis) in the VEGAN package (Oksanen et al., 2018) to determine the variation in fungal and bacterial community composition according to site and *H. fraxineus* infection category, and produced PCoA plots using

Bray-Curtis dissimilarity matrices in phyloseq. We calculated alpha-diversity measures (species richness and community evenness) for each sample by subsampling the raw ESV count table to a standardized number of reads (equal to the sample with the lowest number of reads) using an iterative approach (100 times), and averaged the diversity estimates from each trial. In addition, as a measure of beta-diversity, we extracted PCoA scores for axes 1 and 2 obtained from ordinating relative abundance data for each sample, as described previously. To determine the relationship between these microbial community measures and *H. fraxineus* infection intensity and tree infection score, we used separate linear mixed models in the LME4 package (Bates, Mächler, Bolker, & Walker, 2014), with tree ID and site as random factors and log *H. fraxineus* infection or tree score as the response variable. We used the *associate* function in the MICROBIOME package (Lahti & Shetty, 2017) to identify cross-correlation between the centred log ratios of microbial genera and log *H. fraxineus* infection using Spearman's rank correlation. We constructed a heatmap in ggplot2 (Wickham, 2009) to visualise statistically-significant taxa (that were successfully identified to genus level) according to their correlation coefficients.

As samples from Manchester included both infected ( $n = 36$ ) and uninfected ( $n = 27$ ) leaves (whereas all samples from Stirling were infected; see Results), we subsetted the Manchester samples for further analyses that aimed to identify microbial genera associated with the presence or absence of infection. We agglomerated microbial data to genus level and calculated the relative abundance of each taxon, then conducted an indicator analysis using the *multipatt* function in the INDICESPECIES package (Cáceres & Legendre, 2009) to identify microbial genera associated with the presence or absence of *H. fraxineus* in leaves. Finally, we conducted a DESeq2 analysis (Love, Huber, & Anders, 2014) to identify ESVs with significantly different abundances according to infection status of the leaves.

## 2.7 | Functional analysis of fungal communities

To identify the trophic modes and functional guilds of fungal ESVs, we extracted the ESV table of all samples complete with taxonomic annotation and uploaded this to the online FUNGuild tool (Nguyen et al., 2016). We plotted stacked bar charts to visualize the variation in relative abundance of trophic mode and guild representations according to *H. fraxineus* infection category.

## 2.8 | Relationships between fungal and bacterial communities

To identify relationships between fungal and bacterial communities, we extracted Jensen-Shannon divergence matrices between all samples for both fungal and bacterial communities in the phyloseq and VEGAN packages. We used Mantel tests to correlate fungal and bacterial community distances and visualized the relationship using a scatter plot.

To identify co-occurrence networks between taxa according to *H. fraxineus* infection category in the Manchester samples, we

rarefied fungal communities to 14,080 reads and bacterial communities to 800 reads (resulting in the loss of three samples). We merged these rarefied phyloseq objects for bacterial and fungal communities and converted them to binary presence/absence data. We then calculated the co-occurrence between each pair of ESVs by constructing a Spearman's correlation coefficient matrix in the `bioDist` package (Ding, Gentleman, & Carey, 2018; Williams, Howe, & Hofmockel, 2014). We calculated the number of associations with  $p < .05$  for each infection category (absent, low, medium and high), and those with  $-0.50 > \rho > 0.50$ , and  $-0.75 > \rho > 0.75$ . We visualized those with  $\rho > 0.75$  (positive associations only) using network plots for the four infection categories.

## 2.9 | Relationships between tree genotype and phyllosphere composition, and tree genotype and *H. fraxineus* infection

For the Stirling samples, we used the `merge_samples` function in phyloseq to calculate the mean phyllosphere composition across the three leaf samples collected per tree and converted the per-tree values to relative abundance (for the Manchester samples we only collected one leaf per tree and so this step was not necessary). To measure pairwise microbial community dissimilarities among trees, we extracted Jensen–Shannon divergence matrices between trees for both fungal and bacterial communities using phyloseq and `vegan`. We created separate datasets for each site, as well as a combined dataset. We then used Mantel tests to test for correlations between the microbial distance matrices and tree genetic distance matrices (as calculated above).

To identify relationships between *H. fraxineus* infection and host genotype, we used individual GLMM in `lme4` (with site as a random factor) to determine whether multiple measures of genetic diversity (proportion of heterozygous loci, observed heterozygosity, expected heterozygosity, internal relatedness and homozygosity by locus) influenced tree infection score and average log *H. fraxineus* infection intensity.

## 3 | RESULTS

### 3.1 | *H. fraxineus* prevalence

We found variable *H. fraxineus* infection prevalence between sites. All samples collected at Stirling were infected, including trees that showed no visible signs of infection (i.e. tree infection score of 0), whereas in Manchester, 20 out of the 33 (60.6%) trees sampled were infected.

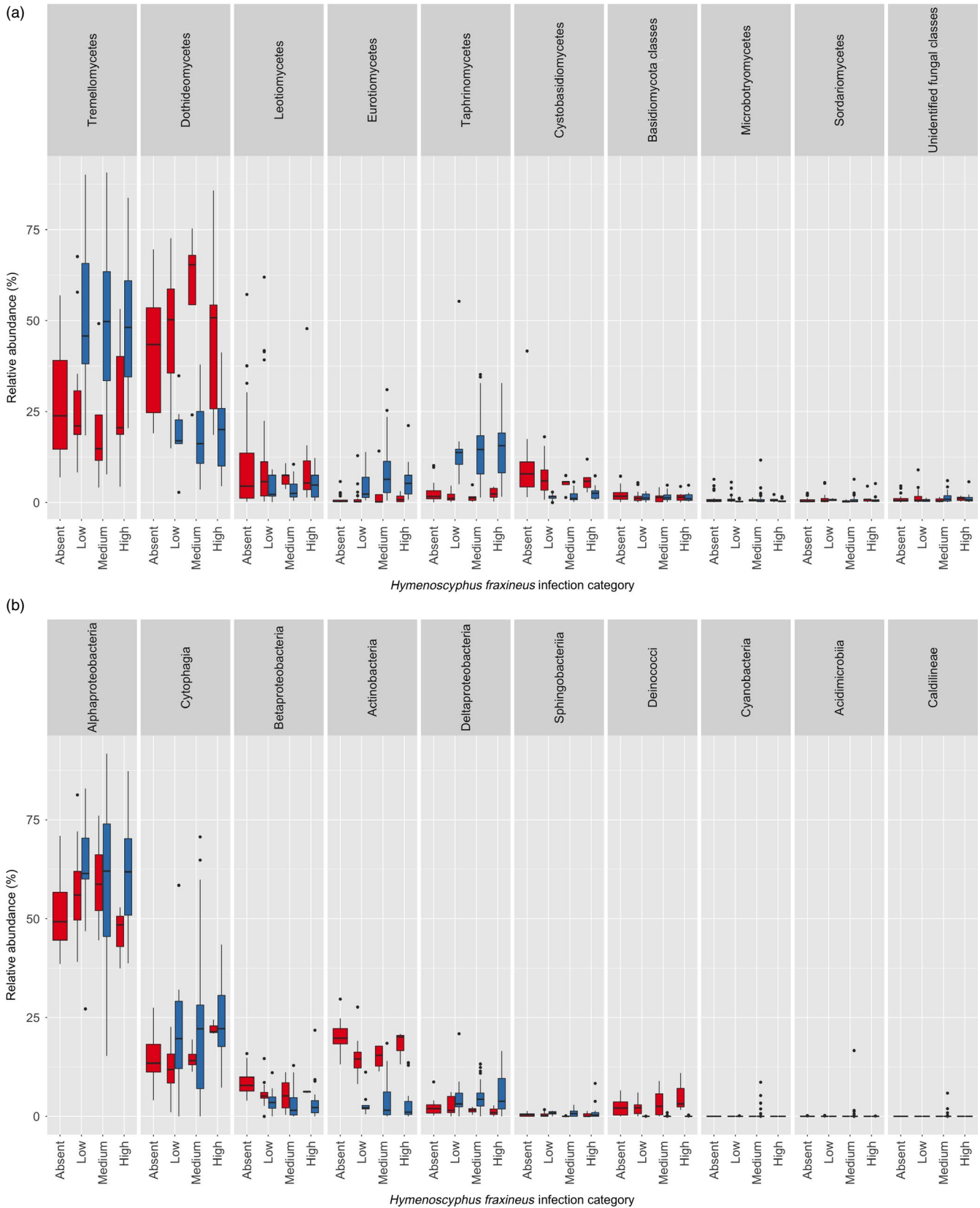
### 3.2 | Phyllosphere composition by site and *H. fraxineus* infection

The most abundant fungal classes across all samples were Tremellomycetes, Dothideomycetes, Leotiomycetes, Eurotiomycetes, Taphrinomycetes and Cystobasidiomycetes (Figure 1a). The most

abundant bacterial classes were Alphaproteobacteria, Cytophagia, Betaproteobacteria, Actinobacteria, Deltaproteobacteria, Sphingobacteria and Deinococci (Figure 1b). PERMANOVA (adonis) analysis showed a significant effect of site (i.e. Manchester or Stirling;  $F_{1,136} = 34.615$ ,  $R^2 = 0.204$ ,  $p = .001$ ) but not *H. fraxineus* infection category (i.e. 'absent', 'low', 'medium' or 'high') ( $F_{3,136} = 1.061$ ,  $R^2 = 0.019$ ,  $p = .342$ ) (Figure 2a) on fungal community composition. Similarly, site had a significant effect on bacterial community composition ( $F_{1,105} = 25.968$ ,  $R^2 = 0.199$ ,  $p = .001$ ) but *H. fraxineus* infection category did not ( $F_{3,105} = 1.088$ ,  $R^2 = 0.025$ ,  $p = .301$ ) (Figure 2b). Site explained a similar proportion of the variation in fungal (20.4%) and bacterial (19.9%) communities, whereas *H. fraxineus* infection category explained only 1.9% and 2.5% of fungal and bacterial community composition respectively. The relative abundance of the top 10 most abundant fungal (Figure 1a) and bacterial (Figure 1b) classes were considerably different between sites. Within sites, there were also differences in the relative abundance of different taxa according to *H. fraxineus* infection category, however, there were no clear patterns in how these groups varied between these categories, either within sites or across sites (Figure 1a,b).

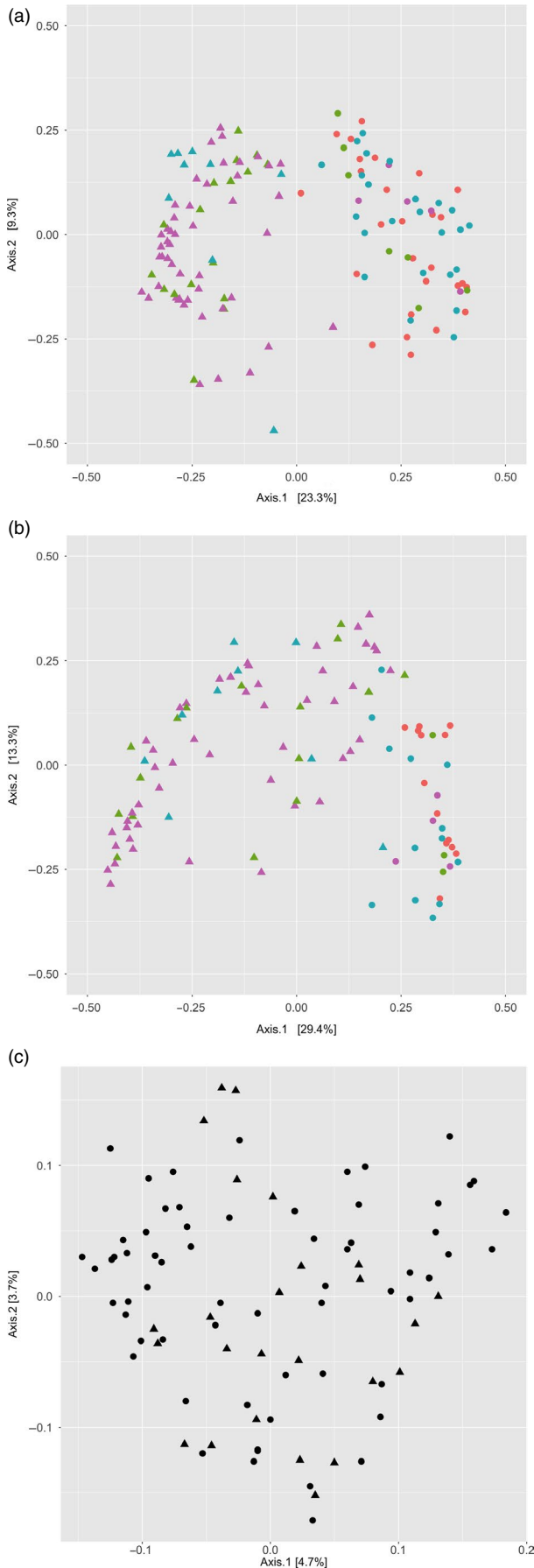
In the linear mixed models, fungal community alpha-diversity significantly predicted *H. fraxineus* infection intensity in terms of both community richness ( $X^2 = 4.560$ ,  $p = .033$ ; Figure 3a) and evenness ( $X^2 = 3.932$ ,  $p = .047$ ; Figure 3b). In both cases, as fungal community alpha-diversity increased, so did *H. fraxineus* infection. Relationships were not statistically significant between log *H. fraxineus* infection and bacterial community alpha-diversity (richness,  $X^2 = 0.787$ ,  $p = .375$ ; evenness,  $X^2 = 0.509$ ,  $p = .475$ ). There was a significant relationship between log *H. fraxineus* infection and fungal community beta-diversity (PCoA axis 1 score,  $X^2 = 39.528$ ,  $p < .001$ , Figure 3c; PCoA axis 2 score,  $X^2 = 5.511$ ,  $p = .019$ ), and log *H. fraxineus* infection and bacterial community beta-diversity (PCoA axis 1 score;  $X^2 = 5.4606$ ,  $p = .019$ ; Figure 3d). However, tree infection score was not significantly predicted by any microbial diversity measure (all  $p > .05$ ).

We identified 26 fungal genera (out of 390) and six bacterial genera (out of 255) with significant positive correlations with log *H. fraxineus* infection intensity (all  $p < .05$ ; Figure 4). We also identified 217 fungal genera and four bacterial genera with a significant negative correlation with log *H. fraxineus* infection intensity (all  $p < .05$ ; Figure 4). Indicator analysis only identified one fungal genus (*Neofabraea*,  $\text{IndVal} = 0.378$ ,  $p = .025$ ) and one bacterial genus (*Pedobacter*,  $\text{IndVal} = 0.643$ ,  $p = .005$ ) that were significantly associated with the absence of *H. fraxineus* infection (i.e. these genera were much more commonly found in the absence of infection). Association analysis identified two fungal genera significantly associated with the presence of *H. fraxineus* infection (*Hannaella*,  $\text{IndVal} = 0.525$ ,  $p = .050$ ; *Keissleriella*,  $\text{IndVal} = 0.450$ ,  $p = .020$ ). DESeq2 analysis did not identify any differentially abundant bacterial ESVs between infected and uninfected leaves (Figure S2), but did for fungal ESVs; *Phyllactinia fraxini* was significantly more abundant in uninfected leaves ( $\log_2\text{FoldChange} = -24.429$ ,  $p < .001$ ) and one *Genolevuria* sp. was significantly more abundant in infected leaves



**FIGURE 1** Relative abundance of (a) fungal classes and (b) bacterial classes in ash tree leaves from Manchester (red) and Stirling (blue) across four different *Hymenoscyphus fraxineus* infection categories





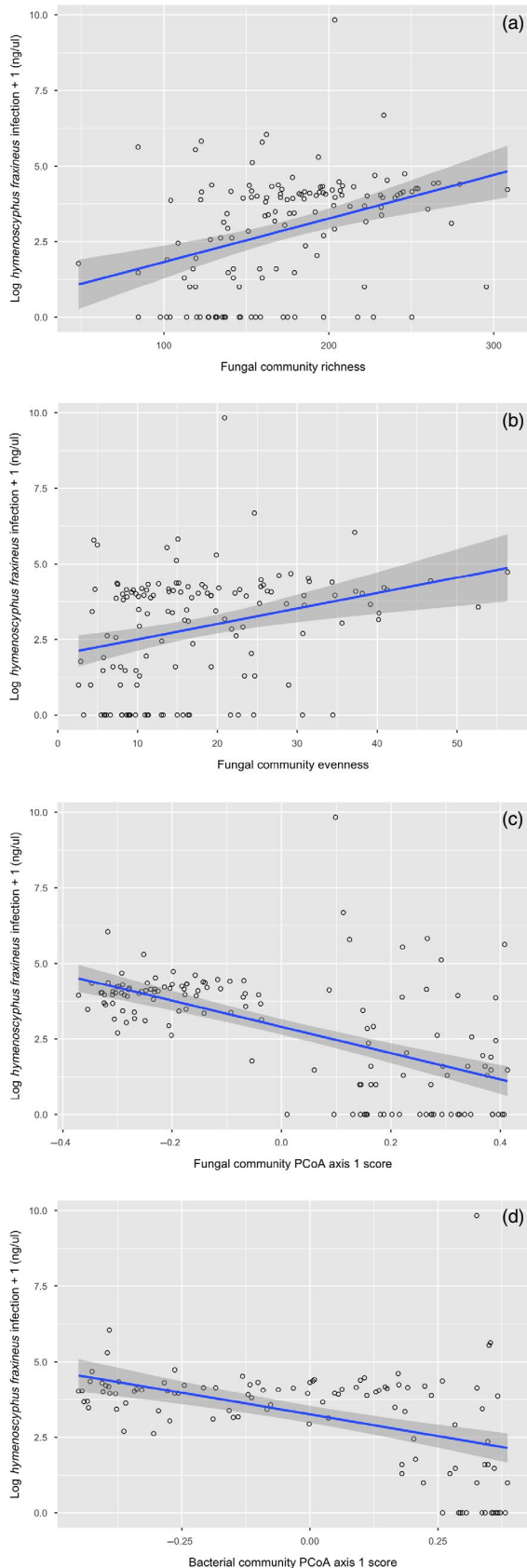
**FIGURE 2** PCoA plots for Bray–Curtis distances among (a) fungal communities and (b) bacterial communities of ash tree leaves collected from Manchester (circles) and Stirling (triangles) with either absent (red), low (blue), medium (purple) or high (green) *Hymenoscyphus fraxineus* infection; (c) PCoA plot of Euclidean genetic distances between ash trees in Manchester (circles) and Stirling (triangles), calculated using multilocus microsatellite genotypes

(log<sub>2</sub>FoldChange = 3.753,  $p < .001$ ; Figure S3). For both fungi and bacteria, however, the DESeq2 analysis indicated there was no clear pattern of ESVs within genera showing particular patterns in abundance according to *H. fraxineus* infection. That is, genus is not an accurate indicator of anti-pathogen capabilities (Figures S2 and S3).

The genus *Hymenoscyphus* had a significant positive correlation with *H. fraxineus* infection intensity ( $r = 0.375$ ,  $p < .001$ ; Figure 4). Although six species of *Hymenoscyphus* were identified (*H. scutula*, *repandus*, *menthae*, *albidus*, *kathiae*, *caudatus*) to species level through ITS rRNA amplicon sequencing, as well as one other unidentified *Hymenoscyphus* sp. that was found at low prevalence and abundance, *H. fraxineus* itself was not found in our ITS rRNA dataset. However, the amplicon produced by ITS rRNA sequencing of DNA extracted from a pure *H. fraxineus* culture was not identified by UNITE (UNITE, 2017). Further NCBI BLAST searches of all the unidentified *Hymenoscyphus* and *Chalara* sequences in addition to unidentified sequences belonging to Fungi, Ascomycota, Leotiomycetes, Helotiales or Helotiaceae identified an additional 18 ESVs in our dataset as *H. fraxineus* (E value  $< e^{-20}$ , bit score  $> 80$ ). However, five of these were removed during filtering of low read numbers, and the remainder did not sum up to more than 0.001% in any of the samples. Therefore, despite high infection loads quantified through targeted qPCR, *H. fraxineus* did not appear to be present in our ITS rRNA amplicon sequencing data to any substantial degree.

### 3.3 | Functional analysis of fungal communities

We obtained functional hypotheses for 65% of ITS rRNA ESVs. Functional analysis of fungal communities indicated that the relative abundance of pathotrophs (fungi causing disease and receiving nutrients at the expense of host cells; Nguyen et al., 2016; Tedersoo et al., 2014) increased as *H. fraxineus* infection intensity increased (Figure 5 and Figure S4). However, the proportion of fungal species with unidentified trophic modes were higher in the absent and low infection categories (Figure 5). Despite this, the most abundant pathogen, *Phyllactinia fraxini*, had a relatively high abundance in leaves absent of infection (7.0%) and with low infection levels (6.2%), compared to medium (0.1%) and high (1.7%) infection levels. The genus *Phyllactinia* also had a significant negative correlation with log *H. fraxineus* infection intensity ( $r = -0.378$ ,  $p < .001$ ) although the negative relationship between log *H. fraxineus* (+1) and log *P. fraxini* (+1) was only approaching significance ( $r = -.15$ ,  $p = .077$ ). Overall, *P. fraxini* was the most abundant pathogen and



**FIGURE 3** Relationship between *Hymenoscyphus fraxineus* infection and (a) fungal community richness, (b) fungal community evenness (Inverse Shannon), (c) fungal community beta-diversity, and (d) bacterial community beta-diversity

the fifth most abundant fungus across all samples (*Vishniacozyma foliicola*, *V. victoriae* and two species of Venturiales were more abundant; Table S2). The second most abundant pathogen was the yeast *Itersonilia pannonica* (formerly *Udeniomyces pannonicus*; Niwata, Takashima, Tornai-Lehoczki, Deak, & Nakase, 2002), which in contrast to *P. fraxini*, had lower abundance in leaves with absent (0.2%) and low (1.2%) *H. fraxineus* infection than in those with medium (6.5%) and high (8.1%) infection levels. Correlation analysis indicated a significant positive relationship between log *H. fraxineus* (+1) and log *I. pannonica* (+1) ( $r = .49, p < .001$ ). In addition, the relative abundance of symbiotrophs (which receive nutrients through exchange with host cells), primarily lichens, also increased on infection by *H. fraxineus* (Figure 5; Figure S4).

### 3.4 | Relationships between fungal and bacterial communities

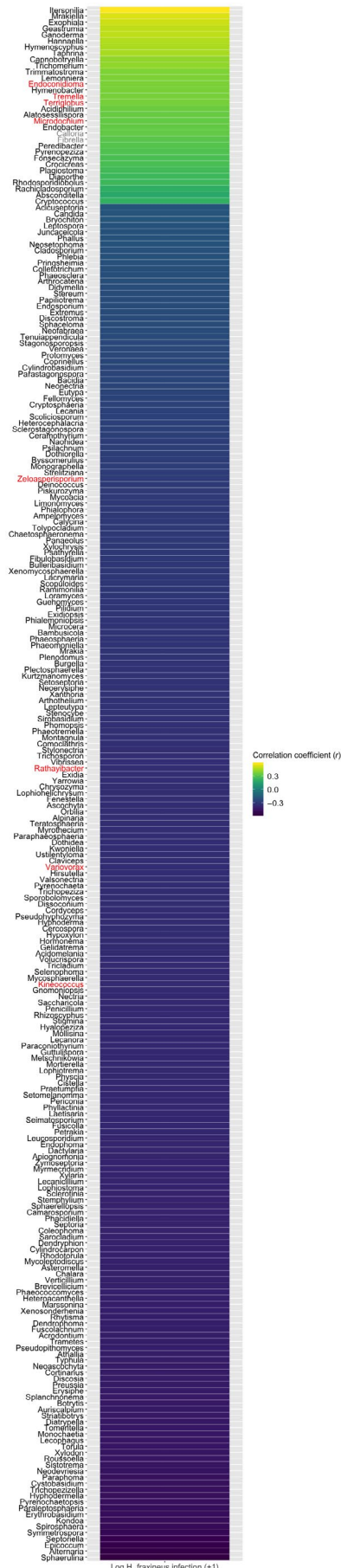
Mantel tests identified significant correlations between fungal and bacterial communities of leaves across both sites ( $r = .552, p = .001$ ; Figure 6a). Co-occurrence analysis indicated that leaves highly infected with *H. fraxineus* had fewer statistically significant ( $p < .05$ ) cross-kingdom microbial connections than the other infection categories (Table 1). The majority of microbial associations in the uninfected categories were of medium strength ( $-0.50 > \rho$  and  $\rho > 0.50$ ) rather than strong ( $-0.75 > \rho$  and  $\rho > 0.75$ ), and were characterized by sprawling, less well-connected hubs with a considerable number of members (Table 1; Figure 7). The proportion of strong microbial connections increased as *H. fraxineus* infection increased, and in highly infected leaves, 100% of associations were strong and positive ( $\rho > 0.75$ ), but characterized by very few, strongly associated larger hubs involving relatively few members (Table 1; Figure 7; Table S3).

### 3.5 | Effects of host genotype on phyllosphere composition and *H. fraxineus* infection

We found very little genetic differentiation between trees in Stirling and Manchester;  $F_{ST}$  between the sites was 0.034, while an AMOVA showed that only 2.4% of total genetic variation was found between sites (Table S3), with little clustering of sites in the PCoA (Figure 2c).

Across sites, there was a significant correlation between genetic distance and fungal community composition ( $r = .106, p = .005$ ; Figure 6b), but no significant relationship between genetic distance and bacterial community composition ( $r = .013, p = .365$ ). Within sites, the correlation between tree genetic distance and fungal community composition was statistically significant for Manchester ( $r = .155, p = .002$ ) but not Stirling ( $r = .042, p = .372$ ). Genetic distance was not significantly correlated with bacterial community composition at either site (Manchester:  $r = -.065, p = .749$ ; Stirling:  $r = .151, p = .091$ ).

No heterozygosity measures significantly predicted *H. fraxineus* infection intensity or tree infection score (all  $p > .05$ ).



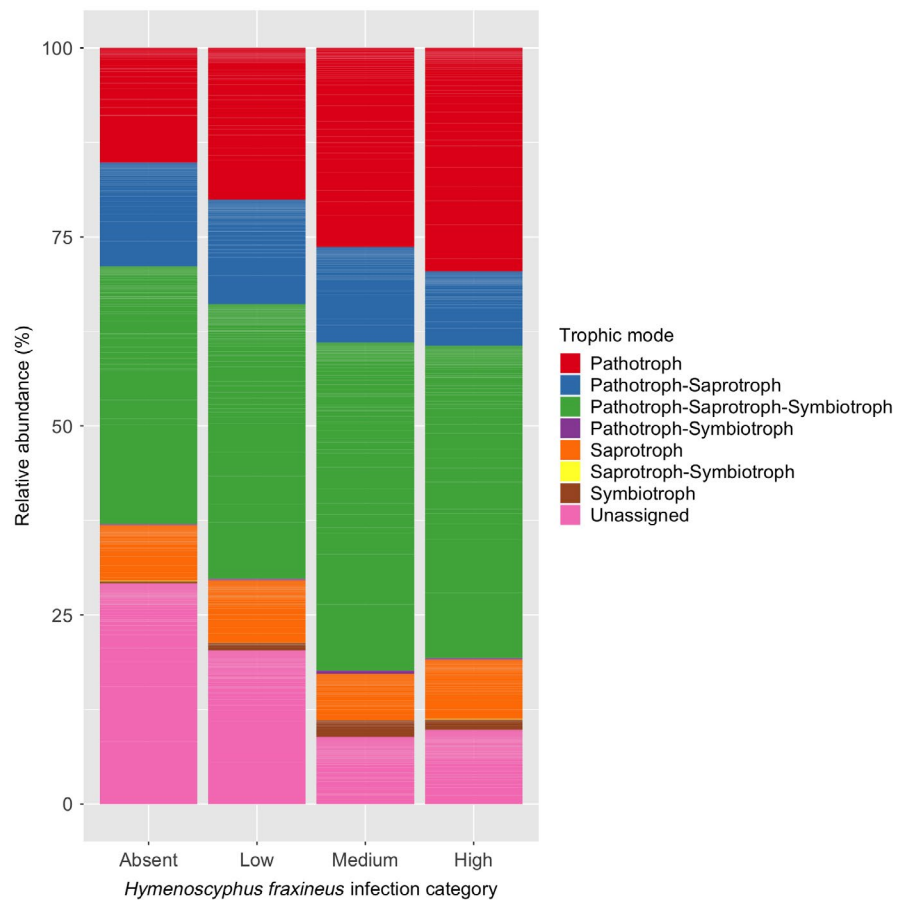
**FIGURE 4** Heatmap of fungal (black text) and bacterial (red text) genera significantly associated with *Hymenoscyphus fraxineus* infection intensity in ash tree leaves

## 4 | DISCUSSION

Our results show that both fungal and bacterial community composition, as well as a considerable number of microbial genera, are significantly correlated with *H. fraxineus* infection intensity. Cross et al. (2017) previously demonstrated that fungal community composition altered as the season progressed and *H. fraxineus* infection intensified, although it was not clear whether these changes resulted from seasonal effects or infection intensity. We extend this work to show that at a given time point, differences in both fungal and bacterial phyllosphere communities relate to *H. fraxineus* infection, even in the absence of phenotypic signs of infection. These effects were apparent in our mixed model analysis, but not significant in the PERMANOVA analysis; this may be due to a loss of statistical power from the use of infection categories (i.e., ‘absent’, ‘low’, ‘medium’ or ‘high’) in the PERMANOVA rather than the continuous log *H. fraxineus* data used in the linear mixed model analysis. Changes in microbiome composition that correlate with pathogenic infection have also been identified in other tree species. For example, the bacterial microbiome of horse chestnut bark was altered by bleeding canker disease caused by the bacterium *Pseudomonas syringae* pv *aesculi* (Koskella et al., 2017). Similarly, Jakuschkin et al. (2016) found evidence of cross-kingdom endophytic dysbiosis in pedunculate oak (*Quercus robur* L.) on infection by *Erysiphe alphitoides*, the causal agent of oak powdery mildew.

Fungal alpha-diversity was positively correlated with *H. fraxineus* infection intensity, although bacterial alpha-diversity was not. Although it may be expected that higher microbiome diversity would increase microbiome-mediated resistance to invasive pathogens through competitive exclusion, the relationship between microbiome diversity and pathogen susceptibility actually varies considerably among host taxa (e.g. Bates et al., 2018; Dillon, Vennard, Buckling, & Charnley, 2005; Johnson & Hoverman, 2012; Nöpflin & Schmid-Hempel, 2018; Upreti & Thomas, 2015; Wehner, Antunes, Powell, Mazukatow, & Rillig, 2010). Our results suggest that low diversity may reflect a stable and resilient microbiome that resists infection, or that *H. fraxineus* infection is associated with dysbiosis that allows for the proliferation of many new members in the microbiome. Indeed, co-occurrence analysis showed that medium-strength, minimally connected networks in leaves with absent or low *H. fraxineus* infection become a few, high-strength, highly connected hubs under medium or high infection. The co-occurrence analysis indicates that although *H. fraxineus* infection is associated with strong microbial networks, these are relatively depauperate in members and so the stability of phyllosphere communities in infected leaves may be compromised. Conversely, leaves with absent or low infection rates have more complex co-occurrence hubs with more medium-strength

**FIGURE 5** Functional analysis of trophic modes of fungal ESVs associated with ash leaves with varying degrees of *Hymenoscyphus fraxineus* infection

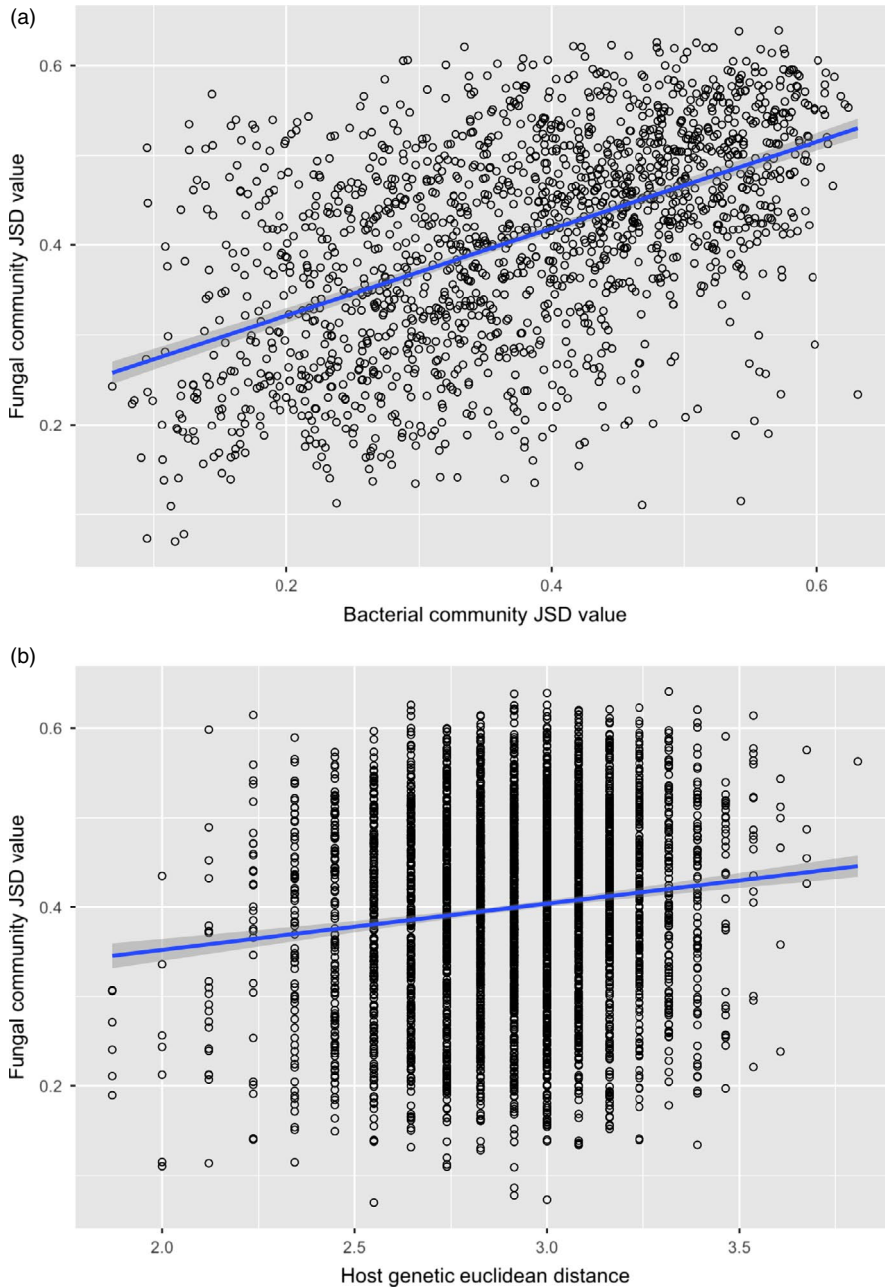


connections involving more members. Together with the higher fungal diversity observed as *H. fraxineus* infection intensity increased, these results suggest *H. fraxineus* infection is associated with dysbiosis in ash leaves that allows for the proliferation of microbial phyllosphere endophytes. Furthermore, Cross et al. (2017) suggested that phyllosphere communities are not significantly altered by *H. fraxineus* until a particular infection density is reached, and our findings support this. We also show that even leaves with high infection intensities can appear asymptomatic but exhibit evidence of phyllosphere dysbiosis, although it is not clear whether such dysbiosis is a result of infection, or in fact facilitates infection. Although causality can be hard to identify without explicit infection trials, leaves in this study were collected late in the season in areas of epidemic infection. This suggests leaves that were clear of infection at the time of sampling may have been able to resist infection up to that point, and so patterns identified here may be representative of real-world infection trials.

There is other evidence that associations between plants and microbes become stronger when the host is stressed, with positive effects for the host (Mendes et al., 2011; Pineda, Dicke, Pieterse, & Pozo, 2013). For example, plants can exploit beneficial microbes when under water or nutrient stress, with positive effects on plant growth and insect attack (Pineda et al., 2013). How the networks identified in our data influence the host to improve resistance to *H. fraxineus* remains to be explored. We also identify

considerable covariation between bacterial and fungal communities and extensive cross-kingdom associations in the leaves of ash trees. Syntrophy (i.e. cross-feeding between microbial species) is phylogenetically and environmentally widespread throughout microbial taxa and leads to high connectedness between members of the microbiome (Hassani et al., 2018; Kouzuma, Kato, & Watanabe, 2015; McInerney et al., 2008). Furthermore, nutrient and pH modulation by fungal communities can influence bacterial colonization (Hassani et al., 2018; Johnston et al., 2018). Thus, such interactions between these two kingdoms may be expected, and the importance of these in the context of disease resistance warrants considerable attention.

The functional analysis identified an overall increase in fungal pathogens as *H. fraxineus* infection increased. Disruption to the natural endosymbiont community by *H. fraxineus* infection may break up previously filled niches, thus allowing co- or secondary infections. Alternatively, prior infection by other pathogens may allow *H. fraxineus* to proliferate. In particular, we found convincing evidence of co-infection by *Itersonilia pannonica*, a likely yeast pathogen (Nguyen et al., 2016). Other secondary infections have previously been documented in ash dieback outbreaks, including *Alternaria alternata*, *Armillaria* spp., *Cytospora pruinosa*, *Diaporthe eres*, *Diplodia mutila*, *Fusarium avenaceum*, *Fusarium lateritium*, *Fusarium solani*, *Phoma exigua*, *Phytophthora* spp. and *Valsa ambiens* (Kowalski, Kraj, & Bednarz, 2016; Marçais, Husson, Godart, & Caël, 2016; Orlikowski



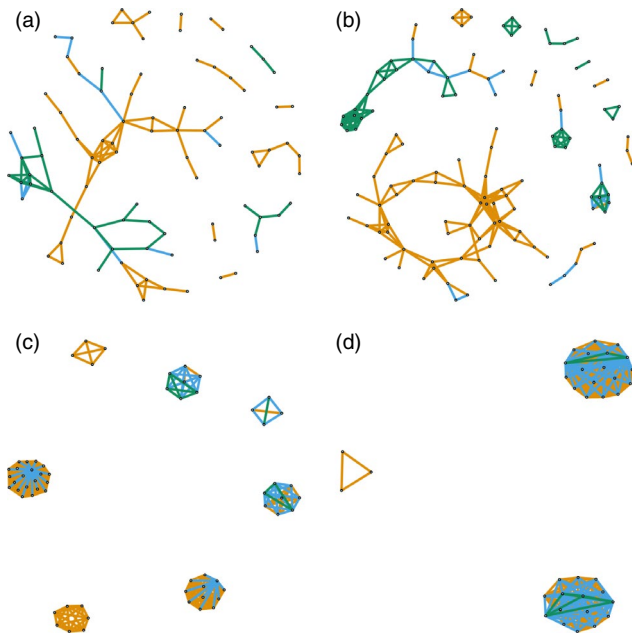
**FIGURE 6** Relationship between (a) Jensen-Shannon divergence values of fungal communities and bacterial communities associated with ash tree leaves, and (b) Jensen-Shannon divergence values of fungal communities and Euclidean genetic distance of ash trees

**TABLE 1** Number of statistically significant ( $p < .05$ ) microbial associations (proportions in brackets) and number of hubs (small = 10 or less members, large = more than 10 members) for various correlation strengths identified using co-occurrence analysis for leaves across four different *Hymenoscyphus fraxineus* infection categories

<i>H. fraxineus</i> infection category	Number of associations	Number of associations ( $-0.50 > \rho$ and $\rho > 0.50$ )	Number of associations ( $-0.75 > \rho$ and $\rho > 0.75$ )	Number of associations ( $\rho > 0.75$ )	Number of hubs ( $\rho > 0.75$ )
Absent	604	604 (100%)	136 (23%)	107 (18%)	12 (10 small, 2 large)
Low	892	892 (100%)	254 (28%)	212 (24%)	13 (11 small, 2 large)
Medium	505	505 (100%)	505 (100%)	347 (69%)	7 (5 small, 2 large)
High	261	261 (100%)	261 (100%)	261 (100%)	3 (1 small, 2 large)

et al., 2011). Co-infection can have considerable implications for host fitness and the evolution of pathogens (Tollenaere, Susi, & Laine, 2016), and may well contribute to the progression of ash

dieback. Similar findings have been shown in other study systems, whereby disruption of the resident microbiome allows other microbial groups to proliferate (Antwis, Garcia, Fidgett, & Preziosi, 2014;



**FIGURE 7** Co-occurrence networks between fungi and bacteria in the leaves of ash trees with varying degrees of *Hymenoscyphus fraxineus* infection; (a) absent; (b) low; (c) medium and (d) high. Edges coloured orange indicate fungi-fungi associations, those coloured green indicate bacteria-bacteria associations, and those in blue are fungi-bacteria associations

Erkosar & Leulier, 2014; Kamada, Chen, Inohara, & Núñez, 2013; Liu, Liu, Ran, Hu, & Zhou, 2016). *Hymenoscyphus fraxineus* infection also appeared to be associated with the growth of fungal symbiotrophs, particularly lichens. Mitchell et al. (2014) identified 548 lichen species associated with *F. excelsior*, indicating that such associations are common for this host. Converse to these positive associations between *H. fraxineus* and other microbes, we saw a reduction in the pathogen *Phyllactinia fraxini* as *H. fraxineus* increased, suggesting the latter may displace the former. *Phyllactinia fraxini* is an ecto-parasitic fungus that causes powdery mildew in ash trees (Takamatsu et al., 2008). The rapid outcompeting of one pathogen by another has been termed a 'selective sweep' and is well-documented in plant hosts, particularly crops (Zhan & McDonald, 2013). These results are contrary to Cross et al. (2017), who found *Phyllactinia* positively correlated with *H. fraxineus* infection, indicating that further research is required to improve our understanding of the interactions between *H. fraxineus* and other pathogens.

Based on a combination of analyses, we identified *Neofabraea* fungi and *Pedobacter* bacteria as potential antagonists of *H. fraxineus* infection, which may have potential for development of anti-pathogenic inoculants or probiotics. *Neofabraea* has previously been shown to inhibit *H. fraxineus* in vitro (Schlegel et al., 2018). Given the large number of microbial genera present in the leaves, it is surprising that only three genera showed significant association with the absence of *H. fraxineus*, despite widespread and heavy infection in the study sites. This finding may reflect the propensity for wide variation within genera for anti-pathogen capabilities (Antwis & Harrison,

2018; Becker et al., 2015), as indicated by the DESeq2 analysis, in which ESVs within a genus did not necessarily show the same type of response (i.e. positive or negative) to *H. fraxineus*. Thus, although we identify potential genera of interest, a genus-by-genus approach may not be the best method for identifying potential probiotics. In vitro studies have identified over 70 species of fungi that inhibit the growth of *H. fraxineus* (Kosawang et al., 2018; Schlegel et al., 2018; Schulz et al., 2015). In addition, secondary metabolite production by endophytes is generally down-regulated when cultured individually but activated in response to other microbes (Schroeckh et al., 2009; Suryanarayanan, 2013), indicating complex and bi-directional interactions between members of the phyllosphere microbiome. Thus, co-culturing such microbes, potentially identified through co-occurrence hubs, may help guide the development of consortium-based approaches to probiotic development, which may be more effective than single-species probiotics (Antwis & Harrison, 2018; Kaminsky, Trexler, Malik, Hockett, & Bell, 2018; Schulz et al., 2015).

We did not find evidence of host genotype influencing tree infection score or *H. fraxineus* pathogen loads. Host genetic variation has previously been found to influence ash dieback susceptibility (Harper et al., 2016; Sollars et al., 2017). However, these studies used genomic and transcriptomic approaches that give finer resolution than microsatellite markers allow. Furthermore, microsatellites cover non-coding regions of DNA and so may be less likely to directly affect pathogen susceptibility, although they are often physically linked to genes that code for functional traits (Santucci, Ibrahim, Bruzzone, & Hewit, 2007; Gemayel, Vinces, Legendre, & Verstrepen, 2010; Tollenaere et al., 2012). Host genetic distance did, however, predict variation in fungal community composition (both across sites and within Manchester, but not within Stirling alone). Thus, microsatellites used in this study may be linked to functional traits that influence phyllosphere fungal communities. As such, host genetic influence on phyllosphere fungal communities could indirectly influence *H. fraxineus* susceptibility. The expression of a number of MADS box genes varies between susceptible and tolerant genotypes of ash trees, which may influence secondary metabolite production (Gantet & Memelink, 2002; Sollars et al., 2017) and thus, influence microbial community diversity on the leaf. Furthermore, higher iridoid glycoside concentrations were identified from biochemical profiles of leaves from susceptible ash trees, which may alter fungal growth (Sollars et al., 2017; Whitehead, Tiramani, & Bowers, 2016). Identifying genes associated microbiome composition in ash trees will allow us to determine whether these can be used along with host genetic markers to improve selection of tolerant trees and thus increase the pool from which selective breeding could occur.

Sampling site was the main predictor of total community composition for both fungal and bacterial communities of ash leaves. Considerable variation in phyllosphere composition still existed between the sites despite the Stirling and Manchester trees being genetically similar, indicating that site-level variation was not due to population differentiation. Both fine- and broad-scale geographic variation affects microbiome composition in many study

organisms (Antwis, Lea, Unwin, & Shultz, 2018; Griffiths et al., 2018; Yatsunenkov et al., 2012) including plants (Edwards et al., 2015; Peiffer et al., 2013; Wagner et al., 2016). The site-level differences observed in this study may reflect a range of differences in abiotic conditions, given that environmental variables, such as temperature and rainfall are considerable determinants of both microbiome composition and pathogen activity (Barge, Leopold, Peay, Newcombe, & Busby, 2019; Busby, Newcombe, Dirzo, & Whitham, 2014; Busby, Ridout, & Newcombe, 2016; Dal Maso & Montecchio, 2014; Laforest-Lapointe, Messier, & Kembel, 2016; Zimmerman & Vitousek, 2012). Methodological differences could also be responsible – in Stirling, we sampled saplings whereas in Manchester we sampled mature trees. Tree and leaf age both significantly affect phyllosphere microbiome structure, possibly due to microbial community succession patterns, as well as niche variation associated with age-related physiological changes in leaves (Redford & Fierer 2009; Meaden, Metcalf, & Koskella, 2016). Thus, site-level patterns in our data may reflect these considerable drivers of microbiome composition. Alternatively, there may well have been different isolates of *H. fraxineus* at the two sites, which may have differentially affected leaf microbial communities through isolate variation in enzyme profiles and growth rates (Junker, de Vries, Eickhorst, & Schulz, 2017). We also observed variation in the strength of genotype  $\times$  microbiome interactions between sites. This may be due to environmental differences, and thus could indicate the presence of genotype by microbiome by environment ( $G \times M \times E$ ) interactions (Smith et al., 2015).  $G \times M \times E$  interactions may be particularly important for disease susceptibility and mitigation as environment plays a considerable role in pathogenicity. Thus, microbially derived resistance to *H. fraxineus*, in addition to the effectiveness of any microbial treatments, may be population, age or site specific, and may vary between sites based on environmental and biological variables, including abiotic factors as well as pollution, tree density, species mix and herbivore activity. Much more work is required to determine how environmental factors and pathogen strain variation affect microbially derived resilience to *H. fraxineus* infection, and identifying cross-population and cross-isolate microbial signatures of resistance will be key to the success of a microbial-based approach to disease management.

It is worth noting that we did not identify *H. fraxineus* itself to species level using ITS rRNA amplicon sequencing (or through additional BLAST searches), despite qPCR demonstrating widespread and high infection rates. Cross et al. (2017) found similar results when using ITS-1. This may be because *H. fraxineus* (or its many strains) is not fully represented in the UNITE database, or because *H. fraxineus* has a long fragment length (~550 bp) for the primer combination we used, which would be less readily sequenced than shorter reads on the Illumina MiSeq platform (Lindahl et al., 2013). As with all amplicon sequencing, there are limitations to the taxa that can be identified based on the primers used, and wider analysis using multiple markers will identify further genera involved in *H. fraxineus* infection dynamics on ash leaves (Cross et al., 2017; Lindahl et al., 2013).

In conclusion, we show that bacterial and fungal communities of ash leaves are strongly associated with one another, and the composition of both is associated with *H. fraxineus* infection dynamics. Leaves with absent or low infection rates have more complex microbial co-occurrence hubs characterized by medium-strength connections involving many members, whereas under medium to high infection levels, microbial networks were characterized by stronger associations between fewer members and with fewer hubs. This suggests that after a particular infection pressure is reached, phyllosphere communities become disrupted. Although host genotype did not affect *H. fraxineus* infection directly, it did have a significant effect on fungal community composition and thus, may have indirect consequences for pathogen susceptibility. Identifying host genes that determine microbiome composition in ash trees may improve selection of trees with more resistant microbiomes, which in combination with host genetic markers of tolerance, may increase the proportion of ash trees from which selective breeding could occur.

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## AUTHORS' CONTRIBUTIONS

R.E.A., S.M.G., J.H. and D.O'B. conceived the study. R.E.A., S.M.G., M.G. and J.R. produced the data. R.E.A., S.M.G. and I.G. analysed the data. All authors wrote and approved the manuscript.

## DATA AVAILABILITY STATEMENT

Sequence data for this project are available from the NCBI Sequence Read Archive (project numbers PRJNA515030 and PRJNA515031) and microsatellite genotypes are available at: <https://doi.org/10.6084/m9.figshare.7599902>. All analysis code has been provided as RMarkdown files.

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## SUPPORTING INFORMATION

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