

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

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16

17 **KEYWORDS:** bacteria, fungi, microbiome, tree pathogen, *Hymenoscyphus fraxineus*, *Fraxinus*

18 *excelsior*, amplicon sequencing, genetic diversity

19 **ABSTRACT**

- 20 1. Tree pathogens are a major threat to forest ecosystems. Conservation management  
21 strategies can exploit natural mechanisms of resistance, such as tree genotype and host-  
22 associated microbial communities. However, fungal and bacterial communities are rarely  
23 looked at in the same framework, particularly in conjunction with host genotype. Here, we  
24 explore these relationships and their influence on ash dieback disease, caused by the  
25 pathogen *Hymenoscyphus fraxineus*, in European common ash trees.
- 26 2. We collected leaves from UK ash trees and used microsatellite markers to genotype trees,  
27 qPCR to quantify *H. fraxineus* infection load, and ITS and 16S rRNA amplicon sequencing to  
28 identify fungal and bacterial communities, respectively.
- 29 3. There was a significant association between *H. fraxineus* infection intensity and ash leaf  
30 fungal and bacterial community composition. Higher infection levels were positively correlated  
31 with fungal community alpha diversity, and a number of fungal and bacterial genera were  
32 significantly associated with infection presence and intensity. Under higher infection loads,  
33 leaf microbial networks were characterised by stronger associations between fewer members  
34 than those associated with lower infection levels. Together these results suggest that *H.*  
35 *fraxineus* disrupts stable endophyte communities after a particular infection threshold is  
36 reached, and may enable proliferation of opportunistic microbes. We identified three microbial  
37 genera associated with an absence of infection, potentially indicating an antagonistic  
38 relationship with *H. fraxineus* that could be utilised in the development of anti-pathogen  
39 treatments.
- 40 4. Host genotype did not directly affect infection, but did significantly affect leaf fungal  
41 community composition. Thus, host genotype could have the potential to indirectly affect  
42 disease susceptibility through genotype x microbiome interactions, and should be considered  
43 when selectively breeding trees.
- 44 5. *Synthesis.* We show the diversity, composition and network structure of ash leaf microbial  
45 communities are associated with the severity of infection from ash dieback disease, with  
46 evidence of disease-induced dysbiosis. We also show that host genotype influences leaf  
47 fungal community composition, but does not directly influence tree infection. These findings  
48 help to elucidate relationships between host genetics, the microbiome, and a tree pathogen,

49 highlighting potential resistance mechanisms and possible co-infection concerns that could  
50 inform ash tree management.  
51

52 **INTRODUCTION**

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

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

74 A range of silvicultural and arboricultural management practices have been suggested for ash  
75 dieback mitigation, such as increasing local tree species diversity, removing infected tissue and/or  
76 autumn leaf fall, reducing tree density, and applying fungicides (Hrabětová, Černý, Zahradník, &  
77 Havrdová, 2017; Skovsgaard et al., 2017). However, such methods may be expensive, labour-  
78 intensive, and damaging to the environment. Exploiting natural host resistance mechanisms offers a  
79 promising alternative, which may provide a more long-term solution whilst avoiding some of these  
80 disadvantages.

81 Ash dieback resistance has a strong host genetic component; nearly 50% of phenotypic  
82 variation in crown damage is based on host genotype (McKinney et al., 2014; McKinney, Nielsen,  
83 Hansen, & Kjær, 2011; Muñoz, Marçais, Dufour, & Dowkiw, 2016). Furthermore, progeny from low-  
84 susceptibility mother clones exhibit lower symptoms of disease, indicating a heritable basis for  
85 tolerance (Lobo, McKinney, Hansen, Kjær, & Nielsen, 2015). The specific genetic drivers of tolerance  
86 are still unclear, but may be linked to genetically-induced variation in phenology (McKinney et al.,  
87 2011; Stener, 2018). In addition, a suite of 20 gene expression markers associated with low  
88 susceptibility to *H. fraxineus* have been identified (Harper et al., 2016; Sollars et al., 2017),  
89 demonstrating that coding regions of the host genome are intrinsically involved in disease resistance.

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

102 The plant microbiome forms an important component of disease tolerance. Host-microbiome  
103 interactions encompass a range of types from antagonistic to mutualistic, however, the overwhelming  
104 benefits of a healthy microbiome are now clear, including protection from infectious diseases (Turner  
105 et al., 2013). In several tree species, changes in microbiome composition in response to pathogenic  
106 infection have been observed (Busby, Peay, & Newcombe, 2016; Cross et al., 2017; Koskella,  
107 Meaden, Crowther, Leimu, & Metcalf, 2017), suggesting an interaction between the host microbiome  
108 and invasive pathogens. As such, interest is growing in the potential to engineer host microbiomes to  
109 enhance or induce microbially-mediated traits (Foo, Ling, Lee, & Chang, 2017; Mueller & Sachs,  
110 2015; Quiza, St-Arnaud, Yergeau, & Rey, 2015; Sheth, Cabral, Chen, & Wang, 2016; Yergeau et al.,

111 2015). Identifying particular leaf endophytes that limit *H. fraxineus* infection may allow us to  
112 manipulate the leaf microbiome (i.e. the phyllosphere) for tree resistance. This could be achieved  
113 through a number of mechanisms including; selection of individuals based on microbial communities  
114 associated with host tolerance (Becker et al., 2015); addition of microbial inoculants that inhibit  
115 pathogenic growth (Marcano, Díaz-Alcántara, Urbano, & González-Andrés, 2016); alteration of  
116 environmental conditions that promote a desirable microbiome (Bender, Wagg, & van der Heijden,  
117 2016; Thijs, Sillen, Rineau, Weyens, & Vangronsveld, 2016); or genetic modification of trees that  
118 alters signalling or selection traits that determine microbial community composition and function  
119 (Beckers et al., 2016). Culturing studies have identified a number of endophytic fungi of ash trees that  
120 inhibit the growth or germination of *H. fraxineus* and thus could be used as potential micro-biocontrol  
121 agents (Haňáčková, Havrdová, Černý, Zahradník, & Koukol, 2017; Kosawang et al., 2018; Schlegel,  
122 Dubach, Buol, & Sieber, 2016; Schulz, Haas, Junker, Andrée, & Schobert, 2015).

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

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

149

## 150 **MATERIALS AND METHODS**

### 151 *Tree Scoring, Leaf Sampling and DNA extraction*

152 We conducted sampling and transport of ash material under Forestry Commission licence number  
153 FCPHS2/2016. We collected leaves from ash trees in semi-natural stands during the summer months  
154 from two areas. We sampled saplings from Balquhiddelock Wood in Stirling, Scotland (25<sup>th</sup> July 2016)  
155 and mature trees from multiple sections of the off-road National Cycle Route 6 in Manchester,  
156 England (the Fallowfield Loop, River Irwell and Drinkwater Park; 19<sup>th</sup> – 25<sup>th</sup> August 2017). We  
157 sampled later in the season to maximise the potential for trees to have been exposed to *H. fraxineus*,  
158 and at both sites, widespread and epidemic levels of ash dieback were evident. We selected and  
159 scored trees displaying a range of ash dieback infection signs, from visibly clear of infection (infection  
160 score = 0) through to heavily infected with extensive signs of ash dieback (infection score = 5). We  
161 collected leaves that were visibly clear of infection from 25 trees in Stirling (three leaves per tree) and  
162 63 trees in Manchester (one leaf per tree) in sterile bags and froze these immediately in the field  
163 using dry ice. We transferred samples to a -20°C freezer within 12 hours of collection, where they  
164 remained until DNA extraction. We weighed 50mg of leaf material and disrupted samples in a  
165 TissueLyser bead beater (Qiagen) for two minutes. We extracted DNA using the Qiagen DNeasy  
166 Plant MiniKit (along with two extraction blanks) according to the manufacturers protocol, and used this  
167 DNA for all downstream molecular analyses.

168

### 169 *Hymenoscyphus fraxineus* quantitative PCR

170 To quantify *H. fraxineus* infection, we conducted quantitative PCR (qPCR) on leaves according to a  
171 modified version of loos et al. (loos, Kowalski, Husson, & Holdenrieder, 2009) and loos & Fourrier  
172 (loos & Fourrier, 2011). Based on preliminary assessments of Ct values obtained during qPCRs  
173 (Cross et al., 2017), we diluted our DNA by a factor of 10. We conducted 10µl reactions using 0.4µl  
174 each of 10µM forward (5'-ATTATATTGTTGCTTTAGCAGGTC-3') and reverse (5'-  
175 TCCTCTAGCAGGCACAGTC- 3') primers, 0.25µl of 8µM dual-labelled probe (5'-FAM-  
176 CTCTGGGCGTCGGCCTCG-MGBNFQ-3'), 5µl of QuantiNova PCR probe kit (Qiagen), 1.95µl of  
177 molecular grade water and 2µl of template DNA (~2ng). We used the following thermocycler  
178 conditions; initial denaturation of 95°C for 2 minutes followed by 50 cycles of 95°C for 10 seconds and  
179 65°C for 30 seconds, using the green channel on a RotorGene Q real-time PCR machine (Qiagen).  
180 We included *H. fraxineus* standards ranging from 0.1 to 100 ng. We ran samples, standards and  
181 extraction blanks in duplicate and used the mean average of these for subsequent analyses. We  
182 multiplied the concentrations obtained from the qPCRs by the dilution factor of 10, and normalised the  
183 data for further analyses by calculating log concentrations using  $\log(H. fraxineus \text{ infection})+1$   
184 (henceforth "log *H. fraxineus* infection"). Additionally, based on the distribution of *H. fraxineus* qPCR  
185 data (Figures S1a-c), we assigned samples with infection categories of "absent" for samples with 0  
186 ng/µl; "low" for <200ng/µl; "medium" for 200 < 2000 ng/µl and "high" for > 2000 ng/µl.

187

#### 188 *Host genotype characterisation*

189 To characterise tree genotype, we used 10 previously developed *F. excelsior* microsatellite markers  
190 (Brachet, Jubier, Richard, Jung-Muller, & Frascaria-Lacoste, 1998; Lefort, Brachet, Frascaria-Lacoste,  
191 Edwards, & Douglas, 1999; Harbourne, Douglas, Waldren & Hodkinson, 2005) (Table S1). We used a  
192 three-primer approach to fluorescently label PCR products (Neilan, Wilton, & Jacobs, 1997) using  
193 universal primers (Blacket, Robin, Good, Lee, & Miller, 2012; Culley et al., 2013) tagged with the  
194 fluorophores FAM, NED and PET (Table S1). We carried out PCRs in 10µl singleplex reactions using  
195 5µl MyTaq Red Mix (Bioline), 1-10 ng DNA, 1µM of the 5' modified forward primer and 4µM each of  
196 the reverse primer and universal primer. PCR cycling conditions varied in annealing temperature  
197 among loci (Table S1), but otherwise consisted of an initial denaturation of 95°C for 3 minutes, 30  
198 cycles of 95°C for 15 seconds, 46-60°C for 15 seconds and 72°C for 15 seconds, followed by a final  
199 extension step at 72°C for 5 minutes. PCR products for certain loci were then multiplexed for



200 automated capillary electrophoresis, and the remaining loci were analysed separately (Table S1).  
201 Capillary electrophoresis was carried out at the University of Manchester Genomic Technologies Core  
202 Facility using a 3730 DNA Sequencer (Thermo Fisher Scientific) with GeneScan 500 LIZ (Thermo  
203 Fisher Scientific).

204 We scored and binned alleles using GeneMapper v3.7 (ThermoFisher Scientific) and  
205 MsatAllele v1.05 (Alberto, 2009). One locus, CPFRA6, was monomorphic and was therefore  
206 removed from subsequent analyses. We estimated null allele frequency using the Expectation  
207 Maximization algorithm (Dempster, Laird, & Rubin, 1977) as implemented in FreeNA (Chapuis &  
208 Estoup, 2007). We removed loci with null allele frequencies above 20% for each site for individual-  
209 level heterozygosity analyses to reduce bias associated with false homozygotes. We also removed  
210 locus CPFRA5 from the Manchester data file as this was monomorphic. This made datasets of five  
211 loci and eight loci for Manchester and Stirling, respectively. Five measures of individual-level  
212 heterozygosity (proportion of heterozygous loci, observed heterozygosity, expected heterozygosity,  
213 internal relatedness and homozygosity by locus) were calculated using the *genhet* function (Coulon,  
214 2010) in RStudio (v1.2.1335) (RStudio Team, 2016) for R (v3.4.1) (R Core Team, 2017).

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

224

#### 225 *ITS 1F-2 and 16S V4 rRNA amplicon sequencing*

226 To identify leaf fungal communities, we amplified DNA for the ITS 1F-2 rRNA gene (White, Bruns,  
227 Lee, & Taylor, 1990) using single indexed reverse primers and a modified protocol of Smith & Peay  
228 (Smith & Peay, 2014) and Nguyen et al. (Nguyen, Smith, Peay, & Kennedy, 2014). Briefly, we ran  
229 PCRs in duplicate using Solis BioDyne 5x HOT FIREPol® Blend Master Mix, 2µM primers and 1.5µl of

230 sample DNA. Thermocycling conditions used an initial denaturation at 95°C for 10 minutes, with 30  
231 cycles of 95°C for 30 seconds, 52°C for 20 seconds, and 72°C for 30 seconds, and a final extension  
232 at 72°C for 8 minutes. We combined PCR replicates into a single PCR plate and cleaned products  
233 using HighPrep™ PCR clean up beads (MagBio) according to the manufacturers' instructions. We  
234 quality checked the PCR products using an Agilent TapeStation 2200. To quantify the number of  
235 sequencing reads per sample, we constructed a library pool using 1 µl of each sample. We ran a  
236 titration sequencing run with this pool using an Illumina v2 nano cartridge (paired end reads; 2 x  
237 150bp) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) on an Illumina MiSeq at the  
238 University of Salford. Based on the percentage of reads sequenced per library, we calculated the  
239 volume required for the full sequencing run and pooled these accordingly. Full ITS rRNA amplicon  
240 sequencing was conducted using paired-end reads with an Illumina v3 (2 x 300bp) cartridge on an  
241 Illumina MiSeq. We also included negative (extraction blanks) and positive (fungal mock community  
242 and *H. fraxineus*) controls.

243 To identify bacterial communities in leaves, we amplified DNA for the 16S rRNA V4 region  
244 using dual indexed forward and reverse primers according to Kozich et al. (Kozich et al., 2013) and  
245 Griffiths et al. (Griffiths et al., 2018). We ran PCRs in duplicate as described above, using  
246 thermocycling conditions of 95°C for 15 minutes, followed by 28 cycles of 95°C for 20 seconds, 50°C  
247 for 60 seconds, and 72°C for 60 seconds, with a final extension at 72°C for 10 minutes. To quantify  
248 individual libraries, we again pooled 1 µl of each library and sequenced this using an Illumina v2 nano  
249 cartridge as described above, then pooled samples according to read coverage and conducted a full  
250 paired-end sequencing run using Illumina v2 (2 x 250 bp) chemistry. We included extraction blanks  
251 and a mock bacterial community as negative and positive controls, respectively.

252

### 253 *Pre-processing of amplicon sequencing data*

254 We trimmed remaining adapters and primers for ITS rRNA sequencing data using cutadapt (Martin,  
255 2011). This step was not required for 16S rRNA sequencing data. Unless otherwise stated, we  
256 conducted all subsequent data processing and analysis in RStudio (see supplementary files for full  
257 code).

258 A total of 6,346,506 raw sequence reads from 139 samples were generated during ITS  
259 sequencing. We conducted ITS rRNA gene amplicon sequence processing in DADA2 v1.5.0

260 (Callahan et al., 2016). Modal contig length was 181bp (range 75-315bp) once paired-end reads were  
261 merged. We did not conduct additional trimming based on sequence length as the ITS region is highly  
262 variable (Schoch et al., 2012). No contaminants were identified in the negative controls. We removed  
263 chimeras and assigned taxonomy using the UNITE v7.2 database (UNITE, 2017). We obtained a  
264 median of 29,043 reads per sample. We exported the final exact sequence variant (ESV) table,  
265 taxonomy table and sample metadata to the phyloseq package (McMurdie & Holmes, 2013). DADA2  
266 identified 12 unique sequence variants in the sequenced mock community sample comprising 12  
267 fungal isolates.

268 A total of 4,055,595 raw sequence reads from 139 samples were generated during 16S rRNA  
269 sequencing. As with ITS rRNA amplicon data, we conducted 16S rRNA gene amplicon sequence  
270 processing in DADA2 v1.5.0. Modal contig length was 253bp once paired-end reads were merged.  
271 We removed ESVs with length >260bp (78 ESVs; 0.026% of total sequences) along with chimeras  
272 and two ESVs found in the negative controls. We assigned taxonomy using the SILVA v128 database  
273 (Quast et al., 2013; Yilmaz et al., 2014). We stripped out chloroplasts and mitochondria from ash leaf  
274 samples, and removed 31 samples for which no sequence data remained, leaving a median of 2930  
275 reads per sample. We exported the final ESV table, taxonomy table and sample metadata to  
276 phyloseq. DADA2 identified 20 unique sequence variants in the sequenced mock community sample  
277 comprising 20 bacterial isolates.

278

#### 279 *Phyllosphere composition by site and H. fraxineus infection*

280 We converted the ESV abundance data of individual samples to relative abundances for fungi and  
281 bacteria separately. We produced box plots visualising the variation in relative abundance of the top  
282 10 most abundant classes according to site and *H. fraxineus* infection category as described above  
283 (i.e. “absent”, “low”, “medium”, or “high”). We conducted a permutational ANOVA (PERMANOVA;  
284 adonis) in the vegan package (Oksanen et al., 2018) to determine the variation in fungal and bacterial  
285 community composition according to site and *H. fraxineus* infection category, and produced PCoA  
286 plots using Bray-Curtis dissimilarity matrices in phyloseq. We calculated alpha-diversity measures  
287 (species richness and community evenness) for each sample by subsampling the raw ESV count  
288 table to a standardised number of reads (equal to the sample with the lowest number of reads) using  
289 an iterative approach (100 times), and averaged the diversity estimates from each trial. In addition, as

290 a measure of beta-diversity, we extracted PCoA scores for axes 1 and 2 obtained from ordinating  
291 relative abundance data for each sample, as described previously. To determine the relationship  
292 between these microbial community measures and *H. fraxineus* infection intensity and tree infection  
293 score, we used separate linear mixed models in the lme4 package (Bates, Mächler, Bolker, & Walker,  
294 2014), with tree ID and site as random factors, and log *H. fraxineus* infection or tree score as the  
295 response variable. We used the *associate* function in the microbiome package (Lahti & Shetty, 2017)  
296 to identify cross-correlation between the centred log ratios of microbial genera and log *H. fraxineus*  
297 infection using Spearman's rank correlation. We constructed a heatmap in ggplot2 (Wickham, 2009)  
298 to visualise statistically-significant taxa (that were successfully identified to genus level) according to  
299 their correlation coefficients.

300 As samples from Manchester included both infected (n = 36) and uninfected (n = 27) leaves  
301 (whereas all samples from Stirling were infected; see Results), we subsetted the Manchester samples  
302 for further analyses that aimed to identify microbial genera associated with the presence or absence  
303 of infection. We agglomerated microbial data to genus level and calculated the relative abundance of  
304 each taxon, then conducted an indicator analysis using the *multipatt* function in the indicpecies  
305 package (Cáceres & Legendre, 2009) to identify microbial genera associated with the presence or  
306 absence of *H. fraxineus* in leaves. Finally, we conducted a DESeq2 analysis (Love, Huber, & Anders,  
307 2014) to identify ESVs with significantly different abundances according to infection status of the  
308 leaves.

309

### 310 *Functional analysis of fungal communities*

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

315

### 316 *Relationships between fungal and bacterial communities*

317 To identify relationships between fungal and bacterial communities, we extracted Jensen-Shannon  
318 divergence matrices between all samples for both fungal and bacterial communities in the phyloseq

319 and vegan packages. We used Mantel tests to correlate fungal and bacterial community distances  
320 and visualised the relationship using a scatter plot.

321 To identify co-occurrence networks between taxa according to *H. fraxineus* infection category  
322 in the Manchester samples, we rarefied fungal communities to 14080 reads, and bacterial  
323 communities to 800 reads (resulting in the loss of three samples). We merged these rarefied phyloseq  
324 objects for bacterial and fungal communities and converted them to binary presence/absence data.  
325 We then calculated the co-occurrence between each pair of ESVs by constructing a Spearman's  
326 correlation coefficient matrix in the bioDist package (Ding, Gentleman, & Carey, 2018; Williams,  
327 Howe, & Hofmockel, 2014). We calculated the number of associations with  $p < 0.05$  for each infection  
328 category (absent, low, medium and high), and those with  $-0.50 > \rho > 0.50$ , and  $-0.75 > \rho > 0.75$ .  
329 We visualised those with  $\rho > 0.75$  (positive associations only) using network plots for the four  
330 infection categories.

331

332 *Relationships between tree genotype and phyllosphere composition, and tree genotype and H.*  
333 *fraxineus infection*

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

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

347

348

349 **RESULTS**

350 *H. fraxineus* prevalence

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

354

355 *Phyllosphere composition by site and H. fraxineus* infection

356 The most abundant fungal classes across all samples were Tremellomycetes, Dothideomycetes,  
357 Leotiomycetes, Eurotiomycetes, Taphrinomycetes and Cystobasidiomycetes (Figure 1a). The most  
358 abundant bacterial classes were Alphaproteobacteria, Cytophagia, Betaproteobacteria,  
359 Actinobacteria, Deltaproteobacteria, Sphingobacteriia, and Deinococci (Figure 1b). PERMANOVA  
360 (adonis) analysis showed a significant effect of site (i.e. Manchester or Stirling;  $F_{1,136} = 34.615$ ,  $R^2 =$   
361  $0.204$ ,  $p = 0.001$ ) but not *H. fraxineus* infection category (i.e. “absent”, “low”, “medium” or “high”)  
362 ( $F_{3,136} = 1.061$ ,  $R^2 = 0.019$ ,  $p = 0.342$ ) (Figure 2a) on fungal community composition. Similarly, site  
363 had a significant effect on bacterial community composition ( $F_{1,105} = 25.968$ ,  $R^2 = 0.199$ ,  $p = 0.001$ )  
364 but *H. fraxineus* infection category did not ( $F_{3,105} = 1.088$ ,  $R^2 = 0.025$ ,  $p = 0.301$ ) (Figure 2b). Site  
365 explained a similar proportion of the variation in fungal (20.4%) and bacterial (19.9%) communities,  
366 whereas *H. fraxineus* infection category explained only 1.9% and 2.5% of fungal and bacterial  
367 community composition, respectively. The relative abundance of the top 10 most abundant fungal  
368 (Figure 1a) and bacterial (Figure 1b) classes were considerably different between sites. Within sites,  
369 there were also differences in the relative abundance of different taxa according to *H. fraxineus*  
370 infection category, however, there were no clear patterns in how these groups varied between these  
371 categories, either within sites or across sites (Figures 1a and 1b).

372 In the linear mixed models, fungal community alpha-diversity significantly predicted *H.*  
373 *fraxineus* infection intensity in terms of both community richness ( $X^2 = 4.560$ ,  $p = 0.033$ ; Figure 3a)  
374 and evenness ( $X^2 = 3.932$ ,  $p = 0.047$ ; Figure 3b). In both cases, as fungal community alpha-diversity  
375 increased, so did *H. fraxineus* infection. Relationships were not statistically significant between log *H.*  
376 *fraxineus* infection and bacterial community alpha-diversity (richness,  $X^2 = 0.787$ ,  $p = 0.375$ ;  
377 evenness,  $X^2 = 0.509$ ,  $p = 0.475$ ). There was a significant relationship between log *H. fraxineus*  
378 infection and fungal community beta-diversity (PCoA axis 1 score,  $X^2 = 39.528$ ,  $p < 0.001$ , Figure 3c;

379 PCoA axis 2 score,  $X^2 = 5.511$ ,  $p = 0.019$ ), and log *H. fraxineus* infection and bacterial community  
380 beta-diversity (PCoA axis 1 score;  $X^2 = 5.4606$ ,  $p = 0.019$ ; Figure 3d). However, tree infection score  
381 was not significantly predicted by any microbial diversity measure (all  $p > 0.05$ ).

382 We identified 26 fungal genera (out of 390) and six bacterial genera (out of 255) with  
383 significant positive correlations with log *H. fraxineus* infection intensity (all  $p < 0.05$ ; Figure 4). We also  
384 identified 217 fungal genera and four bacterial genera with a significant negative correlation with log  
385 *H. fraxineus* infection intensity (all  $p < 0.05$ ; Figure 4). Indicator analysis only identified one fungal  
386 genus (*Neofabraea*, IndVal = 0.378,  $p = 0.025$ ) and one bacterial genus (*Pedobacter*, IndVal = 0.643,  
387  $p = 0.005$ ) that were significantly associated with the absence of *H. fraxineus* infection (i.e. these  
388 genera were much more commonly found in the absence of infection). Association analysis identified  
389 two fungal genera significantly associated with the presence of *H. fraxineus* infection (*Hannaella*,  
390 IndVal = 0.525,  $p = 0.050$ ; *Keissleriella*, IndVal = 0.450,  $p = 0.020$ ). DESeq2 analysis did not identify  
391 any differentially abundant bacterial ESVs between infected and uninfected leaves (Figure S2), but  
392 did for fungal ESVs; *Phyllactinia fraxini* was significantly more abundant in uninfected leaves  
393 ( $\log_2\text{FoldChange} = -24.429$ ,  $p < 0.001$ ) and one *Genolevuria sp.* was significantly more abundant in  
394 infected leaves ( $\log_2\text{FoldChange} = 3.753$ ,  $p < 0.001$ ; Figure S3). For both fungi and bacteria,  
395 however, the DESeq2 analysis indicated there was no clear pattern of ESVs within genera showing  
396 particular patterns in abundance according to *H. fraxineus* infection. That is, genus is not an accurate  
397 indicator of anti-pathogen capabilities (Figures S2 and S3).

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

409 Therefore, despite high infection loads quantified through targeted qPCR, *H. fraxineus* did not appear  
410 to be present in our ITS rRNA amplicon sequencing data to any substantial degree.

411

#### 412 *Functional analysis of fungal communities*

413 We obtained functional hypotheses for 65% of ITS rRNA ESVs. Functional analysis of fungal  
414 communities indicated that the relative abundance of pathotrophs (fungi causing disease and  
415 receiving nutrients at the expense of host cells; Nguyen et al., 2016; Tedersoo et al., 2014) increased  
416 as *H. fraxineus* infection intensity increased (Figure 5 and S4). However, the proportion of fungal  
417 species with unidentified trophic modes were higher in the absent and low infection categories (Figure  
418 5). Despite this, the most abundant pathogen, *Phyllactinia fraxini*, had a relatively high abundance in  
419 leaves absent of infection (7.0%) and with low infection levels (6.2%), compared to medium (0.1%)  
420 and high (1.7%) infection levels. The genus *Phyllactinia* also had a significant negative correlation  
421 with log *H. fraxineus* infection intensity ( $r = -0.378$ ,  $p < 0.001$ ) although the negative relationship  
422 between log *H. fraxineus* (+1) and log *P. fraxini* (+1) was only approaching significance ( $r = -0.15$ ,  $p =$   
423  $0.077$ ). Overall, *P. fraxini* was the most abundant pathogen and the fifth most abundant fungus across  
424 all samples (*Vishniacozyma foliicola*, *V. victoriae* and two species of Venturiales were more abundant;  
425 Table S2). The second most abundant pathogen was the yeast *Itersonilia pannonica* (formerly  
426 *Udeniomyces pannonicus*; Niwata, Takashima, Tornai-Lehoczki, Deak, & Nakase, 2002), which in  
427 contrast to *P. fraxini*, had lower abundance in leaves with absent (0.2%) and low (1.2%) *H. fraxineus*  
428 infection than in those with medium (6.5%) and high (8.1%) infection levels. Correlation analysis  
429 indicated a significant positive relationship between log *H. fraxineus* (+1) and log *I. pannonica* (+1) ( $r$   
430  $= 0.49$ ,  $p < 0.001$ ). In addition, the relative abundance of symbiotrophs (which receive nutrients  
431 through exchange with host cells), primarily lichens, also increased on infection by *H. fraxineus*  
432 (Figure 5; Figure S4).

433

#### 434 *Relationships between fungal and bacterial communities*

435 Mantel tests identified significant correlations between fungal and bacterial communities of leaves  
436 across both sites ( $r = 0.552$ ,  $p = 0.001$ ; Figure 6a). Co-occurrence analysis indicated that leaves  
437 highly infected with *H. fraxineus* had fewer statistically significant ( $p < 0.05$ ) cross-kingdom microbial  
438 connections than the other infection categories (Table 1). The majority of microbial associations in the



439 uninfected categories were of medium strength ( $-0.50 > \rho$  and  $\rho > 0.50$ ) rather than strong ( $-0.75$   
440  $> \rho$  and  $\rho > 0.75$ ), and were characterised by sprawling, less-well-connected hubs with a  
441 considerable number of members (Table 1; Figure 7). The proportion of strong microbial connections  
442 increased as *H. fraxineus* infection increased, and in highly infected leaves, 100% of associations  
443 were strong and positive ( $\rho > 0.75$ ), but characterised by very few, strongly associated larger hubs  
444 involving relatively few members (Table 1; Figure 7; Table S3).

445

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

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

450 Across sites, there was a significant correlation between genetic distance and fungal  
451 community composition ( $r = 0.106$ ,  $p = 0.005$ ; Figure 6b), but no significant relationship between  
452 genetic distance and bacterial community composition ( $r = 0.013$ ,  $p = 0.365$ ). Within sites, the  
453 correlation between tree genetic distance and fungal community composition was statistically  
454 significant for Manchester ( $r = 0.155$ ,  $p = 0.002$ ) but not Stirling ( $r = 0.042$ ,  $p = 0.372$ ). Genetic  
455 distance was not significantly correlated with bacterial community composition at either site  
456 (Manchester:  $r = -0.065$ ,  $p = 0.749$ ; Stirling:  $r = 0.151$ ,  $p = 0.091$ ).

457 No heterozygosity measures significantly predicted *H. fraxineus* infection intensity or tree  
458 infection score (all  $p > 0.05$ ).

459

460

## 461 **DISCUSSION**

462 Our results show that both fungal and bacterial community composition, as well a considerable  
463 number of microbial genera, are significantly correlated with *H. fraxineus* infection intensity. Cross et  
464 al. (2017) previously demonstrated that fungal community composition altered as the season  
465 progressed and *H. fraxineus* infection intensified, although it was not clear whether these changes  
466 resulted from seasonal effects or infection intensity. We extend this work to show that at a given time  
467 point, differences in both fungal and bacterial phyllosphere communities relate to *H. fraxineus*  
468 infection, even in the absence of phenotypic signs of infection. These effects were apparent in our

469 mixed model analysis, but not significant in the PERMANOVA analysis; this may be due to a loss of  
470 statistical power from the use of infection categories (i.e., “absent”, “low”, “medium” or “high”) in the  
471 PERMANOVA rather than the continuous log *H. fraxineus* data used in the linear mixed model  
472 analysis. Changes in microbiome composition that correlate with pathogenic infection have also been  
473 identified in other tree species. For example, the bacterial microbiome of horse chestnut bark was  
474 altered by bleeding canker disease caused by the bacterium *Pseudomonas syringae* pv *aesculi*  
475 (Koskella et al., 2017). Similarly, Jakuschkin et al. (2016) found evidence of cross-kingdom  
476 endophytic dysbiosis in pedunculate oak (*Quercus robur* L.) on infection by *Erysiphe alphitoides*, the  
477 causal agent of oak powdery mildew.

478 Fungal alpha-diversity was positively correlated with *H. fraxineus* infection intensity, although  
479 bacterial alpha-diversity was not. Although it may be expected that higher microbiome diversity would  
480 increase microbiome-mediated resistance to invasive pathogens through competitive exclusion, the  
481 relationship between microbiome diversity and pathogen susceptibility actually varies considerably  
482 among host taxa (e.g. Bates et al., 2018; Dillon, Vennard, Buckling, & Charnley, 2005; Johnson &  
483 Hoverman, 2012; Näpflin & Schmid-Hempel, 2018; Upreti & Thomas, 2015; Wehner, Antunes, Powell,  
484 Mazukatow, & Rillig, 2010). Our results suggest that low diversity may reflect a stable and resilient  
485 microbiome that resists infection, or that *H. fraxineus* infection is associated with dysbiosis that allows  
486 for the proliferation of many new members in the microbiome. Indeed, co-occurrence analysis showed  
487 that medium-strength, minimally-connected networks in leaves with absent or low *H. fraxineus*  
488 infection become a few, high-strength, highly-connected hubs under medium or high infection. The  
489 co-occurrence analysis indicates that although *H. fraxineus* infection is associated with strong  
490 microbial networks, these are relatively depauperate in members and so the stability of phyllosphere  
491 communities in infected leaves may be compromised. Conversely, leaves with absent or low infection  
492 rates have more complex co-occurrence hubs with more medium-strength connections involving more  
493 members. Together with the higher fungal diversity observed as *H. fraxineus* infection intensity  
494 increased, these results suggest *H. fraxineus* infection is associated with dysbiosis in ash leaves that  
495 allows for the proliferation of microbial phyllosphere endophytes. Furthermore, Cross et al. (2017)  
496 suggested that phyllosphere communities are not significantly altered by *H. fraxineus* until a particular  
497 infection density is reached, and our findings support this. We also show that even leaves with high  
498 infection intensities can appear asymptotic but exhibit evidence of phyllosphere dysbiosis, although it

499 is not clear whether such dysbiosis is a result of infection, or in fact facilitates infection. Although  
500 causality can be hard to identify without explicit infection trials, leaves in this study were collected late  
501 in the season in areas of epidemic infection. This suggests leaves that were clear of infection at the  
502 time of sampling may have been able to resist infection up to that point, and so patterns identified  
503 here may be representative of real-world infection trials.

504         There is other evidence that associations between plants and microbes become stronger  
505 when the host is stressed, with positive effects for the host (Mendes et al., 2011; Pineda, Dicke,  
506 Pieterse, & Pozo, 2013). For example, plants can exploit beneficial microbes when under water or  
507 nutrient stress, with positive effects on plant growth and insect attack (Pineda, Dicke, Pieterse, &  
508 Pozo, 2013). How the networks identified in our data influence the host to improve resistance to *H.*  
509 *fraxineus* remains to be explored. We also identify considerable co-variation between bacterial and  
510 fungal communities, and extensive cross-kingdom associations in the leaves of ash trees. Syntrophy  
511 (i.e. cross-feeding between microbial species) is phylogenetically and environmentally widespread  
512 throughout microbial taxa and leads to high connectedness between members of the microbiome  
513 (Hassani et al., 2018; Kouzuma, Kato, & Watanabe, 2015; McInerney et al., 2008). Furthermore,  
514 nutrient and pH modulation by fungal communities can influence bacterial colonisation (Hassani et al.,  
515 2018; Johnston et al., 2018). Thus, such interactions between these two kingdoms may be expected,  
516 and the importance of these in the context of disease resistance warrants considerable attention.

517         The functional analysis identified an overall increase in fungal pathogens as *H. fraxineus*  
518 infection increased. Disruption to the natural endosymbiont community by *H. fraxineus* infection may  
519 break up previously filled niches, thus allowing co- or secondary infections. Alternatively, prior  
520 infection by other pathogens may allow *H. fraxineus* to proliferate. In particular, we found convincing  
521 evidence of co-infection by *Itersonilia pannonica*, a likely yeast pathogen (Nguyen et al., 2016). Other  
522 secondary infections have previously been documented in ash dieback outbreaks, including *Alternaria*  
523 *alternata*, *Armillaria* spp., *Cytospora pruinosa*, *Diaporthe eres*, *Diplodia mutila*, *Fusarium avenaceum*,  
524 *Fusarium lateritium*, *Fusarium solani*, *Phoma exigua*, *Phytophthora* spp. and *Valsa ambiens*  
525 (Kowalski, Kraj, & Bednarz, 2016; Marçais, Husson, Godart, & Caël, 2016; Orlikowski et al., 2011).  
526 Co-infection can have considerable implications for host fitness and the evolution of pathogens  
527 (Tollenaere, Susi, & Laine, 2016), and may well contribute to the progression of ash dieback. Similar  
528 findings have been shown in other study systems, whereby disruption of the resident microbiome

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

542         Based on a combination of analyses, we identified *Neofabraea* fungi and *Pedobacter* bacteria  
543 as potential antagonists of *H. fraxineus* infection, which may have potential for development of anti-  
544 pathogenic inoculants or probiotics. *Neofabraea* has previously been shown to inhibit *H. fraxineus in*  
545 *vitro* (Schlegel et al., 2016). Given the large number of microbial genera present in the leaves, it is  
546 surprising that only three genera showed significant association with the absence of *H. fraxineus*,  
547 despite widespread and heavy infection in the study sites. This finding may reflect the propensity for  
548 wide variation within genera for anti-pathogen capabilities (Antwis & Harrison, 2018; Becker et al.,  
549 2015), as indicated by the DESeq2 analysis, in which ESVs within a genus did not necessarily show  
550 the same type of response (i.e. positive or negative) to *H. fraxineus*. Thus, although we identify  
551 potential genera of interest, a genus-by-genus approach may not be the best method for identifying  
552 potential probiotics. *In vitro* studies have identified over 70 species of fungi that inhibit the growth of *H.*  
553 *fraxineus* (Kosawang et al., 2018; Schlegel et al., 2016; Schulz et al., 2015). In addition, secondary  
554 metabolite production by endophytes is generally down-regulated when cultured individually but  
555 activated in response to other microbes (Schroeckh et al., 2009; Suryanarayanan, 2013), indicating  
556 complex and bi-directional interactions between members of the phyllosphere microbiome. Thus, co-  
557 culturing such microbes, potentially identified through co-occurrence hubs, may help guide the  
558 development of consortium-based approaches to probiotic development, which may be more effective

559 than single-species probiotics (Antwis & Harrison, 2018; Kaminsky, Trexler, Malik, Hockett, & Bell,  
560 2018; Schulz et al., 2015).

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

580 Sampling site was the main predictor of total community composition for both fungal and  
581 bacterial communities of ash leaves. Considerable variation in phyllosphere composition still existed  
582 between the sites despite the Stirling and Manchester trees being genetically similar, indicating that  
583 site-level variation was not due to population differentiation. Both fine- and broad-scale geographic  
584 variation affects microbiome composition in many study organisms (Antwis, Lea, Unwin, & Shultz,  
585 2018; Griffiths et al., 2018; Yatsunenکو et al., 2012) including plants (Edwards et al., 2015; Peiffer et  
586 al., 2013; Wagner et al., 2016). The site-level differences observed in this study may reflect a range of  
587 differences in abiotic conditions, given that environmental variables, such as temperature, and rainfall  
588 are considerable determinants of both microbiome composition and pathogen activity (Barge,

589 Leopold, Peay, Newcombe, & Busby, 2019; Busby, Newcombe, Dirzo, & Whitham, 2014; Busby,  
590 Ridout, & Newcombe, 2016; Dal Maso & Montecchio, 2014; Laforest-Lapointe, Messier, & Kembel,  
591 2016; Zimmerman & Vitousek, 2012). Methodological differences could also be responsible – in  
592 Stirling, we sampled saplings whereas in Manchester we sampled mature trees. Tree and leaf age  
593 both significantly affect phyllosphere microbiome structure, possibly due to microbial community  
594 succession patterns, as well as niche variation associated with age-related physiological changes in  
595 leaves (Redford & Fierer 2009, Meaden, Metcalf, & Koskella, 2016). Thus, site-level patterns in our  
596 data may reflect these considerable drivers of microbiome composition. Alternatively, there may well  
597 have been different isolates of *H. fraxineus* at the two sites, which may have differentially affected leaf  
598 microbial communities through isolate variation in enzyme profiles and growth rates (Junker, de Vries,  
599 Eickhorst, & Schulz, 2017). We also observed variation in the strength of genotype x microbiome  
600 interactions between sites. This may be due to environmental differences, and thus could indicate the  
601 presence of genotype by microbiome by environment (G x M x E) interactions (Smith et al., 2015). G x  
602 M x E interactions may be particularly important for disease susceptibility and mitigation as  
603 environment plays a considerable role in pathogenicity. Thus, microbially-derived resistance to *H.*  
604 *fraxineus*, in addition to the effectiveness of any microbial treatments, may be population, age, or site  
605 specific, and may vary between sites based on environmental and biological variables, including  
606 abiotic factors as well as pollution, tree density, species mix, and herbivore activity. Much more work  
607 is required to determine how environmental factors and pathogen strain variation affect microbially-  
608 derived resilience to *H. fraxineus* infection, and identifying cross-population and cross-isolate  
609 microbial signatures of resistance will be key to the success of a microbial-based approach to disease  
610 management.

611           It is worth noting that we did not identify *H. fraxineus* itself to species level using ITS rRNA  
612 amplicon sequencing (or through additional BLAST searches), despite qPCR demonstrating  
613 widespread and high infection rates. Cross et al. (2017) found similar results when using ITS-1. This  
614 may be because *H. fraxineus* (or its many strains) is not fully represented in the UNITE database, or  
615 because *H. fraxineus* has a long fragment length (~550bp) for the primer combination we used, which  
616 would be less readily sequenced than shorter reads on the Illumina MiSeq platform (Lindahl et al.,  
617 2013). As with all amplicon sequencing, there are limitations to the taxa that can be identified based

618 on the primers used, and wider analysis using multiple markers will identify further genera involved in  
619 *H. fraxineus* infection dynamics on ash leaves (Cross et al., 2017; Lindahl et al., 2013).

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

632

633

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643

#### 644 **AUTHOR CONTRIBUTIONS**

645 RA, SMG, JH and DO'B conceived the study. RA, SMG, MG and JR produced the data. RA, SMG  
646 and IG analysed the data. All authors wrote and approved the manuscript.

647

648

649 **DATA ACCESSIBILITY**

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

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## 1035 **FIGURE LEGENDS**

1036

1037 **Figure 1**

1038 Relative abundance of (a) fungal classes and (b) bacterial classes in ash tree leaves from Manchester  
1039 (red) and Stirling (blue) across four different *Hymenoscyphus fraxineus* infection categories.

1040

1041 **Figure 2**

1042 PCoA plots for Bray-Curtis distances among (a) fungal communities and (b) bacterial communities of  
1043 ash tree leaves collected from Manchester (circles) and Stirling (triangles) with either absent (red),  
1044 low (blue), medium (purple) or high (green) *Hymenoscyphus fraxineus* infection; (c) PCoA plot of  
1045 Euclidean genetic distances between ash trees in Manchester (circles) and Stirling (triangles),  
1046 calculated using multilocus microsatellite genotypes.

1047

1048 **Figure 3**

1049 Relationship between *Hymenoscyphus fraxineus* infection and (a) fungal community richness, (b)  
1050 fungal community evenness (Inverse Shannon), (c) fungal community beta-diversity, and (d) bacterial  
1051 community beta-diversity.

1052

1053 **Figure 4**

1054 Heatmap of fungal (black text) and bacterial (red text) genera significantly associated with  
1055 *Hymenoscyphus fraxineus* infection intensity in ash tree leaves.

1056

1057 **Figure 5**

1058 Functional analysis of trophic modes of fungal ESVs associated with ash leaves with varying degrees  
1059 of *H. fraxineus* infection.

1060

1061 **Figure 6**

1062 Relationship between (a) Jensen-Shannon divergence values of fungal communities and bacterial  
1063 communities associated with ash tree leaves, and (b) Jensen-Shannon divergence values of fungal  
1064 communities and Euclidean genetic distance of ash trees.

1065

1066 **Figure 7**

1067 Co-occurrence networks between fungi and bacteria in the leaves of ash trees with varying degrees of  
1068 *Hymenoscyphus fraxineus* infection; (a) absent; (b) low; (c) medium and (d) high. Edges coloured  
1069 orange indicate fungi-fungi associations, those coloured green indicate bacteria-bacteria associations,  
1070 and those in blue are fungi-bacteria associations.

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