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Research Article

The interplay between scion genotype, root microbiome, and *Neonectria ditissima* apple canker

Hamish McLean [1,2,*], Alexey Mikaberidze[2], Greg Deakin[1], Xiangming Xu[1], Matevz Papp-Rupar[1]

[1]Niab, New Road, East Malling, Kent ME19 6BJ, United Kingdom
[2]School of Agriculture, Policy, and Development, University of Reading, Whiteknights, Reading RG6 6BZ, United Kingdom
*Corresponding author. Niab, New Road, East Malling, Kent ME19 6BJ, UK. E-mail: Hamish.McLean@niab.com, HSCMcLean@gmail.com
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Abstract

Severity of European apple canker caused by *Neonectria ditissima* can vary between locations and apple genotypes. We investigated how location, cold storage/planting season, and apple scion genotype affect root-associated microbial communities. Additionally, we investigated whether differences in abundance of specific taxa could be associated with canker lesion counts. Seven scion cultivars grafted onto M9 rootstocks were inoculated with *N. ditissima* in the nursery and then planted in December 2018 or stored at 2°C until planting in April 2019 at three sites in Kent, UK. We assessed canker lesions and collected root samples in June 2021. Quantitative polymerase chain reaction (qPCR) and internal transcribed spacer (ITS)/16S rRNA gene amplicon sequencing was used to analyse microbial communities. Site was the primary factor affecting microbiome size, diversity, and composition. Cold storage/planting season had small but significant effects, indicating that differences in the microbiome at planting can persist long-term. Scion genotype had a limited effect on diversity but did influence the abundance of specific root-associated taxa. Bacterial α-diversity was associated with canker count in a site-dependent manner. Increased abundances of particular fungal (*Rhizophagus irregularis* and *Epicoccum nigrum*) and bacterial (*Amycolatopsis* and *Bradyrhizobium*) root-associated taxa were associated with fewer cankers.

Keywords: apple root microbiome assembly; European apple canker; *Neonectria ditissima*; planting site; planting timing; root microbiome–canker association; scion genotype

Introduction

European apple canker, caused by the fungal pathogen *Neonectria ditissima* (Tul. & C. Tul.) Samuels & Rossman, is a devastating disease of apple and causes considerable economic losses (Saville and Olivieri 2019). *Neonectria ditissima* spores are wind (ascospores) and splash (conidia) dispersed and infect through wounds in stems and leaf scars leading to large lesions, which can girdle the tree, killing distal tissues (Weber and Børve 2021). Canker susceptibility varies between apple cultivars and is an important target when breeding new cultivars (Gómez-Cortecero et al. 2016). However, canker susceptibility is a complex trait, involving many genetic loci, slowing the development of cultivars with robust canker resistance (Karlström et al. 2022). Canker expression is also affected by the environment and varies widely between geographic locations (sites) (Xu et al. 2022). Revealing the source of this variation could contribute to better management of canker.

The relative abundance of above-ground apple endophytes in leaf scars is partially correlated with canker susceptibility, and specific endophytes are partially associated with the host scion genotype (Papp-Rupar et al. 2022). Apple leaf scar endophyte communities also vary significantly between apple scion genotypes with different canker susceptibility status (Olivieri et al. 2021). Many studies have identified site as a significant source of variation in above-ground apple endophyte communities (Liu et al. 2020, Olivieri et al. 2021, Papp-Rupar et al. 2023a). This variation may contribute to the variation in canker lesion counts observed between sites (Xu et al. 2022).

Research on the role of the microbiome in *N. ditissima* infection has so far focused on endophytes in above-ground parts of the plant. The role of root-associated microbial communities in canker susceptibility is not well understood. Rhizosphere microorganisms and root endophytes form mutualistic or even symbiotic relationships with the host plant that promote plant health (Bais et al. 2006, Nadeem et al. 2014). In apple, rhizosphere and root endophyte communities vary between sites and between rootstock genotypes on the same site (Van Horn et al. 2021, Liu et al. 2022). Scion genotype also influences the rhizosphere bacterial community by altering sugar exudates from roots (Chai et al. 2022). Understanding the factors affecting the variation in apple root-associated communities and their impact on disease could facilitate improved disease management.

This study is an extension of a previous study (Xu et al. 2022) which measured the effects of design factors—geographic location (site), cold storage combined with delayed planting, and apple scion genotype—on the severity of *N. ditissima* apple canker. In this study, we aim to assess how these factors affect fungal and bacterial communities associated with apple roots and explore the relationships between root microbial communities and canker severity. We collected root samples from across the treatments and analysed their microbial community size using quantitative polymerase chain reaction (qPCR) and composition using internal transcribed spacer (ITS)/16S rRNA gene amplicon sequencing. Our

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first objective was to investigate the effects of the design factors and canker severity on the size, diversity, and composition of root-associated fungal and bacterial communities. Our second objective was to investigate whether the observed variation in canker severity could be associated with differences in abundances of specific root-associated taxa. We hypothesize that site, cold storage/planting season, and scion genotype significantly affect diversity and composition of root-associated fungal and bacterial communities. Additionally, we hypothesize that root-associated microbes are associated with canker lesion counts.

Materials and methods

Experimental design and field layout

The full experimental design and field layout as well as a detailed description of the sites are provided in previous work (Xu et al. 2022). Seven apple scion genotypes ('Royal Gala', 'Braeburn', 'Scifresh', 'Nicoter', 'Civni', 'Grenadier', and 'Golden Delicious') grafted onto M9 apple rootstocks were planted at three commercial orchard sites in Kent, UK: site 1 (51.2144101°, 0.6110800°), site 2 (51.1495230°, 0.4074939°), and site 3 (51.1756250°, 0.7611569°). Each site contained five blocks with one plot of each genotype, positioned randomly within the block. Each plot was split into two subplots each containing six trees. One subplot of each plot was planted in December 2018 immediately after lifting from the nursery, and the other in April 2019 after being stored at 2°C for ∼4 months. The trees were uniformly inoculated with *N. ditissima* in the nursery before lifting. Canker lesions on the main trunk and peripheral branches were counted in May 2021 shortly before root sampling was conducted.

Root sampling and processing

We randomly selected four out of five blocks from each site for analysis. From these, we randomly assigned two blocks to each planting season and selected the corresponding subplots for analysis. As such, planting season is no longer nested within blocks (as in Xu et al. 2022). Two representative trees were selected from each subplot and fine root samples were collected at a depth of ∼5 cm from three points around each tree, which were then pooled into one sample per subplot for a total of 84 samples. Soil was separated from the roots by gentle brushing before the roots were stored at −80°C. Roots were washed in sterile distilled water to remove soil, lyophilized for 24 h, and ground to a fine powder in a Geno/Grinder2010 (SPEX SamplePrep, Stanmore, UK) at 1500 rpm for 90 s. Aliquots of root powder were weighed before DNA extraction.

DNA extraction, amplicon sequencing, and qPCR

DNA was extracted from powdered root samples using the E.Z.N.A Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). DNA was quantified and assessed for purity spectrophotometrically (Nanodrop™ 1000, Thermo Fisher Scientific, USA). PCR amplification, library preparation, and metabarcoding sequencing were carried out by Novogene UK (Cambridge, UK) on the Illumina NovaSeq platform in the 250 nt paired end mode. The target regions were ITS1-1F: [ITS1-1F-F: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ (Gardes and Bruns 1993); ITS1-1F-R: 5′-GCTGCGTTCTTCATCGATGC-3′ (White et al. 1990)] and 16S V5-V7: [799F: 5′-AACMGGATTAGATACCCKG-3′ (Chelius and Triplett 2001), 1193R: 5′-ACGTCATCCCCACCTTCC-3′ (Bodenhausen et al. 2013)].

Fungal and bacterial community sizes were estimated using a published protocol (Papp-Rupar et al. 2022). In brief, we used quantitative PCR (qPCR) to calculate ITS and 16S rRNA gene theoretical copy numbers, which we then used to estimate relative fungal and bacterial community sizes. Standard curves were made by pooling 2 µl of extracted DNA from each sample and diluting to five steps of a five-fold serial dilution. Samples from each site were run on a separate 384-well plate at two dilutions (100× and 500×), each in triplicate. The standard curve and a non-template control were run in triplicate alongside samples on each plate. Reactions (10 µl each) were performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and 2 µl of template DNA. The same primers as in the metabarcoding sequencing were used (ITS1-1F and 16S V5-V7) at a final concentration of 100 nM for ITS-1F and 200 nM for 16S V5-V7. The cycling protocol was initial denaturation for 3 min at 98°C, followed by 40 cycles of 15 s at 98°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C and melt curve analysis.

Total ITS or 16S rRNA gene theoretical copy numbers per gram of the lyophilized root sample were calculated using the standard curve for each plate separately according to the published protocol (Papp-Rupar et al. 2022). The theoretical copy number was calculated for the bacterial and fungal standard curves such that the quantification cycle (Cq) intercepts were ∼35 (the theoretical detection limit estimated from standard curves). Samples with efficiency below 0.8 or above 1.2 were repeated at 5× greater dilution factor (500× and 2500×) to improve PCR efficiency. Means of efficiency-corrected qPCR results of three technical replicates for each dilution were included in analysis.

Processing amplicon sequencing data

The bioinformatics and computational analyses were performed on CropDiversity-HPC (Percival-Alwyn et al. 2024). Sequence data were processed as described previously (Papp-Rupar et al. 2023a) with the following modifications. Filtered and merged reads of all 84 samples were dereplicated to generate unique sequences, which were then denoised to generate amplicon sequence variants (ASVs) and remove chimeras. Fungal and bacterial taxonomy was assigned to ASVs at the 80% confidence level using SINTAX (Edgar 2016) and a modified version of the RDP Warcup V2 database, which was updated for consistency with NCBI taxonomy as of 2 September 2022. Bacterial taxonomy was assigned using the 'RDP training set v18' database for the 16S rRNA gene (Cole et al. 2014). Finally, all sequences—including those removed by denoising—were aligned with the ASVs at 97% similarity to generate ITS and 16S rRNA gene ASV count tables. ASVs identified as chloroplast or mitochondria were removed before analysis. The most abundant ASVs accounting for 99% of total reads were retained for analysis.

Statistical analysis

All statistical analyses were carried out with R 4.3.3 (R Core Team 2024). Mean canker lesion counts for the two trees per sample were calculated and rounded to the nearest integer to preserve the discrete count nature of this data. Four plots (samples) had one out of the two sampled trees partly damaged, in which case we used total counts on the remaining undamaged tree in the analysis. In five plots we were not able to obtain accurate canker counts due to both sampled trees being partly broken/damaged. All subsequent analysis was therefore performed on a total of 79 samples. Each model was constructed with the following independent variables: the design factors (site, cold storage/planting season, and scion genotype), canker lesion counts, and all

the one-way interactions of design factors and canker lesion counts.

To assess the effects of design factors and canker lesion counts on microbiome size, fungal and bacterial theoretical copy numbers were base 10 logarithm transformed to achieve normality and homoscedasticity of residuals before analysis of variance (ANOVA) was performed.

Chao1, Shannon, and Simpson indices of α-diversity were calculated using the vegan package in R (Oksanen et al. 2024). Permutation ANOVA of the rank of the α-diversity indices was used to assess the statistical significance of design factors and canker counts on these indices using the lmPerm package in R (Wheeler and Torchiano 2016).

For analysis of β-diversity, the ASV counts were first normalized for community size using qPCR theoretical copy number data as follows. The proportional difference in theoretical copy number between samples and the mean across samples was calculated for ITS and 16S separately. These values were used as size factors for DESeq2 (Love et al. 2014) normalization. qPCR-normalized ASV counts were transformed using DESeq2 variance-stabilizing transformation before principal component (PC) analysis to focus on the major axes of variation in community composition. ANOVA was used to assess the proportion of total variation in each of the first six PCs explained by design factors and canker counts. The Bray–Curtis (BC) dissimilarity index was calculated from qPCR-normalized ASV counts as a measure of β-diversity. Permutational multivariate analysis of variance (PERMANOVA) with 1000 permutations was used to assess the statistical significance of design factors and canker counts on BC indices using the adonis function of the vegan package.

ANOVA was used to assess differential abundance of ASVs in relation to the design factors and canker counts. qPCR-normalized ASV read counts were natural logarithm (ln) transformed to achieve normality and homoscedasticity of residuals. *P* values were adjusted to correct for multiple comparisons using the Benjamini–Hochberg false discovery rate method (Benjamini and Hochberg 1995).

Analysis of deviance of nested models was performed to assess the variance in canker counts explained by the abundance of each ASV as follows. Two generalized linear models (GLMs) with negative binomial distributions were fit to the canker lesion count data using the MASS package in R (Venables and Ripley 2002). GLMs with negative binomial distributions were found to fit the canker count data well and performed better than Poisson distributions. The base model included the design factors and their interactions. The full model first accounted for the variance of the design factors (and their interactions) with the addition of the ASV read counts (separately for each ASV) as the final main effect. ASV read counts were ln-transformed [$\ln(ASV_{count} + 1)$]. Analysis of deviance of nested models was used to assess the association of the each ASV with canker lesion counts after accounting for the effects of the design factors. *P* values were calculated by the likelihood ratio (LR) test and adjusted for multiple testing using the Benjamini–Hochberg method. Due to the high variability in microbiome composition observed between sites, this analysis was repeated for each site separately. *P* values were adjusted for each site separately using the Benjamini–Hochberg method. ASVs for which models failed to converge were removed from analysis. The coefficient (*c*) of the ASV predictor in the full model was reported as a measure of effect size where a unit change in the predictor variable results in a change of *c* in the response variable.

Results

Overview of fungal and bacterial root-associated microbiomes

The 10 most abundant fungal and bacterial ASVs accounting for 50% and 18% of total reads, respectively, are given in Table S1. The frequency of each class (at 80% confidence) on each site is illustrated in Fig. S2.

For fungi there were 2401 ASVs in total. The top 10, 171, and 995 most abundant ASVs accounted for 50%, 90%, and 99% of total reads, respectively. Of all ASVs, 84%, 70%, 54%, 42%, 38%, and 24% could be assigned to the rank of phylum, class, order, family, genus, and species, respectively, at ≥80% confidence. The top two ASVs were Ascomycota (phylum) and *Dactylonectria macrodidyma*, which each accounted for 12% of total reads. The top 10 ASVs—together accounting for 50% of total reads—are shown in Table S1.

For bacteria there were 7261 ASVs in total. The top 169, 2185, and 5880 most abundant ASVs accounted for 50%, 90%, and 99% of total reads, respectively. Of all ASVs, 94%, 84%, 65%, 5%1, and 35% could be assigned to the rank of phylum, class, order, family, and genus, respectively, at ≥80% confidence. The top ASV was *Streptomyces*, which accounted for 3% of total reads. The top 10 ASVs—together accounting for 18% of total reads—are shown in Table S1.

Fungal and bacterial community size

A summary of factors affecting the total fungal and bacterial community size is given in Table 1. Box plots illustrating community size of fungal and bacterial microbiomes across sites and cold storage/planting season treatments are shown in Fig. 1. For fungi, estimated community size was significantly affected by site ($F = 4.66$, $P = .015$), where site 2 had smallest fungal community overall. Cold storage/planting season also had a significant effect ($F = 5.43$, $P = .025$) where the community was overall larger in samples planted in winter with no cold storage. Cold storage also had a site-dependent effect ($F = 3.70$, $P = .034$) where the community was smaller in cold storage/spring planting samples only at site 2. Scion genotype and canker lesion count did not have significant effects. For bacteria, estimated community size was significantly affected by site where it was largest overall at site 2 ($F = 26.1$, $P < .001$). This contrasts with fungal community size, which was smallest in site 2. Scion genotype also had a significant effect ($F = 2.42$, $P = .044$). Cold storage/planting season and canker lesion counts did not have significant effects on bacterial community size.

Within-sample α-diversity

A summary of factors affecting within-sample α-diversity indices is given in Table 1. Box plots illustrating α-diversity indices across sites and cold storage/planting season treatments are shown in Fig. 1. For fungi, site was the primary factor affecting Chao1 richness, which was highest at site 2 ($P < .001$). Site was also the primary factor affecting Shannon diversity ($P < .001$) and Simpson evenness ($P < .001$), which were both highest overall at site 1. Cold storage/planting season had a small but significant site-dependent effect on Shannon diversity ($P = .029$) and Simpson evenness ($P = .037$) both of which were higher for winter planting with no cold storage at sites 1 and 2, but lower at site 3. Scion genotype and canker lesion counts did not significantly affect any α-diversity indices.

For bacteria, site was also the primary factor affecting Chao1 richness ($P < .001$), Shannon diversity ($P < .001$), and Simpson

Table 1. Percentage of variance in community size and α-diversity indices explained by the design factors and canker lesion counts (Canker) for fungal and bacterial root-associated communities based on ANOVA of community size and permutation ANOVA of α-diversity index ranks.

				α-Diversity					
		Community size		Fungi			Bacteria		
ANOVA terms	*df*	Fungi	Bacteria	Chao1	Shannon	Simpson	Chao1	Shannon	Simpson
Site	2	9.16*	40.28***	24.93**	31.82***	31.82***	44.78***	50.93***	45.82***
Storage	1	6.76*	1.29	4.66	2.88*	1.63	0.13	0.24	0.03
Scion	6	6.82	11.21*	3.13	1.75	3.28	3.01	2.26	4.46
Canker	1	0.59	0.01	0.01	0.43	0.50	0.01	0.40	0.26
Site × Storage	2	7.21	1.79	2.57	4.06	4.62	7.16**	1.87	3.01
Site × Scion	12	11.77	4.73	6.50	6.04	4.38	12.52*	8.66	8.45
Storage × Scion	6	4.90	3.87	5.67	6.29	5.89	2.05	1.60	2.21
Site × Canker	2	2.20	1.24	0.17	1.87	3.76	2.65	5.97*	6.57**
Storage × Canker	1	0.11	1.01	3.05	1.26	0.03	1.34	0.83	0.36
Scion × Canker	6	3.77	3.65	1.95	5.20	8.70	6.20	5.27	6.27
Residuals	39	46.70	30.91	47.36	38.40	35.38	20.15	21.97	22.57

*$P < .05$, **$P < .01$, and ***$P < .001$.

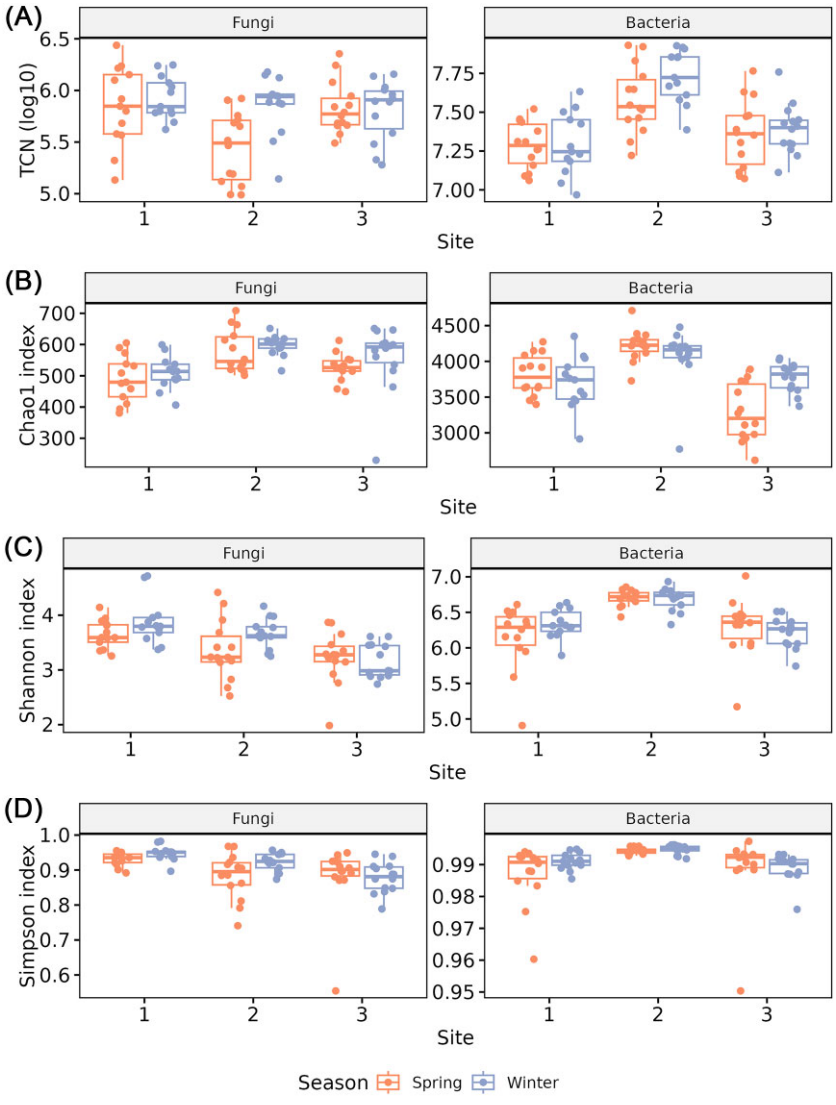

Figure 1. Box plots illustrating fungal and bacterial (A) estimated community size based on qPCR-derived theoretical copy number (TCN), (B) Chao1 richness, (C) Shannon diversity, and (D) Simpson evenness across sites and cold storage/planting season treatments.

evenness ($P < .001$), which were all highest at site 2. Cold storage/planting season had a significant site-dependent effect on Chao1 richness ($P = .002$), which was higher for spring planting with cold storage at sites 1 and 2, but lower at site 3. Scion genotype had a significant site-dependent effect on bacterial Chao1 richness ($P = .041$). Canker lesion counts were significantly related to Shannon diversity ($P = .011$) and Simpson evenness ($P = .005$) in a site-dependent manner. Higher bacterial Shannon and Simpson indices were associated with higher canker counts at sites 1 and 3 but not at site 2 (Fig. S3).

Between-sample β-diversity

A summary of factors affecting the first six PCs (PC1–6) is given in Table 2. For fungi, the first six PCs together explained 66.8% of the total variance in the community. Site explained 38.5% of the total variance in PC1–6 and significantly affected PC1 ($F = 230$, $P < .001$), PC2 ($F = 72.6$, $P < .001$), PC3 ($F = 4.05$, $P = .025$), and PC4 ($F = 4.32$, $P = .020$). Cold storage/planting season did not have a significant effect on PC1–6 alone but had a significant site-dependent effect on PC1 ($F = 5.84$, $P = .006$) and PC2 ($F = 8.57$, $P < .001$) and explained in total 3.32% of the total variance. Scion genotype and canker lesion counts did not significantly affect any of the first six PCs.

For bacteria, the first six PCs together explained 47.5% of the total variance in the community. Site explained 27.2% of the total variance and significantly affected PC1 ($F = 203$, $P < .001$), PC2 ($F = 277$, $P < .001$), and PC4 ($F = 6.25$, $P = .004$). Cold storage/planting season explained 0.53% of the total variance and significantly affected PC5 ($F = 9.88$, $P = .003$) and PC6 ($F = 4.80$, $P = .034$). Cold storage/planting season also had a significant site-dependent effect on PC3 ($F = 9.04$, $P = .001$) and PC5 ($F = 5.72$, $P = .007$), which explained 2.04% of the total variance. Scion genotype explained 2.38% of the total variance in PC1–6 and significantly affected PC3 ($F = 3.07$, $P = .015$) and PC6 ($F = 3.02$, $P = .016$). Scion genotype also had a site-dependent effect on PC4 ($F = 2.49$, $P = .015$) and explained 3.4% of the total variance in PC1–6. Canker lesion counts did not have a significant effect on the first six PCs.

Plots of the first two non-metric multidimensional scaling (NMDS) dimensions of BC dissimilarity for fungal and bacterial communities are shown in Fig. 2. A summary of factors affecting BC dissimilarity is given in Table 2. For fungi, BC dissimilarity was primarily affected by site ($F = 17.53$, $P = .001$) with distinct clusters. Cold storage/planting season had a small but significant effect ($F = 2.28$, $P = .011$) with overlapping clusters. Cold storage also had a small site-dependent effect ($F = 1.87$, $P = .008$) with more separation between storage treatment clusters within site 2 than that within sites 1 and 3. Scion genotype and canker lesion counts did not have significant effects.

For bacteria, BC dissimilarity was primarily affected by site ($F = 18.9$, $P = .001$) shown as distinct site clusters (Fig. 2). Cold storage/planting season had a site-dependent effect ($F = 2.90$, $P = .001$) with more separation between storage treatment clusters within site 3 than sites 1 and 2. Scion genotype and canker lesion counts did not have significant effects.

Abundance of fungal and bacterial ASVs

Of the 995 fungal ASVs, site significantly affected the abundance of 677. Cold storage affected 11 ASVs as a main effect and 94 ASVs in a site-dependent manner. Scion genotype only affected the abundance of 4 ASVs in a site-dependent manner. Canker lesion counts affected the abundance of nine ASVs alone, two dependent on site, and three dependent on scion genotype. Of the 5883 bacterial ASVs, site significantly affected the abundance of 3698 ASVs. Cold storage affected 61 ASVs alone and 399 ASVs dependent on site. Scion genotype affected the abundance of 101 ASVs alone, 65 ASVs dependent on site, and 19 ASVs dependent on cold storage. Canker lesion counts affected 31 ASVs alone, 61 dependent on site, 8 dependent on cold storage, and 57 dependent on scion genotype. A summary of the top 10 most abundant fungal and bacterial ASVs significantly affected by canker lesion counts, or its interactions with the design factors, is given in Table 3. A full list of factors affecting abundances of bacterial and fungal ASVs is available in the Supplementary Information.

Associations of canker lesion counts with root microbiome

After taking into account the effects of site, scion genotype, and cold storage/planting season, we found multiple fungal and bacterial ASVs that were significantly associated ($P < .05$) with canker lesion counts across all sites or within each site separately. The top five most abundant fungal and bacterial ASVs with a significant positive or negative association with canker lesion counts across all sites and within each site are given in Table 4. A full list is available in the Supplementary Information. There were 995 fungal ASVs tested across sites, 820 at site 1, 915 at site 2, and 822 at site 3. Canker lesion counts were significantly associated with 3 fungal ASVs across all sites, 43 at site 1, 3 at site 2, and 1 at site 3. There were 5883 bacterial ASVs tested across all sites, 5713 at site 1, 5750 at site 2, and 5587 at site 3. Canker lesion counts were associated with 273 bacterial ASVs across all sites, 276 at site 1, 401 at site 2, and 77 at site 3.

Discussion

In this experiment, we investigated the effect of site, cold storage/planting season, scion genotype, and canker lesion counts on community size, diversity, and composition of fungal and bacterial apple root-associated communities. We also explored the relationships between abundance and composition of root-associated microbes and canker lesion counts.

Variation in the root microbiome was dominated by site

Site was the primary factor affecting fungal and bacterial community size, α-diversity, β-diversity, and the abundance of most ASVs. Site is well known to be the dominant factor in apple microbiome diversity and composition, including in bulk soil (Lauber et al. 2009, Griffiths et al. 2011, Tedersoo et al. 2014, Prober et al. 2015), apple rhizosphere (Deakin et al. 2019, Van Horn et al. 2021), and endophytes in the above-ground parts of apple trees (Liu et al. 2020, Olivieri et al. 2021, Papp-Rupar et al. 2023a) and consistently dwarfs the contribution of the host genotype. Site encompasses multiple associated factors, which affect microbial communities including local variation in climate, soil characteristics, and vegetation. Apple rhizosphere microbial communities vary significantly between trees grown in soils from different sites (Van Horn et al. 2021). They also vary within orchards between neighbouring rows (Deakin et al. 2019, Papp-Rupar et al. 2023a). The sites in this experiment were at different commercial orchards and therefore subjected to different historical and present soil and orchard management practices, which may have contributed to observed differences in the microbiomes.

Table 2. Percentage of variance in BC dissimilarity and the total community for the first six PCs (PC1–6) explained by design factors and canker lesion counts (Canker) for fungal and bacterial root-associated communities based on PERMANOVA of BC and ANOVA of PCs.

	df	BC	PC1	PC2	PC3	PC4	PC5	PC6	PC1–6 total
					Fungi				
Site	2	31.44***	23.02***	13.98***	0.87*	0.47*	0.07	0.05	38.46
Storage	1	2.05*	0.03	0.20	0.07	0.03	0.19	0.09	0.61
Scion	6	4.65	0.26	0.08	0.48	0.14	0.11	0.07	1.14
Canker	1	0.62	0.16	0.14	0.01	<0.01	0.10	0.02	0.44
Site × Storage	2	3.36**	0.58**	1.65***	0.54	0.34	0.10	0.11	3.32
Site × Scion	12	10.35	0.62	0.58	0.89	0.38	0.34	0.15	2.96
Storage × Scion	6	5.77	0.2	0.21	0.42	0.21	0.16	0.17	1.37
Site × Canker	2	1.82	0.01	0.21	0.10	0.08	0.08	0.2	0.68
Storage × Canker	1	0.60	0.04	0.03	0.14	0.07	0.13	<0.01	0.42
Scion × Canker	6	4.37	0.23	0.37	0.7	0.38	0.09	0.25	2.02
Residuals	39	34.97	1.95	3.76	4.19	2.11	1.92	1.48	15.41
Total variance			27.10	21.20	8.40	4.20	3.30	2.60	66.8
					Bacteria				
Site	2	31.37***	15.46***	11.08***	0.19	0.48**	<0.01	<0.01	27.23
Storage	1	1.55	<0.01	0.02	<0.01	0.04	0.34**	0.11*	0.53
Scion	6	6.64	0.31	0.11	1.21*	0.24	0.11	0.40*	2.38
Canker	1	0.73	<0.01	<0.01	0.02	0.02	0.08	<0.01	0.15
Site × Storage	2	4.80***	0.25*	0.05	1.19***	0.04	0.40**	0.11	2.04
Site × Scion	12	10.10	0.67	0.37	0.92	1.14*	0.20	0.20	3.50
Storage × Scion	6	4.95	0.13	0.07	0.73	0.17	0.17	0.24	1.51
Site × Canker	2	2.11	0.05	0.04	0.06	0.18	0.17	<0.01	0.51
Storage × Canker	1	0.63	0.01	<0.01	<0.01	<0.01	0.04	<0.01	0.09
Scion × Canker	6	3.93	0.19	0.16	0.14	0.17	0.10	0.14	0.90
Residuals	39	33.18	1.53	0.80	2.63	1.53	1.40	0.89	8.78
Total variance			18.60	12.70	7.10	4.00	3.00	2.10	47.50

*$P < .05$,**$P < .01$, and ***$P < .001$.

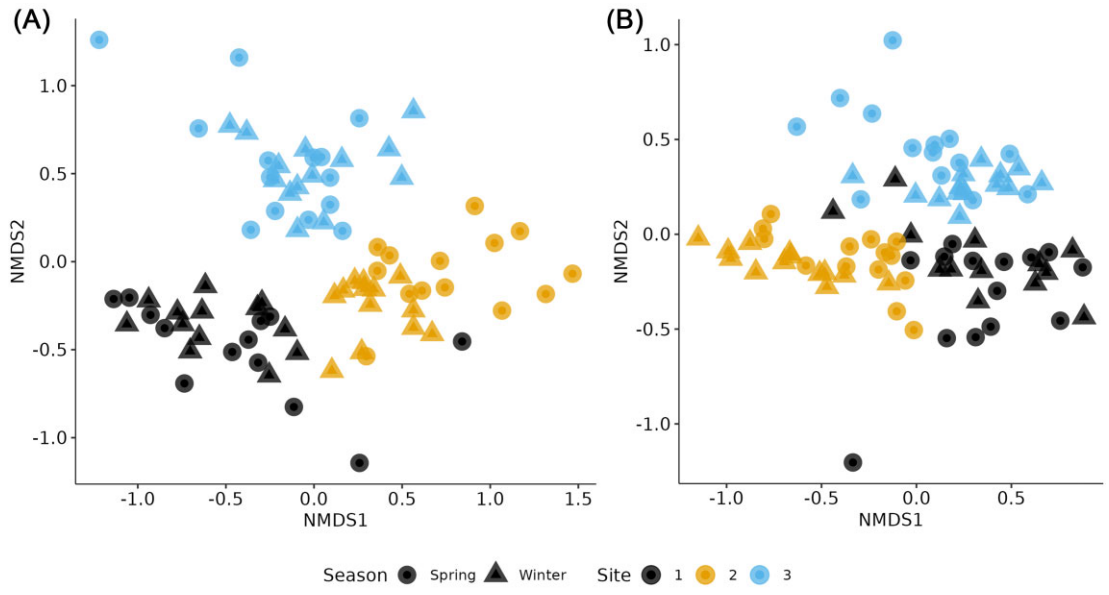

Figure 2. Plots of the first two NMDS dimensions, illustrating BC dissimilarity of fungal (A) and bacterial (B) root-associated communities. Colours are assigned to sites (Site) and shapes to cold storage/planting season (Season).

Cold storage combined with delayed planting affected the root microbiome

In this study, half of the trees were planted immediately after lifting in December, while the other half were cold stored in a commercial facility and planted in the following April. Thus, we cannot isolate the effects of cold storage of trees from the effect of planting season on the root microbiome. We found that cold storage/planting season affected fungal community size and diversity and affected the abundance of particular fungal and bacterial ASVs. These effects were largely site-dependent, i.e. site by storage interaction explained more variance than storage as a main effect.

No research has been done to date on the effects of cold storage or planting season on the apple root microbiome. The cold stor-

Table 3. Summary of the 10 most abundant fungal and bacterial ASVs significantly affected by canker lesion counts as main effect or in interactions with design factors.[a]

ASV	Taxonomy	Mean abundance	Canker	Site × Canker	Storage × Canker	Scion × Canker	Coefficient
			Fungi				
ASV80	*Neonectria* (g)	159.5	0.05	8.54*	0.84	2.32	
ASV262	Basidiomycota (p)	135.5	1.35	1.95	0.26	24.59***	
ASV195	*Trichoderma* (g)	87.8	4.88*	1.51	0.58	1.28	−0.0277
ASV824	Hypocreales (o)	44.9	4.61*	0.93	0.01	1.18	0.0042
ASV269	Helotiales (o)	41.1	6.95*	0.19	0.72	8.00	0.0041
ASV237	Sordariomycetes (c)	24.8	5.06*	2.13	0.12	8.24	0.0052
ASV530	Agaricales (o)	19.6	11.61**	0.14	5.27	1.67	0.0111
ASV574	*Parasola leiocephala* (s)	7.1	<0.01	1.2	2.14	16.63*	
ASV638	Agaricomycetes (c)	6.4	7.29*	6.07	4.37	8.47	0.0119
ASV1128	*Trichoderma* (g)	2.4	0.21	16.2*	0.22	1.50	
			Bacteria				
ASV5468	Rhizobiales (o)	41.7	1.85	5.63*	0.01	1.97	
ASV5197	Sphingomonadales (o)	26.3	0.86	10.35*	0.18	3.60	
ASV3489	Bradyrhizobiaceae (f)	17.3	<0.01	12.69*	0.07	5.02	
ASV1114	*Streptomyces* (g)	16.3	0.76	3.82*	0.12	2.48	
ASV309	*Saccharibacteria_genera_incertae_sedis* (g)	15.9	0.01	1.32	0.12	5.86*	
ASV1011	Pseudonocardiaceae (f)	12.7	0.20	1.24	0.12	16.84**	
ASV5457	*Actinoplanes* (g)	11.0	0.46	1.14	1.01	8.24*	
ASV6145	*Streptomyces* (g)	10.2	3.43	7.75*	0.48	2.76	
ASV549	Micropepsaceae (f)	9.9	0.73	3.68	0.25	15.14*	
ASV1206	Gammaproteobacteria (c)	9.3	0.49	12.62*	0.13	4.69	

[a]Percentage of variance of ASV abundance explained is reported with approximate *P* values indicated. *P* values adjusted for multiple testing using the Benjamini–Hochberg method. The coefficient corresponds to the effect of canker lesion count on the ASV abundance as a main effect.
*$P < .05$,**$P < .01$, and ***$P < .001$.

age treatment may have directly affected the root microbiome on the tree roots (Borowik and Wyszkowska 2016, Alkorta et al. 2017) such that at planting, the microbiomes of the two groups have diverged considerably. The seasonal variation in the bulk soil microbiome (Lauber et al. 2013, Uksa et al. 2014, Docherty et al. 2015) at planting may have also contributed to the difference in the initial microbiome. Further, the difference in host phenology in winter versus spring planting may have differentially influenced the evolution of initial microbiome.

The combination of these factors resulted in sufficiently different initial root microbiome, which persisted for 3 years. Cold storage/planting season was previously found to have a small but significant effect on canker severity, both independently and dependently of site (Xu et al. 2022). Together, these results suggest that the founder effect is important for the long-term development of the root microbiome, supporting the possibility that artificial influence of the microbiome at planting may have long-term effects and may influence plant health. Further research is required to unravel the specific contributions of cold storage and planting season on root microbiome assembly and succession.

Scion genotype had a limited effect on the root microbiome

Scion genotype did not significantly affect fungal or bacterial community size or α-diversity but did have a limited site-dependent effect on bacterial communities as well as the abundance of some individual fungal and bacterial ASVs.

Colonization of roots by artificial inoculation of arbuscular mycorrhizal fungi (AMF) has been found to vary between scion genotypes grafted on the same rootstock genotype in both apple (Berdeni et al. 2018) and citrus (Song et al. 2015). This effect is thought to be caused by differences in root exudates synthesized in the scion and transported to the roots, which are known to affect AMF colonization (Kiers et al. 2011). Rhizosphere bacterial diversity and composition have also been found to vary between scion genotypes, and the abundance of some rhizosphere bacteria correlated with differential exudation of root sugars (Chai et al. 2022).

The limited effect of scion genotype on the root-associated microbiome may be due to our sampling of microbes closely associated with the roots after removal of rhizosphere soil. Apple root endophyte and rhizosphere microbiomes have very different microbial composition, and fungal and bacterial rhizoplane/endophyte populations are much smaller than rhizosphere populations (Liu et al. 2022). Such closely associated microbes may be under strict control of the rootstock, which were M9 in all treatments, rather than scion-derived compounds. The scion genotype, however, has been observed to have a greater effect on the rhizosphere microbiome than the rootstock genotype (Chai et al. 2022). More research is needed to ascertain why the scion seems to affect rhizosphere more than root endophytes.

Canker severity was associated with the root microbiome

The previous study (Xu et al. 2022) found that site, cold storage, and scion genotype had a significant effect on canker severity. This study extends this by exploring the relationships between root-associated microbiomes and canker severity. Our experimental design allowed us to infer associations between microbiome metrics and canker lesion counts, but we could not establish causality due to potential confounding variables and bidirectional influence. Bacterial Shannon diversity and Simpson evenness had significant site-dependent associations with canker lesion counts (Table 1). We also identified specific ASVs, which were associated with canker lesion counts across and within sites (Tables 3 and 4).

Table 4. Summary of the top five ASVs (by mean abundance) with a significant negative or positive association with canker lesion counts either across all sites or within a specific sites.[a]

ASV	Taxonomy	Mean abundance	c	Deviance (%)	P	Site
		Fungi				
ASV530	Agaricales (o)	20.1	−0.416	8.83	.040	All
ASV395	*Flagelloscypha minutissima* (s)	300.8	−0.452	4.88	.046	1
ASV540	*Rhizophagus irregularis* (s)	54.7	−1.009	16.66	.038	1
ASV1105	Chaetomiaceae (f)	45.1	−3.692	16.74	.047	1
ASV759	*Rhizophagus* (g)	16.8	−4.269	18.35	.047	1
ASV921	*Apiospora aurea* (s)	13.3	−0.933	10.02	.047	1
ASV154	*Epicoccum nigrum* (s)	8.1	−0.969	1.99	.047	1
ASV56	*Mortierella alpina* (s)	118.4	0.731	3.17	.042	1
ASV187	*Fusarium* (g)	71.6	0.876	6.19	.021	1
ASV133	*Linnemannia elongata* (s)	46.6	0.436	5.38	.032	1
ASV290	Nectriaceae (f)	45.1	0.816	15.78	.032	1
ASV452	*Flagelloscypha minutissima* (s)	19.0	1.057	0.16	.029	1
ASV573	*Rhizophagus irregularis* (s)	11.4	−0.509	19.57	.048	2
ASV539	*Fusarium cuneirostrum* (s)	1.4	−1.257	7.71	.024	2
ASV644	*Podospora tetraspora* (s)	6.4	2.475	4.59	.013	3
		Bacteria				
ASV5	*Streptomyces* (g)	1169.6	−0.659	2.91	.013	All
ASV7068	*Streptomyces* (g)	82.2	−0.617	2.03	.028	All
ASV3339	*Streptomyces* (g)	78.0	−0.529	1.84	.042	All
ASV103	*Amycolatopsis* (g)	72.6	−0.795	1.38	.019	All
ASV7112	*Bradyrhizobium* (g)	70.0	−0.725	1.92	.045	All
ASV60	Methylophilaceae (f)	70.0	0.650	0.37	.036	All
ASV410	*Pseudomonas* (g)	11.2	0.430	7.88	.014	All
ASV381	*Pedomicrobium* (g)	10.5	0.604	1.05	.019	All
ASV651	Rhizobiales (o)	6.8	0.557	3.75	.014	All
ASV5	*Streptomyces* (g)	892.3	−0.844	10.30	.049	1
ASV7	*Bradyrhizobium* (g)	731.1	−1.936	6.60	.016	1
ASV11	*Actinoplanes* (g)	725.7	−1.278	4.09	.028	1
ASV63	*Povalibacter* (g)	310.7	−2.242	19.13	.010	1
ASV44	*Aquabacterium* (g)	144.8	−1.304	10.76	.011	1
ASV1430	*Pseudomonas* (g)	12.6	0.822	11.02	.048	1
ASV930	*Pseudomonas* (g)	10.0	0.792	24.60	.049	1
ASV1143	Rhizobiales (o)	7.2	−0.361	26.40	.042	2
ASV60	Methylophilaceae (f)	150.9	0.692	6.40	.038	2
ASV49	*Novosphingobium* (g)	84.8	0.744	11.13	.032	2
ASV151	*Flavobacterium* (g)	30.8	0.532	5.36	.031	2
ASV263	*Flavobacterium* (g)	27.2	0.553	13.97	.000	2
ASV1505	*Streptomyces* (g)	23.3	0.734	15.41	.029	2
ASV497	Micromonosporaceae (f)	115.2	−2.335	15.37	.019	3
ASV4343	*Actinoplanes* (g)	34.5	−1.582	13.01	.044	3
ASV750	*Kribbella* (g)	21.4	−1.682	13.14	.036	3
ASV497	Micromonosporaceae (f)	115.2	−2.335	15.37	.019	3
ASV4343	*Actinoplanes* (g)	34.5	−1.582	13.01	.044	3
ASV750	*Kribbella* (g)	21.4	−1.682	13.14	.036	3

[a]Taxonomy at >80% confidence is given. The mean abundance of each ASV across samples is reported. *c* is the coefficient of ASV abundance in the model, i.e. negative *c* corresponds to decreased canker count with increased ASV abundance. Percentage of deviance in canker count explained by each ASV is reported. *P* values calculated by LR were adjusted using the Benjamini–Hochberg method.

Most of the ASVs with a significant effect on canker lesion counts depended on site.

We found high abundance of *Rhizophagus* (ASVs 540, 759, 573), *Epicoccum nigrum* (ASV 154), and *Trichoderma* (ASV 195) associated with fewer canker lesions. *Rhizophagus* is an arbuscular mycorrhizal fungal symbiont that enhances nutrient uptake (Wang et al. 2022) and improves apple tree health, alleviating replant disease (Wang et al. 2024) and increasing resistance to *N. ditissima* apple canker (Berdeni et al. 2018). *Epicoccum nigrum* has biocontrol activity and increases resistance to *N. ditissima* apple canker (Papp-Rupar et al. 2023b). *Trichoderma* are well known for their biological control of agricultural pathogens (Zin and Badaluddin 2020) and have been found to increase resistance to apple Valsa canker (Valetti et al. 2022). More research is needed to confirm whether increased abundance of the above fungi could reduce the impact of canker.

The association of *Flagelloscypha minutissima* with canker counts is unclear, as ASV 395 was associated with fewer cankers and ASV 452 with more cankers. High abundance of the fungi *Fusarium* (ASV 178), *Mortierella alpina* (ASV 56), and *Linnemannia elongata* (ASV 133) was associated with more cankers. Some *Fusarium* species cause root rot (Astapchuk et al. 2020) and are implicated in apple replant disease (Duan et al. 2022). A shift in *Fusarium* communities is associated with a change in apple health (Caputo et al. 2015). The association of *Fusarium* species with more canker lesions could be either causal, where they en-

able *N. ditissima* infection by weakening the host, or opportunistic, where the host weakened by *N. ditissima* infection is more susceptible to *Fusarium*. *Mortierella alpina* has been reported to have antimicrobial activity and is antagonistic to various phytopathogens (Melo et al. 2014). *Mortierella* species have been found to suppress *Fusariam* wilt in cucumber (Li et al. 2016). Similarly, *L. elongata* (Mortierellaceae family) is a plant symbiont reported to stimulate growth and defence in *Arabidopsis* (Vandepol et al. 2022). It is therefore unexpected that high abundance of *M. alpina* and *L. elongata* was associated with more canker lesions. This could be due to strain-specific or environment-specific interactions.

High abundance of the bacteria *Amycolatopsis* (ASV 103), *Bradyrhizobium* (ASVs 7, 7112), *Povalibacter* (ASV 63), *Aquabacterium* (ASV 44), *Actinoplanes* (ASVs 11, 4343), and *Kribella* (ASV750) was associated with fewer cankers. Of these, *Amycolatopsis*, *Bradyrhizobium*, and *Actinoplanes* are known as plant growth-promoting rhizobacteria (PGPR). *Amycolatopsis* also have biocontrol activity for a range of crop diseases (Gandham et al. 2024) and induce plant defence responses in cucumber (Alipour Kafi et al. 2021). *Bradyrhizobium* are nitrogen-fixing bacteria (Kuykendall 2015) and can improve pathogen inhibition of host plants (Mehboob et al. 2012). *Actinoplanes* promote plant growth through synthesis of phytohormones (Solans et al. 2011). *Streptomyces* ASVs were associated with either more or fewer canker lesions. Some *Streptomyces* promote plant growth or antagonise pathogens (Nazari et al. 2023). Other *Streptomyces* are pathogenic (Loria et al. 2006, Viaene et al. 2016). In apple, *Streptomyces* are enriched in soils affected by apple replant disease but their role as either causal agents of disease or opportunists is unknown (Mahnkopp-Dirks et al. 2021). High abundance of *Pseudomonas* (ASVs 410, 930, and 1430), *Novosphingobium* (ASV 49), and *Flavobacterium* (ASVs 151 and 263) was associated with more cankers. Some *Pseudomonas* are plant pathogenic (Höfte and De Vos 2006); others promote plant growth or have biocontrol activity (Kumar et al. 2017). Here, pathogenic *Pseudomonas* may be exacerbating canker by weakening the host or may be opportunistic. *Novosphingobium* promote growth in rice (Zhang et al. 2016, Krishnan et al. 2017, Rangjaroen et al. 2017) and can improve salt tolerance in citrus (Vives-Peris et al. 2018). *Flavobacterium* can also promote plant growth (Seo et al. 2024) and can improve salt and drought stress (Kim et al. 2020). The association of high abundance of *Novosphingobium* and *Flavobacterium* with high canker counts is unexpected and could again be due to strain-specific or environment-specific interactions.

The causal relationships between these taxa and canker disease are unclear from this analysis. Further research using specific microbial amendments prior to *N. ditissima* inoculation is required to establish causality and to uncover the roles of microorganisms or functional groups in canker susceptibility.

Conclusion

This study offers a new perspective on the factors affecting the apple root microbiome and its relationship with apple canker. As shown previously, site accounted for most of the variation in the microbiome indices tested. The small but significant effects of cold storage/planting season and scion on microbiome metrices indicated that differences in the initial conditions at planting can persist in the long term and may be partially scion specific. Scion genotype and number of cankers had very small effects on very few microbiome indices and ASV abundances. We identified associations between specific taxa and canker lesion counts, including beneficial associations with known plant growth-promoting or biocontrol fungi *Rhizophagus irregularis*, *E. nigrum*, *Trichoderma*, and bacteria *Amycolatopsis* and *Bradyrhizobium*. This research demonstrates that orchard establishment characteristics and components of fungal and bacterial communities on apple roots may be associated with canker disease outcomes.

Data availability

Sequence data are available at ENA project accession PRJEB79650.

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Author contributions

Hamish McLean (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing—original draft), Alexey Mikaberidze (Supervision, Writing—review & editing), Greg Deakin (Data curation, Software, Validation, Writing—review & editing), Xiangming Xu (Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing—review & editing), and Matevz Papp-Rupar (Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing—review & editing)

Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

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