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Profiling the *Lolium perenne* Microbiome: From Seed to SeedIan Tannenbaum,^{1,2,†} Jatinder Kaur,¹ Ross Mann,¹ Timothy Sawbridge,^{1,2} Brendan Rodoni,^{1,2} and German Spangenberg^{1,2}¹ Agriculture Victoria, AgriBio, Centre for AgriBioscience, Bundoora, Victoria, Australia² School of Applied Systems Biology, La Trobe University, Bundoora, Victoria, Australia

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ABSTRACT

Lolium perenne (perennial ryegrass) is a common temperate pasture grass species and is favored by dairy farmers. Commercially, the known association with the mutualistic endophytic fungus *Epichloë festucae* subsp. *lolii* is used to enhance insect resistance of host plants. Knowledge of other members of the microbiome and their functions are limited. Sequencing of the V4 region of the 16S ribosomal RNA gene was used to examine the bacterial microbiome of perennial ryegrass (Alto) seed (generation 1 [G1]), subsequent mature plants grown in soil and sand, and seed (generation 2 [G2]) from crosses of these mature plants. The G1 microbiome was dominated by the class *Gammaproteobacteria*. The mature plant microbiomes were far more diverse, comprising up to 37 classes inclusive of *Gammaproteobacteria*. Different growth media yielded different

microbiome profiles in mature plants. The G2 microbiome, similar to the G1 microbiome, was dominated by *Gammaproteobacteria* as the primary constituent, with additional supplementation from class *Bacilli*. This suggests the continuation of a core microbiome which persists from seed through plant maturation to seed. This study sheds new light on the heritability of perennial ryegrass bacterial microbiomes and has identified some operational taxonomic units of potential commercial significance due to their seed transmissibility, and their roles are currently being explored.

Keywords: 16S rRNA, bacterial recruitment, endophytes, *Lolium perenne*, metagenomics, microbial diversity, microbiome, microorganism, symbiosis, transmissibility

Plant microbiomes have piqued interest in agricultural research and have a range of commercial applications. Several microbes have been associated with certain beneficial functions within and around plants such as the well-established link between nitrogen fixation and *Rhizobium* spp. (Mabood et al. 2014). In return for providing nutrients, plants release exudates which provide a variety of metabolites that can be utilized by bacteria and fungi (Steinauer et al. 2016). Microorganisms have been utilized by their host for different uses such as nutrient acquisition and uptake, bioprotection, and bioremediation (Chaudhary and Khan 2018; de Souza et al. 2015; Wang et al. 2018).

Perennial ryegrass (*Lolium perenne*) is the most commonly used grazing grass in temperate Australian and New Zealand pastures, and symbiotic *Epichloë* fungal endophytes are commercially exploited to provide insect resistance in some environments

(Ekanayake et al. 2012; Hettiarachchige et al. 2019; Kaur et al. 2015; Reed 2014). These *Epichloë* endophytes are also seed transmissible, allowing their benefits to persist through seed generations. This can be viewed as a commercial product to microbially enhance perennial ryegrass seed and also an example of the holobiont concept. This conceptualizes plant functions being outsourced to members of the microbiome (Gilbert et al. 2012; Hassani et al. 2018). Resident fungi are only one part of the total holobiont, with other members including the bacterial epiphytes and endophytes, a far less studied collection of microorganisms (Khaksar et al. 2017).

Thus far, several plants have been evaluated to determine their microbiome structure. *Arabidopsis thaliana*, a model plant, was extensively characterized across root exudates, rhizosphere, phyllosphere, and transcriptome (Bai et al. 2015; Bulgarelli et al. 2012; Kerr et al. 2019; Lundberg et al. 2012; Strehmel et al. 2014). There has also been characterization of a variety of crop plants such as rice, maize, soybean, and wheat (Edwards et al. 2015; Gdanetz and Trail 2017; Hassani et al. in press; F. Liu et al. 2019; Peiffer and Ley 2013). Recent microbiome research around perennial ryegrass involved profiling root-associated bacteria exclusively or fungal endophytes, or was related to plant health and disease (Chen et al. 2016; Heineck et al. 2018; Ma et al. 2015; Nissinen et al. 2019).

All plants have multiple subcompartments comprising their microbiomes, the core components being the phyllosphere (aerial surfaces), endosphere (internal tissue and vasculature), and rhizosphere

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(root surface) (Shade et al. 2017; Turner et al. 2013). Microbial profiles have proven to be quite different between compartments (Edwards et al. 2015; Fernández-González et al. 2019). For instance, whereas the rhizosphere and root endosphere are in proximity, not all microorganisms have the mechanisms to enter the plant for colonization. This can yield different profiles between compartments with some shared constituents (Fernández-González et al. 2019). Frank et al. (2017) briefly discussed the modes of microbial transmission, horizontally from the environment and vertically from the parent plant.

During seed generation, a new compartment of the microbiome is created. Seed contain initial reservoirs of bacterial endophytes within the endosphere, inherited from their parent (Eyre et al. 2019; Raj et al. 2019). Some of these bacterial endophytes have been associated with germination, early plant health, and development (Felestrino et al. 2017; Huang et al. 2016). Such bacterial endophytes would be selected for during seed production and would be transmitted vertically, carried along with the vascular tissue to the seed heads. Once the seed microbiome assembly completes and becomes encapsulated within the seed coat, the microbial consortia become more difficult to influence until germination (Kim et al. 2020).

In this study, we described the bacterial microbiome of perennial ryegrass seed from two commercial batches of the Alto cultivar; one batch contained the commercially available fungal endophyte *Epichloë festucae* subsp. *lolii* and the other was an endophyte-free batch. Comparison of batches allowed us to infer the effect of resident endophytes on the host bacterial microbiome. An initial seedborne microbiome for perennial ryegrass was determined by assessing surface-sterilized seed; identified microbiota were presumed to originate from the seed. In addition, we monitored the microbiomes of our plants through their maturation and profiled the subsequent-generation seed to determine microbiome transmissibility across two different soil types (Walters et al. 2018). This study revealed a persistent microbiome of perennial ryegrass, from seed to seed, with accompaniments provided during plant maturation. The implications from these findings may assist in how pasture and crop health will be approached in the future, with the potential for reducing use of chemical additives and fertilizers.

MATERIALS AND METHODS

Ryegrass seed selection. Two seed batches of perennial ryegrass (*L. perenne* ‘Alto’) were sourced from Agriseeds New Zealand. Of the batches, one contained a standard endophyte (SE) (resident fungal endophyte *E. festucae* subsp. *lolii*) and one was without an endophyte (WE); these were referred to as Alto-SE and Alto-WE, respectively (Kaur et al. 2015). These batches were harvested in March 2015 and stored at 4°C until germination in November 2016. To determine SE presence or absence, a kompetitive allele-specific (KASP) assay (Supplementary Methods S1) was performed on each batch (Qureshi et al. 2018).

Sample preparation. Seed were surface sterilized as per Kaur et al. (2015), with the following modifications; the H₂SO₄ step was removed and seed was washed in 3% NaClO for 3 min, then washed five times in sterile Milli-Q H₂O for 1 min. Batches were germinated on moistened filter paper in sterile Petri dishes and incubated in the dark at room temperature for 7 days. Seedlings of similar size were harvested and sectioned into shoots and roots (Supplementary Fig. S3a), which represented the first-generation seed microbiome (G1). G1 seedlings from each batch were transplanted into two different soil types (a potting mix and an autoclaved mixture of sand and vermiculite, ratio 3:2) and incubated under glasshouse conditions for 10 weeks. Mature plants from each batch were harvested

by removing three tillers with their respective roots, washed in sterile phosphate-buffered saline (PBS) (roots only), then sectioned into shoots and roots (Supplementary Fig. S3b), which represented the mature plant microbiome. The remaining plants from each batch were vernalized in a Conviron A1000 growth chamber (Conviron, Canada) with a cycle of 5°C for 16 h (night) and 14°C for 8 h (day) for 10 weeks. To induce flowering, the cycle was adjusted to 24°C for 20 h (day) and 16°C for 4 h (night), following which plants were allowed to cross-pollinate. The soil and sand/vermiculite pots remained separate during the vernalization process. SE and WE batches were allowed to vernalize together because SE is not known to be carried by pollen (J. Liu et al. 2017). The seed was harvested from plants of each batch, germinated as above, and sectioned into shoots and roots (Supplementary Fig. S3a), which represented the second-generation seed microbiome (G2). Full details regarding all treatment combinations are provided in Table 1. All plant sections were processed for DNA extraction and 16S amplicon sequencing.

DNA extraction and 16S amplicon sequencing. For profiling the bacterial communities in seed (G1 and G2), 50 to 100 seedlings were selected from each batch. Parental soil type for G2 was recorded (Supplementary Table S1). Root and shoot sections were pooled into sets of 5, creating 10 replicates for each batch in G1 and 5 replicates of 5 pooled organs for G2 per parental plant. For profiling the bacterial communities in mature plants, shoots and roots were harvested from five plants per growth medium. DNA extraction was performed using the QIAGEN MagAttract 96 DNA Plant Core Kit according to the manufacturer’s instructions, with minor modifications for use with a Biomek FX liquid handling station.

The bacterial microbiome was profiled targeting the V4 region of the 16S ribosomal RNA (rRNA) gene according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Supplementary Methods S2), with minor modification to include the use of peptide nucleic acid (PNA) PCR blockers to reduce amplification of 16S rRNA gene sequences derived from the plant organelle genome (Lundberg et al. 2013; Wagner et al. 2016). Paired-end sequencing was performed on the G1 and G2 libraries on an Illumina MiSeq using a 2 × 300 bp v3 chemistry and an Illumina HiSeq3000 using 2 × 150 bp, respectively. The mature plant bacterial libraries were prepared as previously stated and were sequenced on both aforementioned platforms using the same specifications. All Illumina sequences have been submitted to the NCBI Short Read Archive (SRA accession PRJNA577477).

Data processing and diversity analysis. Sequence data were trimmed and joined using PANDAseq (Massela et al. 2012). HiSeq3000 and MiSeq data were processed as described in the supporting information (Supplementary Method S2). Post PANDAseq reads were imported via manifest, as single read FASTQ sequences, into QIIME2 (release 2019.10) for further processing and organelle read removal (Bolyen et al. 2019). The q2-deblur plugin was used for dereplication and denoising (Amir et al. 2017). The q2-feature-classifier plugin was used for the taxonomic assignment of operational taxonomic units (OTUs) against the SILVA 1.32 database (released 12 December 2017). The q2-phylogeny plugin was used to generate rooted and unrooted phylogenetic trees by implementing MAFFT and FastTree 2 (Katoh and Standley 2013; Price et al. 2010). Comparative analysis of treatments was conducted using QIIME2 and Genedata Expressionist, Analyst Module (Genedata AG, Basel, Switzerland).

α-Rarefaction plots were created using QIIME2 to optimize sample rarefaction to 5,000 reads per sample. The q2-diversity plugin was used to generate Jaccard dissimilarity plots between all treatments. Comparative analysis was conducted on the G1 Alto-SE and Alto-WE seed to profile the effects of fungal endophyte and

organ on the bacterial microbiome. A brief analysis of PNA clamp use was conducted to ensure the prevention of organelle amplification in the 16S PCR and is available in the supporting information (Lundberg et al. 2013). Similar analyses were undertaken on the mature plants grown in sand and soil, as was G2. Genedata Expressionist, Analyst Module (Genedata AG, Basel, Switzerland) was used for the production of Venn diagrams to demonstrate commonalities between treatment and to infer transmissibility of microbial constituents.

Microbiome isolation. For G1 bacterial isolations, 50 seedlings were selected from Alto-WE and 25 seedlings were selected from Alto-SE. Each seedling was sectioned into shoots and roots (Supplementary Fig. S3a). Each section was pooled into a set of 5, creating 10 replicates for Alto-WE and 5 replicates for Alto-SE. Seedlings were ground two times in PBS in a Qiagen TissueLyser II for 1 min at 30 Hz, inverted after each cycle. Macerates were serially diluted to create a 10^{-2} -to- 10^{-4} series, plated onto Reasoner's 2A agar (R2A) (Oxoid, Australia), and incubated at room temperature for up to 4 weeks. Individual colonies were purified on R2A. Pure subcultures were further grown in Reasoner's 2A broth (Amyl, Australia) for 24 to 48 h and stored in 20% glycerol at -80°C . For fungal isolations, 15 seedlings were selected from Alto-WE and 15 seedlings were selected from Alto-SE. Seedlings were sectioned as above and plated directly onto half potato dextrose agar with tetracycline hydrochloride at 0.5% wt vol $^{-1}$, yielding only *E. festucae* subsp. *lolii* from Alto-SE seed only (data not shown).

Identification of bacterial isolates was performed by sequencing the full-length 16S rRNA gene for 83 isolates. DNA was extracted from each isolate using a DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. Further details, including the primer sequence and PCR protocols, are available in the supporting information (Supplementary Methods S4). Forward and reverse sequences were examined for quality, assembled using

Sequencher (Gene Codes Corporation 2017), compared with the NCBI 16S rRNA (Bacteria and Archaea) database, and taxonomically assigned by the greatest sequence homology. The 16S rRNA sequences were assembled into a database for the mapping of Illumina V4 sequence data using NINJA-OPS (Al-Ghalith et al. 2016).

RESULTS

Bacterial microbiome profiling (class level). The most prominent class in both the G1 and G2 microbiomes was *Gammaproteobacteria*, accounting for 88.5 to 99.9% of the OTU abundance, with *Alphaproteobacteria* accounting for 0 to 2% (Fig. 1). The major difference between the G1 and G2 microbiomes was the presence of class *Bacilli*, which accounted for up to 10.9% in G2 and was absent in G1 (Fig. 1). For G1, shoots were dominated by *Gammaproteobacteria* (99.80 to 99.96%), whereas roots contained *Gammaproteobacteria* (97.86 to 99.28%), *Alphaproteobacteria* (0.70 to 2.00%), and *Actinobacteria* (0.02 to 0.04%) (Fig. 1). *Bacteroidetes* accounted for 0.10% of the OTU abundance in G1 shoots containing SE (Fig. 1). For G2, roots contained *Gammaproteobacteria* (96.42 to 99.89%), *Bacilli* (0.07 to 1.48%), and up to 2.02% of *Alphaproteobacteria*, whereas shoots contained less *Gammaproteobacteria* (88.5 to 98.36%) and greater abundances of *Bacilli* (1.62 to 10.88%) and *Alphaproteobacteria* (0.62 to 2.02%) (Fig. 1). Presence of SE was associated with a greater abundance of *Bacilli* and *Alphaproteobacteria* in G2 shoots only (Fig. 1).

The mature plant microbiomes contained 18 to 37 classes (Fig. 1), predominantly comprising *Gammaproteobacteria* (18.03 to 31.64%), *Alphaproteobacteria* (12.50 to 23.54%), *Bacteroidia* (11.72 to 23.01%), and *Deltaproteobacteria* (2.68 to 6.97%). The remaining classes encompassed 6.28 to 18.91% of the OTU abundances (Fig. 1). Plants grown in soil had a greater association with *Actinobacteria* (19.38 to 30.82%) when compared with

TABLE 1
Additional details describing the assessed treatments

Sample name ^a	Status ^b	Organ	Age (days)	Plant type	G ^c	Growth media	Samples per pool	Total pools
Sand SE root	+	Root	90	Mature plant	1	Sand	3	11
Sand SE shoot	+	Shoot	90	Mature plant	1	Sand	3	11
Sand WE root	–	Root	90	Mature plant	1	Sand	3	10
Sand WE shoot	–	Shoot	90	Mature plant	1	Sand	3	10
G1 SE root	+	Root	7	Seedling	1	Filter paper	5	10
G1 SE shoot	+	Shoot	7	Seedling	1	Filter paper	5	8
G1 WE root	–	Root	7	Seedling	1	Filter paper	5	10
G1 WE shoot	–	Shoot	7	Seedling	1	Filter paper	5	10
G2 SE root	+ ^d	Root	7	Seedling	2	Filter paper	5	36
G2 SE shoot	+ ^d	Shoot	7	Seedling	2	Filter paper	5	25
G2 WE root	– ^d	Root	7	Seedling	2	Filter paper	5	25
G2 WE shoot	– ^d	Shoot	7	Seedling	2	Filter paper	5	23
Soil SE root	+	Root	90	Mature plant	1	Soil	3	10
Soil SE shoot	+	Shoot	90	Mature plant	1	Soil	3	9
Soil WE root	–	Root	90	Mature plant	1	Soil	3	7
Soil WE shoot	–	Shoot	90	Mature plant	1	Soil	3	11

^a G1 = generation 1, G2 = generation 2, SE = standard endophyte *Epichloë festucae* subsp. *lolii*, and WE = without endophyte.

^b Endophyte status.

^c Generation.

^d Not confirmed by kompetitive allele-specific (KASP) assay.

plants grown in sand (1.93 to 3.20%) (Fig. 1). Roots from soil-borne plants contained additional classes when compared with shoots, with 12 extra classes observed with SE presence and 9 with SE absence (Fig. 1). Plants grown in sand had a greater association with *Oxyphotobacteria* (15.62 to 19.89%) than plants grown in soil (0.92 to 0.15%) (Fig. 1). Roots from sandborne plants contained seven additional classes when compared with shoots (Fig. 1). There were no observable differences in the bacterial microbiome of sandborne plants with respect to SE presence or absence (Fig. 1).

To assess the presence or absence of bacterial taxa between treatments, we conducted a qualitative Jaccard similarity principal component analysis using the q2-diversity module of QIIME2 (Fig. 2). When comparing all treatments, primary separation was observed between the seed (G1 and G2) and the mature plant (sand and soil) microbiomes along axis 1, accounting for 16.63% of the microbiome variation (Fig. 2A and B). Secondary separation was observed between the G1 and G2 microbiomes along axis 2, accounting for 10.35% of the microbiome variation (Fig. 2a). Tertiary separation was observed between the sand and soil mature plant microbiomes along axis 3, accounting for 7.043% of the microbiome variation (Fig. 2B). Quaternary separation was observed between the SE and WE treatments within G1 only (Fig. 2A). Quinary separation was observed between the shoots and roots of G1 only (Fig. 2A). There was an observable separation of G2 by the SE presence or absence status of the parental plant (Fig. 2A and D).

When comparing only the mature plant microbiomes, primary separation was observed between plants grown in sand and soil, accounting for 25.77% of the microbiome variation along axis 1 (Fig. 2C). Secondary separation was observed between the roots and shoots of both the sand and soil treatments, accounting for

4.215% of the microbiome variation along axis 2 (Fig. 2C). Tertiary separation was observed between SE and WE treatments in the roots from plants grown in sand only (Fig. 2C). When comparing only the G1 and G2 microbiomes, primary separation was observed between the generation treatments, accounting for 19.26% of the microbiome variation along axis 1. Secondary separation was observed between SE and WE treatments, accounting for 11.24% of the microbiome variation along axis 2 (Fig. 2D).

Bacterial microbiome profiling (genus level). The seed microbiome (G1 and G2) consisted of 115 unique genera, 8 of which were unique to G1 and 92 of which were unique to G2. Fifteen genera were vertically transmitted from G1 to G2 (Fig. 3). The microbiome of mature plants grown in soil consisted of 496 genera, 21 of which persisted from G1. The microbiome of mature plants grown in sand consisted of 342 genera, 19 of which persisted from G1. When considering only the mature plant microbiomes, 216 genera were unique to the soil treatment and 62 genera were unique to the sand treatment. In all, 19 and 20 OTUs persisted from G1 into the mature plants grown in sand and soil, respectively (Fig. 3), and 280 genera were shared between the sand and soil treatments (Fig. 3). The G2 microbiome shared 86 and 89 genera with the mature plants grown in soil and sand, respectively (Fig. 3). In total, 9 genera were shared between the G2 and mature plants grown in soil exclusively and 12 genera were shared between the G2 and the mature plants grown in sand exclusively (Fig. 3).

Bacterial microbiome isolations. In total, 284 bacteria were isolated from the G1 seed of Alto-SE ($n = 131$) and Alto-WE ($n = 153$). The SE presence or absence status of each batch was confirmed before bacterial isolation (Supplementary Fig. S1). A greater number of bacteria were isolated from roots ($n = 171$) than shoots ($n = 115$).

Fig. 1. Relative operational taxonomic unit abundance (class level) of the generation 1 (G1) mature plants grown in sand or soil and generation 2 (G2) *Lolium perenne* microbiomes by organ (root and shoot) and fungal endophyte presence (standard endophyte *Epichloë festucae* subsp. *lolii* [SE]) or absence (without endophyte [WE]).

Sequencing of the 16S ribosomal RNA gene. In all, 83 isolates were sequenced for the target 16S rRNA gene (Supplementary Method S4). There were determined to be 24 unique genera (Fig. 4). Alto-WE had greater diversity than SE at the genus level. Both seed batches had similar proportions of classes *Gammaproteobacteria*, *Actinobacteria*, *Betaproteobacteria*, and *Bacilli*. Isolates from family *Bacteroidia* were only observed in the absence of SE. Illumina V4 sequence data mapped to the full-length 16S sequences from isolated bacteria (Supplementary Table S2). In total, 18 of the 24 culturable genera were detectable, where 93.6% of Illumina data mapped at 97% homology and 74.1% mapped at 99% homology (Supplementary Table S2). Further descriptions of the isolates are available in the supporting information (Supplementary Table S3).

DISCUSSION

The seed microbiome. The seed microbiome of perennial ryegrass (*L. perenne*) was dominated by *Alphaproteobacteria* and *Gammaproteobacteria*, similar to previous findings in a variety of alpine (Wassermann et al. 2019), *Oryza* spp. (Edwards et al. 2015),

Brassica spp., and other cropping species (Barret et al. 2015). Noticeably, the seed microbiome of genus *Triticum*, another member of the Poaceae family (Solanki et al. 2019), also included a pronounced *Bacilli* community. These higher classifications have been frequently observed in many other plant species' seed microbiomes, differing only in abundance between species (Turner et al. 2013). Shade et al. (2017) hypothesized that the seed microbiome reflects a key component of the previous parental plants' microbial consortia that may be used as a starting point for the establishment of a new microbiome assemblage in a daughter plant. Interestingly, the G2 microbiome was reflective of the G1 seed microbiome profile, with the addition of class *Bacilli*. The continuation of the prominent classes suggests the importance of the seedborne consortia in the viability of the seed for early development and germination (Torres-Cortés et al. 2018).

The mature plant microbiome. The mature plant microbiomes were far more complex than the seed microbiome. The proportion of *Gammaproteobacteria* was depleted in the mature plants and *Alphaproteobacteria* were enriched. Most of the OTUs detected in the mature plants were not detected in the G1 seed, suggesting that

Fig. 2. Jaccard similarity plots describing the differences between the *Lolium perenne* bacterial microbiomes of seed (generation 1 [G1] and generation 2 [G2]) and mature plants (sand and soil). SE = standard endophyte *Epichloë festucae* subsp. *lolii* and WE = without endophyte. **A**, All treatments (axis 1 and axis 2); **B**, all treatments (axis 1 and axis 3); **C**, mature plants (axis 1 and axis 2); and **D**, seed (axis 1 and axis 2) roots only.

they were recruited from the environment and incorporated into the microbiome. In this study, the environment was observed to have the greatest effect on the microbiome of the mature plants. Similar

observations were made by Schlatter et al. (2015) in their assessments of plant–soil community structures. When considering the differences between the sand and soil treatments, there were dramatic differences in the OTU populations in terms of the relative abundance reflecting the difference in media. This demonstrates that the plants likely actively recruited some OTUs which met their specific needs in each media type, as suggested by Gaiero et al. (2013) and Frank et al. (2017). From the loss of microbial richness in the progeny seed, we conclude that most of the microorganisms detected in the mature plants were transient in the evolution of the microbiome across the generation (Wani et al. 2015).

The intergenerational microbiome. This study has shown evidence of transmissible members of the bacterial microbiome between the parental plants and progeny seed. Similar to *Epichloë* fungal endophytes, some of the bacterial OTUs appeared to be conserved between generations. Most of the detected genera in this study from the G1 microbiome were conserved in the G2 microbiome. Many of the seedborne genera were depleted in the mature plants and were reenriched in the G2 microbiome. This suggests that a small proportion of the mature plant microbiome is particularly relevant for seed viability and establishment. Additionally, there was evidence of horizontal transmissibility from the environment, shown by OTUs observed in the G2 that were first detected in the mature plants. These observations imply that some bacteria taxa could be deployed in seed alongside *Epichloë* endophytes. The observed reduction of diversity between the mature plant and G2 microbiomes suggests that a strict set of selection criteria is applied by the mature plant during seed generation. If an

Fig. 3. Venn diagrams representing the number of identified genera between each of the assessed *Lolium perenne* microbiomes. G1 = generation 1 and G2 = generation 2.

Fig. 4. Proportion of genera as determined through **A**, 16S ribosomal RNA sequencing of 83 isolates and **B**, a summary at the class level. SE = standard endophyte *Epichloë festucae* subsp. *lolii* and WE = without endophyte.

existing endophyte provides an indispensable function, the literature suggests that microorganisms will be more likely selected for during the reproductive stage and seed generation (Moran 2006).

The effect of organ and substrate on the microbiome. The major differences between the bacterial profiles of the mature plants were observed between the roots and shoots (Fig. 2C). These differences were driven by the presence or absence of some minor classes and the differential abundance of major classes. The roots were associated with greater microbial diversity, each being more reflective of the growth media. Because the sand used here was initially autoclaved, there was no preexisting microbiome within the growth medium. After 3 months of growth in a nonsterile greenhouse, the sterile sand likely became colonized by a wide array of microbes from multiple potential sources such as the seedling, water, airborne transmission from neighboring plants, and physical contact with neighboring plants. These results were similar to the demonstrated impacts of different soil types on the rhizosphere and endosphere populations of *Populus* spp. (Gottel et al. 2011). Because some rhizospheric bacteria possess mechanisms for plant colonization, they were capable of accessing and colonizing the endosphere (H. Liu et al. 2017). From our observations, the bacterial profiles of shoots were largely subsets of the roots (Edwards et al. 2015; Naylor et al. 2017). This suggests that some of the bacterial endophytes were transported from the roots, possibly by means of the plant vasculature and xylem vessels, to the shoots (Frank et al. 2017). A subset of the mature plant microbiome was observed in the G2 microbiome, suggesting that a bacterial subset was selected for during the reproductive phase and seed generation (Frank et al. 2017). Interestingly, the growth medium of the mature plants did not produce a clear separation in a Jaccard dissimilarity plot dependent on the parent plants' growth media, emphasizing the return on the seed microbiome (Supplementary Fig. S4b).

The effect of a resident fungal endophyte on the microbiome. There was an observed effect of the known seed-transmissible fungal endophyte *E. festucae* subsp. *lolii* on the G1 and G2 microbiomes within a cultivar; however, the difference were not observable in the mature plants (Figs. 1 and 2A and B) (Yang et al. 2013). One possible explanation for this could be related to the relative effect of a fungal endophyte, which primarily resides near the meristem, on the bacterial microbiome with respect to the host plant size. This could be further examined by finer sampling of the mature plant organs. The lack of endophyte effect was also observed by Nissinen et al. (2019) in their elucidation of fungal endophyte effects on the leaf microbiome of tall fescue. Although there is a clear, bulk effect in G1 and G2 (Fig. 1), the Jaccard plot shows more variation among the seed pools (Fig. 2A and D). Although the SE presence or absence status of G2 was unconfirmed by KASP, the differences seen in the Jaccard dissimilarity between SE and WE in G1 were along the same axis and direction as the G2 seedlings. If a portion of the microbiome can be transmitted via pollen, there may have been a mixture of the microbiomes during the vernalization process because the plants were matured together (Zasloff 2017). This implies that, within the *Lolium* genus, *Epichloë* endophyte status should be recorded when describing seed microbiomes.

Culturability of the microbiome. We obtained 284 isolates from the G1 microbiome from both shoots and roots of SE present and absent batches. The assessed subset of isolates aligned with 24 distinct genera. At class level, the isolations largely aligned to the 16S rRNA amplicon data, although *Gammaproteobacteria* were preferentially sequenced from plant tissue (Supplementary Table S2); more than 53 to 56 and 15 to 19% of the short reads mapped to isolates corresponding to the genera *Pseudomonas* and *Erwinia*,

respectively. When also considering the low abundance reads, we found that the culture-based approach largely represented the seed microbiome of G1 (Supplementary Table S2); 18 of the 24 genera were detectable at 97% homology using next-generation sequencing methods. Some close relatives of the culturable species have been described as plant growth promoting. Most notably, members of the genera *Pseudomonas*, *Paenibacillus*, *Rhizobium*, and *Pantoea* have been characterized with an array of beneficial effects, including nitrogen fixation, indole-3-acetic acid production, and inhibitory effects toward other bacteria and fungi (David et al. 2018; X. Liu et al. 2019; Patil et al. 2017; Silini-Cherif et al. 2012). Our isolates are currently being characterized to elucidate their contribution to the function of the *L. perenne* microbiome. The ability to culture members of a seed microbiome allows for screening processes to address the potential applications of bacterial endophytes and commercialization of those with beneficial effects (Sarhan et al. 2019).

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