*Article*

# *Epichloë* Endophytes Shape the Foliar Endophytic Fungal Microbiome and Alter the Auxin and Salicylic Acid Phytohormone Levels in Two Meadow Fescue Cultivars

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**Abstract:** Plants harbor a large diversity of endophytic microbes. Meadow fescue (*Festuca pratensis*) is a cool-season grass known for its symbiotic relationship to the systemic and vertically- via seeds - transmitted fungal endophyte *Epichloë uncinata* but the effect of the endophyte on the microbial endophyte community and phytohormones is largely unexplored. Here, we sequenced the endophytic bacterial and fungal communities in the leaves and roots, analyzed phytohormone concentrations and plant performance parameters in *Epichloë*-symbiotic (E+) and *Epichloë*-free (E-) individuals of two meadow fescue cultivars. The endophytic microbial community differed between leaf and root tissues independent of *Epichloë*-symbiosis while the fungal community was different in leaves of *Epichloë*-symbiotic and *Epichloë*-free plants in both cultivars. At the same time, *Epichloë*-symbiosis decreased salicylic acid and increased auxin concentrations in leaves. *Epichloë*-symbiotic plants showed a higher biomass, chlorophyll content (SPAD) and higher seed mass at the end of the season. Our results demonstrate that *Epichloë*-symbiosis alters the leaf fungal microbiome, which coincides with changes in phytohormone concentrations, indicating that *Epichloë* endophytes affect both, plant immune responses and other fungal endophytes. Whether the effect of *Epichloë* endophytes on other fungal endophytes is connected to changes in the phytohormone concentrations remains to be elucidated.

**Keywords:** grass endophyte; *Festuca*; symbiosis; microbiome; plant hormone; defense response; plant-fungal interactions; holobiont

## 1. Introduction

Microbes are essential organismal partners inhabiting every living being on the earth contributing to vital life-sustaining functions of their host organism [1,2]. Host-associated microbes interact not only with their host but also with coexisting microbes including bacteria, fungi and viruses [3,4], thus forming a complex 'microbial community'. The importance of the microbiome constituting the genetic component of these microbial communities and their metabolic activities has been acknowledged widely. Current sustainable approaches to improve fitness and health of host plants and animals comprises modifying parameters of their microbial partners [5,6].

Plants are increasingly studied for their microbiome and the associated improvement of performance and fitness in particular under extreme events driven by climate change [7]. The plant holobiont theory defines plants and their microbiome as one entity and plant performance and fitness can be enhanced by association to certain microbial partners [8–

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10]. The plant microbiome can be composed of microbes, residing on plant surfaces (epiphytes) or inside the tissues (endophytes). The endophytes can be systemic endophytes which exist in the plant seed and vertically transmitted in the host plants. The non-systemic endophytes such as soil-borne microbes, microbes introduced via rain and wind from the aerial environment and virtually every biotic and abiotic factor can introduce interact with or enter the plant, and modify the plant microbiome [7,11,12]. These microbes can range from mutualistic, commensalistic to parasitic with plant-beneficial microbes promoting plant growth, nutrition and stress resistance [13,14].

Plants respond to interacting microbes often via immune responses. The resistance to pathogenic microbes depends on plant immune responses, which, on the other hand, can be boosted and primed by plant-associated beneficial microbes [15–18]. Most studied examples of microbes improving plant performance are root-associated bacteria in legumes (nitrogen fixing), endo-and ectomycorrhizal fungi and systemically growing endophytic fungi in grasses. The interactions of these microbes with the plant immune system are increasingly uncovered. Plant hormones are considered as key players in shaping the plant microbiome [19]. Often plant immune responses are mediated by the induction of phytohormones, which can be affected by the presence of microbes or in turn regulate the growth and metabolism of endophytic microbes [20]. In general, plants respond to biotrophic microbes with an induction of salicylic acid and necrotrophic microbes induce jasmonic acid and associated defense pathways [21]. However, an increasing number of studies confirm the involvement of other phytohormones in plant responses to microbial partners, some of which are capable of producing phytohormones themselves, which enable the symbiotic nature between plant and microbe [22].

Systemic *Epichloë* endophytes are a model organism for studying plant symbiotic microbes [23–25]. These fungi are obligate endosymbionts of various cool-season grasses and reproduce strictly vertically via the plant seeds. Asexual *Epichloë* endophytes have been shown to benefit the host plant in high nutrient ecosystems by improving drought tolerance, herbivore resistance and pathogen resistance [26]. The beneficial properties are attributed to *Epichloë*-conferred alkaloids but recent literature aims to unravel the role of plant hormones in growth and defense promoted by *Epichloë* endophytes [27–29]. While some studies suggest *Epichloë* endophytes protect the plant against fungal intruders, such as the ergot fungus in plant seeds, others demonstrate an increased seed infection with ergot fungi on *Epichloë* symbiotic plants [30,31]. Further, it has been shown that *Epichloë* symbiotic grass seeds show a higher endophytic bacterial diversity compared to *Epichloë*-free seeds, which demonstrates their ability to interact with other endophytic microbes [32]. In leaf tissue, on the other hand, *Epichloë* endophytes have shown different trends on shaping the microbial communities. *Epichloë* in perennial ryegrass (*Lolium perenne*) did not significantly affect fungal community structure [33] but *Epichloë* in tall fescue (*Festuca arundinaceae* = *Schedonorus phoenix)* altered the community of only fungal endophytes and not bacteria in leaves of the host plants [34]. In *Festuca rubra*, the presence of *Epichloë festuca* along with habitat of the host plant affected the infection frequencies of non-systemic fungal endophytes [35]. Studies from the grass species *Achnatherum inebrians* showed that the presence of symbiotic fungal partner *Epichloë gansuensis* reduced the diversity of root-associated bacterial community [36] while increasing the diversity of endophytic and epiphytic bacterial and fungal phyllosphere communities [37]. However, it remains largely unknown how *Epichloë* endophyte affects the leaf and root microbiome and whether altered microbiomes can be linked to hormonal and performance attributes of its host plant.

In the present study, we analyzed whether *Epichloë* endophyte symbiosis alters the endophytic microbiota of above and belowground parts of two meadow fescue (*Festuca pratensis*) cultivars ('Valtteri' & 'Kasper') and whether changes coincide with altered plant performance and phytohormone concentrations. The endophytic bacterial and fungal community compositions of leaves and root tissues in the *Epichloë* endophyte symbiotic (E+) and non-symbiotic (E-) plants were determined using targeted sequencing of 16S

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rRNA gene and ITS (Internal Transcribed Sequence) regions. Additionally, plant performance and phytohormone concentrations of the E+ and E- host cultivars were studied to understand their role in shaping the structure and composition of endophytic microbial communities in association with *Epichloë*. We hypothesize that *Epichloë* endophytes (1) shape the endophytic microbiome particularly aboveground, (2) which coincides with changes in phytohormone concentrations and (3) improve plant performance.

## 2. Materials and Methods

### *2.1. Plant material and study setup*

We choose two meadow fescue (*Festuca pratensis* L.) cultivars ('Kasper', and 'Valtteri') as our model, because meadow fescue is widely used pasture and forage grass in Europe and commonly harbors the systemic, seed transmitted fungal endophyte, *Epichloë uncinata* [(W.Gams, Petrini & D.Schmidt) Leuchtm. & Schardl.]. We obtained the seeds of the cultivars from seed producers via the Finnish Food Authority (www.ruokavirasto.fi). We verified by staining and microscopy of 100 seeds that all the seed lots of cultivars had both *Epichloë*-free (E-) and endophyte symbiotic (E+) seeds [38]. The endophyte frequency in the seed lots varied between 30% - 90%. We verified the final endophyte status (E- or E+) of each experimental plant in the field using the results from ITS-targeted PCR for fungal community analysis. Due to mortality of plants, at the end of the experiment we had 7 E- and 5 E+ 'Kasper' plants, as well as 5 E- and 5 E+ 'Valtteri' plants.

We first grew plants in pots (May 2017) in the greenhouse and then transferred 20 plants of each cultivar to the experimental field in the Turku University Botanical Garden (60°26N, 22°10.4E) (June 2017) in a randomized design. The plants were planted 0.5 m apart from each other, hand-weeded to avoid competition from other plant species and watered if needed during the first growing season. The field was fenced with a metal net to keep out mammalian herbivores. The soil in the experimental area is ~ 90% clay with some sand and peat. The soil pH was 6.2, and content of phosphorus 4.2 mg/l, potassium 250 mg/l, calcium 1900 mg/l, magnesium 570 mg/l, sulfur 10.6 mg/l, zinc 2.74 mg/l, copper 7.5 mg/l, manganese 15 mg/l. We did not use fertilizers or pesticides in the area.

### *2.2. Plant performance parameters*

We recorded growth, reproduction and chlorophyll content of the experimental plants in the beginning of August 2019. To estimate growth, we measured average height of the plant, height of the longest leaf, circumference of the tuft at 5 cm above the ground, and number of tillers. For reproduction estimate, we counted the number of flowerheads and for chlorophyll content measured SPAD values (Minolta SPAD-502 Plus meter) from three randomly chosen leaves per experimental plant.

### *2.3. Sample collection*

We collected plant samples for phytohormone and microbiome analysis from the experimental field in mid-August 2019. For phytohormone analysis, we sampled 2-3 healthy leaves from each plant, which were weighed and immediately stored in liquid nitrogen after sampling until further processing.

About 2-3 healthy leaves and approximately 100mg of root samples from 5-7 replicates each of E+ and E- 'Valtteri' and 'Kasper' cultivars in sterile plastic bags, stored on ice and brought to the lab where the samples were washed with tap water, dried and weighed to ensure 100 mg. The samples were then surface sterilized in laminar air flow hood using 70% ethanol (1 min) followed by 3% sodium hypochlorite solution (3 min), rinsed thrice with sterile distilled water (3x1 min) and air-dried. The samples were transferred to 2 ml microcentrifuge tubes and stored in -80°C until further processing.

### *2.4. Microbiome analysis*

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The frozen samples were homogenized using bead mill homogenizer (Bead Ruptor 96 Well Plate Homogenizer, OMNI International US) and DNA was extracted using Invisorb Spin Plant Mini Kit (STRATEC Biomedical AG, Germany). Following DNA extraction, the 16S rRNA gene and ITS (Internal Transcribed Sequence) regions from the DNA samples were PCR amplified. The detailed protocol for PCR is provided in the Supplementary Material.

The PCR products were quantified on Agilent 2100 Bioanalyzer system, pooled to obtain sequence library, size-fractionated on 2% Agarose gel cassette (Marker B) using Pippin Prep (Sage Science, MA, USA) and sequenced on Ion 314™ Chip v2 in Ion Personal Genome Machine™ (ThermoFisher Scientific Ltd.).

### *2.5. Plant hormone extraction and quantification*

We analyzed plant hormones as described in Dobrev and Vankova (2012) [39] and Fuchs et al. (2022) [40]. In brief, approx. 100 mg fresh plant material was homogenized under constant liquid nitrogen supply, followed by an extraction with cold (−20 °C) methanol/water/formic acid (15/4/1, v/v/v) in an 2 ml reaction tube (Eppendorf GmbH). The following isotope-labeled internal standards (10 pmol per sample) were added: $^{13}C_6$IAA, $^{2}H_4$-OxIAA (Cambridge Isotope Laboratories); $^{2}H_4$-SA [Sigma-Aldrich); $^{2}H_3$-PA, $^{2}H_3$-DPA, $^{2}H_4$-7OH-ABA, $^{2}H_5$-ABAGE (NRC-PBI); $^{2}H_6$-ABA, $^{2}H_5$-JA, before subsequent centrifugation (17,000 × g, 4 ◦C, 20 min). The extract was centrifuged (17,000 g, 4°C, 20 min) to remove solid debris. It was then concentrated using an Alpha RVC vacuum centrifuge (Christ; 40 ◦C, 15 mbar, 1.5 h). Phytohormones were purified using a reverse-phase–cation exchange solid-phase extraction (SPE) column (Oasis-MCX, Waters) and eluted with methanol, concentrated to dryness and resuspended in 30 µl acetonitrile (15%). Hormones were analyzed on an HPLC (Ultimate 3000, Dionex) coupled to 3200 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems) and quantified by an isotope dilution method with multilevel calibration curves (r2 > 0.99). Data were processed with the Analyst 1.5 software package (Applied Biosystems). The analyses covered the plant hormones abscisic acid (ABA), indole acetic acid (IAA), phenyl acetic acid (PAA), salicylic acid (SA) and jasmonic acid (JA). Furthermore, we quantified a potential SA precursor benzoic acid (BzA) and the main product in ABA metabolism phaseic acid (PA).

### *2.6. Bioinformatics and Statistical Analyses*

The sequence reads from microbiome analysis were processed using CLC Genomics Workbench 11.0 with a Microbial Genomics Module (Qiagen, Denmark). After filtering low-quality and <150 bp sequence reads, high-quality reads were aligned and clustered into OTUs (Operational Taxonomic Units) at 97% sequence identity. The OTUs were taxonomically classified using reference databases RDP 16S rRNA training set 16 for bacteria and UNITE Fungal ITS trainset 7.1 for fungi (https://rdp.cme.msu.edu) (Wang et al. 2007). OTUs representing plant genes and with less than a total count of 10 reads were eliminated. Statistical analyses were conducted separately for bacterial and fungal communities using PRIMER-7 software with PERMANOVA+ add-on (https://www.primer-e.com/). Permutational Multivariate Analysis of Variance (PERMANOVA) tests and Principal Co-ordinate Analysis to find the effect of *Epichloë* on overall microbial structures community were performed on Bray-Curtis distance matrices of square root transformed abundance data. With Similarity Percentages – species contribution (SIMPER) analysis, we identified OTUs or species majorly contributing to differences between community structures. The statistical analyses were repeated on fungal datasets after removing OTUs assigned as *Epichloë*.

Plant performance parameters showed normal distribution and were analyzed with student's t-test. Phytohormone concentrations were not normally distributed and analyzed with Wilcoxon test. Graphical illustration was done with the ggplot2 package in the software R.

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## 3. Results

### *3.1. Epichloë shapes endophytic fungal community composition in leaves and not in roots*

A total of 276,216 good quality fungal sequence reads were obtained and classified into 438 OTUs representing 3 phyla (Ascomycota, Basidiomycota and Glomeromycota) and belonging to 75 families and 103 genera. The genus *Epichloë* dominated 65% and 39% of the relative abundance of the fungal communities in leaves of *Epichloë*-symbiotic (E+) plants of 'Valtteri' and 'Kasper' cultivars, respectively (Figure 1). The next major genera were *Mycosphaerella* and *Cadophora*. For *Mycosphaerella*, the relative abundance was lower in E+ plants ('Valtteri' 13%; 'Kasper' 8%) and higher in E- plants for both cultivars ('Valtteri' 31%; 'Kasper' 47%). The relative abundance of *Cadophora* was lower in E+ plants compared to E- plants in 'Valtteri' (E+ leaves 5%; E- leaves 20%), while in E+ 'Kasper' plants, their relative abundance was higher (E+ leaves 31%; E- leaves 0.02%) (Figure 1). The presence of *Epichloë* clearly impacted the composition of the endophytic fungal communities in leaves for both 'Valtteri' and 'Kasper' cultivars as evidenced by PERMANOVA analysis (Table 1) and visualized by PCoA (Figure 2a). The PERMANOVA and PCoA analysis on *Epichloë*-depleted datasets was performed to ensure that the fungal community structures were significantly different in E+ and E- leaves of both cultivars (Table 2, Figure 2b).

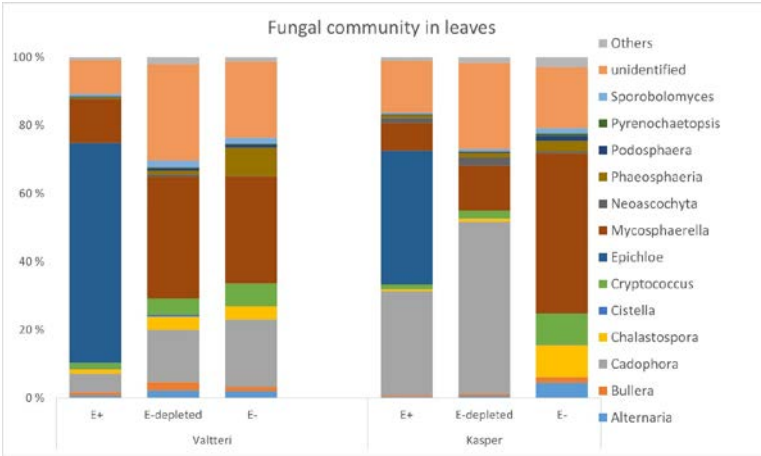


**Figure 1.** Taxonomic distribution of fungal communities at genus level in leaves of *Epichloë*-symbiotic (E+) and *Epichloë*-free (E-) plants of 'Valtteri' and 'Kasper' cultivars of meadow fescue (*Festuca pratensis*). For each cultivar, the X-axis shows taxonomic distribution in E+ plants for total fungal communities (E+), fungal communities in E+ plants after removal of *Epichloë* taxa (E-depleted) and fungal communities in E- plants (E-).

**Table 1.** PERMANOVA results for total fungal communities in meadow fescue.

| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
|---|---|---|---|---|---|---|
| Tissue | 1 | 70639 | 70639 | 41.18 | 0.001 | 996 |
| Endophyte | 8 | 20651 | 2581.4 | 1.51 | 0.001 | 996 |
| TissuexEn | 8 | 17049 | 2131.1 | 1.24 | 0.02 | 997 |
| PERMANOVA analysis on total fungal communities for leaves | | | | | | |
| En | 8 | 25466 | 3183.3 | 2.0446 | 0.001 | 997 |
| PERMANOVA analysis on total fungal communities for roots | | | | | | |
| En | 8 | 24029 | 3003.6 | 1.1204 | 0.241 | 999 |

**Table 2.** PERMANOVA results for *Epichloë*-depleted fungal communities in meadow fescue.

| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
|---|---|---|---|---|---|---|

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| | | | | | | |
|---|---|---|---|---|---|---|
| Tissue | 1 | 34398 | 34398 | 27.47 | 0.001 | 998 |
| Endophyte | 3 | 7297.7 | 2432.6 | 1.9426 | 0.001 | 995 |
| TissuexEn | 3 | 5175.1 | 1725 | 1.3776 | 0.028 | 998 |

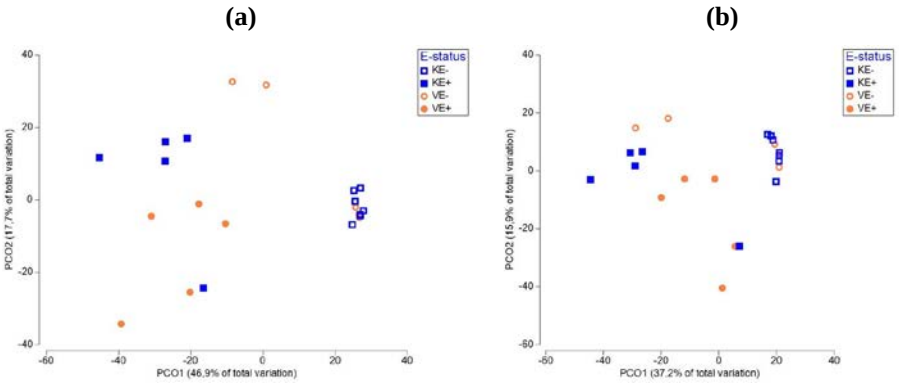


**Figure 2.** PCoA analysis on (**a**) total endophytic fungal communities and (**b**) *Epichloë*-depleted fungal communities in 'Kasper' *Epichloë*-symbiotic (KE+), 'Kasper' *Epichloë*-free (KE-), 'Valtteri' *Epichloë*-symbiotic (VE+) and 'Valtteri' *Epichloë*-free leaves of meadow fescue (*Festuca pratensis*).

With the SIMPER analysis on leaves, the major species that contributed to dissimilarity between E+ and E- plants in both cultivars were Epichloë, Mycosphaerella tassiana, Cadophora and Heliotales sps. In the Epichloë-depleted datasets, in addition to the above taxa other than Epichloë, the relative abundances of Phaeosphaeria triglochinicola and Pleosporales sps. were relatively lower in Valtteri E+ plants. Another interesting observation was that the relative abundance of Pleosporales sps. was higher in VE+ plants compared to VE- plants but in contrast, the relative abundance of Pleosporales sps. was lower in KE+ plants. Furthermore, the relative abundance of Vishniacozyma victoriae was remarkably lower in KE+ leaves (Table 3). A detailed table on SIMPER analysis is provided in Table S1 in the Supplementary Material.

**Table 3.** SIMPER (Similarity Percentages) analysis in leaves of E+ and E- plants in total and *Epichloë*-depleted fungal communities (VE- 'Valtteri' plants without *Epichloë,* VE+ - 'Valtteri' with *Epichloë,* KE- 'Kasper' without *Epichloë* and KE+ 'Kasper' with *Epichloë*) showing micobial taxa with difference in average relative abundances (Av.Abund – Average Abundance).

| **Total fungal communities** | | |
|---|---|---|
| **VE-** | **VE+** | |
| **Av.Abund** | **Av.Abund** | **Taxa** |
| 0.02 | 59.43 | *Epichloë* |
| 28.53 | 8.75 | *Mycosphaerella_tassiana* |
| **KE-** | **KE+** | |
| **Av.Abund** | **Av.Abund** | |
| 0.03 | 36.83 | *Epichloë* |
| 30.87 | 7.06 | *Mycosphaerella_tassiana* |
| 0.03 | 18.91 | *Cadophora* |
| 0.01 | 7.82 | Heliotales_unidentified |
| ***Epichloë*-depleted communities** | | |
| **VE-** | **VE+** | |
| **Av.Abund** | **Av.Abund** | **Taxa** |
| 28.60 | 19.39 | *Mycosphaerella_tassiana* |
| 12.11 | 7.81 | *Cadophora* |
| 5.27 | 3.08 | Heliotales_sps. |
| 5.93 | 7.75 | Pleosporales_sps. |
| 4.25 | 0.72 | *Phaeosphaeria triglochinicola* |
| 4.70 | 2.01 | Capnodiales sps. |

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| KE- Av.Abund | KE+ Av.Abund | |
|---|---|---|
| 0.03 | 29.32 | *Cadophora* |
| 31.6 | 11.49 | *Mycosphaerella_tassiana* |
| 0.01 | 12.1 | Heliotales_sps. |
| 6.48 | 4.06 | Pleosporales_sps. |
| 7.36 | 1.83 | *Vishniacozyma victoriae* |

For roots, the *Epichloë* status did not impact the overall structure of the fungal communities. The taxonomic distribution of the fungal communities in roots (Figure 3) showed major genera including *Cadophora*, *Ophiosphaerella*, *Phaeosphaeria*, *Rhexocercosporidium* and *Scytalidium* in both cultivars of E+ and E- plants. We also compared E+ leaves of 'Valtteri' and 'Kasper' and E- plants of both cultivars to analyze whether cultivar-specific changes occurred in the communities but found no significant differences.

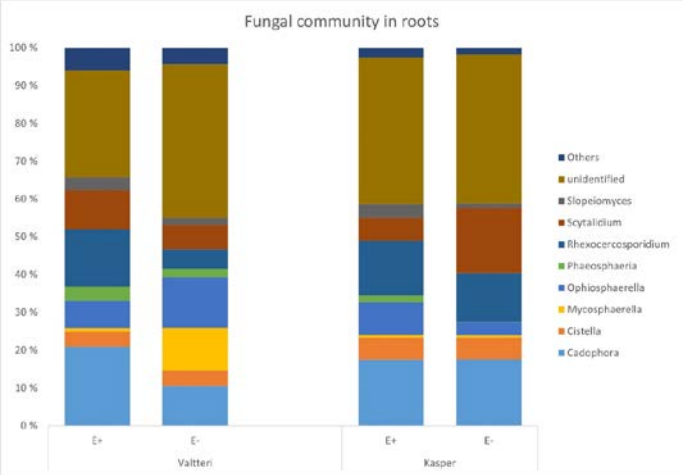
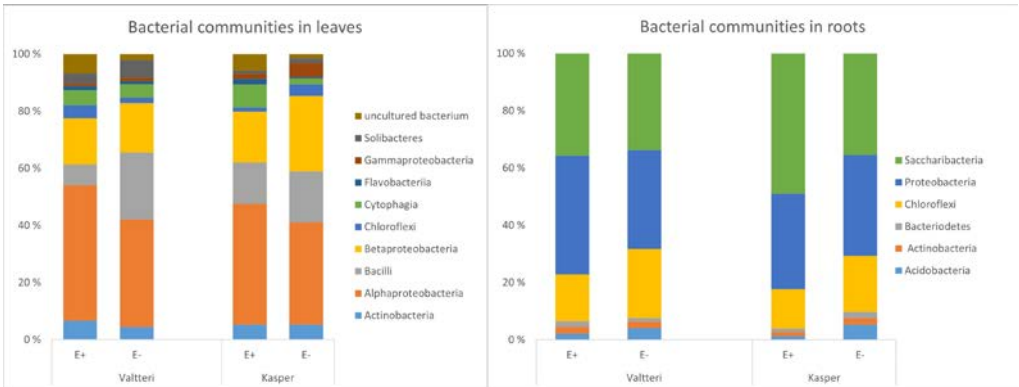



**Figure 3.** Taxonomic distribution of fungal communities at genus level in *Epichloë*-symbiotic (E+) and *Epichloë*-free (E-) roots of 'Kasper' and 'Valtteri' cultivars of meadow fescue (*Festuca pratensis*).

*3.2. Epichloë does not impact endophytic bacterial community structure*

The bacterial community structure was deciphered from 153,824 final sequence reads. The community structure consisted of 12 phyla represented by 110 families and 212 genera. The major genera in the leaves were *Sphingomonas*, *Hymenobacter*, *Massilia*, and *Methylobacterium* and in the roots *Roseiflexus*, *Shinella*, *Rhizobium* and *Rhizobacter*. The taxonomic distribution of bacterial communities in leaves (Figure 4a) and roots (Figure 4b) of the two cultivars ('Valtteri' and 'Kasper') was not different between E+ and E- plants. The PERMANOVA and PCoA analysis (Figure 5) showed there were no significant differences in the bacterial community structures of E+ and E- plants indicating *Epichloë* does not impact endophytic bacterial community structure in leaves or roots.



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**(a)** **(b)**

**Figure 4.** Taxonomic distribution of bacterial communities at phyla level in (**a**) leaves and (**b**) roots of 'Valtteri' and 'Kasper' cultivars of meadow fescue (*Festuca pratensis*).

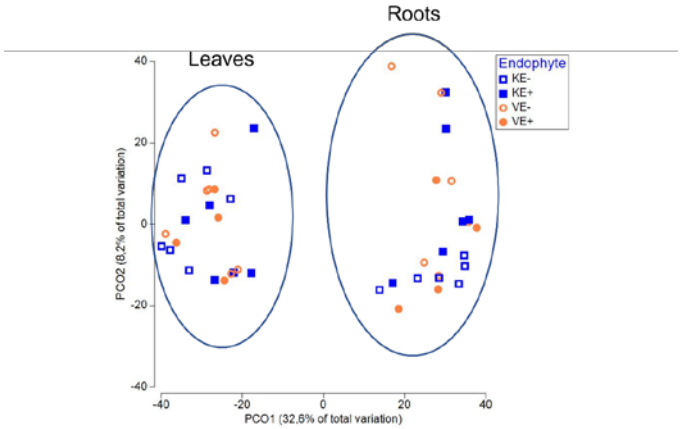


**Figure 5.** PCoA analysis of bacterial communities in leaves and roots of meadow fescue (*Festuca pratensis*) cultivars 'Kasper' and 'Valtteri'. 'Kasper' *Epichloë* -free plants (KE-), 'Kasper' *Epichloë* -symbiotic plants (KE+), 'Valtteri' *Epichloë* -free plants (VE-), 'Valtteri' *Epichloë* -symbiotic plants (VE+).

### *3.3. Epichloë symbiosis alters plant parameters*

The *Epichloë* endophyte (E+) symbiotic plants were taller and had a higher number of flower heads compared to their non-symbiotic (E-) counterparts in both cultivars (Table 4, Figure 6). In contrast, plant circumference was smaller in E+ plants of the cultivar 'Kasper' and showed a trend to be smaller in E+ 'Valtteri' cultivar (Table 4, Figure 6). Chlorophyll content was measured as SPAD value and by trend higher in E+ plants compared to E- plants (Table 4, Figure 6).

**Table 4.** The effect of *Epichloë* symbiosis on plant parameters in the two meadow fescue cultivars analyzed with a student's t-test. Significant p-values are highlighted bold. N=5-6.

| | 'Kasper' | | | 'Valtteri' | | |
|---|---|---|---|---|---|---|
| | t | df | p-value | t | df | p-value |
| Plant height | -3.172 | 9.939 | **0.01** | -2.937 | 7.793 | **0.02** |
| Longest leaf | -4.23 | 8.993 | 0.68 | -0.148 | 7.203 | 0.89 |
| Circumference | 2.474 | 9.019 | **0.04** | 1.961 | 5.119 | 0.11 |
| Tiller no. | -0.308 | 9.981 | 0.76 | -0.468 | 7.387 | 0.653 |
| Flower head no. | -2.895 | 6.592 | **0.02** | -2.539 | 7.859 | **0.04** |
| SPAD value | -1.982 | 9.951 | 0.08 | -2.144 | 6.163 | 0.07 |

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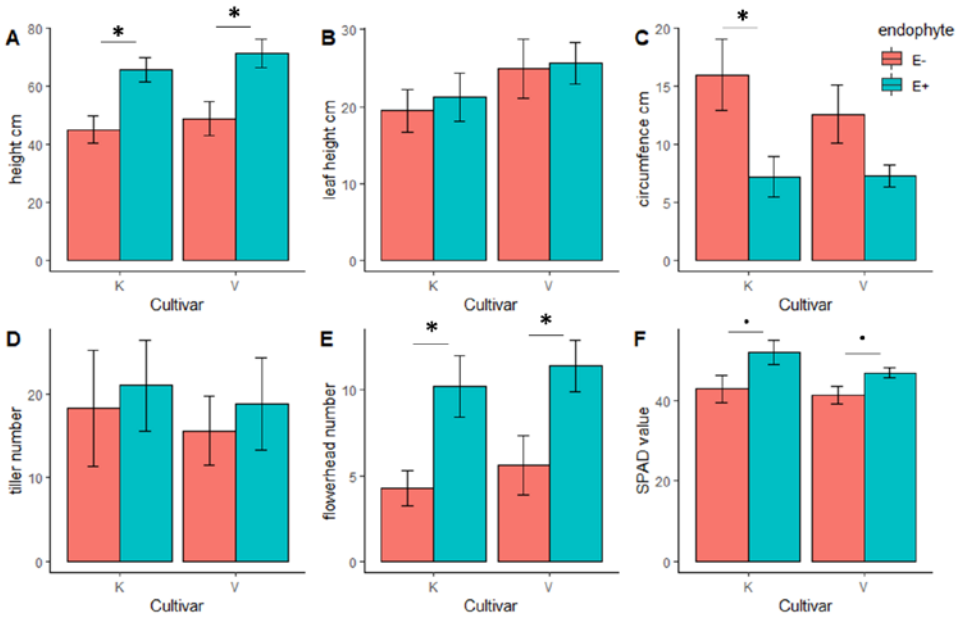


**Figure 6.** Plant parameters of meadow fescue (*Festuca pratensis*) cultivars 'Kasper' (K) and 'Valtteri' (V) were compared between *Epichloë* -symbiotic (E+) and *Epichloë* -free (E-) plants affected by *Epichloë* symbiosis were analyzed with student's t-test for both cultivars separately. N = 5-6. For significance levels, see Table 4.

### *3.4. Epichloë symbiosis alters plant hormone concentrations*

Plants symbiotic to *Epichloë* showed higher auxin concentrations (IAA and PAA - by trend in 'Kasper' cultivar) compared to non-symbiotic plants in both cultivars (Table 5, Figure 7). Furthermore, ABA increased in *Epichloë*-symbiotic plants from the Kasper cultivar. In contrast, the phytohormone SA was lower in *Epichloë*-symbiotic plant from both cultivars (Table 5, Figure 7).

**Table 5.** Phytohormone concentrations were compared between *Epichloë* –symbiotic (E+) and *Epichloë* -free (E-) plants (Wilcoxon test) for both meadow fescue (*Festuca pratensis*) cultivars ('Kasper' and 'Vatteri'). Significant p-values are highlighted in bold. The analyses covered the plant hormones abscisic acid (ABA), indole acetic acid (IAA), phenyl acetic acid (PAA), salicylic acid (SA) and jasmonic acid (JA) and a potential SA precursor benzoic acid (BzA) and the main product in ABA metabolism phaseic acid (PA).

| Phyto- hormone | 'Kasper' p-value | 'Valtteri' p-value |
|---|---|---|
| ABA | **0.05** | 1 |
| PA | 0.88 | 0.69 |
| IAA | **<0.01** | **0.03** |
| PAA | 0.07 | **<0.01** |
| SA | **0.05** | **<0.01** |
| BzA | 0.27 | 0.31 |
| JA | 0.64 | 0.84 |

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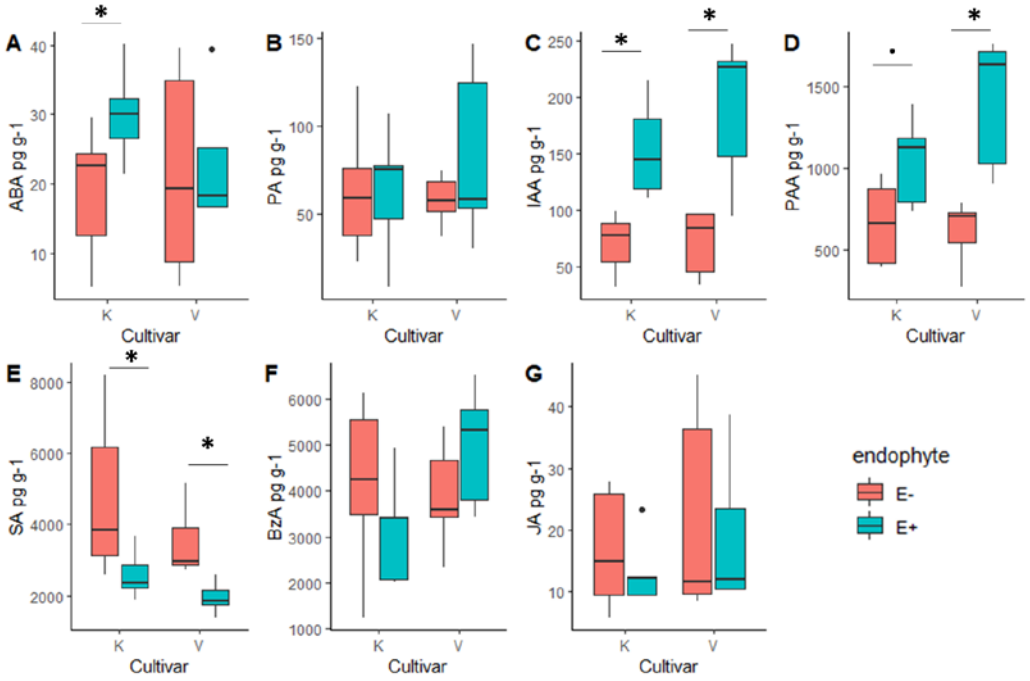


**Figure 7.** Phytohormone concentrations of meadow fescue (*Festuca pratensis*) cultivars 'Kasper' (K) and 'Valtteri' (V) were compared between *Epichloë* -symbiotic (E+) and *Epichloë* -free (E-) plants (Wilcoxon test). Median and 95% confidence interval are shown. The analyses covered the plant hormones abscisic acid (ABA), indole acetic acid (IAA), phenyl acetic acid (PAA), salicylic acid (SA), jasmonic acid (JA), and a potential SA precursor benzoic acid (BzA) and the main product in ABA metabolism phaseic acid (PA).

## 4. Discussion

In this study, *Epichloë*-symbiosis (E+) in the host plant meadow fescue, *F. pratensis*, clearly impacted the structure of endophytic fungal community in leaves but not in roots. This confirms our hypothesis that *Epichloë* symbiosis is one of the major factors shaping the endophytic fungal communities in the aboveground parts of its host plants [34]. *Epichloë* was the dominant genus in the leaves of E+ plants of both cultivars, 'Valtteri' and 'Kasper'. The endophytic fungal community structures of *E+* leaves considerably differed from the *Epichloë*-uninfected (E-) leaves. *Epichloë*-symbiosis did not alter the structure of the endophytic bacterial community in leaves or roots of the host plant. Finally, E+ plants were taller with more flower heads in both cultivars. Both cultivars showed higher auxin and lower salicylic acid concentrations in E+ plants, and ABA concentrations were increased in E+ plants of the 'Kasper' cultivar.

*Epichloë* endophytes grow in the intercellular space of the leaves, stems and reproductive tissue of several cool-season grasses and are typically absent in the roots [41,42], which indicates that their effect on shaping fungal community structure is limited to aboveground plant parts. This is in agreement with our previous study where *Epichloë*-symbiosis in tall fescue (*Schedonorus phoenix*) impacted only the endophytic fungal community in leaves and not the bacterial community [34]. The significant difference in the structures of fungal communities were observed in the dataset, with and without depleting *Epichloë*-taxa, confirming the role of *Epichloë* in shaping the endophytic mycosphere. The most abundant genera after *Epichloë* were *Mycosphaerella* and *Cadophora*. *Epichloë*-symbiosis in *Lolium perenne* similarly impacted its foliar fungal composition with *Mycosphaerella* being the second-most dominant fungi apart from *Epichloë* [33]. In general, the impact of *Epichloë*-symbiosis on the foliar fungal community was more pronounced in the 'Kasper' cultivars compared to 'Valtteri'.

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The relative abundance of *Epichloë* OTUs belonged to *Epichloë uncinata*, specifically forming symbiotic relations with meadow fescue [43] slightly differed between the cultivars. *Epichloë* symbiosis is common but frequencies of infection can vary among cultivars depending on the infection status of the mother plants and genetic and biotic and abiotic environment [44–47]. The noticeable pattern of foliar endophytic fungal assemblage observed in 'Kasper' cultivar reflects the complexity of the relation of mutualistic endophyte *Epichloë* with its specific host cultivar. From the SIMPER analysis of the fungal community in leaves, the relative abundance of *Mycosphaerella tassiana* was lower in the E+ plants compared to the E- plants in both varieties, but more prominent in Kasper. *M. tassiana* is a commonly known plant pathogen in wheat [48] and other grass species [49]. Our results indicate that the *Epichloë* symbiosis may be an advantage keeping pathogen levels such as *M. tassiana*, inactive. Several species of *Epichloë* restrict or inhibit plant pathogens through fungi-fungi interactions, such as producing fungistatic chemicals, compete for favorable niche or by eliciting changes in the host plant endosphere [50–52].

The relative abundance of *Cadophora* was slightly higher in uninfected 'Valtteri' leaves but significantly lower in uninfected 'Kasper' leaves. The complex cultivar-specific compatibility of each cultivar with *Epichloë* possibly explains this contrasting trend of relative abundance of *Cadophora*, which is considered as pathogenic fungus. The relative abundance of another microbial species *Vishniacozyma victoriae* was higher in non-symbiotic leaves of 'Kasper'. *Epichloë*-symbiotic plants generally have more fitness than non-symbiotic counterparts, which then may harbor beneficial microbes such as *V. victoriae* as it is one of the cold-adapted endophytic fungi producing various bioactive and antimicrobial metabolites, enzymes and hormones helping in plant growth and ecological adaptation to cold environments [53].

*Epichloë*-symbiotic plants showed a higher plant performance with increased plant height, number of flower heads and higher chlorophyll content. Similarly to our study, better plant growth of *Epichloë*-symbiotic grasses is commonly documented and may be attributed to increased concentrations of auxins, which are common growth-promoting phytohormones throughout the plant kingdom and connected to physiological changes in endophyte symbiotic grasses [54]. An inducing effect of endophyte symbiosis on salicylic acid concentrations has been connected to a better defense response against biotrophic pathogens and piercing sucking insects [28,55]**.** On the other hand, SA reduction in symbiotic plants has earlier been reported in *Epichloë*-symbiotic plants and can be an *Epichloë*-mediated suppression of the host immune response for a better intercellular establishment [56]. Due to the key role of SA for interactions with biotrophic pathogens, the observed reduction in SA concentrations in endophyte symbiotic plants is likely to be responsible for changes in the endophytic mycosphere [27]. On the other hand, the *Epichloë*-symbiosis may first alter the endophytic microbial community, which leads to altered plant responses at hormonal level [18]. Hence, the *Epichloë*-symbiosis shapes the endophytic fungal community and because of this tripartite symbiont-host plant-endophyte, they mutually co-exist and co-evolve.

## 5. Conclusions

In conclusion, our results demonstrate the central role of systemic *Epichloë* endophytes in cool-season grasses shaping the community of the fungal leaf microbiome is likely mediated by changes in plant hormone concentrations related to resistance against biotrophic pathogens. The endophyte features of improving plant resistance, biomass and reproduction are increasingly unraveled to be linked to multiple transcription factors and hormone regulation. It remains to be elucidated whether the effect presented here on the fungal microbiome is caused by direct effects of the *Epichloë* fungi on other fungal species or whether it is indirectly caused by *Epichloë*-mediated changes in the plant physiology such as phytohormone concentration causes.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1. Method S1: PCR protocol for microbiome analysis. Table S1: SIMPER (Similarity Percentages) analysis in leaves of E+ and E- plants in total and *Epichloë*-depleted fungal communities (VE- 'Valtteri' plants without *Epichloë,* VE+ - 'Valtteri' with *Epichloë,* KE- 'Kasper' without *Epichloë* and KE+ 'Kasper' with *Epichloë*) showing micobial taxa with difference in average relative abundances. OTUs – Operational Taxonomic Units, Av.Abund – Average Abundance, Av.Diss. – Average Dissimilarity, Diss/SD – Dissimilarity/ Standard Deviation, Contrib% - Contibution percentage, Cum% - Cumulative percentage

**Author Contributions:** The following are the contributions of authors in the manuscript. Conceptualization. MH, KS, SD and BF; validation. SM, BF, RV and PD; formal analysis. SM, BF, RV and PD; writing—original draft preparation. SM and BF; writing—review and editing. SM, BF, MH, KS RV, PD and SD; funding acquisition. MH, KS and BF; All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Data will be made publicly available once the manuscript is accepted.

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