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Bacterial communities associated with fungal fruitbodies

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Bacterial communities associated with fungal fruitbodies

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LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following publications that are referred in the text by their Roman numerals:

I. **Pent M**, Põldmaa K, Bahram M. 2017. Bacterial Communities in Boreal Forest Mushrooms Are Shaped Both by Soil Parameters and Host Identity. Frontiers in Microbiology 8, 836.

II. Bahram M, Vanderpool D, **Pent M**, Hiltunen M, Ryberg M. 2018. The genome and microbiome of a dikaryotic fungus (*Inocybe terrigena*, Inocybaceae) revealed by metagenomics. Environmental Microbiology Reports 10, 155–166.

III. **Pent M**, Hiltunen M, Põldmaa K, Furneaux B, Hildebrand F, Johannesson H, Ryberg M, Bahram M. 2018. Host genetic variation strongly influences the microbiome structure and function in fungal fruiting-bodies. Environmental Microbiology 20, 1641–1650.

IV. **Pent M**, Bahram M, Põldmaa K. 2020. Fruitbody chemistry underlies the structure of endofungal bacterial communities across fungal guilds and phylogenetic groups. The ISME Journal 14, 2131–2141.

Author's contribution to each publication:

I involved in developing the idea and the experimental design, performed sampling, molecular analysis and data analysis, wrote the manuscript with contributions from coauthors.

II involved in data analysis, wrote a part of the manuscript with contributions from coauthors.

III involved in data analysis, wrote the manuscript with contributions from coauthors.

IV involved in developing the idea and the experimental design, performed sampling, molecular analysis and data analysis, wrote the manuscript with contributions from coauthors.

1. INTRODUCTION

Bacteria play an important role in the functioning, development and health of animals, plants and humans (Berendsen et al., 2012; Goh et al., 2013; Kohl et al., 2014; Sharon et al., 2014; Compant et al., 2019; Dominguez-Bello et al., 2019). The significant impact of microbial communities on the quality of host life and the fitness of the host has led to numerous studies on the subject (e.g. Turnbaugh et al., 2006; Kohl et al., 2014; Sharon et al., 2014; Requena and Velasco, 2019). Increasing evidence suggests that each plant and animal together with the associated microbial community forms a functional entity, referred to as a holobiont (e.g. Hacquard et al., 2015; Agler et al., 2016; Rosenberg and Zilber-Rosenberg, 2016; Apprill, 2017). Accordingly, the hologenome includes genomes of all members of the holobiont, in which the microbial community extends the phenotype of the host organism (Berendsen et al., 2012; Hacquard et al., 2015; Carthey et al., 2018; Deveau et al., 2018). The structure of eukaryote-associated microbiomes may depend on both host-specific and environmental factors. Namely, alongside habitat conditions, the animal and plant-associated microbiome structure has been shown to be affected by host identity, genotype, health, nutrition and lifestyle (Hacquard et al., 2015; Adair and Douglas 2017; Pratte et al., 2018; Knowles et al., 2019; Dastogeer et al., 2020). In addition, stochastic processes can also drive the structure of microbial communities, independently of host and environmental factors (Adair and Douglas 2017) and microbe-microbe interactions may have an additional impact on microbial community structure in different hosts (Berendsen et al., 2012; Hacquard et al., 2015; Agler et al., 2016; Adair and Douglas 2017; Baltrus, 2017; Dastogeer et al., 2020).

Fungi are one of the most species-rich groups of eukaryotes that significantly contribute to nutrient cycling in terrestrial ecosystems while associating with various organisms (Blackwell, 2011; Tedersoo et al., 2014). Many fungi form conspicuous structures, referred to as fruitbodies, that are not only essential for their reproduction and dissemination but also widely used by humans due to the culinary value of many species. Although each fungus forms a holobiont together with the associated microbes (Carrasco and Preston, 2019), like in case of other eukaryotes, bacterial communities inhabiting fungal fruitbodies have remained little explored. Whereas the structure of the eukaryotic microbiome is shaped both by host-specific and environmental factors, the relative contribution of these factors and the underlying mechanisms of bacterial community assembly may be different for fungi compared to other eukaryotes.

Host taxonomy and phylogeny are known to shape bacterial communities associated with different animals and plants, likely due to the co-evolution between microbes and the host (e.g. Amato, 2016; Nelson et al., 2013; Naylor et al., 2017; Nishida and Ochman 2018; Knowles et al., 2019; Alt et al., 2020; Dastogeer et al., 2020). Co-evolution between mammals and microbes has been shown in case of the gut microbiome, including that of humans (Ley et al., 2008; Spor et al., 2011; Goodrich et al., 2014; Amato et al., 2016; Knowles et al., 2019).

Likewise, co-evolution has been suggested to occur between plants and their microbes (Bouffaud et al., 2014; Baltrus, 2017; Tian et al., 2018) as well as lichens and their characteristic bacteria (Grube et al., 2009). Similarly to plants and animals, it is plausible that fungal-bacterial interactions have also evolved along the diversification of both partner groups (Mondo et al., 2012; Pawlowska et al., 2018).

In addition to the variability of microbial communities between host species, these may also vary between host genotypes (Spor et al., 2011; Hacquard et al., 2015; Smith et al., 2015; Wagner et al., 2016; Qian et al., 2018). However, the host genotype effect on fungal associated microbiome has never been studied in case of basidiomycetes. Studies on the eukaryotic holobionts have shown that the degree of the impact of the environment on the microbes depends on host genotypes, thus pointing to the interaction between environmental factors and host genotypes (Spor et al., 2011; Agler et al., 2016; Baltrus, 2017; Liu et al., 2019). The effect of host genotypes may be mediated by the variation in the production of different metabolic substances, although several microbes can manipulate the production of these metabolites by the host, e.g. by affecting the transcription of particular genes in plants (Tian et al., 2018).

The host nutrient-acquisition strategy has often been determined as a significant factor shaping the microbiota of plants and animals, respectively (Nelson et al., 2013; Hacquard et al., 2015; Pii et al., 2016; Phillips et al., 2017; Nishida and Ochman, 2018; Pratte et al., 2018). Plants with different nutrient-acquisition strategies are able to differently alter pH and the availability of particular carbon compounds as well as macro- and micronutrients in the surrounding environment (Hacquard et al., 2015; Pii et al., 2016; Bahram et al., 2020), thereby changing the conditions of the respective habitats. Similarly, carbon and nitrogen content vary in lichen thalli depending on their photobionts and such variation can shape the lichen-associated bacterial community structure (Hodkinson et al., 2012). Habitat-specific physico-chemical conditions, for example in human intestine (Ley et al., 2006; Hacquard et al., 2015) or in plant-related habitats (Bouffaud et al., 2014; Dastogeer et al., 2020), significantly affect the microbial community composition in there and the physico-chemical conditions may vary among host genotypes, taxa as well as functional guilds (Kashyap et al., 2013; Bouffaud et al., 2014; Kembel et al., 2014; Pii et al., 2016). Apart from pathogens/parasites, decomposers and symbionts represent prevalent but contrasting nutritional strategies among fungi. Thus it is plausible that saprotrophic (SAP) and ectomycorrhizal (EcM) fungi, including most of the species with macroscopic fruitbodies, harbour distinct bacterial communities due to differences in their nutrition and habitat conditions they provide to bacteria.

The start inoculum of host-associated microbiomes largely originates from the environment (Song et al., 2013; Zarraonaindia et al., 2015; Amato et al., 2016; Adair and Douglas 2017; Compant et al., 2019). For instance, the source community of plant root, human gut and fish gill microbiomes is known to originate from soil, food and water, respectively (Berendsen et al., 2012; Cotillard et al., 2013; Hacquard et al., 2015; Pratte et al., 2018; Sylvain et al., 2020). The

mechanisms of microbial uptake in fungi could be similar to plants which have limited mobility and a microbial community that is largerly derived from the surrounding soil (Bulgarelli et al., 2013; Baltrus, 2017). Thus, as a subset of the environmental microbiome, the host microbiome may be affected by the variation in the habitat conditions and microbial community of the surrounding environment (Bulgarelli et al., 2013; Zarraonaindia et al., 2015; Apprill, 2017; Compant et al., 2019). Certain environmental parameters have been shown to affect the host-associated microbial community composition, including habitat pH, oxygen level, chemical parameters and climatic variables (Skovgaard et al., 2015; Amato et al., 2016; Naylor et al., 2017; Kers et al., 2018; Dastogeer et al., 2020; Zarraonaindia et al., 2015). Nevertheless, specific host characteristics most directly influence the filtration of certain bacterial associates from the environment (Adair and Douglas 2017; Dastogeer et al., 2020). pH, oxygen level and host-derived exudates, such as organic carbon compounds but also inhibitory metabolites, have been identified as important host factors that shape the root and gut microbiomes (Spor et al., 2011; Berendsen et al., 2012; Bulgarelli et al., 2013; Hacquard et al., 2015). Although a few studies have examined the origin of bacteria in fungus-affected habitats and the factors influencing the selection of their bacterial community members (Boersma et al., 2009, 2010; Warmink et al., 2009), there is virtually no data concerning fruitbody-inhabiting endofungal bacteria. In the current thesis the term endofungal refers to bacteria located within fungal fruitbodies regardless of their occurrence in or outside the host cells.

1.1. Fungal-bacterial interactions

Similarly to other organisms, both positive and negative interactions may occur between fungi and bacteria (de Boer et al., 2005; de Menezes et al., 2017; Deveau et al., 2018). Fungal-bacterial interactions offer a model system to study host/pathogen interactions and different forms of mutualism, with implications for biotechnological research and biocontrol strategies to combat mushroom diseases (Kobayashi and Crouch, 2009; Mondo et al., 2012; Efimenko et al., 2016; Deveau et al., 2018). Increasing evidence points to the importance of fungal-bacterial interactions in structuring microbial communities (Ballhausen and de Boer, 2016; Bahram et al. 2018). Bacteria and fungi may also complement one another in substrate degradation and other soil biochemical processes (de Menezes et al., 2017; López-Mondéjar et al., 2018). Together fungi and bacteria may promote the decomposition of complex compounds, such as cellulose or lignin (de Boer et al., 2005; Zhang et al., 2014a; de Menezes et al., 2017; López-Mondéjar et al., 2018; Deveau et al., 2018). In addition, bacteria may supply fungi with nitrogen and phosphorus or remove the harmful compounds during the decay, and in turn, benefit from the fungal enzymes, exudates, water redistribution capacity, or hyphae used for crossing distances in soil (Guhr et al., 2015; Ballhausen and de Boer, 2016; de Menezes et al., 2017; López-Mondéjar et al., 2018). It has been suggested that fungus-associated bacteria may better resist environmental stress

compared to free-living bacteria due to the more stable habitat provided by host fungus (de Menezes et al., 2017).

Fungi may respond to the presence of some bacteria through the expression of certain genes, while bacteria themselves affect the transcription of particular metabolism- or antagonism-related genes in the fungal host (Schrey et al., 2005; Deveau et al., 2007; Kombrink et al., 2019; Scherlach and Hertweck, 2020). In addition, the functional capacity of the fungus-associated microbiome may be reduced as a result of fungal-bacterial symbiosis (Quandt et al., 2015; Bonfante et al., 2019), whereas some bacteria may be specialized to use certain fungal metabolites (Bonfante et al., 2019). Some bacterium-responsive genes are characteristic only for certain fungal species and no orthologous genes have been identified even in the genomes of the closely related fungal species (Deveau et al., 2007), reflecting specific adaptation of fungal-bacterial associations. In addition, accumulating evidence supports frequent horizontal gene transfer between fungi and bacteria which expand the resource utilization capability of mycosphere inhabitants and also refers to the co-evolution between fungi and bacteria (Zhang et al., 2014a; Bonfante et al., 2019; Pratama and van Elsas, 2019). The tight and long-term co-evolution between these groups of organisms has also been demonstrated by several specific bacteria that live inside fungal cells (endobacteria; Deveau et al., 2018; Pawlowska et al., 2018).

1.2. Fungus-affected bacterial habitats

The structure of bacterial communities in the mycosphere has been studied in a number of fungal species, such as *Tricholoma matsutake*, *Laccaria proxima, Russula exalbicans, R. griseocarnosa, Phanerochaete chrysosporium*, *Lyophyllum* sp. and *Chroogomphus rutilus* (Boersma et al., 2009, 2010; Nazir et al., 2010a; Wang et al., 2011; Hervé et al., 2014; Halsey et al., 2016; Oh et al., 2016; Yu et al., 2020). There are also studies on the bacterial communities of the ecto-mycorrhizosphere of *Xerocomus pruinatus*, *Scleroderma citrinum*, *Suillus bovinus, S. granulatus*, *Paxillus involutus*, *Amanita pantherina*, *Russula mariae*, *Hebeloma mesophaeum*, *Rhizopogon roseolus* and *Tuber indicum* (Timonen et al., 1998; Timonen and Hurek 2006; Uroz et al., 2007, 2012; Li et al., 2017; Shirakawa et al., 2019). The fungal growth substrate of commercially valuable mushrooms can also be considered as a mycosphere with potential implications for mushroom cultivation (Henry et al., 1991; Noble et al., 2009; Zarenejad et al., 2012; Kertesz and Thai, 2018). Microbial communities have been compared also inside and outside of the fairy rings or the truffle brûlés (Bonanomi et al., 2012; Mello et al., 2013; Xing et al., 2018). In addition, different bacteria have been identified as the third component of lichens with the majority being closely related to the mycobiont in the lichen thalli (Cardinale et al., 2008; Grube and Berg, 2009; Grube et al., 2009). Despite the upmentioned studies documenting bacteria in various habitats affected by different fungal species, bacterial

communities inside fruitbodies (especially those of basidiomycetes) have deserved mush less attention.

Fruitbody-inhabiting bacterial communities have been widely described in the fruitbodies of several Ascomycota species, such as *Elaphomyces granulatus* (Quandt et al., 2015), different *Tuber* species (Barbieri et al., 2005, 2007, 2010; Antony-Babu et al., 2014; Ye et al., 2018b; Splivallo et al., 2019; Monaco et al., 2020; Perlińska-Lenart et al., 2020) and some other Pezizales species (Benucci and Bonito, 2016; Benucci et al., 2019). Among Basidiomycota, bacterial communities have been analyzed for *Cantharellus cibarius* (Danell et al., 1993; Rangel-Castro et al., 2002a; Kumari et al., 2013) and *Tricholoma matsutake* (Li et al., 2016b; Oh et al., 2018) fruitbodies and for a few other Basidiomycota species (Dahm et al. 2005; Zagriadskaia et al. 2013; Efimenko et al. 2016). However most of these studies have used culture-dependent methods which have important limitations. The cultivation-dependent studies are often irreplaceable describing the antimicrobial activity or the mushroom growth promoting effect of bacteria isolated from fruitbodies (Tsukamoto et al., 2002; Efimenko et al., 2016; Aslani et al., 2018; Oh et al., 2018), but only a small proportion of microbes is cultivable. High-throughput sequencing (HTS) can complement culture-based methods, offering unique opportunities to study fungal inhabiting bacterial communities to a greater extent than before. So far, this method has only been used for studying Ascomycota-inhabiting microbial communities (Antony-Babu et al., 2014; Quandt et al., 2015; Benucci and Bonito, 2016; Ye et al., 2018b; Benucci et al., 2019; Splivallo et al., 2019) but to much lesser extent in Basidiomycota fruitbodies (Li et al., 2016a; Koskinen et al. 2018; Liu et al. 2018; Rinta-Kanto et al. 2018).

1.3. Bacterial groups detected in fungal fruitbodies using different methods

The composition of bacterial taxa uncovered from fruitbodies strongly depends on the methodology used – cultivation of bacteria in pure culture *versus* culture-independent methodologies like HTS (Barbieri et al., 2005, 2007; Perlińska-Lenart et al., 2020). For example, Alphaproteobacteria appear to be highly dominant in the ascocarps of *Tuber* species based on cultivation-independent methods but strongly underrepresented among the bacteria isolated into culture (Barbieri et al., 2005, 2007; Splivallo et al., 2015a; Perlińska-Lenart et al., 2020). Several high-throughput and metagenomic analyses of bacterial communities also confirm the high relative abundance of the class Alphaproteobacteria (mainly the family Bradyrhizobiaceae), but also the class Sphingobacteriia (Bacteroidetes) in different ascomycetes (Antony-Babu et al., 2014; Quandt et al., 2015; Li et al., 2017; Ye et al., 2018b; Splivallo et al., 2019; Perlińska-Lenart et al., 2020). Culture-dependent methods have rarely revealed the presence of Alpha-proteobacteria and Bacteroidetes in basidiomycetes (Dahm et al., 2005; Kumari

et al., 2013; Zagriadskaia et al., 2013; Aslani et al., 2018) similarly to that observed in ascomycetes. Nevertheless, different *Pseudomonas* species and enterobacteria, both belonging to Gammaproteobacteria, but also the genera *Bacillus* (Bacilli) and *Burkholderia* (Betaproteobacteria), have been shown to be highly represented among the cultivable bacteria in *Cantharellus* species (Rangel-Castro et al., 2002a; Kumari et al., 2013) and in several other taxa of basidiomycetes (Dahm et al., 2005; Zagriadskaia et al., 2013; Efimenko et al., 2016; Aslani et al., 2018). Studies of matsutake-associated bacteria also support that although the genus *Pseudomonas* and members of Enterobacteriaceae, Betaproteobacteria and Actinobacteria are detectable both by culture-dependent and -independent methods, the relative abundances of Alphaproteobacteria and Bacteroidetes can be underestimated using only the culturing method (Li et al., 2016a,b; Oh et al., 2018). Nevertheless, the study on microbial communities of *Shiraia bambusicola* (Ascomycota) showed that the relative abundances of most bacterial groups are largerly consistent among the two datasets obtained with the HTS and culturing (Ma et al., 2019). However, a critical evaluation of these methods for studying the microbial communities in fruitbodies is still lacking.

1.4. The impact of soil in the formation of bacterial communities in fungus-affected habitats

Environmental filtering affects the distribution and the composition of the fungal and bacterial communities in soil at the global scale (Bahram et al., 2018). Several abiotic factors, such as pH, availability of nitrogen and phosphorus, carbon quality and quantity, moisture and root exudates may affect the structure of the local fungal and bacterial communities (Rousk et al., 2010; Lakshmanan et al., 2014; Fierer, 2017). It is also known that besides environmental conditions, the combination of microbial strains and their antagonistic effect on each other may affect the spread of certain fungi and bacteria in soil (de Boer, 2017; de Menezes et al., 2017). Similarly to the host plant effect on the rhizosphere and root microbiome (Bulgarelli et al., 2013; Hacquard et al., 2015; Pii et al., 2016), fungi are able to shape the microbiome in fungus-affected habitats, such as the mycosphere and the mycorhizosphere (Nazir et al., 2010b). Namely, fungal hyphae in the mycosphere/hyphosphere soil can alter pH, the concentration of carbon, nitrogen, phosphorus, calcium, magnesium, $N\text{-}NH_4^+$ and organic compounds, but also the profile of carbohydrates, enzymes and antimicrobial compounds (Danell et al., 1993; Boersma et al., 2009, 2010; Nazir et al., 2010a,b; Bonanomi et al., 2012; Ghodsalavi et al., 2017; Li et al., 2017; Xing et al., 2018; Shirakawa et al., 2019), thereby filtering certain bacteria from the soil bacterial community.

In addition, the structure of bacterial communities differ significantly between fungal fruitbodies and the ectomycorrhizosphere or hyphosphere which likely reflect the biochemical characteristics and contrasting host effect in these habitats (Rangel-Castro et al., 2002a; Antony-Babu et al., 2014; Liu et al., 2018). Such

variables likely select for certain bacterial groups from the surrounding soil. The tight interactions between the soil and ascocarp microbiomes have been shown for some *Tuber* species (Antony-Babu et al., 2014; Splivallo et al., 2019; Monaco et al., 2020), although it has not directly been demonstrated for basidiomycetes. Assuming that most fruitbody-inhabiting bacteria originate from the surrounding soil, soil properties likely affect the bacterial community composition in fruitbodies. Indeed, certain soil properties correlate with the relative abundance of bacterial taxa associated with *Tricholoma matsutake* fruitbodies (Li et al., 2016a). Similarly, the previously reported locality effect on the microbiome structure of Pezizales (Benucci and Bonito, 2016; Splivallo et al., 2019) and *Tricholoma matsutake* (Li et al., 2016a,b) can be ascribed to the variation in the conditions of soils under fruitbodies across different geographic regions. Although the fruitbody microbiome likely originates from the surrounding soil, there may be differences across EcM and SAP fungi in this regard. In particular, EcM fungi are directly connected to living plants, whereas SAP fungi colonize the decaying organic material and these specific relationships may also affect their fruitbody microbiomes.

1.5. Host-derived factors determining the microbial communities in fungal fruitbodies

Compared to mycosphere/hyphosphere bacteria, fruitbody-inhabiting bacteria may interact more directly with their host fungi, resulting in a stronger effect of host-related factors on their community structure. Here hosts' genotype, phylogenetic relationships, functional guild and fruitbody compartments were considered as the host-related factors. The observed host genotype effect on human-, animal- as well as plant-associated microbiome (Zoetendal et al., 2001; Berendsen et al., 2012; Goodrich et al., 2014; Hacquard et al., 2015) suggests that host genotypes may also shape bacterial community structure in fungi (Splivallo et al., 2019). So far, the effect of host genotypes, taxonomy/phylogeny, functional guild or fruitbody compartment on fruitbody-inhabiting microbiome remain poorly known. Nevertheless, the host fungus identity effect has been described for *Cantharellus* (Kumari et al. 2013) and some other basidiomycete species (Koskinen et al., 2018; Liu et al., 2018; Rinta-Kanto et al., 2018) as well as for different hypogeous Pezizales (Ascomycota; Benucci and Bonito 2016; Ye et al., 2018b). The effect of fungal functional guild on the fruitbody-inhabiting microbiome has been addressed only by Liu et al. (2018). In addition, the structure of bacterial communities varies across different fruitbody compartments of several ascomycetes (Antony-Babu et al., 2014; Splivallo et al., 2015a; Benucci et al., 2019) and also in *Tricholoma matsutake* (Basidiomycota) fruitbodies (Oh et al., 2018).

Host-related factors may be reflected in the chemical characteristics of fruitbodies, and thus fruitbody chemistry could be an important determinant of fruitbody-inhabiting microbiome. Namely, the variation of the carbon and macroelement content in fruitbodies has been observed between fungal taxa, functional guilds and even between different fruitbody compartments (Taylor et al., 1997, 2003; Rudawska and Leski 2005; Trocha et al., 2016; Kranabetter et al., 2019). Thus, it is plausible that the concentration of microelements, vitamins, proteins, certain antibiotics or carbon compounds, which all vary among different fungal taxa (Sanmee et al., 2003; Boersma et al., 2009, 2010; Alves et al., 2012; Vieira et al., 2014; Olagbemide and Ogunnusi, 2015; Shirakawa et al., 2019), alter the bacterial community composition in or around the fruitbodies, favouring or disfavouring certain bacteria. For instance, the concentration of macronutrients and organic compounds show some level of phylogenetic conservatism (Barros et al., 2008a; Kalač, 2009; Vieira et al., 2014; Ruthes et al., 2016) as well as similarities within fungal functional guilds (de Carvalho et al., 2015). SAP fungi have been shown to alter the associated bacterial communities by secreting certain secondary metabolites (de Carvalho et al., 2015). Secondary metabolites may be specifically affecting particular fungal-bacterial interactions in various habitats (Scherlach and Hertweck, 2020). It has also been found that SAP fungi usually produce more extracellular enzymes to degrade various organic compounds than do EcM fungi (Zanne et al., 2020). Thus, the variation of the content and the profile of the carbohydrates or other compounds in fruitbodies, which are specific for different fungal groups (Sanmee et al., 2003; Barros et al., 2007; Ruthes et al., 2016; Trocha et al., 2016) may explain the host effect on bacterial community structure.

1.6. Putative and identified functions of fungus-associated bacteria

The microbial symbionts often provide nutrients, promote defence mechanisms against opportunistic pathogens or improve niche adaptation of their hosts (Bulgarelli et al., 2013; Hacquard et al., 2015; Liu et al., 2019). Similarly to other eukaryotic organisms, fungi have their own characteristic microbes, where bacteria are attached to the hyphae or reside inside the hyphae and may perform particular functions (Bonfante et al., 2019). Several fungal associated bacteria are able to suppress pathogens or detoxify harmful compounds in their host fungi (Tsukamoto et al., 2002; Tarkka et al., 2015; Aslani et al., 2018; Oh et al., 2018; Scherlach and Hertweck, 2020), and may thus be functionally similar to the beneficial bacterial symbionts in other eukaryotes (Bulgarelli et al., 2013; Kohl et al., 2014; Hacquard et al., 2015; Liu et al., 2019). A recent study demonstrates that bacterial community composition significantly differs between healthy *Agaricus bisporus* fruitbodies and those with disease symptoms, suggesting the effect of bacteria on disease development (Martins et al., 2020). Several members of *Pseudomonas*

are resistant to heavy metals (Singh et al., 2010) and are able to degrade polycyclic aromatic hydrocarbons (Kamath et al., 2004). In addition, pollutant-degrading bacteria, including *Pseudomonas* spp., use fungal hyphae to spread in polluted soil (Kohlmeier et al., 2005; Wick et al., 2007) which may lead to their high relative abundance in fruitbodies. However, despite the evidece on the role of certain *Pseudomonas* in promoting fungal growth and fruitbody formation (Deveau et al., 2007; Noble et al., 2009; Chen et al., 2013), the genus also contains numerous pathogenic species and strains (Henry et al., 1991; Aslani et al., 2018; Martins et al., 2020).

Several mycorrhiza-associated bacterial groups, such as genera of Proteobacteria, Firmicutes and Actinomycetes, have been identified as mycorrhiza-helper bacteria (MHB; Deveau et al., 2007; Frey-Klett et al., 2007). MHB promote the formation of mycorrhizae and support the functioning of the symbiotic relationship (Poole et al., 2001; Frey-Klett et al., 2007). MHB may enhance the mycelial growth, the formation of root-fungus contacts and spore germination, producing several growth factors, supressing antagonists and reducing the effect of environmental stress (Frey-Klett et al., 2007; Pavić et al., 2013). Plants are able to choose rhizosphere bacteria that are able to effectively participate in the soil biogeochemical cycles, such as Actinobacteria that solubilize phosphorus or Beta – and Gammaproteobacteria that help to cope with Fe limitations (Pii et al., 2016). Similarly, MHB with high mineral weathering potential may be selected by fungi from surrounding soil, contributing to the nutrition of both host fungi and plants (N, P, Fe; Frey-Klett et al., 2007; Uroz et al., 2007; Pavić et al., 2013; Bonfante et al., 2019). Bacteria that supply the fungus with the mineral compounds, vitamins and volatiles are likely to receive the host fungus-exudated, energy-rich carbon compounds, such as trehhalose, raffinose and mannitol, in return (Duponnois and Kisa, 2006; Riedlinger et al., 2006; Frey-Klett et al., 2007; Deveau et al., 2010; Pavić et al., 2013). Similarly, bacteria as microsymbionts may have several functions in the lichen symbiotic assembly, such as participation in nutrient cycling (nitrogen fixation, phosphate mobilization), hormone production and antagonistic activities against pathogens while using carbohydrates produced by other members forming the lichen (Grube et al., 2009).

The presence of nitrogen-fixing bacteria and nitrogenase activities, including the transcription of nifH genes, have been determined in ectomycorrhizae (Li et al., 1992; Izumi et al., 2006). EcM fungi supply plant with nitrogen, the main limiting factor for plant nutrition and thus the EcM-associated nitrogen-fixing bacteria may contribute to plant N nutrition (Izumi et al., 2006; Timonen and Hurek, 2006; Frey-Klett et al., 2007). The functional gene array of *Tuber melanosporum* fruitbodies shows that the fruitbody-inhabiting bacteria may be involved in sulphur and nitrogen cycling (Antony-Babu et al., 2014). In addition, several bacterial groups among Rhizobiales are highly represented in fruitbodies of EcM ascomycetes (Barbieri et al., 2005, 2007; Antony-Babu et al., 2014; Quandt et al., 2015). The potential function of these bacterial groups has been associated with the nitrogen supply to their host fungi, because several species among Rhizobiales can fix nitrogen and the nitrogenase expression genes and activity has been

detected in *Tuber magnatum* ascocarps (Barbieri et al., 2005, 2007, 2010). The fruitbody-inhabiting nitrogen-fixing bacteria may affect the development and maturation of truffle ascocarps (Barbieri et al., 2007; Antony-Babu et al., 2014). Similarly, nitrogen-fixing bacteria and nitrogen fixation have also been detected in decaying wood, vegetative hyphae of *Pleurotus ostreatus* and in fruitbodies of several other wood-decaying fungi, likely reflecting the interaction between these bacteria and SAP fungi (Larsen et al., 1978; Spano et al., 1982; Jayasinghe-arachchi and Seneviratne, 2004; Hoppe et al., 2014). SAP fungi need large quantities of nitrogen during fruitbody formation and while growing on low nitrogen substrate they may use the atmospheric nitrogen fixed by bacteria and supply the bacteria, in turn, with the low molecular weight organic compounds (Spano et al., 1982; Jayasinghearachchi and Seneviratne, 2004; Hoppe et al., 2014). Similarly, lichens have often been found to be inhabited by nitrogen-fixing bacteria, which may supply the fungal and algal partners with nitrogen on nutrient-poor substrates that the lichens often colonize (Grube et al., 2009; Hodkinson and Lutzoni, 2009).

Fungus-associated bacteria contribute to the cultivation success of several valuable fungi by inducing their fruitbody formation (Li et al., 2016b; Kertesz and Thai 2018; Oh et al., 2018; Carrasco and Preston, 2019; Mediavilla et al., 2019; Suarez et al., 2020; Sun et al., 2020) and affecting the morphology, aroma and pigment formation of fruitbodies (Splivallo and Ebeler, 2015b; Saidi et al., 2016; Zhou et al., 2017; Tauber et al., 2018; Ma et al., 2019). For instance, some members of *Pseudomonas* contribute to fruitbody formation in *Agaricus bisporus* and *Pleurotus ostreatus* (Cho et al., 2003; Noble et al., 2009; Zarenejad et al., 2012; Chen et al., 2013). In addition, certain microbes participate in specific processes such as cellulose degradation, thus supporting the growth of fruitbodies (Carrasco and Preston, 2019) and shortening their maturation period (Suarez et al., 2020; Sun et al., 2020). Fruitbodies have also been highlighted as the best source for isolation of mycelial growth promoting bacteria (Suarez et al., 2020).

1.7. The main aims and hypotheses

The main aims of the current PhD thesis were **1)** to characterize the bacterial community structure and functions and **2)** to estimate the relative contribution of various host-specific vs environmental factors underlying the bacterial community variation in fungal fruitbodies across genotypes, phylogenetic groups and functional guilds of mushroom-forming members of Basidiomycota.

As the main hypothesis it was proposed that **1)** host related features (taxonomy/ phylogeny, functional guild, fruitbody chemistry, genotype) are the primary determinants of the fruitbody microbiome structure and functions. In addition, as the fruitbodies of the studied fungi arise from the mycelium in soil, we hypothesized that **2)** soil is the primary source for the fruitbody microbiome and therefore soil characteristics play an additional role in shaping the structure of fruitbody bacterial communities.

2. MATERIALS AND METHODS

2.1. Study sites and sampling

Fungal fruitbodies were collected in boreal forests at three nature reserves in Eastern Estonia (**I**), including a site at Meenikunno (Figure 1), from where most of the fruitbodies of the main dataset of study **IV** were collected. Fruitbodies belonged to species from four main orders of mushroom-forming fungi in Basidiomycota (**I**, **IV**) and were identified to 15 EcM species in study **I**. In study **IV**, the main dataset contained three SAP and three EcM species (**IV**). In order to have a comparable number of EcM and SAP fruitbodies, 15 species of SAP fungi and 12 species of EcM fungi were sampled from other areas in Estonia. The extended dataset is referred to as the validation dataset (**IV**). A dried collection of *Inocybe terrigena* was analyzed in study **II**. The fruitbodies of *Marasmius oreades*, analyzed in study **III** were collected from six fairy rings growing on tended lawns in park areas in southern Sweden (Uppsala and Vadstena regions).

Figure 1. Sampling site at Meenikunno.

Only mature fruitbodies were sampled, excluding damaged or decaying fruitbodies from our selection. All fruitbodies were packed individually in foil, transported to the lab in a cooled container and kept in fridge at 4 °C until being handled in a laminar flow chamber (**I**, **IV**). The fruitbodies were cut lengthwise under the laminar flow using a sterile scalpel (**I**, **IV**). The cut surface was sterilized under UV light for 5 min, followed by a second incision into the exposed inner tissue while avoiding contact with the fruitbody outer surface (**I**, **IV**). Two 5 mm^3 pieces were taken from each fruitbody compartment – the cap, the middle part and the lower part of the stipe (**I**, **IV**). In study **III**, fruitbodies were kept in airtight tubes for up to one hour before freezing and subsequent lyophilization. Using sterile forceps and needles, the cap was removed (**III**). Soil samples originated from the same sites as the fruitbodies, collected according to the methodology described by Tedersoo et al. (2014; **I**). In study **IV**, soil samples were taken as 28 subsamples from 10 cm depth within 50 cm radius from each fruitbody.

2.2. Chemical analysis of soil and fruitbody samples

The concentration of carbon (C), nitrogen (N) and phosphorus (P) and pH_{KCl} were measured from soil samples (**I**, **IV**). In addition, the concentration of organic matter, $\delta^{15}N$, potassium (K), calcium (Ca), and magnesium (Mg) were evaluated in study **I** and the content of ammonium nitrogen (NH_4-N) in study **IV**. Soil pH was measured at a distance of 20 cm from inside and outside each fairy ring growth front, close to the cardinal points where fruitbodies had been collected (**III**). Total phosphorus (P), nitrogen (N) and carbon (C) content and pH_{KCl} were measured from fruitbody samples (**IV**).

2.3. Culturing

The homogenate of three fruitbody pieces and 0.1 M phosphate buffer (1 M SmartMix, pH 7, Naxo OÜ, Estonia) was plated to one Petri dish with R2A low nutrient agar or in some cases onto twice diluted tryptic soybean agar (TSA, Liofilchem, Italy). The plates were incubated at 25 °C for 30 days not to miss the slow growing bacteria. From each Petri dish, colonies with a different size, shape, elevation, color, margin, texture, surface, or opacity were transferred to a new Petri dish with TSA. Pure isolates were preserved at –80 °C in 50% glycerol in the Tartu Fungal Culture Collection (TFC; **I**).

2.4. DNA extraction and 16S rRNA gene amplification

DNA was extracted from the fruitbody pieces using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany; **I**) and the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, CA, USA; **IV**) following manufacturer's instructions. In studies **II** and **III** DNA was extracted from lamellae of the dried *I. terrigena* fruitbodies using Plant mix DNeasy DNA Isolation kit and from the whole stipe of the fruitbodies of *Marasmius oreades* with the ZR Fungal/Bacterial DNA MiniPrep TM (Zymo Research), respectively. PowerMax Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) and ZR Soil Microbe DNA Kit (Zymo Research, CA, USA) were used for the extraction of DNA from soil samples in studies **I** and **IV**, respectively.

The 16S rRNA gene variable regions V3-V4 were amplified using bacterial primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806RB (5′-GGAC TACNVGGGTWTCTAAT-3′) (**I**, **IV**). The PCR products were sequenced at the Estonian Biocentre (Tartu, Estonia) using Illumina MiSeq technology (**I**, **IV**). Library preparations in study **II** (PCR free, paired-end, 300bp insert libraries) and in study **III** (38 samples with TruSeq PCR free and 2 with TruSeq Nano; Illumina) and sequencing (Illumina HiSeq 2500) was done by the Science for Life Laboratory (SciLifeLab) in Uppsala, Sweden.

To extract DNA from culture strains, the bacterial cells were transferred into lysis buffer containing 0.8 M Tris–HCl, 0.2 M $(NH_4)_2SO_4$, 0.2% w/v Tween-20 (10 × Reaction Buffer B, Solis Biodyne, Tartu, Estonia) and proteinase K (20 mg/ml, Fermentas, Lithuania). The mixtures were incubated at 56 °C for 15–16 h, followed by incubation at 98 °C for 15 min to inactivate proteinase K. From each sample the almost full-length 16S rRNA gene was amplified using the universal bacterial primers 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CTACGGCTACCTTGTTACGA-3'). Purified PCR products were sequenced using the Sanger method at the Macrogen Inc. (Amsterdam; **I**).

2.5. Bioinformatics

Illumina sequences from soil and fruitbody samples were processed using the software package LotuS (Hildebrand et al., 2014), including demultiplexing, quality-filtering and chimera-checking (**I**, **IV**). Sequences were clustered into OTUs at the 97% similarity level using UPARSE and USEARCH v10.0.240 (Edgar, 2010) was used for generating consensus OTU sequences (**I**, **IV**). Representative sequences from each bacterial OTU were classified using the SILVA (https://www.arb-silva.de) database (**I**, **IV**) and compared with most similar sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), RDP (http://rdp.cme.msu.edu) and in GreenGenes (http://greengenes.lbl.gov) databases (**I**). All chloroplast, mitochondria, taxonomically unidentified at the kingdom level, eukaryotic OTUs (**I**, **IV**) and Archaea (**I**) sequences were removed. In addition, we excluded all OTUs containing <5 reads or OTUs that occurred in high numbers in positive or negative controls (**I**, **IV**).

Unmapped sequences (reads without significant matches to the *M. oreades* or *I. terrigena* draft genome), obtained from fruitbodies of *M. oreades* and *I. terrigena*, were uploaded to the MG-RAST metagenomics analysis server 4.0.3 (Meyer et al., 2008), including quality-filtering and taxonomic assignment as the built-in pipeline functions (**II**, **III**). Reads were taxonomically and functionally annotated based on RefSeq database and SEED subsystems, respectively (**II**, **III**).

The sequences obtained from culture isolates were assembled and manually edited using Sequencher 5.1 (Gene Codes Corporation, USA) and subsequently aligned using MAFFT (http://mafft.cbrc.jp/alignment/server/; **I**). Sequence similarity searches were performed using the SILVA, the GenBank and the GreenGenes databases (**I**).

2.6. Statistical analyses

The datasets were rarefied and further normalized using Hellinger transformation before statistical analyses (**I**, **III**, **IV**). R packages of the versions 3.2.2 (R Development Inc., 2013) and 3.6.1 (R Development Core Team, 2019) were used for statistical analyses (**I**, **III**, **IV**). Permutational Multivariate Analysis of Variance (PERMANOVA) was performed using the program Primer 6 (Primer-E, Plymouth, UK) with the PERMANOVA+ add-on package or the *adonis* function in vegan (**I**, **III**, **IV**). The best model was selected based on forward selection with F-value as the selection criterion (**I**, **III**, **IV**). Canonical analysis of principal (CAP) coordinates, based on Bray–Curtis dissimilarity (Anderson and Willis, 2003), was performed using the program Primer 6 (Primer-E Ltd., Plymouth, UK) with the PERMANOVA+ add-on package (**I**, **III**). Non-metric multidimensional scaling (NMDS) plots were generated using the vegan package of R (**IV**) or using the program Primer 6 (Primer-E, Plymouth, UK; **III**). The phylogenetic tree was constructed using the perl script taxonomy_to_tree.pl following Tedersoo et al. (2018), and phylogenetic distance between fungi was calculated using the picante package in R (vers.3.6.1, R Development Core Team, 2019; **IV**). The Mantel and Partial Mantel tests were performed using the vegan package of R (**III**, **IV**). Principal Coordinates of Neighbourhood Matrix (PCNM; Borcard et al., 2004) were constructed using the *pcnm* function of vegan and added stepwise to the model in order of significance until no further improvement was obtained using the *forward.sel* function of the packfor (version 0.0–8) package (Dray et al., 2007) with default parameters (**III**). The function *betadisper* in the vegan package was used for analysis of homogeneity of groups dispersions followed by Tukey's honestly significant difference (HSD) tests (**I**). General and pairwise comparisons were determined based on Kruskal-Wallis, Wilcoxon or t-tests (**IV**). In addition, multivariate analysis of variance (MANOVA) and analysis of variance (ANOVA) were applied in R packages dplyr and vegan (vers.3.6.1). The heatmaps were prepared using the R function *heatmap* in study **III** and ggplot2 package of R in study **IV**. The indicator OTUs were determined using the Dufrene-Legendre Indicator Species Analysis in labdsv package of R (**IV**).

3. RESULTS AND DISCUSSION

3.1. Factors determining fruitbody-inhabiting microbial community composition

Host-specific factors, including host genotype, phylogeny/taxonomy (hereinafter identity), fruitbody chemistry and functional guild have higher impact on bacterial community structure in fungal fruitbodies compared to environmental parameters such as soil properties and habitat type (I, III, IV). Similarly to our finding, both environment and host factors have been shown to affect the structure of bacterial communities in truffle fruitbodies (Benucci and Bonito 2016; Splivallo et al., 2019) as well as in the mycorrhizosphere (Timonen et al., 1998; Li et al., 2017). However, studies on the factors determining the microbial community structure and functions in other eukaryotic hosts have yielded different results. For instance, host identity has been determined as the most important factor shaping the structure of the gut microbiome in primates (Amato et al., 2016) and small mammals (Knowles et al., 2019) as well as the root microbiome of grasses (Naylor et al., 2017) while in all cases some other host-specific or environmental factors were also significant. Numerous studies have also shown that both host-specific and environmental factors are involved in the development of the microbial community in eukaryotes, but the most important factor has often not been singled out (Nelson et al., 2013; Pii et al., 2016; Apprill, 2017; Dastogeer et al., 2020).

3.1.1. Host-specific factors

Among host related factors, host identity is the primary determinant of the microbial community structure across different fungal taxa (I, IV). Host phylogeny was found to have a strong effect on the microbial community structure in study **IV** (Mantel: r=0.496, p=0.001). Study **I** showed that the host fungal order (PERMANOVA: R^2adj. = 0.09; $p \leq 0.001$) and also host fungal genus (PERMANOVA: R^2adj. = 0.03; $p = 0.002$) explained a significant part of the variability in the bacterial community composition in eight fungal genera from four orders of Agaricomycetes, Basidiomycota (Figure 2). Similarly, fungal species strongly affected the microbial community composition in fungal fruitbodies (PERMANOVA: R^2adj. = 0.15; $p \leq 0.001$) of six species from three fungal orders (**IV;** Figure 3). The host identity has been found to affect the microbial community structure in fruitbodies of various members of Basidiomycota (Rinta-Kanto et al., 2018). Similarly, the significant effect of the host identity on the microbiome structure has been documented in the Pezizales, Ascomycota (Benucci and Bonito, 2016; Ye et al., 2018b). The host species specificity of bacterial fungiphiles identified in the mycospheres or mycorrhizospheres of various basidiomycetes has been explained by the ability of these bacteria to use

particular carbon compounds (e.g. mannitol, trehalose, inositol or xylitol), organic acids (e.g. oxalate, acetate) or amino acids, released by the host fungi (Timonen et al., 1998; Warmink et al., 2009; Nazir et al., 2010b) and by the tolerance to antibacterial compounds produced by fungi (de Carvalho et al., 2015; Shirakawa et al., 2019). The variability of the aforementioned compounds in different fungal taxa may be one of the main selection mechanism behind the host fungus identity effect on fruitbody-inhabiting bacterial community. For instance, *Cantharellus cibarius*, phylogenetically distant from most other mushroom forming basidiomycetes, contains higher amounts of proteins, selenium, β-carotene and vitamin C than several other fungal species and are characterised by a specific carbohydrate and essential amino acid profile (Rangel-Castro et al. 2002a,b; Agrahar-Murugkar and Subbulakshmi, 2005; Barros et al., 2008a,b; Kalač, 2009). The characteristic biochemical composition of golden chanterelle fruit-bodies might explain the distinctness of its microbial community structure compared to other studied fungal taxa (**I**, **IV**; Figure 2, 3; Rinta-Kanto et al., 2018; Gohar et al., 2020).

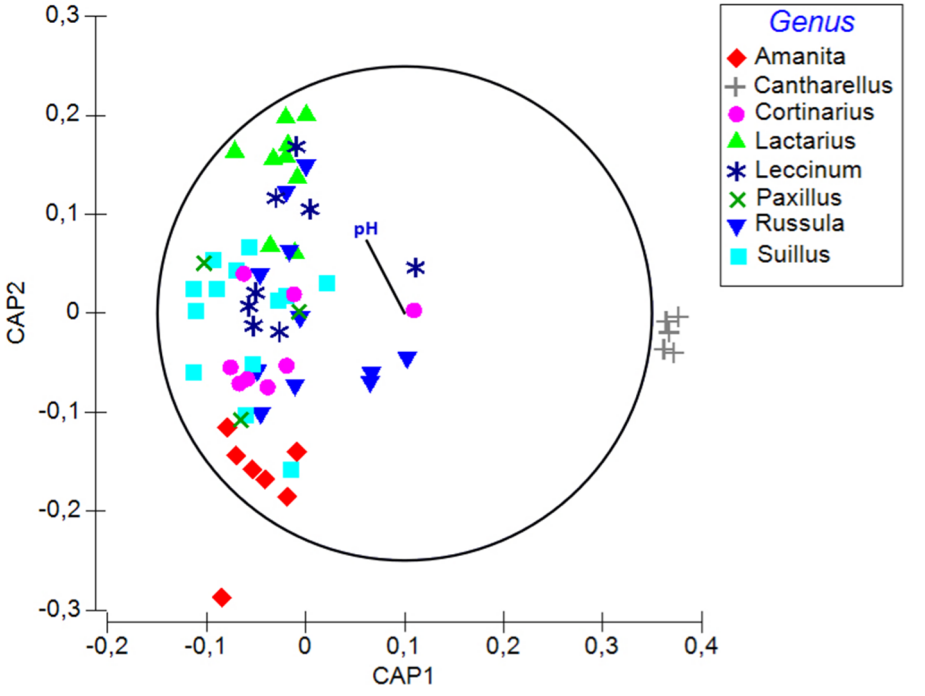

Figure 2. A plot of the canonical analysis of principal (CAP) coordinates visualizing the differences in bacterial communities among fungal genera and the effect of soil parameters underlying the observed variation based on Bray-Curtis dissimilarity of HTS data. Vector shows Pearson correlations with soil pH along the second axis of CAP (variables with correlations ≥ 0.3 are presented). Correlation coefficient for pH is –0.26. Figure from Publication I.

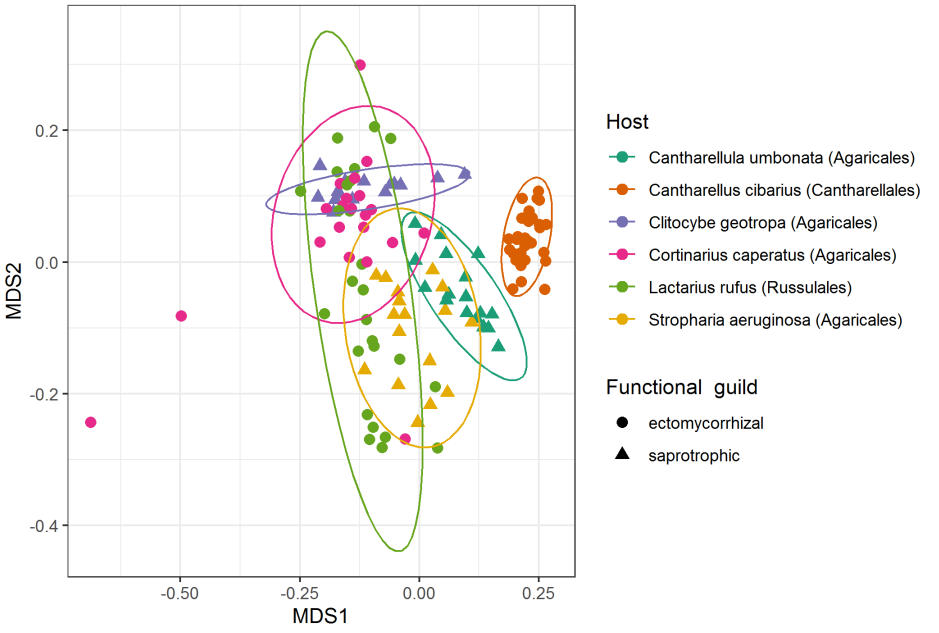

Figure 3. Non-metric multidimensional scaling (NMDS) ordination illustrating compositional differences in bacterial communities associated with different host taxa and functional guilds of host fungi. The Bray-Curtis dissimilarity matrix was calculated based on rarified and Hellinger transformed OTU matrix, containing fruitbody samples, rarefied to ≤2000 reads. Samples containing less than 1000 reads were removed. Each symbol represents one of the three fruitbody parts. Both functional guild and phylogenetic group had a significant effect on the bacterial community composition in fruitbodies (PERMANOVA $p \leq 0.001$). Figure from Publication IV.

Fungal genotype strongly shapes the intraspecific variation of fruitbody microbiome structure and function. Study **III** indicated that host genotype explained 25% and 37% of the variation among the taxonomic and functional gene composition of *Marasmius oreades* microbiome, respectively (Figure 4). To our knowledge, this was the first study investigating fungal genotype effect on the fruitbody-inhabiting microbiome. However, studies on the fungal-bacterial interactions at the fine genetic level have demonstrated the priming effect of certain bacterial strains on the gene expression of their fungal associates (Schrey et al., 2005; Deveau et al., 2007; Lastovetsky et al., 2020). For instance, in *Amanita muscaria* some *Streptomyces* strains are able to change the expression levels of fungal genes, most of which are involved in signalling pathways, metabolism, cell structure and the cell growth as well as in stress response (Schrey et al., 2005). The host genotype effect may result from the ability or inability of the host fungus to produce particular enzymes, vitamins, proteins or secondary metabolites and the involvement of some specific bacteria in the metbolism of these compounds. The variability of bacterial genotypes, especially in terms of

metabolic characteristics, mineral-weathering efficacy and pathogenicity have often been identified in fungus-related habitats (Rangel-Castro et al., 2002a; Uroz et al., 2007; Ye et al., 2018a). For instance, the fungal growth promoting auxofuran is produced by *Streptomyces* strain AcH 505 (Riedlinger et al., 2006) and the genome of *Streptomyces* strain 150FB encodes several genes involved in the production of extracellular enzymes and secondary metabolites which inhibit mycoparasitic fungi (Tarkka et al., 2015). Several mycotoxins, such as rhizoxin and rhizonin as well as several antibiotics are also produced by bacterial endosymbionts (Partida-Martinez et al., 2005, 2007; Efimenko et al., 2016; Pawlowska et al., 2018). In general, the specificity of fungal-bacterial interactions may be largely determined by the secondary (symbiosis-specific) metabolites, which mediate the interactions, providing signals and protecting each other from pathogens and competitors as well as affecting the gene expression of the other party (Scherlach and Hertweck, 2020). Similarly, some cellulolytic, chitinolytic, lignolytic or proteolytic bacterial strains may support their host fungal growth or functionality (Rangel-Castro et al., 2002a; Sbrana et al., 2002; de Boer et al., 2005; Zhang et al., 2014a; Saidi et al., 2016). Thus, the endofungal bacteria with certain genotypes, which are able to produce specific compounds and which can allow the absence or inactivity of some certain genes in the host genome, may be preferred in the selection of the fruitbody microbial community to perform particular functional tasks in their host fungus or in fungus-affected habitats.

One of the possible mechanisms for fungi harbouring specific bacteria may be the production and release of certain carbon compounds by the host as mentioned above. Namely, some carbohydrates, such as trehalose, mannitol, fructose and glycerol may induce the spread of specific, fungal growth stimulating or functionally complementing bacteria in fungus-affected habitats (Timonen et al., 1998; Duponnois and Kisa 2006; Uroz et al., 2007; Boersma et al., 2010; Pavić et al., 2013; Saidi et al., 2016). Warmink et al. (2009) have shown that the selection of mycosphere fungiphiles is strongly dependent on their capacities to use particular fungal exudates. Such specific interactions in which host fungi are able to release particular carbon compounds and the bacteria are adapted to effectively metabolize these compounds, are largerly driven by both bacterial and fungal genomes. For instance, the variability of the proportions of certain amino acids and the excretable carbon compounds has been demonstrated in two different *C. cibarius* strains (Rangel-Castro et al., 2002b). Similarly, the genetics of host fungi determines the production of certain antimicrobial compounds involved in the development of the bacterial communities in fungus-affected habitats (Alves et al., 2012; de Carvalho et al., 2015; Shirakawa et al., 2019). However, as the knowledge about the variability in the production of particular fungal exudates and the corresponding genomic changes is quite limited for most fungal genotypes, it is difficult to declare whether such variations are more pronounced among different host taxa or genotypes.

Study **III** revealed that host fungal genotypes may select for microbes with certain functions in fungal fruitbodies. Similarly, bacterial communities in the bulk soil and mycosphere have also been identified to have different functional

traits, suggesting that some specific traits have been selected or favoured in the mycosphere (Yu et al., 2020). Several functional traits, such as the metabolism of amino acids, vitamins and cofactors, were remarkably more common for microbial communities in *Russula griseocarnosa* mycosphere than for the microbial community in bulk soil (Yu et al., 2020). The bacterial communities associated with the hyphae of *Penicillium* species and inhabiting ectomycorrhizosphere have been identified to have increased phosphorus cycling potential and higher mineral weathering potential, respectively, compared to the bulk soil microbes (Uroz et l., 2007; Hao et al., 2020). The potential importance of secondary metabolites in fungal-bacterial interactions is supported by the fact that the majority of the bacterial genome, especially in the case of symbiotic bacteria, usually encodes a large number of diverse secondary metabolites (Tarkka et al., 2015; Scherlach and Hertweck, 2020). Studies **II** and **III** also indicated that most of functional genes of fruitbody-inhabiting bacteria in *Marasmius oreades* and *Inocybe terrigena* were related to the metabolism, more specifically to the metabolism of carbohydrates, amino acids, proteins, cofactors, vitamins and pigments. The high proportion of these functional genes may also reflect their higher response rate to the variability of host genotypes and to the involvement in genotype-genotype interactions.

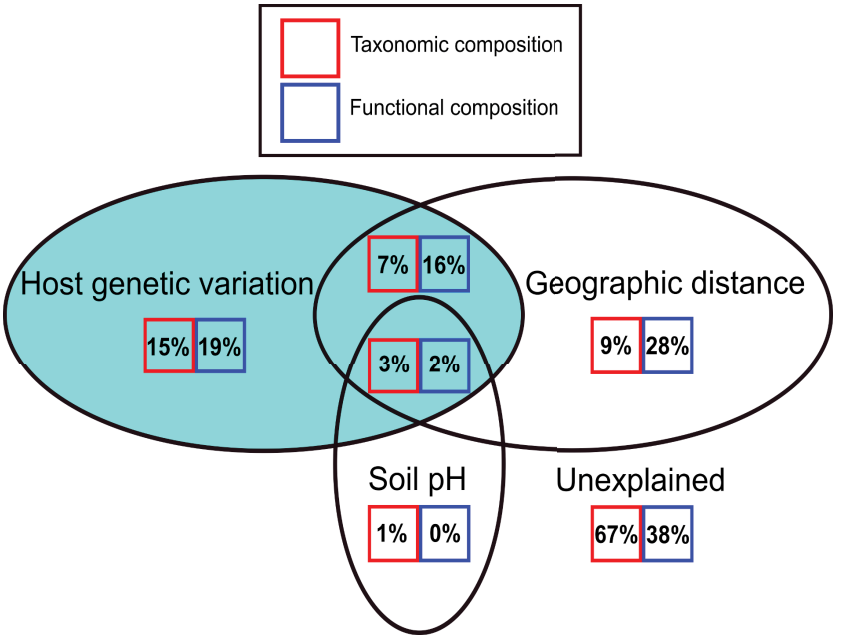

Figure 4. Venn diagram of variation partitioning analysis of the taxonomic composition and function of the microbiome in *Marasmius oreades* fruitbodies. The figure shows the relative importance of host genetic, geographic and pH and their shared importance in explaining of the variation in the microbiome structure and function. Note that the effect of each individual component is significant. Host genetic and geographic distance matrices were converted to PCNM eigenvectors which were forward prior to variation partitioning analysis. Figure from Publication III.

Fungal functional guilds affect the structure of bacterial communities in fruitbodies (IV). Fungal functional guilds had an additional effect, next to the host identity, explaining 5% of the variation of bacterial community structure across EcM and SAP fruitbodies (**IV;** Figure 3). This finding is in line with that from a recent study on EcM and SAP fruitbodies (Liu et al., 2018), but differs from the observations in the mycosphere showing no significant functional guild effect on bacterial community structure (Warmink et al., 2009). In general, bacterial communities have rarely been studied in SAP fungi. However, de Carvalho et al. (2015) identified that certain secondary metabolites affect the structure of bacterial communities in fruitbodies of SAP basidiomycetes. In addition, the variation of bacterial community structure between EcM and SAP fungi may result from the host-specific differences in the contents and profile of carbohydrates. For instance, certain species-specific fungal bacteria have been selected with respect to their ability to use particular fungal-released carbonaceous compounds in fungus-affected habitats (Timonen et al., 1998; Duponnois and Kisa 2006; Warmink et al., 2009; Boersma et al., 2010; Saidi et al., 2016). Furthermore, the profile of carbohydrate-active enzyme of soil bacteria and fungi differ significantly among habitats with different mycorrhizal types (Bahram et al., 2020). This supports the hypothesis that the specificity of the microbial community structure and function may be due to their ability to use particular carbon compounds and the variability of available carbon compounds in turn may be related to the functional guilds of dominant fungi in a given environment.

Most of the characteristics that vary among different functional guilds of fungi are physiological (Zanne et al., 2020), resulting in differences in fungal exudates and fruitbody chemistry. Whereas SAP fungi degrade various organic compounds, EcM fungi are involved in symbiosis, transferring several nutrients to the host plant in exchange for the photosynthates (Taylor et al., 2003; de Boer et al., 2005; Trocha et al., 2016; Zanne et al., 2020). Evidence suggests that the mineral-weathering efficacy of EcM fungi is probably improved by certain bacterial strains in the EcM symbioses and several potentially nitrogen-fixing bacteria have been isolated from mycorrhizal roots (Izumi et al., 2006; Timonen and Hurek 2006; Uroz et al., 2007). In addition, several ectomycorrhizosphere bacteria are able to promote mycorrhiza formation (Poole et al., 2001; Frey-Klett et al., 2007). Thus, such "mycorrhiza-helper bacteria" that are widely represented and perform specific functions in the ectomycorrhizosphere and ectomycorrhizas are more likely reaching the fruitbodies of EcM fungi than SAP fungi. Nevertheless, SAP fungi maintain a greater degree of nutritional homeostasis than EcM fungi (Kranabetter et al., 2019), offering more stable habitats which may also shape their bacterial communities. However, the traits defining various functional guilds of fungi, their development in different phylogenetic groups and their effect on fungal inhabiting microbes or vice versa remain to be explored (Zanne et al., 2020).

Chemical properties of fruitbodies that vary significantly among fungal taxa and functional guilds shape the structure of bacterial communities in fungal fruitbodies (IV). This finding suggests that chemical parameters may, at least to some extent, underlie the observed effects of the host identity and functional guild (IV). Study **IV** revealed that the fruitbody C:N ratio (ADONIS adj. R^2=0.107; p<0.001) in the main dataset and pH (ADONIS adj. R^2=0.108; p<0.001) in the validation dataset strongly affect the structure of microbial communities in fungal fruitbodies, more so than host identity and functional guilds (Table 1). Significant variation in chemical characteristics was also found between EcM and SAP fungi (MANOVA, approx. $F_{1,36}$=12.26; p<0.001) and among studied fungal species (MANOVA, approx. $F_{5,32}$=11.26; p<0.001; **IV**; Figure 5). Our study (**IV**) was the first to show that fruitbody chemistry significantly shapes the structure of bacterial communities in fungal fruitbodies, although the distinction of chemical composition among fungal functional guilds and taxonomic groups has been acknowledged previously (Taylor et al., 1997, 2003; Rudawska and Leski 2005; Trocha et al., 2016; Kranabetter et al., 2019; Zanne et al., 2020). Therefore, bacterial community turnover among host fungi may relate to fungal chemical compounds, content and profile, which vary among different host taxa (Taylor et al., 2003; Rudawska and Leski, 2005; Barros et al., 2008a; Kalač, 2009; Vieira et al., 2014). These data point to the importance of fungal chemistry in driving fungal associated microbiomes.

Our study showed that N% and P% were significantly higher and C% was significantly lower in SAP than EcM fungi (**IV**) which is in line with other studies (Vogt et al., 1981; Sanmee et al., 2003; Trocha et al., 2016; Kranabetter et al., 2019). The C:N ratio was significantly higher in EcM fungi than in SAP fungi, being especially high in *Cantharellus cibarius* fruitbodies (**IV**). By contrast, pH was significantly higher in SAP fungi than in EcM fungi, especially in *Infundibulicybe geotropa* (=*Clitocybe geotropa*) fruitbodies (**IV**). In EcM fungi that transfer particular nutrients from soil to plants and receive carbon compounds from their host plants, the content of nutrients, especially of C and N, are most likely related to their interaction with their host plants (Taylor et al., 2003; Trocha et al., 2016). EcM and SAP fungi also respond differently to changes in soil properties and some chemical properties of EcM fungi, such as C:P ratio and N:P ratio, are additionally affected by the corresponding properties of their host plants (Taylor et al., 2003; Kranabetter et al., 2019). Thus, differences in the concentration and stoichiometry of C and macronutrients as well as in fruitbody pH values are significantly affected by fungal functional guilds. These properties may lead to the development of specific habitats in fruitbodies of different fungal groups, each suitable for particular bacterial taxa.

Table 1. Effect of fruitbody parameters, host taxonomic identity and functional guild on bacterial community composition in fungal fruitbodies as revealed by *adonis* function of the Hellinger transformed main dataset (rarefied to ≤2000 reads) and the validation dataset (rarefied to ≤5000 reads). Samples containing less than 1000 reads have been removed. One sample represents one of the three fruitbody parts in the main dataset and the whole fruitbody in the validation dataset. Table from Publication IV.

	Df	SS	MS	F	R^2	R^2adjusted	p
Main dataset							
Fruitbody C/N	1	4.694	4.6939	18.6410	0.11546	0.1074	0.001 ***
Functional guild	1	2.371	2.3710	9.4161	0.05832	0.0498	0.001 ***
Species	4	7.148	1.7870	7.0967	0.17583	0.1450	0.001 ***
Residuals	105	26.440	0.2518		0.65038	–5.4680	
Total	111	40.652			1	1	
Validation dataset							
Fruitbody pH	1	1.6820	1.68201	7.3979	0.13259	0.1078	0.001 ***
Order	3	2.4296	0.80988	3.5620	0.19152	0.1180	0.001 ***
Functional guild	1	0.5243	0.52427	2.3059	0.04133	0.0139	0.014 *
Genus	11	3.5030	0.31846	1.4007	0.27613	–0.0424	0.009 **
Residuals	20	4.5473	0.22736		0.35844	–0.4435	
Total	36	12.6862			1	1	

* p≤0.05
*** p≤0.001

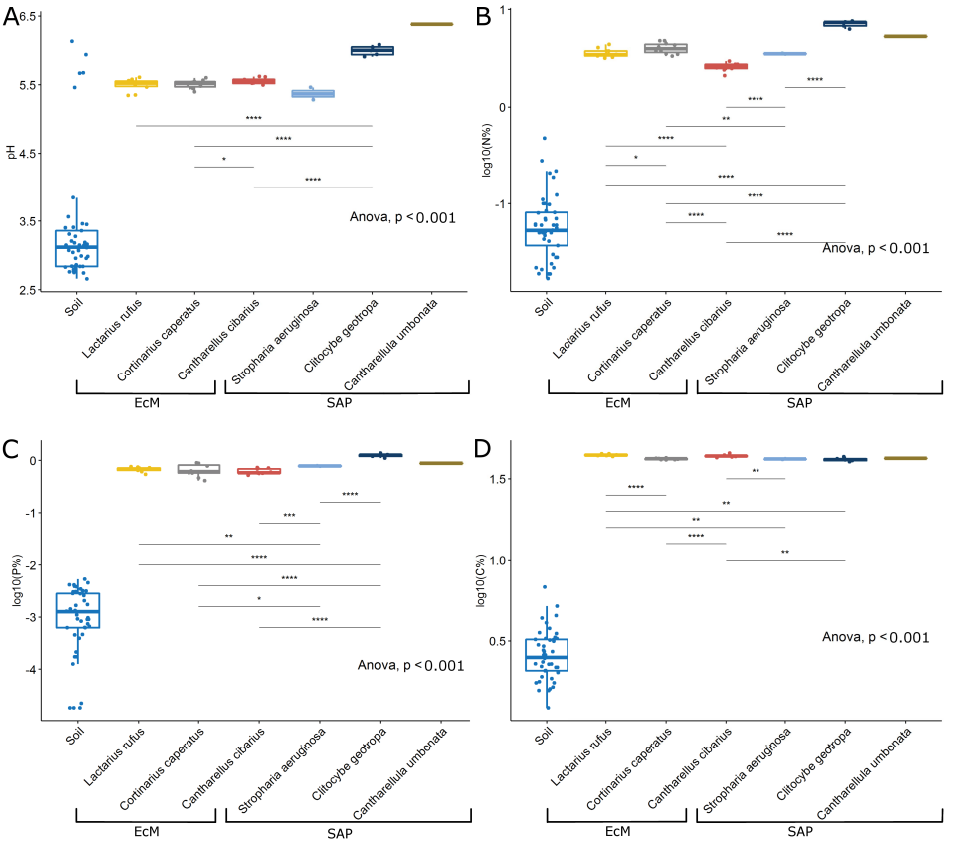

Figure 5. The relationship between the chemical properties of soil and fungal fruitbodies. Boxplots illustrating the variation of pH (**A**), N% (**B**), P% (**C**), and C% (**D**) in fruitbodies of six fungal species and in soil. P values are given for general differences of chemical properties among soil and fungi (ANOVA) and for pairwise significant differences among five fungal species (t-test; *p≤0.05; **p≤0.01; ***p≤0.001; **** p≤0.0001); differences between each fungal species and soil were all significant ($p < 0.001$). The N, P, and C values were log-transformed. Each symbol represents a fruitbody or a soil sample. Because of the small size of fruitbodies of *Cantharellula umbonata* these were pooled for chemical analyses and are presented as one replicate that was not included in these statistical analyses. Figure from Publication IV (slightly modified).

Fruitbody compartment has a negligible ($p > 0.05$) effect on the structure of fruitbody-inhabiting bacterial communities (IV). A few studies have reported that different fruitbody compartments can harbour distinct bacterial communities. For example in truffle ascocarps, the relative abundance of the major bacterial taxa was different in gleba and peridium (Antony-Babu et al., 2014; Splivallo et al., 2015a). In other studies, bacterial community structure varied significantly between the outside and inside parts of fruitbodies (Oh et al., 2018; Gohar et al., 2020) and between the stipe and the pileus of *Tricholoma matsutake* (Oh et al., 2018). These significant differences may result from contrasting structural and physico-chemical properties of different fruitbody compartments. For instance, peridium consists of sclerified and melanized cells with pores, thus creating a specific barrier between the external environment and the interior of fruitbody (Perlińska-Lenart et al., 2020) and the pileus and gills are protein and lipid rich, whereas the stipe has high content of carbohydrates and fibers (Alam et al., 2008). However, fruitbody surface, including gills, were not analyzed in our study.

3.1.2. Environmental factors

Soil properties have a significant effect on the structure of endofungal bacterial communities (I, III, IV). PERMANOVA revealed that soil pH (adj. R^2=0.062; $p<0.001$) and to a lesser degree $\delta^{15}N$ (adj. R^2=0.014; p=0.009) significantly explained the variation in bacterial community composition across fungal fruitbodies (**I**). Soil pH also explained 4% and 2% of the taxonomic and functional composition of the microbial communities, respectively, in the fruitbodies of *Marasmius oreades* (**III;** Figure 4). Soil pH (ADONIS adj. R^2=0.002; p=0.045) was also one of the few soil properties that next to NH_4N% (ADONIS adj. R^2=0.002; p=0.043) explained the microbial community composition in the fruitbodies of three SAP and three EcM species (**IV**). Soil pH and nitrogen content, and to a lesser extent organic carbon, available phosphorus and several available microelements have also been identified as important determinants of microbial community structure in hyphae, ectomycorrhizae, mycosphere and ectomycorrhizosphere (Nazir et al., 2010b; Li et al., 2017; Liu et al., 2017; Hao et al., 2020; Yu et al., 2020). Similarly, Li et al. (2016a) found significant correlations between the relative abundance of certain bacterial taxa associated with matsutake fruitbodies and some soil properties, such as the contents of N, P, K and Zn. Soil origin and properties have been identified as the main determinants of bacterial communities associated with hyphae of two *Penicillium* species, whereas the fungal identity effect was negligible (Hao et al., 2020). The strong soil effect observed in Hao et al. (2020) may be related to the direct and continuous exposure of the hyphae-associated bacterial community with the bulk soil, unlike in the fruitbody-associated bacterial communities.

The influence of soil properties on the fruitbody microbiome may be mediated by fruitbody characteristics. Some chemical properties, such as P content and N:P ratio, correlate between the fruitbodies of EcM fungi and the surrounding soil (**IV**). Kranabetter et al. (2019) also found that the N content of fruitbodies of SAP fungi and P content of fruitbodies of EcM fungi were significantly correlated to these values in soil. Thus, it can be assumed that the effect of the site on the fruitbody chemistry may be due to the variation of soil properties between locations and the variable responses of the fungal taxa to these changes (Taylor et al., 2003). For instance, the soil mineral and organic N sources and their availability may affect $\delta^{15}N$ values in fruitbodies, especially in EcM fungi (Taylor et al., 2003). In addition, the content of microelements (Fe, Zn, Mn) in morels (*Morchella* spp., Ascomycota) have been found to be correlated with the corresponding values in their growth substrate (Liu et al., 2017). The exudation and storage of various carbohydrates as well as the production of different secondary metabolites may also vary depending on the fungus growth conditions, such as the temperature, the pH, the nutrition source and the CO_2 level in growth media (Rangel-Catro et al., 2002b; Tibbett et al., 2002; Saidi et al., 2016). Thereby, the surrounding environment may indirectly affect the structure of fungus-associated bacterial communities through the availability of carbon compounds or through shaping the chemical properties in the fruitbody.

In addition, fungi may affect soil properties in the mycosphere, thereby selecting specific fungal bacteria. For instance, the mycelium of fungi may raise soil pH or release some specific carbohydrates and antibiotics and increase the availability of the organic compounds, carbon, nitrogen, phosphorus, calcium and magnesium in soil (Danell et al., 1993; Boersma et al., 2009, 2010; Warmink et al., 2009; Nazir et al., 2010a,b; Li et al., 2017; Shirakawa et al., 2019). Such changes in the soil chemistry may be particularly significant after the decomposition of fruitbodies followed by incorporation of the dead matter into the humus layer (Ingelög and Nohrstedt, 1993).

Habitat type and soil type have negligible effect on fruitbody microbiome structure (I). After accounting for soil properties, soil type or habitat type had no significant impact on bacterial community structure ($p > 0.05$). A previous study that did not account for soil properties reported soil type as the main determinant of cultivable bacterial populations in the ectomycorrhizosphere of *Suillus bovinus* and *Paxillus involutus* (Timonen et al., 1998). In addition, it has been found that the dominant vegetation and plant nutrient-acquisition strategies may affect the soil fungal and bacterial community structure through altering the soil properties (Shen et al., 2013; Pii et al., 2016; Bahram et al., 2020) that confirms the close link between the soil properties and the vegetation type (habitat type). The effect of soil properties, especially soil pH, has been determined as the most important factor shaping the soil bacterial community structure, by far superseding the effect of other habitat properties (Bahram et al., 2018, 2020). Similarly, it was found that soil pH affects the structure of bacterial communities in fruitbodies, whereas the effect of soil and habitat type remained negligible. Therefore, it can

be assumed that the effect of soil type and habitat type as independent factors remain negligible after accounting for soil characteristics or other environmental characteristics.

3.1.3. Site effect

Sampling site has much weaker effect on the microbial community structure than host identity or genotype (I, III). However, our results still showed that the geographic distance explained 9% of the taxonomic composition and even 28% of the functional composition of the microbiome in *Marasmius oreades* fruitbodies (**III**; Figure 4). The strong site effect on the microbiome functionality suggests that the function of the microbiome may react on some geographically variable properties and/or the same microbial taxa could perform different functions depending on the specific local conditions. Similarly, various factors, such as spatial location have been shown to affect the microbiome of *Tuber aestivum,* depending on the particular orchard (Splivallo et al., 2019). The studies involving only one fungal species, as in the two aforementioned studies (**III**; Splivallo et al., 2019), allowed the sampling site effect to be estimated without the dominating host identity effect.

The site effect was not significant in study **I** that revealed the strong effect of host identity on microbial communities in eight basidiomycete genera sampled from three areas. By contrast, geographic distance effect has been previously identified as an important determinant of bacterial community structure in fruitbodies of different species of Pezizales (Benucci and Bonito 2016). In that study, unlike in our study (**I**), the fruitbodies were collected from very distant areas (different continents) and soil properties were not included into the analyses, presumably reinforcing the sampling site effect. Namely, since variation in soil properties is likely to be one of the most important differences between sampling sites, their inclusion may make the effect of sampling site negligible (**I**). The topsoil microbiome is also mainly shaped by the environmental variables rather than geographic distance (Fierer and Jackson, 2006; Bahram et al., 2018). Thus, given that the source communities of endofungal bacteria largerly originate from soil (**I**; **IV**; Antony-Babu et al., 2014; Benucci et al., 2019) and considering the strong effect of soil conditions on bacterial community structure (Fierer et al., 2007; Eilers et al., 2010; Rousk et al., 2010; Bergmann et al., 2011; Lakshmanan et al., 2014; Fierer, 2017), the observed site effect can be explained by the spatial heterogeneity of soil properties and bacterial community structure.

3.1.4. Soil microbiome effect

The soil microbiome – the main source of fruitbody-inhabiting bacteria (I, IV) has a significant effect on the structure of fruitbody bacterial community (IV). Studies **I** and **IV** showed that 41% and 68.5% of bacterial OTUs inhabiting fruitbodies were also present in the surrounding soil, respectively. Comparing the results of these studies reveals that the level of overlap between soil and fruitbody microbiomes depends on whether soil samples were collected beneath each fruitbody (**IV**) or from random spots of the same area (**I**). The origin of endofungal bacteria from the surrounding soil has also been shown in several studies on ascomycetes (Antony-Babu et al., 2014; Benucci et al., 2019; Monaco et al., 2020). However, to the best of our knowledge, our study (**I**) is the first showing the overlap of a significant part of the bacterial community in basidiomycete fruitbodies and their surrounding soil. Later study by Liu et al. (2018) showed that more than half of the bacterial OTUs in basidiomycete fruitbodies also occurred in their hyphosphere soil. In addition, the proportion of shared OTUs was found to be higher between SAP fungi and the corresponding soil samples (65.6%) compared to EcM fungi and their soil samples (50.4%; **IV**), as observed for six other fungal species in China (Liu et al. 2018). The greater overlap of bacterial communities between SAP fungi and the corresponding soil is probably due to the closer association of SAP fungi with soil and decaying material, while EcM fungi are tightly associated with their host plants. This is supported by the overlap of several of the most abundant bacterial taxa between SAP fruitbodies and the decaying material, but also between EcM fruitbodies and the ectomycorrhizosphere (Timonen and Hurek, 2006; Uroz et al., 2012; Bulgarelli et al., 2013; Hervé et al., 2014; Hoppe et al., 2015). We also found significantly lower bacterial diversity in EcM than SAP fruitbodies (**IV**). EcM fungi could have a negative effect on bacterial phylogenetic diversity, suppressing, for example, Gram-positive bacteria (Shirakawa et al., 2019), which may explain the lower bacterial richness in EcM-dominated ecosystems (Bahram et al., 2020) and the lower bacterial diversity in EcM fruitbodies and their soils than in SAP-affected habitats (**IV**).

The proportions of certain bacterial taxa are likely to be influenced by similar parameters in both fungi and soil (**IV**; Fierer et al., 2007; Eilers et al., 2010; Rousk et al., 2010; Bergmann et al., 2011). This, in turn, may affect the structure of bacterial communities across fruitbodies and their surrounding soil. For instance, several studies of the soil microbiome, have identified soil pH as the main determinant of bacterial community structure (Fierer and Jackson, 2006; Lauber et al., 2009; Rousk et al., 2010; Shen et al., 2013; Bahram et al., 2018), although the strong effect of soil C:N ratio on the bacterial community structure has also been observed in soil (Shen et al., 2013; Zhang et al., 2014b). Similarly, fruitbody C:N ratio and fruitbody pH were the most important properties shaping the bacterial community structure in fungal fruitbodies (**IV**). Thus, the soil properties and their contrast with the fungal chemistry may affect the overlap of endofungal and soil bacterial communities.

3.2. Dominant bacterial taxa in fruitbodies and their host specificity

Most of the bacteria that inhabit the interior of fungal fruitbodies belong to the phyla Proteobacteria (classes Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria) and Bacteroidetes (class Sphingobacteriia; Figure 6). Dominant genera include *Pseudomonas*, *Rhizobium* and *Pedobacter* as well as members of the *Burkholderia-Caballeronia-Paraburkholderia* complex (I, II, III, IV). Proteobacteria, in particular Beta- and Gammaproteobacteria, appear to involve general bacterial "fungiphiles" that are commonly detected from fungus-affected habitats (e.g. Dahm et al., 2005; Warmink et al., 2009; Uroz et al., 2012; Kumari et al., 2013; Halsey et al., 2016; Li et al., 2016a). Various members of Proteobacteria were identified in different EcM (**I, II, IV)** and SAP fungi (**III**, **IV)**. By contrast, Sphingobacteriia (Bacteroidetes) and Alphaproteobacteria, especially the order Rhizobiales, were found as rather as specific to *Cantharellus cibarius* (**I**, **IV**). Rinta-Kanto et al. (2018) also found that the orders Sphingobacteriales and Rhizobiales were more highly represented in *C. cibarius* fruitbodies than in other fungal species. Interestingly, members of the order Rhizobiales and especially one of its families – Bradyrhizobiaceae, have been previously identified as dominant bacterial groups in various hypogeous ascomycetes, such as *Tuber* spp. and *Elaphomyces granulatus* (Barbieri et al., 2005, 2007; Quandt et al., 2015; Benucci and Bonito 2016; Ye et al., 2018b; Splivallo et al., 2019; Monaco et al., 2020; Perlińska-Lenart et al., 2020). Nevertheless, the bacterial communities in different fungus-affected habitats are generally dominated by the same taxa, which shows that their variability is mainly due to the differences in the relative abundances of these bacterial taxa and not their presence/absence (**I**, **IV**). Bacteroidetes and Alpha-, Beta- and Gammaproteobacteria are common bacterial taxa in various fungus-affected habitats, being often identified as the most abundant bacterial groups in the mycosphere, ectomycorrhizosphere and mycorrhizae (e.g. Frey-Klett et al., 2007; Uroz et al., 2012; Halsey et al., 2016; Shirakawa et al., 2019) as well as in fruitbodies (e.g. Dahm et al., 2005; Kumari et al., 2013; Antony-Babu et al., 2014; Quandt et al., 2015; Li et al.,2016a,b; Rinta-Kanto et al., 2018).

In general, members of Proteobacteria and Bacteroidetes prefer high carbon content and pH in soil (Fierer et al., 2007; Eilers et al., 2010; Rousk et al., 2010). In addition, the classes Beta- and Gammaproteobacteria as well as particular taxa of Alphaproteobacteria and Bacteroidetes are known to associate with copiotrophic environments with high substrate levels (Ho et al., 2017). Thus, the high relative abundance of Proteobacteria and Bacteroidetes in fungal fruitbodies may be stimulated by their higher nutritional status compared to the surrounding environment. For example, high concentrations of variable carbon compounds are characteristic of fungus-associated habitats, including fruitbodies (**IV**; Sanmee et al., 2003; Fierer et al., 2007; Warmink et al., 2009; Hodkinson et al., 2012; Hao et al., 2020; Yu et al., 2020). In addition, fungal mycelium may increase soil pH in the mycosphere (**III**; Danell et al., 1993; Nazir et al., 2010a) as observed based on higher pH of fruitbodies compared to their surrounding soil (**IV**).

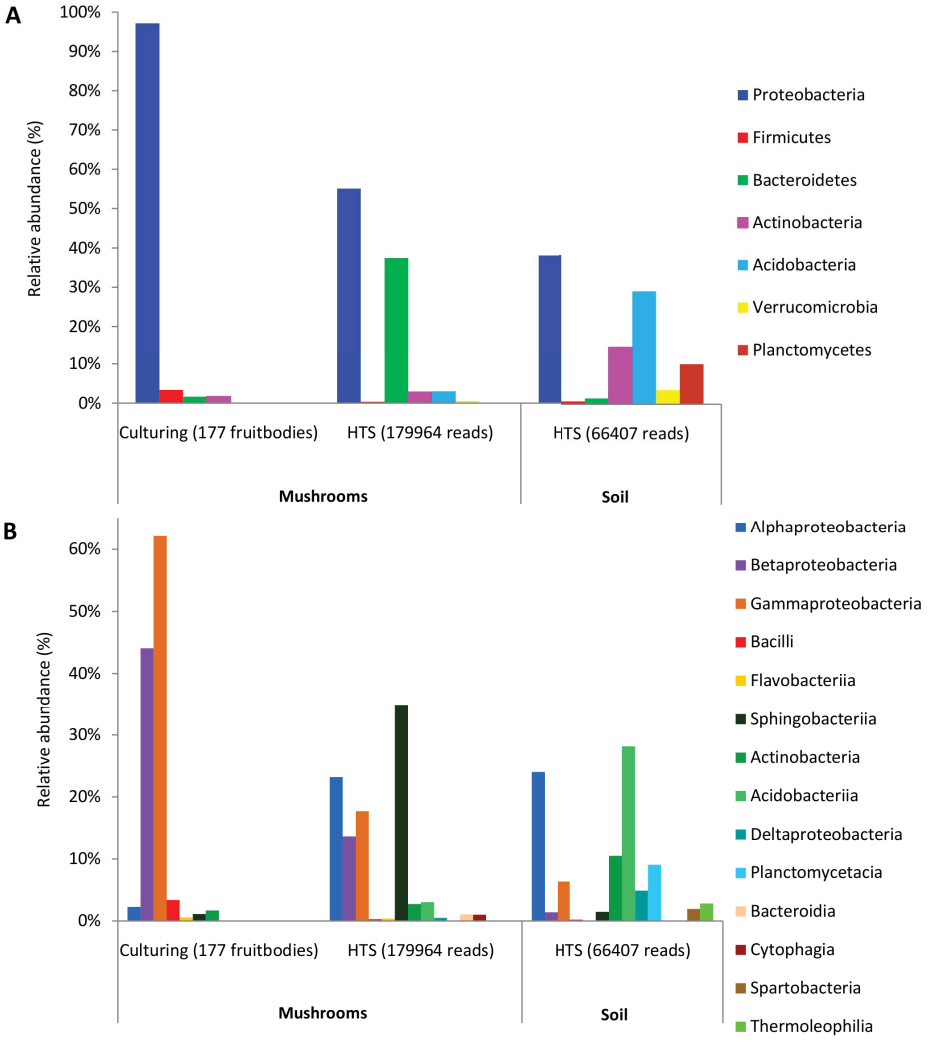

Figure 6. Relative abundance of dominant bacterial phyla (**A**) and classes (**B**) in fruitbodies based on culturing and high-throughput sequencing (HTS), and in soil based on HTS. The ratios show the share of fruitbodies inhabited by the bacterial taxon or of Illumina read numbers from that of the total (in parentheses) for culture isolates and HTS data, respectively. Figure from Publication I.

On the contrary, the phyla Actinobacteria, Verrucomicrobia, Planctomycetes, Chlamydiae and especially Acidobacteria were more abundant in the bulk soil than in fruitbodies or in fungus-affected habitats (**I**; **IV**; Uroz et al., 2012; Antony-Babu et al., 2014; Halsey et al., 2016; Liu et al., 2018; Monaco et al., 2020; Figure 6). Most of these phyla contain predominantly oligotophic bacteria that are able to inhabit environments containing relatively low nutrients (Fierer et al., 2007; Eilers et al., 2010; Bergmann et al., 2011; Ho et al., 2017) which probably also explains the higher relative abundance of these bacterial taxa in soil

compared to the fruitbodies. Although Acidobacteria, Actinobacteria and Verrucomicrobia are able to degrade several fungal and bacterial polysaccharides, their low relative abundance in carbon-rich conditions have been often related to their slow growth rate (Lopez-Mondéjar et al., 2018).

3.3. Methodological considerations

Comparing results obtained using HTS and culturing revealed that certain bacterial groups, such as Alphaproteobacteria and Sphingobacteriia, were underrepresented, whereas members of Firmicutes prevailed among the cultured isolates (I; Figure 6). The classes Gammaproteobacteria, Betaproteobacteria and Actinobacteria were detectable regardless of the method used, while members of the class Alphaproteobacteria and the phylum Firmicutes were found either by HTS or culturing, respectively (**I**; Barbieri et al., 2005, 2007; Splivallo et al., 2015a; Perlińska-Lenart et al., 2020). Previous studies support these findings. Namely, Burkholderiaceae (Betaproteobacteria) and Pseudomonadaceae (Gammaproteobacteria) have been identified through the use of HTS or some other culture-independent approaches (Uroz et al., 2012; de Carvalho et al., 2015; Quandt et al., 2015; Halsey et al., 2016; Li et al., 2016a; Li et al., 2017; Oh et al., 2018; Splivallo et al., 2019), as frequently as in studies applying only culturing of bacteria from fungus-affected habitats (Poole et al., 2001; Bending et al., 2002; Sbrana et al., 2002; Dahm et al., 2005; Timonen and Hurek 2006; Uroz et al., 2007; Kumari et al., 2013). However, while the members of Rhizobiales including Bradyrhizobiaceae are detectable by the culture-independent methods (Uroz et al., 2012; de Carvalho et al., 2015; Benucci and Bonito 2016; Halsey et al., 2016; Li et al., 2017; Splivallo et al., 2019; Perlińska-Lenart et al., 2020), several Gram-positive genera, such as *Bacillus* and *Paenibacillus,* both belonging to Firmicutes, are more widely represented among the cultured bacterial isolates (e.g. Poole et al., 2001; Barbieri et al., 2005; Kumari et al., 2013; Zagriadskaia et al., 2013; Oh et al., 2018; Perlińska-Lenart et al., 2020; Suarez et al., 2020). In addition, the genera *Bacillus* and *Paenibacillus* were not found in lichens using HTS, although they appeared to be common in cultured bacterial isolates from the same hosts (Grube et al., 2009; Hodkinson et al., 2012).

The limitations of HTS, such as short read lengths produced by most current platforms and the sequencing errors, especially in GC-rich regions and long homopolymer stretches (Reuter et al., 2015), or the primers used in particular study may to some extent explain the variation in the proportion of bacterial groups detected by HTS and culture-based methods. For instance, the genera *Bacillus* and *Paenibacillus* that are strongly underrepresented using HTS, are easily detectable by some other culture-independent methods, such as cloning of the 16S rRNA genes or fluorescence *in situ* hybridization (Splivallo et al., 2015a; Suarez et al., 2020). However, these genera were detected using different primers for HTS (Illumina MiSeq: Rinta-Kanto et al., 2018; Ma et al., 2019; Illumina HiSeq: Sun et al., 2020). It is likely that the phylum Firmicutes is indeed

underrepresented in most of the fungus-affected habitats and the choice of identification method does not play a major role for their detection. For instance, the antibacterial activity of EcM fungi exerts selective pressure on Gram-positive bacteria, including thus also most of the members among the phylum Firmicutes, in the ectomycorrhizosphere (Shirakawa et al., 2019). In general, I believe that the culture-dependent and HTS methods complement each other, resulting in a better overview of the bacterial community in fruitbodies. Although a very small proportion of bacteria can be cultivated, isolates are needed for the phenotypic characterization of bacteria, to test their antifungal and other biochemical activities and to demonstrate the potential functionality of bacteria in co-cultures with fungi (Poole et al., 2001; Sbrana et al., 2002; Duponnois and Kisa 2006; Uroz et al., 2007; Efimenko et al., 2016; Oh et al., 2018).

4. CONCLUSIONS

The following conclusions can be drawn from my thesis:

- The structure of bacterial communities in fungal fruitbodies depend mostly on host-derived factors and to a lesser extent on environmental factors and sampling site.
- Hosts phylogenetic affiliation, reflected in the taxonomy of fungi, is the most important factor shaping the structure of bacterial communities in fungal fruitbodies. These results indicate the need to study possible co-evolution between fungi and certain bacteria that has been documented in case of many other eukaryotes and their associated microbes.
- Similarly to plants and animals, the host genotype has a strong effect on the intraspecific variation of microbiome structure and potential function in fungal fruitbodies.
- Fungal functional guild has an additional impact on the structure of bacterial communities across mushroom-forming fungi. Differences in the relative abundance of bacteria with contrasting functional roles in ectomycorrhizal and saprotrophic fungi suggest that bacteria can participate in the decomposition or nutrient release processes, respectively, thereby supporting the functionality of these fungi.
- The chemical properties of fruitbodies, in particular fruitbody pH and C:N ratio, have remarkably strong effect on the bacterial community structure, explaining to some extent the observed effects of fungal identity and functional guild. The same chemical properties, especially pH, strongly shape the microbial community structure in other environments, such as soil and decaying wood.
- Among environmental factors, several soil properties, especially pH, significantly affect the structure of bacterial communities in fungal fruitbodies. In addition, soil forms the main source in the assembly of endofungal bacterial communities, as indicated by the high overlap of bacterial OTUs between soil and fruitbodies.
- The selection of bacteria from the surrounding soil is likely quite similar in case of fungi and plants. Whereas the original source community is affected by the soil parameters, the main selection step is mediated by the host exudates, determined by the host identity.
- Fungal fruitbodies, representing nutrient-rich islands arising from the soil, are inhabited mainly by phyla Proteobacteria and Bacteroidetes and classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Sphingobacteriia, all known to contain mainly copiotrophic species. On the contrary, bacterial phyla Actinobacteria, Verrucomicrobia, Planctomycetes, Chlamydiae and Acidobacteria, containing mainly oligotrophs, were more abundant in soil than in fruitbodies.

- Most of the functional genes of fruitbody-inhabiting bacteria contribute to the metabolism of carbohydrates, amino acids, proteins, cofactors, vitamins and pigments. However, further studies are needed to understand the functions of the fruitbody-inhabiting microbiome. Such knowledge could be applied for improving mushroom cultivation, in line with recent advances in plant microbiome engineering.

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SUMMARY IN ESTONIAN

Seente viljakehadega seotud bakterikooslused

Eukarüootsed organismid on elupaigaks väga mitmekesise struktuuri ja funktsiooniga mikroobikooslustele, kusjuures mikroobid (bakterid, arhed, seened, viirused ja protistid) võivad asustada nii nende organismide pinda kui ka elada peremehe rakkudes või kudedes. Interaktsioonid mikroobide ja nende eukarüootsete peremeeste vahel võivad olla nii positiivsed kui negatiivsed. Lisaks peremeest ohustavatele patogeenidele on järjest enam tuvastatud interaktsioone, kus mikroobid osalevad peremehele oluliste funktsioonide täitmises, soodustades seeläbi peremehe kasvu, arengut ja kohasust (Berendsen *et al.*, 2012; Goh *et al.*, 2013; Kohl *et al.*, 2014; Sharon *et al.*, 2014; Compant *et al.*, 2019; Dominguez-Bello *et al.*, 2019). Seesuguste interaktsioonide tuvastamine aitab paremini mõista ökoloogilisi seoseid erinevate organismirühmade vahel ja nende rolli kogu ökosüsteemis laiemalt.

Peremehe ja temaga seotud mikroobikoosluse kujunemise ja seoste iseloomu mõistmiseks on oluline selgitada välja tegurid, mis mikroobikoosluse arengut enim mõjutavad. Eukarüootsetel organismidel võivad need tegurid lähtuda nii peremehest kui ka keskkonnast (Hacquard *et al.*, 2015; Adair ja Douglas 2017; Pratte *et al.*, 2018; Knowles *et al.*, 2019; Dastogeer *et al.*, 2020). Peremehest lähtuvatest faktoritest ilmneb sageli peremehe taksonoomilise kuuluvuse mõju, mis omakorda põhineb organismide sugulussuhetel, viidates seega eukarüootide ja neile iseloomulike mikroobikoosluste koevolutsioonile (e.g. Nelson *et al.*, 2013; Bouffaud *et al.*, 2014; Amato *et al.*, 2016; Nishida ja Ochman 2018; Tian *et al.*, 2018; Knowles *et al.*, 2019; Alt *et al.*, 2020). Peremehega seotud faktorite seas on liigisisesel tasandil oluline mõju ka peremehe genotüübil (Hacquard *et al.*, 2015; Spor *et al.*, 2011; Smith *et al.*, 2015; Wagner *et al.*, 2016; Qian *et al.*, 2018). On ka leitud, et lisaks peremehe identiteedile ja genotüübile mõjutab peremeestaime ja -looma toitainete omastamise viis ja toitumistüüp peremehega seonduvat mikroobikooslust (Nelson *et al.*, 2013; Hacquard *et al.*, 2015; Pii *et al.*, 2016; Phillips *et al.*, 2017; Nishida ja Ochman, 2018). Mikroobikooslust võivad kujundada ka peremehe poolt mõjutatud elupaikade spetsiifilised füüsikalis-keemilised tingimused, näiteks erinevate süsinikuühendite ja teiste metaboliitide ning mikro- ja makorelementide olemasolu ja hulk (Ley *et al.*, 2006; Hacquard *et al.*, 2015; Dastogeer *et al.*, 2020). Samad näitajad varieeruvad sageli ka peremehe erinevate genotüüpide, taksonite ning funktsionaalsete rühmade hulgas (Kashyap *et al.*, 2013; Bouffaud *et al.*, 2014; Kembel *et al.*, 2014; Pii *et al.*, 2016).

Keskkonnategurite mõju peremehe mikroobikooslusele sõltub suuresti sellest, milline on peremehe elupaik ning kust pärinevad peremehega seotud mikroobid. Mitmed eukarüootidega tehtud uuringud on näidanud, et mikroobikooslus sõltub vahetust peremeest ümbritsevast keskkonnast (Hacquard *et al.*, 2015; Song *et al.*, 2013; Amato *et al.*, 2016; Adair ja Douglas, 2017; Compant *et al.*, 2019). Näiteks on peremeestaimega seotud mikroobid valdavalt pärit mullast (Berendsen *et al.*, 2012; Zarraonaindia *et al.*, 2015) ja kaladega seotud mikroobid veest (Pratte

et al., 2018; Sylvain *et al.*, 2020), samas inimene omastab mikroobid suuresti toiduga, aga ka ümbritsevast keskkonnast (Cotillard *et al.*, 2013; Song *et al.*, 2013; Hacquard *et al.*, 2015). Tingimused, mis peremehe elupaikades varieeruvad, võivad mõjutada temaga seotud mikroobikooslust nii lähtekooslust kujundades kui ka peremehe iseloomulikke omadusi mõjutades.

Seened on üks liigirikkamaid rühmi eukrüootide hulgas ja täidavad olulist ökoloogilist rolli, olles peamised lagundajad maismaa ökosüsteemides ning interakteerudes mitmete teiste organismidega (Blackwell, 2011; Tedersoo *et al.*, 2014). Võrreldes teiste eukarüootidega on aga seentega seotud mikroobikooslusi ja nende funktsioone vähe uuritud. Seni on peamist tähelepanu pööratud seenefarmides suurt majanduslikku kahju põhjustavatele patogeensetele bakteritele ja nende biokontrolli võimalustele (Henry *et al.*, 1991; Aslani *et al.*, 2018; Martins *et al.*, 2020). Samas on siiski teada, et seente ja bakterite vahel esineb ka positiivseid interaktsioone ja bakterid võivad täita mitmeid seenele olulisi funktsioone. Leitud on, et bakterite ja seente koostöös toimuvad erinevad biokeemilised protsessid mullas, seejuures mitmete komplekssete ühendite lagundamine (de Boer *et al.*, 2005; de Menezes *et al.*, 2017; López-Mondéjar *et al.*, 2018; Zhang *et al.*, 2014a). Lisaks võivad seentega seotud bakterid pärssida seenele ohtlikke patogeene ja vähendada keskkonnas leiduvate toksiliste ühendite mõju (Tsukamoto *et al.*, 2002; Tarkka *et al.*, 2015; Aslani *et al.*, 2018; Oh *et al.*, 2018; Scherlach ja Hertweck, 2020). Mitmed bakterirühmad on tuntud ka niinimetatud mükoriisa-abistaja bakteritena, kes soodustavad mükoriisa teket ja vähendavad kahjulike keskkonnamõjude toimet (Deveau *et al.*, 2007; Frey-Klett *et al.*, 2007; Pavić *et al.*, 2013). Seenekasvatuses on eriti oluline bakterite funktsioon soodustada viljakehade teket ja arengut (Li *et al.*, 2016b; Oh *et al.*, 2018; Kertesz ja Thai, 2018; Carrasco ja Preston, 2019; Mediavilla *et al.*, 2019; Suarez *et al.*, 2020).

Seened ja bakterid puutuvad tihedalt kokku paljudes elupaikades. Näiteks mükosfääris ja mükorisosfääris, kus seeneniidistik mullakeskkonda mõjutab, kujuneb sageli elupaigale iseloomulik bakterikooslus. Mitmetes töödes on kirjeldatud iseloomulikke bakterikooslusi erinevate seeneliikide mükosfääris ja mükorisosfääris (e.g. Timonen ja Hurek 2006; Boersma *et al.*, 2009, 2010; Halsey *et al.*, 2016; Oh *et al.*, 2016; Li *et al.*, 2017; Shirakawa *et al.*, 2019; Yu *et al.*, 2020). Seente, eriti kandseente, viljakehadega seotud bakterikooslusi on märksa vähem uuritud. Nii on bakterikooslusi kirjeldatud vaid üksikute kandseeneliikide viljakehades, valdavalt kultuuri viimise meetodit kasutades (e.g. Danell *et al.*, 1993; Dahm *et al.* 2005; Kumari *et al.*, 2013; Zagriadskaia *et al.*, 2013; Li *et al.*, 2016b; Oh *et al.*, 2018). Kultuuris kasvatamine annab väärtuslikku lisateavet bakterite omaduste ja interaktsioonide kohta, samas aga jääb suur osa bakterikoosluse mitmekesisusest sel viisil siiski tuvastamata, kuna enamikke baktereid ei õnnestu kultuuris kasvatada. Järjest enam koguvad populaarsust järgmise põlvkonna sekveneerimismeetodid, mis annavad võimaluse saada seene viljakehaga seotud mikroobikoosluse struktuurist ja funktsioonist täielikum ülevaade.

Seente viljakehadega seotud bakterikoosluste struktuuri ja funktsiooni kõrval on väga vähe teada teguritest, mis seentega seotud bakterikooslusi kujundavad. Võib oletada, et sarnaselt teiste eukarüootidega mängivad ka seente bakteri-

koosluste kujunemisel olulist rolli nii peremehest kui ka keskkonnast sõltuvad faktorid. Sarnaselt taimedega pärineb tõenäoliselt suur osa seenega seotud bakteritest ümbritsevast mullast, nagu seda on näidatud kottseente puhul (Antony-Babu *et al.*, 2014; Splivallo *et al.*, 2019; Monaco *et al.*, 2020). Sellest tulenevalt on seenega seotud bakterikooslus vähemalt kaudselt mõjutatud erinevate mullafaktorite poolt, mis kujundavad lähtekoosluse struktuuri ja funktsiooni: eelkõige mulla pH, niiskusetase, lämmastiku, fosfori ja teatud süsinikuühendite kättesaadavus ning erinevad juureeritised (Rousk *et al.*, 2010; Lakshmanan *et al.*, 2014; Fierer *et al.*, 2017). Peremeesseene bakterite valik ümbritsevast mullakeskkonnast võib toimuda sarnaselt risosfääris toimuva bakterite filtreerimisega, kus taim on kujundanud spetsiifilised tingimused, mis soodustavad teatud bakterirühmade levikut taime lähiümbruses (Bulgarelli *et al.*, 2013; Hacquard *et al.*, 2015; Pii *et al.*, 2016). Nimelt on leitud, et mitmed keskkonnatingimused, nagu pH, süsinikuühendite hulk ja koosseis, mikro- ja makroelementide sisaldus ja ka antimikroobsete ühendite sisaldus, erinevad mükosfääris/hüfosfääris oluliselt võrreldes ümbritseva mullaga (e.g. Danell *et al.*, 1993; Boersma *et al.*, 2009, 2010; Nazir *et al.*, 2010a,b; Bonanomi *et al.*, 2012; Xing *et al.*, 2018; Shirakawa *et al.*, 2019), pakkudes seeläbi spetsiifilist elupaika teatud bakterikooslusele.

Seente viljakehi, eriti sisemust, asustavaid baktereid mõjutavad tõenäoliselt kõige vahetumalt siiski viljakeha omadused. Lähtudes teiste eukarüootidega tehtud uuringuist, võivad seene bakterikooslust oluliselt mõjutada peremehe genotüüp ja taksonoomilisse ning funktsionaalsesse rühma kuulumine, kus viimane on võrreldav teiste organismide puhul käsitletud toitumisstrateegiaga. Omadused, mis erinevate genotüüpide, seenetaksonite ja funktsionaalsete rühmade vahel varieeruvad ja võivad seega viljakeha asustavat bakterikooslust vahetult kujundada, on näiteks viljakeha keemiline koostis, sealhulgas süsinikuühendite, mikro- ja makroelementide, vitamiinide, valkude ja mitmete sekundaarmetaboliitide sisaldus (Taylor *et al.*, 1997, 2003; Sanmee *et al.*, 2003; Rudawska ja Leski 2005; Alves *et al.*, 2012; Vieira *et al.*, 2014; Trocha *et al.*, 2016; Kranabetter *et al.*, 2019; Scherlach ja Hertweck, 2020). Kasu, mida bakter omakorda seenelt saab, ja põhjus, miks teatud bakterid seenest mõjutatud elupaiku eelistavad, ongi tõenäoliselt suuresti seentes sisalduvad või seene eritatavad ühendid, näiteks energiarikkad süsinikuühendid, nagu trehhaloos, raffinoos ja mannitool (Duponnois ja Kisa, 2006; Riedlinger *et al.*, 2006; Frey-Klett *et al.*, 2007; Deveau *et al.*, 2010; Pavić *et al.*, 2013).

Käesolevas doktoritöös uuriti viljakehi asustavate bakterikoosluste struktuuri ja funktsioone erinevates kandseentes. Töö eesmärgiks oli selgitada peremehe ja keskkonnafaktorite suhteline tähtsus seenega seotud bakterikoosluse kujunemisel. Hüpotees oli, et peremehega seotud faktorid mõjutavad seente viljakehade bakterikooslusi olulisemal määral kui teeb seda ümbritsev keskkond, mis pole viljakeha asustavate bakteritega niivõrd vahetus kontaktis. Erinevate seeneliikide viljakehad (I, IV), kes kuulusid ka erinevatesse funktsionaalsetessse rühmadesse (IV), koguti Eesti boreaalsetest metsadest, peamiselt kolmelt looduskaitsealalt (I, IV). Peremehega seotud tegurid, mis analüüsidesse kaasati, olid peremehe genotüüp, taksonoomiline/fülogeneetiline kuuluvus (identiteet), funktsionaalne

rühm, viljakeha keemilised omadused ja viljakeha osa, kust proov võeti. Seene viljakeha asustava mikroobikoosluse funktsionaalset struktuuri ja peremeesseene genotüübi mõju analüüsiti aasnööbiku (*Marasmius oreades*) näitel, mille viljakehad koguti kahest erinevast piirkonnast ja kuuest erinevast seeneringist Lõuna-Rootsis (III). Üheks eesmärgiks oli saada infot bakterite võimalikest ülesannetest seente viljakehades ja seega kaudselt ka põhjustest, et miks mõned bakterirühmad on antud elupaigas levinud, teised aga mitte. Kõigi viljakehade siseosast võeti koetükid, et eraldada DNA ning järgmise põlvkonna sekveneerimismeetodeid (Illumina) või kultuuri viimist kasutades teha kindlaks viljakeha asustav bakterikooslus. Ülejäänud sama viljakeha materjali kasutati viljakeha keemiliste omaduste määramiseks (IV). Keskkonnateguritest analüüsiti elupaigatüübi ja erinevate mullaomaduste mõju, kuna võis arvata, et enamik viljakeha asustavast bakterikooslusest pärineb ümbritsevast mullast. Selle hüpoteesi kontrollimiseks hinnati ümbritseva mulla ja viljakehade bakterikoosluse koosseisu sarnasust. Mullaproovid bakterikoosluse analüüsimiseks (I, IV) ja/või mulla keemiliste omaduste hindamiseks (I, III, IV) koguti kas viljakehade vahetust ümbrusest (III, IV) või juhuslikest kohtadest samalt alalt (I).

Tulemused näitavad, et viljakeha asustava bakterikoosluse struktuuri mõjutavad tugevamalt peremehega seotud faktorid (peremehe identiteet, genotüüp, funktsionaalne rühm, keemilised omadused), mitte keskkonnategurid (mullaomadused, elupaigatüüp). Peremeesseene faktoritest avaldas omakorda kõige tugevamat mõju peremehe identiteet – iga seenetaksoniga oli seotud talle omane bakterikooslus. Bakterite valik või teatud bakterite eelistamine peremeesseene poolt võib toimuda spetsiifiliste omaduste ja ühendite vahendusel, mille sisaldused omakorda varieeruvad erinevates seenetaksonites (Taylor *et al.*, 2003; Rudawska ja Leski, 2005; Barros *et al.*, 2008a, Kalač, 2009; Vieira *et al.*, 2014). Näiteks on leitud, et erinevate kandseente mükosfäärides ja mükorisosfäärides leiduvad mõned bakterirühmad tänu nende võimele kasutada peremeesseene vabastatavaid süsinikuühendeid, orgaanilisi happeid või aminohappeid (Timonen *et al.*, 1998; Warmink *et al.*, 2009; Nazir *et al.*, 2010b) või hoopis nende resistentsuse tõttu peremeesseene toodetud antibakteriaalsete ühendite suhtes (Shirakawa *et al.*, 2019). Viljakeha osal (kübar, jala keskmine ja alumine osa) statistiliselt olulist mõju bakterikoosluse struktuurile viljakeha sees ei leitud.

Teadaolevalt tuvastati käesolevas töös esmakordselt, et ühe seeneliigi piires mõjutab peremeesseene genotüüp oluliselt nii viljakeha mikroobikoosluse struktuuri kui ka funktsiooni. Oluline mõju võib olla tingitud erineva genotüübiga seente võimest toota teatud ühendeid, mis omakorda soodustavad viljakehade koloniseerimist kindlate, neid ühendeid tolereerivate või metaboliseerivate bakterite poolt. Samas võivad peremeesseene funktsionaalsed omadused varieeruda sõltuvalt genotüübist ja seetõttu võivad erineva genotüübiga seened eelistada kindlaid funktsioone täitvaid baktereid, kelle olemasolu võimaldab peremeesseenes teatud geenide puudumist või inaktiivset olekut. Näiteks on tuvastatud, et mitmeid seenes sisalduvaid mükotoksiine ja antibiootikume toodavad hoopis neis elavad endobakterid (Partida-Martinez *et al.*, 2005, 2007; Efimenko *et al.*, 2016; Pawlowska *et al.*, 2018). Lisaks võivad erinevad

bakteritüved toota seenele kasulikke ensüüme ja metaboliite, soodustades seeläbi seene elutegevust (Rangel-Castro *et al.*, 2002a; Sbrana *et al.*, 2002; de Boer *et al.*, 2005; Riedlinger *et al.*, 2006; Zhang *et al.*, 2014a; Scherlach ja Hertweck, 2020). Seentega seotud elupaikades on täheldatud ka bakterite genotüüpide märkimisväärset varieeruvust ja sellest tulenevalt ka nende metaboolsete omaduste, mineraalide lagundamisefektiivsuse ja patogeensuse varieeruvust (Rangel-Castro *et al.*, 2002a; Uroz *et al.*, 2007; Ye *et al.*, 2018a). Uuringud näitasid, et enamik seene viljakeha asustavate bakterite funktsionaalsetest geenidest olid seotud metabolismiga, sealhulgas süsivesinike, aminohapete, valkude, kofaktorite, vitamiinide ja pigmentide metabolismiga. Metabolismiga seotud geenide oluliselt suuremat osakaalu võrreldes ümbritseva mullaga on täheldatud ka näiteks pilvikuliigi *Russula griseocarnosa* mükosfääris (Yu *et al.*, 2020), mis viitab nende funktsionaalsete geenide tähtsusele seenega seotud elupaikades. Kokkuvõttes võib oletada, et genotüüp-genotüüp interaktsioonid, kus kindla genotüübiga peremeesseen eelistab omakorda kindla genotüübiga ja sellest tulenevalt ka teatud funktsioonidega baktereid, on seente elupaikades laialdaselt levinud.

Peremeesseene kuulumine funktsionaalsesse rühma, antud juhul ektomükoriisaseente või saprotroofide hulka, mõjutab samuti bakterikoosluse koosseisu. Samas ilmnes, et viljakehade ektomükoriisaseente või saprotroofide hulka kuuluvusel pole viljakehade bakterikooslustele siiski nii tugev mõju kui seda on peremeesseene identiteedil. Sarnaselt viimasele võib peremehe funktsionaalse grupi mõju olla tingitud nende viljakehade erinevast keemilisest koostisest. Näiteks vabastavad mitmed saprotroofsed seened spetsiifilisi sekundaarseid metaboliite, mis mõjutavad bakterikoosluse kujunemist nende viljakehades (de Carvalho *et al.*, 2015). Lisaks on elupaikades, kus üksteise suhtes domineerivad erinevad mükoriisatüübid, tuvastatud, et sealse bakterikoosluse süsivesinike lagundamisega seotud ensüümide profiil varieerub sõltuvalt mükoriisatüübist (Bahram *et al.*, 2020). See kinnitab, et domineeriva seene eluviis võib kujundada ümbritsevat mikroobikooslust erinevate süsinikuühendite vabastamise kaudu. Omadused, mis erinevatesse funktsionaalsetesse rühmadesse kuuluvates seentes varieeruvad ongi keemilis-füsioloogilised (Zanne *et al.*, 2020), avaldudes ensüümide ja erinevate ühendite sisalduses.

Kooskõlas varasemate uuringutega (Vogt *et al.*, 1981; Sanmee *et al.*, 2003; Trocha *et al.*, 2016; Kranabetter *et al.*, 2019) tuvastati, et näiteks lämmastiku ja fosfori sisaldus on saprotroofide viljakehades märkimisväärselt kõrgem ja süsiniku sisaldus oluliselt madalam kui ektomükoriisaseentes. Need variatsioonid on osaliselt tingitud ektomükoriisaseente tihedast seosest peremeestaimega, keda seen varustab toitainetega ja kellelt saab vastutasuks süsinikuühendeid (Taylor *et al.*, 2003; Trocha *et al.*, 2016). Lisaks võivad ektomükoriisaseened ja saprotroofid reageerida erinevalt mulla keemilise koostise muutustele ning ektomükoriisaseened reageerivad ka keemiliste omaduste muutustele peremeestaimes (Taylor *et al.*, 2003; Kranabetter *et al.*, 2019). Erinevate seeneliikide erinev reageerimine mullaparameetrite muutustele võibki seejuures vähemalt osaliselt tingida mullaparameetrite mõju viljakeha mikroobikooslusele ja seda just viljakeha keemiliste omaduste mõjutamise kaudu (Taylor *et al.*, 2003). Näiteks

korreleeruvad mulla fosfori sisaldus ning lämmastiku ja fosfori suhe vastavate näitajatega ektomükoriisaseente viljakehades ja lämmastiku sisaldus ümbritsevas mullas ja saprotroofides (IV, Kranabetter *et al.*, 2019). Viljakeha keemiline koostis ongi üheks võimalikest mehhanismidest, mis bakterikooslust viljakehas otseselt kujundab, varieerudes märkimisväärselt nii erinevate seeneliikide kui ka funktsionaalsete rühmade vahel. Kõige olulisemad keemilised näitajad on seejuures viljakeha pH ning süsiniku ja lämmastiku suhe. Kuigi viljakehade keemilise koostise varieerumist erinevate seenetaksonite ja funktsionaalsete rühmade vahel on ka varasemalt näidatud (Taylor *et al.*, 1997, 2003; Rudawska ja Leski, 2005; Trocha *et al.*, 2916; Kranabetter *et al.*, 2019; Zanne *et al.*, 2020), on käesolev töö esimene, kus tuvastati, et viljakeha keemiline koostis mõjutab olulisel määral seent asustava bakterikoosluse struktuuri. Eelpool nimetatud keemilised parameetrid, eriti pH, aga ka süsiniku ja lämmastiku suhe, on peamised bakterikooslusi mõjutavad keemilised näitajad ka mullas ja kõdupuidus (Fierer ja Jackson, 2006; Lauber *et al.*, 2009; Rousk *et al.*, 2010; Shen *et al.*, 2013; Zhang *et al.*, 2014b; Hoppe *et al.*, 2015). Seega kujundavad bakterikooslusi erinevates keskkondades peamiselt ühed ja samad keemilised omadused, mille varieerumisest ja kontrastidest võib sõltuda ka bakterikoosluste koosseisu kattumine erinevates keskkondades.

Üle poole seente viljakehadest tuvastatud bakteritest olid olemas ka ümbritsevas mullakeskkonnas, mille mikrobioom on seega peamiseks lähtekoosluseks seente viljakehi asustavale bakterikooslusele. Võrreldes ektomükoriisaseentega oli mulla ja viljakehade bakterikoosluste ühisosa suurem saprotroofsetel seentel. See võib olla tingitud saprotroofide tihedamast seotusest mulla ja laguneva orgaanilise materjaliga mullas, samas kui ektomükoriisaseened sõltuvad toitainete omastamisel rohkem peremeestaimest. Tulenevalt seosest ümbritseva mullaga on ka peamisteks viljakehade bakterikooslusi mõjutavateks keskkonnateguriteks just erinevad mullaparameetrid. Mullaomaduste mõju bakterikooslusele on tuvastatud ka varasemalt seentega seotud elupaikades, nagu hüüfid, ektomükoriisa, mükosfäär ja mükorisosfäär (Nazir *et al.*, 2010b; Li *et al.*, 2017; Hao *et al.*, 2020; Yu *et al.*, 2020). Kuna mullaparameetrid on teiste keskkonnaparameetrite kõrval peamised potentsiaalselt varieeruvad tegurid erinevates geograafilistes piirkondades ja ka elupaigatüüpides, võib eeldada, et see määrab suuresti ka mullatüübi, elupaigatüübi ja prooviala mõju. See selgitab ka käesoleva töö tulemusi, kus mullaparameetrite kaasamisel mullatüüp ja elupaigatüüp bakterikoosluste struktuuri kujunemisele viljakehades märkimisväärset mõju ei avaldanud. Lisaks on leitud, et mulla mikrobioomi kujundavad peamiselt keskkonnatingimused, mitte geograafiline kaugus (Fierer ja Jackson, 2006; Bahram *et al.*, 2018). Siiski tuvastati proovialade geograafilise vahemaa mõju aasnööbiku viljakehade mikroobikoosluse struktuuris ja funktsioonides. See ilmnes tõenäoliselt seetõttu, et ühe seeneliigi analüüsil ei mängi rolli seene identiteet ning keskkonna- ja mullaparameetritest oli analüüsidesse kaasatud vaid mulla pH. Eriti tugevalt mõjutas proovialade geograafiline distants mikrobioomi funktsiooni, näidates, et mikroobikoosluse funktsioon sõltub proovialade vahel varieeruvatest keskkonnaparameetritest tugevamalt kui mikroobikoosluse

struktuur. See omakorda võib olla seletatav asjaoluga, et samad bakteritaksonid võivad sõltuvalt keskkonnatingimustest täita erinevaid ülesandeid. Samas osutus proovialade geograafilise vahemaa mõju viljakeha mikroobikooslusele siiski nõrgemaks kui peremehe genotüübi mõju.

Domineerivad bakteritaksonid seente viljakehades kuulusid hõimkondadesse *Proteobacteria* (klassid *Alphaproteobacteria*, *Betaproteobacteria* ja *Gammaproteobacteria*) ning *Bacteroidetes* (klass *Sphingobacteriia*). Kõik need taksonid on laialt levinud seentega seotud elupaikades, nagu mükosfäär, mükorisosfäär ja mükoriisad (e.g. Frey-Klett *et al.*, 2007; Uroz *et al.*, 2012; Halsey *et al.*, 2016; Shirakawa *et al.*, 2019) ning viljakehad (e.g. Antony-Babu *et al.*, 2014; Quandt *et al.*, 2015; Dahm *et al.*, 2005; Kumari *et al.*, 2013; Li *et al.*, 2016a,b; Rinta-Kanto *et al.*, 2018). Klassid *Betaproteobacteria* ja *Gammaproteobacteria* sisaldavad seega arvukalt bakteritaksoneid, keda võib pidada niinimetatud üldisteks fungifiilideks, kuna neid on sageli tuvastatud erinevate seeneliikidega seotud elupaikadest (e.g. Dahm *et al.*, 2005; Warmink *et al.*, 2009; Uroz *et al.*, 2012; Kumari *et al.*, 2013; Halsey *et al.*, 2016; Li *et al.*, 2016a). Samas tuvastati ka bakterirühmi, mis olid iseloomulikud pigem kindlatele seenetaksonitele. Näiteks kukeseenele (*Cantharellus cibarius)* on iseloomulik väga spetsiifiline bakterikooslus, kus domineerivad seltsi *Rhizobiales* (*Alphaproteoacteria*) ja klassi *Sphingobacteriia* esindajad (I, IV, Rinta-Kanto *et al.*, 2018; Gohar *et al.*, 2020). Üldiselt tuleneb aga bakterikoosluste varieeruvus erinevate seenerühmade vahel pigem bakteritaksonite osakaalude erinevustest, mitte teatud taksonite esinemisest või puudumisest antud koosluses. Viljakehi asustavate bakterihõimkondade esindajad eelistavad pigem kõrgema süsiniku sisalduse ja pH-ga keskkondi (Fierer *et al.*, 2007; Eilers *et al.*, 2010; Rousk *et al.*, 2010), mida seentega seotud elupaigad, sealhulgas viljakehad, ka on (Danell *et al.*, 1993; Sanmee *et al.*, 2003; Warmink *et al.*, 2009; Nazir *et al.*, 2010a; Yu *et al.*, 2020; Hao *et al.*, 2020; III; IV). Seevastu ümbritsevas mullas olid viljakehadega võrreldes suhteliselt arvukamad hoopis bakterihõimkonnad *Actinobacteria*, *Verrucomicrobia*, *Planctomycetes*, *Chlamydiae*, aga iseäranis hõimkond *Acidobacteria* (Uroz *et al.*, 2012; Antony-Babu *et al.*, 2014; Halsey *et al.*, 2016; Liu *et al.*, 2018; Monaco *et al.*, 2020; I, IV), kuhu kuuluvad valdavalt oligotroofsed bakterid, kes asustavad madala toitainete sisaldusega elupaikasid, milleks muld sageli on (Fierer *et al.*, 2007; Eilers *et al.*, 2010; Bergmann *et al.*, 2011; Ho *et al.*, 2017).

Kasutades paralleelselt järgmise põlvkonna sekveneerimismetoodikat ja kultuuri viimist, ilmnes, et mõningate viljakeha asustavate bakteritaksonite tuvastamine emma-kumma metoodikaga on problemaatiline. Nimelt tuvastati, et klasse *Alphaproteobacteria* ja *Sphingobacteriia* ei õnnestu üldjuhul kultiveerimise meetodiga tuvastada, kuid hõimkond *Firmicutes* on seevastu alaesindatud Illumina MiSeq sekventsiandmetes. Sarnaseid tulemusi on näidanud ka varasemad kottseentega tehtud uuringud (Barbieri *et al.*, 2005, 2007; Splivallo *et al.*, 2015a; Perlińska-Lenart *et al.*, 2020). Teadaolevalt kasvab vaid väga väike osa bakteritest kultuuris, samas kui järgmise põlvkonna sekveneerimismeetodid võivad samuti olla sobimatud mõningate bakteritaksonite tuvastamiseks. Seega täiendavad kultuurist sõltumatud meetodid ja kultiveerimine teineteist, andes

bakterikoosluse koosseisust parema ülevaate. Lisaks võimaldab bakterite kultuuri viimine testida näiteks nende seenevastast aktiivsust ja kirjeldada bakterite fenotüüpe ning uurida nende funktsioone, kasvatades neid kultuuris koos seentega (Poole *et al.*, 2001; Sbrana *et al.*, 2002; Duponnois ja Kisa, 2006; Uroz *et al.*, 2007; Efimenko *et al.*, 2016; Oh *et al.*, 2018).

Käesolev doktoritöö näitab, et sarnaselt teiste eukarüootidega on ka seentel iseloomulikud bakterikooslused. Lisaks on seente bakterikooslusi kujundavad tegurid sarnased teiste eukarüootide puhul tuvastatud teguritega, kus eelkõige peremehest sõltuvad faktorid, nagu peremehe identiteet, genotüüp, funktsionaalne roll ja keemilised omadused osalevad peremehe bakterikoosluse kujunemisel. Seentes elupaiga leidnud bakterid on suuresti pärit peremeest ümbritsevast keskkonnast, nagu on leitud ka teiste eukarüootsete organismide puhul, ja mehhanismid keskkonnast bakterite filtreerimisel võivad eelkõige sarnaneda taimede poolt kasutatavate valikumehhanismidega, sest mõlemal juhul on peamiseks lähtekoosluseks mulla mikroobikooslus. Kokkuvõttes näitab käesolev töö, et seente ja bakterite assotsiatsioonid võivad olla kujunenud välja evolutsioonilise ajaloo vältel, nagu on tuvastatud ka taimede ja loomade ning neile omaste mikroobikoosluste puhul. Sellest tulenevalt on spetsiifilisel mikroobikooslusel seentes, sarnaselt teiste eukarüootidega, tõenäoliselt täita oluline roll, mille selgitamine aga vajab edasisi uuringuid.

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