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WHITE MULBERRY (*MORUS ALBA* L.) FRUIT-ASSOCIATED BACTERIAL AND FUNGAL MICROBIOTA

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Highlights

- Next Generation Sequencing approach reveals *Morus alba* fruit microbial community.
- White mulberry fruits possess high bacterial and fungal microorganism diversity.
- Potentially beneficial and pathogenic microorganisms are distributed on *M. alba*.
- Microbiota structure are important for mulberry development and quality of the berries.

Abstract. *Morus alba* L. has been worldwide cultivated and commercially exploited plant with profound potential in environmental management, food and medicinal industries. Plant-associated microbial communities are playing an essential role in sustainable plant development. In the present study, the bacterial and fungal microorganism populations distributed on the white mulberry fruits harvested in the Czech Republic for the first time were characterized by metagenomics approach. A total of 62 bacterial and 37 fungal families were identified on white mulberry. Bacterial population was represented by the genera *Tatumella*, *Leuconostoc*, *Frateuria* and *Pseudomonas,* while fungal microorganisms – by *Hanseniaspora*, *Cryptococcus*, *Cladosporium* and *Phoma*. Both potentially beneficial, inducing resistance in the hosting plant, and pathogenic, responsible for disease development, microorganisms were detected. The information on the prevalence of bacterial and fungal microorganisms on the carposphere of *M. alba* is highly relevant for the development of strategies for environment-friendly plant cultivation, disease management and prevention.

Keywords: metagenomic analysis, Moraceae, fungal microbiota, bacterial microbiota.

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Introduction

Mulberry trees (*Morus L.*) have been widely cultivated in temperate and subtropical regions of Africa, America, Asia and Europe; their planting steadily increases every year due to realized economic and ecological benefits (Ou et al., 2019; Xu et al., 2019). Mulberry (*Morus spp.*) has been commercially exploited as the host of the silkworm (*Bombyx mori*). Mulberry leaves and fruits contain a variety of nutrient and nutraceutical substances. Fruits have been recognized as a kind of natural nutrition and functional food; they are usually eaten fresh or made into jam, juice and wine (Łochyńska, 2015). Due to the presence of anthocyanins, carotenoids and flavonoids, mulberry fruits possess potential pharmacological properties including antibacterial, antidiabetic, antiobesity and antioxidative effects (Mahboubi, 2019; Rodrigues et al., 2019; Zhang et al., 2018). Rapid development of industry, transportation, introduction of anthropogenic toxic pollutants to the atmosphere, soil and groundwater possess serious threats on the remediation capacity of the environment (da Silva et al., 2017; Jiang et al., 2017; Khoshdel & Vaziri, 2016). Mulberry's developed root system has the potential to absorb heavy metals from contaminated soil and water, and to degrade organic compounds. Mulberry leaves absorb atmospheric pollutants. Planting of mulberry can effectively improve soil permeability and water balance, modify soil chemical and physical properties (Jiang et al., 2017).

Morus alba, rubra and *nigra* are the best-known mulberry species. The white mulberry (*Morus alba* L.) is very

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populated species, however almost forgotten in Europe. The potential of *Morus* L. plants in agriculture, environmental protection, industry and human health is rather huge (Łochyńska, 2015; Ercisli & Orhan, 2007). *M. alba* leaves can be used for tea making and in the silkworm diet for the commercial production of silk (Mahboubi, 2019). Very fast growth of white mulberry tree produces significant amount of plant biomass, which may be used as the renewable source of biofuel (Łochyńska, 2015; Chinnaswamy & Harisparad, 1995). Due to natural dyeing properties, it is applicable in furniture industry (Łochyńska, 2015). The food industry increasingly uses white mulberry in food production to provide valuable bioactive substances (Łochyńska, 2015). The main active constituents of *M. alba* include flavonoids, alkaloids, stilbenes, and polysaccharides, providing health benefits through anti-inflammatory and immunomodulatory effects (Zhang et al., 2018). Fruits, roots, and leaves of *M. alba* are used also for the treatment of dizziness, insomnia, and premature aging (Rodrigues et al., 2019). The resistance to disease and pests, relatively low soil requirements as well as attractive bioremediation properties substantiate great potential of *M. alba* for landscaping and ecological environment management (Jiang et al., 2017).

Economic value of the mulberry fruit is constantly increasing, so is the need for plant cultivation and disease management to produce suitable for consumption berries. Among multiple biotic and abiotic factors affecting plant development, plant-associated microbial communities are playing one of the primary roles. It must be mentioned that very few works concentrated on the characterization of mulberry-associated microbial assemblages are published so far. Some studies are focused on the diversity of endophytic bacteria on white mulberry cultivars by revealing seasonal variation and emphasizing antimicrobial and plant growth-promoting activities of bacteria (Ou et al., 2019; Xu et al., 2019). Only two studies are intended for characterization of soil fungal communities of different white mulberry genotypes and analysis of relationship with fruit sclerotiniosis (Zhang et al., 2019; Yu et al., 2016). To the best of our knowledge, the white mulberry carposphere-associated microbial populations remain undescribed in the scientific literature. The aim of the present study was to characterize the bacterial and fungal microorganism communities found on the *M. alba* fruit surface by applying metabarcoding and Next Generation Sequencing (NGS) technology.

1. Materials and methods

1.1. White mulberry sample preparation

Morus alba L. fruits were aseptically sampled from 30–40 years-old planted trees growing in Ostrava city park (the Czech Republic, GPS coordinates: 49°50′1″N, 18°17′40″E) in July, 2016. The fruits (at medium ripeness stage, without blemish) were randomly collected from reachable branches of the five white mulberry trees growing in distance of more than 30 meters. *M. alba* fruits were combined into sterile plastic bags and within 2 hours after harvesting were transported on ice to the Ostrava University for further processing. Gathered fruits (~300 g) were washed with aseptic 0.05M phosphate buffer, pH 6.8 (500 mL) for 30 min at room temperature. Plant debris was removed by filtration through 420 μm filters. Samples were centrifuged at 12,000×g for 20 min. The collected pellet was transported on ice to Lithuania and kept in –20 °C freezer until DNA extraction and amplification of marker genes (Department of Chemistry and Bioengineering, Vilnius Gediminas Technical University).

1.2. Extraction of DNA and sequencing

The DNA was extracted from 40 mg of collected pellet using a Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). The isolated DNA was quality checked and quantified by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). V3-V4 variable regions of the bacterial 16S rRNA gene were amplified by the primer set S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) and used to classify bacteria. The ITS2 region of ribosomal DNA specific primers ITS3-KYO2 (5′-GATGAAGAACGYAGYRAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were applied for fungal microorganism identification. Amplicon library preparation and sequencing was performed by Macrogen Inc. (Seoul, Korea). The libraries were sequenced using Illumina MiSeq V3 as paired-end 2×300 bp reads. Raw sequencing data was submitted to the NCBI Short Read Archive (SRA) database (Accession Number PRJNA612435).

1.3. Sequence analysis

Bioinformatics analysis was conducted using FLASH v1.2.11 (Magoč & Salzberg, 2011) to filter sequences with a minimum Q score of 25 and merge paired-reads. OTUs were picked by the greedy heuristic clustering algorithm, CD-HIT-OTU v4.5.5 (Li et al., 2012). Alpha diversity was calculated (including Chao1, Shannon, Good coverage) and relative abundance summaries were conducted using QIIME 1.8 (Caporaso et al., 2010). UNITE (Kõljalg et al., 2013) and Ribosomal Database Project (RDP) (Cole et al., 2014) were used as the reference databases for the taxonomy assignments of most abundant sequences. Non-bacterial, non-fungal sequences and singletons were filtered out and taxonomic relative abundance at all classification levels was calculated. The taxonomic results were visualized in Krona (Ondov et al., 2011).

2. Results

2.1. Bacterial and fungal diversity of *M. alba* fruits

The Illumina MiSeq sequencing generated 516,658 and 554,058 paired-end reads from ITS2 and V3-V4 target regions respectively. Following quality filtering and checking of chimera sequences, 302,705 (for ITS2) and 169,313 (for V3-V4) reads were obtained. 97% threshold of sequence similarity was used for clustering into operational

Table 1. Summary of ITS2 and 16S rRNA gene amplicon sequence analysis and microbial community diversity parameters

	Target region	Reads obtained	High quality reads	OTU	Chao1	Goods coverage	Shannon diversity
Morus alba	ITS2	516,658	302,705	206	215	0.99996	2.95
	V3-V4	554,058	169,313	365	370	0.99997	4.56

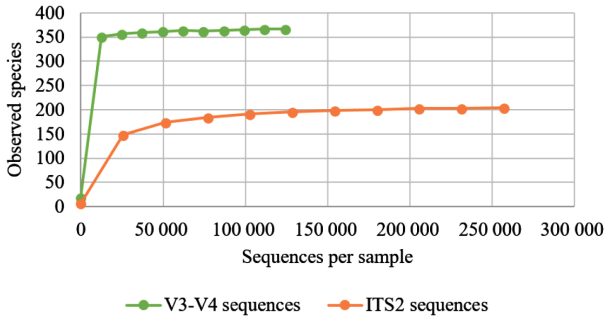

Figure 1. Rarefaction curves describing M. alba fruit-associated species richness at a genetic distance of 3% for each sample. ITS2 sequences from the analysis of eukaryotic microbial community and V3-V4 region sequences from the analysis of prokaryotic microbial community

taxonomic units (OTUs) (Table 1). 365 bacterial and 206 fungal OTUs were represented by identified sequences. Rarefaction curves (Figure 1) and calculated Goods coverage confirmed that the majority of the bacterial (coverage: 99.9%) and the fungal OTUs (coverage: 99.9%) were recovered. In agreement with obtained OTU data, the Chao1 and Shannon indexes demonstrated that white mulberry had higher bacterial community diversity than fungal microorganisms.

2.2. Taxonomic composition of white mulberry bacterial microbiota

The obtained sequences were classified into 14 phyla, 22 classes, 32 orders, 62 families and 87 genera (Table S1). The dominating bacterial phyla were Proteobacteria (61.72%), Firmicutes (24.88%), Bacteroidetes (4.38%), and Actinobacteria (2.36%), collectively accounting for more than 93% of the total bacterial population. Prokaryotic microorganisms essentially belonged to Gammaproteobacteria (50.92%) and Bacilli (24.79%) at the class level. The first class was represented by bacteria from Enterobacteriales, Xanthomonadales and Pseudomonadales orders, while the second – by Lactobacillales. Among 62 bacterial families identified, Enterobacteriaceae (28.43%), Leuconostocaceae (23.43%), Xanthomonadaceae (12.7%) and Pseudomonadaceae (7.72%) were the most abundant, represented mainly by the genera *Tatumella* (19.5%), *Leuconostoc* (21.58%), *Frateuria* (12.45%) and *Pseudomonas* (7.72%) (Table S1, Figure 2). The analysis of unique bacterial OTUs allowed to identify at the species level 10 prokaryotic microorganisms, with the highest abundance *Frateuria aurantia* (12.6%) and *Gluconobacter kanchanaburiensis* (4.37%), while other species were assigned to uncultured bacteria (Table 2, Table S1).

Table 2. List and relative abundance of bacterial and fungal microorganisms identified at species level

Bacterial relative abundance, %	
Frateuria aurantia DSM 6220	12.60
Gluconobacter kanchanaburiensis	4.37
Paenibacillus hordei	0.78
Nodularia spumigena	0.12
Lonsdalea quercina subsp. britannica	0.12
Lysinibacillus sphaericus	0.04
Pseudomonas geniculata	0.04
Rhizobium leguminosarum bv. viciae	0.02
Myxococcus virescens	0.02
Vagococcus teuberi	0.01
Fungal microorganisms relative abundance, %	
Hanseniaspora uvarum	34.04
Cryptococcus laurentii	8.18
Cryptococcus magnus	2.17
Candida railenensis	0.34
Torulaspora delbrueckii	0.24
Auriculibuller fuscus	0.21
Sporobolomyces roseus	0.20
Bulleromyces albus	0.11
Wickerhamomyces anomalus	0.05
Sporobolomyces salicinus	0.04
Sporobolomyces gracilis	0.04
Udeniomyces pannonicus	0.04
Verticillium dahliae	0.04
Erythrobasidium hasegawianum	0.04
Dioszegia catarinonii	0.04
Monographella nivalis	0.03
Sarocladium strictum	0.03
Cryptococcus uzbekistanensis	0.03
Cystofilobasidium lari_marini	0.02
Exophiala psychrophila	0.02
Fusarium culmorum	0.02
Sporobolomyces coprosmae	0.01
Mrakiella aquatica	0.01
Dioszegia aurantiaca	0.01
Knufia cryptophialidica	0.01
Saccharomycopsis crataegensis	0.01
Colletotrichum acutatum	0.01
Septoria lepidii	0.01
Kondoa malvinella	0.01
Botryotinia fuckeliana	0.01
Chaetomium murorum	0.01

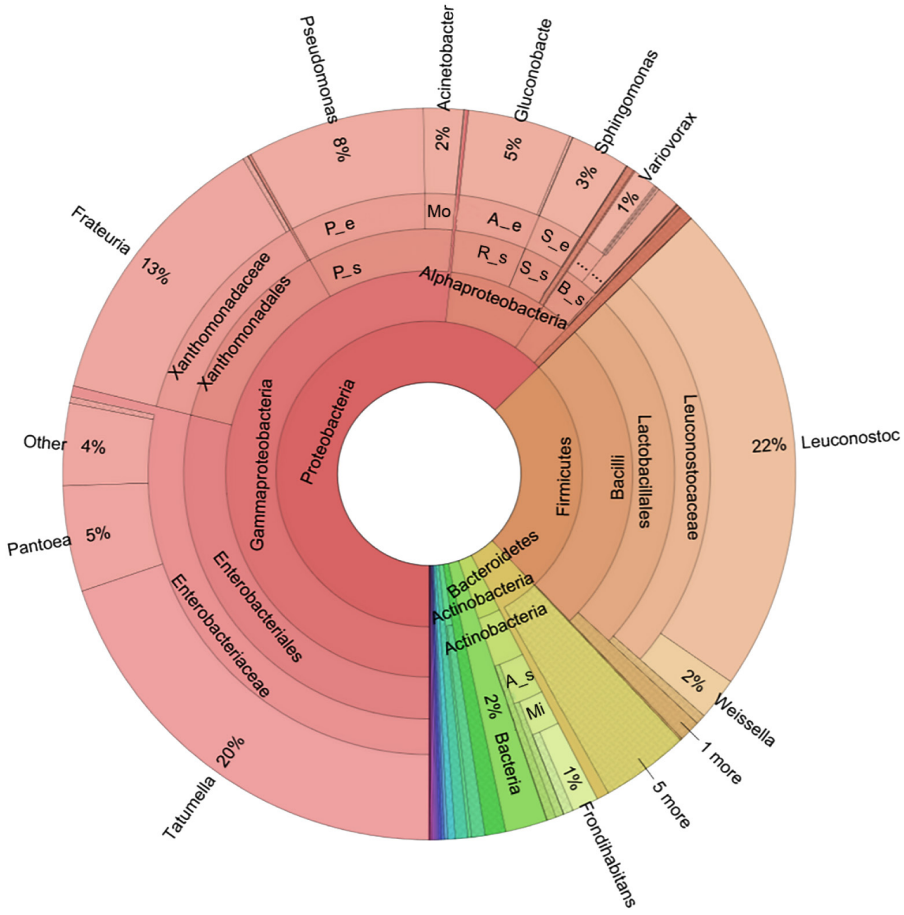

Figure 2. Prokaryotic microbial community distribution on *M. alba* carposphere. A_e – *Acetobacteriaceae*; A_s – *Actinomycetales*; B_s – *Bulkholderiales*; R_s – *Rhodospirillales*; S_e – *Sphingomonadaceae*; S_s – *Sphingomonadales*; P_e – *Pseodomonadaceae*; P_s – *Pseudomonadales*; Mo – *Moraxellaceae*; Mi – *Microbacteriaceae*. Minor OTUs were not labeled. Pie-charts were constructed using KRONA (Ondov et al., 2011)

2.3. Composition of white mulberry carposphere fungal microbiota

In total, 65 genera of fungi from 4 classified phyla, 13 classified classes, 26 classified orders, 37 classified families were detected (Table S2). The fungal microbiota was mostly dominated by Ascomycota phylum (76.42%), followed by Basidiomycota (21.52%) and unidentified microorganisms (1.99%). Dothideomycetes (40.32%), Saccharomycetes (34.83%) and Tremellomycetes (20.79%) have been identified to be the major classes associated with white mulberry fruits. At the family level, the eukaryotic microorganisms were represented by Saccharomycodaceae (34.04%) and Dothioraceae (31.08%). Fungal microorganisms belonging to Dothioraceae family were assigned as *uncultured Aureobasidium* at the species level (31.08%) (Table S2). The dominant genus distributed on mulberry fruits was *Hanseniaspora* (34.04%), followed by *Cryptococcus* (16.84%), *Cladosporium* (4.73%), and *Phoma* (4.06%) (Table S2; Figure 3). Thirty one fungal microorganism species were identified with dominating *Hanseniaspora uvarum* (34.04%), *Cryptococcus laurentii* (8.2%) and *Cryptococcus magnus* (2.2%) (Table 2).

3. Discussion

So far, a few studies were focused on the characterization of bacterial community distributed on different *Morus* L. cultivars using culture-dependent (Xu et al., 2019) and metagenomics (Ou et al., 2019) approaches. It was demonstrated that the endophytic *Morus* L. microorganism assemblies are specific for the host plant and the climatic conditions (Ou et al., 2019). Several aspects aggravate comparative analysis of our data with previous *Morus* L. microbiome studies: different plant site were chosen (in our study – fruits, in in mentioned above studies – stems), the lack of information on exact mulberry species of tested cultivars, and microorganisms analyzed (ours – epiphytic, in mentioned above studies – endophytic bacteria). Nevertheless, analysis of taxonomic composition of prokaryotic microorganisms revealed that Actinobacteria, Firmicutes, and Proteobacteria were the three most abundant bacterial phyla on different *Morus* L. cultivars, mainly represented by *Pantoea*, *Pseudomonas*, and *Methylobacterium* genera (Ou et al., 2019; Xu et al., 2019). In our study, about 90% of *M. alba* fruits were also covered by microorganisms belonging to these phyla but represented by different genera. At genus level, *Leuconostoc* was the most prevalent taxa

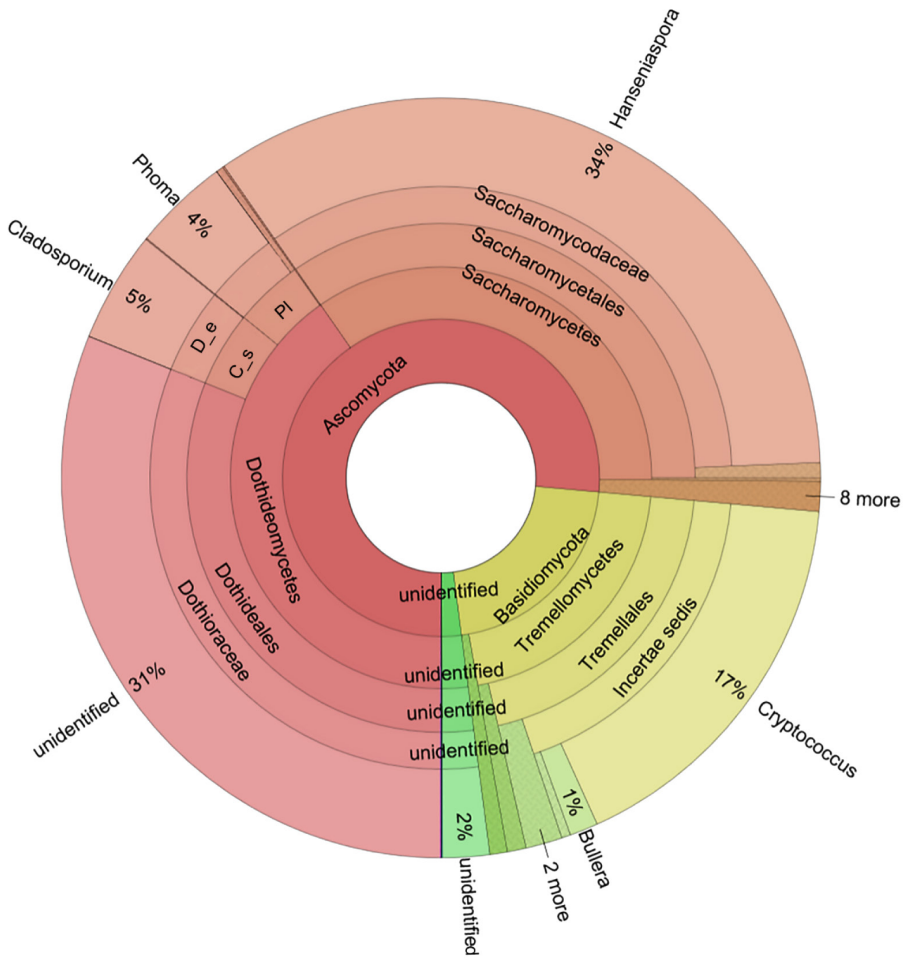

Figure 3. The taxonomic distribution of fungal microorganisms on *M. alba* carposphere. C_s – Capnodiales; D_e – Davidiellaceae; Pl – Pleosporales. Minor OTUs were not labeled. Pie-charts were constructed using KRONA (Ondov et al., 2011)

on white mulberry fruits. *Leuconostoc spp.* are non-pathogenic bacteria frequently found on plants, advantageous in most foods because of their flavor development and preservation abilities, capacity to improve the nutritional and organoleptic quality, possessing beneficial effects as potential probiotics (Shin & Han, 2015; Holland & Liu, 2011). Some *Leuconostoc* species are also capable of causing uncommon human infections or wine ropiness, nevertheless the genus is generally recognized as safe (Holland & Liu, 2011). In our study, *Tatumella* and *Frateuria* were detected among dominating bacterial genera on *M. alba* fruits. Some representatives of *Tatumella* genus are foodborne opportunistic pathogens isolated from various food sources, pineapples and clinical specimens, able to cause numerous infections (Mardaneh et al., 2014). *Frateuria* bacteria, represented in our study by *Frateuria aurantia*, were previously documented as capable to improve the plant growth and control phytopathogens (Lidor et al., 2019). Bacteria from *Pseudomonas* and *Pantoea* genera, observed in high content on white mulberry plants, are reported as plant-associated pathogenic microorganisms (Coutinho & Venter, 2009; Ligon et al., 2000). Nevertheless, both genera contain species possessing antimicrobial features (Trotel-Aziz et al., 2008; Ligon et al., 2000). Among those identified at species level in low frequency, bacteria with known plant growth promoting and biocontrol potential (*Pseudomonas geniculate*, *Lysinibacillus sphaericus*, *Myxococcus virescens*, *Rhizobium leguminosarum bv. viciae*) were observed.

So far, only two studies using molecular techniques have described the fungal communities related to a *M. alba* tree, in the investigation conducted on soil samples (Zhang et al., 2019; Yu et al., 2016). It was demonstrated that representatives of six phyla were observed with three dominant phyla (Ascomycota, Mucoromycota and Basidiomycota) (Zhang et al., 2019), while fruits were mainly covered by representatives of two phyla as revealed in our study. Based on mulberry rhizosphere-associated fungal community analysis, it has been shown that plant genotype has a significant influence on the abundance and composition of soil microbial population (Yu et al., 2016). In addition, the differences in the soil microbial assemblages may affect the plant resistance to mulberry fruit sclerotiniosis (Yu et al., 2016). As observed in our study, *Hanseniaspora* was the most prevalent mulberry fruit-associated taxa at the genus level, represented by *Hanseniaspora uvarum* species. *Hanseniaspora* spp. possess low fermentative activity and have been frequently

Table 3. Most abundant bacterial and fungal microorganisms on the *Morus alba* carposphere in comparison to those on *Malus pumila* and *Ribes nigrum*. Taxonomy analyzed at genus level. Cz -fruits collected in Czech Republic, LT – in Lithuania. Data on distribution of apple and blackcurrant microbiota obtained from Vepštaitė-Monstavičė et al. (2018)

Genus	*M. alba*_CZ	*M. pumila*_LT	*M. pumila*_CZ	*R. nigrum*_LT	*R. nigrum*_CZ
	Bacteria relative abundance, %				
Leuconostoc	21.83	0.00	0.01	0.00	0.00
Tatumella	19.73	0.00	0.01	0.00	18.91
Frateuria	12.60	0.00	0.00	0.00	0.00
Pseudomonas	7.81	32.57	41.26	2.80	7.78
Pantoea	4.66	48.70	34.16	0.38	1.02
Gluconobacter	4.53	0.12	0.12	0.00	5.49
Sphingomonas	2.63	0.57	2.21	1.17	7.41
Weissella	1.88	0.06	0.00	0.00	0.00
Acinetobacter	1.76	0.00	0.00	4.61	0.00
Frondihabitans	1.17	1.60	0.96	0.26	1.79
Hymenobacter	1.10	0.27	0.80	0.49	7.00
Variovorax	1.05	0.04	0.00	0.82	2.32
	Fungal microorganisms relative abundance, %				
Hanseniaspora	34.04	3.26	17.62	0.00	48.53
Cryptococcus	16.86	5.68	3.48	15.22	0.88
Cladosporium	4.73	4.19	1.76	45.40	5.20
Phoma	4.06	0.44	0.66	3.99	1.64
Bullera	1.21	0.06	0.05	0.14	0.00

discovered on the surface of different fruits, e.g. apples, blackcurrants, grapes, strawberries and etc. (Lukša et al., 2018; Vepštaitė-Monstavičė et al., 2018; Graça et al., 2015). It is well-recognized that fungal microorganisms from this genus could be useful to hosting plant by executing antagonistic activity on the development of fruit spoilage-causing mold (Tilocca et al., 2020; Liu et al., 2010). Among other dominating fungal genera, our study on mulberry fruits detected *Cryptococcus, Cladosporium,* and *Phoma*. Ubiquitous fungi *Cryptococcus* and *Cladosporium* are typical members of the yeast population have been often detected on the surface of different plants (Vepštaitė-Monstavičė et al., 2018; Vadkertiová et al., 2012). Some representatives of these genenera enclose species producing antifungal agents against many pathogens (Freimoser et al., 2019; Hashem et al., 2014; Wang et al., 2013), and have been established as biocontrol agents for the management of the postharvest diseases (Liu et al., 2013). On the other hand, certain species can cause plant, human and animal diseases (Sandoval-Denis et al., 2016; Bernal-Martinez et al., 2010). Therefore, the potentially beneficial or pathogenic features of *Cryptococcus laurentii* and *Cryptococcus magnus* fungal microorganisms, identified in our study, could be determined only by culture isolation and further analysis. Fungal microorganisms belonging to the genus *Phoma* are known to be plant pathogens, characterized by parasitic relationships with their host (Aveskamp et al., 2008). *Phoma* spp. have been shown to contaminate seeds, fruits and vegetables (Bennett et al., 2018; Lukša et al., 2018; Vepštaitė-Monstavičė et al., 2018; Oliveira et al., 2017; Termorshuizen, 2007), produce cytotoxic metabolites causing infections for humans and animals (Bennett et al., 2018). Among identified at species level in low frequency, potentially beneficial (*Torulaspora delbrueckii*, *Wickerhamomyces anomalus*, *Candida railenensis*, etc) as well as pathogenic fungi (*Auriculibuller fuscus*, *Bulleromyces albus*, *Sporobolomyces roseus*, *Sp. salicinus*, *Sp. gracilis*, etc) were observed.

The abundance and distribution of prokaryotic and eukaryotic microorganisms on fruits are dependent on the plant origin and ripening stage, geographic location and climatic conditions, application of agrochemicals as well as other biotic and abiotic factors (Vepštaitė-Monstavičė et al., 2018; Pinto et al., 2015). Considering the biogeographic effect, we compared the *M. alba*-associated microbial communities with ones identified previously on *Malus pumila* Mill. and *Ribes nigrum* L. collected in Czech Republic, Ostrava region and Lithuania (Vepstaite-Monstavičė et al., 2018) (Table 3). All tested fruits were sampled in the same season on July-August, 2016. The higher amount of bacteria belonging to *Tatumella*, *Gluconobacter* and *Sphingomonas* genera and fungal microorganisms from *Hanseniaspora* genus were detected on the surface of fruits sampled in Czech Republic, as compared to Lithuanian samples. These results indicate the importance of climatic conditions and geographical distribution

on the prevalence of microorganisms. On the other hand, detection of bacteria from *Leuconostoc* and *Frateuria* genera exceptionally on *M. alba* revealed importance of the plant origin. Higher abundance of *Pseudomonas* spp. and *Pantoea* spp. on *M. pumila* comparing to *M. alba* and *R. nigrum* from both localities as well as dominance of *Phoma* spp. on *M. alba* and *R. nigrum* further highlights the plant-conditioned distribution. The differences between the microbial communities inhabiting various plants are determined by various hosting plant features, including production of volatile organic compounds as well as interactions of microorganisms with the plant and other microbial inhabitants (Deveau et al., 2018; Lindow & Brandl, 2003). In the natural environment, different factors act cumulatively, therefore differentiation of the major and specific impacts driving the divergence of bacterial and fungal microorganism assemblages is rather complicated.

In conclusion, the results of this study demonstrated that *M. alba* carposphere possesses high bacterial and fungal microorganism variety. Among eukaryotic and prokaryotic microbiota, both potentially beneficial and pathogenic microorganisms were identified. The obtained information on the structure of white mulberry-associated fungal and bacterial assemblages, spreading of potentially beneficial and phytopathogenic microorganisms is important to develop effective plant disease control strategy. Due to clear potential of white mulberry berries in food industry, the metagenomics data appear to be significant for the evaluation of the influence of microbiota in general, and specific microorganisms in particular on food quality and human wellbeing.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization, E. S.; collection of plant material, E. S.; metagenomic analysis of microorganisms, J. L., E. S.; bioinformatics analysis, J. L.; data interpretation, E. S., J. L.; writing – original draft, E. S., J. L.

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