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Distribution of apple and blackcurrant microbiota in Lithuania and the Czech Republic

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ABSTRACT

The microbial assemblies on the surface of plants correlate with specific climatic features, suggesting a direct link between environmental conditions and microbial inhabitation patterns. At the same time however, microbial communities demonstrate distinct profiles depending on the plant species and region of origin. In this study, we report Next Generation Sequencing-based metagenomic analysis of microbial communities associated with apple and blackcurrant fruits harvested from Lithuania and the Czech Republic. Differences in the taxonomic composition of eukaryotic and prokaryotic microorganisms were observed between plant types. Our results revealed limited geographic differentiation between the bacterial and fungal communities associated with apples. In contrast, blackcurrant berries harvested from different regions demonstrated high diversity in both bacterial and fungal microbiota structures. Among fungal and bacterial microorganisms, we identified both potentially beneficial (*Cryptococcus, Hanseniaspora*, *Massilia*, *Rhodotorula, Sphingomonas*) and phytopathogenic microorganisms (*Cladosporium*, *Pantoea*, *Phoma*, *Pseudomonas, Septoria, Taphrina*) indicating their important roles in ecological and evolutionary processes.

1. Introduction

Plants host many microorganisms that colonize the surface of fruits, leaves, flowers and stems, as well as within their tissues (Abdelfattah et al., 2016a). The distribution of microorganisms on fruits is defined by a continuum of factors, including plant species, geographic location, climatic conditions, ripening stage and the application of agrochemicals (Pretorius, 2000; Pinto et al., 2014, 2015). The microorganism community year-to-year is characterized by the appearance of many new patterns, indicating that the behavior of most of the strains is not perennial. Fungi and bacteria inhabiting the fruit surface may be transported from the soil to the plants by insects and other animal species (Valero et al., 2007; Stefanini et al., 2015). On the other hand, some microorganisms, particularly yeast, could be permanent residents on fruits employing the latter as depositorium for survival and propagation. Microorganisms naturally associated with fruits may be beneficial and induce resistance in the hosting plant (e.g. *Cryptococcus*, *Sphingomonas*) or phytopathogenic and responsible for significant economic losses (e.g. *Phoma*, *Pantoea*) (Coutinho and Venter, 2009; Liu et al., 2013; Abdelfattah et al., 2016a). The interactions between different microorganism species may influence the structure of microbial communities inhabiting the fruit surface and through either direct or indirect impact on the plant can mediate many ecological and evolutionary processes (Friesen et al., 2011; Alvarez-Perez and Herrera, 2013).

The fungal and bacterial communities can be very diverse and will be defined by the associated plant species (Pinto et al., 2014). However, geographic location and farming practice also significantly influence microbial diversity (Leff and Fierer, 2013). Until now, the biogeographic distribution of microbiota communities has been studied mainly on grapes, the essential resource for wine production (Setati et al., 2012; Pinto et al., 2015; Wang et al., 2015). Only a limited number of studies on microorganisms residing on plums, apples, pears, cherries, and strawberries have been reported (Janisiewicz et al., 2014; Abdelfattah et al., 2016a, 2016b; Clooney et al., 2016; Volschenk et al., 2016). Few of them were dedicated to comparison of fruit-associated fungal communities differing in location (Setati et al., 2012; Bokulich et al., 2014; Taylor et al., 2014).

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The domesticated apple (*Malus pumila* Mill.) is a worldwide-grown major temperate fruit crop. Like many other fruits, apple is colonized by a number of different microorganisms and could be affected by several different phytopathogens (Teixidó et al., 1999; Graca et al., 2015). Current knowledge about the apple microbiota is limited and largely focused on species that cause disease and thus pose economic threats (Teixidó et al., 1999; Abadias et al., 2006). Another research focus is related to natural antagonists that could be used as biological control agents against phytopathogens (Piano et al., 1997). To date, only one comprehensive report on the fungal community associated with organic and conventionally grown apples in the state of Washington, USA, has been published (Abdelfattah et al., 2016b). It was demonstrated that the phylum Ascomycota was dominant on apples, followed by Basidiomycota and Chytridiomycota. Communities of fungal microorganisms differ depending on the parts of the apple fruit (e.g. *Cryptococcus* and *Alternaria* were most abundant on the stem and calyx; *Penicillium* – in peel and wounded flesh; while *Mycosphaerella* was found exclusively in the calyx). Bacterial communities associated with apples (in Colorado, USA) consisted of two most abundant groups – Microbacteriaceae and Sphingomonadaceae (Leff and Fierer, 2013). The apples used for that studies were purchased from a local supermarket or grocery store, thus analysis was conducted not immediately after collection. It is possible therefore that external conditions such as fruit storage and transportation as well as period of time before performing molecular analysis may have impacted the structure of microbiota due to decreasing survival of fruit-associated microorganisms or involving contaminating ones.

Blackcurrant (*Ribes nigrum* L.) is a native temperate crop widely cultivated both commercially and domestically in the major part of Europe and northern Asia. Even in the USA, there is a growing interest in expanding *Ribes* production (Hummer and Dale, 2010). The berries are rich in polyphenols and vitamin C, thus are attractive for regulation of the gut and intestinal microbiota, protecting against anti-inflammatory degenerative disorders or even cancer in humans (Paredes-Lopez et al., 2010; Tabart et al., 2012). The blackcurrants or their extracts are also widely used in food and beverage manufacturing. The broad interest in growing and application of blackcurrants demand investigation of the microbial communities colonizing the surface of these berries. To the best of our knowledge, no reports on the blackcurrant fungal and bacterial microbiota have been presented thus far.

The objective of the present study was to identify the composition of the bacterial and fungal microbiota closely associated with apples and blackcurrants collected in Lithuania and the Czech Republic. The identification and quantification of fruit and berry microflora expanded current knowledge about the structure of plant-associated bacterial and fungal communities, and revealed the biogeographic distribution of microbiota on apples and blackcurrants, as well as provided valuable information on the impact of environmental factors on the distribution of these microbial populations.

2. Materials and methods

2.1. Ethics statement

The collection of samples was carried out on private land and the owner of the land gave permission to conduct the study on site. It did not involve endangered or protected species.

2.2. Sampling of the fruits and DNA extraction

The domesticated apples (*Malus pumila* Mill.) were aseptically collected in the late-August 2016 on the private farms located in the Vilnius region of Lithuania (GPS coordinates: 54°75′20.0″N, 25°27′99.6″E) and Ostrava region of the Czech Republic (GPS coordinates: 49°83′03.9″N, 18°17′47.3″E). Blackcurrants (*Ribes nigrum* L.) were sampled from the Ignalina region of Lithuania (GPS coordinates: 55°34′23.0″N, 26°16′46.8″E) and Ostrava region of the Czech Republic (GPS coordinates: 49°83′03.9″N, 18°17′47.3″E) in the mid-July 2016. The fruits were collected into sterile plastic bags and processed within 2–4 h after harvesting. Fruits of interest (300 g) were placed in 500 mL of sterile 0.05 M phosphate buffer pH 6.8 for 30 min (in the case of blackcurrants) and 2 h (for apples) with shaking at120 rpm. Outwashes were filtered through 420 μm filters, centrifuged at 12,000 g for 20 min, and precipitates were stored at −20 °C until subsequent analysis.

For metagenomic analysis, 40 mg of pellet per sample was used. DNA isolation from collected sediments was performed using a Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) in accordance with the manufacturer's instructions. The quantity and quality of extracted DNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

2.3. Bacterial and fungal DNA amplification and amplicon library preparation

DNA samples from apples and blackcurrant microbiota were amplified using the primers specific for fungi and bacteria. For identification of fungal microorganisms, the ITS2 region of ribosomal DNA was amplified using ITS3-KYO2 (5′-GATGAAGAACGYAGYRAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (Toju et al., 2012). For bacteria identification, the V3-V4 region of the 16S rRNA gene was amplified with primers S-D-Bact-0341-b-*S*-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). Amplicon libraries were prepared using modified Illumina adapters (www.illumina.com), validated on an Agilent Technologies Bioanalyzer DNA 1000 and sequenced using Illumina MiSeq V3 (2 × 300 bp) (Macrogen Inc., Seoul, Korea). All sequences obtained during this work are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), under accession number SRP108314.

2.4. Data processing and analysis

The bioinformatics pipelines, FLASH 1.2.11 (Magoc and Salzberg, 2011), CD-HIT-OTU 4.5.5 (Li et al., 2012), and QIIME v. 1.8 (Caporaso et al., 2010), were used to process and analyze the obtained sequence data. Preliminary processing of the data was performed using the default parameters of FLASH 1.2.11: sequences with a minimum quality score of 25 were filtered and paired-end reads were merged. Sequences were denoised, chimeric sequences were identified and filtered, and the remaining reads were clustered into the Operational Taxonomical Units (OTUs) with a minimum 97% similarity threshold, using the CD-HIT-OTU 4.5.5 (Li et al., 2012). The most abundant sequences in each OTU were used for the taxonomy assignments using the RDP (Ribosomal Database Project) (Wang et al., 2007; Cole et al., 2014) and the UNITE (Koljalg et al., 2013) databases as references. For downstream analysis, the OTU table was rarefied at an even depth to reduce biases in sequencing depth. Alpha diversity was calculated using observed species, Shannon, Good's coverage and Chao1 estimates (Caporaso et al., 2010). Weighted Unifrac algorithm was used to evaluate β-diversity (Lozupone and Knight, 2005). Principal coordinates analysis (PCoA), as implemented in QIIME v. 1.8, related the bacterial and fungal microbiota composition to sample types and examined the distance between different ecosystems.

2.5. Cultivable yeast enrichment and identification

The aseptically collected apple and blackcurrant fruits (30 g each) were kept in 5% dextrose solution for 15 days at a temperature of 22 °C. Serial dilutions were made in a Ringer solution (Merck, Kenilworth, United States), plated on YEPD-agar plates (1% yeast extract, 1% peptone, 2% dextrose, 2% agar) containing 50 μg/mL chloramphenicol