Fungal Microbiota of Sea Buckthorn Berries at Two Ripening Stages and Volatile Profiling of Potential Biocontrol Yeasts

Juliana Lukša 1, Iglė Vepštaitė-Monstavičė 1, Violeta Apšegaitė 2, Laima Blažytė-Čereškienė 2, Ramunė Stanevičienė 1, Živilė Strazdaite-Žielienė 1, Bazilė Ravoitytė 1, Dominykas Aleknavičius 2, Vincas Būda 2, Raimondas Mozūraitis 2 and Elena Servienė 1,*

1 Laboratory of Genetics, Institute of Botany, Nature Research Centre, Akademijos str. 2, LT-08412 Vilnius, Lithuania; julian.luksa@gamtc.lt (J.L.); igle.vepstaite@yahoo.com (I.V.-M.); ramune.staneviciene@gamtc.lt (R.S.); zivile.strazdaite-zieliene@gamtc.lt (Ž.S.-Ž.); bazile.ravoityte@gamtc.lt (B.R.)

2 Laboratory of Chemical and Behavioral Ecology, Institute of Ecology, Nature Research Centre, Akademijos str. 2, LT-08412 Vilnius, Lithuania; violeta.apsegaite@gamtc.lt (V.A.); laima.blazyte@gamtc.lt (L.B.-Č.); dominykas.aleknaviicus@gamtc.lt (D.A.); vincas.buda@gamtc.lt (V.B.); raimondas.mozuraitis@gamtc.lt (R.M.)

* Correspondence: elena.serviene@gamtc.lt; Tel.: +370-5-272-93-63

Received: 29 January 2020; Accepted: 21 March 2020; Published: 23 March 2020

Abstract: Sea buckthorn, Hippophae rhamnoides L., has considerable potential for landscape reclamation, food, medicinal, and cosmetics industries. In this study, we analyzed fungal microorganism populations associated with carposphere of sea buckthorn harvested in Lithuania. An amplicon metagenomic approach based on the ITS2 region of fungal rDNA was used to reveal the ripening-affected fungal community alterations on sea buckthorn berries. According to alpha and beta diversity analyses, depending on the ripening stage, sea buckthorn displayed significantly different fungal communities. Unripe berries were shown to be prevalent by Aureobasidium, Taphrina, and Cladosporium, while ripe berries were dominated by Aureobasidium and Metschnikowia. The selected yeast strains from unripe and mature berries were applied for volatile organic compounds identification by gas chromatography and mass spectrometry techniques. It was demonstrated that the patterns of volatiles of four yeast species tested were distinct from each other. The current study for the first time revealed the alterations of fungal microorganism communities colonizing the surface of sea buckthorn berries at different ripening stages. The novel information on specific volatile profiles of cultivable sea buckthorn-associated yeasts with a potential role in biocontrol is important for the development of the strategies for plant cultivation and disease management, as well as for the improvement of the quality and preservation of the postharvest berries. Management of the fungal microorganisms present on the surface of berries might be a powerful instrument for control of phytopathogenic and potentially antagonistic microorganisms affecting development and quality of the berries.

Keywords: Hippophae rhamnoides; fungal communities; volatile organic compounds

1. Introduction

Sea buckthorn (Hippophae rhamnoides L.) is a garden and landscaping shrub widespread in Europe, Asia, and North America, distinguished for good adaptation to barren soils and harsh climates [1]. The ecological impact of sea buckthorn in preventing soil erosion, improving its properties, and reducing pollution has been demonstrated [2]. Economically, the plant species has been considered as a source of healthy nutritious food, giving rise to an increased demand for medicine and cosmetics [3,4].
Cultivation of sea buckthorn is developing due to the request for its berries with a high content of minerals, different vitamins, specific valuable oils, phenolics, and essential fatty acids, as well as low level of both fructose and glucose [5]. Phytochemicals and essential oil generated from sea buckthorn berries and seeds reduce inflammation; have antibacterial, anticancer, and radioprotective activity; relieve pain; and promote regeneration of tissues [3,6,7].

The microenvironments coherent with plants and fruits are highly colonized by different communities of bacterial and fungal microorganisms [8]. Plants provide nutrient-rich and stable surrounding conditions vital for the development of microbiota. In turn, epiphytic microorganisms produce secondary metabolites that may enhance plant immunity to unfavorable conditions and diseases [8–10]. Numerous microorganisms due to the production of volatile organic compounds (VOCs) have been found to promote plant growth and improve crop productivity [11]. Volatile production, secretion of antifungal compounds, and killer toxins allow for fruit-associated microorganisms to demonstrate increased antagonistic activity, an ability to modulate carposphere microbiota structure, and might mediate interactions with insects [12–16]. The role of bacterial and fungal microorganisms as producers of chemical indicators for the attraction of insects and their communication has also been revealed [17,18]. Microorganisms occupy a trophic level between plants and insects. Insects from several orders are feeding on fruits, while yeasts use insects either as hosts or vectors [17]. The chemical composition of each blend of volatiles may vary depending on the producing yeast and the ecological niche where the cross-talking species are growing [14,16].

The spreading of microorganisms on plants is determined by many factors, such as climatic conditions, application of agrochemicals, plant species, and ripening stage [19–21]. Changes in the planting regime alter microbial habitat and diversity, affect functional traits [22]. Comprehensive next-generation sequencing-based (NGS) microbiome analysis accomplished on several fruits and berries—such as apples, blackcurrants, grapes, olives, strawberries, oranges, etc.—is mainly focused on plant-conditioned prevalence [23–28]. The biogeographic distribution of microbiota communities has been revealed on apple, blackcurrant, and grape [21,28]. The effect of the ripening stage on the development of the microbial population was investigated in grapes, mango, nectarines and plums [8,29–31]. Analysis of grape bacterial microbiota defined changes in the structure and size of the population occurring during the ripening process. The levels of bacteria raised gradually and reached their highest value when the berries became overripe [8]. Yeasts are sparsely observed in the early stage of fruit development, but they increase in number as fruits ripen [32]. The yeast genera occurring on most mature nectarine fruit (e.g. Hanseniaspora, Pichia, and Zygosacharomyces) were significantly different from those on fruit at earlier stages of development [29].

It must be noticed that few works dealing with the characterization of sea buckthorn-associated microbial communities were published [33,34]. By analyzing representative microbial operational taxonomic units (OTUs) prevalent on sea buckthorn berries, the plant-defined composition of bacterial and fungal microbiota was recorded [33]. The impact of plant age and season was investigated in sea buckthorn plantation on the rhizosphere microbial community only [34,35].

To the best of our knowledge, there is no information on the ripening affected sea buckthorn carposphere-associated fungal community’s alterations and release of volatile organic compounds by the yeasts isolated from this plant. Therefore, the aims of this study were (i) to investigate the changes of the sea buckthorn-associated fungal microbiota depending on the ripening stage of the berries, (ii) to isolate and cultivate potential biocontrol yeasts distributed on unripe and ripe sea buckthorn berries, and (iii) to identify pattern of volatiles produced by the selected cultivable yeast species. The obtained information on the ripening-affected changes of sea buckthorn-associated fungal microbiota composition, and distribution of potentially beneficial and phytopathogenic microorganisms, may be important to develop effective plant disease control strategies. Due to high demand of sea buckthorn berries in food industry and increased request of unprocessed food, the metagenomics data appear to be highly relevant for the evaluation of the impact of microbiota and specific microorganisms on food quality and human health. Isolated cultivable yeasts might encourage new opportunities for
developing natural tools for disease management, postharvest protection, and the quality of the sea buckthorn berries with potential in food production. The data on volatile profiling of selected yeasts could be relevant for improving plant productivity and generating new environmentally friendly formulations for pest control and plant disease management.

2. Materials and Methods

2.1. Ethics Statement

The collection of sea buckthorn berries was authorized by the private owner, fully acknowledged in a paper. Endangered or protected species were not involved; therefore, no specific permissions were required.

2.2. Sample Collection

Hippophae rhamnoides L. berries were aseptically sampled from the organic private plantation located in the Molėtai region of Lithuania (GPS coordinates: 55°15′12.2″N, 25°26′23.1″E). The sea buckthorn berries were collected from three different places of sea buckthorn plantation separated by more than 50 meters. The berries were randomly selected from three bushes and combined into one biological replicate at each location. The experiment was carried out at two different growth stages: at the beginning of berry ripening (UB) in July and ripe berries (RB) in September 2018. The samples were collected into sterile plastic bags, transported to the laboratory and processed within 2–3 h after harvesting.

For metagenomic analysis, 300 g of the berries were placed into flasks with 500 mL of sterile 0.05M phosphate buffer (pH 6.8) for 30 min and incubated at room temperature with shaking at 120 rpm. Outwashes were filtered through 420 µm filters and followed centrifugation at 12,000 × g for 20 min. The pellets were stored at −20 °C for subsequent DNA extraction.

For cultivable fungal microorganisms isolation, 15 g of the berries were placed in a 30 mL of liquid MD medium (2% dextrose, 1% (NH4)2SO4, 0.09% KH2PO4, 0.05% MgSO4, 0.023% K2HPO4, 0.01% NaCl, 0.01% CaCl2) for 24 h at room temperature with shaking at 100 rpm. Outwashes were serially diluted in MD medium and plated on extract-peptone-dextrose (YPD)-agar plates (1% yeast extract, 1% peptone, 2% dextrose, 2% agar) containing 50 µg mL⁻¹ chloramphenicol and incubated for 3–5 days at 25 °C. Morphologically distinct yeast-like colonies were applied for molecular identification.

2.3. Metagenomic Analysis of Sea Buckthorn Fungal Microbiota

For metagenomic analysis, DNA was isolated from collected sediments (40 mg of pellet per sample) using a Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and following the manufacturer’s protocol. The quantity and quality of extracted DNA were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA samples of fungal microorganism populations were amplified using the ITS2 region-specific primers: ITS3-KYO2 (5′-GATGAAGAACGYAGYRAA-3′) and ITS4 (5′-TCCCTCGCTTATTGATATGC-3′) [36]. Amplicon libraries were prepared using modified Illumina adapters (www.illumina.com), validated on an Agilent Technologies Bioanalyzer DNA 1000 and paired-end sequenced (2 × 300 bp) using Illumina MiSeq platform (BaseClear B.V. Leiden Netherlands). Complete data sets were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Accession number PRJNA590349).

2.4. Bioinformatics Analysis

Sequences were pre-processed, quality filtered, and analyzed using QIIME2 version 2018.4 [37]. Forward and reverse reads were trimmed with Cutadapt 2.8 [38]. Primer-free fastq files were processed using Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin implemented in QIIME2. Reads with higher than 2.6 expected error rates were discarded. Chimeric sequences were eliminated using
‘consensus’ method. Alpha and beta-diversity analyses were performed with the q2-diversity plugin in QIIME2 at a sampling depth of 43458. Principal coordinates analysis (PCoA) was performed based on non-phylogenetic Bray–Curtis distances and visualized with the make_2d_plots.py script. To assign taxonomy of obtained Amplicon sequence variants (ASV) we used the QIIME2 q2-feature-classifier plugin and the v7.2 UNITE database as the reference at 97% identity [39].

2.5. Cultivable Yeast Identification

Yeast strains were streak-plated from single colonies onto YPD-agar medium supplemented with 50 µg mL⁻¹ chloramphenicol and incubated for 3 days at 25 °C. The colors and the structure of the colonies were recorded. For the microscopy study, slides were prepared from 1-week-old cultures and observed under the light microscope (Leica DM750, Wetzlar, Germany), by recording micrographs with the digital camera (Leica ICC50 HD, Wetzlar, Germany).

DNA was isolated from fresh yeast culture (24 h) by using the Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) following the manufacturer’s instructions. For identification of yeast, PCR amplification of the region between the 18S rRNA and 28S rRNA genes was performed using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers. The reaction mixtures (50 µL) contained 5 µL of DreamTaq green buffer, 1 µL of 2 mM dNTP mix, 1 µL of each primer (10 µmol L⁻¹), 2.5 unit of DreamTaq DNA polymerase (all from Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and 1 µL of DNA template (5 ng). The PCR conditions were as follows: initial denaturation at 94 °C for 5 min; 25 cycles of 94 °C for 1 min, 53 °C for 1 min 30 s, 72 °C for 2 min; a final elongation at 72 °C for 10 min. For preliminary yeast species identification, PCR products were digested with CfoI and HinfI enzymes and restriction profiles were checked by 1% agarose gel electrophoresis. PCR products were purified using a GeneJet PCR purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and sequenced using ITS1 and/or ITS4 primers at BaseClear (Leiden, Netherlands). The generated sequences were compared with those found in the FASTA network service of the EMBL-EBI database (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) and deposited in the National Center for Biotechnology Information (NCBI) under accession numbers MN700626, MN700627, MN700628, and MN700629.

2.6. Sampling and Identification of Volatile Organic Compounds (VOCs) Produced by Yeasts

The solid-phase micro-extraction (SPME) technique was applied to collect the headspace volatiles of yeasts. Yeasts were cultivated in polystyrene Petri dishes (Ø 55 mm × 14 mm) on YPD-agar (14 mL) for 2 days at 25 °C. For sampling background volatiles, YPD-agar plates without yeast were used as control samples. SPME fiber coated with a polydimethylsiloxane-divinylbenzene polymer (DVB/PDMS, 65 mm coating layer thickness, Supelco, Pennsylvania, USA) was used. Before each collection, purification of SPME fibers was conducted at 240 °C for about 10 min in a GC injector. The needle of SPME syringe was placed above the yeast layer through a small hole made in a Petri dish; the fiber was exposed to the headspace for 60 min at room temperature. After sampling, the fiber was inserted for 2 min into the injection port of gas chromatograph for desorption of the volatiles.

The volatiles collected were analyzed using Shimadzu gas chromatograph GC-2010 coupled with Shimadzu mass selective detector MS-QP 2010 Plus (Kyoto, Japan). In the GC, Restek Stabil-Wax column (30 m × 0.25 mm × 0.25 µm, Bellefonte, PA, USA) was used. The oven temperature was programmed as follows: the initial temperature was maintained isothermally at 40 °C for 1 min; afterward, it was raised to 200 °C at a rate of 5 °C min⁻¹, then increased to 240 °C at a rate of 10 °C min⁻¹, and maintained isothermally for 11 min. The GC injector temperature was set at 240 °C. Helium was used as carrier gas (1.5 mL min⁻¹). The relative amount of each of the compounds was counted based on the area of the chromatographic peak. The volatile compounds were identified according to their mass spectra and their retention indexes in a NIST version 2.0 mass spectral library (National Institute
of Standards and Technology, USA) as well as co-chromatography of synthetic standards. Software GCMS solution version 2.71 (Shimadzu, Kyoto, Japan) was applied for data analysis.

2.7. Statistical Data Analysis

Nonparametric Mann–Whitney U test was applied to evaluate differences of volatile amounts between yeast and control samples, i.e., YPD-agar plates without yeast, using the Statistica 6.0 program package (StatSoft, Inc., Tulsa, OK, USA). To assess and visualize the associations between odor blends of four yeast species and volatile compounds, the principal component analysis (PCA) was performed using Canoco 4.5 software (Biometris Plant Research International, Wageningen, The Netherlands). Amounts of styrene, 2,5-dimethyl pyrazine, 2-ethylhexanol and methoxy-phenyl-oxime derived from yeast samples did not differ from those collected from the control; hence, they were not included in PCA analysis. Significantly smaller amounts of the compounds collected from the control samples were subtracted from the amounts released by yeasts. By Canoco 4.5 software, absolute amounts expressed as area under chromatographic peak were log-transformed, scaled dividing each value by its standard deviation and centered.

3. Results

3.1. Diversity and Richness of Fungal Microorganism Communities

To characterize the diversity of fungal microorganism community distributed on sea buckthorn at different ripening stages, we harvested berries from the three distinct shrubs at private organic plantation and generated a combined sample as one biological replicate. For metagenomics analysis, three biological replicate samples of unripe berries (UB) obtained from different locations of the sea buckthorn plantation and three corresponding to ripe berries (RB) were used. Sequencing of the ITS2 rRNA partial gene region of DNA samples generated a total of 726,634 reads with an average of 121,105 sequences per sample (range 897,20–164,922) (Table 1). Following quality filtering, 454,538 sequences were recovered. The clustering of the sequences generated a total of 1615 unique amplicon sequence variants (ASVs) (1014 [338±70, hereafter median for 3 samples ± standard deviation] for fungal ITS2 from unripe berries and 601 [200±35.6] ASV for ripe berries). The observed number of ASV reveals a significant difference in fungal community richness between UB and RB sample groups (ANOVA p=0.038).

|             | UB1  | UB2  | UB3  | RB1  | RB2  | RB3  | Total |
|-------------|------|------|------|------|------|------|-------|
| Reads Obtained | 103,024 | 164,922 | 89,720 | 114,814 | 135,098 | 119,056 | 726,634 |
| High Quality Reads | 49,891 | 110,782 | 43,458 | 76,641 | 92,822 | 80,944 | 454,538 |
| ASV         | 300  | 419  | 295  | 190  | 240  | 171  | 1615  |
| Shannon Diversity | 5.26  | 5.00  | 5.37  | 3.71  | 3.795 | 3.21  | |
| Pielou Eveness | 0.64  | 0.58  | 0.66  | 0.49  | 0.48  | 0.44  | |
| Simpson Index | 0.94  | 0.91  | 0.94  | 0.86  | 0.85  | 0.79  | |

The microbial community analysis according to the nonparametric Kruskal–Wallis test showed a statistically significant decrease (p-value 0.046) in fungal microorganism diversity amongst RB samples in comparison to UB samples. In agreement with ASV data, Shannon’s diversity and Simpson indexes estimate that unripe sea buckthorn berries have a higher fungal microorganism diversity than ripe berries. Rarefaction curve approached plateaus indicating that the sequencing depth was sufficient to capture the microbial diversity of each sample (Figure S1). Principal coordinate analysis (PCoA) accomplished with the representative ASVs demonstrated a clear separation of UB and RB samples.
samples, indicating a difference in the composition of the fungal microbiota (Figure 1). Clustering of triplicate sample sequence libraries using the Bray–Curtis dissimilarity index revealed consistency of microbial community structure among replicates.

![Figure 1](image-url)

**Figure 1.** Principal coordinate analysis (PCoA) of the relative abundance of fungal microorganism amplicon sequence variants (ASVs) associated with unripe (UB) and ripe (RB) sea buckthorn berries. PCoA plot is based on unweighted UniFrac distance metrics.

3.2. Composition of Fungal Microbiota on Unripe and Ripe Sea Buckthorn Berries

A total of four phyla, 69 orders, and 144 families were identified in the present study (Table S1). Based on the average abundance analysis, the dominating phyla on both sea buckthorn samples were Ascomycota (85.0% for UB and 97.6% for RB, respectively) followed by the Basidiomycota (10.6% and 1.7% in UB and RB, respectively) and other fungi (Figure 2A; Table S1). The impact of ripening on the fungal microorganism community was notable starting at the class level. Dothideomycetes were present in large numbers in both samples (54.0% for UB and 44.6% for RB, respectively) (Figure 2B). However, on unripe berries (UB) the most abundant classes were Taphrinomycetes (18.4%) and Tremellomycetes (7.6%) when in RB samples Saccharomycetes (47.6%) prevailed. At the family level, in UB samples we found dominating Aureobasidiaceae and Taphrinaceae, while RB samples were prevalent by Aureobasidiaceae and Metschnikowiaceae (Figure 2C). In total, 196 genera were identified in this study; the most abundant were *Aureobasidium* (31.4%), *Taphrina* (18.4%), and *Cladosporium* (8.1%) for UB samples, and *Metschnikowia* (44.5%) with *Aureobasidium* (40.6%) for RB samples (Figure 2D).
Figure 2. Fungal microorganism community distribution on sea buckthorn unripe (UB) and ripe (RB) berries. Relative abundance of sequences classified at the phylum (A), class (B), family (C), and genus (D) level. The taxonomic groups comprising less than 1% of the total composition were assigned to “Other”.

The Venn diagram presents the distribution of unique fungal ASVs in UB and RB samples (Figure 3). A total of 887 unique ASVs found in this study, 198 were shared by both UB and RB samples, while 493 ASVs were exclusive to UB and 196 to RB samples, respectively.

Figure 3. Venn diagram illustrating the number of unique and shared amplicon sequence variants (ASVs) among sea buckthorn samples at different ripening stages. UB – unripe sea buckthorn berries, RB – ripe berries.

A heatmap diagram illustrates the distribution of the most abundant fungal ASVs (Figure 4). Hierarchical cluster analysis showed that the microbial community structure displays different patterns
between UB and RB groups, while samples collected within the same ripening stage feature similar community structure. The core microbiomes detected in both samples comprised of ASV1 and ASV3 assigned to *Aureobasidium pullulans* (Figure 4; Table S2). At the beginning of ripening, besides the *Aureobasidium pullulans* the most abundant ASVs were ASV4 (7.6%) and ASV14 (2.4%) (both *Taphrina carpini*), ASV10 (4.7%) (Taphrina), ASV6 (6.8%) (*Cladosporium*), and ASV20 (1.6%) (*Filobasidium wieringae*). During the ripening of the berries, their prevalence diminished to less than 1%. A dramatic change was observed between the beginning and the end of the ripening period, mainly due to the increase of fungal microorganisms belonging to *Metschnikowia* spp. (ASVs 2, 5, 7, 9, 11, 22, and 30). The overall abundance of ASVs assigned to *Metschnikowia* in RB berries reached 44%, while on the unripe berries they consisted only 0.7%.

![Figure 4. Heatmap of fungal microorganism’s unique amplicon sequence variants (ASVs) abundance on sea buckthorn. The color intensity is proportional to the relative abundance of fungal microorganism ASVs.](image)

3.3. Sea-Buckthorn-Associated Cultivable Potential Biocontrol Yeasts and Their Produced Volatiles

By applying cultivation techniques, more than 20 yeast strains were isolated from the surface of sea buckthorn berries. Yeasts were subjected for primary species identification by morphological colony analysis and bright field microscopy with following fingerprinting of PCR-amplified sequences of internal transcribed spacers 1 and 2 including 5.8S ribosomal RNA gene. For further analysis of VOC production, selected strains proceeded sequencing-based species level confirmation and deposition of sequences in NCBI.

Four yeast strains, belonging to different genera identified by metagenomics analysis of UB and RB microbiota with referred potential biocontrol properties [15,16,40] (Table S1, Figure 5), were selected for VOCs analysis. Morphologically different SB-18-2 and SB-18-25 yeast strains were recovered from UB berries while SB-18-34 and SB-18-31 yeast strains were isolated from RB berries. Basing on the electrophoretic analysis, similar size (about 600 bp) ITS regions were PCR-amplified from SB-18-2 and SB-18-25 strains, and differed in *HinfI* and *CfoI* restriction patterns (Figure 5). The sequence identity
match of SB-18-2 yeast was 99.64%, comparing to that of *Aureobasidium pullulans* culture YY11 deposited in GenBank. SB-18-25 yeast strain was assigned to *Cryptococcus wieringae* (syn. *Filobasidium wieringae*) basing on RFLP profile and 99.48% similarity to *C. wieringae* CBS:11709 culture. Following the genomic DNA purification, 380 bp and 700 bp PCR products were amplified from SB-18-34 and SB-18-31 yeast strains, respectively. Their PCR-RFLP profiles corresponded to *Metschnikowia* and *Hanseniaspora* yeast species [41]. According to the high similarity to sequences deposited in GenBank, SB-18-34 strain was identified as *Metschnikowia pulcherrima* and SB-18-31 as *Hanseniaspora uvarum* yeast species (Figure 5).

![Figure 5.](image)

Aureobasidium pullulans SB-18-2 belongs to genus, which is the most abundant in UB samples (31.4%), and of significant representation (40.6%) in RB samples. Metschnikowia pulcherrima SB-18-34 represents genus, which is the most abundant (44.5%) in RB samples. Cryptococcus wieringae SB-18-25 belongs to genus, more prevalent (1.9%) on the surface of UB berries than on the RB berries (0.2%). Hanseniaspora uvarum SB-18-31 is a member of genus, which is the least dominant among the selected genera with only 0.5% and 0.2% occurrence in UB and RB samples, respectively.

The gas chromatographic-mass spectrometric analysis revealed 29 compounds that were exclusively present in the headspace of four yeast species and occurred significantly more abundant compared to those of control samples. The yeast released volatiles were composed of 11 esters, 6 alcohols, 5 volatile fatty acids, 3 ketones, 2 aromatics, and 2 compounds bearing both ester and aromatic moieties (Table 2).
Table 2. Odor blends of four yeast species and control sampled by SPME technique.

| No. | Compound                   | CAS No. | RI   | GR   | H. uvarum   | M. pulcherrima | A. pullulans | A. wieringae | Control |
|-----|----------------------------|---------|------|------|-------------|----------------|--------------|--------------|---------|
| 1   | Ethyl acetate              | 141-78-6| 898  | ES   | 26.26 ± 4.63 | 8.98 ± 0.28    | 0            | 0            | 0.06 ± 0.02 |
| 2   | Ethanol                    | 64-17-5 | 902  | OH   | 4.58 ± 0.36  | 4.89 ± 0.42    | 6.26 ± 0.82  | 0.39 ± 0.03  | 0.10 ± 0.02 |
| 3   | Ethyl propionate           | 1105-37-3| 915  | ES   | 1.00 ± 0.05  | 0.24 ± 0.01    | 0            | 0            | 0.01 ± 0.001|
| 4   | 2-Methylprop-1-yl acetate  | 110-19-0| 985  | ES   | 0.31 ± 0.09  | 0.07 ± 0.01    | 0            | 0            | 0         |
| 5   | Ethyl butanoate            | 105-54-4| 1013 | ES   | 0.05 ± 0.03  | 0              | 0            | 0            | 0         |
| 6   | Ethyl 2-methylbutanoate    | 7452-79-1| 1033 | ES   | 0.02 ± 0.01  | 0              | 0            | 0            | 0         |
| 7   | Ethyl 3-methylbutanoate    | 108-64-5| 1050 | ES   | 0              | 0.04 ± 0.01    | 0            | 0            | 0         |
| 8   | Butyl acetate              | 123-86-4| 1063 | ES   | 0.28 ± 0.03   | 0              | 0            | 0            | 0.10 ± 0.02 |
| 9   | 2-Methylpropanol           | 78-83-1 | 1095 | OH   | 0.42 ± 0.07  | 0.98 ± 0.08    | 1.04 ± 0.13  | 1.70 ± 0.11  | 0         |
| 10  | 3-Methylbutyl acetate      | 123-92-2| 1105 | ES   | 13.28 ± 0.5  | 0.40 ± 0.02    | 0.15 ± 0.05ns| 0.07 ± 0.01ns| 0.10 ± 0.07 |
| 11  | Butanol                    | 71-36-3 | 1149 | OH   | 0              | 0.13 ± 0.03    | 0.09 ± 0.01  | 0            | 0         |
| 12  | 2-Heptanone                | 110-43-0| 1170 | KT   | 0              | 0.07 ± 0.01    | 0.06 ± 0.01  | 0            | 0         |
| 13  | 3-Methylbutyl propionate   | 105-68-0| 1176 | ES   | 0.62 ± 0.09   | 0              | 0            | 0            | 0         |
| 14  | 2-Methylbutanol            | 137-32-6| 1207 | OH   | 1.63 ± 0.28   | 1.77 ± 0.24    | 0.84 ± 0.18  | 6.47 ± 1.21  | 0         |
| 15  | 3-Methylbutanol            | 123-51-3| 1213 | OH   | 3.28 ± 0.37   | 5.95 ± 0.56    | 4.07 ± 0.51  | 10.39 ± 0.36 | 0.21 ± 0.11 |
| 16  | Ethyl hexanoate            | 123-66-0| 1224 | ES   | 0.02 ± 0.01   | 0              | 0            | 0            | 0         |
| 17  | Styrene*                   | 100-42-5| 1238 | AR   | 0.20 ± 0.04ns | 0.13 ± 0.03ns  | 0.07 ± 0.01ns| 0.16 ± 0.01ns| 0.14 ± 0.06ns|
| 18  | 2-hydroxy-3-butanol        | 513-86-0| 1273 | KT   | 0.15±0.04   | 0.14 ± 0.01    | 0.06 ± 0.01  | 0            | 0         |
| 19  | 2,5- Dimethyl pyrazine*    | 123-32-0| 1316 | PY   | 0.02±0.01ns | 0.01 ± 0.001ns| 0.03 ± 0.01ns| 0.04 ± 0.01ns| 0.03 ± 0.02ns|
| 20  | 6-Methyl 5-hepten-2-one    | 110-93-0| 1327 | KT   | 0              | 0.93 ± 0.10    | 0            | 0            | 0         |
| 21  | Ethyl octanoate            | 106-32-1| 1430 | ES   | 0.12 ± 0.01  | 0              | 0            | 0            | 0         |
| 22  | Acetic acid                | 64-19-7 | 1449 | FA   | 1.15 ± 0.25   | 0              | 0.01 ± 0.008 | 0.07 ± 0.02  | 0         |
| 23  | 6-Methyl-5-hepten-2-ol     | 1569-60-4| 1461 | OH   | 0              | 0.08 ± 0.02    | 0            | 0            | 0         |
| 24  | 2-Ethylhexanol*            | 104-76-7| 1488 | OH   | 0.02 ± 0.001ns| 0.02±0.01ns | 0.02±0.001ns| 0.02±0.0004ns| 0.03±0.01ns|
| 25  | Propionic acid             | 1979-09-4| 1528 | FA   | 0              | 0              | 0            | 0.03 ± 0.004 | 0         |
| 26  | 2-Methylpropionic acid     | 79-31-2 | 1562 | FA   | 0              | 0              | 0            | 0.18 ± 0.04  | 0         |
| 27  | Butanoic acid              | 107-92-6| 1634 | FA   | 0.03 ± 0.01   | 0              | 0            | 0.03 ± 0.01  | 0         |
| 28  | 3-Methylbutanoic acid      | 503-74-2| 1703 | FA   | 0.07 ± 0.02   | 0.01 ± 0.001   | 0.06 ± 0.04  | 0.71 ± 0.25  | 0         |
| 29  | Methoxy-phenyl-oxime*      | 1767   | IM   | 0.03±0.01ns | 0.01 ± 0.003   | 0.01 ± 0.004  | 0.01 ± 0.01ns| 0.03 ± 0.01ns| 0         |
| 30  | 2-Phenylethyl acetate      | 103-45-7| 1795 | ES/AR| 3.52 ± 0.64   | 0.04 ± 0.01    | 0            | 0            | 0         |
| 31  | 2-Phenylethyl propionate   | 122-70-3| 1858 | ES/AR| 0.11 ± 0.02   | 0              | 0            | 0            | 0         |
| 32  | Phenylmethanol             | 1960-12-8| 1856 | AR   | 0              | 0.03 ± 0.01    | 0            | 0            | 0         |
| 33  | 2-Phenylethanol            | 1960-12-8| 1894 | AR   | 3.60±0.26     | 3.45±0.65     | 2.28±0.45    | 0.21±0.03    | 0.03±0.01  |

Compounds indicated by * mark were excluded from PCA; CAS No.—chemical abstract service number; RI—retention index (DB-Wax fused silica capillary column 30 m x 0.25 mm i.d., 0.25 µm film thickness); GR—group of chemical compound; ES—ester; OH—alcohol; KT—ketone; AR—aromatic; PY—pyrazine; FA—fatty acid; IM—imine; all values in the columns headed by yeast names are the absolute amounts expressed as areas under the chromatographic peaks and have to be read as numbers times 100000 ± standard error of the mean followed by the "ns" are not significantly different compared to control (nonparametric Mann–Whitney U test, P < 0.05); control samples were obtained by collecting background volatiles from YPD-agar plates without yeast.
The principal component analysis revealed that the volatile blend of any of the four yeast species was clearly separated from each other. The first principal component axis accounted for 56% of the total variation and to a large extent explained separation between volatile blends of *C. wieringae* versus *H. uvarum* yeasts and *A. pullulans* versus *M. pulcherrima* yeasts (Figure 6A). Volatile profiles of *C. wieringae* yeasts were characterized by five alcohols (9, 11, 14, 15, and 23), three volatile fatty acids (25, 26, and 28), and two ketones (12 and 20) which were exclusively present or occurred in the large amounts compared to those released by *H. uvarum* yeasts (Figure 6B, Table 2). Ten esters were unique for the volatile blends of *H. uvarum* yeasts in addition to acetic acid (22), 2-hydroxy-3-butanone (18), and 2-phenylethanol (33) with amounts larger compared to those of *C. wieringae* yeasts (Figure 6B, Table 2). *M. pulcherrima* yeasts released the larger amounts of esters (1, 3, 4, and 10), while *A. pullulans* yeasts released the higher amounts of two alcohols (ethanol (2), butanol (11)) (Table 2).

![Figure 6](image-url) Associations between blends and their components of four species of yeasts populating sea buckthorn berries. Associations are visualized by principal component analysis (PCA). (A) Distribution of odor blends released by *C. wieringae* (Cw, diamond shape), *A. pullulans* (Ap, triangle shape), *M. pulcherrima* (Mp, square shape), and *H. uvarum* (Hu, circle shape). n=5 for each species. (B) Distribution of volatile compounds composing the blends. Stars represent the volatile compounds indicated by numbers. Names of the compounds are listed in Table 2.

The second PC axis accounted for 25% of the total variation and explained the separation between volatile blends of *A. pullulans* and *C. wieringae* species and in part between volatile blends of *M. pulcherrima* and *H. uvarum* yeasts (Figure 6A). 6-Methyl 5-hepten-2-one (20), 6-methyl-5-hepten-2-ol (23), propionic acid (25) and 2-methylpropionic acid (26) were unique volatiles released by *C. wieringae*, while only ethyl 3-methylbutanoate (7) was specific for *A. pullulans* (Figure 6B, Table 2). Ethyl butanoate (5), ethyl 2-methylbutanoate (6), 3-methylbutyl propionate (13), ethyl hexanoate (16), ethyl octanoate (21), and 2-phenylethyl propionate (31) were exclusively present in volatile blends of *H. uvarum* yeasts, and phenylmethanol (32) was unique for *M. pulcherrima* (Figure 6B, Table 2).

4. Discussion

The current study, for the first time, uncovered the structure of fungal microorganism communities colonizing the surface of sea buckthorn berries at different ripening stages in natural habitat. For a comprehensive analysis, we have applied next generation sequencing and observed the wide diversity of eukaryotic microorganisms on tested berries. The total abundance of fungal microorganisms changed significantly during the period of fruit ripening. The microbial alpha-diversity—estimated by the Simpson’s index, Shannon’s diversity index, and Pielou’s evenness index—was highest among the fungal community on unripe berries. The analysis of the fungal microbiota structure revealed clear differentiation between unripe and mature berries, as demonstrated by the Bray–Curtis beta-diversity
analysis. According to our data, unripe sea buckthorn berries were dominated by *Aureobasidium*, *Taphrina*, *Cladosporium*, and *Filobasidium* (syn. *Cryptococcus*) genera microorganisms, while on ripe berries prevailed *Aureobasidium* and *Metschnikowia* (Table S1, Figure 2). It was previously demonstrated that *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, and *Aureobasidium pullulans* yeasts were associated with the early stage of maturation of fruits and berries, such as apples, grapes, nectarines, etc. [29,32,42]. In our previous work, it was demonstrated that Dothioraceae family fungal microorganisms prevailed on sea buckthorn berries, which at the lower taxonomic level were attributed to uncultured *Aureobasidium* [33]. Our results were partially consistent with previous findings, since most fungal microorganisms present on sea buckthorn were represented by *Aureobasidium*. However, the overall structure of fungal microbiota varies most likely depending on the differences in climatic conditions, berry harvesting period (previously—September 2016; in this study—July 2018), localization of sea buckthorn plantation, etc.

Various microorganisms act as biological control agents of phytopathogens and have a role in postharvest decay and toxin contamination by emitting volatile organic compounds [40,43]. Several biocontrol yeasts (e.g., *Candida oleophila*, *Aureobasidium pullulans*, *Metschnikowia fructicola*, *Cryptococcus albidus*, *Saccharomyces cerevisiae*) have already been used for the production of commercial plant protection products, encouraging the deeper exploration of the fascinating traits and application possibilities of other yeasts as well [16,44,45]. Even the different strains of the same yeast species are able to produce highly different amounts of the same VOCs (e.g., *A. pullulans* [43]), thus it is important to investigate volatiles of yeasts strains within the species that are already proven to possess biocontrol traits (like *Aureobasidium pullulans* [43,46], *Metschnikowia pulcherrima* [46], and *Hanseniaspora uvarum* [47]), or are less investigated, but closely related to bioactive yeasts (e.g., potential biocontrol yeast *Cryptococcus wieringae* which belongs to the same genus as *C. albidus*, *C. laurentii*, and *C. flavus* [16,48–50]).

*Aureobasidium pullulans* is well-known yeast-like fungus hosting on plants, mostly present in a cooler climate like that of central Europe and less related to grapes grown in warm areas [51]. It was revealed that some *Aureobasidium* species demonstrated biological control activity against phytopathogens, such as *Botrytis*, *Bacillus*, *Colletotrichum*, *Penicillium* [43,52,53]. The antagonistic activity of *A. pullulans* mainly includes nutrient competition and production of volatile organic compounds or glucanases, chitinases, and extracellular proteases [40,46]. *Taphrina* and *Cladosporium* were the next numerous fungal microorganisms observed on unripe sea buckthorn berries in our study (Table S1, Figure 2). *Taphrina* genus fungi are known as biotrophic plant pathogens that cause plant and fruit disease symptoms, which may be ascribed to the ability to synthesize and modulate different plant hormones [54]. *Cladosporium* is considered as ubiquitous fungi, with some species providing numerous antifungal agents [55], while others causing plant or human diseases [56,57]. *Cryptococcus* encloses species producing biocontrol agents against many pathogens [16,50], while certain species can cause infections in humans and animals [58].

During the ripening of different fruits and berries, the oxidative or weakly fermentative yeast populations—such as *Candida*, *Hanseniaspora*, *Metschnikowia*, and *Pichia*—increased in frequency [42]. *Hanseniaspora* spp. have been frequently found on the surface of different fruits, e.g. apples, grapes, or strawberries [59,60]. Numerous yeasts belonging to the *Hanseniaspora* genus display antagonistic properties against the development of fruit spoilage causing molds [40,61]. *Metschnikowia* spp. isolated from fruit are sugar-loving yeasts and their populations are most likely stimulated by juices diffusing from the damaged surface of mature fruit [42]. This yeast genus includes several biocontrol agents, which are very effective against fruit spoilage fungi [62]. The biocontrol abilities of *M. pulcherrima* have been mainly assigned either to competition for nutrients or to the production of volatile organic compounds [15,46]. The competition of *M. pulcherrima* for iron was reported to play a substantial role in biocontrol interactions [63]. *Metschnikowia* spp. are linked to several groups of nectarivorous insects, suggesting that this specificity could be correlated to the services provided by the yeast to the insect or vice versa [64,65].
Yeasts produce chemo-diverse blends of volatiles comprised of aldehydes, ketones, alcohols, volatile fatty acids, esters, aromatics, heterocyclic compounds, hydrocarbons, terpenoids, and some others [66,67]. Ethanol is probably the most known volatile metabolite of yeasts derived from carbohydrate catabolism and fermentation [66]; it is one of the major volatiles collected from the headspace of H. uvarum, M. pulcherrima, and C. wieringae yeasts. Amounts of ethyl acetate, another metabolite of carbohydrate catabolism, prevailed in the VOCs produced exclusively by H. uvarum and M. pulcherrima yeasts. It is a valuable solvent for extracting compounds with antimicrobial properties [68] and possess minor inhibitory effect for Botrytis cinerea [69]. In addition to ethyl acetate, 10 esters were unique for the volatile blends of H. uvarum yeasts; these compounds originated from amino acid synthesis and degradation pathways [66]. Prevalence of esters in volatile blends of H. uvarum has been reported in previous studies [70,71]; however, ethanol being the third largest component of the blend in our experiments, has not been determined by Babcock et al. [71] or Piper et al. [70]. High production of ethanal, 3-methylbutanol, 2-phenylethanol, ethyl acetate, and 3-methylbutyl acetate were reported for M. pulcherrima and A. pullulans yeasts [15]. The data published are comparable with the results of our study in terms of large production of alcohols by both yeast species; however, we were not able to detect ethyl acetate in the headspace of A. pullulans. To date, there is no report on VOCs of C. wieringae yeast. Volatile organic compounds produced by A. pullulans that were found in our study (namely 2-phenylethanol, 2-methylpropanol, 2-methylbutanol, and 3-methylbutanol) had been previously shown to control postharvest fruit pathogens (B. cinerea, Colletotrichum acutatum, Penicillium expansum, Penicillium digitatum, and Penicillium italicum) [43]. All these alcohols were produced by A. pullulans SB-18-2, M. pulcherrima SB-18-34, C. wieringae SB-18-25, and H. uvarum SB-18-31 but in different amounts, suggesting the potential biocontrol capabilities of these strains. 2-Phenylethanol is one of the main VOCs produced by various yeast species (e.g. Cyberlindnera jadinii, Lachancea thermotolerans, Candida intermedia, Candida friedrichii, Saccharomyces cerevisiae, Wickerhamomyces anomalus) affecting numerous pathogens (e.g. Aspergillus carbonarius, Aspergillus flavus, Aspergillus ochraceus, Penicillium digitatum, Phyllosticta citricarpa) (see [40] and references therein). Among tested strains, H. uvarum SB-18-31 and M. pulcherrima SB-18-34 produced the highest amount of 2-phenylethanol, whereas A. pullulans SB-18-2 excelled at ethanol generation, while volatilome of C. wieringae SB-18-25 was dominated by 3-methylbutanol. Ethyl acetate was the dominating volatile of H. uvarum SB-18-31 and M. pulcherrima SB-18-34. 2-Phenylethyl acetate was produced in significant amounts (comparing to other tested yeast strains) by H. uvarum SB-18-31 and had been previously reported to contribute to growth inhibition of A. ochraceus growth and ochratoxin A production during processing of coffee [47].

Due to the extremely sensitive olfaction system, insects are capable to use microbial volatiles to search for suitable feeding and oviposition sites [18,72]. Emissions from microorganisms associated with different development stages of fruits and berries contribute to the information about habitat suitability [73] and it was demonstrated that insects recognized yeast communities based on their specific volatile profiles [74–76]. Most of the volatile compounds (2-phenyl ethanol, 2-methylbutanol, 3-methylbutanol, ethyl acetate, 2-phenylethyl acetate, ethyl propionate, and others) produced by yeast in our study had been suggested to play a role in modulating behavior of Rhagoletis batava flies [67]. Examples of microbial VOC application for pest control already demonstrated their high potential for use in integrated pest management programs [77]. Identification of attractive and repellent odors from the yeasts associated with berries at preferred and unsuitable stages for pest insect feeding and oviposition would provide a base for the development of environment-friendly pull-push pest control for Rhagoletis batava flies, a major pest of the sea buckthorn.

5. Conclusions

The metagenomic approach based on the sequencing of ITS2 region of fungal rDNA revealed changes in the fungal community associated with the ripening of sea buckthorn berries. Investigation of alpha and beta diversity of the fungal community, analysis of representative microbial ASVs
showed a clear separation among inhabitants of unripe (UB) and ripe berries (RB). Based on results of metagenomics analysis, unripe berry samples prevalently had *Aureobasidium*, *Taphrina*, and *Cladosporium* genera yeasts; while ripe berries were dominated by *Aureobasidium* and *Metschnikowia*. Potential biocontrol yeasts *Aureobasidium pullulans* and *Cryptococcus wieringae* were isolated from sea buckthorn surface at the early harvesting stage, while *Metschnikowia pulcherrima* and *Hanseniaspora uvarum* were amplified from ripe berries and applied for the analysis of produced volatile organic compounds. Eleven esters, six alcohols, five volatile fatty acids, three ketones, two aromatics, and two compounds bearing both ester and aromatic moieties were isolated from the headspaces of four yeast species and identified by gas chromatography and mass spectrometry techniques. Principal component analysis revealed that volatile blends of all four yeast species were clearly separated from each other.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2076-2607/8/3/456/s1, Figure S1: Rarefaction curves at a genetic distance of 3% for each sample, Table S1: Fungal taxonomy abundance count for sea buckthorn samples at different ripening stages from phylum to species level, Table S2: Relative abundance of the most common ASVs.

**Author Contributions:** Conceptualization, E.S., V.B., and R.M.; Collection of plant material, I.V.M., D.A., and B.R.; Metagenomic analysis of fungal microorganisms, I.V.-M., J.L., and B.R.; Bioinformatic analysis, J.L. and E.S.; Cultivable yeast isolation and identification, I.V.-M., R.S., Ž.S.-Ž., and B.R.; Sampling and identification of volatiles, V.A., L.B.-Č., and D.A.; Data interpretation, E.S., R.M., and V.B.; Funding acquisition, R.M.; Writing—original draft, E.S., J.L., and R.M.; Writing—review and editing, E.S., B.R., R.M., and V.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by European Social Fund/European Regional Development Fund under grant agreement with the Research Council of Lithuania (LMTLT), grant number 09.3.3-LMT-K-712-01-0099.

**Acknowledgments:** We would like to thank to Povilas Šukys for kind permission to collect sea buckthorn berries in his plantation.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

**References**

1. Ruan, C.J.; Rumpunen, K.; Nybom, H. Advances in improvement of quality and resistance in a multipurpose crop: sea buckthorn. *Crit. Rev. Biotechnol.* 2013, 33, 126–144. [CrossRef] [PubMed]
2. Li, G.; Du, S.; Guo, K. Evaluation of limiting climatic factors and simulation of a climatically suitable habitat for Chinese sea buckthorn. *PloS ONE* 2015, 10, e0131659.
3. Li, T.S.C.; Schroeder, W.R. Sea buckthorn (*Hippophae rhamnoides* L.): a multipurpose plant. *Hort. Technol.* 1996, 6, 370–380. [CrossRef]
4. Patel, C.A.; Divakar, K.; Santani, D.; Solanki, H.K.; Thakkar, J.H. Remedial Prospective of *Hippophae rhamnoides* Linn. (Sea Buckthorn). *ISRN Pharmacol.* 2012, 2012, 436857. [CrossRef] [PubMed]
5. Zheng, J.; Yang, B.; Trépanier, M.; Kallio, H. Effects of genotype, latitude, and weather conditions on the composition of sugars, sugar alcohols, fruit acids, and ascorbic acid in sea buckthorn (*Hippophaë rhamnoides ssp. mongolica*) berry juice. *J. Agric. Food Chem.* 2012, 60, 3180–3189. [CrossRef]
6. Olas, B. The beneficial health aspects of sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) oil. *J. Ethnopharmacol.* 2018, 213, 183–190. [CrossRef]
7. Ulanowska, K.; Skalski, B.; Olas, B. Sea-buckthorn (*Hippophae rhamnoides* L.) as a source of compounds with antitumor and radioprotective activity. *Postepy Higieny i Medycyny Doświadczalnej.* 2018, 72, 240–252. [CrossRef]
8. Martins, G.; Miot-Sertier, C.; Lauga, B.; Claissé, O.; Lonvaud-Funel, A.; Soulas, G.; Masneuf-Pomarède, I. Grape berry bacterial microbiota: Impact of the ripening process and the farming system. *Int. J. Food Microbiol.* 2012, 158, 93–100. [CrossRef]
9. Taqarort, N.; Echairi, A.; Chaussod, R.; Nouaim, R.; Boubaker, H.; Benoumar, A.A.; Boudyah, E. Screening and identification of epiphytic yeasts with potential for biological control of green mold of citrus fruits. *World J. Microbiol. Biotechnol.* 2008, 24, 3031–3038. [CrossRef]
10. Janisiewicz, W.J.; Jurick, W.M.; Peter, K.A.; Kurtzman, C.P.; Buyer, J.S. Yeasts associated with plums and their potential for controlling brown rot after harvest. *Yeast* 2014, 31, 207–218. [CrossRef]

11. Li, Z.T.; Janisiewicz, W.J.; Liu, Z.; Callahan, A.M.; Evans, B.E.; Jurick, W.M. II; Dardick. C. Exposure in vitro to an Environmentally Isolated Strain TC09 of Cladosporium sphaerospermum Triggers Plant Growth Promotion; Early Flowering; and Fruit Yield Increase. *Front. Plant Sci.* 2019, 9, 1999. [CrossRef]

12. Droby, S.; Wisniewski, M.; Macarisin, D.; Wilson, C. Twenty years of postharvest biocontrol research: is it time for a new paradigm? *Postharvest Biol. Technol.* 2009, 52, 137–145. [CrossRef]

13. Buser, C.C.; Newcomb, R.D.; Gaskett, A.C.; Goddard, M.R. Niche construction initiates the evolution of mutualistic interactions. *Ecol. Lett.* 2014, 17, 1257–1264. [CrossRef] [PubMed]

14. Parafati, L.; Vitale, A.; Restuccia, C.; Cirvilleri, G. Performance evaluation of volatile organic compounds by antagonistic yeasts immobilized on hydrogel spheres against gray; green and blue postharvest decays. *Food Microbiol.* 2017, 63, 191–198. [CrossRef] [PubMed]

15. Contarino, R.; Brighina, S.; Fallico, B.; Cirvilleri, G.; Parafati, L.; Restuccia, C. Volatile organic compounds (VOCs) produced by biocontrol yeasts. *Food Microbiol.* 2019, 82, 70–74. [CrossRef] [PubMed]

16. Freimoser, F.M.; Rueda-Mejía, M.P.; Tilocca, B.; Migheli, Q. Biocontrol yeasts: mechanisms and applications. *World J. Microbiol. Biotech.* 2019, 35, 154. [CrossRef] [PubMed]

17. Becher, P.G.; Flick, G.; Rozpedowska, E.; Schmidt, A.; Hagman, A.; Lebreton, S.; Larsson, M.C.; Hansson, B.S.; Piškur, J.; Witzgall, P.; et al. Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. *Funct. Ecol.* 2012, 26, 822–828. [CrossRef]

18. Davis, T.S.; Crippen, T.L.; Hofstetter, R.W.; Tomberlin, J.K. Microbial volatile emissions as insect semiochemicals. *J. Chem. Ecol.* 2013, 39, 840–859. [CrossRef]

19. Pretorius, I.S. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 2000, 16, 675–729. [CrossRef]

20. Pinto, C.; Pinho, D.; Sousa, S.; Pinheiro, M.; Egas, C.; Gomes, A.C. Unravelling the diversity of grapevine microbiome. *PLoS ONE* 2014, 9, e85622. [CrossRef]

21. Pinto, C.; Pinho, D.; Cardoso, R.; Custódio, V.; Fernandes, J.; Sousa, S.; Pinheiro, M.; Egas, C.; Gomes, A.C. Wine fermentation microbiome: A landscape from different Portuguese wine appellations. *Front. Microbiol.* 2015, 6, 905. [CrossRef] [PubMed]

22. Saleem, M.; Moe, L.A. Multitrophic microbial interactions for eco- and agro-biotechnological processes: theory and practice. *Trends Biotechnol.* 2014, 32, 529–537. [CrossRef] [PubMed]

23. Abdelfattah, A.; Li Destri Nicosia, M.G.; Cacciola, S.O.; Droby, S.; Schena, L. Metabarcoding Analysis of Fungal Diversity in the Phyllosphere and Carposphere of Olive (Olea europaea). *PLoS ONE* 2015, 10, e0131069. [CrossRef] [PubMed]

24. Abdelfattah, A.; Wisniewski, M.; Droby, S.; Schena, L. Spatial and compositional variation in the fungal communities of organic and conventionally grown apple fruit at the consumer point-of-purchase. *Hortic Res.* 2016, 3, 16047. [CrossRef] [PubMed]

25. Abdelfattah, A.; Wisniewski, M.; Nicosia, M.G.L.D.; Cacciola, S.O.; Schena, L. Metagenomic analysis of fungal diversity on strawberry plants and the effect of management practices on the fungal community structure of aerial organs. *PLoS ONE* 2016, 11, e0160470. [CrossRef] [PubMed]

26. Clooney, A.G.; Fouhy, F.; Sleator, R.D.; O’ Driscoll, A.; Stanton, C.; Cotter, P.D.; Claesson, M.J. Comparing apples and oranges?: Next Generation Sequencing and its impact on microbiome analysis. *PLOS ONE* 2016, 11, e0148028. [CrossRef]

27. Droby, S.; Wisniewski, M.; Teixidó, N.; Spadaro, D.; Jijakli, M.H. The science, development, and commercialization of postharvest biocontrol products. *Postharvest Biol. Tech.* 2016, 122, 22–29. [CrossRef]

28. Vepštaitė-Monstavičiūtė, I.; Lukša, J.; Stanevičienė, R.; Strazdaite-Žielenė, Ž.; Jurkeno, V.; Serva, S.; Servienė, E. Distribution of apple and blackcurrant microbiota in Lithuania and the Czech Republic. *Microbiol. Res.* 2018, 206, 1–8.

29. Janisiewicz, W.J.; Kurtzman, C.P.; Buyer, J.S. Yeasts associated with nectarines and their potential for biological control of brown rot. *Yeast* 2010, 27, 389–398. [CrossRef] [PubMed]

30. Paramithiotis, S.; Kouretas, K.; Drosinos, E.H. Effect of ripening stage on the development of the microbial community during spontaneous fermentation of green tomatoes. *J. Sci. Food Agric.* 2014, 94, 1600–1606. [CrossRef] [PubMed]
31. Diskin, S.; Feygenberg, O.; Maurer, D.; Droby, S.; Prusky, D.; Alkan, N. Microbiome alterations are correlated with occurrence of postharvest stem-end rot in mango fruit. *Phytoomiomes J.* 2017, 1, 117–127. [CrossRef]

32. Vagkertiova, R.; Molnarova, J.; Vranova, D.; Slavikova, E. Yeasts and yeast-like organisms associated with fruits and blossoms of different fruit trees. *Can. J. Microbiol.* 2012, 58, 1344–1352. [CrossRef] [PubMed]

33. Lukša, J.; Vepštaitė-Monstaviči, I.; Yurchenko, V.; Serva, S.; Servienė, H. High content analysis of sea buckthorn, black chokeberry, red and white currants microbiota – A pilot study. *Food Res. Int.* 2018, 111, 597–606. [CrossRef] [PubMed]

34. Parafati, L.; Vitale, A.; Restuccia, C.; Cirvilleri, G. Biocontrol ability and action mechanism of food-isolated *Lactobacillus* strains used as postharvest biocontrol agents of postharvest fruit pathogens. *Front. Microbiol.* 2020, 11, 1–13. [CrossRef] [PubMed]

35. Toju, H.; Tanabe, A.S.; Yamamoto, S.; Sato, H. High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS ONE* 2012, 7, e40863. [CrossRef]

36. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. *PeerJ Preprints* 2018, 6, e27295v2. [CrossRef]

37. Esteve-Zarzoso, B.; Belloch, C.; Uruburu, F.; Querol, A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* 1999, 49, 329–337. [CrossRef] [PubMed]

38. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 2011, 17, 10–12. [CrossRef]

39. Diskin, S.; Feygenberg, O.; Maurer, D.; Droby, S.; Prusky, D.; Alkan, N. Microbiome alterations are correlated with occurrence of postharvest stem-end rot in mango fruit. *Phytoomiomes J.* 2017, 1, 117–127. [CrossRef]

40. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. *PeerJ Preprints* 2018, 6, e27295v2. [CrossRef]

41. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 2011, 17, 10–12. [CrossRef]

42. Nilsson, R.H.; Larsson, K.H.; Taylor, A.F.S.; Bengtsson-Palme, J.; Jeppesen, T.S.; Schigel, D.; Kennedy, P.; Picard, K.; Glöckner, F.O.; Tedersoo, L.; et al. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* 2018, 47, D259–D264. [CrossRef]

43. Tilocca, B.; Cao, A.; Migheli, Q. Scent of a Killer: Microbial Volatilome and Its Role in the Biological Control of Plant Pathogens. *Front. Microbiol.* 2020, 11, 1–13. [CrossRef] [PubMed]

44. Parafati, L.; Vitale, A.; Restuccia, C.; Cirvilleri, G. Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. *Food Microbiol.* 2015, 47, 85–92. [CrossRef]

45. Masoud, W.; Poll, L.; Jakobsen, M. Influence of volatile compounds produced by yeasts predominant during processing of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*. *Yeast* 2005, 22, 1133–1142. [CrossRef]

46. Elad, Y.; Köhl, J.; Fokkema, N. Control of infection and sporulation of *Botrytis cinerea* on bean and tomato by saprophytic yeasts. *Phytopathol.* 1994, 84, 1193–1200. [CrossRef]

47. Zhang, H.Y.; Zheng, X.D.; Yu, T. Biological control of postharvest diseases of peach with *Cryptococcus laurentii*. *Food Control.* 2007, 18, 287–291. [CrossRef]

48. Hashem, M.; Alamri, S.A.; Hesham, A.E.L.; Al-Qahtani, F.M.H.; Kilany, M. Biocontrol of apple blue mould by new yeast strains: *Cryptococcus albidus* KKUY0017 and *Wickerhamomyces anomalus* KKUY0051 and their mode of action. *Biocontrol. Sci. Technol.* 2014, 24, 1137–1152. [CrossRef]

49. Lederer, M.A.; Nielsen, D.S.; Toldam-Andersen, T.B.; Herrmann, J.V.; Arneborg, N. Yeast species associated with different wine grape varieties in Denmark. *Acta Agr. Scand. B-S.* 2013, 63, 89–96. [CrossRef]

50. Grube, M.; Schmid, F.; Berg, G. Black fungi and associated bacterial communities in the phyllosphere of grapevine. *Fungal Biol.* 2011, 115, 978–986. [CrossRef] [PubMed]
53. Parsa, S.; Garcia-Lemos, A.M.; Castillo, K.; Ortiz, V.; López-Lavalle, L.A.B.; Braun, J.; Vega, F.E. Fungal endophytes in germinated seeds of the common bean, Phaseolus vulgaris. *Fungal Biol.* 2016, 120, 783–790. [CrossRef] [PubMed]

54. Tsai, I.J.; Tanaka, E.; Masuya, H.; Tanaka, R.; Hirooka, Y.; Endoh, R.; Sahashi, N.; Kikuchi, T. Comparative genomics of taphrina fungi causing varying degrees of tumorous deformity in plants. *Genome Biol. Evol.* 2014, 6, 861–872. [CrossRef]

55. Wang, X.; Radwan, M.M.; Taráwwneh, A.H.; Gao, J.; Wedge, D.E.; Rosa, L.H.; Cutler, H.G.; Cutler, S.J. Antifungal activity against plant pathogens of metabolites from the endophytic fungus cladosporium cladosporioides. *J. Agric. Food Chem.* 2013, 61, 4551–4555. [CrossRef] [PubMed]

56. Wit, P.J.G.M.; van der Burgt, A.; Ökmen, B.; Stergiopoulos, I.; Abd-Elsalam, K.A.; Aerts, A.L.; Bahkali, A.H.; Beenen, H.G.; Chettri, P.; Cox, M.P.; et al. The genomes of the fungal plant pathogens *Cladosporium fulvum* and *Dothistroma septosporum* reveal adaptation to different hosts and lifestyles but also signatures of common ancestry. *Plas Genetics* 2012, 8, e1005775.

57. Sandoval-Denis, M.; Gené, J.; Sutton, D.A.; Wiederhold, N.P.; Cano-Lira, J.F.; Guarro, J. New species of *Cladosporium* associated with human and animal infections. *Persoonia.* 2016, 36, 281–298. [CrossRef]

58. Bernal-Martinez, L.; Gomez-Lopez, A.; Castelli, M.V.; Mesa-Arango, A.C.; Zaragoza, O.; Rodriguez-Tudela, J.L.; Cuenca-Estrella, M. Susceptibility profile of clinical isolates of non-*Cryptococcus neoformans/*non-*Cryptococcus gattii* Cryptococcus species and literature review. *Med. Mycol.* 2010, 48, 90–96. [CrossRef]

59. Santo, D.E.; Galego, L.; Gonçalves, T.; Quintas, C. Yeast diversity in the Mediterranean strawberry tree (*Arbutus unedo* L.) fruits’ fermentations. *Food Res. Int.* 2012, 47, 45–50. [CrossRef]

60. Graça, A.; Santo, D.; Esteves, E.; Nunes, C.; Abadías, M.; Quintas, C. Evaluation of microbial quality and yeast diversity in fresh-cut apple. *Food Microbiol.* 2015, 51, 179–185. [CrossRef]

61. Liu, H.M.; Guo, J.H.; Cheng, Y.J.; Luo, L.; Liu, P.; Wang, B.Q.; Deng, B.X.; Long, C. Control of gray mold of grape by *Hansemiaspora wavorum* and its effects on postharvest quality parameters. *Ann. Microbiol.* 2010, 60, 31–35. [CrossRef]

62. Janiszewicz, W.J.; Tworkoski, T.J.; Kurtzman, C.P. Biocontrol potential of *Metschnikowia pulcherrima* strains against blue mold of apple. *Phytopathol.* 2001, 91, 1098–1108. [CrossRef] [PubMed]

63. Saravanakumar, D.; Ciavarella, A.; Spadaro, D.; Garibaldi, A.; Gullino, M.L. *Metschnikowia pulcherrima* strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum* in apples through iron depletion. *Postharvest Biol. Technol.* 2008, 49, 121–128. [CrossRef]

64. Suh, S.O.; McHugh, J.V.; Pollock, D.D.; Blackwell, M. The beetle gut: a hyperdiverse source of novel yeasts. *Mycol. Res.* 2005, 109, 261–265. [CrossRef] [PubMed]

65. Pozo, M.I.; Lachance, M.A.; Herrera, C.M. Nectar yeasts of two southern Spanish plants: The roles of immigration and physiological traits in community assembly. *FEMS Microbiol. Ecol.* 2012, 80, 281–293. [CrossRef] [PubMed]

66. Ebert, B.E.; Halbfe1d, C.; Blank, L.M. Exploration and exploitation of the yeast volatilome. *Curr. Metabolomics* 2017, 5, 102–118. [CrossRef]

67. Mozúraitis, R.; Aleknavičius, D.; Vepštaitė-Monstavičė, I.; Stanevičienė, R.; Emamí, S.N.; Čepšaite, V.; Radžiutė, S.; Blažytė-Cereškienė, L.; Servienė, E.; Būda, V. *Hipposphaerachromaticus* berry related *Pichia kudriavzevii* yeast volatiles modify behaviour of *Rhagoletis batava* flies. *J. Adv. Res.* 2020, 21, 71–77. [CrossRef]

68. Bakht, J.; Noor, N.; Iqbal, A.; Shafi, M. Antimicrobial activity of different solvent extracted samples from the leaves and fruits of *Capsicum annuum*. *Pak. J. Pharm. Sci.* 2020, 33, 27–32.

69. Huang, R.; Li, G.Q.; Zhang, J.; Yang, L.; Che, H.J.; Jiang, D.H.; Huang, H.C. Control of post-harvest botrytis fruit rot of strawberry by volatile organic compounds of *Candida intermedia*. *Phytopathol.* 2011, 101, 859–869. [CrossRef]

70. Piper, A.M.; Farnier, K.; Linder, T.; Speight, R.; Cunningham, J.P. Two gut-associated yeasts in a tephritid fruit fly have contrasting effects on adult attraction and larval survival. *J. Chem. Ecol.* 2017, 43, 891–901. [CrossRef]

71. Babcock, T.; Borden, J.H.; Gries, R.; Carroll, C.; Lafontaine, J.P.; Moore, M.; Gries, G. Inter-kingdom signaling - symbiotic yeasts produce semiochemicals that attract their yellowjacket hosts. *Entomol. Exp. Appl.* 2019, 167, 220–230. [CrossRef]
72. Becher, P.G.; Hagman, A.; Verschut, V.; Chakraborty, A.; Rozpedowska, E.; Lebreton, S.; Bengtsson, M.; Flick, G.; Witzgall, P.; Piškur, J.; et al. Chemical signaling and insect attraction is a conserved trait in yeasts. *Ecol. Evol.* 2018, 8, 2962–2974. [CrossRef] [PubMed]

73. Versace, E.; Eriksson, A.; Rocchi, F.; Castellan, I.; Sgado, P.; Haase, A. Physiological and behavioral responses in *Drosophila melanogaster* to odorants present at different plant maturation stages. *Physiol. Behav.* 2016, 163, 322–331. [CrossRef] [PubMed]

74. Dobzhansky, T.; Cooper, D.M.; Phaff, H.J.; Knapp, E.P.; Carson, H.L. Studies on the ecology of *Drosophila* in the Yosemite region of California differential attraction of species of *Drosophila* to different species of yeasts. *Ecol.* 1956, 37, 544–550. [CrossRef]

75. Ganter, P.F. Yeast and invertebrate associations. In *Biodiversity and Ecophysiology of Yeasts*; Augusto, R.C., Gábor, P., Eds.; Springer: Berlin, Germany, 2006; pp. 303–370.

76. Scheider, N.H.; Liu, C.; Hamby, K.A.; Zalom, F.G.; Syed, Z. Volatile codes: Correlation of olfactory signals and reception in *Drosophila*-yeast chemical communication. *Sci. Rep.* 2015, 5, 14059. [CrossRef] [PubMed]

77. Holighaus, G.; Rohlfs, M. Fungal allelochemicals in insect pest management. *Appl. Microbiol. Biotechnol.* 2016, 100, 5681–5689. [CrossRef] [PubMed]