**Fruit microbiome: A powerful tool to study the epidemiology of dry lenticel rot and white haze – Emerging postharvest diseases of apple**

**Fruit microbiome: a powerful tool to study the epidemiology of dry lenticel rot and white haze – emerging postharvest diseases of apple**

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**Abstract**

With the introduction of new apple varieties, emerging diseases have been recorded including dry lenticel rot and white haze. *Ramularia mali* has been identified as the causal agent of dry lenticel rot, whereas species of the *Golubevia*, *Tilletiopsis* and *Entyloma* genera have been associated to white haze, but the epidemiology of these pathogens remains unclear. In the present study, we measured fruit disease incidence and quality parameters, and we used metabarcoding to characterize both epiphytic and endophytic microbial communities of apple fruit of two commercial cultivars, 'Opal' and 'Ambrosia', across six time points from early fruit development up to the end of shelf life. *R. mali* first develops in both cultivars as an endophyte at BBCH (Biologische Bundesanstalt, Bundessortenamt and CHemical industry) phenological phase 73 (10-11 % relative abundance), BBCH 77 (26-33 % relative abundance) and BBCH 81 (1-7 % relative abundance), then it appears as an epiphyte from BBCH 87 onward (1-2 % relative abundance), when symptoms start to be visible. This was confirmed in endophytic samples through qPCR specific for *R. mali*. Among the genera associated to white haze, *Golubevia* was the most abundant epiphyte (2-4 %) from BBCH 81 to the end of shelf life. Alpha and beta diversity analyses unveiled the presence of significant difference both in richness and composition among different tissue, time points and cultivars. In conclusion, the study helps to explain the epidemiology of white haze and dry lenticel rot, and to design a targeted crop protection strategy, reinforcing the hypothesis that fruit metabarcoding could be a valuable tool for assessment and prediction of postharvest diseases, before symptoms occurrence in fruit.

**Keywords**

Apple, Dry Lenticel Rot, Microbiome, Metabarcoding, White haze, Postharvest Disease

## 1. Introduction

Apples are one of the world's most consumed fruit, with annual production estimated at over 87 million tonnes (FAOSTAT, 2020). In recent years, the apple market has undergone rapid change as an increasing number of new cultivars have been released and cultivated for local and international markets (Brown and Maloney, 2013). The introduction of new apple varieties is accompanied by the appearance of diseases with negative impact on fruit quality and commercial value, including dry lenticel rot and white haze.

Dry lenticel rot symptoms are brown-to-black spots, 0.1 to 0.8 cm in diameter occurring on the fruit peel (Gianetti et al., 2012). The disease is caused by *Ramularia mali* Videira & Crous, previously named *R. eucalypti* (Videira et al., 2015). It was first reported on 'Ambrosia' (Gianetti et al., 2012; Giordani et al., 2012) and later 'Golden Delicious' apples harvested in northern Italy (Lindner, 2013). Observations in Piedmont (northern Italy) suggest that this pathogen infects the fruit in orchard and then undergoes a latency period followed by active infection and development of symptoms during storage.

White haze is a waxy, opaque white film that forms on the cuticle, mostly around the peduncle and the stem end (Spadaro et al., 2019). It has been associated with saprophytic yeast-like basidiomycetes of the Entylomatales and Golubeviales orders (in particular, belonging to the genera *Tilletiopsis, Golubevia* and *Entyloma*). It has been reported in several European countries, including Italy (Baric et al., 2010), the Netherlands (Boekhout et al., 2006), Germany (Weber and Zabel, 2011) and Croatia (Prencipe et al., 2016). In Italy, and in particular in the Piedmont region, white haze occurred in the same orchards where dry lenticel rot was reported (Giannetti et al., 2016), though no biological correlation has been found until now between the diseases.

The occurrence and role of the causal agent/s in the development of white haze during the phenological phases of apple is not understood yet.

Moreover, the epidemiology of both diseases – white haze and dry lenticel rot – needs further clarification. Different methods, including cultural and molecular ones, can be used to study the epidemiology of postharvest pathogens. In recent years, metabarcoding is emerging as a flexible tool to elucidate the biology and epidemiology of pathogens (Franco Ortega et al., 2020; Piombo et al., 2020; Bösch et al., 2021). Several meta-barcoding studies have been performed on the composition of apple microbiota as it is affected by cultivation systems, cultivar, geography and postharvest processing (Abdelfattah et al., 2016; Shen et al., 2018; Vepštaitė-Monstavičė et al., 2018; Wessermann et al., 2019; Al Riachy, 2021; Bösch et al., 2021). A recent study demonstrated that geographic location has a significant effect on the composition and structure of fungal and bacterial communities of apple fruit (Abdelfattah et al., 2021). The same study also showed the presence of a global core microbiota across all studied geographical locations consisting of six fungal (*Aureobasidium, Cladosporium, Alternaria, Filobasidium, Vishniacozyma, Sporobolomyces*) and two bacterial genera (*Sphingomonas* and *Methylobacterium*), accounting for over 63 % of fungal and 13 % of bacterial communities. The composition of fruit microbiota over time in various parts of the fruit permits a better understanding of the effects of biotic and abiotic factors on the development of postharvest diseases (Bösch et al., 2021), as well as on the potential antagonistic effect of the microbiota in disease establishment and development.

This study aimed at better understanding the epidemiology of the causal agents of the dry lenticel rot and white haze, by considering two recently released apple varieties ‘Opal’ and ‘Ambrosia’. ‘Opal’ is a yellow scab resistant crossbreed between cultivars ‘Topaz’ and ‘Golden Delicious’

(Tupy et al., 2005). This cultivar does not require the application of chemical treatments against apple scab every 7 to 10 d during the cropping season, a common practice in susceptible varieties (Fiaccadori et al., 2011), and for this reason it is suitable for organic farming practise.

'Ambrosia', on the other hand, is a reddish-pink, scab susceptible cultivar (Mennell and Mennell, 1997), which is commercialized through farmers clubs, a commercial formula where farmers must pay a licensing fee and/or join an exclusive group to be allowed to grow this cultivar (Brown and Maloney, 2009).

The occurrence of *R. mali* and fungal genera involved in white haze was determined in apple during their phenological phases in orchards and during postharvest storage. We used meta-barcoding, combined with disease incidence in orchard and after harvest to establish a correlation between the fungal genera and disease development at six time points within the context of the epiphytic and endophytic fruit microbiota. The presence of *R. mali* was quantified in the endophytic microbiota with a qPCR approach.

## 2. Materials and methods

### *2.1 Apple orchards and treatments*

Fruit was sampled from two apple orchards in northwest Italy: an older (established in 2010) 'Ambrosia' cultivar orchard located in the Caraglio municipality (N 44°25'55"; E 7°18'27") and a more recent (established in 2017) 'Opal' cultivar orchard located in the Lagnasco municipality (N 44°35'15"; E 7°31'24"). Both orchards were with the same planting density (1 m x 4 m), and phytosanitary treatments carried out throughout the season on 'Ambrosia' and 'Opal' orchards are shown in **Suppl. Tables 1 and 2**, respectively. The 'Ambrosia' orchard was cultivated

following an integrated control strategy, whereas the 'Opal' orchard followed an organic farming cropping system. On 'Ambrosia' apples, 20 fungicide treatments were carried out throughout the cropping season, including the following active ingredients (**Suppl. Table 1**): mancozeb (1 treatment), ditianon(3), ditianon + pyrimethanil (1), calcium polysulphide(1), difenoconazole (4), fluxapyroxad (2), trifloxystrobin (1), fluazinam (3), dodine (2) and potassium bicarbonate (2). On 'Opal' apples, 12 fungicide treatments were carried out, including 2 copper treatments to control apple scab and 10 sulphur treatments to control powdery mildew (**Suppl. Table 2**). A relatively low number of copper treatments was applied against *Venturia inaequalis* as 'Opal' is an apple scab resistant cultivar.

Weather data (temperatures and precipitation) are provided for both sites, as shown in **Suppl. Fig. 1 and 2** for 'Ambrosia' and 'Opal' orchards, respectively.

### *2.2 Disease incidence and fruit quality*

Disease incidence in the fruit was measured at harvest, after 60 d of storage and then after another 15 d of shelf life. For every time point and cultivar, 4 boxes, each containing 50 fruits, were measured. The number of apples showing symptoms was compared to the total number of apples examined and the incidence of rots was expressed as a percentage. Fungal isolation was performed from fruit showing disease symptoms. Isolation was done by transferring small pieces (0.2x0.2 cm) of symptomatic fruit tissues, after surface disinfection with 1 % sodium hypochlorite and rinsing in sterile distilled water, onto potato dextrose agar (PDA, Merck, Darmstadt, Germany) plates amended with 25 mg $L^{-1}$ streptomycin sulphate (Merck, Darmstadt, Germany). Fungal cultures were observed under optical microscope for the shape of structures such as fruiting bodies, spores, conidiophores and conidia.

For selected isolates, molecular identification was carried out by extracting DNA according to previously published protocols (Santoro et al., 2018) and performing a PCR with universal primers ITS1 and ITS4 (White et al., 1990). PCR products were paired-end sequenced at Macrogen (Amsterdam, the Netherlands), the resulting chromatograms polished with Chromas v2.6.6 (Technelysium, Brisbane, Australia) and then merged with DNA baser (Heracle Biosoft, Arges, Romania). Identity of resulting sequences was confirmed by comparison with reference sequences in the GenBank database using BLASTn.

Apple fruit firmness, total soluble solids, and titratable acidity were measured according to previously described protocols (Oraguzie et al., 2009). These values were determined at harvest in September, after 60 d of storage at 1±1 °C and after 15 d of shelf life at 24±1 °C. Analyses were performed using 3 replicates of 5 apples for each cultivar.

Fruit firmness values were determined, using a 5300 penetrometer (Turoni, Forlì, Italy) with an 11 mm diameter probe at two points of the equatorial region of each fruit after skin removal.

Total soluble solids were determined using the NR151 (Rose Scientific Ltd, Edmonton, Canada) refractometer. Values obtained from the measurement were expressed as percentage of soluble solid content for each fruit. Juice from each fruit was extracted from the pulp, filtered and one drop was placed in a refractometer to determine the soluble solids content. Two measurements were taken for each fruit, and from the two values obtained an average was then calculated to obtain a representative measurement of the entire fruit.

Titratable acidity was determined using the 6 mL of the extracted juice. Each sample was diluted by adding 50 mL distilled water and then titrated with a 0.1 M NaOH up to pH 8.2. The Five Easy Plus pH meter FP20-Std-Kit (Mettler Toledo Italia, Milan, Italy) was used to check the exact point of pH change. The total acidity value was calculated using the following equation:

$$\frac{VNaOH \times 0.0067 \times 100}{6\ mL}$$

where 0.0067 indicates the acidity factor of the malic acid and 6 are the millilitres of juice used for the solution titration.

### *2.3 Microbiome sampling*

Fruit samples were collected at four time points in orchard during 2019: June, July, August and September, which roughly corresponded to BBCH growth stages 73, 77, 81 and 87, respectively. At each sampling time, five biological replicates were collected, and each replicate consisted of 30 fruit harvested from 10 trees (three apples per tree) in the same orchard row. Biological replicates were collected from different tree rows in the orchard**.** The selected trees were marked and the same trees were used for the subsequent sampling time points. During September, 45 apples were collected and divided in three groups of 15 fruit: one group was sampled immediately (harvest time point), the second group was kept at 1±1 °C for 60 d (storage time point) and the last one was kept at 1±1 °C for 60 d and followed by shelf life of 15 d at 24±1 °C. For each time point, biological replicates were kept at 2 °C for one night, and then epiphytes and endophytes were immediately collected.

Epiphytes were recovered by swabbing the entire fruit surface with sterile cotton buds dipped in PBS (phosphate-buffered saline) buffer. Cotton buds' tips belonging to the same replication were cut and stored in falcon tube with 8 mL of PBS solution. Tubes were shaken at room temperature for 20 min and then sonicated at 40 kHz for 5 min. All bud tips, lifted with sterile tweezers and squeezed, were later transferred to empty falcon tubes containing 4 mL of PBS solution and shaken and squeezed manually. Suspensions of the first and second eppendorf were pooled and centrifuged at maximum speed (12,578 g) for 30 min. The resulting pellet was re-suspended in 2

mL of PBS buffer and centrifuged again at 12,578 g for 30 min. The pellet was frozen in liquid nitrogen and stored at -20 °C.

For endophytes sampling, apples were washed in a 5 % Na-hypochlorite solution for 2 min, then rinsed twice in tap water for 2 min. Fruit peel was swabbed with cotton dipped in 90 % ethanol to remove residual epiphytic contamination, then peel section (1 cm wide) was removed from the equatorial region of each fruit. Peel tissue sections were lyophilized, frozen in liquid nitrogen and stored at -20 °C.

### *2.4 Genomic DNA extraction and sequencing*

The DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Biotech AB, Finnboda Varvsväg, Sweden) for the epiphytes, following the protocol suggested by the manufacturer. The endophytes were extracted from the dry powder of fruit peel tissue with the kit DNeasy Power Soil (Qiagen, Hilden, Germany), following a modified version of the manufacturer's protocol. In this protocol, the 10 min vortex step was replaced with a 30 min shake with a Tissue Lyzer (Qiagen). Moreover, the initial shake, centrifuge and supernatant recovery were repeated after the addition of 500 µL of lysis buffer SL2 (Macherey-Nagel, Düren, Germany).

ITS2 sequences were amplified using primers ITS3_KYO2 (Toju et al., 2012) and ITS4 (White et al., 1990), while a PNA oligonucleotide blocker was used to reduce the quantity of apple-derived amplicons (**Suppl. Table 3**). The ITS Amplicon PCR was performed in a volume of 25 µL containing 12.5 ng of template DNA (volume 2.5 µL), 1 µL of a 10 µM solution for both primers, 1 µL of a 10 µM ITS Blocking Oligo solution, 12.5 µL of KAPA Ready Mix (Kapa Biosystems Inc., Cape City, South Africa) (2x) and 7 µL of nuclease-free water. The ITS

Amplicon PCR reaction consisted of an initial denaturation at 95 ºC for 5 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and a final extension at 72 °C for 5 min.

16S sequences were amplified using the primers 16s 515f (Parada et al., 2016) and 16s 806r (Apprill et al., 2015). Two PNA oligonucleotide blockers were used to reduce the quantity of mitochondria- and plastidia-derived amplicons (**Suppl. Table 3).** The 16S Amplicon PCR was performed using 12.5 ng of template DNA (volume 2.5 µL), 1 µL of 10 µM solution for both primers, 1 µL of two10 µM 16S plastid and mitochondrial PNA solutions, 12.5 µL of 2X KAPA ReadyMix (Kapa Biosystems Inc.) and 3µL of nuclease-free water. The 16S Amplicon PCR followed this program: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s at 95 °C, 5 s at 78 °C, 30 s at 55 °C, 30 s at 72 °C and a final extension at 72 °C for 5 min. Adapters were added with Illumina Nextera Index Kit (Illumina, San Diego, California) according to the manufacturer's protocol. After adding adapters, libraries were quantified through a QUBIT dsDNA Assay kit (Life Technologies, Monza, Italy), diluted at 4 nM and regrouped and denatured as described in the manufacturer's protocol. Sequencing was performed with a final library concentration of 10 pM, using the MiSeq Reagent Kit V3 600-cycle (MS-102-3003, Illumina) following manufacturer's instructions.

### *2.5 Bioinformatic analyses*

Data analysis was performed by using both the QIIME2 suite (Bolyen et al., 2019) and a series of custom Python scripts. Fungal sequences were trimmed down to the ITS2 region using the ITSxpress plugin (Rivers et al., 2018), while bacterial sequences were trimmed using the Cutadapt plugin (Martin, 2011). In both cases, trimmed sequences were processed using the DADA2 plugin (Callahan et al., 2016) to obtain amplicon sequence variants (ASVs). Truncation

position for forward and reverse reads was chosen based on quality profile and overlap constraints, with truncation at position 283 for forward reads and 235 for reverse reads in fungi and truncation at position 150 for forward reads and 130 for reverse reads in bacteria.

Taxonomic classification of ASVs was carried out using the q2-feature-classifier plugin (Bokulich et al., 2018). For fungi, a Naïve-Bayes predictor was trained on version 8.2, dynamic, singletons set as RefS of the UNITE database (Abarenkov et al., 2020). For bacteria, another Naïve-Bayes predictor was trained on version 138 of the SILVA 16S database (Quast et al., 2013), with redundant sequences removed using the RESCRIPt plugin (Robeson et al*.,* 2021). A preliminary trimming of reference sequences was carried out for the 16S database in order to isolate the V4 region, as this step has been shown to increase prediction accuracy (Werner et al., 2012). Based on taxonomic assignments from this step, mitochondrial and plastidial contamination were detected for the bacterial sequences and removed from the samples. At the same time, sequences found in a single replicate were filtered out, as they could weight down the analysis for no benefit, given their nature as likely contaminants. Data normalization for alpha and beta diversity analyses was carried out using the scaling with ranked subsampling approach as implemented in the SRS module (Beule and Karlovsky, 2020). Normalization values were set at 9,241 for fungi and 27,000 for bacteria, based on a compromise between sample size and number of retained replications for each thesis. For alpha diversity, we chose the Shannon index and the number of observed features as metrics, while for beta diversity we chose the Bray-Curtis metric. Pairwise comparisons of alpha diversity measurements were carried out using a non-parametric Kruskal-Wallis with Benjamini-Hochberg FDR (false discovery rate) correction (Benjamini and Hochberg, 1995).

Beta diversity results were used to carry out a Permutational Multivariate Analysis of Variance (or PERMANOVA) with 999 permutations, using the Adonis plugin (Oksanen et al., 2018), and a Principal Coordinates Analysis (or PCoA), using the implemented PCoA algorithm (Halko et al., 2011) and the Emperor plugin (Vázquez-Baeza et al., 2013)

Compositional analyses were carried out using a combination of available software and custom scripts. ASVs absolute frequencies were aggregated based on genus and relative abundances were calculated for each sample, then samples were collapsed together based on tissue, cultivar and time point through simple mean. A preliminary filtering was carried out and genera without at least 2 % relative abundance in at least one of the considered time points had their relative abundances pooled into the "other" category.

Beside taxonomic composition, co-occurrence and co-exclusion networks were generated by using custom python scripts, as well as Cytoscape v3.8.2 (Shannon et al., 2003) in combination with the CoNet 3.0 plugin (Faust and Raes, 2016), while differential abundance tables were calculated using ANOVA-Like Differential Expression tool (ALDEx 2; Fernandes et al., 2013). Relative frequencies for the target genera (e.g.: *Golubevia*, *Tilletiopsis, Entyloma* and *Ramularia*, with the addition of *Alternaria*), regardless of relative abundance, were retrieved and used to create a histogram, in order to follow their abundance over the observation period.

**2.6 Quantitative PCR**

To validate the data of the metabarcoding analysis, the quantification of *R. mali* was obtained through a newly developed qPCR protocol (Prencipe et al., 2022). Analyses were performed using 1 µL of DNA extracted from apples for endophytes metabarcoding analysis, 5 µL of SSO Advance Supermix 5x (Bio-Rad, USA) and 0.25 µL of each primer (1µM). Amplifications were

conducted using thermal cycler Real Time CFX Connect™ (Bio-Rad, USA) with 96 well-plates (Bio-Rad) sealed with Microseal® B seal adhesive film (Bio-Rad). The quantification of the pathogen was obtained using DNA extracted from the reference strain of *R. mali* CBS 129581 serially diluted from 10 ng to 100 fg.

## 3. Results

### 3.1 Disease incidence and fruit quality

Apples were cultivated by following two different crop protection strategies. On 'Ambrosia' apples, an integrated pest management (IPM) strategy was adopted, whereas on 'Opal' apples an organic disease management strategy was used.

During storage and shelf life, 'Ambrosia' apples were mainly affected by white haze and dry lenticel rot **(Fig. 1**), whereas 'Opal' apples showed black spots. At harvest, the incidence of dry lenticel rot on 'Ambrosia' was 0.5 % and 11.0 % and 15.0 % after 60 d of cold storage and shelf life, respectively. The incidence of white haze was 5.0 % at harvest, 10.5 % after 60 d of cold storage, and 20.7 % after shelf life (**Table 1**). On 'Opal', dry lenticel rot was not detected at all the period starting from harvest until the end of storage and shelf life. White haze was not observed and quantified because the yellow skin of this cultivar of apples does not permit to easily observe this cosmetic defect (**Table 1**). On the other hand, the incidence of black spots was 7.5 % at harvest, 52.5 % after 60 d of cold storage, and 83.5 % after shelf life on 'Opal'. Fungi belonging to the species *Golubevia pallescens*, *Tilletiopsis washingtonensis* and *Entyloma belangeri* were isolated from white haze, *R. mali* from dry lenticels and *Alternaria alternata* from black spots.

Measurements of fruit quality parameters were made at harvest, after 60 d of storage and 15 d of shelf life. Results showed a normal maturation profile over time, with a decrease in firmness and acidity and an increase in soluble solids (**Table 2**). Fruit of 'Opal' cv. showed significantly higher levels of total soluble solids and titratable acidity at harvest and throughout storage and shelf life. 'Opal' apples were firmer than 'Ambrosia' at harvest and after storage, but there was no difference between the two cultivars in the quality parameter after shelf life.

### 3.2 Sequencing results

After paired-end alignments, quality filtering and deletion of chimeras, singletons, and mitochondrial and chloroplast sequences, a total of 6.7 million bacterial 16S rRNA and 13.6 million fungal internal transcribed spacer (ITS) reads were recovered and assigned to 2,774 bacterial and 1,849 fungal amplicon sequence variants (ASVs) in a total of 240 samples. Sample heterogeneity was removed by normalizing to an even depth of 9,421 (ITS) and 27,000 (16S) reads per sample. After both steps, only three of the twelve treatments would have retained at least three replicates associated with bacterial endophytes, which were considered the minimum acceptable number. For this reason, we dropped the samples associated with endophytic bacteria. In total, 170 out of 240 samples were used in the subsequent analyses after normalization.

### 3.4 **Bacterial diversity**

For endophytic bacteria, an alpha diversity analysis could not be carried out due to very low sequence recovery from associated samples. For epiphytic bacteria (**Fig. 2A; Fig. 2B)**, alpha diversity analyses showed that 'Opal' had significantly higher Shannon index than 'Ambrosia' for the six sampling time points, while significantly higher number of observed features were

recorded in June (BBCH 73), during storage and during shelf life for ‘Opal’ compared to ‘Ambrosia’ **(Suppl. Table 4**).

No significant difference was found between consecutive time points in the same cultivar, the only exception to this was that ‘Opal’ and ‘Ambrosia’ apples presented a significant reduction in Shannon Index values comparing the end of storage to the end of shelf life (**Suppl. Table 5**).

The PERMANOVA analysis showed that sampling time point alone explained 44.4 % of the total variance, almost twice compared to the 24.7 % explained by the cultivar (**Table 3**). An additional 10.2 % of total variance was explained by the combination of time point sampling and cultivar, while 20 % of the total variance remained unexplained.

PCoA analysis results showed that samples belonging to different cultivars formed two clearly distinct groups, which indicates a different composition of these two populations (**Fig. 3A**). Separation of samples based on sampling time points was less defined (**Fig. 3B**): with the exception of samples associated with shelf life in ‘Opal’ and ‘Ambrosia’, samples did not form clearly defined clusters, but were distributed along a gradient.

### 3.4 Fungal diversity

For fungi, alpha diversity analyses at the six time points (**Fig. 2C; Fig. 2D**) showed statistically significant differences for both Shannon Index and number of observed features in epiphytic fungi at almost all considered time points, with higher values for ‘Opal’ compared to ‘Ambrosia’ (**Suppl. Table 6**). Within the endophytic communities, only sporadic statistically significant differences between the two cultivars were found for both metrics. We observed significant differences for the number of observed features between epiphytic and endophytic communities at the six time points and for both cultivars, whereas Shannon index values were significantly

different for every time point only for ‘Opal’ (**Suppl. Table 7**). For both metrics, values are higher for epiphytes compared to endophytes. Finally, comparison between consecutive sampling times showed only sporadic differences for both metrics (**Suppl. Table 8**).

The PERMANOVA analysis (**Table 3**) showed that none of the parameters or their combinations had a predominant role in explaining the overall variance, with explained variance percentages fluctuating between 4.2 % and 14.2 %. The sampling time point, which explains 9.9 % of total variance, was less important than the cultivar (14.2 %) or the tissue (12.5 %), differently from what seen in bacteria. In addition, several combinations of parameters show a statistically significant effect on variance: the combination of cultivar and sampling time point (5.6 %), the combination of cultivar and tissue (4.7 %), the combination of time point and tissue (7.7 %) and the combination of cultivar, time point and tissue (4.2 %).

PCoA analysis showed that samples belonging both to epiphytic and endophytic communities form distinct clusters based on cultivar (**Fig. 3C**), although the difference is less clean cut for endophytes. On the other hand, when considering different sampling time points, we did not observe any clustering or trend, which reflects the lower influence of time point in explaining the total variance for fungi (**Fig. 3D**).

### 3.5 **Microbial populations composition**

The composition of epiphytic bacterial samples is presented in **Suppl. Fig. 3** and differential composition data are shown in **Suppl. Table 9**. The main genera *Sphingomonas* (11.0 % in ‘Opal, 19.8 % in ‘Ambrosia)*, Pseudomonas* (10.0 % in ‘Opal’, 25 % in ‘Ambrosia’)*, Massilia* (7.1 % in ‘Opal’, 7.6 % in ‘Ambrosia’)*, Pandoraea* (7.1 % in ‘Opal’, 11.7 % in ‘Ambrosia’) and *Methylobacterium-Methylorubrum* (6.0 % in ‘Opal’, 5.2 % in ‘Ambrosia’) are common in both

cultivars. Among the main genera, *Hymenobacter* (7.2 %) is only reported in 'Opal', while *Pantoea* (8.1 %) only in 'Ambrosia'. The main genera are more abundant in 'Ambrosia', whereas the total number of detected genera is higher in 'Opal'.

Although the bacterial endophytes could not be analyzed for diversity, the most represented genera (**Suppl. Table 10**) were *Cutibacterium* (10.6 %), *Lactobacillus* (10.5 %), *Pandoraea* (8.0 %), *Pseudomonas* (6.4 %) and *Staphylococcus* (5.1 %).

Compositions of epiphytic and endophytic fungal communities are presented in **Fig. 4A** and **Fig. 4B**. Differential composition data for fungal communities are shown in **Suppl. Table 11** (for epiphytes) and **Suppl. Table 12** (for endophytes). Among the most abundant epiphytic taxa, *Filobasidium* (12.0 % in 'Opal, 6.2 % in 'Ambrosia'), *Cladosporium* (9.7 % in 'Opal', 10.5 % in 'Ambrosia') and *Alternaria* (5.2 % in 'Opal', 5.0 % in 'Ambrosia'), as well as Capnodiales (12.0 % in 'Opal', 8.0 % in 'Ambrosia') are present in both cultivars. *Aureobasidium* (19.0 %), *Epicoccum* (8.0 %) and *Vishniacozyma* (7.4 %) are the main genera only for 'Opal', while *Sporobolomyces* (20.5 %) and *Rhodotorula* (5.8 %), in addition to Entylomatales (5.6 %), are the main taxa only in 'Ambrosia'. On the other hand, among the most abundant endophytic genera, *Cladosporium* (19.5 % in 'Opal', 9.6 % in 'Ambrosia'), *Epicoccum* (12.0 % in 'Opal', 5.6 % in 'Ambrosia'), *Ramularia* (8.0 % in 'Opal', 8.6 % in 'Ambrosia') and *Alternaria* (6.8 % in 'Opal', 7.6 % in 'Ambrosia'), in addition to Capnodiales (15.7 % in 'Opal', 7.5 % in 'Ambrosia'), are present in both cultivars. Among the most abundant genera, *Aureobasidium* (7.0 %) was included in 'Opal', while *Mortierella* (11.2 %) and *Malassezia* (5.1 %) in 'Ambrosia'.

### 3.6 Abundance patterns and co-occurrence networks of the genera of interest

The abundance pattern of the fungal genera involved in white haze (*Tilletiopsis, Entyloma, Golubevia*) and dry lenticels rot (*Ramularia*) are shown in **Fig. 5**, while abundance pattern of *Alternaria* is provided in **Suppl. Fig.4A**. Among the white haze genera, *Golubevia* is present in epiphytic samples of both cultivars in September (1.8 % in 'Ambrosia', 0.4 % in 'Opal'), during storage (4.1 % in 'Ambrosia', 0.7 % in 'Opal') and after shelf life (2.2 % in 'Ambrosia', 2.2 % in 'Opal'), while a very low number of reads is present in the endophytic samples. In total, 14 ASVs were associated with this genus, three of them representing 98 % of overall detected *Golubevia* ASVs, with a Spearman correlation value of 0.69-0.79. *Tilletiopsis*, however, was detected in 'Ambrosia' endophytic samples from June (0.5 %) to August (0.4 %), while in 'Opal' endophytic samples it can be observed from August (0.6 %) up to the end of shelf life (1.4 %). It is also present as an epiphyte on 'Opal' apples from July (0.3 %) to the end of shelf life (1.7 %), but it was at the detection limit in all 'Ambrosia' epiphytic samples. Finally, *Entyloma* is present only in June (1.1 %) as an 'Ambrosia' epiphyte. *Ramularia*, the causal agent of dry lenticels rot, appears as an endophyte in both cultivars during June (9.9 % in 'Ambrosia', 10.6 % in 'Opal'), with a peak in July (33.4 % in 'Ambrosia', 25.6 % in 'Opal'). Its endophytic levels diminish from August onward, when the pathogen starts to appear as an epiphyte in both cultivars (0.5-2 %, depending on cultivar and time point). A total of 198 ASVs were associated with this genus, six of them representing 95 % of all occurrences. A positive Spearman correlation was observed only for the three most abundant ASVs (0.6-0.68).

In addition to metabarcoding data, presence of *R. mali* in endophytic samples was also investigated through qPCR. The quantification of *R. mali* was possible for 3 of the six analyzed time points (**Suppl. Fig. 5**). Samples of 'Ambrosia' and 'Opal' cultivars had a mean quantification of $3.72 \times 10^6$ cells $g^{-1}$ and $7.80 \times 10^3$ cells $g^{-1}$ for June samples, $5.62 \times 10^3$ cells $g^{-1}$

and $1.36\times10^3$ cells $g^{-1}$ for July samples, and $5.52\times10^2$ cells $g^{-1}$ and $1.89\times10^3$ cells $g^{-1}$ for August samples, respectively. The other time points analyzed could not be quantified, as the value was below the detection limit.

A closer analysis of interactions between the fungal genera involved in white haze and dry lenticel dry rot and other fungal and bacterial genera for the epiphytic and endophytic component are shown in **Fig. 6A and 6B**, respectively. For the epiphytic component, *Golubevia* has a negative correlation with bacterial genera *Ralstonia, Curvibacter* and *Duganella*, but a positive correlation with the genus *Ramularia*, which has also a positive correlation with fungal genera *Kalmanozyma, Nigrospora* and *Pseudopithomyces*. *Tilletiopsis* has positive correlations with fungal genera *Knufia, Xenoramularia, Bengsintonia, Neosetophoma*, *Rachicladosporium* and *Microstroma*, as well as with the bacterial genus *Friedmanniella,* whereas *Entyloma* has no correlation with other genera. For the endophytic component, *Tilletiopsis* has a positive correlation with the fungal genus *Aureobasidium* and a negative correlation with the fungal genus *Trichoderma*, whereas it has a negative correlation with bacterial genera *Staphylococcus* and *Corynebacterium*. *Ramularia*, on the other hand, has a positive correlation with the fungal genus *Plectosphaerella* and a negative correlation with the fungal genus *Kalmanozyma*.

## 4. **Discussion**

The apple market is characterised by the introduction of new varieties, some of them aiming at improving the organoleptic profile, such as ‘Ambrosia’, others introducing tolerance to pathogens, in particular to *V. inaequalis,* such as ‘Opal’. With the introduction of new apple varieties, new phytopathological issues are emerging. In the case of ‘Opal’, the reduction of fungicide treatments favours minor pathogens, in orchard and during storage, such as *Alternaria*

spp. causing black spots. On the other hand, ‘Ambrosia’ is particularly susceptible to dry lenticel rot and white haze.

There is a lack of information about the epidemiology of dry lenticel rot, caused by *R. mali*, and of white haze, caused by Golubeviales and Entylomatales. The apple fruit microbiome has been explored to investigate the development of *Neofabraea alba* (Bösch et al., 2021), the causal agent of bull’s eye rot, and of *Penicillium expansum*, the causal agent of blue mould and producer of the mycotoxin patulin (Al Riachy et al., 2021).

Similarly, the aim of the present work was to study the epidemiology of dry lenticel rot and white haze, using metabarcoding analysis as a tool. In particular, the occurrence and role of each of the microbial genera in the development of white haze during the phenological phases of apple is incomplete. Both dry lenticel rot and white haze occur on the fruit during the postharvest phase, i.e., during storage and subsequent shelf life. To understand if *R. mali* and the causal agents of white haze occur at harvest or in earlier stages of fruit development, we included four sampling time points in orchard: June, July, August and September (90, 60, 30 d before harvest and at harvest time in September).

Furthermore, to explore the origin of *R. mali* and the causal agents of white haze, the epiphytic and endophytic population of the microbiome were sampled, to determine if the fungal agents reach the apple skin from an external source or if they are already present as endophytes in the fruit pulp.

The incidence of dry lenticel rot and white haze in ‘Ambrosia’ was particularly high, reaching 15.0 % and 20.7 % after shelf life, respectively (**Table 1)**. ‘Opal’ apples provided another set of interesting results, as they were not affected by dry lenticel rot and white haze, but they showed black spots on over 50 % of the fruit at the end of shelf life (**Table 1**).

Using a metagenomics approach, we showed that it is possible to identify a broad variety of bacterial and fungal species including the target postharvest pathogens. By investigating the abundance of *Ramularia*, this pathogen was detected as endophyte during June (average temperature 21.6 °C, average humidity 72.5 % for 'Opal'; 21.7 °C and 65.3 % for 'Ambrosia'), July (23.2 °C and 87.9 % for 'Opal'; 22.0 °C and 71.6 % for 'Ambrosia') and August (22.1 °C and 77.6 % for 'Opal'; 20.9 °C and 77.2 % for 'Ambrosia'), whereas it occurred as an epiphyte in September (17.4 °C and 87.6 % for 'Opal'; 16.5 °C and 81.7 % for 'Ambrosia') and after storage and shelf life, when symptoms of dry lenticel rot were visible (Giannetti et al., 2012; Giordani et al., 2012; **Fig**. **5 and Fig. 7**).

The presence of *R. mali* in the metabarcoding samples was validated with a specific qPCR assay (Prencipe et al., 2022). The amount of *R. mali* detected with qPCR follows a similar trend of abundance observed with metabarcoding, with a higher amount of the pathogen in June which decreases in the months of July and August and is not quantifiable in the last three time points (**Suppl. Fig 5**). These results suggest that *Ramularia* is an endophytic fungus already present three months before harvest in the peel of 'Ambrosia' apples, and its abundance increases as epiphyte at harvest and during storage. This progression from an endophytic to an epiphytic presence over time is similar to the data of another species of *Ramularia*, *R. collo-cygni*, a barley pathogen that has a first growth phase intracellular and later emits conodiophores through stomata (Walters et al., 2008). *Ramularia* is also present as endophyte in 'Opal' apples, but on this cultivar, dry lenticel rot does not occur during storage and shelf life. These findings would suggest that *Ramularia* is present endophytically in apples, but it becomes pathogenic only on particularly susceptible varieties, such as 'Ambrosia'. The presence of high *Ramularia* levels in

June could suggests an earlier establishment on the apple fruit, therefore an earlier sampling during the cropping season is advisable, starting from early flowering (BBCH 61).

Regarding white haze, the abundance of the genera *Golubevia*, *Tilletiopsis* and *Entyloma* was investigated at six time points (four in orchard and two during the postharvest phase). Among the three genera, *Golubevia* had the highest abundance in epiphytic communities found on 'Ambrosia' apples at harvest, after storage and shelf life, the three time points when the disease becomes evident (**Fig. 5**). The abundance of the other two genera was relatively low compared to *Golubevia*. In particular, both *Tilletiopsis* and *Entyloma* were detected as endophytes, particularly in 'Ambrosia' apples during June (21.6 °C and 72.5 % for 'Opal'; 21.7 °C and 65.3 % for 'Ambrosia'), but their abundance was very low at harvest (17.4 °C and 87.6 % for 'Opal'; 16.5 °C and 81.7 % for 'Ambrosia') and after storage and shelf life (**Fig. 5**). It should be noted that *Golubevia* is particularly abundant also on 'Opal' apples characterized by a yellow skin, which does not favour an evident manifestation of white haze. Although ITS2 alone cannot provide a reliable identification at species level, it is worth mentioning that all 14 recovered *Golubevia* ASVs could be assigned to the species *Golubevia pallescens.*

Very low amounts of reads mapped to *Venturia* reflecting the fact that although fungal spores can be present on apple fruit, no disease symptoms developed, both on the resistant 'Opal' and on the susceptible 'Ambrosia' apples. In the latter, a complete crop protection strategy permitted avoiding *V. inaequalis* infection and apple scab development.

Compositional variance indicates that sampling time point, cultivar and tissue have high impact on microbial diversity. Time point is the parameter with the highest impact on compositional variance for bacteria (44.4 %), but, except for shelf life, samples belonging to different time point do not form distinct clusters. Instead, their distribution describes a gradual shift over time,

likely as a result of short sampling intervals compared to the rate of compositional evolution. The effect of time point could not be seen for community richness, where almost no significant differences in Shannon Index and observed features were detected.

By considering fungi, time point has the lowest impact (9.9 %) among the considered parameters and samples associated to different time points do not form clear clusters, nor are distributed according to time gradients. The effect on richness was also limited and statistically significant differences did not show a clear pattern. The presence of a significant effect of the time points on compositional variance of both fungi and bacteria could be explained by the changes in environmental parameters, such as temperature and relative humidity, which alter microbiota composition (Cordier et al., 2012; Gomes et al., 2018; Frindte et al., 2019). The higher impact of these factors on bacteria compared to fungi was previously observed (de Vries et al., 2018; Costa et al., 2022).

Cultivar, on the other hand, had the highest impact on compositional variance of fungi (14.2 %) and it explained almost a quarter of total variance for bacteria (24.7 %). The cultivar was associated with a clear clustering of bacterial epiphytes. A cultivar related effect could also be observed in community richness, where ‘Opal’ communities showed significantly higher Shannon index for all time points compared to ‘Ambrosia’. For epiphytic fungi, the cultivar effect permitted a clear clustering, while endophytic fungi presented a less clear clustering based on the cultivars. Among epiphytic communities, the cultivar impact was mirrored by significantly higher number of observed features and Shannon index for most ‘Opal’ samples, compared to ‘Ambrosia’ ones.

The marked differences in microbiota composition found in the two tested cultivars are in accordance with previous studies on the cultivar effect (Liu et al., 2018). A possible explanation

of the cultivar effect could be associated to different crop protection strategies adopted (Bösch et al., 2021). ‘Ambrosia’ apples were grown using an integrated crop protection strategy, whereas ‘Opal’ apples were grown in organic farming. As previously shown in **Suppl. Table 1** and **Suppl. Table 2**, this resulted in a lower number of fungicide treatments in ‘Opal’ compared to ‘Ambrosia’, therefore we could hypothesize that a lower pesticide use could correlate with a higher fungal and bacterial diversity, as previously observed by Fournier et al. (2020).

The last parameter, tissue, could only be studied for fungal communities, where it explained 12.5 % of total variance and it was associated with a clear clustering. Regarding community richness, the number of observed features was significantly higher in epiphytic communities compared to endophytic ones for both cultivars at all sampling time points, while Shannon Index was higher in all epiphytic samples only in ‘Opal’. The subepidermal region of fruit was shown to present lower oxygen levels compared to the surface (Ho et al., 2008); furthermore, apple pulp contains high amounts of sugars (Hecke et al., 2006), and sugar-rich environments showed to significantly condition microbial communities (Lievens et al., 2015). Combined, these factors could select the smaller spectrum of microorganisms in the fruit pulp, thus explaining the tissue effect.

Overall microbial compositional data for fungi and bacteria points to the presence of a shared apple microbiome fraction across different cultivars and geographical locations.

The genera *Pseudomonas*, *Sphingomonas, Pantoea, Massilia* and *Methylobacterium* represent the main components of both ‘Opal’ and ‘Ambrosia’ carposphere microbiome, and they were found as core species also in ‘Arlet’ apples, regardless of crop protection strategy (Wassermann et al., 2019). The genus *Kineococcus* was found as part of the core microbiome only in organically grown ‘Arlet’ apples, while it was not present in conventionally grown fruit. This is in accordance with data presented in this study, which show that this genus was detected only in

organically grown ‘Opal’ apples, whereas it was absent in ‘Ambrosia’ apples, grown using an IPM strategy. On apples resistant to scab such as ‘Enterprise’ cultivar (Abdelfattah et al., 2020), *Pseudomonas, Sphingomonas* and *Methylobacterium* were the main components of the microbiome of conventionally grown fruit, together with several unknown organisms belonging to the Microbacteriaceae and Comamonadaceae, which were found in ‘Ambrosia’ and, to a lower degree, in ‘Opal’ apples. *Sphingomonas* and *Methylobacterium* were present in scab resistant cultivars ‘Ariane’, ‘Otava’ and ‘Topaz’ (a parental of cultivar ‘Opal’) (Bösch et al., 2021), but also in the cultivar ‘Royal Gala’, where they are part of a core microbiome shared across several countries (Abdelfattah et al., 2021). Both *Sphingomonas* and *Methylobacterium*, as well as *Pseudomonas* and *Pantoea*, are known as frequently occurring genera in the phyllosphere and some of their species have been shown to possess potential as biocontrol agents against foliar and fruit pathogens (Legein et al., 2020).

The main fungal genera found in the present study were previously detected in other apple metabarcoding studies. For example, *Aureobasidium*, *Cladosporium, Alternaria, Sporobolomyces, Malassezia* and *Penicillium* occurred with an overall relative abundance higher than 1 % in a study carried out on ‘Red Delicious’ apples (Abdelfattah et al., 2016). *Cladosporium*, *Sporobolomyces* and *Rhodotorula* were found on the peel of apples of an unspecified cultivar in Lithuania (Vepštaitė-Monstavičė et al., 2018), while *Aureobasidium* and *Cryptococcus* were identified on the peel of ‘Fuji’ apples (Shen et al., 2018). *Vishniacozyma*, *Alternaria, Cladosporium, Ramularia, Penicillium* and *Aureobasidium* as well as several unidentified organisms belonging to order Capnodiales, were detected in the scab resistant cultivar ‘Enterprise’ (Abdelfattah et al., 2020; Al Riachy et al., 2021). *Aureobasidium, Cladosporium, Alternaria, Filobasidium, Vishniacozyma* and *Sporobolomyces* were suggested as

core species for ‘Royal Gala’ apples across several countries around the world (Abdelfattah et al., 2021).

Among the most common genera found in the present study, there are some known for their potential as biocontrol agents, such as *Aureobasidium* (Di Francesco et al., 2020)*, Rhodotorula* (Li et al., 2016), *Epicoccum* (Taguiam et al., 2021), *Vishniacozyma* (Lutz et al., 2020) and *Cryptococcus* (Kheireddine et al., 2018). Other common genera are known as postharvest rot pathogens, such as *Penicillium* (Luciano-Rosario et al., 2020), *Alternaria* (Oettl et al., 2021) and *Fusarium* (Wenneker et al., 2016a). Co-presence/Co-exclusion data between recovered fungal and bacterial genera could provide new insight on potential biocontrol agents, present in co-exclusion with the target pathogens, which could be isolated and developed to be applied in crop protection (Zheng et al., 2021). For instance, in the endophytic community, *Ramularia* shows a negative correlation with *Kalmanozyma,* which includes strains with antifungal activity (Kulakovskaya et al., 2005), while *Tilletiopsis* has a negative correlation with *Trichoderma*, a well-known biocontrol agent (Tyśkiewicz et al., 2022). These data also confirm previous observations regarding community dynamics in apple fruit. In the endophytic community, *Alternaria* showed a positive correlation with *Methylobacterium*, in accordance with previous studies (Abdelfattah et al., 2021).

## 5. **Conclusions**

In this study, we compared microbial communities over six time points on two cultivars, with a focus on fungal genera involved in emerging postharvest diseases. Bacterial and fungal epiphytic communities of ‘Opal’ apples boasted a higher richness compared to ‘Ambrosia’ apples, and these communities showed clear compositional differences between cultivars. On the other hand,

there was no significant difference in richness between ‘Opal’ and ‘Ambrosia’ fungal endophytic communities, which are also compositionally similar.

The most abundant bacterial and fungal genera detected in this study are similar to those observed in the cultivars ‘Golden Gala’, ‘Arlet’, ‘Enterprise’, ‘Ariane’, ‘Otava’, ‘Fuji’, ‘Bedan’ and ‘Topaz’. As these studies were carried out in different geographical locations, they reinforce the idea of a core microbiota associated with apple fruit (Abdelfattah et al., 2021).

Epidemiological data for white haze genera (*Golubevia*, *Entyloma* and *Tilletiopsis*) gathered through microbiome analysis seem to implicate *Golubevia*, in particular *G. pallescens,* as the main causal agent of this postharvest defect, as previously reported (Prencipe et al., 2016). Microbiome analysis data showed that *R. mali*, the causal agent of dry lenticel rot, first appears as an endophyte in orchard in June, but it gives rise to visible symptoms starting from September and during storage, when its abundance in the epiphytic population increases.

These findings help to explain the epidemiology of white haze and dry lenticel rot, and to design a targeted crop protection strategy. Similar patterns were observed when comparing the incidence of fruit showing disease symptoms with the abundance of reads belonging to specific pathogens, reinforcing the hypothesis that apple metabarcoding could be a valuable tool for assessment and prediction of postharvest diseases, before symptom occurrence in fruit.

In general, data gathered in this study provide a better understanding of the close connection of pre-harvest microbiota composition on post-harvest dynamics. Moreover, the research permitted to underline the presence of a clear difference between the endophytic and epiphytic microbial dynamics at play on fruit.

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**Figures**

**Figure 1.** Apple fruit of cultivar ‘Ambrosia’ with white haze signs (left) and dry lenticel rot symptoms (right) at the end of storage.

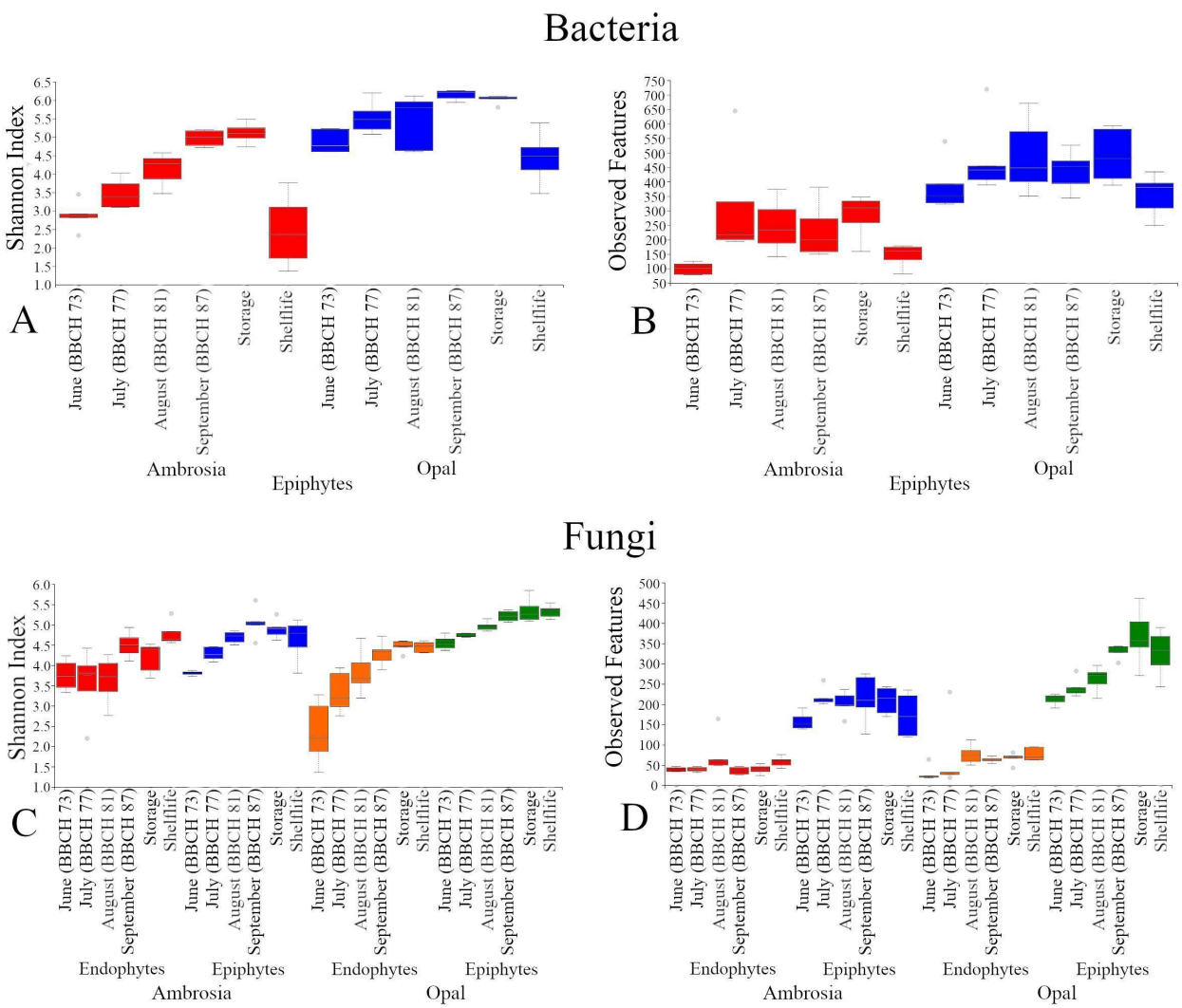



**Figure 2.** Shannon index (A for bacteria, C for fungi) and number of observed features (B for bacteria, D for fungi) for 'Ambrosia' epiphytic (blue) and endophytic (red) samples, as well as 'Opal' epiphytic (green) and endophytic (orange) samples. Each group is

ordered based on sampling time point. Lower and upper box bounds indicate the 25$^{th}$ and 75$^{th}$ percentile of the distribution, while lower and upper whiskers indicate the 9$^{th}$ and 91$^{st}$ percentile of the distribution.

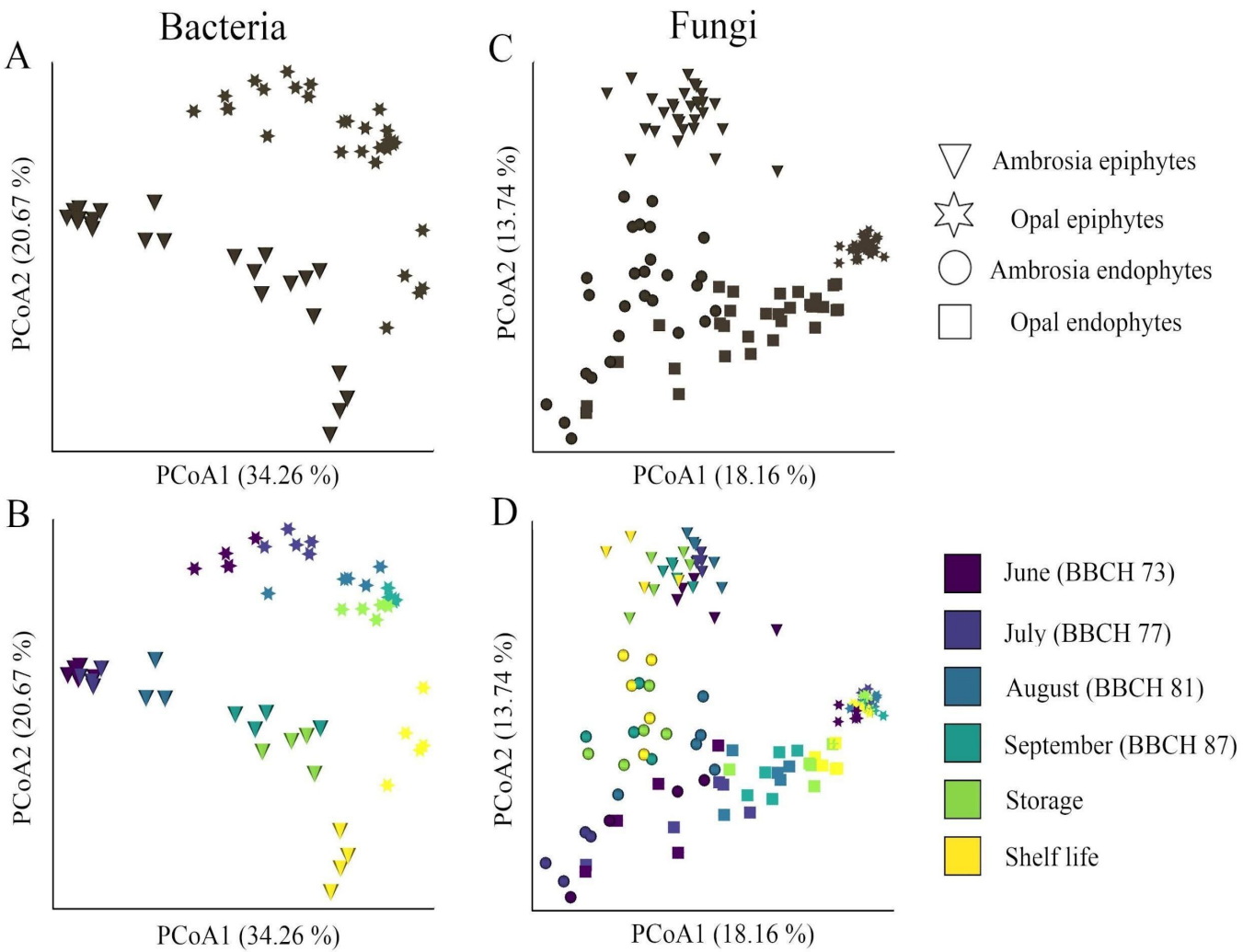
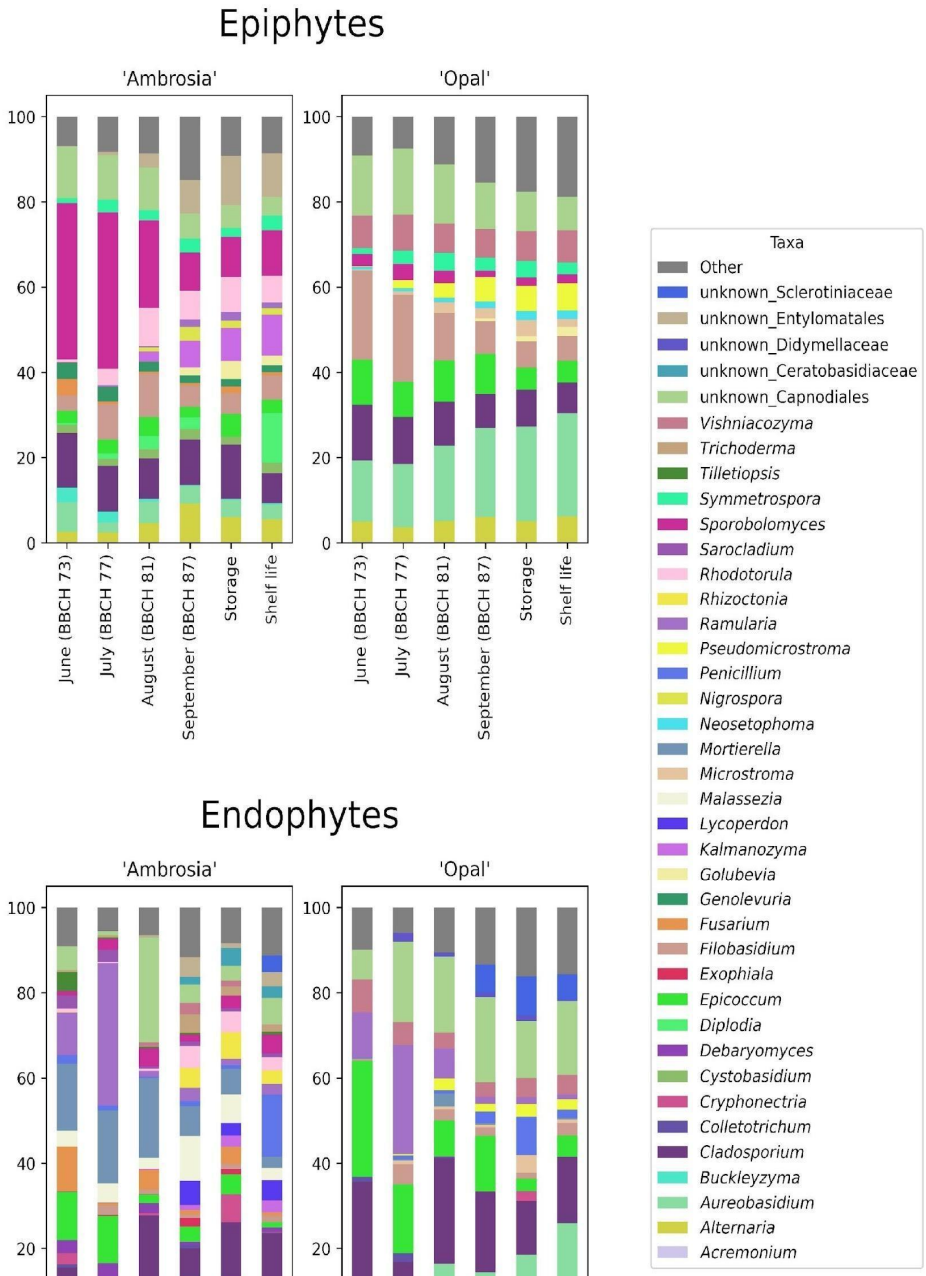



**Figure 3**. PCoA analysis of Bray-Curtis dissimilarity results for bacteria and fungi. A and C are monochrome and show samples from different cultivars and tissues by different shapes. B and D have in addition been color mapped based on sampling time point.

A


Epiphytes
'Ambrosia'
'Opal'
100
80
60
40
20
0
June (BBCH 73)
July (BBCH 77)
August (BBCH 81)
September (BBCH 87)
Storage
Shelf life
Taxa
Other
unknown_Sclerotiniaceae
unknown_Entylomatales
unknown_Didymellaceae
unknown_Ceratobasidiaceae
unknown_Capnodiales
Vishniacozyma
Trichoderma
Tilletiopsis
Symmetrospora
Sporobolomyces
Sarocladium
Rhodotorula
Rhizoctonia
Ramularia
Pseudomicrostroma
Penicillium
Nigrospora
Neosetophoma
Mortierella
Microstroma
Malassezia
Lycoperdon
Kalmanozyma
Golubevia
Genolevuria
Fusarium
Filobasidium
Exophiala
Epicoccum
Diplodia
Debaryomyces
Cystobasidium
Cryphonectria
Colletotrichum
Cladosporium
Buckleyzyma
Aureobasidium
Alternaria
Acremonium

B


Endophytes
'Ambrosia'
'Opal'
100
80
60
40
20
0
June (BBCH 73)
July (BBCH 77)
August (BBCH 81)
September (BBCH 87)
Storage
Shelf life

**Figure 4**. Genus composition of epiphytic (A) and endophytic (B) fungi in ‘Ambrosia’ and ‘Opal’ apples, calculated as a mean of all repetitions in each time point. The ‘other’ category groups all genera with less than 2 % relative frequency in all time points. For all time points prior to harvest, the BBCH phenological stage is shown.

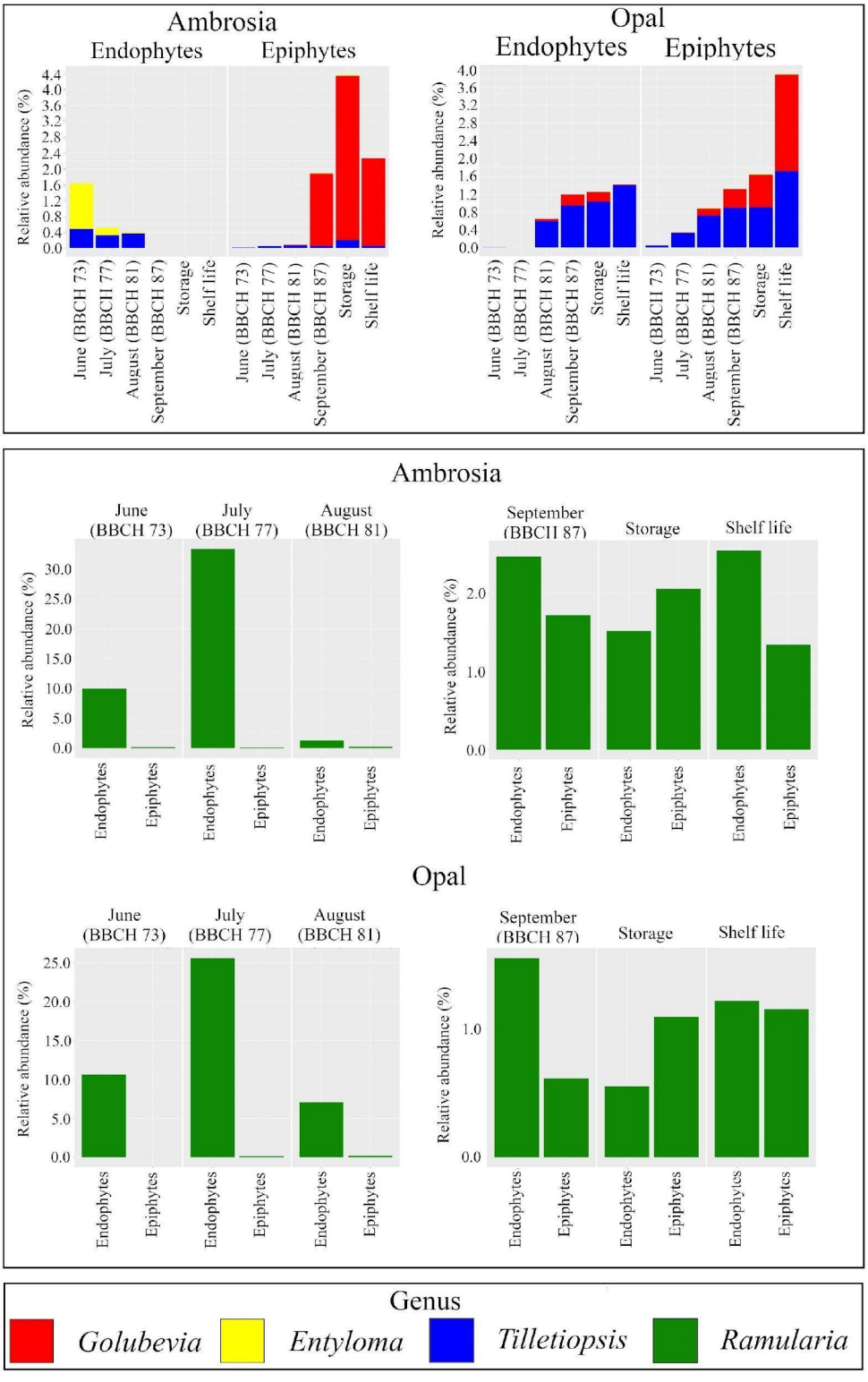



**Figure 5**. Relative abundance of white haze and dry lenticels rot genera in 'Ambrosia' and 'Opal' over all sampling time point and tissues

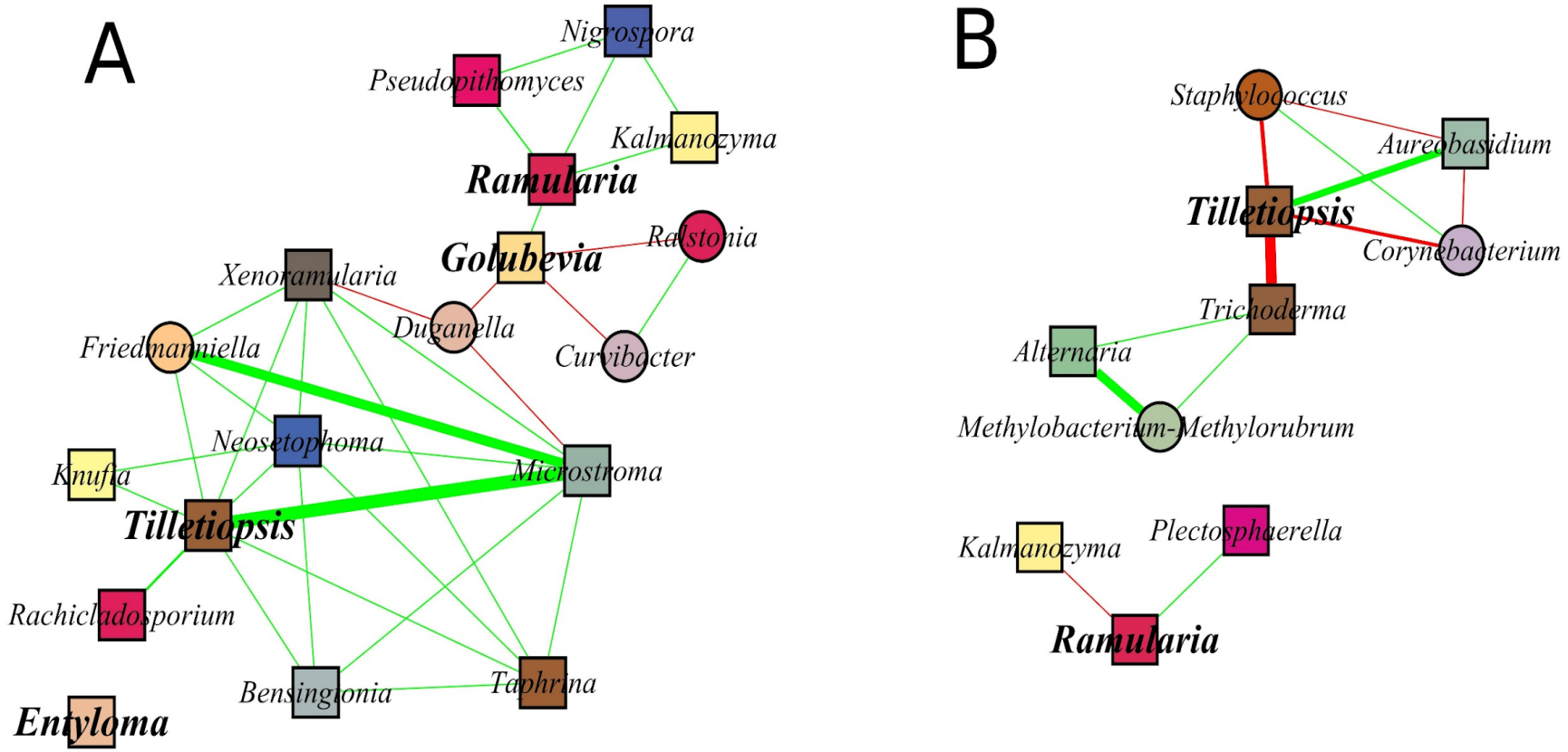



**Figure 6.** Interaction networks between *Entyloma*, *Golubevia*, *Tilletiopsis*, *Ramularia* and other epiphytic (A) and endophytic (B) microbial genera. Round nodes indicate bacterial genera, while square nodes indicate fungal genera and octagonal nodes metavariables. Red edges indicate co-exclusion, while green edges indicate co-presence. Width of each edge is proportional to association strength. Considered metrics are Bray-Curtis dissimilarity, Pearson correlation, Spearman correlation, Kullnack-Leibler

distance and mutual information. P-values were calculated using 100 repetitions and merged with the Brown method, while multiple testing correction was carried out with Benjamini-Hochberg. Resulting values were considered significant if <0.05

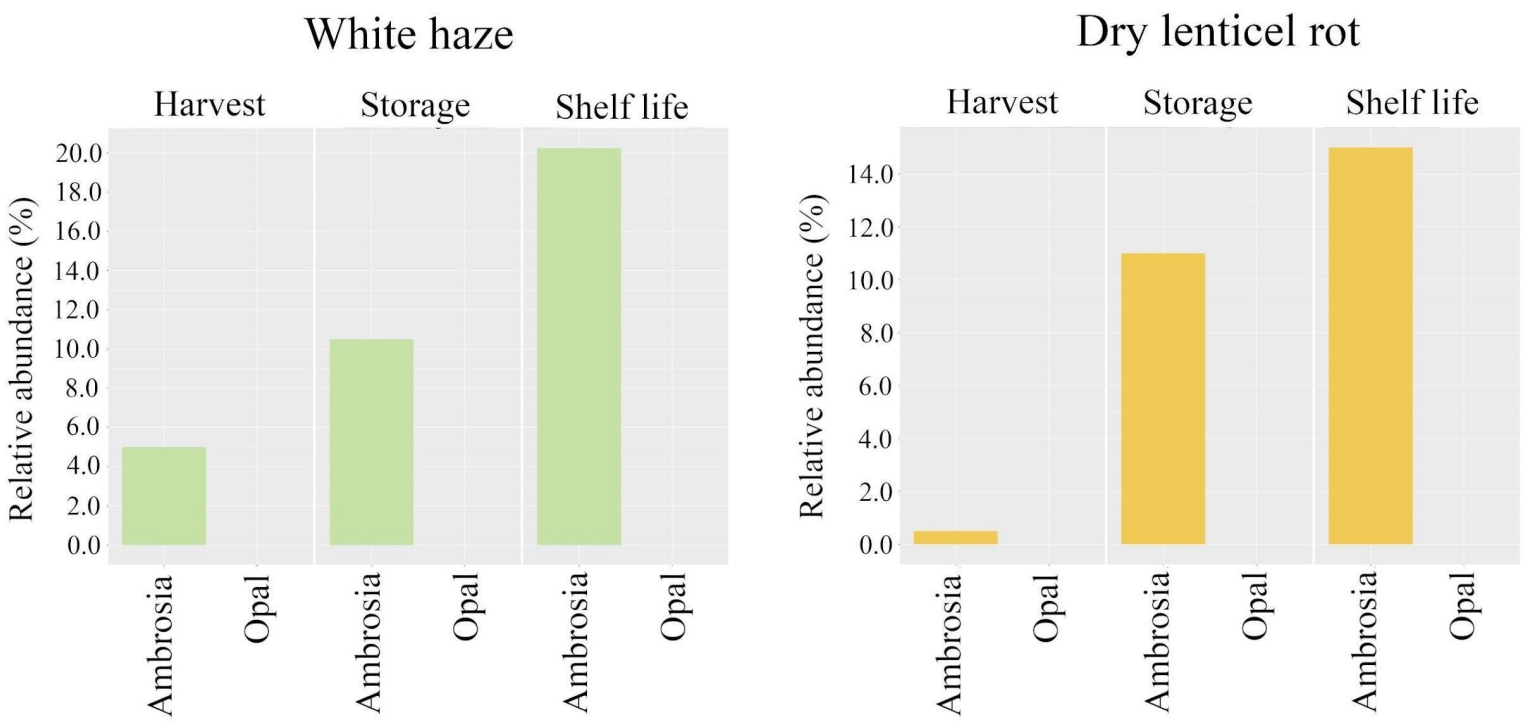



**Figure 7**. Incidence of white haze and dry lenticels rot measured at harvest, after cold storage and at the end of shelf life for 'Ambrosia' and 'Opal'.

**Tables**

| Cultivar | Incidence at harvest (%) | | | Incidence after storage (%) | | | Incidence after shelf life (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | **White haze** | **Dry lenticel rot** | **Black spot** | **White haze** | **Dry lenticel rot** | **Black spot** | **White haze** | **Dry lenticel rot** | **Black spot** |
| 'Ambrosia' | 5.00±5.00 | 0.50±1.00 | 0.0±0.0 | 10.50±10.00 | 11.00±10.00 | 0.0±0.0 | 20.24±14.00 | 15.00±6.00 | 0.0±0.0 |
| 'Opal' | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 7.50±4.00 | 0.0±0.0 | 0.0±0.0 | 52.5±10.00 |
| T-test | * | n.s. | n.s. | * | * | * | * | * | *** |

**Table 1.** Incidence of white haze, dry lenticel rot and black spot on 'Ambrosia' and 'Opal' apples at harvest, after storage and after shelf life. Statistical analyses were conducted using Student's t-test with alpha<0.05. Values are expressed as mean ± standard deviation. For significance levels: * = P< 0.05; ** = P<0.01; *** = P< 0.001; n.s. = not significant

| Cultivar | Firmness (N) | | |
|---|---|---|---|
| | **At harvest** | **After storage** | **After shelf life** |
| 'Ambrosia' | 35.40±0.88 | 31.58±1.47 | 30.20±0.78 |
| 'Opal' | 43.93±1.27 | 36.68±2.06 | 34.22±4.41 |
| T-test | *** | n.s. | n.s. |
| | **Total soluble solids (%)** | | |
| | **At harvest** | **After storage** | **After shelf life** |
| 'Ambrosia' | 10.75±1.23 | 10.9±0.38 | 11.48±0.90 |
| 'Opal' | 14.09±0.36 | 14.54±0.53 | 15.09±0.70 |
| T-test | * | ** | ** |
| | **Titratable acidity (%)** | | |
| | **At harvest** | **After storage** | **After shelf life** |
| 'Ambrosia' | 0.28±0.07 | 0.20±0.03 | 0.17±0.02 |
| 'Opal' | 0.60±0.06 | 0.40±0.03 | 0.36±0.03 |
| T-test | ** | *** | *** |

**Table 2.** Firmness, total soluble solids and titratable acidity for 'Ambrosia' and 'Opal' apples at harvest, after storage and after shelf life. Statistical analyses were carried out using Student's t-test with alpha<0.01. Values are expressed as mean ± standard deviation. For significance levels: * = P< 0.05; ** = P<0.01; *** = P< 0.001; n.s. = not significant.

| Taxon | Parameter | R2 | Pr (>F) |
|---|---|---|---|
| **Bacteria** | Cultivar | 0.247 | *** |
| | Time point | 0.444 | *** |
| | Cultivar×Time point | 0.102 | *** |
| | Residuals | 0.207 | NA |
| | Total | 1.000 | NA |
| **Fungi** | Cultivar | 0.142 | *** |
| | Time-point | 0.099 | *** |
| | Tissue | 0.125 | *** |
| | Cultivar×Time point | 0.056 | *** |
| | Cultivar×Tissue | 0.047 | *** |
| | Time point×Tissue | 0.077 | *** |
| | Cultivar×Time point×Tissue | 0.042 | ** |
| | Residuals | 0.411 | NA |
| | Total | 1.000 | NA |

**Table 3.** PERMANOVA analysis results for bacteria and fungi. R2 value indicates the total variance fraction explained by the parameter or combination of parameters, while Pr (>F) is the FDR adjusted p-value using the Benjamini-Hochberg correction. For significance levels: * = P< 0.05; ** = P<0.01; *** = P< 0.001; n.s. = not significant.