**Characterization of the lenticel damage of avocado cv. Hass and its associated fungal communities**

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**Abstract**

The lenticel damage reduces the quality of avocado cv. Hass, leading to high fruit rejection rates and losses for the growers. The causes of this affectation are not fully understood, but mechanical stress and phytopathogens have been associated with the damage, suggesting that abiotic and biotic factors are involved. This study evaluated several aspects of the lenticel damage of avocado cv. Hass to unravel the nature of its causality. Precisely, we followed the lenticel damage in two Colombian farms for two years and found that spatiotemporal factors influence the damage as it is non-uniform and progressive. Then, we used NGS to characterize fungal communities of fruits with severe and mild injuries and found that these communities varied between farms. We also found that the damage severity influenced the composition of the fungal communities as some taxa were more common in avocados with severe damage. Finally, we isolated different fungi from necrotic lenticel to see whether we found some of these enriched taxa. Still, the isolated fungi did not coincide, showing that standard culturable techniques fail to capture the complexity of the fungal communities associated with the lenticel damage.

**Key words**

Persea americana, Next-generation sequencing, lenticelosis, *Pseudocercospora*, *Colletotrichum*

**Introduction**

Colombia has nearly 26.0 thousand ha of avocado cv Hass producing 155.3 thousand t per year, as reported by Procolombia in 2021[1]. Most of this production goes to international markets, with the most relevant consumers being Canada, the United States, countries of the European Union, and Russia [1, 2]. The yield of the avocado cv. Hass crops isaround 9.0 t/ha in Colombia, below 30 t/ ha, which is the potential yield estimated for these crops [2]. Several factors are responsible for these low yields. Among these factors are those affecting the quality of the fruit, such as lenticel damage [3, 4].

The lenticel damage consists of 1 mm to 5 mm-long necrotic spots that develop on the exocarp of the fruits around the lenticels [5, 6], affection similar to others such as black spot, pepper spot, anthracnosis, pox, and speckle [4, 7–10]. The lenticel damage affects avocado cv. Hass but can also affect other cultivars, including the avocado cv. Lorena and cv. Choquette [11]. It can affect the fruit during the harvest but is more limiting during the post-harvest. The percentage of fruits with lenticel damage varies from 2.0% to 35.0% during harvest, but it can be between 10.0% and 62.0 % during the post-harvest [3, 4]. The damage severity influences the value and destination of the fruit [12], and according to processing plants, it is responsible for 5% to 30% of the rejected fruit. Furthermore, fruit rejection may increase as lenticel damage facilitates the entry of other plant pathogens, resulting in more severe affections [6].

The causes of the lenticel damage are controversial, but two hypotheses seem possible. The first one relates to mechanical stress suffered by the fruit during harvest and post-harvest [4, 13]. According to this hypothesis, impact injury promotes polyphenols production leading to tissue oxidation and necrosis [4]. The other hypothesis argues that pathogenic fungi are responsible for the lenticel damage. Different studies have isolated fungi such as *Pseudocercospora purpurea*, *Colletotrichum* spp., *Neofusicoccum parvum, Phomopsis* spp.and *Dothiorella* spp. from necrotic lenticels of avocado fruits [6, 7, 11, 14–16]. Also, fungicide applications with benzimidazoles, dithiocarbamates and copper-base fungicides during the harvest reduced the damage severity [10, 11]. The involvement of pathogenic bacteria besides fungi cannot be discarded, as one study isolated *Pseudomonas syringae* pv. *syringae* from symptomatic lenticels in avocado [17].

Despite the attempts of these studies to clarify the lenticel damage causality, their results are not conclusive. The necrosis resulting from mechanical stress and fungal infections differ at the cellular level, but these differences are not evident to the naked eye and can be mistaken [4, 18]. Also, most of the studies reporting the isolation of pathogenic fungi and bacteria have failed to replicate the infection on healthy fruits, which is critical to attribute causality [7, 8, 11, 15]. High humidity during harvest and post-harvest associate with severe lenticel damage [5, 6, 9, 13]. Those who support the mechanical-stress theory argue that the increased severity results from cells in hyperplasia being more vulnerable to mechanical stress. However, the increased humidity also favors the proliferation of pathogenic fungi, including *C. gloeosporioides* [6, 9, 13, 19].

All the above show that the nature and causality of the lenticel damage of avocado cv. Hass are poorly understood. This lack of understanding complicates the design of strategies that mitigate the loses resulting from this affectation. Here, we evaluated some of the unknown aspects of the damage. We hypothesized that plant pathogens have a role in causing the damage. We expected the damage to be non-uniform and progressive, with spatiotemporal components determine its occurrence. This behavior would be in line with the plant pathogenic-fungi hypothesis. We also expected that fungal communities associated with fruits with lenticel damage would differ from those of healthy fruits. This communities would be enriched in plant pathogenic taxon which could be isolated. To test these hypotheses, we first characterized the behavior of the damage across harvest in two farms with distinctive agroclimatic characteristics. Then, we assessed the damage progression during the post-harvest. Finally, we characterized the fungal communities associated with mild and severe lenticel damages using next generation sequencing and contrasted the findings with fungal isolations.

**Material and Methods**

Study area and sampling

Lenticel damage was evaluated in two commercial farms with different levels of affection in Colombia from June 2019 to June 2021. La Escondida Farm, located in de department of Antioquia, has low levels of lenticel affection, while El Sinaí farm, located in the department of Caldas, has high incidence levels (Supplementary Table S1).

Thirty trees with similar height, phenology, and age, were selected from five and nine plots in La Escondida and in El Sinai, respectively (Supplementary Table 2). To assess the lenticel damage, 10 mature avocado from each tree (i.e., fruits with reached a dry matter above 24 %) were collected with their peduncles in each harvest, for a total of 300 fruits per farm. Twenty additional fruits were sampled from each farm during the principal harvest of 2019 and the traviesa harvest of 2021 to isolate fungi associated with healthy and necrotic lenticels. Similarly, 12 additional fruits were sampled from each farm in the traviesa harvest of 2020 for the microbial ecology analysis. Six of these fruits had severe lenticel damage and the other six mild lenticel damage. All the sampled fruits were packed in punnets and carried to the laboratory, where they were processed.

Lenticel damage estimation

The lenticel damage was evaluated by analyzing the photographs of each face of the fruit in a macro developed in FIJI [20]. Specifically, the fruits were photographed on each of their faces (two photos per fruit). The macro used the photographs to estimate the incidence (i.e., the number of necrotic spots) and severity of the damage (i.e., percentage of necrotic area (area of necrosis/fruit surface area)). Then, the macro averaged the data of the two faces for each of the variables.

Lenticel damage was evaluated upon fruit arrival (0 days post-harvest; 0dph). Then, fruits were immersed in Timorex Gold Ⓡ (Stockton, Israel) at a concentration of 2 ml/l and stored at 6°C to simulate the post-harvest conditions of commercial fruits. A second measurement of the lenticel damage was performed after 21 days of cold storage (21 days post-harvest; 21 dph).

Differences in the severities and incidences of lenticel damage between farms and between the two measurements over time (0 dph and 21 dph) were evaluated using mixed-effect analyzes. The models assessing differences between farms included the interaction between farm and harvest as the fixed effect and, as random effects, the intercepts for the nested effect of tree in the plot (1 | pt) and plot (1 | plot) (Supplementary Table 3). The models evaluating the difference between the two measurements included the interaction between measurement and farm as a fixed effect and the intercepts for harvest (1|harvest) and fruit (1 | fruit) as random effects (Supplementary Table 4).

Linear mixed-effects models (lmer) were used for the severity analysis, and the data were transformed with the logarithm of the severity plus one (log (severity +1)). Generalized linear mixed models with the Poisson family (glmer (family = Poisson)) were used for the incidence analysis with no data transformation. Visual inspection of the models showed no deviation from linearity, homogeneity of variance, or normality. Complex models (including the fixed effect) were compared with simpler models (without the fixed effect) to assess the contribution of the fixed effect, using the *likelihood* *ratio* test with a confidence level of 95% (p-value: 0.05). These analyses used the *lmer* and *glmer* functions of the R library *lme4* (version 1.1-26) [21] and the *anova* function of the R library *stats* (version 4.0.4) [22]. The results were visualized using the R library *ggplo2* (version 3.3.3) [23].

DNA extraction and sequencing

DNA was extracted from the exocarp of avocado fruits with mild or severe lenticel damage to characterize the fungal communities associated with the lenticel damage using a modified version of a protocol reported elsewhere [24]. Specifically, exocarp samples were taken from the fruits using surgical knives and were macerated in a mortar with liquid nitrogen. One hundred mg of the macerated material were transferred to 2 ml-Eppendorf tubes. Samples were mixed with one ml of a prewash buffer (100.0 mM Tris-HCl (Thermo Fisher Scientific, Massachusetts) pH 8.0, 0.35 M Sorbitol (ProtoKimica, Colombia), 5.0 mM EDTA (Thermo Fisher Scientific) pH 8.0, 1% (W / V) polyvinylpyrrolidone (PVP-40) (Amresco, Texas) and 1% (V / V) β-mercaptoethanol (Acros Organics, Belgium)). The β-mercaptoethanol was added to buffers before the DNA extraction, while the other components were premixed. The tubes were centrifuged at 5000 g for 5 min, and the supernatant was discarded. The washing was repeated two times or until the supernatant was translucent**.**

One ml of lysis buffer (100 mM Tris-HCl pH 8.0, 3.0 M NaCl (ProtoKimica), 3% prewarmed cetyltrimethylammonium bromide (CTAB) (Amresco), 20 mM EDTA, 1% (P / V) PVP-40 and 1% (V / V) of β-mercaptoethanol were added to the tubes and mixed with vortex. Tubes were incubated at 65°C for 1 h, mixing the samples during the incubation. Samples were let stand for 5 min at room temperature and centrifuged at 5000 g for 5 min. The supernatant was transferred to a new tube and mixed with 1 ml of chloroform: isoamyl alcohol (24: 1) (Sigma-Aldrich, Missouri). Tubes were centrifuged at 5000 g for 10 min, and the upper aqueous phase was transferred to a new tube. 0.1 volume of 3 M sodium acetate pH 5.2 (Amresco) and a 0.66 volume of cold isopropanol (ITW Reagents, Germany) were added. Tubes were mixed by inversion and incubated overnight at -20°C. The DNA was precipitated by centrifugation at 15000 g for 10 min and washed twice with 0.6 ml of 70% ethanol in water (Sigma-Aldrich). The DNA pellet was recovered by centrifugation at 15000 g for 10 min and vacuum dried in a vacuum concentrator (Eppendorf) (alcoholic volume) at 30°C for 5 min. Finally, the pellet was resuspended in 50 µl of TE buffer (Biobasic, Canada) with RNase A (Thermo Fisher Scientific) at 0.05 mg/ml and incubated at 37°C for 30 min. The enzyme was inactivated at 65°C for 5 min, and the DNA suspensions were stored at -20°C until needed. DNA concentration was quantified using a Qubit fluorometer (Thermo Fisher Scientific) with the Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific). The DNA quality and integrity were verified by spectrophotometry and electrophoresis.

The DNA was sent to BaseClear (Holland) for paired-end sequencing of fragments of the internal transcribed spacer (ITS) of the ribosomal DNA region in the Ilumina's Miseq platform. The fragments were nearly 400 bp long and were limited by the forward primer 5'-GCATCGATGAAGAACGCAGCGAAA-3' and the reverse primer 5'-TCCTCCGCTTATTGATATGCTTAA-3 '. The company performed the quality controls and normalization of the DNA samples and prepared the Illumina libraries. Chloroplast and mitochondrial blocking primers were employed during the sequencing, and the sequences demultiplexed in the FASTAQ format were obtained from the company.

ITS amplicon analysis

Filtering, de-replication, removal of chimeras, and pairing of the forward and reverse ITS sequences were done using the opensource program DADA2 (version 1.18.0) [25]. These processes ensured a minimum of 10 bp overlapping between the two sequences and using a quality score higher than 30. The sequences of the primers were removed using Cutadap (version 3.1) [26]. Sequences were assigned to amplicon sequence variants (ASVs), retaining unique sequences occurring in several samples. The taxonomic identity was assigned to the ASVs in Qiime2 (version 2020.11) using the *q2-feature-classifier* with the *classify-sklearn* method and the Unite databases with 97% dynamic grouping and 99% (version 8.3) [25, 27, 28]. The dataset was trained with the Qiime2 (version 2020.11) *q2-feature-classifier* using the Naive Bayes classifier method [28]. The ASVs tables were filtered to exclude mitochondrial, chloroplast, and arcuate archaeal sequences with the *filter-table* functionality of Qiime2 (version 2020.11) [28], and the resulting ASVs tables were used for all the following evaluations.

Composition and diversity of fungal communities

The alpha diversity metrics (Richness, Simpson, Chao, and Faith's phylogenetic diversity indexes) were calculated using the *core-metrics-phylogenetic* and alpha diversity methods of Qiime2 (version 2020.11) with a rarefication depth of 18145 sequences considering the rarefaction curves [28]. A unidirectional anova was used to compare the alpha diversity of fungal communities of avocado fruits with mild and severe lenticel damage using the *anova* function of the R library *stats* (version 4.0.4) [22].

A principal coordinate analysis (PCoA) and a principal coordinate canonical analysis (CAP) analysis constrained to the strength of the lenticel damage (mild and severe) were used to compare the fungal communities (beta diversity). The weighted-UniFrac-distance metric and the ordinate function of the R library *phyloseq* (version 1.34.0) were used for these analyses [29]. The ASVs table was normalized using the *cumNorm* function of the R library *metagenomeSeq* (version 3.4) with the CSS (cumulative-sum scaling) method [30]. Differences between fungal communities were evaluated with permutational multivariate analysis of variance with the *adonis* and *anova.cca* functions of the R library *vegan* (version 2.5-7) [31]. The visualization of the principal component biplanes was done using the *plot\_ordination* function of the R library *phyloseq* (version 1.34.0)[29].

To assess whether some fungal taxa were differentially abundant in the fungal communities of fruits with mild and severe lenticel damage. The ASVs table was filtered using the function *calculateEffectiveSamples* from the R library *metagenomeSeq* (version 3.4). The ASVs table was normalized with the CSS method as before. A Zero-Inflated Gaussian Distribution Mixture Model was applied using the *fitZig* function from the R library *metagenomeSeq*. The model coefficients were compared with a moderated t-tests using the functions *makeContrasts* and *eBayes* from the R library *Limma* (v.3.46.0)[30]. P-values were adjusted with the Benjamini–Hochberg correction method, and taxa were considered differentially abundant when adjusted P-values were lower than 0.05. The taxonomic relation and relative abundance of the enriched ASVs were visualized using the *plot\_tree* of the R library *phyloseq* (version 1.16.2) and using the *ggplo2* library of R (version 3.3.3) [23, 29].

Isolation of fungal strains from healthy and necrotic lenticel

Between three to five samples of nearly 25 mm2 containing healthy or necrotic lenticels were taken from the exocarp of fruits for fungal isolation. The surface of the samples were disinfected with sodium hypochlorite (ProtoKimica, Colombia) at 2% for 5 min and ethanol (ProtoKimica, Colombia) at 70% for 1 min. Then, samples were washed three consecutive times with sterile distilled water and grown in plates containing 50% potato dextrose agar (PDA) (Alpha Bioscience, USA). The plates were incubated at 30°C, and the growing mycelium was subjected to multiple passages in 50% PDA until obtaining pure colonies. The isolates were stored in 20% glycerol (ITW reagents, Colombia) in water at –80°C and activated in 50% PDA for eight days at 30°C when needed.

Molecular identification of fungal isolates

 Genomic DNA of the fungal isolates was extracted from 48 h old mycelia grown in Sabroud broth (Merck, Germany) cultures using DNeasy Powersoil kit following the manufacturer's indications (Qiagen, Germany). The extracted DNA (2.5 µl) was used to amplify a 550 bp fragment of the ITS of the ribosomal DNA region using the ITS1 (5'-TCCGTAGGTGAACCCTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC-3') primers and 25 µl PCR reactions of GoTaq® Green Master (Promega, Madison, WI) according to the manufacturer's instructions. The PCR products were sequenced with Sanger sequencing using the same primers (ITS1 and ITS4). The sequences were processed, and their taxonomic identity was assigned using he Basic Local Alignment Search Tool (BLAST) and the NCBI database. Both processes were carried using the platform *Geneious Prime* (version 2020.2.4) (https://www.geneious.com).

**Results**

**Lenticel damage varies between farms and harvest**

To characterize the behavior of lenticel damage, avocado cv. Hass fruits were collected during principal and traviesa harvests between June 2019 through June 2021 in two commercial farms. The severity and incidence of the damage at 0 days post-harvest (dph) depended on the farm and the harvest (p-value < 0.001), with fruits from La Escondida having overall lower (0.6-fold or below) incidences and severities than those from El Sinai in most harvests (Fig 1 and Supplementary Table S3). The exception was the principal harvest of 2020, in which fruits from El Sinai had severities and incidences slightly lower than those of La Escondida (Fig 1 and Supplementary Table S3). Overall, at 0 dph, fruits from La Escondida had severities of 0.5% on average, with a maximum severity of 5.3%. On the other hand, fruit from El Sinai had severities around 1.0 %, with a maximum of 8.1%. Regarding the incidence, fruits from La Escondida had on average 46 necrotic spots, with the most affected fruit having 300, while those from El Sinai had on average 85 necrotic spots and a maximum of 520.

The lenticel damage was most damaging during both traviesa harvests in fruits from the El Sinai (Fig 1 and Supplementary Table S3). These harvests also had the highest daily precipitation and relative humidity (Supplementary Table S1). In La Escondida, the traviesa harvest of 2020 also had fruits with high affections, high daily precipitation, and relative humidity. However, this farm's traviesa harvest of 2021 had mild lenticel damage despite having high daily precipitation and relative humidity. These results show that the lenticel damage behavior changes across harvest and depends on the farm, suggesting a spatiotemporal component affected by climatic variables. This spatiotemporal component was also present at the tree and plot level, with 7 % and 5 % of the variation in the incidences and severities of the lenticel damage not explained by the farm and the harvest resulting from differences between fruits coming from different trees within a plot and different plots, respectively (Supplementary Table S1, Fig 1).

**Lenticel damage increases during cold storage**

Comercial avocados cv. Hass undergo cold storage (6⁰C) during the post-harvest to prevent the fruit from maturing before reaching the marketers. The storage time depends on the client but is nearly 21 d for fruits exported to EEUU and EU. Some anecdotical evidence shows that the damage increases during the storage as marketers report receiving fruits with lenticel damages more severe than those evidenced by the packing plants [13].

The severity and the incidence of lenticel damage increased in the fruits of both farms and all harvests after 21 d at 6⁰C. The increments were higher for fruits from El Sinai (4.0-fold for the severity and 2.6-fold for the incidence) than those of fruits from La Escondida (x 2.9 and x 2.2, respectively) (Fig 2 and Supplementary Table S4). The harvest influenced the lenticel damage to some extent, as 20% of the lenticel damage differences unexplained by the farm and the cold storage, came from differences between fruits coming from different harvests (Supplementary Table S4).

**Fruits from different farms have distinct fungal communities and the lenticel damage influences their structure**

Lenticel damage has been associated with mechanical stress suffered by the fruit during harvest and post-harvest [4]. However, the spatiotemporal component of the lenticel damage and its increase during cold storage suggests that other factors might also be involved. Fungal pathogens might be among those factors as they have been associated with the damage [4, 11, 14]. We characterized the fungal communities of fruits with mild and severe lenticel damage in the traviesa harvest of 2020 of both farms to assess this association. Although these fruits were selected attempting to maximize the differences between severe and mild lenticel damage, the differences were more pronounced for fruits from El Sinai (Supplementary Fig S3).

Fungal communities of avocado cv. Hass consisted of 370 to 583 AVSs with an average of nearly 450 AVSs (Fig 3a).

These communities had a Shannon diversity between 4.4 and 6.5, with a mean of 5.3, and a Fath's PD between 43.3 and 67.1, with a mean of 52.2 (Fig 3b and 3c). Farm or severity of the lenticel damage did not affect the richness, Shannon diversity, or Fath's PD of these communities (p-value > 0.050). In La Escondida, the severity of the damage did not affect the alpha-diversity of the fungal communities either (Fig 3). However, in El Sinai, severe lenticel damage was associated with richer fungal communities (Severe: 447 vs. Mild: 406; p-value: 0.020) (Fig 3a) but equally diverse (p-value > 0.050) in terms of the Shannon diversity and Fath's PD (Fig 3b and 3c).

Fungal communities were separated into distinct groups in the PCoA biplane (Fig 4a). The PCoA axis captured 72.6% of the community’s variation (Fig 4a), and 70.1% of this variation could be attributed to the combined effect of the farm and the severity of the lenticel damage of the fruit according to the permutational multivariate analysis of variance (p-value: 0.001). Nonetheless, the farm was the main driver of the separation, explaining 56.6% of the overall variation between populations (p-value: 0.001; Fig 4a and Fig 4b). The effect of the lenticel damage severity, on the other hand, was barely significant (p-value: 0.097) and less pronounced. The extent of the damage explained only 9.0% of the community's variation (Fig 4a and Fig 4c).

**Some fungal taxa are common in fruits with lenticel damage influences**

Two phyla, 23 classes, 67 orders, 168 families, and 278 genera composed the fungal communities of avocados cv. Hass. Many of these taxa (i.e., 17 classes (74 %), 34 orders (51 %), 74 families (44 %), and 97 genera (36%)) were shared between the fruits regardless of the farm or lenticel damage severity (Supplementary figure 2). On the other hand, few taxa (< five taxa) were unique to the fungal communities of fruits with severe lenticel damages (Supplementary figure 2). For example, only five genera, including Bannoa, Calonectria, Coprinus, Orbilia, and Pleopassalora, were shared between severely damaged fruits of both farms. These genera had low abundances (< 10 ASV), which might not be biologically relevant. However, these fruits had increased relative abundances of Ascomycetes (Fig 5a). The relative abundance of Ascomycetes in the fungal communities of fruits with severe damage was between 36.2 % and 73.7 % (mean: 53.3 %) compared with 17.8 % and 59.9 % (mean: 28.9 %) observed in fruits with mild lenticel damage (p-value: 0.001). The opposite occurred for basidiomycetes (Fig 5a). The relative abundances of Basidiomycetes in fruits with severe damages was between 2.6 % to 21.5 % (mean: 12.1 %) which was lower (p-value: 0.004) than 15.4 % and 49.6 % (mean: 29.3 %) observe for fruits with mild damage.

These ascomycetes and basidiomycetes communities consisted of different genera depending on the farm and the severity of the lenticel damage (Fig 5b and 5c). However, some trends occurred between farms. The ascomycete genera *Trichomerium*, *Pseudocercospora*, and *Colletotrichum* had increased relative abundances in fruits with severe damages, while *Hyphozyma* and *Cladosporium* in mildly damaged fruits (Fig 5b). When considering each farm, the ascomycetes *Pestalotiopsis*, *Geastrumia*, *Cyphellophora* and *Chaetothyrina*, and the basidiomycetes *Saitozyma*, *Meira*, *Ceraceosorus*, and *Bulleribasidium* were increased in El Siani fruits with severe damage (Fig 5b and 5c). Whereas ascomycetes *Setophoma*, *Pleurophoma*, *Meyerrozyma* and *Diaporthe*, and the Basidiomycetes *Moniliopthora*, *Kockovaella*, *Itersonilia*, *Gjaerumia*, *Derxomyces*, and *Cystobasidium* were prevalent in La Escondida (Fig 5b and 5c). For fruits with mild damages, the ascomycetes *Temphureobicoium*, *Perribasccium*, and *Aspergillus* and the basidiomycetes *Symmeterspora*, *Sporobolomyces*, *Mycrostroma*, and *Gebolevuria* were common in El Sinaí (Fig 5b and 5c). Whereas, in La Escondida, the ascomycete *Zasmidium* and the Basidiomycete *Vishidiomicema* were common (Fig 5b and 5c).

Several ASVs were also enriched (p-value> 0.05) in fruits with severe lenticel damage (Fig 6 and 7, Supplementary Table S5 and S6). Nine ASVs were enriched in the fruits from La Escondida and 40 in fruits from El Siani. Among those from La Escondida, enriched ASVs included one basidiomycete of the genus Cystobasidium and three ascomycetes of the genera Setophoma, Bacidina, and Neopestalotiopsis. The remaining six were not classified, but 3 of them clustered with ASVs of the Ascomycota class Dothideomycetes (Cluster III) and one with the ASVs classified as Cystobasidium sp. (Cluster XIII). The other not classified ASVs did not relate to any classified ASVs (Cluster IV) (Fig 6 and Supplementary Table S5). In El Sinai, most of the enriched ASVs (n: 22) belonged to two clusters (clusters III and IV) composed of ASVs belonging to the Ascomycota class Dothideomycetes. Several of the enriched ASVs in these clusters belonged to the genera Microcyclospora, Pseudocercospora, and Geastrumia. The Ascomycota classes Eurotiomycetes (Cluster II) and Sordariomycetes (Cluster V) were also common among the ASVs enriched in the severely damaged fruits of the El Sinai. The former had six enriched ASVs, with three of them classified as Trichomerium spp. The latter included four enriched ASVs, all classified as Colletotrichum spp. Seven of the remaining enriched ASVs of the El Sinai cluster with other AVSs belonging to several Basidiomycetes classes (Cluster IX). Two of them belonged to the Agaricomycetes class, and the other four were not classified but grouped closely together with the Agaricomycetes. Only one of the enriched ASVs in the El Sinai belonged to a phylum other than Ascomycota and Basidiomycota. However, this ASV was not classified (Fig 7 and Supplementary Table S6).

**The communities of cultivable fungi are poor predictors of the fungal communities associated with the lenticel damage**

We performed isolation from healthy and necrotic lenticels of avocado cv. Hass to determine whether some of the enriched ASVs could be recovered. Forty-nine fungal strains were isolated, and 39 were identified. All isolates were *Ascomycetes*, and the most common genera were *Colletotrichum*, with 19 isolates, and *Cytospora*, with ten. These most common genera were isolated from both farms. *Alternaria*, *Diaporthe*, *Neofusicoccum*, *Neurospora*, and *Phyllosticta* were also among the genera isolated, and they had one to three representatives. These genera were restricted to one farm (Table 1; Supplementary Table 7). We found no clear trend between the isolated taxon and the health status of the lenticel. Also, no isolated taxon besides *Colletotrichum* coincided with the ASVs enriched in severely damaged fruits. However, *Colletotrichum* spp. were isolated from healthy and necrotic lenticel.

**Discussion**

The lenticel damage in avocado cv. Hass is poorly understood, and its causality is unknown. Two hypotheses are considered the most likely, one related to mechanical stress suffered by the fruits during harvest and post-harvest and the other to plant pathogens. We found that the lenticel damage has a non-uniform pattern and is progressive. The severity and incidence of the damage changed across trees, plots, farms, and harvest and increased during cold storage. We also found that fungal communities associated with the fruits varied from one farm to another, and the lenticel damage influenced to some extent the composition of these communities. Some taxa were more prevalent in fruits with severe lenticel damages. However, most of these enriched taxa were not among the fungal strains isolated from necrotic lenticels.

Damages resulting from non-living and living factors differ in their patterns. Those caused by non-living factors such as impact injury have a uniform pattern. On the other hand, damage caused by a living factor such as fungal pathogens has non-uniform patterns [32]. A spatial-temporal component influenced the severity and incidence of the damage at different scales. The damage varied not only between farms but between trees within a plot, and plots within farms. Some trees have fruits more damaged than those of other trees in the same plot. Also, some plots have fruits more damaged than those of other plots on the same farm. This scales variability suggests that the physical environment restrains whatever is causing the lenticel damage [33]. Then, we observe what are likely patterns of aggregation supporting the involvement of a plant pathogen in the damage. The lenticel damage also varied across the harvest, with the most severe damages occurring in harvests with the highest humidity and precipitation. These observations further suggest that the damage responds to the physical environment [33]. Other evaluations have also evidenced the temporal variation of the lenticel damage and its association with high humidity and precipitation [9, 11]. The authors of these evaluations attribute this association to climatic conditions that favor pathogen proliferation. The association between high humidity, rain, and *Colletotrichum* proliferation is well documented for several crops including avocado [34–36]. However, other evaluations have also demonstrated that high humidity makes avocado fruits more vulnerable to lenticel damage by mechanical injury [6].

We saw an increase in the lenticel damage during the cold storage. The damage almost double during this period in which the fruits were not subjected to further mechanical stress. The progressive nature of the lenticel damage has been reported before and is another piece of evidence suggesting the involvement of a plant pathogen or a plant pathogen complex in the damage [13]. Damage caused by a living organism is progressive, while those caused by non-living factors are not [32]. The observed increments varied depending on the harvest and were more pronounced for fruits coming from El Sinai. These observations are also not consequent with the uniform pattern of damages caused by non-living factors [32].

We found variation in the fungal communities associated with the exocarp of the fruits between the two farms. The observation of geographical location affecting microbial communities of fruits is not novel [37, 38]. A study evaluating the microbial communities of “Royal Gala” apple at different spatial scales demonstrated that these communities were similar across different tissues of the fruit but varied between fruits from different growing regions and orchids, with regions being the most influential [38]. It is likely that environmental conditions influence the assembly of fungal communities. These communities can be deferentially conducive for disease development and influence the health status of the fruit [38, 39]. The different fungal communities observed between the two farms might be in part responsible for the different levels of lenticel damage observe in the fruits coming from these farms. However, more evidence is necessary.

In line with the above mentioned, we saw that fungal communities differed between fruits with severe and mild lenticel damage. However, the differences were less evident than those observed between farms. Several studies have evaluated the association between microbial communities assembling and plant health. These studies have shown that diseased fruits and other plant tissue have distinct communities enriched with particular taxa [39–43]. An interesting finding was the relatively low and proportion of Ascomycetes (close to 29 %) and Basidiomycetes (close to 29 %) found in avocados with mild lenticel damages. These proportions seem uncommon as other studies report proportions of Ascomycota ranging between 50 to 100 % and proportions of Basidiomycota below 10 % [38, 41, 43]. Fruits with severe damages had Ascomycota and Basidiomycota proportions close to 53% and 12 %, respectively. The dominance of Basidiomycota might be an indication of a low probability of lenticel damage development. Some fungal genera such as *Trichomerium*, *Pseudocercospora*, and *Colletotrichum* were more common in the fruits from both farms with severe lenticel damages. These observations are in agreement with several studies that have isolated *Pseudocercospora*, and *Colletotrichum* from necrotic lenticels [6, 7, 11, 16]. Studies about *Trichomerium* are less common but this genus was recently included in the fungal complex responsible for the sooty blotch and flyspeck [44]. This complex comprises over 100 fungal species and produces symptoms like those of the lenticel damage in several crops [45]. Avocado might be among these crops, but more evidence is necessary [46].

The extent of the association between the lenticel damage and fungal community composition and diversity varied between farms and was most pronounced in El Sinai. The fruits severely damaged in this farm had distinct fungal communities, which were more diverse. This finding was unexpected as higher diversity is usually associated with healthy plants [39, 40]. The association between this increased diversity and the lenticel damage is unknown. However, the increase might come from saprophyte or other plant pathogenic fungi colonizing the most advanced damages [4, 11]. We found that these distinct communities were enriched in several Dothideomycetes, Eurotiomycetes, Sordariomycetes, and Agaricomycetes. These fungal classes have been associated with diseased plants in other systems [40, 42]. The taxonomy of most of these fungi is limited to the class level as we could not assign them higher ranks. The exception was *Trichomerium*, *Pseudocercospora* and *Colletotrichum,* mentioned before*,* and Microcyclospora and Geastrumia. The finding of these two Dothideomycetes among the enriched taxa associated with severe lenticel damages is interesting as species belonging to these genera are also part of the sooty blotch and flyspeck fungal complex [45].

The differences between fungal communities of fruits with mild and severe lenticel damage were less pronounced in La Escondida. These communities were comparable in their alfa diversities and some of the communities from fruits with severe damages were indistinguishable from those of mildly damaged fruits. However, it is important to point that the differences between the damage severity of the fruits from this farm were not as remarkable as those of fruits from El Sinai. It is likely that the greater the damage the greater the effect on the fungal communities, but more evaluations are necessary to test this hypothesis. Some Dothideomycete and one *Sordariomycetes, i.e., Neopestalotiopsis* were also enriched in the fungal communities of fruits with more severe damages. No evidence to our knowledge exists of Neopestalotiopsis spp. causing disease in avocado but some species belonging to this genus are part of the avocado endophytic-fungal community [47]. Among Dothideomycete, a fungus from the Setophoma genus was also enriched in these communities*.* Fungi from this genus cause leaf spots and necrosis in several hosts, but we lack evidence of whether avocado is among them [48]. Other fungi, including some *Cystobasidiomycetes* and *Lecanoromycetes*, were enriched in the fungal communities of fruits with more severe damages in this farm but their possible connection with plant disease is unknown.

We isolated different fungi from healthy and necrotic lenticel to see whether some of the taxa enriched in the fungal communities of avocado with severe and mild lenticel damage could be isolated. These isolated fungi did not coincide with the enriched taxa, with *Colletotrichum* being the exception*.* We also found no clear trend between the lenticel health status and the isolated taxon. Several of the isolated fungi are the same ones associated by other authors with a variety of avocado disease symptoms, including lenticel damage [6, 7, 11, 14–16, 49–52]. Our results show that the fungal communities associated with this damage are complex, and it is likely that more than one species cause the damage. This scenario would help to explain the impossibility of several works to recreate the lenticel damage symptoms in healthy fruits [7, 11, 15]. Most of these studies have been based on culturable techniques like those used in this study, making it likely that the culturable methods in these evaluations fail to capture the complexity of the fungal communities responsible for the lenticel damage. Our study presents evidence supporting the hypothesis that lenticel damage has a biotic component. The damage cannot be fully explained by mechanical stress suffered by the fruit during the post-harvest. However, further evaluations including more comprehensive isolation techniques and pathogenicity tests are still necessary to fully prove the participation of pathogenic fungi causing the lenticel damage of avocado cv. Hass.

**Acknowledgments**

We would like to acknowledge the Universidad EAFIT (Colombia), the avocado exporters CARTAMA, and the Department of Science, Technology and Innovation (COLCIENCIAS) for funding this project (contract number XXX). We also thank the supercomputing resources made available by the Centro de Computación Científica Apolo at Universidad EAFIT.

Sta**tements & Declarations**

**Author contributions**

Conceptualization: Mosquera, S., Villegas-Escobar, V., and Saavedra, S.; Methodology: Mosquera, S., Villegas-Escobar, V., and Saavedra, S.; Investigation: Mosquera, S., CatañoC., and Saavedra, S.; Formal analysis: Mosquera, S., and Villegas-Escobar, V.; Writing-original draft preparation: Mosquera, S., and Villegas-Escobar, V.; Review and editing: Mosquera, S., and Villegas-Escobar, V.; Funding acquisition: Villegas-Escobar, V., and Saavedra, S.; Resources: Villegas – Escobar, V.; Project administration:  Villegas – Escobar, V.; Supervision: Villegas – Escobar, V.

**Funding**

the avocado exporters CARTAMA, and the Department of Science, Technology and Innovation (COLCIENCIAS) for funding this project (contract number XXX).

**Conflict of Interest**

The authors declare no competing interests

**Data availability**

Raw sequence data have been deposited at the European Nucleotide Archive (ENA) under accession numbe XXX. Any additional datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**Ethics approval**

This study was performed in line with the approval of the Ethics Committee of Universidad EAFIT































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**Figure Captions**

**Fig 1**. Lenticel damage for avocado cv. Hass fruits collected from the La Escondida and El Sinai farms between 2019 and 2021 at harvest time (0 days post-harvest, 0dph). Bars and error bars represent mean and standard deviation for the severity (A) and incidence (B) of the damage observed in the farms for each harvest at 0 dph. The points show the severity and incidence means for the fruits collected from the different plots, and the letter after the year denotes main (m) and traviesa (t) harvest..

**Fig 2**. Lenticel damage for avocado cv. Hass fruits collected from the La Escondida and El Sinai farms between 2019 and 2021 at harvest time (0 days post-harvest, 0 dph) and after 21 days of storage at 6⁰C (21 days post-harvest, 21 dph). Shown are the means and standard deviations for the severity (A) and incidence (B) of the damage for the two evaluatin points (0 dph and 21 dph). The points show the severity and incidence means for the fruits collected in the four harvest, and the letter after the year denotes main (m) and traviesa (t) harvest.

**Fig 3**. Alfa diversity for the fungal communities of the avocado cv. Hass fruits with different severities of lenticel damage (Mild and Severe) collected from the La Escondida and El Sinai farms during the traviesa harvest of 2020. Bars and error bars represent mean and standard deviation for the alfa diversity index richness (A), Shannon (B), and Faith pd (C) alpha-diversity (n: 6). The points show the alfa diversity metrics the fruits and the asterisk denote statistic differences at the 95.0 % confidence level (\*) according to the t test.

**Fig 4**. Fungal communities’ structure for fruits of avocado cv. Hass fruits with different severities of lenticel damage (Mild and Severe) collected from the La Escondida and El Sinai farms during the traviesa harvest of 2020. Cumulative sum scaling (CSS) transformed reads were used to calculate weighted unifrac distance. Shown are the bidimensional planes of the principal coordinates analysis (PCoA) (72.6 % of the overall variance) (A) and canonical analysis of principal coordinates (CAP) constrained by the farm (La Escondida and El Sinai) (68.8 % of the overall variance; p-value: 0.001) (B). Each point represents the fungal community of a fruit, and the colors show the origin (La Escondida o El Sinai) and damage severity (Severe or Mild) of the fruit. The ellipsis are the 95 % confidence level ellipses.

**Fig 5.** Taxonomic composition of the fungal communities of the avocado cv. Hass fruits with different severities of lenticel damage (Mild and Severe) collected from the La Escondida and El Sinai during the traviesa harvest of 2020. Shown are the relative abundances of the fungal families of the entire communities (A) and the genus for the communities of Ascomycota (B) and Basidiomycota (C).

**Fig 6.** Taxonomy and relative abundance of the amplicon sequence variants (ASVs) enriched (p-value < 0.05) in the fungal communities of avocado cv. Hass fruits with different severities of lenticel damage (Mild and Severe) collected from the La Escondida during the traviesa harvest of 2020. Shown are the taxonomic relation (A) and the relative abundances (B) of the ASVs group by color according to the clade (Supplementary Table 5).

**Fig 7.** Taxonomy and relative abundance of the amplicon sequence variants (ASVs) enriched (p- value < 0.05) in the fungal communities of avocado cv. Hass fruits with different severities of lenticel damage (Mild and Severe) collected from the La Sinai during the traviesa harvest of 2020. Shown are the taxonomic relation (A) and the relative abundances (B) of the ASVs group by color according to the clade (Supplementary Table 6).

**Tables**

**Table 1.** Fungal isolates originated from healthy and necrotic lenticels of avocado cv. Hass fruits collected from the La Escondida and El Sinai during the main harvest of 2019 and traviesa harvest of 2021.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Isolates** | **La Escondida** | | **El Sinai** | | **Total** |
| **Necrotic** | **Healthy** | **Necrotic** | **Healthy** |
| *Alternaria* sp. | 1 | 1 | 0 | 0 | 2 |
| *Colletotrichum* sp. | 6 | 5 | 7 | 1 | 19 |
| *Cytospora* sp. | 6 | 1 | 0 | 3 | 10 |
| *Diaporthe* sp. | 0 | 0 | 1 | 1 | 2 |
| *Neofusicoccum* sp. | 0 | 1 | 0 | 0 | 1 |
| *Neurospora* sp. | 2 | 0 | 0 | 0 | 2 |
| *Phyllosticta* sp. | 0 | 0 | 3 | 0 | 3 |
| **Total** | 15 | 8 | 11 | 5 | 39 |