Fungal microbiome shifts on avocado fruit associated with a combination of postharvest chemical and physical interventions

Running head: Postharvest avocado fungal microbiome shifts

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Abstract

Aim of the study: To characterise the baseline microbial population of the avocado carposphere and understand shifts in community structure from the harvest to ready-to-eat stages.

Methods and Results: The changes in surface or stem-end fungal microbiomes at the postharvest stage of avocado fruit were studied using next-generation sequencing of the internal transcribed spacer (ITS) region. Avocado fructoplane and stem-end pulp fungal richness differed significantly between postharvest stages with a decline following prochloraz dip treatments. Known postharvest decay-causing genera, *Colletotrichum*, *Fusarium*, *Alternaria*, *Epicoccum*, *Penicillium* and *Neofusicoccum* were detected, with *Papiliotrema*, *Meyerozyma* and *Aureobasidium* confirmed as the most dominant potentially beneficial genera. Postharvest interventions such as prochloraz had a negative non-target effect on the presence of *Papiliotrema flavescens* on the avocado fructoplane.

Conclusion: Our findings reveal a core community of beneficial and pathogenic taxa in the avocado fructoplane, and further highlights the reduction of pathogenic fungi as a consequence of fungicide use.

Significance and impact of the study: The current study provides important baseline data for further exploration of fungal population shifts in avocado fruit driven by chemical (fungicide) as well as physical (cold storage) interventions.

Keywords: Non-target fungicidal effect; Pathogenic and beneficial fungi; Plant microbiome; Stem-end pulp; Postharvest treatment

INTRODUCTION

The avocado (*Persea americana* Mill.) is a single-seeded fruit from the Lauraceae family (Silva and Ledesma, 2014). The avocado is highly sought after because of its delicate taste and health characteristics which is attributed to its high content of fatty acids and fat-soluble vitamins (Duarte *et al.*, 2016). The postharvest quality of avocados is affected by preharvest fungal pathogens, storage conditions and agronomical practices that result in postharvest development of fruit disease symptoms at the market-end of the supply chain (Korsten *et al*., 1997; Hernández *et al*., 2016; Ramírez-Gil *et al*., 2019). Some of the major postharvest diseases that cause serious losses during avocado export include anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., stem-end rot (SER) caused by *Phomopsis perseae* Zerova, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Dothiorella aromatica* (Sacc.) Petr. & Syd. and *Thyronectria pseudotrichia* (Schwein.) Seeler. (Zerova, 1940; Darvas and Kotze, 1987; Schaffer *et al*., 2013; Diskin

et al., 2017). These postharvest diseases threaten fruit industries facing restrictions on copper-based fungicides and postharvest disease control formulations such as prochloraz (due to its unfavourable toxicological properties) (Shimshoni *et al*., 2020). Prochloraz has traditionally been used in several fruit industries including the South African avocado industry for anthracnose control at the postharvest stage (Darvas, 1984). Hence, the development of alternative disease control strategies is urgently warranted.

Precisely for this reason, Wisniewski *et al*. (2016) have recommended that there should be a greater focus on alternative methods of integrated disease control. Strategies include augmentative control, conservation biocontrol, as well as the use of biopesticides and plant defence stimulators (Lamichhane *et al.*, 2017). Biocontrol use and integration with early-season chemical sprays decrease both reliance on and detrimental effects of conventional chemicals (Korsten *et al.*, 1997; Lamichhane *et al.*, 2017). The use of natural biocontrol was previously reported not to be commercially viable (Wisniewski and Wilson, 1992). Ease of use, efficacy and low cost of chemical treatments were amongst other factors contributing to apprehension towards biocontrol agents (Korsten, 1995). The development of biocontrol agents was also regarded as a complex and time-consuming approach that required extensive knowledge on the host-pathogen relationship and associated microflora (Korsten, 1995). However, the restrictions on chemical fungicide use coupled with recent advances in technology associated with biocontrol development (Leung *et al*., 2020), has necessitated revisiting the use of biocontrol products. These changes have hence resulted in a lot of interest and investment in bioproduct development in countries like South Africa.

To secure sustainable future avocado production, regenerative farming practices, wherein holistic disease management is part of futuristic farming, are crucial. The first step towards a more sustainable pest management system is the gathering of information on all aspects of the ecosystem. In consideration of the host, pathogen, and environmental interactions, host microflora has for many years been overlooked (Peñuelas and Terradas, 2014). Agricultural microbiome studies (Volschenk *et al.*, 2016; Carmicheal *et al.*, 2017; Abdelfattah *et al*., 2021) have provided an advantageous baseline knowledge for the development of postharvest control strategies. Focusing on an understanding of ecological balances, a more balanced long-term fruit health focus can be achieved (Korsten and de Jager, 1995; Wisniewski *et al.*, 2016; Wassermann *et al*., 2019). The insight and understanding of microbial population dynamics in commercial agricultural production systems presents new opportunities to approach disease control in a more comprehensive way (Sébastien *et al.*, 2019).

Previously, culture-dependent methods were used to assess microbial population dynamics, however, these approaches have several limitations in depicting the core and total microbial community. In reality, it could not be ascertained how far the cultured isolates mirrored the indigenous community on the host plant (Stefani *et al.*, 2015). On the other hand, culture-independent approaches provide powerful new tools to investigate the total microbiome of plants and in the context of fruit; it can provide valuable information on population dynamics, diversity, density, persistence and dominance (Mayo et al., 2014). In this context, Xue *et al*. (2015) and Wassermann *et al*. (2019) have for instance used DNA sequencing studies of the fruit microbiome to unravel the interaction between pathogens and natural biological control populations. Using microbiomics to provide more

information on the avocado fructoplane and stem-end pulp populations in response to chemical and physical interventions at the postharvest stage will aid in the development of targeted disease control strategies. It is in this context that the present study was embarked upon understanding the baseline microbial population of the avocado fruit from harvest to the ready-to-eat stage. Special emphasis was placed on understanding the existing commercial postharvest handling practices (prochloraz treatment and cold storage to simulate shipping) effect on pathogenic fungi known to cause anthracnose and stem-end rot as well as potential biocontrol (fungal) agents and the core microbiome. With this in mind, we hypothesized that fungal diversity and composition differs at various production and processing stages, and that both chemical and physical interventions influence the presence of potential beneficial fungal taxa at the postharvest stage of avocado.

MATERIALS AND METHODS

Study site, sample collection and processing

Experimental sampling was conducted at a commercial avocado orchard in Tzaneen in the Limpopo Province, South Africa during the 2018/19 production season. A total of 54 'Hass' avocado trees were randomly selected as mature fruit (early season and ~27% dry matter content) sources in the commercial orchard. Sampling involved the collection of 18 fruit per tree at the final stages of production (at harvest), sampled at the top (n = 6), middle (n = 6) and lower (n = 6) regions of the tree canopy. In total, 972 individual fruit were collected to determine postharvest fungal shifts and the effect of postharvest physical and chemical interventions on these communities; comparisons were made between either the fructoplane or stem-end pulp samples at six different postharvest

stages: (i) freshly harvested and untreated fruit (AH), (ii) after commercial prochloraz (Chronos® 45 EC, ADAMA, Brackenfell, South Africa) dip treatment (APD), (iii) two weeks cold storage (2WCS), (iv) three weeks cold storage (3WCS), (v) four weeks of cold storage during simulated shipping (4WCS), and (vi) at the ripe and ready-to-eat stage (RE) (Table S1). All samples were stored at 4 °C during transport and processed within 24 h of collection from the commercial packhouse.

Epiphytic (fructoplane) microbiomes

For epiphytic micro-flora, a total of 108 individual fruit were collected per postharvest developmental stage. These were divided into six batches of 18 individual fruit per developmental stage. Within each developmental stage, these were further divided into three samples comprising six individual fruit per sample, with a total of 18 final samples for DNA extraction across the developmental stages. Briefly, each 225 g of fruit was suspended in 500 ml sterile peptone buffered water supplemented with 0.1% Tween80 (Associated Chemical Enterprises, Johannesburg, SA), before sonicating in an ultrasonic water bath (Labotec, Johannesburg) to dislodge microbes from fruit surfaces, as previously described by Chidamba and Korsten (2015). The micro-floral wash was concentrated through 0.45 µm pore size cellulose nitrate filters (Sartorius, Gottingen, Germany) yielding 18 samples for each postharvest stage from which DNA was later extracted.

Endophytic (stem-end pulp) microbiomes

To isolate the endophytic populations of the internal stem-end area, 54 fruit were collected per postharvest developmental stage. These were divided into three of 18 individual fruit per developmental stage. As with the epiphytic samples, these were further divided into three samples containing six individual fruit per sample per developmental stage, providing a total of 18 final samples for DNA extraction across all developmental stages. Samples were processed according to Diskin *et al*. (2017). Briefly, the fruit were surface disinfected with 70% ethanol for five minutes and then rinsed three times with sterile distilled water to remove epiphytic microorganisms. The surface disinfected fruit were aseptically peeled at the stem-end with a disinfected peeler, and the internal portion of the stem-end (stem-end pulp) of the fruit, sampled for analysis. This was immediately snap-frozen in liquid nitrogen, ground with a coffee grinder and stored at -80 °C for DNA extraction.

DNA extraction, amplification and analysis

Total community DNA was extracted from the avocado micro-floral surface wash filters (n = 18) and processed fruit pulp (n = 18) at different postharvest stages using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (ZymoResearch, Irvine, CA, USA), according to the manufacturer's instructions, before storing at -20 °C. The DNA concentration in each sample was quantified using the Nanodrop™ ND2000 spectrophotometer. Thereafter, DNA samples from either the fructoplane or stem-end pulp for each postharvest stage were pooled to equivalent concentrations, generating 36 bulk DNA samples for sequencing. All DNA samples were quantified and assessed for DNA purity

using the NanoDrop™ 2000 UV-Vis spectrophotometer (Thermo Scientific™). The mycobiome was sequenced at the Molecular Research DNA sequencing facility (MR DNA, Shallowater, TX, USA) using the Illumina MiSeq platform, targeting the fungal specific internal transcribed spacer (ITS) rRNA gene region. The ITS1 loci were amplified using ITS1-2 primers (ITS1F (5′- CTTGGTCATTTAGAGGAAGTAA -3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC -3′)). Raw sequence data were pre-processed using the MR DNA analysis pipeline and freeware for demultiplexing and trimming of barcodes (www.mrdnalab.com, MR DNA, Shallowater, TX, USA). Sequences are available on NCBI-SRA under the BioProject accession number: PRJNA774453.

Amplicon sequences were processed using the open-source software pipeline Qualitative Insights for Microbial Ecology 2 (QIIME 2) version 2020.2 (Bolyen *et al*., 2019). Sequences were demultiplexed and quality screened before dereplication, chimera and singleton detection and removal using the DADA2 workflow (Callahan *et al*., 2016). Amplicon sequence variants (ASVs) generated were taxonomically resolved using the classify-sklearn naïve Bayes taxonomy classifier against the pre-trained UNITE sequence database version 8.2 (Abarenkov *et al*., 2020) using the q2-feature-classifier plugin (Bokulich *et al*., 2018). The feature table output of relative read abundances was used to compare stem-end and surface fruit mycobiomes. Non-rarefied ASVs were used for alpha diversity (Observed ASV richness, Shannon's diversity index, and Pielou's measure of species evenness) and compositional analysis, while rarefied (15 124 reads per sample) ASVs were used to determine beta diversity. Core microbiome analysis was performed using the core-features plugin in QIIME2 using the default parameters.

Postharvest fruit quality and decay parameters of avocado fruit

Physiological maturity parameters i.e., fruit pH and firmness were measured at harvest, two weeks after cold storage (5.5 °C), three weeks after cold storage (5.5 °C), four weeks after cold storage (5.5 °C) and at the ripe and ready-to-eat avocado of 15 individually selected fruit representing each sampling stage. The fruit pH was measured according to Montesinos-Herrero *et al*. (2009) with minor modifications, using a Jenway 3510 pH meter (Bibby Scientific Ltd, UK) with a flat surface electrode combination (Extech Instruments, Waltham, USA). Flesh firmness (kg) was determined on two points at the equatorial point of the fruit using a Chitillon Penetrometer, Model DFM50 (Ametek, Largo, Florida, USA) according to Woolf *et al*. (2005). All fruit tested during the study were visually assessed for development of any postharvest diseases. The disease incidence of stem-end rot and anthracnose was determined as a percentage of decayed fruit in a batch at the ripe and ready and ready-to-eat stage.

Data analysis

The R software (http://cran.r-project.org/; R version 3.6.3) was used for statistical analysis of the fungal diversity and relative abundance of taxonomic groups data. Data distribution was verified for normality using the Shapiro-Wilk test before comparing alpha diversity indices and changes in the relative abundance of different taxonomical levels (non-rarefied data) between the postharvest sampling stages. Where data met the assumption of normality, a two-way analysis of variance (ANOVA) was conducted and followed by a Tukey's HSD posthoc test in *vegan*. Otherwise, a Kruskal-Wallis test was applied for multiple comparisons. Statistical differences in beta diversity were inferred with

permutational multivariate analysis of variance (PERMANOVA, 999 permutations), using the *adonis* function in *vegan* R package. Principal coordinate analyses (PCoA) was performed with Unweighted UniFrac and Bray-Curtis distance matrix estimation to visualize spatial patterns between samples. To determine the differences in avocado fruit flesh pH and firmness as well as disease (anthracnose and stem-end rot) incidence between sampling stages (harvest to ripe and ready-to-eat stages), the data was subjected to General Linear Models procedure of Statistical Analysis Systems (SAS) version 9.4 (Institute Inc., Carry NC, USA). Test results with $P < 0.05$ were considered statistically significant.

Results

A total of 3 180 280 ITS sequences were recovered from six avocado postharvest handling stages (36 samples), after paired-end alignments, quality filtering, and deletion of chimeric sequences and singletons. The ITS sequences ranged from 18 380 to 183 645 reads per sample (median 88 341), with 957 fungal taxa identified across all samples. A total of four phyla, 13 classes, 35 families, 39 genera and 35 fungal species were identified in all 36 samples.

Structure and composition

The phylum Ascomycota, composed mostly of known avocado stem-end (SE) pathogens, dominated the fungal data set (65.4% of total reads) irrespective of postharvest sampling stage (Figure 1). Other dominant phyla detected throughout the sampling stages included Basidiomycota (33.6%) and Mucoromycotina (0.4%). A total of 13 fungal families were

recorded across all the sampling stages, of which the *Debaryomecetaceae* (27.2%), *Glomerellaceae* (8.0%), *Cladosporiaceae* (7.3%) *Aspergillaceae* (3.6%), and *Botryospaeriaceae* (2.9%) dominated the Ascomycota phylum. The families *Rhynchogastremataceae* (26.9%), *Sporidiobolaceae* (8.1%), and *Malasseziaceae* (3.0%) dominated the Basidiomycota phylum (Figure 2). The relative abundances of *Aspergillaceae*, *Cladosporiaceae, Botryosphaeriaceae* and *Aureobasidiaceae* declined following the prochloraz dip treatment at the postharvest stage on the fructoplane and in the stem-end pulp. *Glomerellaceae* (47.1%) and *Botryospaeriaceae* (22.2%) dominated the stem-end pulp community at the ripe and ready-to-eat stage.

The fungal ASV richness and diversity of the avocado fructoplane were assessed at different postharvest stages in response to chemical and physical postharvest interventions. The fructoplane fungal ASV richness differed significantly (χ^2 = 14.66, P = 0.01) between the assessed postharvest stages, with 1.4 times decrease in observed richness from 138 to 96 following prochloraz dip treatment. This decrease remained constant for samples subjected to two weeks in cold storage. Similarly, the fungal diversity at harvest differed significantly (Shannon diversity, F = 18.77, P = 0.0002; Pielou's evenness, F = 14.04, P = 0.001) on the fructoplane at different postharvest stages (Table 1). Pairwise comparisons (P = 0.037; [*t*-test] statistic = −0.120) of fungal taxa on the fructoplane indicated a 27% increase in Pielou's evenness from the harvest stage (0.45) to the prochloraz dip treatment stage (0.57). This was followed by a significant decline (P = 0.009; [*t*-test] statistic = −0.076) in evenness to 0.42 at the two weeks cold storage stage. No further significant differences in Pielou's evenness were observed beyond the two weeks cold storage stage on the avocado fructoplane. We also observed significant

Table 1 Summary of alpha diversity analyses of fungal micro-community on avocado fructoplane and stem-end pulp at the postharvest stages of 'Hass' avocado

Sample Type	Postharvest stage	Total number of sequence reads	Observed richness(s)	Pielou's evenness	Shannon diversity index
Fructoplane	At harvest	113 437	138.33*	0.45***	2.21***
	After prochloraz dip treatment	101 143	96.00	0.57	2.59
	Two weeks cold storage (5 °C)	102 338	96.00	0.42	1.92
	Three weeks cold storage (5 °C)	131 077	80.33	0.34	1.51
	Four weeks cold storage (5 °C)	143 423	77.33	0.32	1.38
	Ripe and ready-to-eat	110 954	73.67	0.37	1.61
Stem-end pulp	At harvest	57 474	83.33**	0.61^{NS}	2.60^{NS}
	After prochloraz dip treatment	113 437	52.33	0.64	2.53
	Two weeks cold storage (5 °C)	21 771	52.67	0.80	3.18
	Three weeks cold storage (5 °C)	46 127	50.00	0.63	2.45
	Four weeks cold storage (5 °C)	29 210	40.33	0.61	2.20
	Ripe and ready-to-eat	165 369	63.67	0.48	2.01

*, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$; NS, not significant.

differences (χ^2 = 11.40, P = 0.04) in fungal richness between the assessed sampling stages in the stem-end pulp. The most notable change in the fungal community richness in the stem-end pulp was observed following the prochloraz dip treatment, which saw a sharp decline from 83.33 to 52.33. However, no significant differences were observed in stem-end fungi Shannon diversity (P = 0.092, F = 2.47) and Pielou's evenness (P = 0.122, F= 2.20).

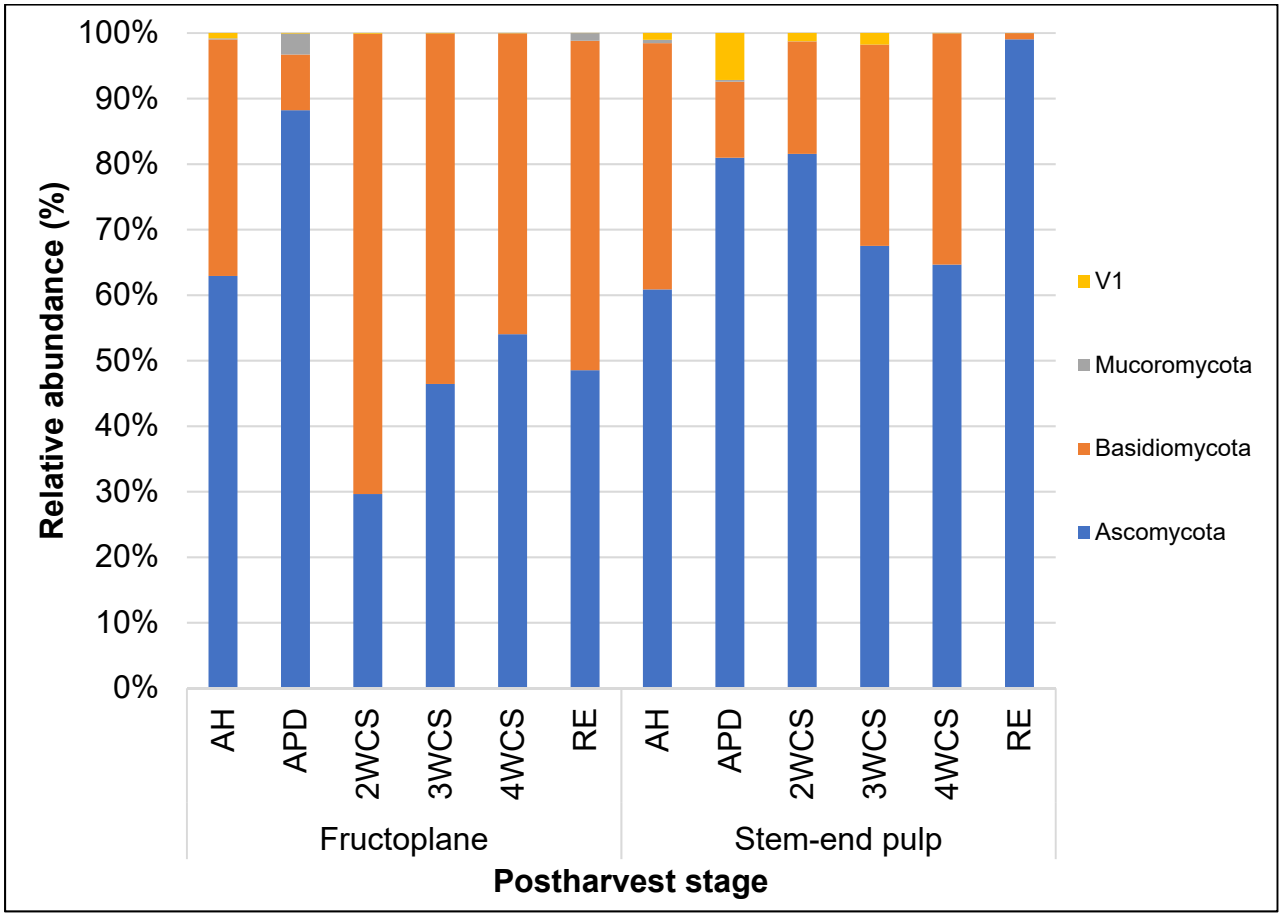

Figure 1 Taxonomic abundance of major fungal phyla on the avocado fructoplane and stem-end pulp and stem at the postharvest stages of 'Hass' avocado. AH, At harvest; APD, After commercial prochloraz dip treatment, 2WCS, Two weeks cold storage (5 °C); 3WCS, Three weeks cold storage (5 °C); 4WCS, Four weeks cold storage (5 °C); RE, Ripe and ready-to-eat.

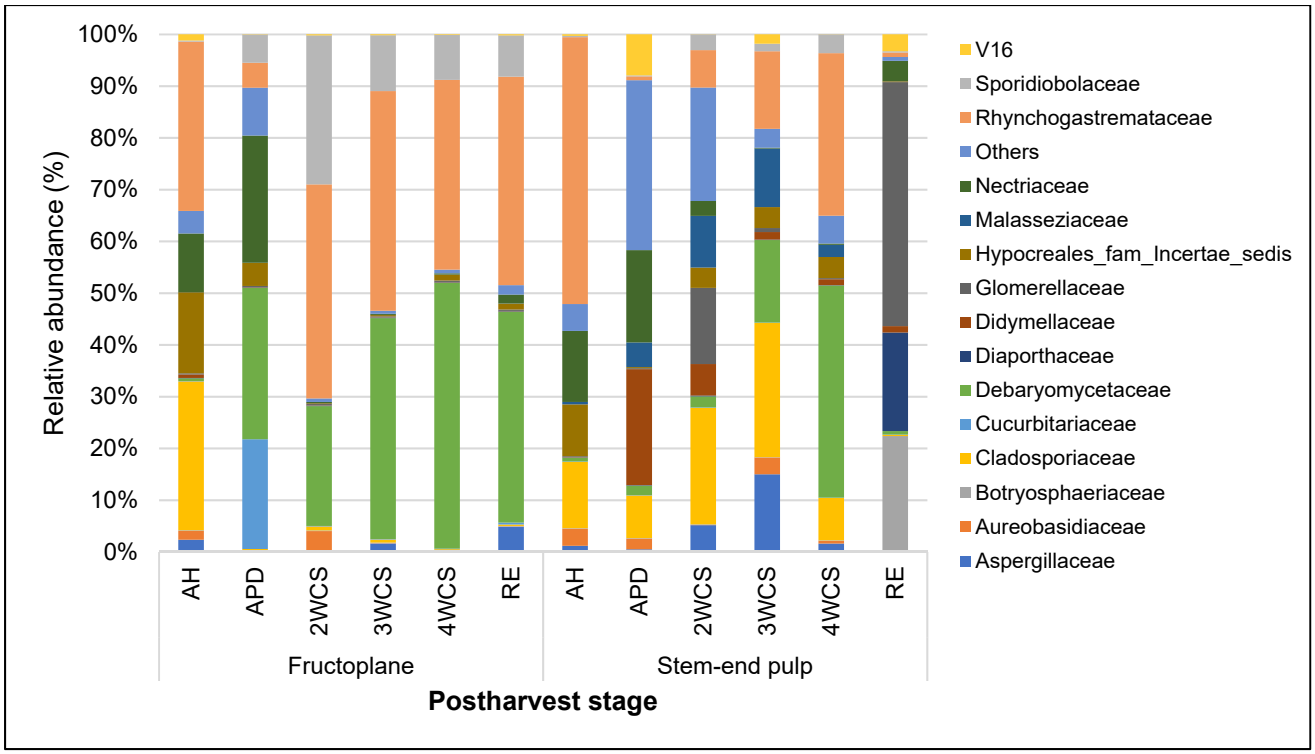

Figure 2 Taxonomic abundance of the top 15 fungal families on the avocado fructoplane and stem-end pulp and stem at the postharvest stages of 'Hass' avocado. AH, At harvest; APD, After commercial prochloraz dip treatment, 2WCS, Two weeks cold storage (5 °C); 3WCS, Three weeks cold storage (5 °C); 4WCS, Four weeks cold storage (5 °C); RE, Ripe and ready-to-eat.

Evaluation of fungal beta diversity showed distinct clustering of the fructoplane and stem-end pulp samples in PCoA (Figure 3) with most of the fructoplane samples clustering tightly compared to stem-end pulp samples. The significant effect of sample type (stem-end pulp or fructoplane) was further supported by PERMANOVA testing ($F = 1.52$; $R^2 = 0.430$; $P = 0.001$). Within the sample types, significant impact on the community structure was observed in the postharvest sampling stages for the fructoplane (F-value = 4.64; $R^2 = 0.659$; $P = 0.001$) and stem-end pulp (F-value = 2.16; $R^2 = 0.474$; $P = 0.001$).

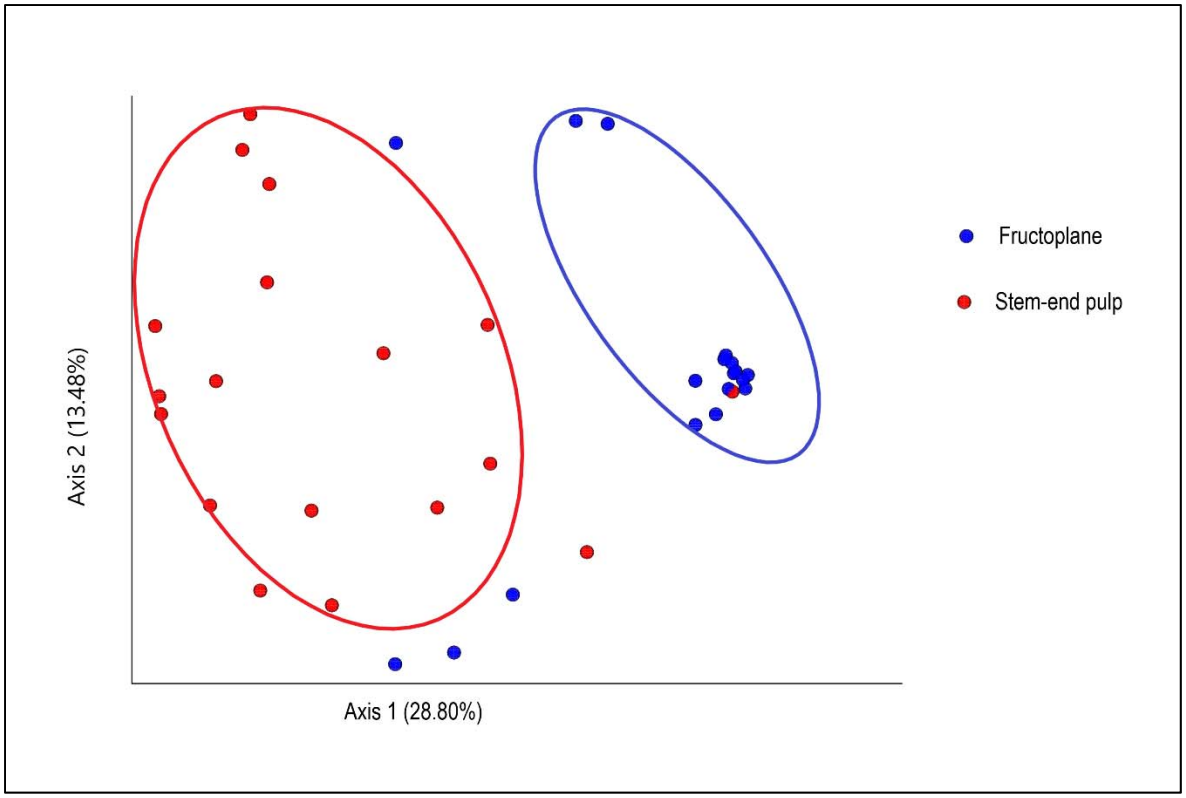

Figure 3 Principal component analysis of the fungal community compositions present on 'Hass' avocado fructoplane and in stem-end pulp at the postharvest stage.

Core fungal microbiome on the fructoplane and stem-end pulp of 'Hass' avocado at the postharvest stage

Sixteen fungal genera were identified as core fungal taxa at a 75% prevalence threshold (Table 2) on the fructoplane of 'Hass' avocado at different postharvest stages. The fructoplane core microbiome primarily consisted of 11 Ascomycota (*Cladosporium Meyerozyma* and *Sarocladium*, *Neofussicocum*, *Fusarium*, *Penicillium*, *Diaporthe*, *Aureobasidium*, *Epicoccum* and two uniclassified) and five Basidiomyacota (*Papiliotrema*, *Rhodotorula mucilaginosa*, *Melassezia* and three unclassified) genera, found in all samples. Of the sixteen core fungal genera identified, four members of Ascomycota

Table 2 Core fungal microbiome of the fructoplane and stem-end pulp of 'Hass' avocado

Taxonomy						Core member	
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Fructoplane**	**Stem-end pulp**
Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	*Neofusicoccum*	*Neofusicoccum parvum*	Y	
		Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium perangustum*	Y (2)[Z]	Y
		Dothideales	Aureobasidiaceae	*Aureobasidium*		Y	
		Pleosporales	Cucurbitariaceae	Unclassified	Unclassified	Y	Y
			Didymellaceae	*Epicoccum*	*Epicoccum nigrum*	Y	
	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium bialowiezense*	Y	
	Sordariomycetes	Diaporthales	Diaporthaceae	*Diaporthe*		Y	
		Hypocreales	Hypocreales fam Incertae sedis	*Sarocladium*		Y	Y
					Sarocladium kiliense	Y	
		Hypocreales	Nectriaceae			Y	
				Fusarium	*Fusarium solani*	Y	
		Saccharomycetales	Debaryomycetaceae	*Meyerozyma*	*Meyerozyma guilliermondii*	Y	Y
Basidiomycota	Exobasidiomycetes	Exobasidiales	Unclassified	Unclassified	Unclassified	Y	
	Malasseziomycetes	Malasseziales	Malasseziaceae	*Malassezia*	*Malassezia restricta*	Y	
	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*Rhodotorula mucilaginosa*	Y (2)	Y
				Unclassified	Unclassified	Y	
	Tremellomycetes	Tremellales	Rhynchogastremataceae	*Papiliotrema*	*Papiliotrema flavescen*	Y	Y

Y, yes; [Z], means there were two amplicon sequence variants for that genus.

(*Cladosporium, Meyerozyma* and *Sarocladium* and one unclassified) and two of Basidiomycota (*Papiliotrema* and *Rodotorula*) were found in all the SE pulp samples. The Ascomycota core genera *Neofusicoccum* and *Penicillium* were found in 94% of the SE pulp samples, *Diaporthe*, *Cladosporium* and *Fusarium* in 89%, *Epicoccum* and one unclassified genus in 83%, *Aureobasidium* and *Sarocladium kiliense* in 56%. Meanwhile, the core Basidiomycota taxa including *Rhodotorula diabovata* was found in 89%, *Malassezia* in 83% and two unclassified genera in 56% of the SE pulp samples.

Variation in pathogenic and potential beneficial fungal taxa at different postharvest stages of 'Hass' avocado

Potential pathogenic fungal taxa

The study revealed six pathogenic fungal genera present at the postharvest sampling stages (Table 3). This included Ascomycota genera previously described to contain species associated with fruit body rot (anthracnose): *Colletotrichum* (*Colletotrichum viniferum*), and stem-end rot (SER): *Fusarium* (*Fusarium solani* (Mart.) Sacc), *Penicillium* (*Penicillium commune* Thom.), *Alternaria* (*Alternaria alternata* (Fr.) Keissl.), *Epiccocum* (*Epiccocum nigrum* Link), *Neofusicoccum* (*Neofusicoccum parvum* Pennycook & Samuels). No significant differences (P = 0.854, F = 0.378) were noted in the abundance of the *Colletotrichum* genus (0.2%) at different postharvest stages on the avocado fructoplane. The same was observed for *Colletotrichum viniferum*, a grape ripe rot related fungal species. Amongst the SER associated genera, *Fusarium* (4.0%) was the most dominant on the fructoplane, followed by *Epiccocum* (3.5%) and *Penicillium* (1.2%), while *Alternaria* (0.5%) was the least abundant. Comparisons of SER genera of *Fusarium*,

Table 3 Relative abundance of pathogenic and beneficial genera detected on avocado fructoplane and in stem-end pulp

Sample type and postharvest stage	Fructoplane						Stem-end pulp					
	AH	APD	2WCS	3WCS	4WCS	RE	AH	APD	2WCS	3WCS	4WCS	RE
Pathogenic fungal genera (%)												
Colletotrichum	0.20^{NS}	0.21	0.24	0.20	0.26	0.30	0.20***	0.18	14.7	0.78	0.21	47.15
Alternaria	0.44*	0.01	0.02	0.02	0.74	2.03	0.27^{NS}	0.00	0.02	0.75	2.76	2.06
Fusarium	0.11**	0.16	7.29	12.68	3.45	0.10	0.10^{NS}	0.14	9.31	5.53	0.09	0.11
Penicillium	0.07^{NS}	0.42	1.50	2.29	1.77	1.20	0.07^{NS}	0.42	2.23	1.75	1.72	0.10
Epiccocum	0.67*	0.03	0.02	0.03	12.01	7.97	0.44^{NS}	0.03	0.03	12.00	19.93	7.96
Neofusicoccum	0.10^{NS}	0.09	0.11	0.11	0.08	0.10	0.08**	0.10	0.12	0.09	0.07	0.12
Beneficial fungal genera (%)												
Aureobasidium	1.79*	7.46	0.67	0.04	2.77	1.22	7.12^{NS}	7.44	0.04	2.75	2.7	1.85
Meyerozyma	0.56***	27.51	23.08	42.19	51.18	39.50	0.72***	2.26	0.92	13.75	37.62	0.66
Papiliotrema	33.33***	23.21	34.05	4.51	1.15	15.40	25.55^{NS}	36.18	29.01	2.53	0.77	31.61

AH, At harvest; APD, After commercial prochloraz dip treatment, 2WCS, Two weeks cold storage (5 °C); 3WCS, Three weeks cold storage (5 °C); 4WCS, Four weeks cold storage (5 °C); RE, Ripe and ready-to-eat. *, P < 0.05; **, P < 0.01; *** P < 0.001; NS, not significant.

Epicoccum, *Penicillium*, *Alternaria* and *Neofusicoccum* changes on the fructoplane in response to chemical and physical postharvest interventions stages showed a significant change in the relative abundance of *Fusarium* (P = 0.005, F = 6.14), *Epiccocum* (P = 0.046, F = 3.196) and *Alternaria* (P = 0.009, F = 3.527). Pairwise comparisons between samples at harvest stage and the prochloraz dip treatment revealed a significant increment in the relative abundance of *Fusarium* (P = 0.009; [*t*-test] statistic = −1.3284) by over 50% (Table 3). This was most probably due to the increase by over 150 times in relative abundance of *Fusarium solani*, after the prochloraz dip treatment (Table 4). In contrast, the *Epiccocum* and *Alternaria* genera declined by as much as 44 and 22 times, respectively, following the prochloraz dip treatment. However, there was no significant difference (P = 0.053), in the relative abundance of *Epicoccum nigrum* after the prochloraz dip treatment. No significant changes were noted in the abundance of the *Penicillium* (P = 0.488) and *Neofusicoccum* (P = 0.219) at the different postharvest stages on the fructoplane of the avocado fruit. This was also the case with *Penicillium commune* (P = 0.062) and *Neofusicoccum parvum* (P = 0.805). The relative abundance of *Fusarium* increased by 46 times between the prochloraz dip treatment stage and the two weeks cold storage stage. Although not significant, the increase in the relative abundance of *Fusarium* from the two weeks cold storage stage (7.29%) to the three weeks cold storage stage (12.68%) was coupled with a very sharp and significant increase in the relative abundance *Fusarium solani* i.e. from 0.07% to 13.88%. Thereafter, a sharp decline was observed in the relative abundance of *F. solani* at both the four weeks cold storage and the ripe and ready-to-eat stages.

Table 4 Relative abundance of pathogenic and beneficial taxa detected on avocado fructoplane and in stem-end pulp

Sample type and postharvest stage	Fructoplane						Stem-end pulp					
	AH	APD	2WCS	3WCS	4WCS	RE	AH	APD	2WCS	3WCS	4WCS	RE
Pathogenic fungal species (%)												
Colletotrichum viniferum	0.20 ^{NS}	0.21	0.24	0.20	0.26	0.30	0.20***	0.18	14.7	0.78	0.21	47.15
Fusarium solani	0.08	0.08	0.07	13.88	0.09*	1.98	0.13^{NS}	0.08	0.08	0.05	0.08	3.02
Penicillium commune	0.01^{NS}	1.55	0.05	0.65	0.02	0.03	0.32^{NS}	1.56	0.01	1.32	0.01	0.01
Epiccocum nigrum	0.68^{NS}	0.03	0.04	0.04	0.04	0.04	0.02^{NS}	19.19	1.38	1.58	1.75	0.03
Neofusicoccum parvum	0.09^{NS}	0.10	0.12	0.11	0.14	0.12	0.09**	0.07	0.07	0.08	0.11	21.06
Beneficial fungal species (%)												
Aureobasidium pullulans	0.16**	0.01	0.53	0.02	0.01	0.01	0.66^{NS}	3.41	1.62	3.03	1.69	0.00
Meyerozyma guilliermondii	0.53***	23.17	22.30	40.98	50.00	38.07	0.65***	2.14	0.62	12.61	35.39	0.54
Papiliotrema flavescens	31.87**	3.82	40.16	41.76	35.46	39.33	33.27^{NS}	0.73	4.78	13.53	22.30	0.71

AH, At harvest; APD, After commercial prochloraz dip treatment, 2WCS, Two weeks cold storage (5 °C); 3WCS, Three weeks cold storage (5 °C); 4WCS, Four weeks cold storage (5 °C); RE, Ripe and ready-to-eat. *, P < 0.05; **, P < 0.01; *** P < 0.001; NS, not significant.

In the SE pulp of ‘Hass’ avocado, significant differences were observed in the abundances of *Colletotrichum* (P = 0.001, F = 8.595) at different postharvest stages (Table 3). Pairwise comparisons at different postharvest stages of the avocado fruit revealed that the highest increase (224 times) in the relative abundance of the *Colletotrichum* genus occurred from the four weeks cold storage stage and the ripe and ready-to-eat stage (P = 0.003). This was marked by a 21% increase in relative abundance between the two stages. Similarly, *Colletotrichum viniferum* significantly (P = 0.001) increased by over 200 times at the ripe and ready-to-eat stage in comparison to the four weeks cold storage stage. No significant differences in relative abundance were noted in *Epiccocum* (P = 0.061, F = 2.904), *Fusarium* (P = 0.455, F= 1.026), *Penicillium* (P = 0.243, F = 1.564), and *Alternaria* (P = 0.557, F = 0.822). This was also true for *Epiccocum nigrum* (P = 0.050, F = 3.103), *Fusarium solani* (P = 0.454, F= 1.007) and *Penicillium commune* (P = 0.186, F = 1.808). Although the relative abundance of the *Epicoccum* genus in the SE pulp did not differ significantly between the different postharvest stages, it was notably the most dominant (8.0%) SER species, especially at the ripe and ready-to-eat stage. The *Neofusicoccum* genus, on the other hand, significantly (P = 0.006) increased in relative abundance by 71% in the SE pulp of ‘Hass’ avocado at the ripe and ready-to-eat stage in comparison to the preceding four weeks cold storage while remaining the least abundant (0.12%) amongst all the detected pathogenic genera. Similarly, *Neofusicoccum parvum* significantly (P = 0.020) increased by 150 times from the four weeks cold storage to the ripe and ready-to-eat stage.

Potential beneficial fungal taxa

We further assessed the impact of the different postharvest stages of 'Hass' avocado on potentially beneficial fungi (Table 4 and 5). Three beneficial genera were detected on the fructoplane of 'Hass' avocado, with *Papiliotrema* being the most dominant (20.9%, P= 0.0005, F = 10.59), followed by *Meyerozyma* (9.7%, P = 0.0003, F = 11.62) and *Aureobasidium* (3.7%, P = 0.014, F = 4.613). The prochloraz dip treatment had a significant effect on the relative abundance of *Papiliotrema,* which decreased by over 30% (P = 0.006), and *Meyerozyma,* which increased by approximately 13% (P = 0.03) genera on the avocado fructopane, following the prochloraz dip treatment. At the species level, *Papiliotrema flavescens* decreased by over eight times, while *Meyerozyma guilliermondii* increased by 44 times following prochloraz dip treatment. Between the two genera, the only significant change on the avocado fructoplane post prochloraz dip treatment was a 47% rise in the relative abundance of the *Papiliotrema* (P = 0.0008) at the two weeks cold storage stage probably due to the increase in the relative abundance of *Papiliotrema flavescens* (10.5 times) at this stage.

Despite being dominant, the relative abundance of the *Papiliotrema* (*Papiliotrema flavescens*) genus in the avocado SE pulp did not differ significantly (P > 0.05) between the postharvest stages of avocado fruit. Similar observations were made for *Aureobasidium* (*Aureobasidium pullulans*). Meanwhile, the abundance of *Meyerozyma* in the SE pulp of 'Hass' avocado differed significantly (P < 0.001) between the postharvest stages, and pairwise comparisons indicated 2.7 times and significant (P = 0.001) increase from three weeks of cold storage to four weeks cold storage. This was followed by a very sharp decrease from 37.62% to 0.66% at the ripe and ready-to-eat stage. Consequently,

Table 5 Physiological maturity parameters and decay incidence of 'Hass' avocado fruit at the postharvest stages

Sampling stage	pH	Firmness (kg)	Anthracnose (%)	Stem-end rot (%)
At harvest/after prochloraz dip treatment	5.1 b[y]	10.1 a	0 b	0 b
Two weeks in cold storage (5 °C)	5.1 b	10.0 a	0 b	0 b
Three weeks cold storage (5 °C)	5.1 b	9.9 a	0 b	0 b
Four weeks cold storage (5 °C)	5.2 b	9.9 a	0 b	0 b
Ripe and ready-to-eat	6.1 a	1.01 b	79 a	80 a

[y], values in the same column with different letters are significantly different ($P < 0.05$).

the relative abundance of *Meyerozyma guilliermondii* decreased by approximately 66 times at the ripe and ready-to-eat stage.

Physiological maturity parameters and disease incidence

Significant ($P < 0.05$) changes were noticed in the fruit firmness and pH from the harvest to the ripe and ready-to-eat stages (Table 5). Fruit firmness decreased successively during storage and shelf-life while the pH increased. Anthracnose was not detected at the pre- or postharvest stages. Anthracnose and SER were only detected in ripe and ready-to-eat fruit at 79% and 80% incidences, respectively (Table 5).

Discussion

The current study using Illumina sequencing to investigate the dynamics of the avocado fungal community (fructoplane and stem-end pulp) provides baseline data on fungal diversity and composition on the fructoplane and stem-end (SE) pulp in response to commercial chemical and physical interventions at the postharvest stage. This data serves as a foundation in the development of effective disease (anthracnose and stem-end rot) management strategies. To our knowledge, there is no study to date, that has comprehensively documented the dynamics in mycobiomes of avocado fruit at the postharvest stage using culture-independent assessments and considering commercial handling practices. Previous studies reflected on culturable organisms and mainly focused on a single organism (Prusky *et al*., 1991; Yakoby *et al*., 2000).

In South Africa, the control of postharvest disease is primarily mediated through fungicidal copper sprays (preharvest) and prochloraz dip treatment (postharvest). The

commercial application of prochloraz dip treatment in this study was associated with a decline in fungal richness on both the fructoplane and SE pulp area of the avocado fruit. Prochloraz is known to target several fungal groups by inhibition of the fatty acid, ergosterol, the synthesis of which is vital to structural formation of fungal cell membranes (Danderson, 1986) hence the observed decrease in fungal richness on the fructoplane. Despite prochloraz being non-systemic, there is a possibility of fungicide internalization (Sudheer *et al.*, 2007) into the stem-end area during commercial dipping, which could account for the reduced presence and richness of target fungi inhabiting the SE pulp area.

The Ascomycota and Basidiomycota phyla have generally been reported to dominate fruit fungal profiles (Shen *et al.*, 2018; Carmichael *et al.*, 2019) and similar observations were made in the current study. Other fungal phyla not reported to be prevalent on avocado such as Mucoromycotina were also detected in the present study. Microbial core communities of plants have so far been rarely investigated (Sauer at al., 2021) and for the first time, the current study endeavoured to distinguish the epi- and endophytic core communities of avocado fruit at the postharvest stage. Our 'Hass' avocado core taxa analysis revealed 16 fungal taxa that were conserved on the fructoplane (epiphyte) of the fruit across the six different postharvest stages. Amongst these core taxa were members of the Ascomycota phylum including *Cladosporium*, *Neofussicocum*, *Fusarium*, *Penicillium* and *Epicoccum*, which were previously described as stem-end rot (SER) causing genera in avocado (Valencia *et al*., 2019, Fourie and Coertzen, 2018; Wanjiku et al., 2021). The core communities tend to be comprised of key microbial taxa that play an essential role in the health and fitness of the plant holobiont (Lemanceau et., 2017; Sauer et al., 2021) e.g., they provide critical ecological functions

such as protection of the host from pathogen attack (Ramos-Garza *et al*., 2015). Supporting this, we observed three core taxa in very high abundances, including two Ascomycota genera *Meyerozyma* and *Aureobasidium* and one Basidiomycota genera, *Papiliotrema,* which have been reported to have biocontrol effect against plant pathogens (Schisler *et al*., 2019; Agirman and Erten, 2020). Of these, two (*Meyerozyma*, *Papiliotrema*) were also core taxa in the stem-end pulp. Another core taxon observed in both the fructoplane and SE pulp of 'Hass' avocado, *Rhodotorula mucilaginosa*, was previously reported to promote plant growth and seed germination in a bioassay using *Brassica juncea* (Ramos-Garza *et al*., 2015). We further observed that there was a decrease in numbers of core taxa from the epiphytic (16) to endophytic (6) compartments of the 'Hass' avocado fruit and this is similar to the findings of Sauer *et al*. (2021) on *Achillea* leaves. Despite not being dominant in the SE pulp at all of the six avocado postharvest stages in the present study, the potential SER causing genera, *Neofussicocum*, *Fusarium*, *Penicillium* and *Epicoccum* were all present in very high abundances at the ripe and ready-to-eat stage, which is a critical point in pathogen infection. From this result, we can corroborate that both beneficial and pathogenic taxa formed part of the core communities in the avocado fructoplane, and that the stem-end pulp core community were less diverse than the fructoplane community. The less diverse SE pulp core community is associated with high abundances of SER causing genera, especially at the ready-to-eat stage of 'Hass' avocados.

The majority (70%) of the families (36) detected in the current study were present on the fructoplane of the avocado fruit at the harvest stage (mature fruit). Most of the families present on the fructoplane were members of the Ascomycota phylum of which

Glomerellaceae (*Colletotrichum*), *Aureobasidiaceae* (*Aureobasidium*), *Pleosporaceae (Alternaria), Cladosporiaceae, Aspergillaceae* (*Penicillium*), and *Botryospaeriaceae* (*Neofusicoccum*) were previously detected as the most dominant fungi using culture-dependent methods (Earecho and Belay, 2019; Wanjiku *et al*., 2020).

Most of the families including *Aspergillaceae*, *Aureobasidiaceae*, *Pleosporaceae*, *Cladosporiaceae* and *Botryosphaeriaceae* decreased in relative abundance on the fructoplane following the prochloraz dip treatment at the postharvest stage. Previous work done by Danderson (1986) on avocado indicated the ability of prochloraz to control a diverse range of fungal genera, including *Penicillium*, *Alternaria*, *Fusarium,* and *Colletotrichum.* In agreement with this observation, the relative abundance of *Epiccocum* and *Alternaria* on the fructoplane decreased sharply following the prochloraz dip treatment. The findings of the present study, however, indicated that prochloraz did not negatively affect the *Fusarium* genus but, it resulted in an increase of over 50% in relative abundance after fruit treatment. Further increases (up to 46 times) in the relative abundance of *Fusarium* on the fructoplane at two weeks of cold storage were also observed. *Fusarium* is an important SE pathogen under specific environmental and physiological conditions (Dita *et al*., 2018). However, this organism is known to rapidly colonise a niche once other competing organisms are removed, representing a typical r-strategist (Louw, 2014). The fungal community structure of avocado fruit at the postharvest stage was also reported to change during storage (Earecho and Belay, 2019). Immediately after postharvest treatment, pesticide concentrations should be higher on the fruit surface but gradually declines as the product breaks down or diffuses

into the fruit (Shiea *et al.*, 2015); this could explain the observed surface population and endophyte recovery in endophytic *Fusarium* at two weeks cold storage.

The fruit SE pulp was dominated by *Epiccocum* followed by *Fusarium*, *Penicillium*, *Alternaria* and *Neofusicoccum*. Most of these genera appeared unaffected by postharvest interventions except for *Neofusicoccum* (*Neofusicoccum parvum*), belonging to the Botryosphaeriaceae family (Valencia *et al*., 2019), which increased in relative abundance by 71% at the ripe and ready-to-eat stage. However, despite this increase, the genus remained the least abundant in SE pulp.

Although the relative abundances of the *Colletotrichum* genus did not differ significantly on the fructoplane, we observed a 21% increase in relative abundance in the stem-end pulp following fruit ripening. This increase was coupled with a very sharp increase in the presence of *Colletotrichum viniferum*, a species previously known to cause grape ripe rot (Echeverrigaray *et al*., 2020). In addition to anthracnose (body rots), the occurrence of the *Colletotrichum* genus and, more specifically, *Colletotrichum gloeosporioides* has been previously reported to be associated with SER development in avocado fruit (Everrete, 1999; Sanders and Korsten, 2003; Guarnaccia *et al*., 2016). However, the current study showed for the first time, that, *C. viniferum* could successfully inhabit the fruit SE pulp as an endophyte, as we observed very high relative abundances especially at the ripe and ready-to-eat stage of avocado.

Prusky *et al*. (1991) indicated that during the ripening process, fruit exhibit biochemical and physiological changes that include a decrease in important defence mechanisms such as phytoanticipin, phytoalexin levels, inducible plant defence mechanisms, and changes in ambient host pH. This decline in the defence mechanisms

as the fruit ripens could have resulted in an increase in pathogenic taxa including *Colletotrichum viniferum*. More so, an increase in the presence of *Neofusicoccum parvum* resulted in a higher SER occurrence in avocado (Twizeyimana et al., 2013; Guarnaccia et al., 2016) hence over 80% incidence was observed at the ready-to-eat stage of the avocado in the current study.

In recent decades, the use of microorganisms for protection against a broad range of plant diseases has been evaluated. Amongst these microorganisms are epiphytic yeasts and yeast-like fungi (Lindow and Brandl, 2003) in the current study, three fungal genera previously identified for biocontrol activity were detected on most postharvest stages, on the fructoplane and in the SE pulp. These included *Papiliotrema* (*Papiliotrema flavescens*), which was the most dominant, followed by *Meyerozyma* (*Meyerozyma guilliermondii*) and *Aureobasidium* (*Aureobasidium pullulans*) (Schisler *et al*., 2019; Agirman and Erten, 2020). Following the prochloraz dip treatment, the relative abundance of *Papiliotrema flavescens* decreased by over 30%, while that of the *Meyerozyma guilliermondii* genus increased by approximately 13% on the fructoplane. To our knowledge, little attention has been placed on understanding the effect of prochloraz on the non-target beneficial microorganism. Our study is therefore able to provide vital first-hand contributions on the effect of prochloraz dip treatment on potential biocontrol fungal taxa on the avocado fructoplane.

Further assessments on the effect of cold storage conditions on potentially beneficial taxa in the SE pulp indicated a negative effect on the presence of *Meyerozyma,* as evidenced by a significant increase in its relative abundance from three weeks cold storage to four weeks cold storage. However, this effect did not persist post cold storage,

and as a result, the relative abundance of the *Meyerozyma* genus sharply decreased (by 57 times) at the ripe and ready-to-eat stage. As a result, the potential biocontrol species *Meyerozyma guilliermondii* (Huang *et al*., 2021), decreased drastically at the ripe and ready-to-eat stage, and this coincided with the increase in the relative abundance of pathogenic genera including *Colletotrichum* (*Colletotrichum viniferum*) and *Neofusicoccum* (*Neofusicoccum parvum*).

The current study provides a number of fundamental insights on the fungal community dynamics of avocado fruit (fructoplane and SE pulp area) at the postharvest stage. The use of fungicides reduced the richness and abundance of pathogenic fungal populations at the postharvest stage, though the effects did not persist to the final ready-to-eat stage. The core fungal communities were composed of both beneficial and pathogenic taxa in the avocado fructoplane, while the stem-end pulp core community was less rich than that of the fructoplane. The non-target effect of prochloraz was observed on potentially beneficial fungal species such as *Papiliotrema flavescens*. Future studies should focus on the total impact on the bacterial microbiome and the potentially beneficial organisms used in biocontrol applications on the overall microbial ecology and anthracnose and SER diseases in avocado.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization, M.B. and L.K.; laboratory and field work, M.B. and F.V.; raw sequence data processing and bioinformatic analysis, J.K.G., data analysis and interpretation, M.B. and J.K.G.; validation, J.K.G. and L.K.; investigation, F.V. and M.B.; data curation, F.V., J.K.G and M.B.; original draft manuscript preparation, M.B.; manuscript revision writing, review and editing, J.K.G. and L.K.; visualization, M.B.; supervision, M.B., J.K.G. and L.K.; project administration, L.K.; funding acquisition, L.K. All authors have read and agreed to the published version of the manuscript.

Data availability statement

The data that support the findings of this study are openly available on NCBI-SRA under the BioProject accession number: PRJNA774453.

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