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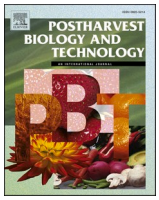

Amplicon sequencing and shotgun metagenomics reveal functional impacts of aminoethoxyvinylglycine-mediated ripening and cold storage on the microbiome of 'NY1' apples

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ABSTRACT

Understanding community-level microbial dynamics provides more holistic insight into the nature of pathogen infection and biocontrol in fruits and vegetables. We investigated the impact of preharvest sprays of aminoethoxyvinylglycine (AVG, 0.25 g L^{-1}), an inhibitor of ethylene production of fruit, to assess the association between ethylene-mediated ripening and the microbiome at harvest and during storage. 'NY1' (Snapdragon®) apples were sprayed on the tree two weeks before first harvest and the microbiome of the fruit was assessed at harvest and at one-week intervals across four weeks. Fruit from harvests 1 and 4 were stored in air at 3 °C for up to 6 months. The bacterial communities changed over harvest time while fungal communities were affected by AVG treatment. In addition, both harvest time and AVG, as well as storage time, were associated with bacterial and fungal variation after 2–6 months of storage. Postharvest fungal microbiomes showed consistent responses to variations in fruit maturation, with the microbial communities of AVG-treated apples being similar to those of early harvested apples. Fungal microbiome variation was linked to the soluble solids concentration and I_{AD}, revealing further links between microbial dynamics and apple quality. Bacterial shifts that occurred from harvest to 6 months of storage were characterized by metagenome changes that resulted in a lower abundance of biofilm formation pathways after cold storage, which may limit the ability of these bacterial communities to block colonization by fungal pathogens. These findings advance our understanding of how the microbiome is connected to fruit quality and functional metagenomics related to biocontrol.

1. Introduction

Fungal decay can cause serious losses of horticultural produce in both developing countries and in industrial supply chains of more developed countries (Ali et al., 2021; Argenta et al., 2021). Fungicides can be used to treat postharvest pathogens of fruit and vegetables, but increasing fungicide resistance combined with the difficulty in discovering new modes of action and increasing regulatory concerns suggest the need for a wider variety of approaches in controlling postharvest decay (Spadaro and Droby, 2016). A potential supplement or alternative to conventional fungicides is biocontrol, which has been researched since the mid-1980s (Wilson and Pusey, 1985). However, biocontrol products have made limited impact on markets due to production issues and inconsistent performance (Droby et al., 2016; Dukare et al., 2019). A potential way to improve biocontrol efficacy and bolster pathogen resistance is to utilize the native microbiota of crops that are theorized to be intertwined with nearly all aspects of host physiology (Wisniewski and Droby, 2019). Therefore, a better understanding of postharvest microbial ecology may pave the way to more effective ways to combat fungal decay.

Apples are an ideal model system for studying postharvest microbiomes due to long storage times and the presence of a core set of microorganisms that remains present regardless of global geography (Abdelfattah et al., 2021). Previous work has found that postharvest apple microbiome composition varies based on conventional vs. organic management (Abdelfattah et al., 2021, 2016; Bartuv et al., 2023; Leff

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and Fierer, 2013; Shen et al., 2022; Vepštaitė-Monstavičė et al., 2018; Wassermann et al., 2019b), host genotype (Abdelfattah et al., 2022; Liu et al., 2018; Zhimo et al., 2022), biocontrol application (Biasi et al., 2021; Shi et al., 2022), postharvest treatments such as waxing and hot water (Abdelfattah et al., 2020; Bösch et al., 2021; Shen et al., 2018; Wassermann et al., 2019a), time spent in cold storage (Abdelfattah et al., 2020; Biasi et al., 2021; Bösch et al., 2021; Lane et al., 2023; Shen et al., 2018; Wassermann et al., 2019a; Zhimo et al., 2022), and storage conditions such as low oxygen controlled atmosphere storage (Bösch et al., 2021; Lane et al., 2023). Together, this body of work suggests that apple microbiomes are malleable, but that there is a core microbiome consistent enough that discovered microbial community dynamics are likely to apply beyond limited geographic contexts.

When attempting to connect the role of the apple microbiome to fruit physiology, the connection between the microbiome and ethylene is less understood. Apples are a climacteric fruit, producing ethylene as a critical component of the ripening process (Johnston et al., 2009). Ethylene production can be suppressed with plant growth regulators (PGRs) such as aminoethoxyvinylglycine (AVG; commercial formulation ReTain™), which inhibits the activity of 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) by binding to pyridoxal-5'-phosphate and therefore suppresses the ability of ACS to catalyze the conversion of methionine to ACC oxidase (Boller et al., 1979). Another PGR is 1-methylcycopropene (1-MCP; commercial formulation Harvista®), which inhibits ethylene perception (Sisler, 2006). In addition to its impacts on ripening and senescence, ethylene is known to affect plant defense signaling (Adie et al., 2007), as well as the signaling of fungal pathogens and bacteria (Gamalero and Glick, 2015; Ravanbakhsh et al., 2018; van Loon et al., 2006), suggesting a link to the microbiome. Our previous work found that preharvest AVG treatments impacted bacterial but not fungal communities (Lane et al., 2023), and other work has linked ethylene-mediated ripening to the microbiome of pear and kiwifruit (Xie et al., 2021; Zhang et al., 2021). However, it is not known how these dynamics are affected by differences of ethylene production and fruit quality across harvest time, nor what functional changes are associated with taxonomic microbiome shifts.

In this study, we investigated the role of preharvest AVG treatment, harvest time, and storage time on the apple microbiome at harvest and during storage. We hypothesized that 1. AVG treatment would shift microbiomes by mediating plant-microbe interactions; 2. The microbiome would shift over harvest time due to microbes responding to maturation and ripening processes, but these effects would be affected by AVG; and 3. Microbiomes would shift throughout storage, but that differences would remain between harvest times due to different at harvest microbial communities and host physiology. We aimed to link these potential changes to fruit quality, and in microbial metagenomes to uncover functional differences associated with taxonomic ones. Our previous work (Lane et al., 2023) utilized metagenome prediction from amplicon sequencing to assess this question, and in this study we utilized shotgun metagenomics to obtain a more cohesive and accurate picture of the metagenome. Functional genomics with shotgun sequencing for apple microbiomes has largely been limited to data mining for biocontrol and antimicrobial genes (Angeli et al., 2019; Wassermann et al., 2022), with limited use to address treatments such as conventional vs. organic management (Bartuv et al., 2023). Our objective was to use metagenomic analysis to provide a key step towards linking microbiome shifts with functional outcomes.

2. Materials and methods

2.1. Field treatments, quality assessment, and storage

'NY1' apples grown at the Cornell Orchards in Ithaca, NY (coordinates 42.45018807, −76.462011341) were used for this experiment. Eight single tree replicates for untreated and AVG treatment were used. AVG (ReTain®, Valent BioScience Corporation, Libertyville, IL) was sprayed at a rate of 0.25 g L^{-1} on the trees 14 d before the initial harvest, with the control treatment receiving water instead. The first harvest took place on September 16, 2021, with three subsequent harvests at one-week intervals. Each replicate consisted of 5 fruit. Nitrile gloves were used for handling of the fruit until the microbiome was sampled to prevent contamination.

The internal ethylene concentration (IEC) of each fruit was sampled on the day of harvest, being sampled before the microbiome so that the wash sampling method described below did not interfere with the gas dynamics in the apple core. Firmness, delta absorbance (I_{AD}), soluble solids concentration (SSC), titratable acidity (TA), and the starch pattern index (SPI) were assessed the day after microbiome sampling due to the destructive nature of this sampling. All procedures were carried out as previously described (Al Shoffe et al., 2021). A total of 64 samples (2 treatments x 4 harvest times x 8 replicates) were assessed for quality and microbiome sampling at each harvest time.

Fruit from the first and fourth harvests were stored in air at 3 °C, and fruit were assessed at 2, 4, and 6 months of storage. The first and fourth harvests (H1 and H4) were chosen to assess the range of maturity from least to most mature. At each time point, IECs were measured 24 h after removal from storage, the microbiome was sampled, and then the quality factors other than the SPI were measured. Apples that showed signs of decay after 6 months of storage were not sampled for the microbiome or for quality assessment. This included two entire replicates, leaving a total of 94 samples assessed throughout storage (2 chemical treatments x 2 harvest times x 3 storage times x 8 replicates = 96), for a total of 158 total samples (64 at harvest + 96 postharvest – 2 decayed) used for quality assessment and microbiome sampling.

2.2. Microbiome sampling and sequencing

Microbiome sampling and DNA extraction was performed as previously described (Lane et al., 2023). Briefly, five apples were placed in a 0.05 M phosphate buffer solution with 0.1 % Tween 80 and were sonicated and placed in a rotary shaker for 20 minutes each before being filtered through a 0.22 μm filter paper for microbiome collection and eventual extraction via Qiagen DNeasy® PowerSoil® DNA extraction kit (Beverly, MA). However, unlike our previous study, we did not pool DNA from multiple replicates prior to sequencing.

At harvest and postharvest samples were sequenced in separate facilities, resulting in different PCR and library preparation steps. At harvest samples were processed as previously described with sequencing done through the Cornell sequencing facility (Bray et al., 2019), with the alteration that 5 μM PNA clamps (5′-GGCAAGTGTTCTTCGGA-3′ and 5′-GGCTCAACCCTGGACAG-3′) were added during 16 S PCR to block amplification of plant plastid and mitochondria 16 S (Lundberg et al., 2013). Postharvest samples were processed as previously described with sequencing done through Novogene (Lane et al., 2023). In both cases, the V3 and V4 subunits of the 16 S were used for bacteria, and the ITS2 gene was used for fungi.

Two of the eight replicates for AVG and control were selected for shotgun metagenomic sequencing from the same extracted microbial DNA. Samples were selected from harvests 1 and 4 at 0 and 6 months for sequencing, for a total of 16 samples (2 preharvest treatments x 2 harvests x 2 storage times x 2 replicates). Samples were shipped to Novogene (Sacramento, CA), who performed library preparation and sequencing. A total amount of 1 μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™II DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, the DNA sample was fragmented by sonication to a size of 350 bp, then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. Finally, PCR products were purified (AMPure XP system) and libraries were analyzed for size distribution by Agilent2100 Bioanalyzer and

quantified using Real Time PCR. Quantified libraries were pooled and sequenced on Illumina Illumina NovaSeq 6000 platform, according to effective library concentration and data amount required.

2.3. Sequence processing and statistics

Amplicon sequence analysis was performed in QIIME2 (Bolyen et al., 2019). Due to different sequence protocols, the at harvest and postharvest amplicon sequencing datasets were analyzed separately. First, sequences were imported, merged, and filtered through DADA2 (Callahan et al., 2016). Default parameters were used for the postharvest samples, while in the at harvest samples reads were trimmed prior to merging due to low quality. The resulting amplicon sequencing variants (ASVs) were clustered de novo into operational taxonomic units (OTUs) based on 97 % similarity, followed by removal of chimeric sequences using uchime (Edgar et al., 2011). OTUs were then assigned taxonomy based on a Naive Bayes Classifier trained on the UNITE_ver8_dynamic database for ITS and the SILVA 138 99% database for 16 S (Abarenkov et al., 2010; Quast et al., 2012). Chloroplast and mitochondrial sequences were then removed from the 16 S dataset. Finally, OTU abundance and taxonomy tables were exported along with representative sequences for subsequent analysis in R version 4.1.2 (R core team, 2021).

For shotgun metagenomic sequence analysis, reads were first filtered and trimmed using Fastp (Chen et al., 2018). These reads were then assembled into a separate metagenome for each sample using MetaSPADES (Nurk et al., 2017). Assembly contigs were taxonomically classified using Kraken2 (Wood et al., 2019), and assemblies were divided into bacterial and fungal assemblies with Kraken tools. Metagenome functional annotation was then performed with emapper-2.1.3 using the eggnog 5.0 database (Huerta-Cepas et al., 2019, 2017), using Prodigal for open reading frame prediction in bacteria and MMseqs2 for sequence searches in bacteria and fungi (Hyatt et al., 2010; Steinegger and Söding, 2017). KEGG orthologs (KOs) and pathway abundances were extracted from the output and assembly coverage estimates for downstream analysis in R (Kanehisa and Goto, 2000).

Table 1 summarizes the sequencing statistics. To maximize the data obtained from each sample, rarefication was not performed.

2.4. Statistical analyses

The association between IECs and harvest time, storage time, and AVG application was assessed with an ANOVA, with $p < 0.05$ being used as the cutoff for significance. The proportion of each sample with apples showing signs of rot, mold, or decay after 6 months was assessed in the same manner across harvest time and storage time.

As with sequence analysis, the at harvest and postharvest amplicon sequencing datasets were analyzed separately due to different sequencing protocols used. Effects of AVG treatment, harvest time, and storage time (for postharvest dataset) on microbial OTU composition were assessed by performing a Permutational Multivariate Analysis of Variance (PERMANOVA) using 999 permutations (Anderson, 2014) with a $p < 0.05$ being used as the cutoff for significance. This test was performed on a Bray-Curtis distance matrix (obtained using the vegan package) of the OTU abundance table (Beals, 1984; Dixon, 2003). The distance matrix was also visualized with a Principal Coordinate Analysis (PCoA). Microbiomes were then compared against treatments and quality metrics using a Distance-based Redundancy Analysis (dbRDA), with certain parameters excluded to keep the variance inflation factor lower than 5. The significance of dbRDA parameters was assessed using a permutational ANOVA using 999 permutations. The Shannon diversity was calculated from the same OTU table and compared across treatment, harvest time, and storage time using an ANOVA. The R package phyloseq was used to create a genera table that was then assessed by the Maaslin2 package to determine genera that were significantly different across treatments (Mallick et al., 2021; McMurdie and Holmes, 2013). A false discovery rate cutoff of 0.05 with the Benjamini-Hochberg method was used to determine significance (Benjamini and Hochberg, 1995).

A similar statistical analysis was performed on the shotgun metagenomics KEGG output, with kos being used for the PERMANOVA and PCoA due to their specificity. When assessing which genomic features significantly changed across predictors of metagenome composition, whole pathways were used in place of KOs to establish clearer connections with biological processes of potential interest. When doing this multiple testing, pathways with less than 0.001 % abundance were excluded.

3. Results

3.1. Internal ethylene concentrations and postharvest decay

AVG treatment, harvest time and storage time were highly significant predictors of IEC with numerous interaction effects between factors (Table 2). In particular, IEC through storage time showed different patterns depending on harvest and AVG treatment, decreasing over time in the harvest 4 control, increasing in the harvest 1 control and AVG, and remaining consistently low in the harvest 4 AVG treatment (Fig. 1). Overall, IEC was lower in AVG-treated fruit, and other patterns it showed relied on a combination of factors.

Disease incidence was assessed after 6 months by looking for clear visual signs of pathogen presence such as areas of rot or visible mold. The proportion of disease incidence in apples was higher in harvest 4 (48 %) than harvest 1 (11 %), with no other significant effects being found (Table 2). No signs of pathogen activity were observed at earlier time points.

3.2. Microbiome composition and diversity

At harvest, bacterial microbiomes were associated with harvest time, while fungal microbiomes were associated with AVG application (Table 2). Notably, the bacterial microbiomes shifted in a consistent direction throughout the harvest season (Fig. 2A), while fungal microbiomes of AVG-treated fruit showed little to no overlap on an ordination compared to control (Fig. 2B). The interaction between harvest time and AVG was not significant for bacteria or fungi.

In contrast, AVG treatment, harvest time and storage time were all

Table 1
Sequencing statistics for different datasets including average raw reads per sample, filtered reads and ASVs after DADA2 (or Fastp for shotgun metagenomics), and number of final OTUs after 97 % clustering and subsequent chimera removal. The final column depicts the number of samples with a final OTU count of fewer than 1000, which were removed from statistical analyses.

	Average raw read count per sample	Average filtered read count per sample	Number of ASVs	Number of OTUs	Number of samples removed from analysis
At Harvest 16 S	28,028	7641	5648	646	4
At Harvest ITS	30,549	15,188	1235	718	3
Postharvest 16 S	96,500	79,340	7464	2742	0
Postharvest ITS	88,716	60,584	3070	884	0
Shotgun Metagenomics	31,227,287	30,991,046	-	-	0

Table 2
p-values of the associations between treatments (column labels) and responses (row labels). IEC and Shannon Diversity were assessed using an ANOVA, while composition and KEGG KOs were assessed using a PERMANOVA. Postharvest and KEGG KO datasets used only harvests 1 and 4, while the KEGG KO dataset also only used 0 and 6 months of storage.

	Harvest Time	Storage Time	AVG	Harvest Time x Storage Time	Harvest Time x AVG	Storage Time x AVG	Harvest Time x Storage Time x AVG
IEC	<0.001	0.096	<0.001	<0.001	0.368	<0.001	<0.001
Decay After 6 Months	<0.001	-	0.132	-	0.897	-	-
At Harvest 16 S Composition	<0.001	-	0.589	-	0.948	-	-
At Harvest 16 S Diversity	0.507	-	0.319	-	0.923	-	-
At Harvest ITS Composition	0.002	-	<0.001	-	0.036	-	-
At Harvest ITS Diversity	0.465	-	0.125	-	0.075	-	-
Postharvest 16 S Composition	<0.001	<0.001	0.003	0.022	0.184	0.229	0.875
Postharvest 16 S Diversity	0.117	0.764	0.837	0.556	0.613	0.807	0.415
Postharvest ITS Composition	<0.001	<0.001	<0.001	0.061	0.984	0.047	0.677
Postharvest ITS Diversity	<0.001	0.874	0.33	0.3	0.002	0.03	0.767
Bacterial KEGG KOs	0.256	<0.001	0.374	0.663	0.443	0.549	0.805
Fungal KEGG KOs	0.94	0.388	0.093	0.818	0.539	0.855	0.732

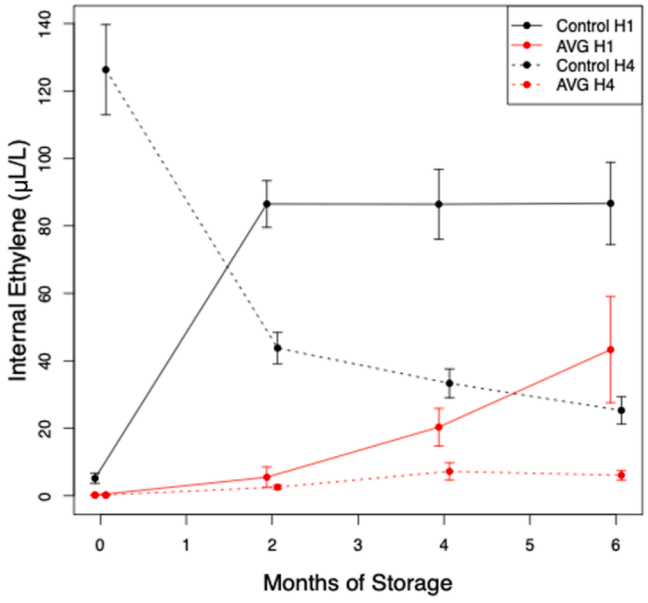

Fig. 1. Internal ethylene concentration by harvest time, storage time, and AVG application on day of harvest or 24 hours after removal from storage. Points represent the average across up to 40 apples per treatment/time combinations, with error bars displaying one standard error from the mean. Table 2 and S1 provide statistical information for these associations.

associated with postharvest bacterial and fungal composition (Table 2). The bacteria showed clear clustering by storage time (Fig. 2C), while fungi showed the clearest clustering by harvest time (Fig. 2D). Additionally, bacterial microbiomes depend on the interaction between harvest time and storage time (Table 2), and it can be observed that bacterial microbiomes separate by harvest more clearly at early stages of storage than late storage (Fig. 2C). This suggests that the effects of harvest time on bacterial microbiomes may be less pronounced the longer the apples are stored.

Additionally, postharvest fungal microbiomes covary with quality parameters such as I_{AD} and SSC (Fig. 3B). Notably, AVG applications along with high I_{AD} and SSC values were associated with different microbiomes than long storage and late harvest, as determined by the primary axis of variation. Similar visual patterns were shown in bacteria (Fig. 3 A), but the only significant factor was storage time. No significant effects of dbRDA parameters were found on at harvest bacterial or fungal microbiomes.

The Shannon Diversity remained largely steady regardless of most factors, with the only observed association being between postharvest fungal diversity and harvest time (Table 2). Within this dataset, microbiomes of late harvested apples had higher diversity than microbiomes of early harvested apples (Fig. 4D). However, significant interactions between harvest time and AVG as well as storage time and AVG highlight that AVG application may influence fungal diversity in a more context-specific manner (Table 2).

3.3. Microbiome taxonomy

The most common bacterial genera on apples at harvest were *Curtobacterium* (26.15 %), *Pseudomonas* (10.35 %), and *Methylobacterium-Methylorubrum* (9.41 %; Fig. 5A). During storage, however, the most common bacterial genera were *Curtobacterium* (19.24 %), *Sphingomonas* (12.44 %), and *Methylobacterium-Methylorubrum* (11.69 %; Fig. 5C).

The abundance of many common bacterial genera depended on experimental factors, with storage time being associated with the abundance of the largest number of genera. Out of 75 bacterial genera identified in the at harvest dataset, 5 increased in relative abundance through the harvest season, and 5 decreased. Genera with increased relative abundance and over 1 % total abundance were *Pseudomonas* and *Bradyrhizobium,* and genera with decreased relative abundance and over 1 % total abundance were *Methylobacterium-Methylorubrum, Massilia, Sphingomonas, Curtobacterium,* and a genus the classifier denoted as *1174–901–12* (Fig. 5A). For the storage samples, 167 bacterial genera were identified. Of these, one had higher relative abundance on the late harvested apples compared to early harvested apples while four had lower relative abundance, including the common (> 1 % total abundance) *Massilia* and *Bradyrhizobium* (Fig. 5C). Thirty eight bacterial genera had increased relative abundance at late storage compared to early storage (including the common *Azorhizobium, Curtobacterium, Kineosporia,* and *Subtercola*), while 27 had decreased relative abundance (including the common *Candidatus Obscuribacter, Caulobacter, Massilia,* and *Sphingomonas)*. One bacterial genus had higher abundance in AVG-treated stored apples than control, while three had decreased abundance. None of these genera had more than 1 % total abundance.

The most common fungal genera on apples at harvest were *Entyloma* (17.07 %), *Vishniacozyma* (9.23 %), and *Sporobolomyces* (8.58 %; Fig. 5B), while the most common fungal genera on the postharvest apples were *Aureobasidium* (32.00 %), *Vishniacozyma* (7.91 %), and *Entyloma* (6.52 % Fig. 5D). While it appears striking that *Aureobasidium* is the most common fungal genus in postharvest samples while not reaching 1 % abundance in the at harvest dataset, this is likely explained by 26.39

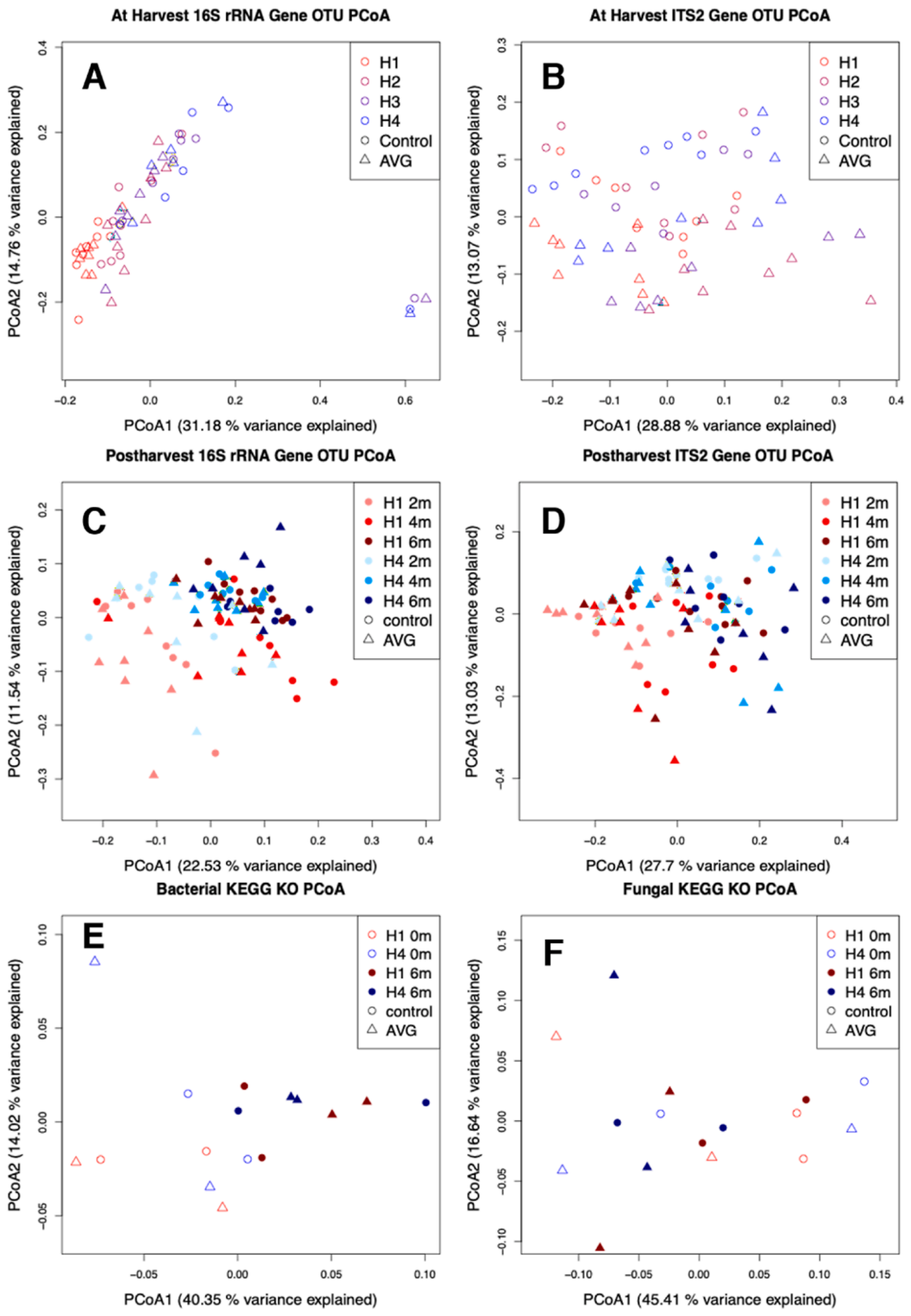

Fig. 2. Principal Coordinate Analysis of bacterial (A, C, E) and fungal (B, D, F) microbiomes, looking at preharvest OTU composition (A, B), postharvest OTU composition (C, D), and metagenomic KEGG ko composition (E, F). Color corresponds to harvest and storage time, while point shape corresponds to AVG application. Table 2 gives the p-values for these associations.

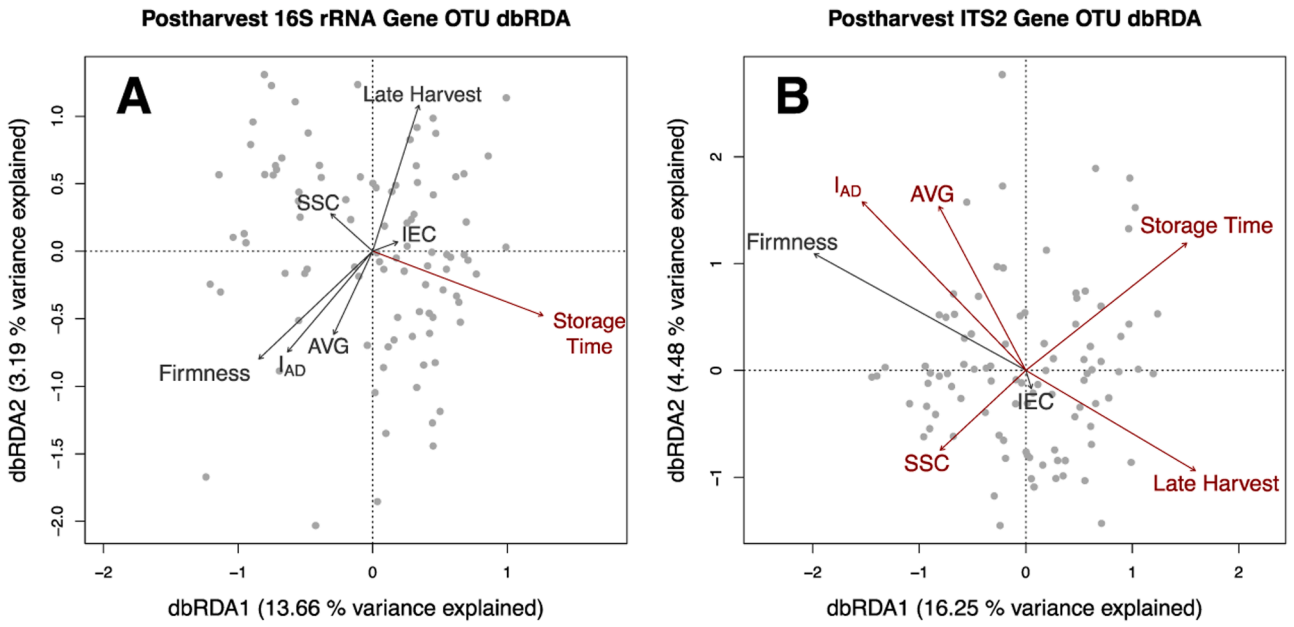

Fig. 3. dbRDA plots of the postharvest bacterial 16 S (A) and fungal (ITS) OTUs (B). Factors colored red are significant ($p < 0.05$) predictors of microbiome variation, while factors colored grey are not.

% of at harvest fungal OTUs being classified into the Aureobasidiaceae family and unclassified at the genus level.

The abundance of several fungal genera also covaried with treatment factors, with storage time having the most associations in similar fashion to bacteria. Of the 70 fungal genera in the at harvest samples, two had increased abundance in AVG-treated apples compared to control while four had decreased abundance. The genera with decreased abundance included the common (> 1 % total abundance) *Golubevia, Sporobolomyces, and Articulospora.* For the storage dataset, 103 genera were identified. Of these, seven had increased abundance in AVG-treated apples compared to control (including the common *Entyloma*), while one (the common *Golubevia*) had decreased abundance. 15 increased in abundance over storage time (including the common *Bulleromyces, Cladosporium, Sarocladium,* and *Niesslia)*, while 12 decreased in abundance (including the common *Entyloma*). 20 had increased abundance in late harvested apples compared to early harvested apples (including the common *Bulleromyces, Cladosporium*, *Entyloma,* and *Vishniacozyma),* while 5 (including the common *Alternaria* and *Sporobolomyces*) had decreased abundance.

3.4. Microbiome metagenomics

Storage time was a predictor of bacterial metagenome KEGG KO composition, while no factors were shown to covary with fungal KEGG KO composition (Table 2). This is visualized by ordination, where clear clustering was observed between at harvest and 6 months storage in bacteria (Fig. 2E), but no clear clustering is observed in fungi (Fig. 2F).

After accounting for multiple testing, we revealed that pathways involving antibiotic resistance and biofilm formation had lower relative abundance after 6 months of storage compared to at harvest. Out of 302 classified KEGG pathways, none had increased relative abundance after 6 months of storage compared to at harvest and 20 had decreased relative abundance. Pathways that decreased in abundance had a variety of functions. These include synthesis of terpenoids, N-glycans, carotenoids, and lipopolysaccharides, as well as the degradation and metabolism of cysteine, methionine, and furfural. In addition, pathways antibiotic resistance and biofilm formation had more consistently decreased relative abundance after storage. Two of the three pathways related to antibiotic resistance, cationic antimicrobial peptide resistance and beta-Lactam resistance, were less abundant after 6 months of storage, while all three pathways related to biofilm formation had decreased abundance (Fig. 6). This does not equate with the abundance of the three bacteria listed. Instead, the consistent pattern of biofilm formation pathways being less abundant after storage suggests that the trend holds broadly true despite differences in the genetics of biofilm formation between taxa.

4. Discussion

4.1. Microbiome composition is associated with storage time and harvest time

Our work was consistent with previous studies showing that the apple microbiome shifts over months of storage (Abdelfattah et al., 2020; Biasi et al., 2021; Bösch et al., 2021; Lane et al., 2023; Shen et al., 2018; Wassermann et al., 2019a; Zhimo et al., 2022), with minor differences in some of the specifics. Our previous work found no effect of storage type and time on the fungal microbiome of 'Gala' apples (Lane et al., 2023), while we found a strong association in the current study (Table 1). This discrepancy could be due to factors such as a different cultivar and different postharvest treatments. Other studies have found that fungal shifts throughout storage are context-dependent, such as observing shifts only in certain parts of the apple (Abdelfattah et al., 2020).

Our study showed that bacterial and fungal microbiomes can shift over the course of a harvest season, a result that supports a recent observation that microbiome shifts throughout apple fruit development and maturation (Zhimo et al., 2022). This effect was pronounced in bacteria but was not observed in fungi at harvest (Table 1, Fig. 2 A-B), similar to previous findings showing larger differences between maturation and harvest periods in bacteria compared to fungi (Zhimo et al., 2022). In addition, we observed a strong association between fungal composition and harvest time, but only after 2 or more months of storage (Table 1, Fig. 2D). The trend held true even during early storage, suggesting that it may not necessarily be related to spoilage by pathogens after long storage. Overall, our hypothesis that microbiome variation associated with harvest time remains pronounced through storage was largely supported by the data, and in the case of fungi we even observed effects during storage that were not detected at harvest. However, we did observe an interaction effect between harvest time and

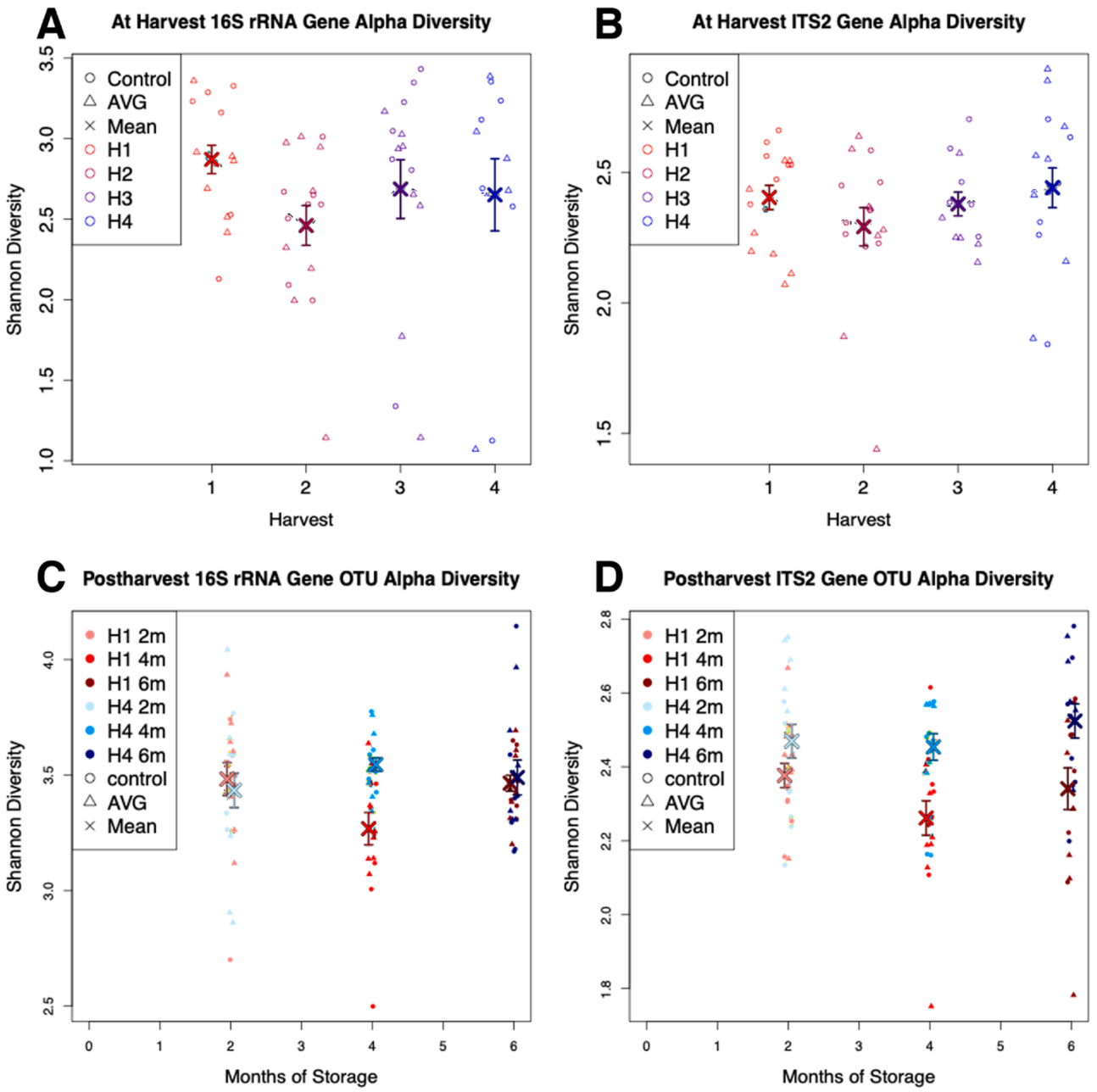

Fig. 4. Shannon diversity of bacterial 16 S (A, C) and fungal ITS (B, D) OTUs, at preharvest (A, B) and postharvest (C, D) time points. X points represent the mean for each harvest/storage time combination, with error bars being one standard error of the mean. Table 2 shows the p-values for these associations.

storage time in bacteria (Table 2), which may be due to samples from different harvests clustering closer together late in storage (Fig. 2C). This suggests that for bacteria, differences in the microbiome due to variation in harvest time may not have been maintained throughout long periods of storage.

4.2. *Microbiome differences can be linked to postharvest fruit quality*

Our work expanded on previous microbial ecology studies on ethylene inhibitors by connecting the effects of AVG on the microbiome to ethylene-mediated ripening. Previous work that showed the effect of ethylene inhibitors on the microbiomes of climacteric fruit (Lane et al., 2023; Xie et al., 2021; Zhang et al., 2021), but it proved difficult to isolate ripening differences as the cause for those shifts. In accordance with our hypothesis, not only was AVG treatment associated with microbiome variation in fungi, but the postharvest microbiomes of AVG-treated apples also resembled the microbiomes of early harvested apples (Fig. 3B). This suggests that the microbiome is linked to the physiology of apple ripening rather than an unrelated effect of AVG. In addition, those patterns being present in storage but not at harvest samples also indicated that microbiome changes across harvest time were likely due to host-microbe interactions rather than turnover, and that these interactions may take time or require the presence of cold storage to induce microbiome shifts. In bacteria we similarly observed that AVG treatment only affected the microbiome during storage rather than at harvest (Table 2), indicating that microbial responses to host ripening may be delayed or mediated by cold storage. Overall, these findings constitute an important step towards understanding the relationship between fruit maturation and ripening with microbial activity.

In addition to treatment effects on microbiomes, we observed that postharvest fungal microbiomes are associated with I_{AD} and SSC (Fig. 3B). Apples with high SSC and high I_{AD} (greener) had microbiomes

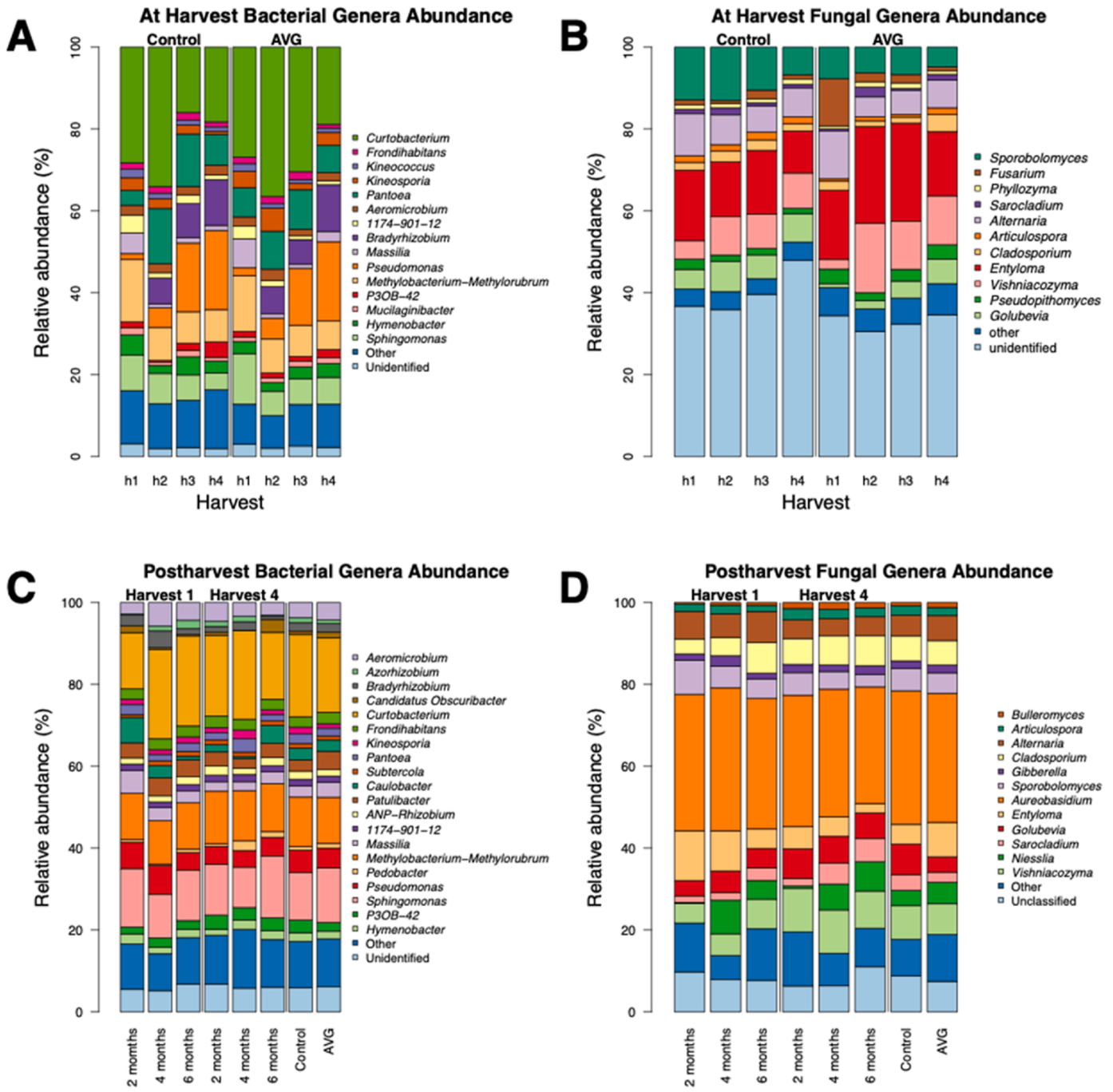

Fig. 5. Abundance of common bacterial (A, C) and fungal (B, D) genera at the at harvest (A, B) and postharvest (C, D) time points derived from the OTU dataset. For postharvest time points, control and AVG bars include samples from all harvest and storage times, while other bars include samples from both control and AVG. Genera with less than 1% abundance are grouped under "Other." For bacteria, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* is shortened to *ANP-Rhizobium.*

that are more similar to early harvested, AVG-treated, or short storage apples along the primary axis of variation. These findings build on the previous literature by revealing that commercially important quality indices are associated with microbiomes in addition to treatments. Further research may allow the microbiome to be integrated into fruit quality management on a practical level.

4.3. Metagenome analyses show a decline in the abundance of biofilm formation pathways throughout cold storage

Metagenomic markers associated with biofilm formation decreased in abundance from harvest to 6 months of cold storage (Fig. 6). The trend was consistent across all three taxa that the KEGG database uses for biofilm formation characterization, suggesting that the abundance of biofilm formation pathways decreases as a whole despite potential differences in pathways across taxonomy. The implications of this finding are multifaceted, as biofilms promote microbial growth with negative implications for food safety (Nathanon, 2003), but can also act as modes of biocontrol against colonizing pathogens (Wallace et al., 2018). In fact, biofilm formation from the same bacterial strain can be beneficial for one crop while pathogenic to another (Giobbe et al., 2007). Nevertheless, bacterial biofilm formation has been identified as an important biocontrol mechanism (Spadaro and Droby, 2016), and the lowered abundance of biofilm formation pathways after cold storage may suggest

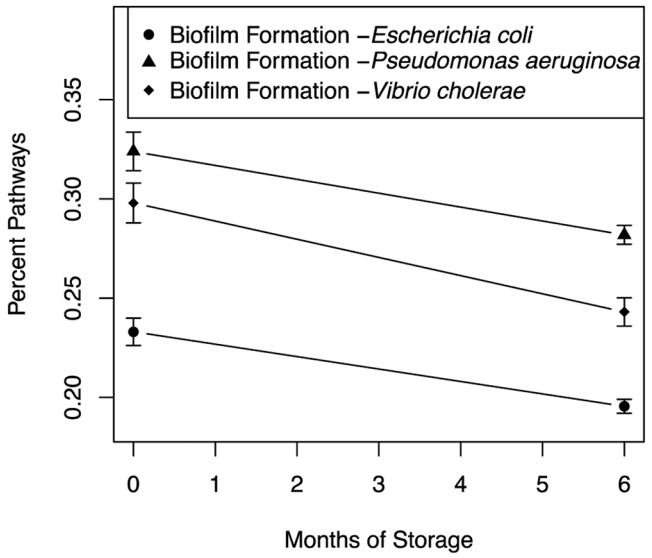

Fig. 6. Bacterial KEGG pathway abundance of three pathways associated with biofilm formation in different bacteria. Points represent the mean, with error bars measuring one standard error from the mean. Differences for all three pathways are significant (FDR < 0.05).

that native microbiota have a lessened ability to protect apples from fungal pathogens using biofilm formation at that time.

However, an important caveat of metagenomic interpretation is the potential discrepancies between gene abundance and expression, along with the possibility that the traits under selection pressure are not part of the database searched. Nevertheless, this shotgun metagenomic analysis highlights the importance of biofilm pathways, providing a key step in linking microbiome shifts with implications for biocontrol. This finding opens up opportunities for further research on the biofilm formation of native microbiota on the fruit surface.

5. Conclusion

In accordance with our hypotheses, we found that AVG treatment, harvest time and storage time impacted the microbiome. In particular, the effect of AVG on slowing maturation and ripening was mirrored by shifting postharvest fungal microbiomes to be more similar to apples found in earlier harvests. Indeed, the postharvest fungal microbiome was also associated with the harvest index of background color (I_{AD}) and the quality index SSC, providing a link between fruit physiology and the microbiome. Notably, many of these effects were observed during storage rather than at harvest, suggesting that microbiome responses to fruit ripening physiology may be delayed or mediated by cold storage. In addition, we were able to link certain microbiome shifts to functional metagenomic changes, such as a reduced abundance in biofilm formation pathways in bacteria after 6 months of cold storage compared to those at harvest. These results build on previous postharvest microbiome research by connecting the microbiome to ripening physiology and functional biocontrol implications through the metagenomic abundance of biofilm formation pathways.

CRediT authorship contribution statement

Peter Schafran: Writing – review & editing, Validation, Formal analysis, Data curation. **Fay-Wei Li:** Writing – review & editing, Validation, Formal analysis, Data curation. **Yosef Al Shoffe:** Writing – review & editing, Investigation. **Jenny Kao-Kniffin:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Conceptualization. **Christopher B Watkins:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Connor Lane:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.postharvbio.2024.112969.

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