# TEMPORAL FRUIT MICROBIOME AND IMMUNITY DYNAMICS IN POST-HARVEST APPLE (*MALUS* X *DOMESTICA*)

By

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## ABSTRACT

The plant immune response plays a central role in maintaining a well-balanced and healthy microbiome for plant health. However, insights into how the fruit immune response and the fruit microbiome influence fruit health after harvest is limited. Additionally, our understanding of microbial succession patterns throughout the full season and the source of microbial communities of the apple fruit is unknown. Here, I examined epiphytic and endophytic microbiota composition over the full developmental time course of apple fruit starting at flowering. I also investigated their temporal dynamics along with the host defense gene expression patterns during post-harvest storage of apple fruits.

My data shows that the endophytic community in ripe fruit are similar to the community at full bloom, which suggests that the endophytic community may be derived from the flower. Conversely, epiphytic communities are more dynamic over time, which could indicate that surface microbes are strongly influenced by environmental factors.

Further, my results demonstrate a temporal dynamic shift in these communities during post-harvest storage that coincides with a steep-decline in host immune response (as monitored by *MdFLS2* and *MdBAK1* gene expression). We observed the emergence of putative pathogenic/spoilage microbes belonging to genera *Alternaria* (fungi) and *Gluconobacter* and *Acetobacter* (bacteria) at the expense of *Sporobolomyces* and other genera suggested to be beneficial for plant hosts. These results suggest that the fruit immune response helps to orchestrate the microbiome composition and that the collapse of the immunity results in the proliferation of spoilage microbes and fruit rot. Consistent with our hypothesis that the fruit immune response plays a role in protection against dysbiosis, I show that by inducing fruit innate immunity with the flg22 peptide I can delay the onset of fungal rot in apple fruit. Future research

is required to determine if this protection is associated with compositional changes of the microbiota.

To further test the influence of fruit immune response on the microbial community composition, especially endophytes that may have a profound impact on postharvest fruit rot, Acibenzolar S methyl (ASM) a systemic acquired resistance inducer, was applied to trees one week before harvest. I hypothesized that ASM application, known to robustly induce defense gene expression, will lead to a shift in microbial community composition and improved shelf life. Through my work, I demonstrated that, indeed, induction of the immune response by ASM application significantly changed the microbiota composition, especially the endophytes, in post-harvest apple fruits. The findings from my PhD research hold significant implications for the development of strategies to increase fruit quality and prolong shelf life in fruits and vegetables.

**I dedicate this work to my Lord and Savior, Jesus Christ. For His glory!**
**And to my daddy, the late Wozamo Kithan, for his inspiration and his unfailing love and support.**

*"For I can do everything through Christ, who gives me strength."*
*Phillipians 4:13 NLT*

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# CHAPTER 1

## LITERATURE REVIEW - APPLE FRUIT MICROBIOME AND IMMUNITY: INTERACTIONS AND RELEVANCE TO POST-HARVEST HEALTH

## THE PLANT MICROBIOME

Plants provide a multitude of niches for the growth of diverse microorganisms including bacteria, fungi, protists, nematodes and viruses, collectively called the plant microbiota (Trivedi et al., 2020; Trivedi et al., 2021). The plant microbiota is a key determinant of plant health and productivity, and its composition is driven by host compartment, environment, host genotype, host immunity and microbe-microbe interactions (Karasov et al., 2018; Grady et al., 2019; Kumar et al., 2021; Almario et al., 2022; Sohrabi et al., 2023). Researchers have identified a highly complex, yet stable, subset of microbial lineages that are associated with given hosts across a range of environments (Fitzpatrick et al., 2018; Hamonts et al., 2018). These microorganisms are in constant contact with plants and play important roles in promoting plant growth, nutrient uptake and pathogen resistance in natural environments (Richardson and Simpson, 2011; Estabrook and Yoder, 2020). The beneficial effect on the host plant conferred by these microorganisms can be direct such as nitrogen fixation and nodulation to translocate essential nutrients from soil to plants, degradation of environmental pollutants, mitigation of environmental stresses and protection from plant pathogens through competition, antibiosis and the production of hormones and antibiotics or lytic enzymes (Richardson and Simpson, 2011; Xie et al., 2016; Gouda et al., 2018). These microorganisms can also indirectly benefit the plant host in promoting plant growth through enhancement of a plant's resistance responses such as induction of systemic resistance (ISR) to lessen or prevent the deleterious effects of phyto-pathogens (Pieterse et al., 2014; Trivedi et al., 2020; Trivedi et al., 2021).

Extensive efforts have been made to develop commercial products based on the use of single microorganisms (antagonists) to improve plant growth and health, as well as to protect postharvest fruits and vegetables from pathogens (Vorholt et al., 2017). However,

implementation of microbial inoculants as biocontrol agents has seen limited success, as the introduced inoculants are often not able to persist in the plants. The main problem with this inundative inoculation approach is likely that an introduced isolate is not the only player in the system; i.e., this strategy neglects the complex interactions that the introduced isolate may experience as part of a microbial network and as a component of a biological system (Wisniewski and Droby, 2019). The identification of a stable microbiota within a particular host, and how they are established, will provide foundational knowledge on developing synthetic communities (SynCom) to manipulate plant-microbiomes to reduce the incidence of plant disease, increase agricultural production and resilience against biotic and abiotic stress, and reduce emissions of greenhouse gases, resulting in more sustainable agricultural practices (Turner et al., 2013).

Microorganisms are found in all compartments of the plant and require unique traits and life strategies to survive in these environments. Niche adaptation and environmental conditions may have a major role, where only successful colonizers that can compete for the available resources, or those that can form stable co-existing communities through mutual cooperation can adapt to the plant environment and flourish (Trivedi et al., 2020). Microbes occur as epiphytes on the surface of plant tissue, and as endophytes within plant tissue, and grow in the rhizosphere (below ground), phyllosphere (above ground), and the carposphere (the fruit). Studies have also noted that community compositions are distinct across rhizosphere, phyllosphere, carposphere, plant tissue types and geographic locations (Niu et al., 2017).

**PLANT MICROBIOTA ASSEMBLY**

To better understand the beneficial effects of plant microbiota at both the individual plant and ecosystem levels we need to have a better understanding of their colonization and potential

route of transfer and dispersal. Host-associated microbiota assembly is a successional, multistep process that is determined by dispersal, microbe-microbe interactions, the environment and the host (Trivedi et al., 2020; Trivedi et al., 2021). Host-associated microbes can colonize vertically, via the parents to the offspring through seed transmission pathways, or horizontally, via the environment, or by mixed modes (Bright and Bulgheresi, 2010; Shade et al., 2013; Frank et al., 2017). Microorganisms that are vertically transmitted through seeds may lack features for active dispersal and are unable to survive in the environment. However, vertical transmission is common in systems and they have been shown to provide indispensable functions to the host and may be key taxa for the health of the next plant generation (Moran, 2006; Frank et al., 2017; Bergna et al., 2018; Malinich and Bauer, 2018).

Once seeds germinate, most microbiota are likely to be horizontally transmitted. Seed-borne microorganisms preferentially become associated with aboveground plant tissues, whereas soil-derived microorganisms are mainly associated with below ground plant tissues (Torres-Cortés et al., 2018). Microbiota of seedling grown under normal soil were shown to have a higher diversity than the seedling grown under sterile condition (Hardoim et al., 2012). This suggests that a substantial number of microbes are acquired after germination. Horizontal transmission of beneficial microbes may be in the best interest of the host plant and is likely to be dynamically recruited and assembled over its lifecycle, providing a means to respond to a changing environment (Carroll, 1988). The temporal dynamic shift in microbiota below the ground and above the ground are consistent across geographic location (Edwards et al., 2018; Abdelfattah et al., 2021). In contrast, some microbes are transmitted both horizontally and vertically, where some microbes are transmitted to the next generation if they provide beneficial effects to their host at a particular circumstance (Frank et al., 2017).

## INTERACTION BETWEEN MICROBIOTA AND HOST IMMUNE SYSTEM IN HOMEOSTASIS

The interactions between a plant and its microbiota are highly complex and dynamic. Plants have evolved a sophisticated innate immune system to protect themselves against pathogen evasion. However, suppression of the plant innate immune system is not only essential for pathogens to successfully infect the hosts but also critical for commensal microbes, meaning that they do not appear to have obvious positive or negative effects on plant health to colonize different plant niches (Wang and Wang, 2014; Colaianni et al., 2021).

*Plant immunity*: In plant innate immunity, pattern-triggered immunity (PTI) is the first line of active defense against most pathogens (Zipfel, 2009). PTI is triggered by receptor recognition of microbial molecular signatures such as flagellin, lipopolysaccharides, chitin and elongation factor TU-derived peptides, among others, referred to collectively as pathogen- (or microbe-) associated molecular patterns (PAMPs/MAMPs), via pattern-recognition receptor (PRRs). Both pathogenic and beneficial microbes can carry these microbial molecular signatures. The perception of PAMPs/MAMPs by plants induces complex signaling pathways that result in numerous cellular changes, including the generation of reactive oxygen species, the activation of mitogen-activated protein kinases (MAPKs) and the induction of salicylic acid-signaling and jasmonic acid-signaling pathways (Han et al., 2014; Trivedi et al., 2020). It has been shown that PTI is an important component that acts as a host barrier to control the level of commensal microbes and opportunistic microbes from excessive proliferation in the host tissue for optimal plant health (Chen et al., 2020; Song et al., 2023; Pfeilmeier et al., 2024).

*Plant innate immunity control of microbiota homeostasis*: Plant innate immunity plays an important role in determining plant microbiota structure. Analysis of an immune-compromised

*A. thaliana* mutant has shed some light on the role of the plant immune system in preventing beneficial, nonpathogenic bacteria from growing inside the leaf (Chen et al., 2020). A dual disruption in PTI signaling and the *A. thaliana* MIN7 gene (involved in intracellular vesicle trafficking), i.e., the *min7 bak-1 bkk-1-1 cerk1* quadruple mutant (*mbbc*), allowed the non-pathogenic Δ*hrcC* mutant of *Pseudomonas syringae* to proliferate under high humidity conditions (Chen et al., 2020). The Δ*hrcC* mutant is defective in the type III secretion system (T3SS) and is incapable of translocating virulence effector proteins into the plant cells to suppress PTI. The nonpathogenic Δ*hrcC* mutant was chosen as it resembles the vast majority of commensal microbes that reside at a modest population in healthy plants. Consistent with this result, the quadruple *mbbc* mutant also allowed excessive proliferation of the endophytic bacterial community when the plants were shifted to high humidity, whereas in the wild type Col-0 plants, a low level of endophytes were detected. Furthermore, *mbbc* leaves (but not roots) showed tissue chlorosis and/or necrosis when shifted to high humidity (Chen et al., 2020). This result persisted when harvested leaves from the *mbbc* mutant and wild type were maintained under high humidity conditions for five days. In a separate study, *A. thaliana* mutant deficient in systemic acquired resistance (SAR) showed a dissimilar rhizosphere bacterial community composition compared with wild type (Hein et al., 2008). All these studies suggest that plant innate immunity plays an important role in establishment of a healthy, eubiotic microbial community (Paasch and He, 2021).

*Evading plant defense:* The plant-associated microbiota must cope with a host immune system that can recognize PAMPs/MAMPs. Studies have shown that some factors implicated in host-pathogen interaction such as T3SS (regulation of virulence, invasion and intracellular resistance) and T6SS (employed in microbe-microbe interactions) are found enriched in healthy

rhizosphere microbiome of citrus as well as barley (Bulgarelli et al., 2015; Zhang et al., 2017; Xu et al., 2018). Another mechanism to evade host innate immunity by plant-associate microbiome could be the ability to disperse after triggering plant defense response (Liu et al., 2018). All this evidence suggests that plants and their microbiomes have evolved together (Berg et al., 2020), and form a composite meta-organism (Trivedi et al., 2020; Trivedi et al., 2021).

## THE POSTHARVEST FRUIT MICROBIOTA

A United Nations Food and Agricultural Organization report stated that food waste and loss is an enormous issue worldwide and estimated that one-third of the food produced for human consumption is either lost or wasted after harvest. (Nations, 2022, 2022). It is estimated that nearly half of the fruits and vegetables produced globally are lost or wasted between production and consumption (Buchholz et al., 2018). Major losses in fruits and vegetables occur during storage, primarily through pathogen infections (Kusstatscher et al., 2020). Current control measures include the use of innovative packaging, physical measures, and chemical pesticides (Li et al., 2015). However, reducing the use of chemical pesticides is necessary for health and environmental concerns and emergence of pathogen resistance.

The growing evidence that there is plant–microbiome co-evolution and an arms-race co-evolution model in natural communities offers a new perspective on microbiome-based innovative strategies to preserve food (Droby and Wisniewski, 2018). However, the study of the postharvest microbiome is only beginning and our knowledge on the functional effect of the microbiome on postharvest food is still very limited. Researchers are starting to explore the presence of a core microbiome in fresh produce that has a potential influence on host-microbiome interactions after harvest. To implement a microbiome-based strategy to preserve food, it is essential that we understand the interaction between the pathogen the host and the

residing microbiota.

The identification of a stable microbiota how it is established, and understanding the functional role and impact of the endophytic and epiphytic microbiota of developing and harvested fruit will provide valuable information needed for the development of a SynCom to manipulate the microbiota of fruits and vegetables to delay postharvest decay or rotting and perhaps even post-harvest physiological disorders. Recent studies have demonstrated that management practices (Abdelfattah et al., 2016; Wassermann et al., 2019), geographical locations (Abdelfattah et al., 2021), post-harvest treatments (Abdelfattah et al., 2020), genetic background (Liu et al., 2018), and cold storage systems (Bosch et al., 2021) impact microbial community composition and diversity on post-harvest apple fruits. It has also been shown that microbial communities of apple fruit undergo temporal changes during cold storage over the course of months. However, the underlying mechanism(s) remain unclear.

*Role of fruit immunity on microbiome structure*: During storage, the fruit goes through physiological changes which could have a significant impact on the microbiome, possibly by decreasing beneficial microbes and increasing pathogenic microbes. A major question that needs to be resolved is whether these changes in physiology, especially the host immunity and senescence, have an impact on microbial community composition that could influence the overall fruit susceptibility to pathogens. In plants, it has been shown that the host immune system orchestrates the maintenance of key features of host-microbe symbiosis, while the microbiome plays critical roles in the training and development of major components of the host's innate and adaptive immune system (Chen et al., 2020; Zheng et al., 2020; Song et al., 2023; Pfeilmeier et al., 2024). Therefore, it is possible that post-harvest losses of fruits could also be in part the result of a breakdown in the relationship between fruit immune responses, the microbiome, and

fruit health

Preliminary data indicate that induction of immune responses by application of acibenzolar-*S*-methyl (ASM), a well-known plant resistance inducer (PRI) and a salicylic acid (SA) functional analog, induces systemic acquired resistance (SAR) in postharvest apple fruits and has a strong correlation in reducing postharvest disease in fruits (Poveda, 2020). However, to my knowledge no published work has investigated how enhanced immune response in a fruit impacts its microbiome. It is important to connect a unique microbiota to a specific physiological change at postharvest, such as the host immune response, and determine how the dynamic of the microbiota at postharvest impact pathogen susceptibility during storage. Functional studies will prove key to understanding the impact of fruit microbiota and fruit immune response on fruit health and decay after harvest. This is the knowledge gap that my work addresses, through a focus on the relationship between fruit immunity, microbiome and health.

**STUDY SYSTEM: APPLE (*MALUS* × *DOMESTICA*)**

Apple is among the most widely consumed fruits in the world (Abdelfattah et al., 2021). It is a temperate fruit adapted to the temperate zone of latitude varying between 35 and 50° (Kellerhals, 2009). The global production of apple is about 83.74 million Metric Tons in 2023/2024 (https://fas.usda.gov/data/production/commodity/0574000). However, post-harvest decay leads to substantial losses with annual losses vary from 5 to 35%; postharvest loss of apple fruit is even more prevalent in developing countries ranging between 20- 50% before reaching the consumers (Porat et al., 2018). Preventing the proliferation and development of postharvest pathogens in storage is an important challenge for maintaining fruit quality and prolonging shelf life.

Disease outbreak in plants often correlates with shifts in the microbiota composition, resulting in a microbial dysbiosis, meaning a state of microbiota homeostasis associated with negative impacts on host health, and a response of specific microbes, which can act as antagonists or synergists towards plant pathogens (Berg et al., 2017). But we have limited knowledge on temporal dynamics of the fruit microbiota during post-harvest disease development (Buchholz et al., 2018). Kõiv *et al.* (2015) demonstrated that a shift in endophytic bacterial community in potato tubers contributed to the development of disease which lead to the pathogen, *Pectobacterium atrosepticum*, infection causing soft rot. Considering this finding along with several other studies, it appears likely that temporal changes in the assembly and composition of microbial communities on and in fruit during storage influences development of disease. The work presented here investigates microbiota assembly in fruit, temporal dynamics of fruit microbiota and immune response at post-harvest and its influence in storage stability of apple fruit. This knowledge could lead to employment of microbial-based solutions to prolong the shelf life of apple fruits and other produce.

**SUMMARY AND OBJECTIVES**

There has been a shift in the life sciences in the past few decades, in which the microbiome (comprising all microbial genomes) is now viewed as a driver of the physiological capabilities of eukaryotic hosts (Cordovez et al., 2019). The plant microbiota confers fitness advantages to the plant host that promote essential processes including growth, health, abiotic stress tolerance, nutrient uptake, and pathogen resilience (Berg et al., 2017; Trivedi et al., 2020). Thus, a proper understanding of how plant microbiomes are established and maintained can facilitate a potentially powerful, untapped approach to enhance plant health, defense, and productivity.

The importance of the plant host immune system in the establishment and maintenance of the microbiome has been illustrated by recent findings (Chen et al., 2020; Song et al., 2023). An immune-compromised *Arabidopsis thaliana* quadruple mutant effected in pattern-triggered immunity (PTI) and the MIN7 gene displayed bacterial dysbiosis of the leaf endophytic microbiome (Chen et al., 2020). Coupled with this dysbiosis is overall poor health of the plant. This finding emphasizes the significance of a balanced microbiome for plant health, and the central role plant immunity can play in maintaining that balanced microbiome.

Post-harvest losses of fruits and vegetables represent an enormous drain on the global food supply. The Food and Agriculture Organization of the United Nations states that over one-third of food produced for human consumption is lost or wasted between production and consumption (Nations, 2022). Considering the findings above, it seems likely that post-harvest losses of many fruits and vegetables could be the result of a breakdown in the relationship between plant immune defenses, the microbiome, and plant health, comparable to what is observed in the immune-compromised *A. thaliana* mutant.

This dissertation aims to address the impact of microbiota and host immune response on postharvest apple fruit health. Chapter 2 examines the epiphytic and the endophytic microbiota composition over the full developmental time course of apple fruit. The aim of this study is to investigate if microbiota, especially endophytes, are vertically transferred from flower to ripe apple fruit. Chapter 3 seeks to investigate the temporal dynamics of the fruit microbiota and fruit immune response during post-harvest storage of apple fruits at room-temperature and to relate these dynamics to fruit decay. This aim is to characterize the relationship between fruit immunity and the fruit microbiota and the influence of that relationship on fruit health during storage. Finally, Chapter 4 tests the influence of fruit immune response(s) induced by ASM on the

microbial community composition, especially endophytes that may have a profound impact on postharvest fruit rot. The goal of this study is to develop a better understanding of host immune-microbiome interactions, as well as their impact on post-harvest fruit diseases. Finding from these studies will enhance our knowledge of the mechanisms underlying i) microbial assembly, ii) host immunity, iii) recruitment of beneficial microbes, and iv) microbial activity. Advancement on this knowledge can be utilized to prolong shelf life in apples and potentially other food crops.

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# CHAPTER 2

## MICROBIAL COMMUNITY SUCCESSION AND DYNAMICS DURING THE SEASON-LONG DEVELOPMENT OF APPLE FRUIT

## INTRODUCTION

The plant microbiota remains in constant contact with plants and represents a key determinant of plant health and productivity (Berendsen et al., 2012). These microorganisms exist as epiphytes on the surface of plant tissue and as endophytes within plants (Turner et al., 2013; Wassermann et al., 2019). The fruit tissue of plants, known as the carposphere, is a rich habitat for microbial life and harbors a distinct microbial community composition and diversity across tissue types such as the skin (epiphytes) and the pulp (endophytes) (Wisniewski and Droby, 2019; Abdelfattah et al., 2020; Chapter 2). However, very little is known about the fruit microbiome and its role in disease resistance and fruit quality during fruit development and storage. The identification of a stable microbiota associated with tree fruit, and how that microbiota is established, will provide fundamental insights regarding engineering of a consortium of microbes that could thrive under various environmental stressors. Such synthetic communities (SynComs) could contain key taxa that render a desired microbiome function, and the community could be applied to plant tissues to promote crop productivity and broad stress resilience.

Apple represents the one of the most consumed fruits worldwide and provides an excellent platform for the study of fruit microbiome assembly. During the development period from anthesis to full fruit maturation, covering approximately 145 days, apple fruit undergoes several physiological and biochemical changes including an increase in starch at early development, a decline in starch during fruit maturation, and an increase in sugars at full maturation and ripening stage (Janssen et al., 2008). The developing flowers and fruits are also exposed to various environmental stressors such as changes in temperature, humidity, radiation, precipitation and management practices. The microbiota in the developing fruit must also adapt

to these constant changes in environment and fruit physiology in order to persist. During flowering and fruit development, phytopathogens colonize the tissue surface and cause disease of both pre- and post-harvest fruits (Barbé et al., 2022). However, we have very little knowledge of the antagonistic activity of the resident microbes toward these phytopathogens. Understanding the dynamics of microbiota composition in a developing fruit, and identification of stable and persistent microbes, can provide a mechanistic understanding of the interactions between the resident microbiota and the phytopathogen. This knowledge could open a new avenue to approach disease control from a more holistic perspective.

Studies of apple have shown a clear successional pattern of microbes across different stages of flower development (Shade et al., 2013) and fruit development (Zhimo et al., 2022). However, these studies did not distinguish endophyte and epiphyte composition, either focusing specifically on epiphytes during fruit development or pooling epiphytic and endophytic tissues prior to the analysis during flower development. Furthermore, neither study spanned the full developmental progression from flower bud to fully mature fruit. This limits our understanding of microbial succession patterns throughout the full season and the source of microbial communities of the harvested apple fruit. Here, we examined epiphytic and endophytic microbiota composition over the full developmental time course of apple fruit. We show that both epiphytic and endophytic communities change from full flower bloom to fruit maturation. Further, our data show that endophytic communities in ripe fruit are more similar to the community at full bloom, which suggests that the endophytic community may be derived from the flower. Conversely, epiphytic communities are more dynamic over time, which could indicate that surface microbes are more strongly influenced by environmental factors. Together our data suggest that the host plant potentially has some level of control on the endophytes

whereas the epiphytes are considerably more impacted by the environmental factors.

## RESULTS

### Microbial communities associated with apple skin and pulp are distinct

To track the bacterial and fungal community succession during fruit development, we performed 16S rRNA gene and ITS region sequencing analysis, respectively. To evaluate the effects of tissue types on microbial succession, data analyses were conducted on skin and pulp samples, separately. Tissue types of both the flower and the fruit showed a significant effect on both bacterial and fungal community composition (PERMANOVA: Bacteria: $R^2 = 0.17$, $p = 0.001$ and Fungi: $R^2 = 0.10$, $p = 0.001$). A Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity distance illustrated the distinct microbial community composition between skin (epiphytes) and pulp (endophytes) when all sampling time points are considered together (**Figure 2.1)**. A significant dissimilarity distance between the tissue types was observed in every development stage.

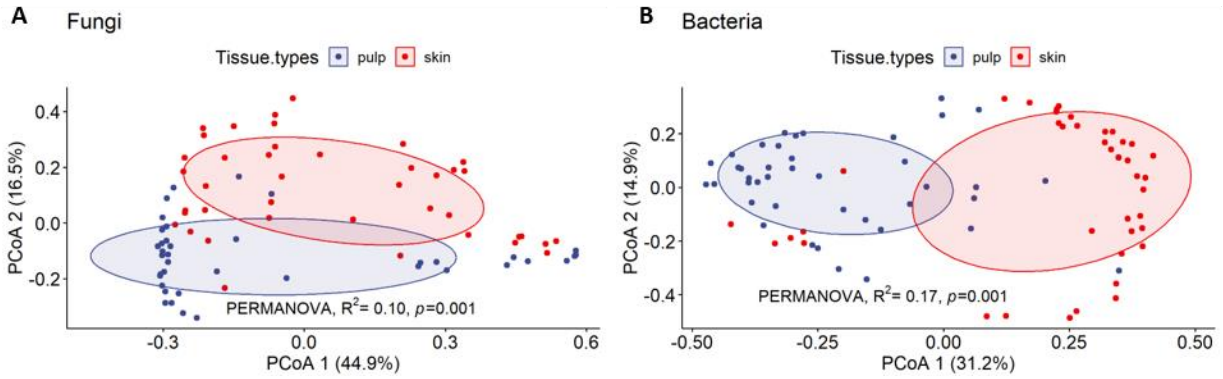


**Figure 2.1** Effects of tissue types on microbial community composition from flower to ripe fruit. **A**, and **B**, principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metrics between pulp and skin in fungal and bacterial community, respectively.

### Dynamic changes in the microbial community during apple fruit development

The α-diversity of both the fungal and bacterial communities in developing apple fruit was highly dependent on the developmental stage. Broadly speaking, the Shannon index of

bacteria and fungi was highest at petal fall both tissues then declined at either the pf or fl1 stage and progressively increased across subsequent time points representing fruit development and maturation, ultimately returning to the level of α-diversity seen at the initial time point. This pattern was most striking for the bacterial composition on the skin (**Figure 2.2 D**) and was quite muted among the bacterial composition in the pulp (**Figure 2.2 C**). It was also notable that the abrupt decline in Shannon index emerged between the fb and pf stages for the bacterial species, while it occurred later, between the pf and fl1 stages, for the fungal species (**Figure 2.2 A & 2B**).

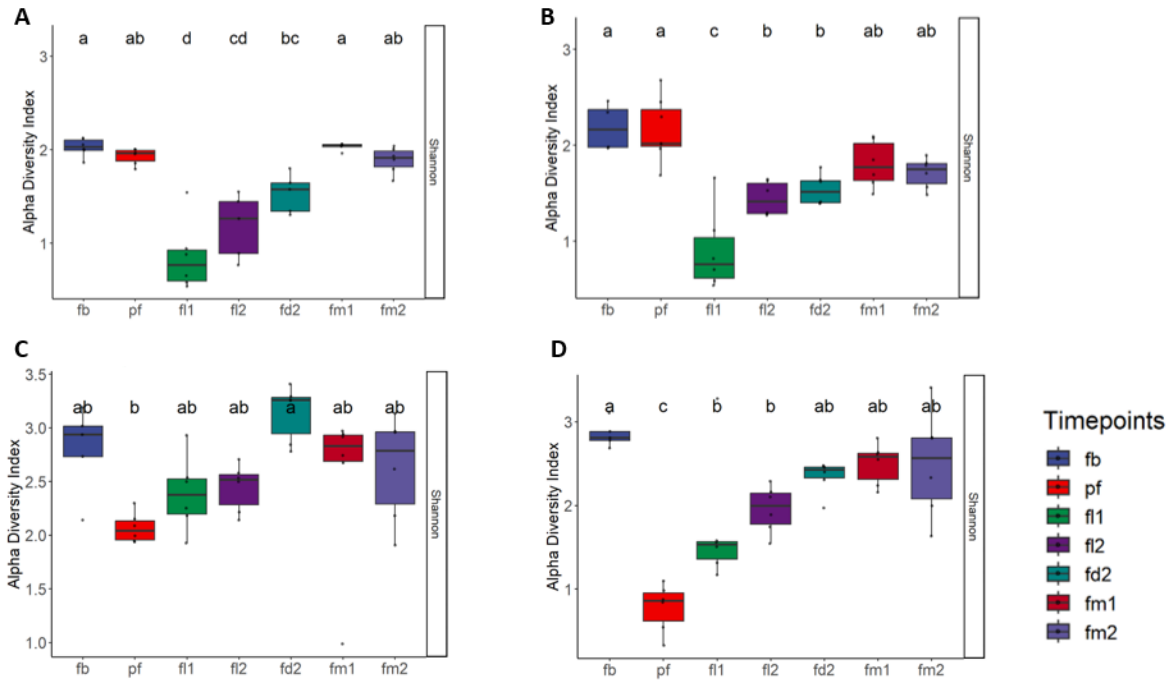


**Figure 2.2.** Box plots showing α-diversity comparison between timepoints based on the Shannon Index. **A,** fungal diversity in pulp, **B,** fungal diversity in skin, **C,** bacterial diversity in pulp, and **D,** bacterial diversity in skin. The different letters above each bar indicate statistically significant differences at $P_{adj} < 0.05$ as calculated by Tukey's HSD test.

Fruit development stages also showed significant effect on β-diversity **(Figure 2.3)**. A Principal Coordinate Analysis (PCoA) illustrated a significant change in fungal community composition in both pulp and skin during fruit development using the Bray-Curtis dissimilarity distance (PERMANOVA; Pulp: $R^2 = 0.72$, $p = 0.001$; Skin: $R^2 = 0.64$, $p = 0.001$) (**Figure 2.3 A & B**). In

pulp samples, the first two sampling stages, (fb) and (pf), and the last two sampling stages, (fm1) and (fm2), were clustered together, indicating that similar fungal composition occurs in the pulp at the early and late time points of our study. In contrast, (fl1) and (fl2) were clustered separately from flower and mature fruit samples. Fruit development 2 (fd2) was isolated from either of the other two clusters **(Figure 2.3 A)**. In skin samples, the two flowering stages showed dissimilar fungal composition. They were also distinct from fm1 and fm2, contrasting with what was seen in the pulp. However, fl1 and fl2 were clustered together as was observed in pulp samples. As expected, the last three stages, fd2, fm1 and fm2 were clustered together and separated from all other timepoints **(Figure 2.3 B)**.

A similar result was observed in bacteria with a significant change in β-diversity in both tissues during apple fruit development (PERMANOVA: Pulp: $R^2 = 0.47$, $p = 0.001$; Skin: $R^2$ =

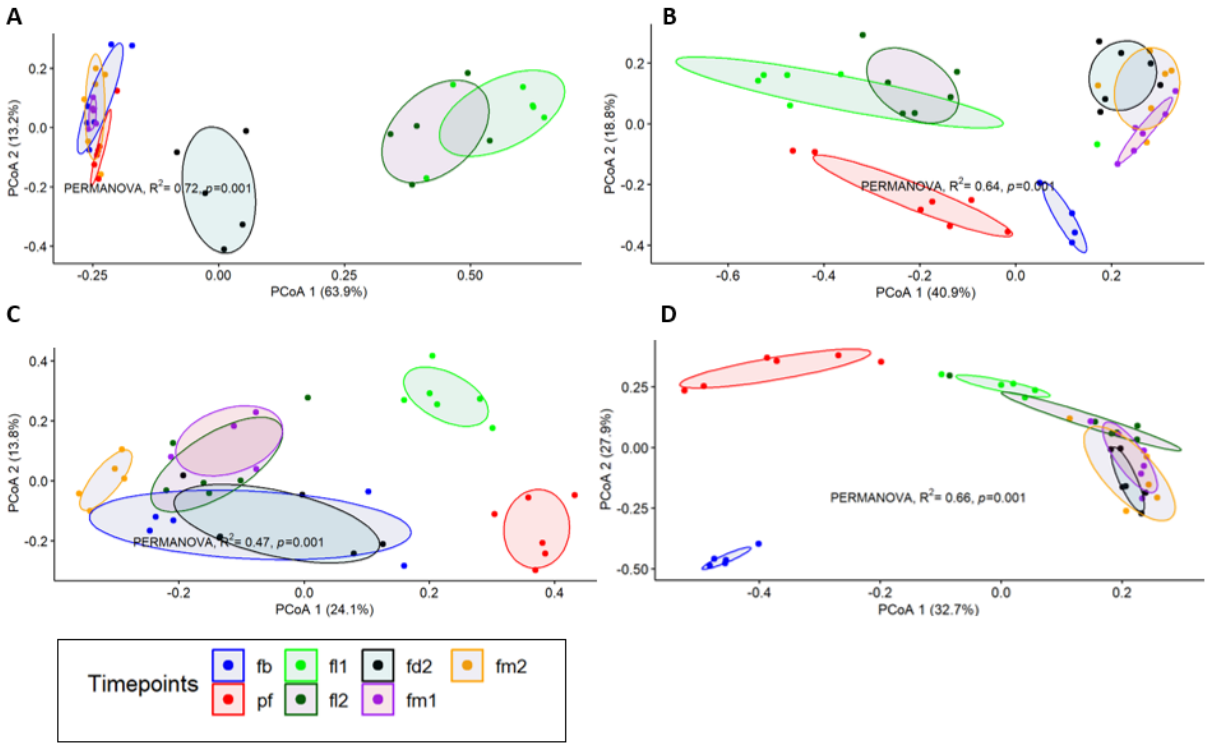


**Figure 2.3.** Principal coordinate analysis (PCoA) plot based on Bray-Curtis dissimilarity metrics comparing fungal and bacterial community across timepoints. **A,** fungal community in pulp, **B,** fungal community in skin, **C,** bacterial community in pulp, and **D,** bacterial community in skin.

0.66, p = 0.001) (**Figure 2.3 C & D**). In pulp samples, PCoA illustrated that fb clusters closely to the last four development stages (fl2, fd2, fm1 and fm2). pf and fl1 showed distinct composition according to Bray-Curtis dissimilarity matrix **(Figure 2.3 C)**. The shift in bacterial community composition in skin was similar to the shift observed in fungal community composition in skin, however fl1 and fl2 were clustered more closely to the last three development stages as illustrated by PCoA **(Figure 2.3 D)**.

**Temporal dynamic change in relative abundance of microbial taxa during apple fruit development**

The relative abundance of fungal and bacterial genera detected across all samples is shown in **Figure 2.4**. There was a shift in relative abundance of microbiota composition during apple fruit development in both tissues. In pulp samples, the relative abundance of fungal genera observed in the first two flowering stages (fb and pf) and the last two fruit maturation stages (fm1 and fm2) were strikingly similar (**Figure 2.4 A**). After the flowering stage, there was a shift in relative abundance of fungal genera in the two fruitlet stages (fl1 and fl2) and the fruit development stage (fd2) but reverted at fruit maturation stage. *Alternaria*, *Sporobolomyces* and *Vishniacozyma* were detected at high relative abundance at the flowering and maturation stages. There was a substantial increase in *Aureobasidium* and a decrease in *Alternaria, Sporobolomyces* and *Vishniacozyma* at fruitlet stage. This is consistent with the results of the fungal β-diversity in pulp samples shown above, where the flowering stages and the maturation stages were clustered together and separated from the fruitlet stages (**Figure 2.3 A**). In contrast, in the skin samples, fb and fm2 showed a dissimilar fungal community composition. In skin, the last three development stages (fd2, fm1 and fm2) showed more similar composition and distinct from both flowering stages. This is consistent with β-diversity analysis in skin samples where the last three

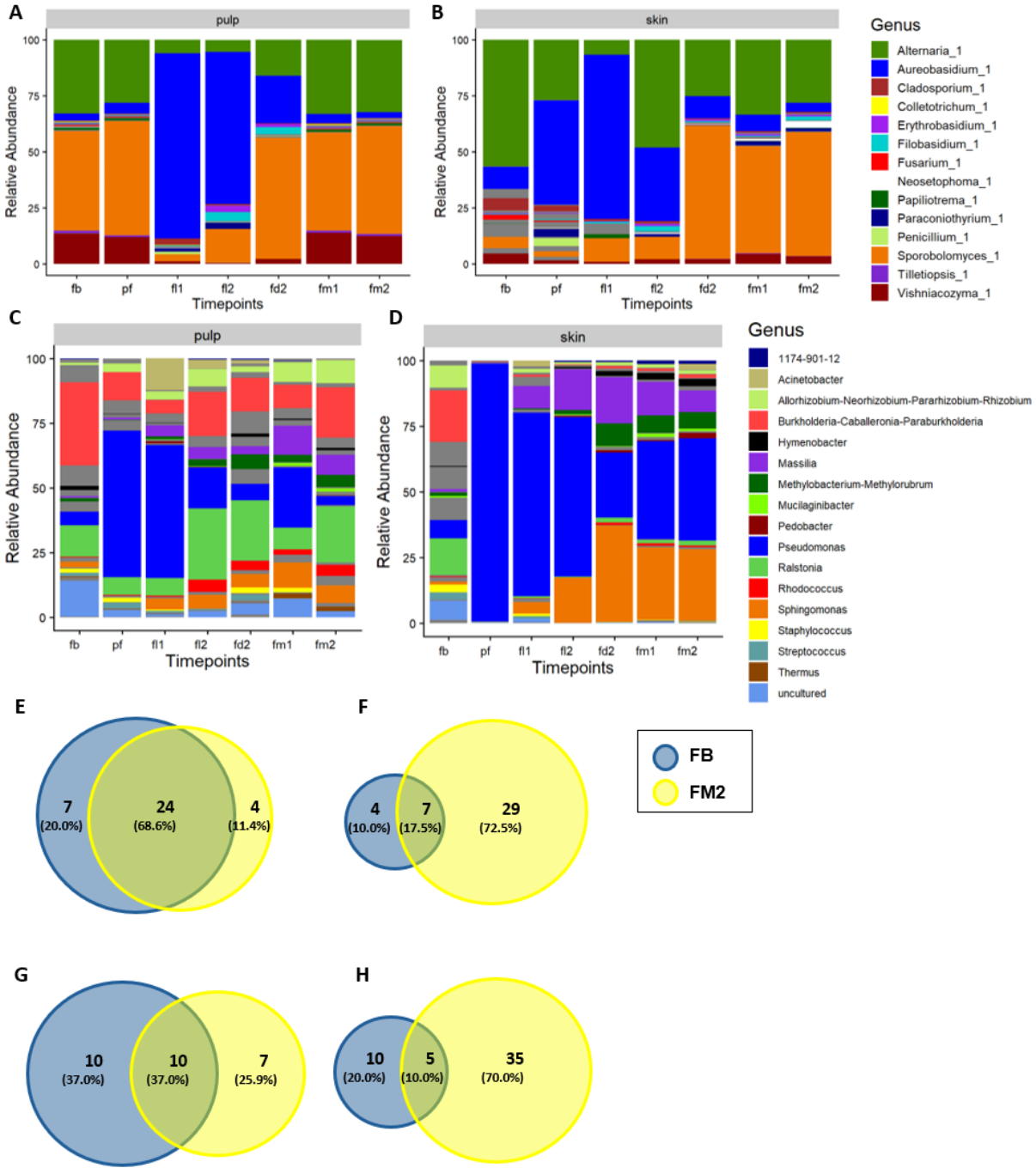


**Figure 2.4.** Bar plots representing mean relative abundance of the most prevalent genera present across timepoints. **A,** fungal genera in pulp. **B,** fungal genera in skin. **C,** bacterial genera in pulp and **D,** bacterial genera in skin. Each bar shows the average composition from all the replicates within a timepoint. Venn diagram showing numbers of shared OTUs between full bloom (fb) and fruit maturation 2 (fm2). **E,** fungal community in pulp, **F,** fungal community in skin, **G,** bacterial community in pulp, **H,** bacterial community in skin.

development stages clustered together and were separated from both flowering stages as illustrated by PCoA **(Figure 2.4 B).** Among fungi, in the last three stages, there was a high relative abundance of *Sporobolomyces* followed by *Alternaria* and then *Aureobasidium*, and *Vishniacozyma*. There was an increase in relative abundance of *Neosetophoma* at the last maturation stage (fm2). At full bloom, there was a low relative abundance of *Sporobolomyces* and high relative abundance of *Alternaria.* The abundances of *Sporobolomyces* and *Alternaria* flipped at fruit maturation stage where *Sporobolomyces* became most dominant. *Cladosporium* was also observed in high relative abundance at full bloom, but its relative abundance decreased over time. There was an increase in *Aureobasidium* during fruitlet stages in skin, this increase in *Aureobasidium* was also observed in pulp. There was also an increase in *Aureobasidium* in pf **(Figure 2.4 B)**.

In bacteria, fb and fm2 also showed a similar pattern of abundance among the different genera in pulp, although the resemblance was not identical as was seen in fungi. There was a shift in bacterial composition at pf and fl1 stages but restored more similar to fb at fl2 stage **(Figure 2.4 C)**. This supports the result observed for β-diversity in bacteria in pulp samples, which shows that fb and fm2 cluster close together and was distinct from pf and fl1 **(Figure 2.3 C)**. *Burkholderia* and *Ralstonia* were detected at high relative abundance followed by *Pseudomonas* in fb and fm2. These taxa were also seen at high abundance in fl2, fd2 and fm1. *Sphingomonas, Allorhizophium, Rhodococcus, Methylobacterium, Hymenobacter* and *Massilia* were also observed at both stages with a slightly higher relative abundance at fm2. Most of these OTUs were present in every development stage but with differing relative abundances. *Actinobacter* was observed only at fruitlet stages and at fruit development stage in substantial abundance. There was also an increase in *Pseudomonas* in petal fall and fruitlet 1 stages. In skin

samples, the shift in relative abundance was consistent to the pattern observed in fungi. This again supports our β-diversity analysis of skin samples as illustrated by PCoA **(Figure 2.3 D).** In skin samples, *Pseudomonas* and *Sphingomonas* were observed at high relative abundance in fruit development and fruit maturation stages followed by *Massilia, Methylobacterium, Ralstonia, Hymenobacter Allorhizophium and Burkholderia*. In contrast, OTU belonging to *Pseudomonas* and *Sphingomonas* were observed at lower relative abundance during fb and instead *Allorhizophium*, *Burkholderia* and *Ralstonia* were the most dominant genera found at this stage. *Massilia, Methylobacterium,* and *Hymenobacter,* which were also observed at substantial abundance in fruit maturations stages, showed low relative abundances at the fb stage. OTU belonging to genera 1174-901-12 and *Pedobacter* were also observed during the later development stages but not at both flowering and fl1 stages **(Figure 2.4 D)**. At petal fall, OTU belonging to *Pseudomonas* dominated in the bacterial community which explains the result that was illustrated in **Figure 2.2 D** where there was a decrease in α-diversity at petal fall.

**More endophytic OTU is shared between full bloom and ripe fruit than epiphytes**

To further explore the fungal and bacterial taxa shared between full bloom and fruit maturation 2 (ready-to-harvest), Venn diagram analysis was performed. A considerable overlap of OTUs was observed between fb and fm2 in pulp but not in skin samples. In fungi, 69% of the OTUs were shared in the pulp samples whereas only 17.5 % were shared in the skin samples **(Figure 2.4 E & F)**. The list of the OTUs shared between fb and fm2 in pulp and skin is shown in the **Supplementary Figure S1A**. In the skin samples, fm2 shared 26.6% of OTUs with fl2 and 20.4% with fd2, separately **(Figure 2.5 B)**. Interestingly, in the pulp samples fm2 did not share any OTU with fl2 and fd2 other than the total shared OTUs, but did share 17 OTUs with fb **(Figure 2.5 A)**.

Similarly, in bacteria the number of OTUs shared between fb and fm2 in pulp is more than in skin. 37.0% of bacterial OTUs are shared in the pulp samples and only 10% of OTUs are shared between fb and fm2 in skin samples **(Figure 2.4 G & H)**. Consistent with fungi, in skin samples, fm2 shared more bacterial OTUs with fl2 with 34.6% and 39% with fd2 **(Figure 2.5 D)**. Apart from the total OTUs shared at the selected sampling points in the pulp samples, fm2 shared 26.1 % of the OTUs with fl2 and fd2 and 4.8% with just fd2 separately **(Figure 2.5 C)**. The list of the bacterial OTUs shared between fb and fm2 in pulp and skin is shown in **Supplementary Figure S1B.**

## DISCUSSION

The goal of this study was to understand the assembly of the microbiota community composition in the apple carposphere. Our results indicate that apple fruit microbiota composition is driven by the host developmental stage, compartment and tissue type. The distinct community compositions in the tissue types (e.g., flower vs. fruit) occurred as early as the

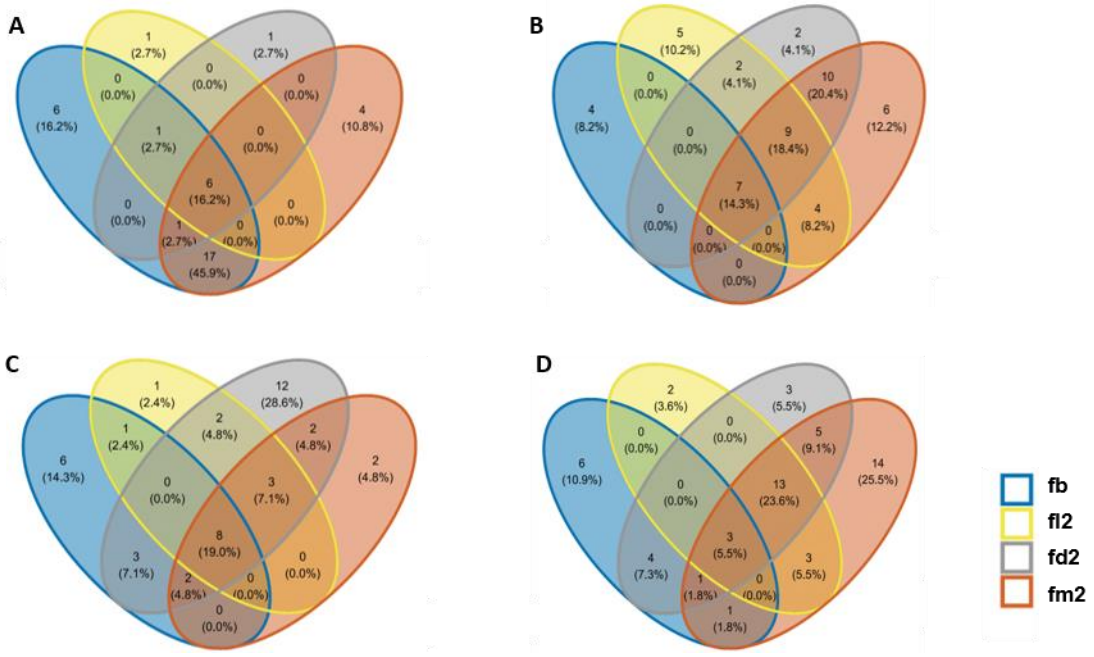


**Figure 5.** Venn diagram showing numbers of shared OTUs between full bloom (fb), fruitlet 2 (fl2), fruit development 2 (fd2), and fruit maturation 2 (fm2). **A,** fungal community in pulp, **B,** fungal community skin, **C,** bacterial community in pulp, **D,** bacterial community in skin.

flowering stage even before the hypanthium had developed into a fruitlet. Additionally, we found that the epiphytic (from skin) and the endophytic (from pulp) compartment communities differed in their $\beta$-diversity responses to changes during fruit development.

The change in the epiphytic community was more dramatic compared to the endophytic community, suggesting the involvement of different microbial assembly and maintenance mechanisms between these two types of communities. Future research should examine if such differences reflect exposure of epiphytic and endophytic communities to different aspects of endogenous fruit physiology or external factors. In our study, we observed that the fungal endophytes residing in the pulp tissue showed similar composition between late fruit maturation stages and the flowering stages as illustrated in $\beta$-diversity analysis. About two-thirds or more of the OTUs found at the flowering stages were shared with fruit maturation stages as shown in the Venn diagram, indicating that a substantial fraction of the endophytic microbial community is transferred from flower to ripe fruits. Amongst these OTUs that were shared between these stages are *Alternaria, Aureobasidium, Cladosporium, Papiliotrema, Filobasidium, Sporobolomyces, Tilletiopsis,* and *Vishniacozyma*. Interestingly, even the relative abundances of these OTUs were nearly identical between the flowering and the fruit maturation stages, which suggests that the endophytic microbial community is likely transferred from flower to ripe fruits.

We noted that flowers and fruits at the maturation stages were both associated with a high fungal diversity. This may be attributed to high levels of sugars and other nutrient content in mature flowers and ripen fruit (Janssen et al., 2008; Aleklett et al., 2014; Palmer, 2014). Flowers exude various types of nutrient-rich secretions including nectar, stigmatic exudate, and pollen exudate that could attract a diverse range of insects, both pollinators and non-pollinators, thereby transferring microbes to the flower (Bill et al., 2021). The high level of sugars, amino acids,

polysaccharides, and glycoproteins in these secretions may also be excellent sources of nutrients for many different types of microorganisms, thus enhancing microbial richness (Aleklett et al., 2014; Steven et al., 2018; Cui et al., 2021).

Notably, we observed a shift in microbiota composition during the fruitlet stages and concurrent to that we also observed a significant dip in α-diversity at fruitlet stages which then increased gradually over time as the fruit matured and ripened. A decline in α-diversity at the early stages of fruit development (fruitlet stages) could be the result of low sugar content which increases gradually as the fruit undergoes a series of biochemical changes that convert starches into more available and attractive compounds, such as sugars (Janssen et al., 2008; Aleklett et al., 2014). This increase in sugar content is perhaps the reason why we saw an increase in diversity in mature and ripe fruit. Our result aligned with those of Bill, *et al.* 2021, who documented a decrease in α-diversity at the fruitlet stage during mango fruit development (Bill et al., 2021). In our case, the decrease in diversity was associated with an increase in relative abundance of *Aureobasidium* at the expense of *Alternaria*, *Sporobolomyces*, *Vishniacozyma* and other fungal genera with low relative abundances. Interestingly, *Aureobasidium pollulans* is ubiquitous in nature and has exceptional tolerance for a broad range of ecological conditions and, as such, it is considered a polyextremotolerant organism (Gostinčar et al., 2010; Gostinčar et al., 2014).

Another potential reason for the fungal community shift at fruitlet could be the impact of the fungicide, such as Roper and Captan, application, which was applied before our first sample collection as part of a normal management practices for apple at the MSU farm. Studies have shown that fungicide application decreases fungal diversity in leaves and flowers (Schaeffer et al., 2017; Noel et al., 2022). Intriguingly, *Aureobasidium pollulans* shows tolerance to several commonly used fungicides (Magoye et al., 2020). In contrast, most fungal endophytes appear to

be intolerant to fungicides with few exceptions such as *Aureobasidium*, which has the ability to thrive in many environmental conditions.

Among the bacterial endophytic community, the fb stage clustered close to the last four development stages as illustrated by PCoA for β-diversity analysis. This result was in parallel with the relative abundance taxa where a similar composition and abundance was observed between fb and the last four stages. However, we observed a change in microbial community structure at pf and fl1 stage. Concurrently, these changes in community shift relate to a decrease in α-diversity similar to what we observed in fungi. A decrease in diversity at the later stage of bloom was also reported by (Cui et al., 2021). As mentioned above, possible reasons for this dynamic shift in bacterial diversity may be due to endogenous fruit physiology changes and/or external antibiotic applications before the first sample collection, which included kasumin for the control *Erwinia amylovara*, a pathogen that causes fire blight in apples (McGhee and Sundin, 2011).

The shift in bacterial diversity was associated with an increase in relative abundance of the genus *Pseudomonas*. It is possible that *Pseudomonas* outcompetes transient taxa that are less competitive in the community. This seems compelling because members of the genus *Pseudomonas are well studied for their metabolic versatility and their capability to adapt, compete and survive in diverse and harsh environments (Moradali et al., 2017; Craig et al., 2021). Pseudomonas* spp. are ubiquitous microorganisms that exhibit intrinsic and acquired resistance to many antimicrobial agents (Silverio et al., 2022). Strains of *Pseudomonas aeruginosa* are also known to utilize their high levels of intrinsic and acquired resistance mechanisms to counter most antibiotics (Pang et al., 2019). However, despite the change in diversity, many prevalent taxa such as *Allorhizobium, Burkholderia, Massillia, Pseudomonas,*

*Ralstonia, Rhodoccocus,* and *Sphingomonas* persisted, and these members dominated the communities at most development stage. Therefore, we attribute the microbial successional pattern to prevalent and persistent taxa that are adaptable to changing environments (e.g., *Allorhizobium, Burkholderia, Massillia, Pseudomonas, Ralstonia, Rhodoccocus,* and *Sphingomonas*), and the background variability to rare and transient taxa. Our studies show that antibiotics have a short-term impact on some non-target prevalent taxa, but the levels of these taxa are restored by fd2, indicating a remarkable level of resilience in the microbiota. This suggests a considerable level of control over the microbiome by the host plant potentially mediated, at least in part, by host PAMP-triggered immunity (Chapter 2).

In contrast with endophytes, for both bacterial and fungal epiphytic communities, less than one-third of the OTUs (17.5% for fungi and 10% for bacteria) were shared between the fb and fm2 stages **(Figure 2.4 F & H).** Correspondingly, a dissimilar composition was observed between full bloom and fruit maturation stages (**Figure 2.3 A & B**). Our results show that there is less microbial epiphyte succession from flower to ripe fruit relative to endophytes. We observed that the epiphytes were more dynamic especially in the early fruit development stages, suggesting a transient imbalance in the microbiota that might have been directly related to external changes in temperature, light, UV radiation, or nutrient availability. Another noteworthy observation was that the abundances of a specific suite of OTUs become relatively constant as the fruit matures, indicating that the epiphytic community reaches a stable level. In parallel to what was observed in pulp samples, there was a significant decrease in α-diversity at the fl1 stage in fungi and pf stage in bacteria but showed a gradual increase in α-diversity in both the communities. This could be due to the same reasons described above. Similar to pulp samples, the shift in community dynamics was observed as an increase in relative abundance in

*Aureobasidium* in fungi and *Pseudomonas* in bacteria. However, unlike the endophytes, the epiphytic community does not revert back but continues to show a gradual shift in composition until reaching the late maturation stage. In fungi, OTUs such as *Alternaria, Aureobasidium*, *Cladosporium, Sporobolomyces*, and *Vishniacozyma* were seen across all developmental stages but with differing relative abundance. In bacteria, *Pseudomonas, Sphingomonas, Mythylobacterium,* and *Massilia* are seen in high abundance in ripe fruit in contrast to full bloom. The inability of the epiphytes to re-establish the microbial composition after the fl1 perturbation, as was seen with the endophytes, indicates that the host may have a less influence on the epiphytic microbiome than the endophytic microbiome. This would be consistent with the fact that the epiphytes are in contact with the external environment and would presumably be impacted more by fluctuations in environmental factors such as temperature, and humidity.

Understanding the succession of a microbial consortia and identification of a stable microbiota within a particular host can provide fundamental knowledge regarding the formation of a consortium of microbes that can be applied to manipulate the microbiome in pre- or post-harvest fruits for resilience against biotic and abiotic stress. Our microbiota succession study has allowed an in-depth look into the dynamics of the microbiota composition at flower-to-fruit transition and during apple fruit development.

## MATERIALS AND METHODS

### Sampling Procedures

Using the 'Gala' apple variety, a total of seven sampling time points from full bloom to ripened fruit were collected from the Michigan State University (MSU) Plant Pathology Farm over the 2022 growing season. Each time point was designed to target key morphological and biochemical transitions during fruit development based on a previous study (Janssen et al.,

2008). The first sample was collected on 13 May at full bloom stage (fb), second on 19 May at petal fall stage (pf), third on 1 June at first fruitlet stage (fl1), fourth on 30 June during fruitlet development (fl2), fifth on 12 August when the fruits were more developed (fruit development - fd2), sixth on 26 August when the fruits were fully developed but not ready for harvest (fm1) and finally on 9 September when the fruits were ready for harvest (fm2).

Six replicate trees were selected at random within an orchard block. Ten flowers or five fruits were sampled from around the circumference of each of the replicate trees at each time points and pooled to make one sample in a sterile whirl-pak bag. The bags were then transported to the laboratory on ice. They were immediately stored at -80°C until DNA extraction. Before DNA extraction, petals were removed from the flowers

To extract endophytic DNA from flowers, the epiphytes were removed from the surface by sonicating the flowers with phosphate buffered saline (PBS), in a bath sonicator for seven minutes. The flowers were removed from the buffer, and the supernatant was reserved then surface-sterilized with 75 % (v/v) ethanol and 10 % (v/v) bleach for 20 secs each and rinsed with sterile water twice. The surface-sterilized samples were then used for endophytic microbial DNA extraction. For DNA extraction from epiphytes, the flower debris from the sonicated PBS buffer (see above) was filtered out and the filtrate was centrifuged to pellet microbial cells. The pellets were used to extract epiphytic microbial DNA.

For the apple fruits, epiphytes were collected with a sterile blade which was used to peel a thin layer of the skin. After removal and collection of the skin tissue a sterile cork-borer was used to collect pulp cores for endophyte samples. Immediately, tissue samples were homogenized in liquid nitrogen. The homogenized samples were stored at -80°C for subsequent DNA extraction.

**DNA Extraction and Sequencing of 16S rRNA gene and ITS genes**

Microbial genomic DNA samples were extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, US) following the manufacturer's recommended protocols. Extracted DNA was used as the template for polymerase chain reaction (PCR) that amplified the bacterial 16S rRNA gene and fungal internal transcribed spacer (ITS) 1 region. The V4 region of the 16S rRNA gene was amplified using the universal 515F/806R primer set (Caporaso et al., 2011), and the ITS1 region was amplified using the ITS1F/ITS2 primer set (Ghannoum et al., 2010; White et al., 2013). Peptide nucleic acid (PNA) clamps (Lundberg et al., 2013) were added to the 16S rRNA gene PCR mix (total of 20 μL) with a concentration of 2.5 μM mitochondrial PNA and 2.5 μM plastid PNA to block amplification of host plastid and mitochondrial DNA.

Sequence processing was performed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) (Bolyen et al., 2019). Primer and adapter sequences were removed using cutadapt, paired reads were joined, samples were denoised with dada2, and chimeric sequences were removed using VSEARCH ((Rognes et al., 2016). Operational taxonomic units (OTUs) were clustered at 97% similarity using QIIME's sklearn classifier and the SILVA database v132 for bacteria (Yilmaz et al., 2014). CONSTAX2 (Liber et al., 2021) was used with the UNITE fungal general release dataset from Nov. 29, 2022 (Abarenkov et al., 2020) to assign fungal taxa with an 80% confidence threshold and recommended settings.

**Data Processing and Analysis**

Microbial community analyses were conducted in the R environment for statistical computing. The OTU table, taxonomy, metadata, and phylogenetic tree were imported into R using the Phyloseq package v.1.24.2 (McMurdie and Holmes, 2013). OTUs assigned to host mitochondria and chloroplasts were discarded. Alpha diversity was estimated using the Shannon

diversity index to determine the evenness based on the presence of rare OTUs (singletons and doubletons) using Phyloseq v.1.24.2 (McMurdie and Holmes, 2013). Statistical significance was calculated by Analysis of Variance (ANOVA). Beta diversity was analyzed to compare the microbiome composition among groups, based on the Bray-Curtis dissimilarity distance matrix. The ordination was calculated by Principal coordinates analysis (PCoA). To compare the microbiome composition between time points, statistical significance was calculated with permutational multivariate analysis of variance (PERMANOVA) using the vegan package v.2.6-4 (Oksanen et al., 2022). To visualize the relative abundances of OTUs/amplicon sequence variants (ASVs), a bar plot was constructed using the ggplot2 package (Wickham, 2016).

Differentially abundant taxa were identified using DESeq2 v.1.38.3 to extract the main effects of time point in each cultivar and tissue type (Love et al., 2014; McMurdie and Holmes, 2014). Taxa with similar trends in abundance over time were grouped using hierarchical and K means clustering. The elbow method was used to identify the optimal number of clusters. Indicator species analysis was performed using indicspecies v.1.7.14 to identify possible unique taxa and compositional signatures at each timepoint (De Cáceres and Legendre, 2009). Venn diagrams were made using the R package ggvenn v.0.1.10 (Yan, 2023). OTUs were considered present in a sample if they had read counts above zero in at least three replicates.

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## **APPENDIX A**: SUPPLEMENTARY FIGURES

**Fungal OTUs Shared between fb and fm2 in pulp**

OTU1 - Alternaria
OTU2 - Aureobasidium
OTU3 - Cladosporium
OTU4 - Cystobasidium
OTU5 - Filobasidium
OTU6 - Fusarium
OTU7- Papiliotrema
OTU8 - Rhodotorula
OTU9 - Sporobolomyces
OTU10 - Sporobolomyces
OTU11 - Symmetrospora
OTU12 - Tilletiopsis
OTU13 - Vishniacozyma
OTU14 - Vishniacozyma
OTU15 - Vishniacozyma
OTU16 - Vishniacozyma
OTU17 - Vishniacozyma
OTU18 - Unknown
OTU19 - Unknown
OTU20 - Unknown
OTU21 - Unknown
OTU22 - Unknown
OTU23 - Unknown
OTU24 - Unknown

**Fungal OTUs Shared between fb and fm2 in Skin**

OTU1 - Alternaria
OTU2 - Aureobasidium
OTU3 - Cladosporium
OTU4 - Sporobolomyces
OTU5 - Vishniacozyma
OTU6 - Vishniacozyma
OTU7 - Unknown

**Bacterial OTUs Shared between fb and fm2 in pulp**

OTU1 - Allorhizobium
OTU2 - Burkholderia
OTU3 - Burkholderia
OTU4 - Massillia
OTU5 - Pseudomonas
OTU6 - Ralstonia
OTU7- Rhodoccocus
OTU8 - Sphingomonas
OTU9 - Sphingomonas
OTU10 - Unknown

**Bacterial OTUs Shared between fb and fm2 in Skin**

OTU1 - Allorhizobium
OTU2 - Burkholderia
OTU3 - Burkholderia
OTU4 - Pseudomonas
OTU5 - Ralstonia

**Figure S2.1. A,** list of fungal genera that are shared between full bloom (fb) and fruit maturation 2 (fm2). **B,** list of bacterial genera that are shared between full bloom (fb) and fruit maturation 2 (fm2).

# CHAPTER 3

## TEMPORAL FRUIT MICROBIOME AND IMMUNITY DYNAMICS IN POST-HARVEST APPLE

## INTRODUCTION

In recent years, the microbiome is viewed as a driver of some of the physiological capabilities of eukaryotic hosts (Cordovez et al., 2019; Berg et al., 2020). In plants, members of the microbiome can live as epiphytes on the surface of the plant tissue and as endophytes within the plant tissue in the rhizosphere (belowground tissue), the phyllosphere (aboveground tissue), and the carposphere (the fruit tissue) (Turner et al., 2013; Wassermann et al., 2019). The plant microbiome is a key determinant of plant health and productivity, and its composition is driven by the surrounding environment, host compartment, host genotype, host immunity and microbe-microbe interactions (Berendsen et al., 2012; Sohrabi et al., 2023). Highly complex, yet stable, microbial communities that are associated with numerous plants have been identified and are distinct across tissue types and geographic locations (Niu et al., 2017). Within the plant microbiome, individual organisms confer fitness advantages that promote essential processes including growth and development, abiotic stress tolerance, nutrient uptake, and pathogen resilience (Berg et al., 2017; Trivedi et al., 2020). Niche adaptation and environmental conditions may play a major role in which microbes have beneficial functions, where only successful colonizers that can compete for the available resources or microbial groups that can adapt to the plant environment will flourish (Trivedi et al., 2020).

The importance of the plant immune system in the establishment and maintenance of the microbiome has been illustrated in recent studies. Pattern-triggered immunity (PTI) is the first line of inducible defense of plants against pathogens (Zipfel, 2009). PTI is triggered via activation of pattern-recognition receptors (PRRs) upon binding of conserved microbial molecular signatures such as flagellin, lipopolysaccharides, chitin and elongation factor TU-derived peptides, referred to collectively as pathogen- (or microbe-) associated molecular

patterns (PAMPs/MAMPs). The perception of MAMPs by PRRs induces complex signaling pathways that result in numerous cellular changes (Zipfel, 2008). Chen et al., 2020 demonstrated that an immune-compromised *Arabidopsis thaliana* quadruple mutant, affected in PTI and MIN7-asscoiated vesicle traffic, displayed bacterial dysbiosis of the leaf endophytic microbiome (Chen et al., 2020). Similarly, studies have shown that Arabidopsis mutants defective in the NADPH oxidase RBOHD (Pfeilmeier et al., 2024) or the phytosulfokine receptor 1 (PSKR1) (Song et al., 2023) display dysbiosis in leaf and root microbiomes, respectively. In all of these cases, dysbiosis was associated with overall poor health of the plant highlighting the significance of a balanced microbiome for plant health and the central role plant immunity can play in maintaining a balanced microbiome.

Post-harvest losses of fruits and vegetables represent an enormous drain on the global food supply and could be in part the result of a breakdown in the relationship between fruit immune responses, the microbiome, and fruit health. Indeed, the Food and Agriculture Organization of the United Nations states that over one-third of food produced for human consumption is lost or wasted between production and consumption (Nations, 2022). Factors contributing to loss or waste include product deterioration, physiological disorders, and excess perishable products due to microbial infection (Kader, 2005). In contrast to leaf and root microbiomes, our knowledge of postharvest fruit immune responses and of the fruit microbiome is very limited. Apple represents the most consumed fruit worldwide and provides an excellent model for the study of fruit microbiomes, fruit immunity and their relationship to post-harvest losses.

We hypothesized that developing and freshly harvested fruits would possess a robust innate immunity response that maintains a healthy microbiota to prevent fruit decay. However,

the resources necessary to support innate immunity in fruit after harvest are likely to be gradually depleted over time. This could lead to a disruption of healthy microbiota, resulting in the proliferation of pathogenic and opportunistic microbes. A proper understanding of how the fruit microbiota is established and maintained could facilitate an untapped approach to enhance fruit health, defense, and productivity. Indeed, recent studies have demonstrated that management practices (Abdelfattah et al., 2016; Wassermann et al., 2019), geographical locations (Abdelfattah et al., 2021), post-harvest treatments (Abdelfattah et al., 2020), genetic background (Liu et al., 2018), and cold storage systems (Bosch et al., 2021) impact microbial community composition and diversity on post-harvest apple fruits. It has also been shown that microbial communities of apple fruit undergo temporal changes during cold storage over the course of months. However, the underlying mechanism(s) remain unclear.

In this study, we sought to investigate the temporal dynamics of the fruit microbiota and fruit defense gene expression during post-harvest storage of apple fruits at room-temperature and to relate these dynamics to fruit decay. By tracking epiphytic and endophytic microbial community composition, as well as host PTI-associated gene expression, we found that the apple fruit microbiota is highly dynamic during post-harvest storage and that expression of PTI-associated genes decreases over time. These patterns were observed in two apple cultivars, 'Gala' and 'Jonathan', despite their differing genetic makeup and microbial community composition. To our knowledge, this study represents the first exploration of the apple fruit microbiota and apple fruit PTI gene expression patterns under the natural progression of fruit spoilage.

## RESULTS

### Fungal Communities Associated with Pulp and Skin Tissues in Gala and Jonathan

To evaluate the effects of apple fruit tissue on fungal diversity, we performed ITS gene amplicon sequencing on the skin and pulp separately. Our result showed that apple fruit tissue type had a significant effect on fungal diversity in both 'Gala' and 'Jonathan', as indicated by the Shannon index (**Figure 3.1 A & B**). The fungal diversity present in the apple skin was significantly higher compared to the pulp tissues.

A Principal Coordinate Analysis (PCoA) also illustrated the distinct fungal community composition in the different tissue types based on Bray-Curtis dissimilarity distance (**Figure 3.1 C & D**). The fungal community composition differed significantly between apple skin and pulp

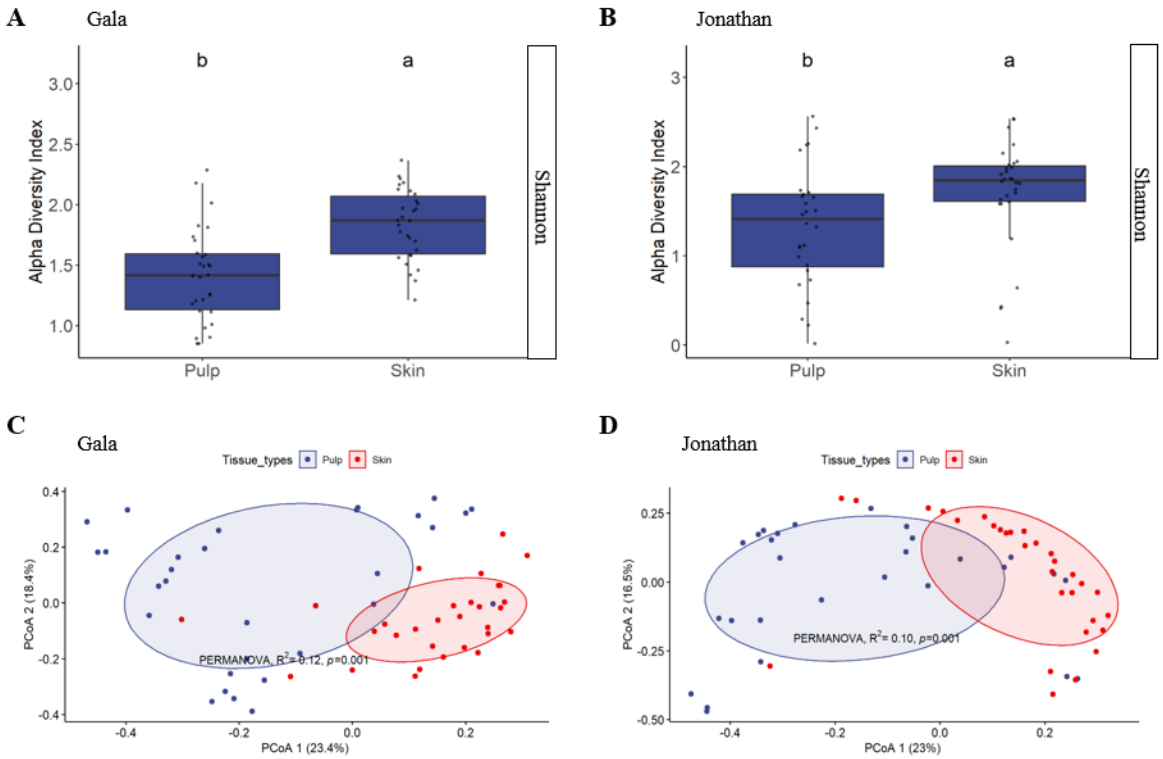


**Figure 3.1. A & B,** box plots showing fungal α-diversity based on the Shannon index between pulp and skin. Superimposed on the box plots are the horizontally jittered data points. The different letters above each bar indicate statistical significance at $P_{adj} < 0.05$ as calculated by Tukey's HSD test. **C & D,** principal coordinates analysis based on Bray-Curtis dissimilarity metrics comparing fungal communities between pulp and skin.

(PERMANOVA: Gala: $R^2 = 0.12$, $p = 0.001$ and Jonathan: $R^2 = 0.10$, $p = 0.001$). Differences in fungal community composition between tissue types were also observed in both cultivars.

**Temporal Dynamics of Fungal Diversity and Community Structures in Post-harvest Apple fruit**

We observed significant alterations in fungal α-diversity over the course of post-harvest storage in 'Jonathan'. In 'Jonathan', apples at the rotten stage in both tissue types harbored the lowest fungal α-diversity according to the Shannon index (**Figure 3.2 B & D**). However, this trend was not observed in 'Gala', where "Gala' skin showed a significant decrease in diversity at day 42, and 'Gala' pulp did not show any change in α-diversity over the course of time according to the Shannon index (**Figure 3.2A & C**).

Additionally, the apple fruit fungal community composition during post-harvest storage showed a significant change in β-diversity over time on both cultivars and in both tissue types

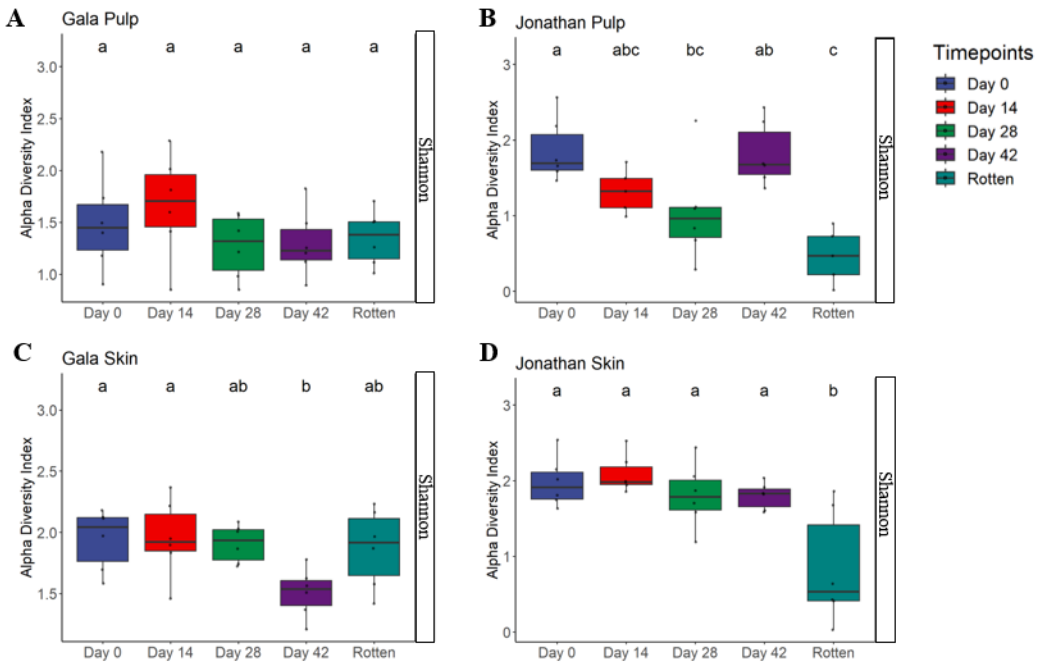


**Figure 3.2.** Box plots showing fungal α-diversity on the Shannon index between time points in Gala pulp (**A**), Jonathan pulp (**B**), Gala skin (**C**), Jonathan skin (**D**). The different letters above each bar indicate statistically significant differences at $P_{adj} < 0.05$ as calculated by Tukey's HSD test.

(PERMANOVA: Gala pulp: $R^2$ = 0.24, p = 0.007, Jonathan pulp: $R^2$ = 0.30, p = 0.002, Gala skin: $R^2$ = 0.22, p = 0.01, and Jonathan skin: $R^2$ =0.31, p = 0.003). A Principal Coordinate Analysis (PCoA) of the fungal community illustrates the distinct fungal community composition in the different tissue types based on Bray-Curtis dissimilarity distance (**Figure 3.3**).

The pairwise comparison between time points showed a significant change in fungal community composition starting at day 42 after harvest in both pulp (**Supplementary Figure S1B**) and skin (**Supplementary Figure S2B**) tissue in 'Jonathan'. Similarly, in 'Gala' pulp a significant difference in fungal community was observed starting at day 42 (**Supplementary Figure S1A**). However, the change in fungal community composition in 'Gala' skin was observed only at rotten stage (**Supplementary Figure S2A**).

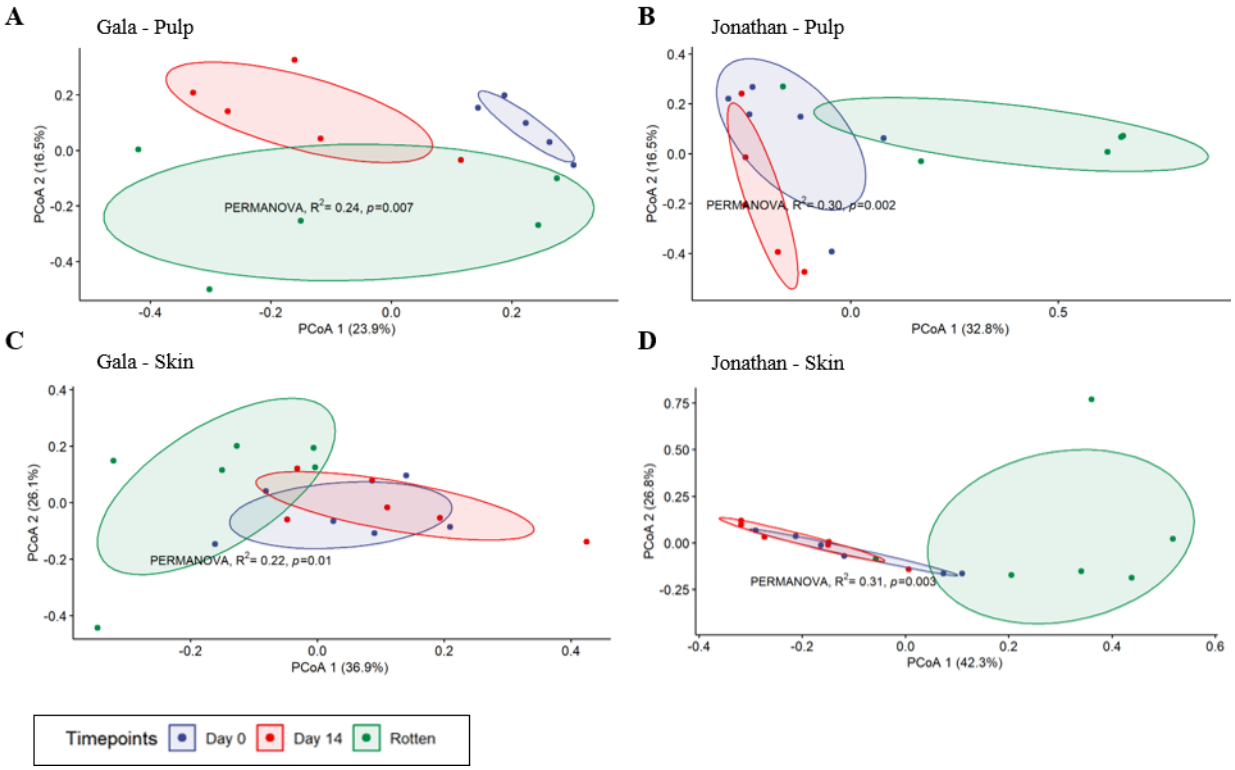


**Figure 3.3.** Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metric comparing the fungal communities between timepoints in Gala pulp (**A**), Jonathan pulp (**B**), Gala skin (**C**), and Jonathan skin (**D**).

**Relative Abundance of Fungal Microbial Taxa Over Time After Harvest**

The relative abundance of fungal genera detected across all samples is shown in **Figure 3.4**. Overall, the abundance of *Alternaria* was seen to gradually increase over the course of post-harvest storage in the skin. The shift in the relative abundance of fungal genera in pulp showed a more rapid change over the experimental time course than in the skin. This observation was very similar in 'Gala' and 'Jonathan' despite being distinct cultivars with Gala having a greatly slower rate of decay. The second most abundant fungal genus in skin was *Sporobolomyces*. The relative abundance of *Sporobolomyces* was also high at several timepoints in Gala pulp and at day 0 in Jonathan pulp. There was a striking decrease in the relative abundance of *Sporobolomyces* that coincided with the increase of *Alternaria* relative abundance over time in Jonathan skin (**Figure 3.4 D**). This was observed also in Gala skin, though the trend is not as strong (**Figure 3.4 B**). In

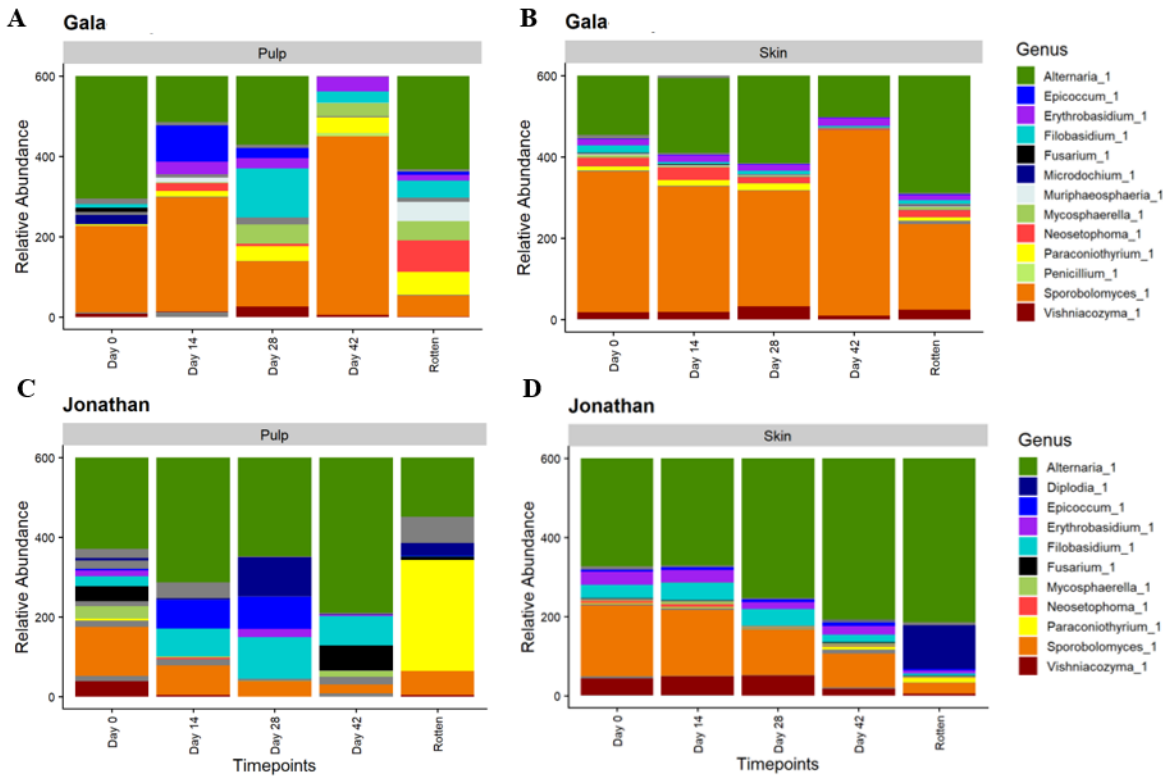


**Figure 3.4.** Bar plots representing mean relative abundance of the most prevalent genera present across timepoints. Gala pulp (**A**), Gala skin (**B**), Jonathan pulp (**C**), and Jonathan skin (**D**). Grey color shade in the bar plot represents fungal taxa with ≤1% abundance.

pulp samples, changes in the relative abundance of *Alternaria* and *Sporobolomyces* were more stochastic, though the relative abundance of *Sporobolomyces* did generally decrease over time (**Figure 3.4 A & B**). *Vishniacozyma* was observed across all samples even though their relative abundance was substantially less than *Alternaria* or *Sporobolomyces*. *Filobasidium* was also observed across all samples with a higher relative abundance in the earlier time points than the later time points in the skin samples. The shift in relative abundance of fungal genera in the skin tissue was slower but steadier and more consistent than in the pulp where the change in abundance and presence and absence of different genera appeared more stochastic.

Hierarchical clustering revealed several taxa with similar trends in abundance over time. Some *Sporobolomyces* OTUs were observed at high relative abundance at earlier timepoints when the apples were healthy in both tissues and cultivar (**Supplementary Figure S3)**. In 'Gala' pulp, *Sporobolomyces patagonicus* and *Filobasidium* decreased in abundance dramatically at the rotten stage while OTUs in the genera *Alternaria*, *Paraconiothyrium*, *Neosetophoma*, and *Mycosphaerella tassiana* all increased in abundance at the rotten stage (**Supplementary Figure S3A**). In 'Jonathan' pulp, *Paraconiothyrium brasiliense, Colletotrichum,* and *Fusarium* increased in abundance at the rotten stage while *Sporobolomyces, Entyloma* and *Filobasidium* all show a relative decrease (**Supplementary Figure S3C**). In 'Jonathan' skin, the genus *Alternaria*, *Colletotrichum*, and *Diplodia* gradually increased in abundance at rotten stage, while *Sporobolomyces patagonicus* and *Filobasidium* gradually decreased in abundance (**Supplementary Figure S3D**). No indicator species were identified for any time point in both 'Gala' and 'Jonathan'.

**Bacterial Communities Associated with Pulp and Skin Tissues in Gala**

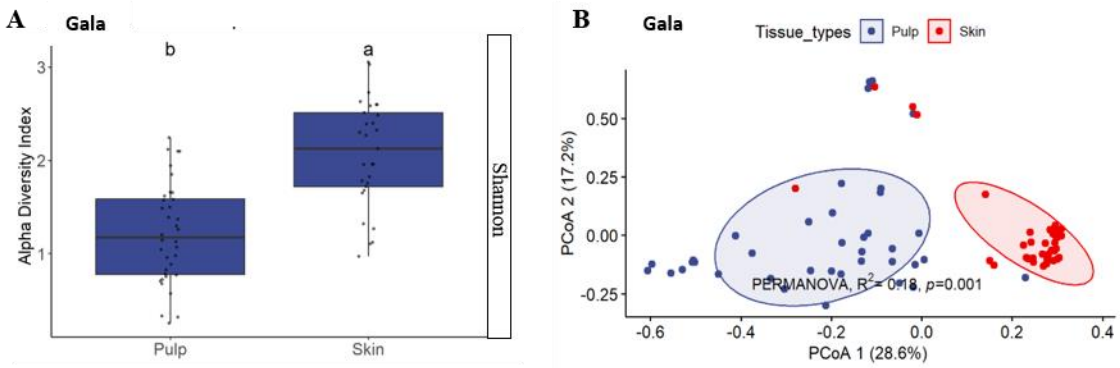


**Figure 3.5.** Bacterial communities associated with pulp and skin tissues in Gala. Box plots showing the bacterial α-diversity based on the Shannon index between pulp and skin tissues (**A**), principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metrics, showing the distance in the bacterial community composition between pulp and skin (**B**).

Apple skin and pulp harbored significantly different levels of bacterial diversity. Like in fungi, apple skin also harbored a significantly higher bacterial alpha diversity than the pulp according to the Shannon index (**Figure 3.5 A**). Additionally, the bacterial community composition differed significantly between apple skin and pulp as analyzed by Bray-Curtis dissimilarity distances (PERMANOVA: Gala: $R^2 = 0.18$, $p = 0.001$). The distinct bacterial community composition between the tissue types in 'Gala' apple is shown in **Figure 3.5 B**.

**Temporal Dynamics of Bacterial Diversity, Community Structures and Relative Abundance in Post-harvest Apple fruit**

The apple fruits at post-harvest did not show any change in bacterial α-diversity over the sampling time points (day 0 to day 49) (**Figure 3.6 A & B**). The bacterial diversity result for 'Gala' skin contrasted with the fungal diversity in that it showed a significant decrease in diversity at day 42 (**Figure 3.2 C**). However, similar to the fungal community composition, there was a dynamic shift in bacterial community composition in both tissues (PERMANOVA: 'Gala' pulp: $R^2 = 0.4101$, $p = 0.001$, 'Gala' skin: $R^2 = 0.3181$, $p = 0.001$). The distinct bacterial community composition over post-harvest storage was illustrated by Principal Coordinate

Analysis (PCoA) based on Bray-Curtis dissimilarity distance (**Figure 6C & 6D**).

There was a shift in relative abundance of bacterial genera over time in both tissues, much like was seen with the fungal community composition. Although the relative abundances

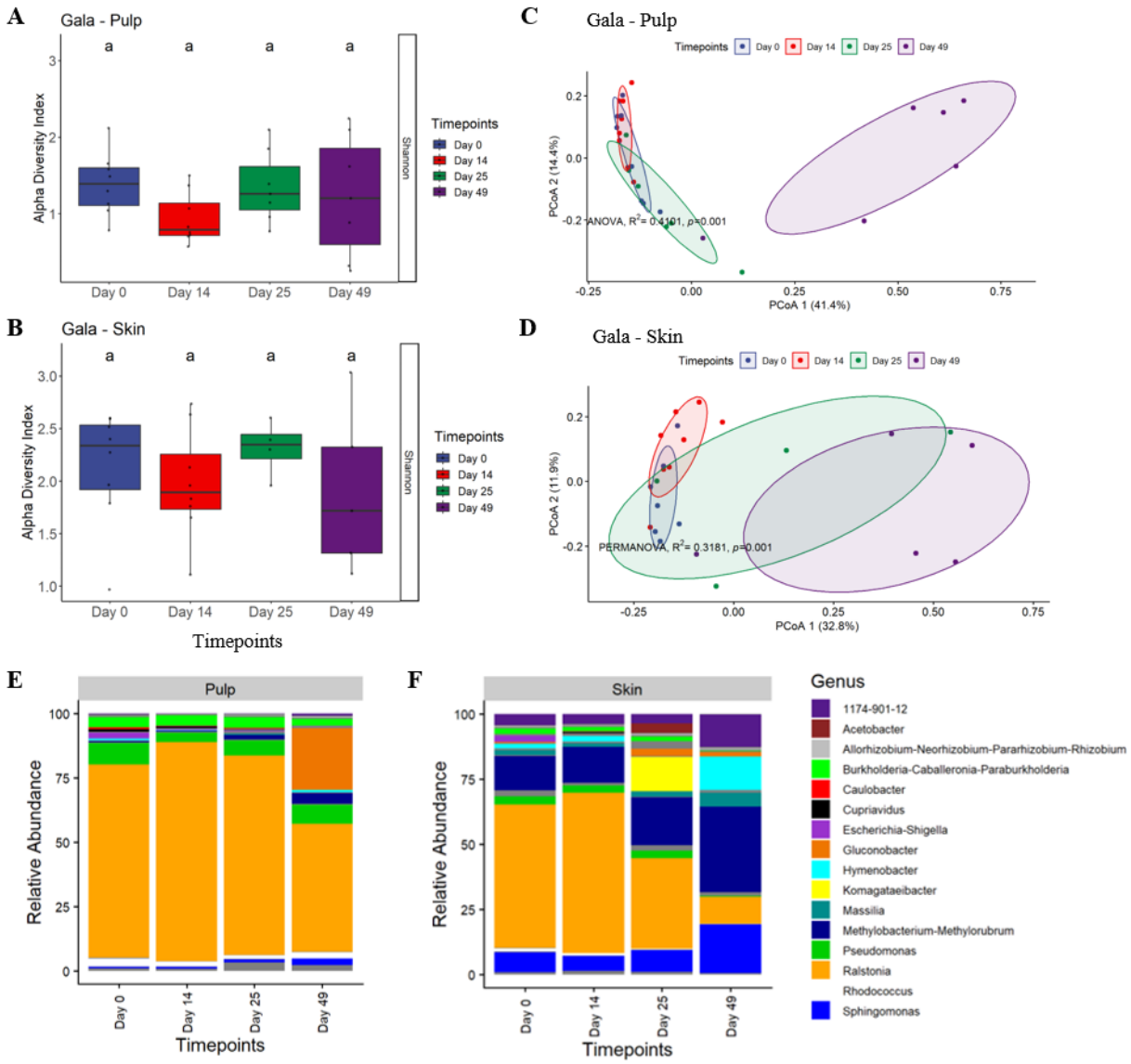


**Figure 3.6.** Effect of post-harvest aging on bacterial diversity, and community structures in Gala fruit. Box plots showing the bacterial α-diversity based on the Shannon index between timepoints in Gala pulp and skin (**A & B**), respectively. The different letters above each bar indicate statistically significant difference at Padj < 0.05 as calculated by Tukey's HSD test. Principal coordiantes analysis (PCoA) based on Bray-Curtis dissimilarity metrics showing the distance in the bacterial communities between timepoints in Gala pulp and skin (**C & D**), respectively. Relative abundance of the most prevalent bacterial genera present across sampling points at post-harvest in Gala pulp and skin (**E & F**), respectively. Grey color shade in the bar plot represents bacterial taxa with ≤1% abundance.

of bacterial genera in the pulp do not change significantly until Day 49, the bacterial community composition changes substantially over time in the skin (**Figure 3.6 E & F**). These findings support the result from the Bray-Curtis dissimilarity distances in skin and pulp, where the community in pulp is most dissimilar at Day 49 (**Figure 3.6 C**) and the skin shows a gradual change across time points (**Figure 3.6 D**). The most abundant genus observed across both tissues was *Ralstonia*. There was also a high abundance of *Methylobacterium* in the skin tissue that showed a gradual increase over time. *Methylobacterium* was also present in the pulp and showed a similar increase in abundance over time in the pulp, albeit with a lower relative abundance. *Sphingomonas* and 1174-901-12 were also observed in moderate abundance in skin with an increase in relative abundance over time. Some other genera that were present across all samples are *Burkholderia*, *Pseudomonas* and *Rhodococcus*. However, some of these showed a decrease in abundance over time, especially in skin. *Gluconobacter* was seen in high relative abundance at day 49 in the pulp sample and also showed an increase in abundance at day 25 and day 49 in the skin samples. Overall, there was a shift in relative abundance of the most prevalent as well as the less prevalent genera overtime.

Hierarchical clustering revealed that *Caulobacter*, *Hymenobacter, Cupriavidus*, and *Pseudomonas* genera were abundant in healthy apples (day 0 and day 14) but steadily declined in relative abundance over the course of post-harvest in both tissues (**Supplementary Figure S4**). Interestingly, OTUs belonging to *Gluconobacter* and *Acetobacter* genera, some members of which were reported to cause apple fruit decay (Van Keer et al., 1981), began appearing at 49 days post-harvest (**Supplementary Figure S4**). In 'Gala' pulp, a total of 24 indicator species were identified at day 25 and 33 indicator species at day 49.

**Temporal Expression of PTI-associated Defense Genes in Apple Fruit**

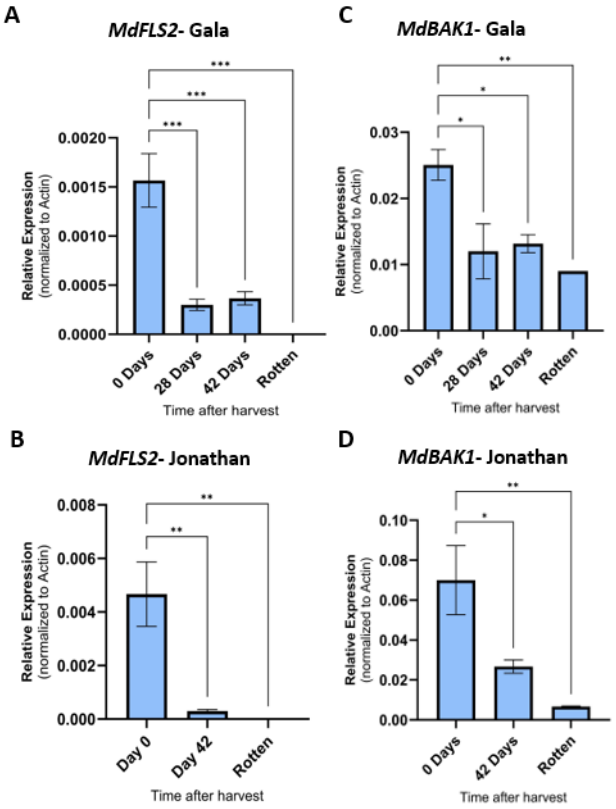


**Figure 3.7.** PTI gene expression in post-harvest apple fruit over time. *MdFLS2* Gala (**A**), *MdFLS* Jonathan (**B**), *MdBAK1* Gala (**C**), *MdBAK1* Jonathan (**D**). RT-qPCR was performed with RNA isolated from apple fruits at the timepoints indicated. For RT-qPCR, transcript levels were normalized to that of the house-keeping gene (*Mdβ-ACTIN*). Data are means of three biological replicates. Error bars represent ± 1 standard error of the mean. Number of stars represent statistically significant differences between timepoints (One-way ANOVA, Tukey's HSD test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

The gene expression pattern of PRR/coreceptor genes *MdFLS2* and *MdBAK1* were investigated over the course of post-harvest storage of Gala and Jonathan cultivars. Expression of

these genes was normalized to the housekeeping gene *Mdβ-ACTIN*. In both cultivars, expression of these genes decreased over time (**Figure 3.7**). On the day of harvest, when the apple fruit was fresh and healthy, *MdFLS2* and *MdBAK1* were both highly expressed. But by day 42 after harvest, when apples had begun to dehydrate and show some signs of decay, the expression level of these genes had become significantly reduced. By the rotten stage (day 96 and day 67 for Gala and Jonathan, respectively), *MdFLS2* expression was no longer detected and *MdBAK1*, although detected, was substantially reduced compared to that at day 0 and day 42 post-harvest **(Figure 3.7)**. In 'Gala', an additional sampling point was added to the experiment at day 28. Interestingly, there was no significant change in gene expression between day 28 and day 42, although at both time-points the expression levels of *MdFLS2* and *MdBAK1* were significantly reduced compared to those at day 0. Overall, changes in *MdFLS2* and *MdBAK1* gene expression in apple fruit were inversely correlated to the shift in the fruit microbiota composition toward dysbiosis. Enhanced Defense Response with Flg22 Treatment on Post-harvest Apple Fruits.

To determine whether apple fruit immunity can be enhanced during post-harvest, apple fruits were treated with the flg22 peptide right after harvest. flg22 is a 22-amino-acid epitope derived from bacterial flagellin and a well-characterized elicitor of PTI (Chinchilla et al., 2006). *MdPathogenesis-related (PR)-4* gene expression was investigated at several time points after flg22 treatment in the skin and the pulp separately. The *PR-4* gene was investigated because this gene has also been shown to have antifungal activities against fungal pathogens such as *Botryosphaeria dothidea* in apple (Bai et al., 2013) and resistance against apple replant disease pathogen (Zhou et al., 2021). RT-qPCR indicated that in the skin *MdPR-4* gene expression was significantly increased at 6 hrs. and further enhanced by 20 hrs. post-flg22 treatment (**Figure 3.8 A**). Similarly, in the pulp samples *MdPR-4* was slightly enhanced at 6 hrs. and very significantly

enhanced by 20 hrs. post-flg22 treatment (**Figure 3.8 B**). Thus, flg22 treatment had a positive impact on *PR-4* defense gene expression of the apple fruit in both tissue types within hours.

To determine the effect of flg22 treatment on apple fruit decay caused by a fungal pathogen, 5 µL of a conidia suspension of *P. expansum*, a known apple rotting fungus, was

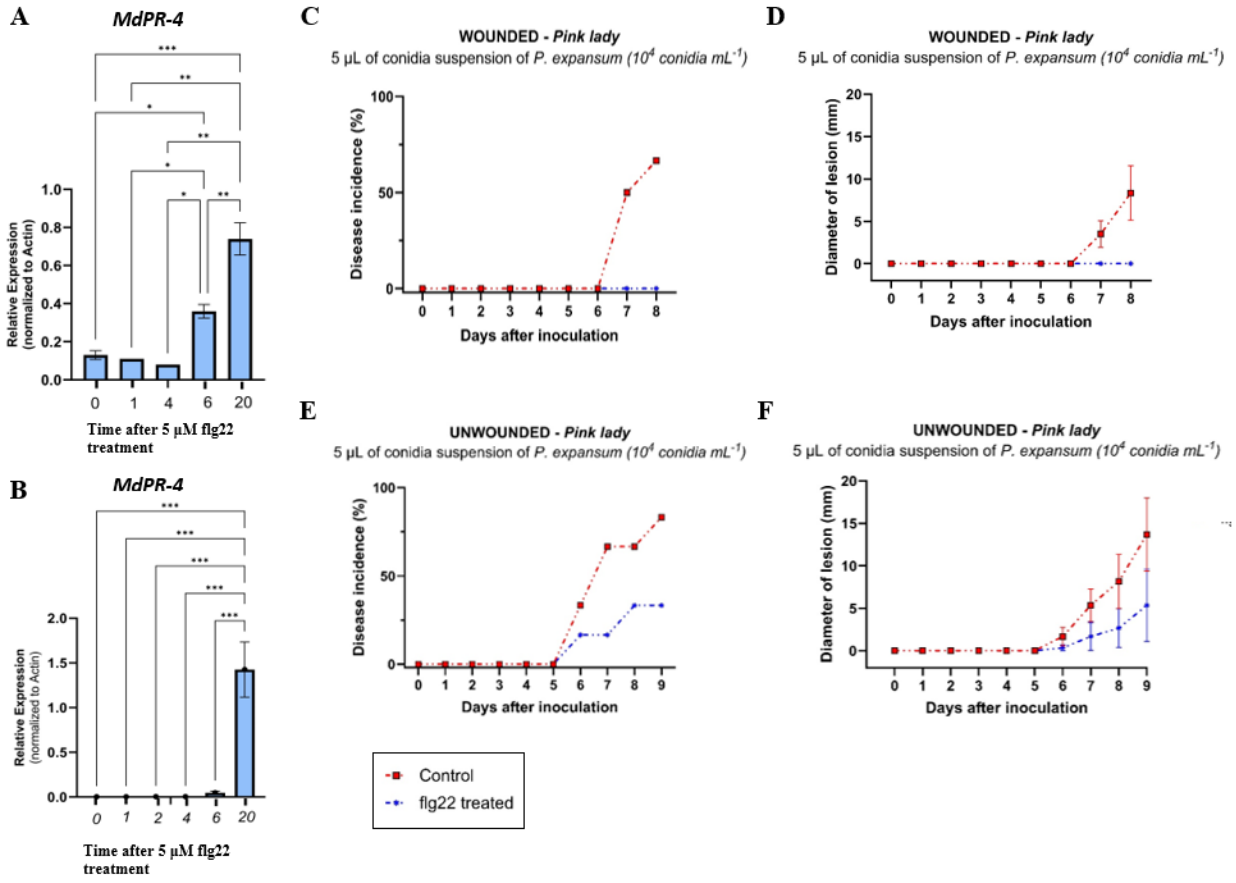


**Figure 8.** Defense genes (MdPR-4) expression in Pink Lady apple fruit post-flg22 treatment in skin and pulp (**A & B**), respectively. The fruits were pre-stabbed with a sterile needle at a width of 1 mm and a depth of 2 mm, prior to flg22 treatment. RT-qPCR was performed with RNA isolated from apple fruits at the timepoints indicated (in hours). For RT-qPCR was performed with RNA isolated from apple fruits at the timepoints indicated (in hours). For RT-qPCR, transcript levels were normalized to that of the house-keeping gene (*Mdβ-ACTIN*). Data are means of the three biological replicates. Error bars represent mean ± 1 standard error of the mean. Number of stars represent statistically significant differences between timepoints (one-way ANOVA with Tukey's test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Infection of wounded apple fruits by *P. digitatum* at 24 hrs post-flg22 treatment illustrating disease incidence and lesion diameter (**C &D**), respectively. Infection of unwounded apple fruits by *P. digitatum* at 24 hrs post-flg22 treatment illustrating disease incidence and lesion diameter (**E & F**), respectively. Each value is the mean of six biological replicates. Error bars represent ± 1 standard error of the mean.

wound-inoculated in the flg22-treated and control apples 24 hours post-flg22-treatment. In this experiment, the flg22 peptide was delivered through surface wounds to reach the pulp (see methods). We found that, in the control samples, lesions at the inoculated site became visible by 7 days post-fungal inoculation. However, the flg22-treated fruits did not show any sign of infection even at eight days post-fungal inoculation (**Figure 3.8 C & D**). This indicates that the induction of immunity by the flg22 peptide has a positive impact on apple fruit resistance to *P. expansum*. An additional experiment was conducted by dipping fruits in flg22-containing solution without wounding the apples. flg22 could still reduce disease incidence in the treated apples although not as effectively as delivering flg22 peptide through wounds to reach the pulp (**Figure 3.8 E & F**).

**DISCUSSION**

Fruit undergoes physiological changes and eventually decay during post-harvest storage, mostly due to microbial infection. The rate of post-harvest decay of apple fruit varies between different cultivars (Spotts et al., 1999; Argenta et al., 2021). In this work, we investigated if there were observable patterns in the apple fruit microbiota that correlated with fruit PTI-associated gene expression after harvest. We first asked if the microbiota of the apple fruit changes during post-harvest storage. We studied the change in both the endophytic (from pulp) and epiphytic (from skin) microbial communities. Consistent with Wasserman et al. (2019), we observed that apple skin and apple pulp harbor distinct microbial communities. Our results also demonstrated that the apple fruit microbiota is dynamic. We observed a shift in beta diversity (Bray Curtis dissimilarity) after harvest when the fruits were stored at room temperature. Among fungi, a greater relative abundance of the genus *Sporobolomyces*, was observed in healthy fruits. Some members of *Sporobolomyces* exhibit antimicrobial activities inhibiting growth of pathogens in

fruits (Janisiewicz, 1994). Over the course of post-harvest storage, we observed decreasing levels of *Sporobolomyces* that coincided with increasing relative abundance of the genus *Alternaria*, members of which are commonly found in a variety of habitats as ubiquitous agents of decay (Thomma, 2003). Interestingly, *Alternaria* became the dominant taxa in the skin of rotting apples (**Figure 3.4**). Several studies have shown that the antagonistic activity of *Sporobolomyces roseus* to other fungal pathogens such as *Penicillium, Alternaria* and *Aspergillus* results from degrading the mycotoxin produced by these pathogens (Janisiewicz, 1994; Ianiri et al., 2017; Sanzani et al., 2021). *Sporobolomyces ruberrimus* was shown to regulate ethylene homeostasis to maintain a well-balanced availability of metals such as Fe and Ni in plants (Domka et al., 2023). Hence, the high relative abundance of the genus *Sporobolomyces* observed in freshly harvested apple fruit could suggest a healthy microbiota that is effectively suppressing the growth of pathogens. This pattern was observed for some *Sporobolomyces* OTUs in both 'Gala' and 'Jonathan'. Thus, an important future research direction is to isolate these strains and to mechanistically understand factors that contribute to the decline of *Sporobolomyces* over time.

A similar pattern was observed in the bacterial community on harvested apples where bacteria such as *Caulobacter, Cupriavidus,* and *Pseudomonas*, which contain strains that have beneficial effects on plants (Estrada-de Los Santos et al., 2014; Luo et al., 2019; Berrios, 2022; Guro et al., 2023), were abundant in healthy apples but steadily declined in relative abundance over time **(Supplementary Figure S4).** *Pseudomonas* species, for example, can be pathogenic, but most plant-associated *Pseudomonas* species are beneficial to plant growth and health (Preston, 2004). *Pseudomonas* has also been shown to increase fruit production and protection against pests and pathogens in blackberries (García-Seco et al., 2012). Members of the *Cupriavidus* and *Caulobacter* genera have been studied for their beneficial role in plant growth-

promotion including production of phytohormones and metabolite synthesis (Estrada-de Los Santos et al., 2014; Luo et al., 2019; Berrios, 2022; Guro et al., 2023). It was also reported that genus *Hymenobacter* was found abundantly in healthy Kiwifruit plants, but its relative abundance was significantly reduced in a diseased plant (Ares et al., 2021). We observed a similar trend in this study where the abundance of the genus *Hymenobacter* was significantly reduced in rotting apple. Our observation of these putative beneficial bacteria in healthy fruit suggests that they may be playing a role in activities contributing to beneficial fruit physiology.

Interestingly, as in fungi, *Gluconobacter* and *Acetobacter* genera began to appear in high abundance at 49 days post-harvest. Members of these genera are commonly found in rotten fruits and spoiled juices and have the ability to grow at relatively low pH and low nutrient levels. These genera can also oxidize sugars and alcohols into acids (Van Keer et al., 1981; Gupta et al., 2001; Gomes et al., 2018). It remains to be determined if the taxa observed in this study are involved in initiating the rotting process by changing the fruit physiology, leading to the growth of opportunistic fungal pathogens that are the ultimate causal agents of fruit rot. Overall, the fact that we observed beneficial microbes in healthy fruit, and conversely pathogens in unhealthy fruit, is consistent with the notion that the fruit microbiota plays a significant role in dictating fruit health. Of note, some of the OTUs, such as *Cupriavidus and Caulobacter* from healthy apples, and *Gluconobacter* and *Acetobacter* from rotting apples are rare taxa and are present in low abundance compared to other taxa in the apple microbial community. Future research is needed to determine if some of these OTUs detected in our study act as keystone taxa that have cascading effects in changing apple fruit microbial community dynamics (Shade et al., 2013; Jousset et al., 2017).

Another important finding of our study is the immune gene expression dynamics in

postharvest apple fruits. Specifically, we determined that expression of the PTI receptor/co-receptor genes, *MdFLS2* and *MdBAK1*, diminished over time during post-harvest storage. In healthy apples, these genes were highly expressed, but expression was significantly reduced by 42 days after harvest. In rotten apples, *MdFLS2* gene expression could not be detected, and *MdBAK1* expression was low as well. In plant innate immunity, PTI is the first line of active defense against most pathogens (Zipfel, 2009). Recently, it has been shown that PTI is an important component that acts as a host barrier to control the level of commensal microbes and opportunistic microbes from excessive proliferation in the host tissue for optimal plant health (Chen et al., 2020; Song et al., 2023; Pfeilmeier et al., 2024). In light of these observations, and our findings reported here, we hypothesize that fruit immunity likely plays an important role in maintaining a balanced and healthy microbiota and, in turn, preventing fruit spoilage. It was interesting that we observed a steep decline in the fruit immune response (as monitored by *MdFLS2* and *MdBAK1* expression) around 42 days post-harvest which coincides with the timing of the emergence of potential pathogenic microbes. Thus, there seems to be an inverse relationship between PTI gene expression and microbiota dysbiosis. Consistent with our hypothesis that the fruit PTI plays a role in protection against dysbiosis, we show that by inducing PTI with the flg22 peptide we can delay the onset of fungal rot in apple fruit. Future research is needed to determine if this protection is associated with composition changes of the microbiota.

To my knowledge, this study represents the first exploration of apple fruit microbiota conducted during the post-harvest period and the patterns in apple fruit PTI gene expression patterns under the natural progression of fruit spoilage. Our results have implications in developing strategies to enhance fruit defense for increased fruit quality and prolonged shelf life

and may motivate future studies to examine if the relationship we observed in apple fruit immunity and microbiome dynamics is generally applicable to other postharvest fruits and vegetables.

## MATERIALS AND METHODS

### Sampling Procedures

Healthy fruits of apple cultivars ‘Gala’ and ‘Jonathan’ were collected at the Michigan State University Plant Pathology farm. Harvesting was done on different dates due to differences in ripening times between the two cultivars; 'Gala' was harvested on 9 September 2022, and 'Jonathan' on 7 October 2022. Six trees were selected at random, and 40 fruits per tree were sampled from around the circumference of the tree from each of six replicate trees. The fruits from each tree were maintained separately in clean plastic bins stored at room temperature in the laboratory. At each sampling point (every two weeks), five fruits were randomly selected from each replicate and pooled to make one biological replicate for a total of six biological replicates per time point per cultivar. From each apple, two tissue types (skin and pulp) were sampled. Fruit pulp was excised using a sterile cork-borer and a sterile blade to peel a thin layer of the skin. Immediately, each sample tissue was homogenized separately in liquid nitrogen. The homogenized samples were stored at -80°C for subsequent DNA and RNA extractions. Sampling of the remaining apple fruits was repeated every two weeks until the $8^{th}$ week after harvest, or when decay was present in all fruit (day 96 and day 67 for Gala and Jonathan, respectively).

### DNA Extractions and Sequencing of 16S rRNA and ITS genes

Microbial genomic DNA samples were extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, United States) following the instructions of the manufacturer. Extracted DNA was used as the template for amplicon PCR reactions that amplified the bacterial

16S ribosomal region and fungal internal transcribed spacer (ITS) 1 region. The V4 region of the 16S rRNA was amplified using the universal 515F/806R primer set (Caporaso et al., 2011), and the ITS1 region was amplified using the ITS1F/ITS2 primer set (Ghannoum et al., 2010; White et al., 2013). Peptide nucleic acid (PNA) clamps (Lundberg et al., 2013) were added to the 16S rRNA PCR mix (total of 20 µl) with a concentration of 2.5 µM mitochondrial PNA and 2.5 µM plastid PNA to block amplification of host plastid and mitochondrial 16S DNA. Raw sequence data preparation and data analysis was performed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) (Bolyen et al., 2019). Primer and adapter sequences were removed using cutadapt, paired reads were joined, samples were denoised with dada2, and chimeric sequences were removed using VSEARCH (Rognes et al., 2016). Operational taxonomic units (OTUs) were identified at 97% similarity using QIIME's sklearn classifier and the SILVA database v132 for bacteria (Yilmaz et al., 2014). CONSTAX2 was used with the UNITE fungal general release dataset from Nov. 29, 2022, to assign fungal taxa (Abarenkov et al., 2020) with 80% confidence threshold and recommended settings (Liber et al., 2021). Finally, sequences assigned to host mitochondria and chloroplasts were discarded.

**Data Processing and Analysis**

The OTU table, taxonomy, metadata, and phylogenetic tree were imported into the R package Phyloseq v.1.24.2 (McMurdie and Holmes, 2013). Host mitochondria and chloroplast OTUs were removed. Alpha diversity was estimated with Shannon diversity index to determine the evenness based on the presence of rare OTUs (singletons and doubletons), respectively using Phyloseq v.1.24.2 (McMurdie and Holmes, 2013). Statistical significance was calculated by Analysis of Variance (ANOVA). Beta diversity was analyzed to compare the microbiome composition among groups, based on the Bray-Curtis dissimilarity distance matrix. The

ordination was calculated by Principal coordinates analysis (PCoA). To compare the microbiome composition between time points, statistical significance was calculated with permutational multivariate analysis of variance (PERMANOVA) using the vegan package v.2.6-4 (Oksanen et al., 2022). To visualize the relative abundances of OTUs/ASVs, a bar plot was constructed using the ggplot2 package (Wickham, 2016).

Differentially abundant taxa were identified using DESeq2 v.1.38.3 to extract the main effects of time point in each cultivar and tissue type (McMurdie and Holmes, 2013; Love et al., 2014). Taxa with similar trends in abundance over time were grouped using hierarchical and K means clustering. The elbow method was used to identify the optimal number of clusters. Abundance trends were visualized using pheatmap v.1.0.12 (Kolde, 2022) and ggplot2 v.3.5.0. Indicator species analysis was performed using indicspecies v.1.7.14 to identify possible unique taxa and compositional signatures at each timepoint (De Caceres and Legendre, 2009).

**RT-qPCR Analysis**

Expression of host defense genes (**Table 1**) was monitored at three time points. RNA extraction (RNeasy plant mini kit- QIAGEN), and reverse-transcription (SYBR Green Real-Time PCR master mix), were performed according to the manufacturer's instructions. Real-time qPCR was performed with 35 cycles using a set of primers for each of the defense genes (**Table 1**). The expression of defense marker genes was normalized to the expression of the *Mdβ-ACTIN* gene. Significant differences ($p < 0.05$) were calculated using one-way ANOVA.

**Table 1: Gene Expression Targets for qPCR and their primer sequences**

| Defense genes | Primer Pairs | Housekeeping genes | Primer Pairs |
|---|---|---|---|
| *MdFLS2* | *5'-TCCCTGCACGACAATGCTT*<br>*5'-GTGGAATTGGACCCGTCAGT* | *Mdβ-ACTIN* | *5'-*<br>*CTATGTTCCCTCGTATTGCAGACC*<br>*5'-GCCACAACCTTGTTTTTCATGC* |
| *MdBAK1* | *5'-CGGGGAGCTACAGTTCCAAA*<br>*5'-GCAGCCTTTCTGTTGGTGTC* | | |
| *MdPR-4* | *5'-GAAGGTGCCTCTTGGTG*<br>*5'CGTCGGTGTCAATTTGG* | | |

**flg22 Treatment and *Penicillium expansum* Pathogenicity Assay**

Each freshly harvested 'Pink Lady' apple was wounded with a sterile needle with 15 injuries randomly around the fruit (each 1 mm x 3 mm). The wounded apples were dipped for 1 min in 2.5 µmol flg22 solution containing the organosilicone surfactant Silwet L-77 (Helena Agri-Enterprises, Collierville, TN), at a concentration of 0.025%. Control apples were wounded, but only dipped in 0.025% Silwet L-77. Samples (both skin and pulp) were collected for RNA extraction at zero-, one-, two-, four-, six-, twelve- and twenty-hour post treatment. At each sampling point, three fruits were randomly selected and pooled to make one biological replicate for a total of three biological replicates. For RT-qPCR, transcript levels were normalized to that of the house-keeping gene *Mdβ-ACTIN*.

A fungal pathogenicity assay was conducted using six apples (biological replicates) per treatment. Fifteen µL of a *Penicillium expansum* spore suspension ($1 \times 10^4$ conidia/mL) was inoculated on two opposite sides of each wound. Conidia suspensions were made in 0.05% (w/v) Tween-20. Control samples were inoculated with 15 µL sterile water containing 0.05% (w/v) Tween-20. After air-drying, the fruits were placed in a clean bin without the fruits touching each other. The bins were then covered with plastic film to maintain a high relative humidity of 80–90% and incubated at 20 ±1° C. Lesion diameters (cm) were regularly monitored and measured when symptoms emerged.

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## **APPENDIX A**: SUPPLEMENTARY FIGURES

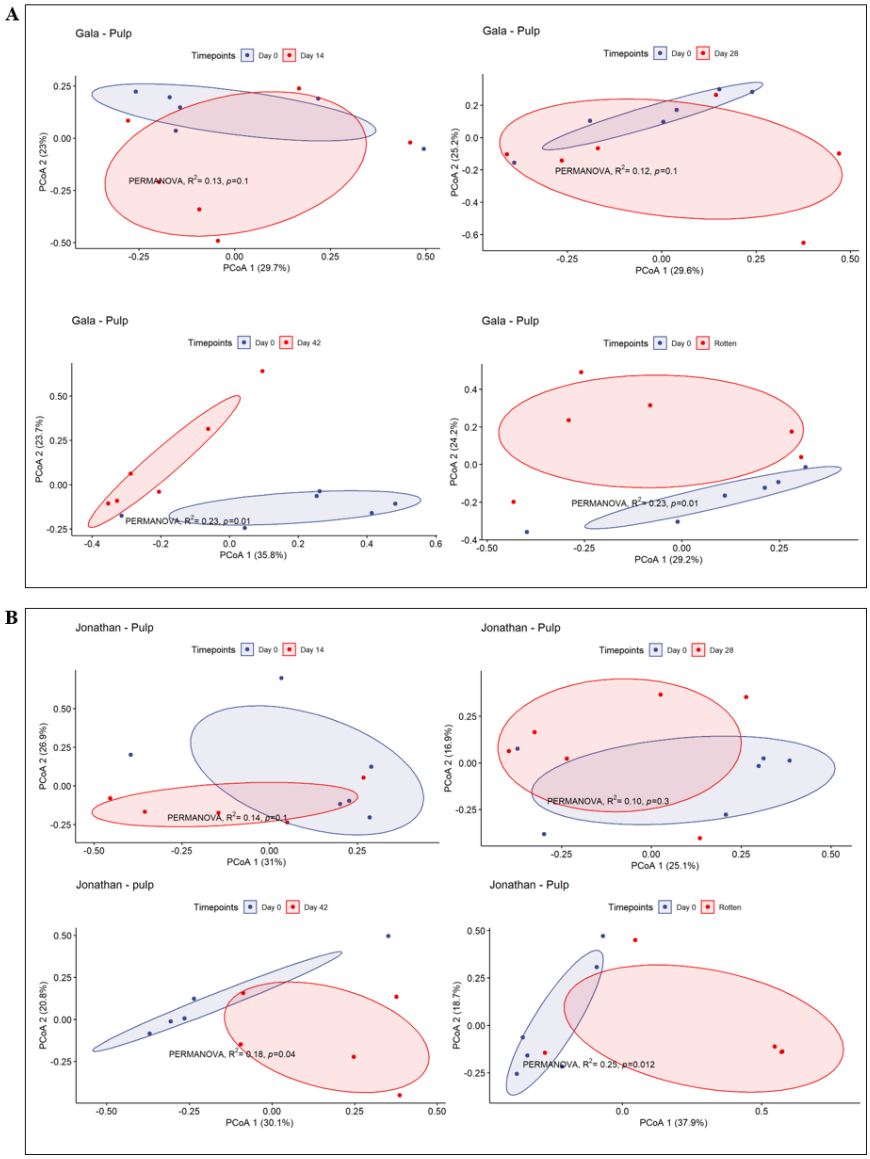


**Figure S3.1.** Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metrics showing the distance in the fungal community composition from day 0 to rotten stage in Gala pulp (**A**) and Jonathan pulp (**B**).

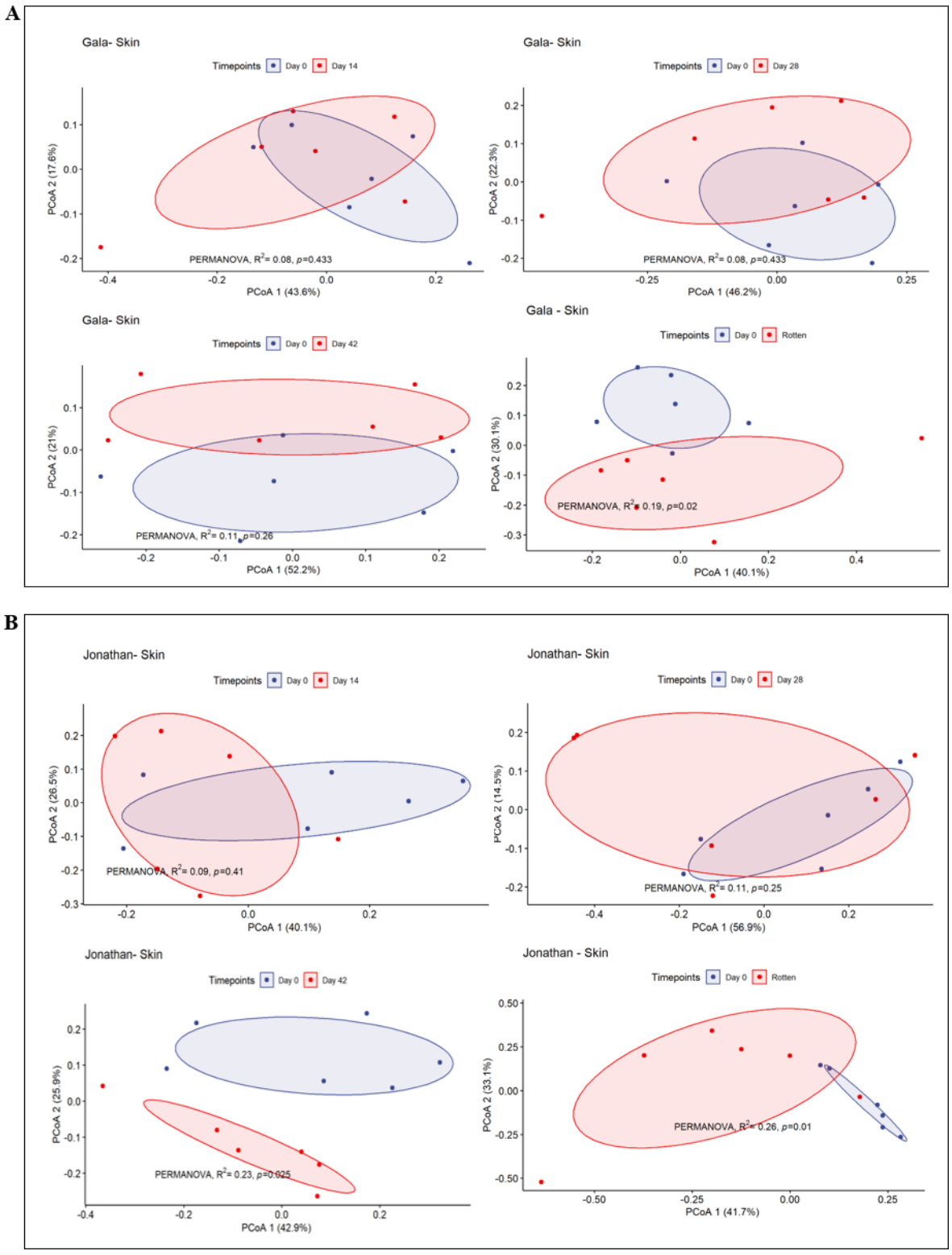


**Figure S3.2.** Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metrics showing the distance in the fungal community composition from day 0 to rotten stage in Gala skin (**A**) and Jonathan skin (**B**).

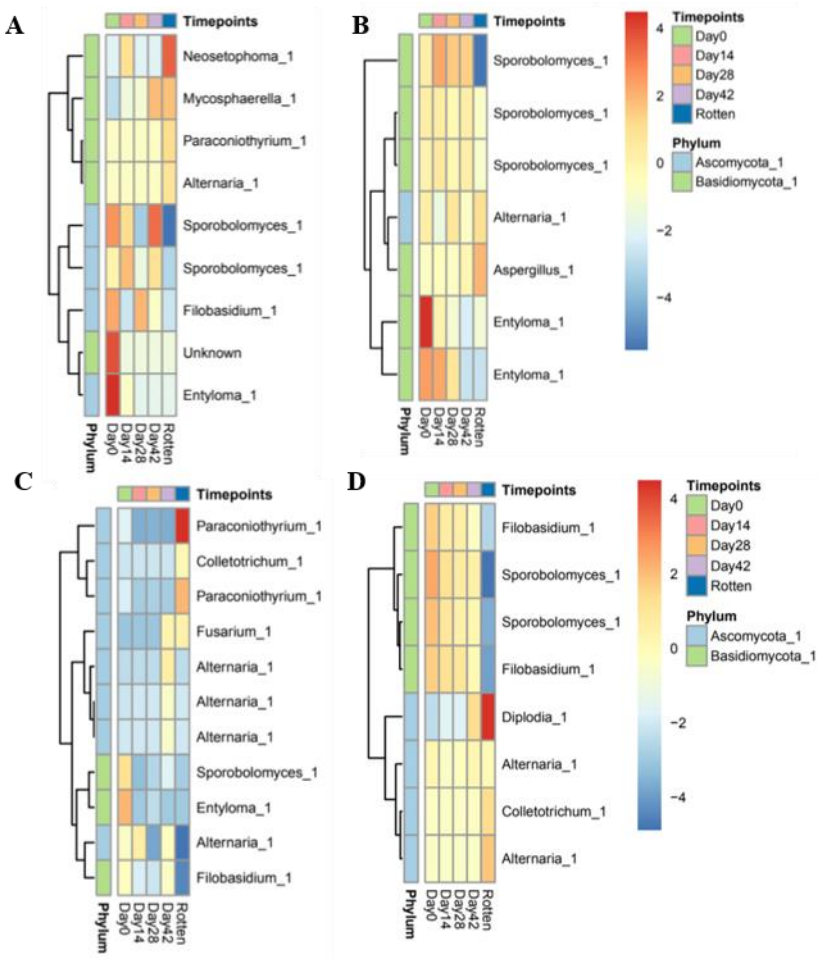


**Figure S3.3.** Clustered heatmap with selected set of fungal operational taxonomic units (OTUs) with differential abundance patterns across timepoints in Gala pulp (**A**), Gala skin (**B**), Jonathan pulp (**C**), and Jonathan skin (**D**). Hierarchical clustering was performed using Euclidean distances on variance stabilized data. Relative abundance was calculated for each OUT across timepoints.

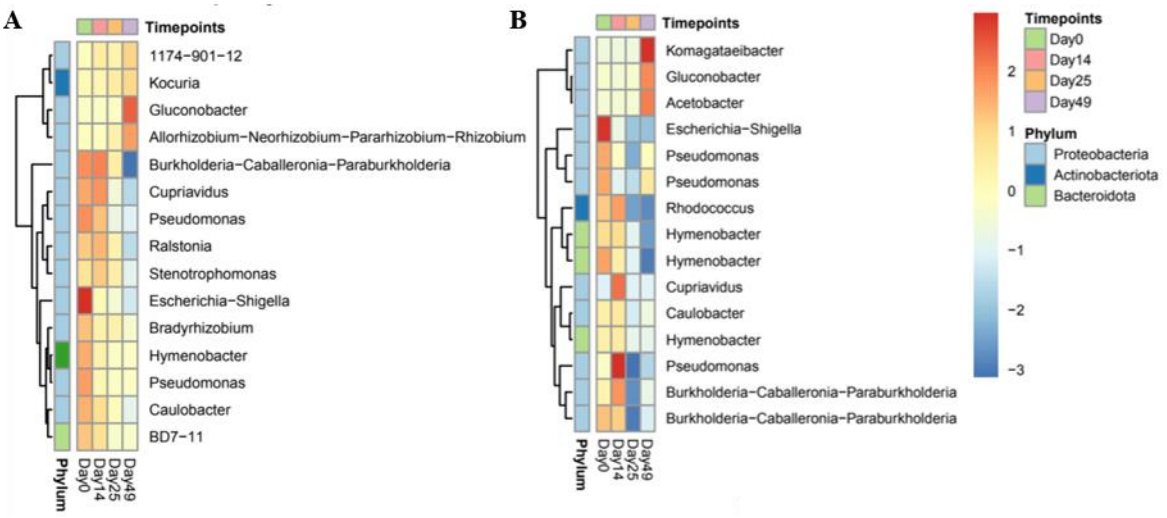


**Figure S3.4.** Clustered heatmap with selected set of bacterial operational taxonomic units (OTUs) with differential abundance patterns across timepoints in Gala pulp (**A**), Gala skin (**B**), Jonathan pulp (**C**), and Jonathan skin (**D**). Hierarchical clustering was performed using Euclidean distances on variance stabilized data. Relative abundance was calculated for each OUT across timepoints.

# CHAPTER 4

## IMPACT OF ACIBENZOLAR-S-METHYL TREATMENT ON POST-HARVEST MICROBIOME COMPOSITION AND HEALTH OF APPLE FRUIT

## INTRODUCTION

Apple is among the most widely consumed fruits in the world, however post-harvest decay leads to substantial losses (Droby and Wisniewski, 2018). Previous evidence has indicated that the microbiome of the fruit plays a role in fruit decay (Zhang et al., 2021; Kuruppu et al., 2023). Fruit microbiome studies assessing populations of epiphytes and endophytes of harvested fruit can advance our understanding of postharvest biocontrol and postharvest health and physiology. Studies have shown that the host immune system orchestrates the maintenance of key features of host-microbe symbiosis, while the microbiome plays critical roles in the training and development of major components of the host's innate and adaptive immune system (Zheng et al., 2020). Chen et al, 2020 also demonstrated that immune response plays an important role in maintaining a well-balanced microbiota in Arabidopsis plants (Chen et al., 2020). I have already demonstrated that healthy apple fruit has a strong immune response which collapses over time at post-harvest, correlating with the emergence of pathogenic microbes (Chapter 2).

ASM is a synthetic analog of salicylic acid (SA) that induces systemic acquired resistance (SAR) and has been used to protect crops from plant diseases by activating plant defense (Brisset et al., 2000; Marolleau et al., 2017). Application of ASM has a strong negative correlation with postharvest apple disease (Poveda, 2020). Dipping fruits in ASM post-harvest prolongs the quality of the fruits by slowing down ethylene production and the respiratory rate (Li et al., 2020). Because ASM does not exhibit a direct antimicrobial activity in vitro, the inhibition is due to an enhanced immune system in postharvest fruit (Cao et al., 2005). Additionally, it has also been demonstrated that exogenous application of ASM on Gala apple trees induces defense response and protects against apple scab and postharvest diseases such as blue mold in apple (Marolleau et al., 2017). However, the effects of enhanced host defenses by

ASM application on the composition of the microbial community has not been explored so far.

This study proposes to test the influence of fruit immune response induced by ASM on the microbial community composition, especially endophytes that may have a profound impact on postharvest fruit rot. I hypothesize that ASM application will lead to a robust induction of defense gene expression, leading to a shift in microbial community composition and improved shelf life. This experiment was conducted on the Gala variety because it is a more desirable cultivar with a broader geographic growth range. The goal of this study is to develop a better understanding of host immune-microbiome interactions, as well as their impact on post-harvest fruit diseases. These insights could translate to the development of microbiome-targeted therapeutic interventions for the prolongation of shelf life of fresh produce.

## RESULTS

### Apple fruit tissue type has an effect on microbiota composition in both control and ASM treated samples

In preceding chapters, I described how apple fruit skin and pulp harbor distinct microbial communities (Chapters 2 & 3). In this study, I found dissimilar microbial community compositions, both fungal and bacterial, between tissue types in both control **(Figure 4.1 A & E)** and treated samples **(Figure 4.1 B & F)**. A Principal Coordinate Analysis (PCoA) illustrated distinct fungal (PERMANOVA: Control: $R^2 = 0.28$, $p = 0.001$ and Treated: $R^2 = 0.22$, $p = 0.001$) and bacterial (PERMANOVA: Control: $R^2 = 0.15$, $p = 0.001$ and Treated: $R^2 = 0.25$, $p = 0.001$) community composition between the tissue types, based on Bray-Curtis dissimilarity distance. In the fungal community, at Day 49 of post-harvest when most apple fruit started to appear dehydrated and exhibit rotting symptoms, the dissimilarity in composition between skin and pulp can no longer be observed (PERMANOVA: Control: $R^2 = 0.04$, $p = 0.7$ and Treated: $R^2 = 0.03$, p

= 0.9) (**Figure 4.1 C & D**). In contrast, the dissimilarity between tissue types was observed in the bacterial community at Day 49.

**Temporal dynamic shift in microbial community in both control and ASM treated samples**

Additionally, I found that there is a temporal dynamic change in β-diversity in apple fruit

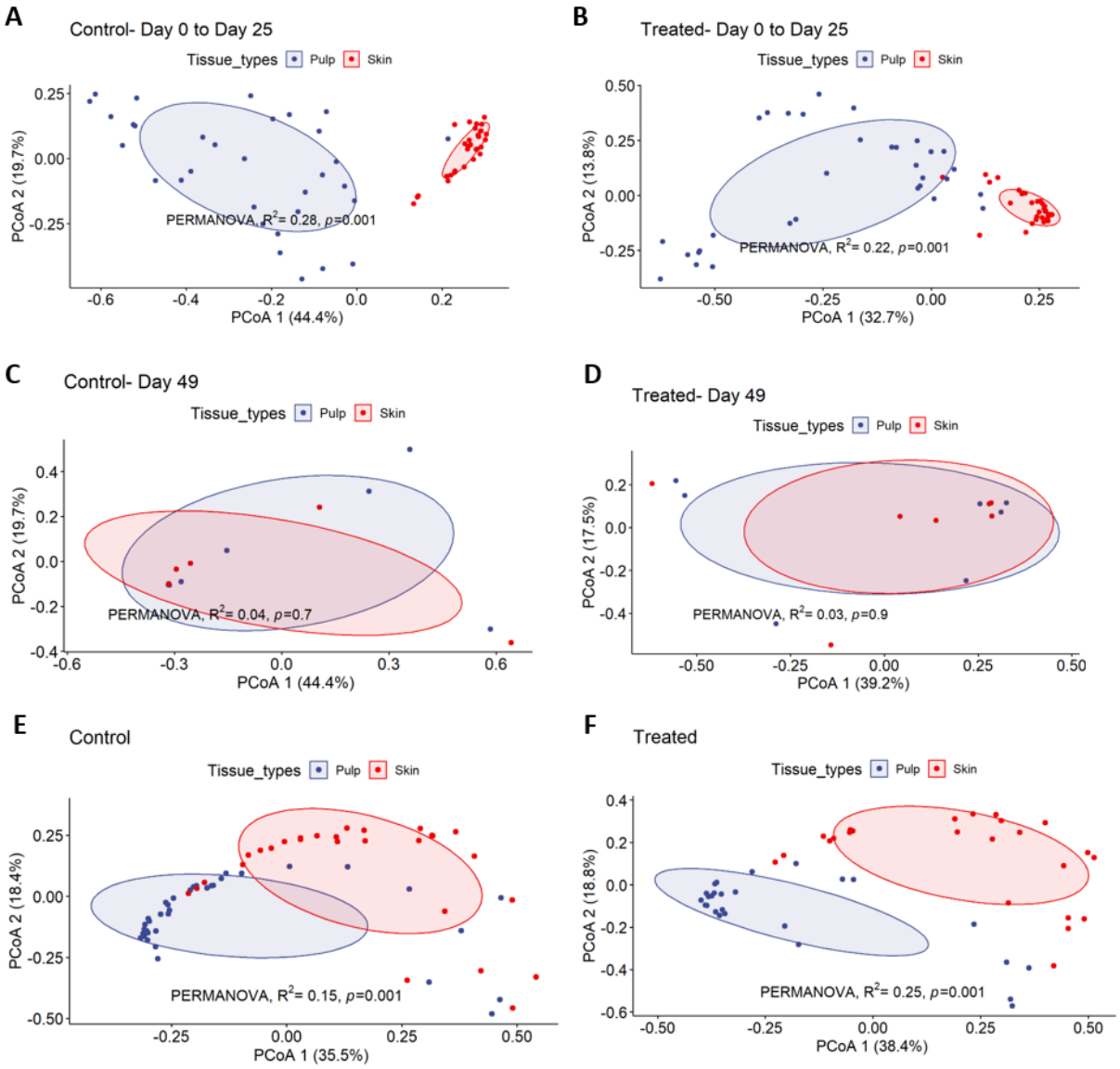


**Figure 4.1.** Effects of tissue types on fungal community composition. Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metrics between pulp and skin from day 0 to day 25 in control and ASM-treated fruits (**A & B**), respectively. PCoA based on Bray-Curtis dissimilarity metrics in fungal communities between pulp and skin on day 49 control and ASM-treated fruits (**C & D**), respectively. Effects of tissue types on bacterial community composition, PCoA based on Bray-Curtis dissimilarity metrics between pulp and skin rom day 0 to day 49 in control and ASM-treated fruits (**E & F**), respectively.

microbial community composition during post-harvest storage at room temperature, which is consistent with the previous study (Chapter 2). I observed a temporal dynamic change in fungal β-diversity in both control and ASM-treated pulp samples (PERMANOVA: Control pulp: $R^2$ = 0.32, p = 0.001, treated pulp: $R^2$ = 0.31, p = 0.001) **(Supplementary Figure 1A, 1B, 1C and 1D)**. In the skin samples we saw a significant shift only at Day 49 but in the pulp samples we observed a significant shift as early as Day 14. This observation was true in both control and treated samples. However, even in the pulp samples, Day 49 clustered far from the earlier time points indicating a significant difference in composition at Day 49 which aligns well with the previous study described in Chapter 2.

Interestingly, there was a trend towards decreased α-diversity by Day 49 in both tissue types and treatments according to Shannon index **(Supplementary Figure 1E, 1F, 1G and 1H)**. The temporal pattern in fungal community shift over time in post-harvest apple fruits was similar irrespective of the treatment with ASM.

Similarly, in bacteria, there was a temporal dynamic shift in community composition in both control and treated samples. The shift in composition was seen especially after Day 25 in both tissues **(Supplementary Figure 2).**

**Dynamic change in relative abundant taxa among endophytic community with ASM treatment**

To test if ASM treatment had a significant effect on microbiota composition, Principal Coordinate Analysis (PCoA) was employed to illustrate the distances among samples of each dataset using Bray–Curtis dissimilarity distance. In pulp samples, we observed that ASM treatments had a significant impact on both the bacterial **(Figure 4.2 A)** and fungal **(Figure 4.3 A)**

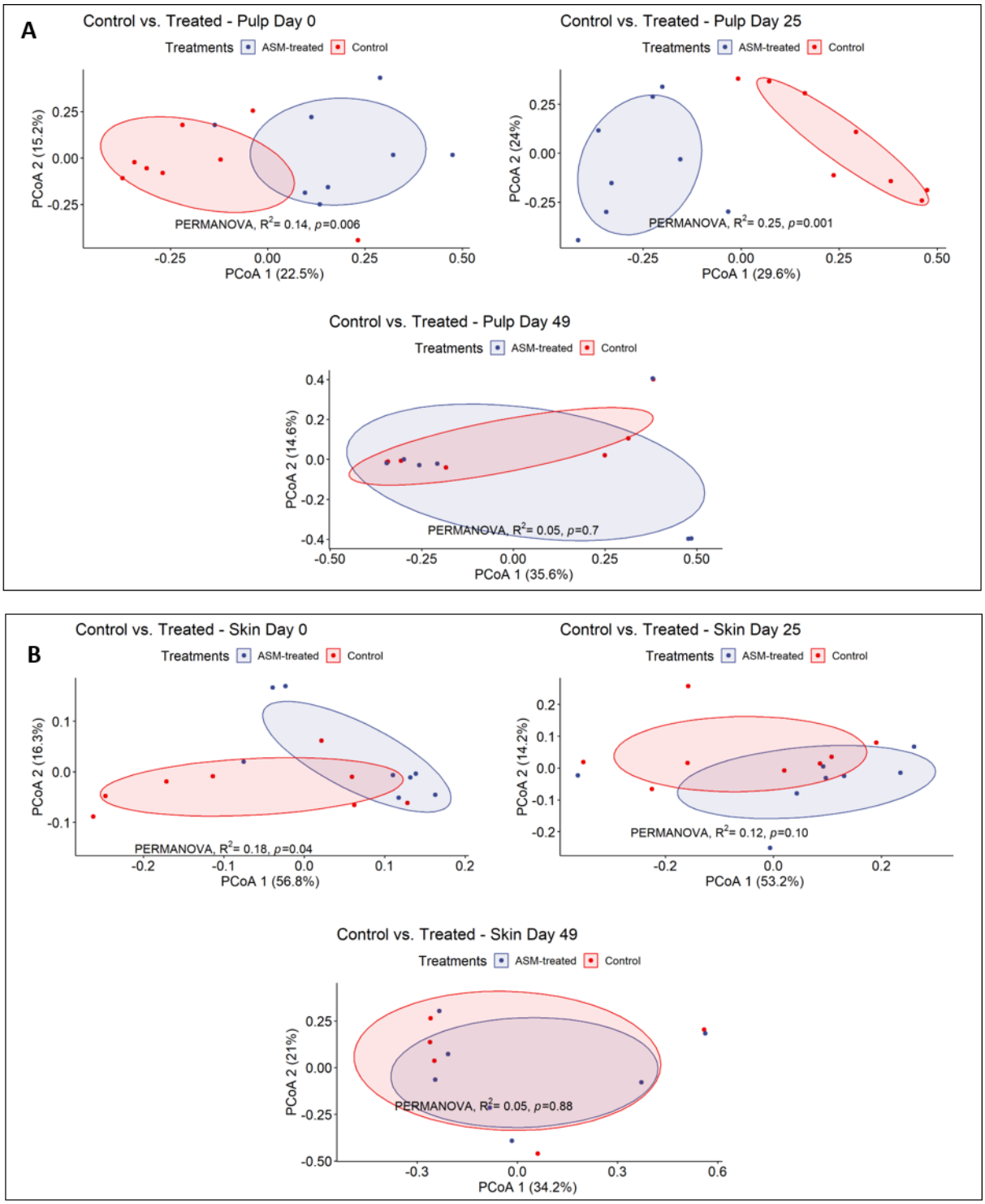


**Figure 4.2.** Principle coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metrics showing the distance between control and treated fungal communities from day 0 and day 49 in pulp (**A**) and skin (**B**).

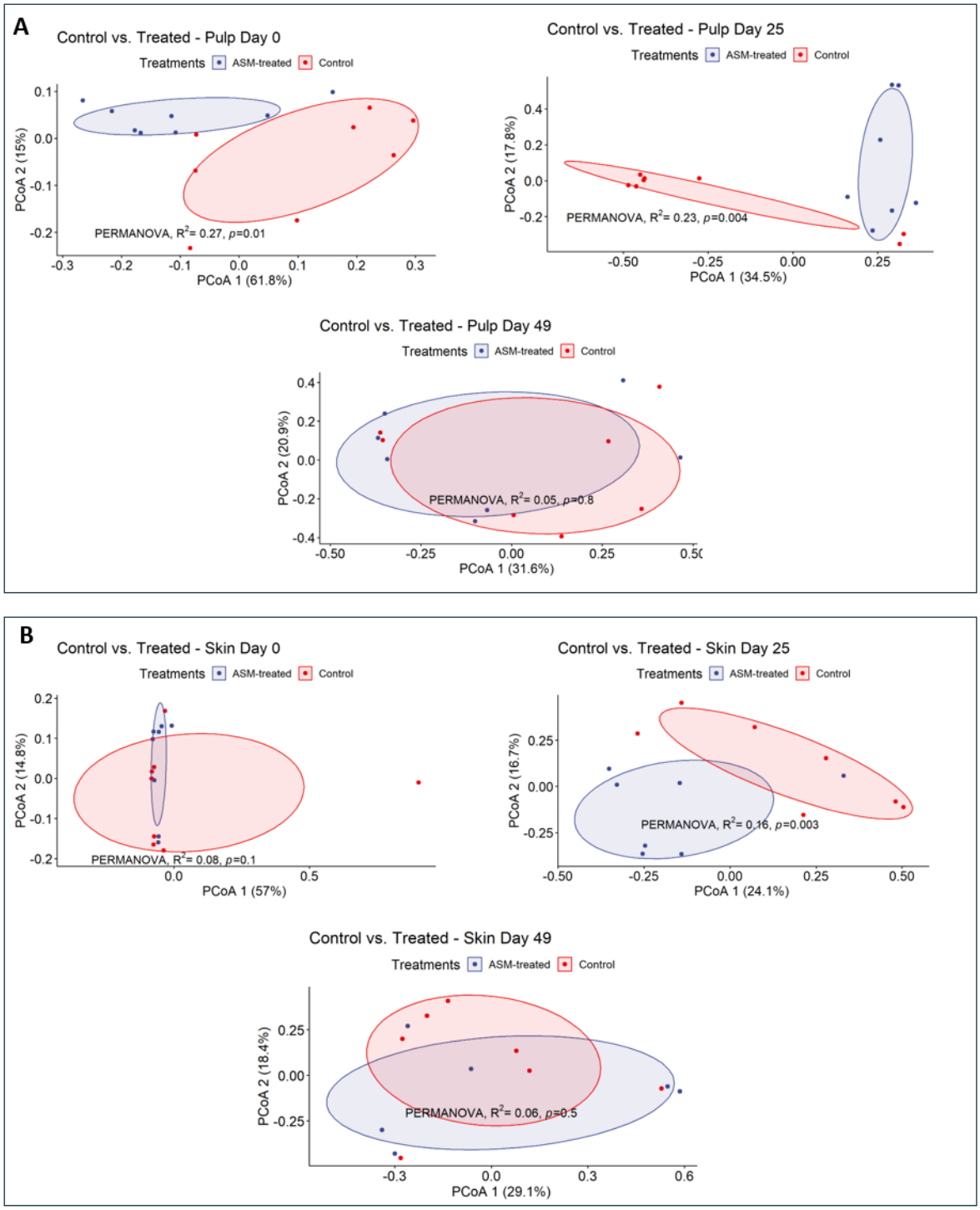


**Figure 4.3.** Principle coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metrics showing the distance between control and treated bacterial communities from day 0 and day 49 in pulp (**A**) and skin (**B**).

microbiota composition. We observed that the treated samples clearly clustered away from the control samples, which was confirmed using PERMANOVA at every sampling point. The distinct fungal community composition was observed until Day 25 sampling point, but interestingly the dissimilar composition between control and treated samples was no longer seen at Day 49 for both fungi **(Figure 4.2 A)** and bacteria **(Figure 4.3 A).**

Among fungi in the pulp samples *Fusarium*, which was seen in high abundance at earlier time points in treated samples, was found in low abundance in the control samples. Conversely, *Naganishia* which was seen in high relative abundance at Day 0 and Day 7 in control samples was not found in Day 0 and Day 7 of treated samples. Moreover, *Erythrobasidium* which was found in treated samples on Day 0, Day 7 and *Thanatephorus* at Day 25 was not found in control samples. Additionally, *Rhodotorula* which was found in high abundance in control samples were seen in low abundance at treated samples indicating that ASM treatment influenced the growth of some fungal endophytes and not just the overall abundance of the total community **(Figure 4.4 A)**. On Day 49 in both control and treated, *Alternaria* became the predominant OTU in the community **(Figure 4.4 A)**.

In Bacteria, although Bray-Curtis dissimilarity distance matrix showed a distinct bacterial community composition between control and treated, we did not observe a substantial difference in the relative abundance of taxa. In both treated and control samples, *Ralstonia* was the dominate taxa at every time point with the exception at Day 25 of treated samples where *Pseudomonas* was the dominant taxa in the community. Interestingly, *Gluconobacter*, a spoilage organism, was found at day 49 in both control and treated samples.

In the skin samples for fungal community analysis, the effects of the ASM treatment were seen only on Day 0 **(Figure 4.2 B),** the opposite of our result in bacteria where no effects

on community composition with ASM treatment was observed on Day 0 but effects were observed on Day 14 and Day 25 **(Figure 4.3 B).** In fungi, there was no significant shift in community composition and abundance as illustrated in the relative abundance plot **(Figure 4.4 B).** However, there was an increase in α-diversity in the treated skin samples based on Shannon index **(Figure 4.4 D).**

In bacteria, there was no strong visible change in community composition at Day 0 supporting the observation in β-diversity (**Figure 4.5**). However, on Day 14, the relative

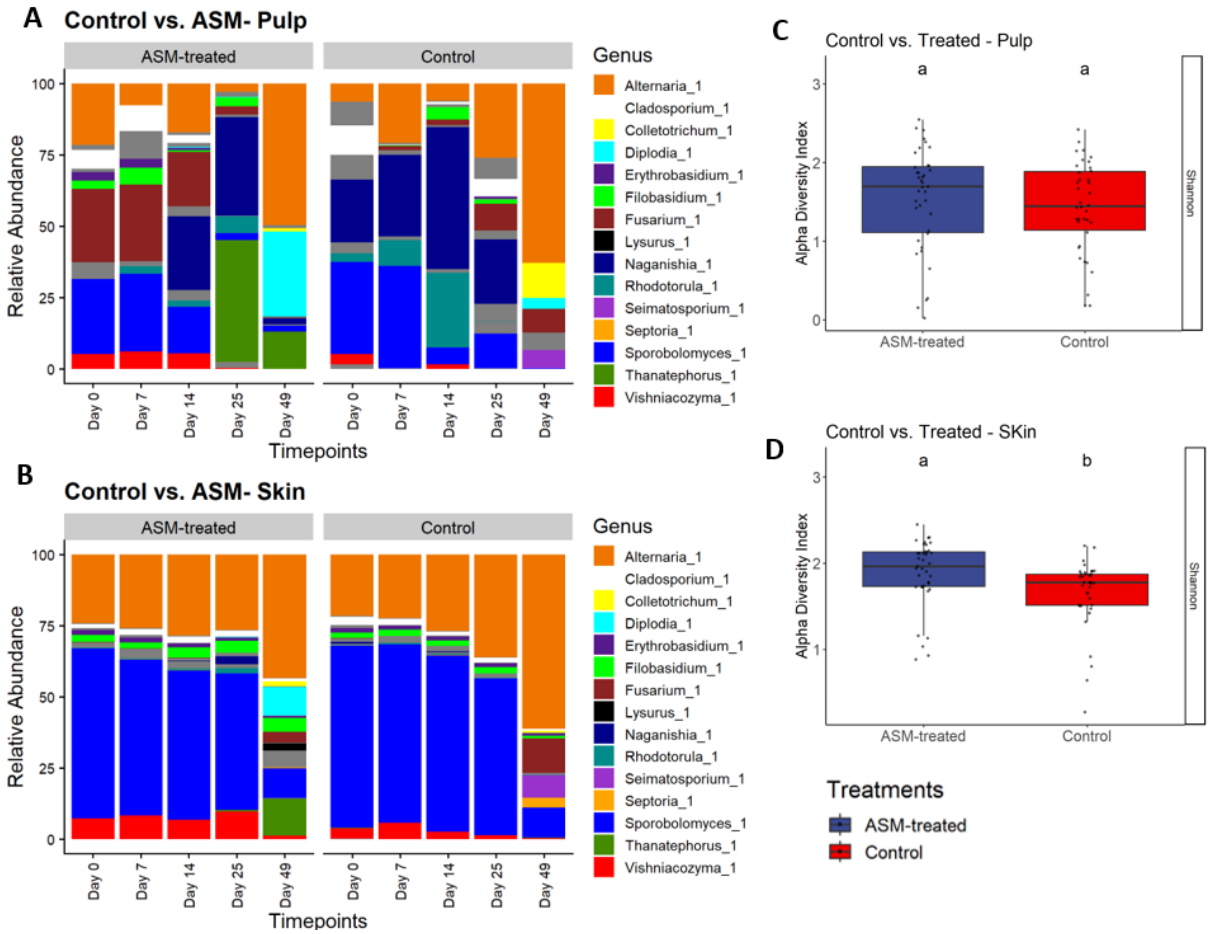


**Figure 4.4.** Stacked bar plots represent mean relative abundance of the most prevalent fungal genera across timepoints in control versus treated pulp and control versus treated skin (**A & B**), respectively. Each bar shows the average composition from all the replicates within timepoint. Box plots showing fungal α-diversity comparison collectively from day 0 to day 49 between control and treated samples based on the Shannon index in pulp and skin (**C & D**), respectively. The different letters above each bar indicate statistically significant differences at $P_{adj} < 0.05$ as calculated by Tukey's HSD test.

abundance of *Sphingomonas* and *Methylobacterium* was higher in treated samples and conversely the relative abundance of *Ralstonia* was lower in treated samples than in the control samples. Additionally, on Day 25, similar to pulp samples, there was relatively high abundance of the *Pseudomonas* in treated samples than in the control. All these changes in the relative abundance of some prevalent taxa may have contributed to a shift in β-diversity. Collectively, this finding indicates that induction of the fruit host immune response with ASM treatment impacts especially the fruit endophytes.

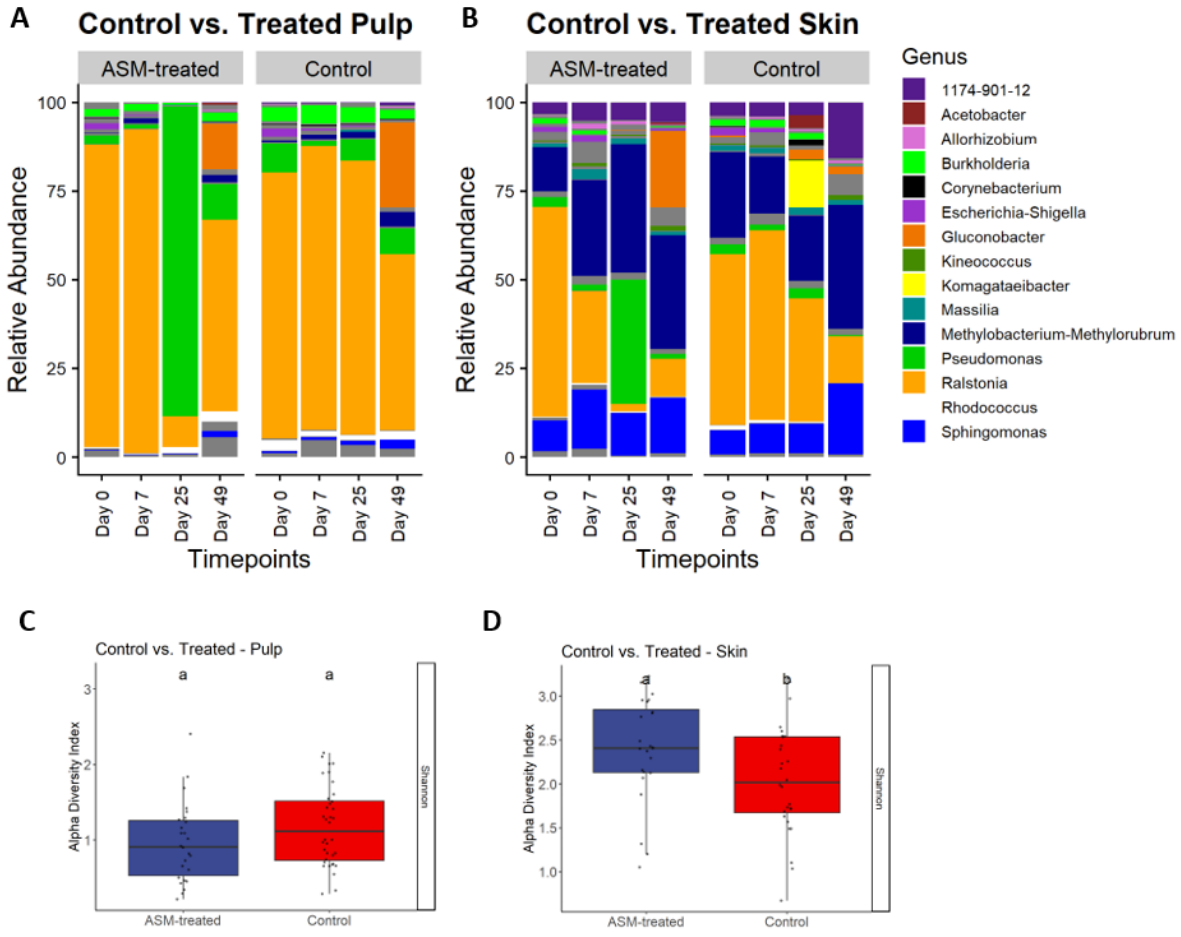


**Figure 4.5.** Stacked bar plots represent mean relative abundance of the most prevalent bacterial genera across timepoints in control versus treated in pulp and skin (**A & B**), respectively. Each bar shows the average composition from all replicates within a timepoint. Box plots showing bacterial α-diversity comparison between control and treated based on the Shannon index in pulp and skin (**C & D**), respectively. The different letters above each bar indicate statistically significant differences at $P_{adj} < 0.05$ as calculated by Tukey's HSD test.

We also observed a decrease in *Ralstonia* on Day 49 in both control and treated samples. In parallel to fungi, the bacterial α-diversity in the treated skin samples was higher than the control samples.

**DISCUSSION**

To test the influence of host immune responses on microbiome assembly, especially the endophytes that may have a profound impact on postharvest apple rot, a systemic acquired resistance inducer, ASM, was applied on Gala trees one week before harvest. We demonstrate that induction of the immune response by ASM application significantly changed the microbiota composition, especially the endophytes, in post-harvest apple fruits.

Our results showed that despite the ASM application, the distinctness in microbiota composition between tissue types and timepoints at postharvest was maintained in both control and treated samples. However, in the fungal community the dissimilarity in the microbiota composition between the tissue types, pulp and skin, was no longer observed in Day 49 in both control and treated samples **(Figure 4.1 C & D)**. The study revealed that induction of immune response had an impact on microbial community composition especially the endophytes. In the pulp samples, we observed that the treated samples clustered away from the control samples until Day 25 of post-harvest as illustrated by PCoA based on Bray-Curtis dissimilarity. This pattern was shown in both bacterial and fungal communities. Interestingly, the dissimilarity in the microbiota composition between treated and control samples was no longer observed in Day 49 of post-harvest **(Figure 4.2 A & 4.3 A)**. This might indicate that at Day 49 stage of post-harvest the microbiota in the fruit is dominated by opportunistic microbes suppressing the growth of beneficial or other commensal microbes. I have previously demonstrated that around Day 49, the PTI immune response of the fruits collapses with increased relative abundance of spoilage

microbes such as *Alternaria* among fungi and *Gluconobacter* and *Acetobacter* among bacteria (Chapter 3). Our result here also showed an increase in those microbes in Day 49 in both treated and control samples which validates the previous observation. This also suggests that the immune response can be induced in healthy apple fruits but that there is a threshold somewhere between 25- and 49-days post-harvest past which the immune response can no longer be sufficiently activated, at which point dysbiosis sets in. This is an important finding for postharvest treatments of apple fruits at post-harvest.

ASM application did not have an equal effect on the epiphytes where a clustering was seen only at Day 0 in fungal community and Day 25 in bacterial community. This signifies that the immune response has more impact on the endophytes than the epiphytes or that the ASM preferentially induces immune response in pulp rather than skin tissue. Endophytes remain in close contact with the host and promote host health by different mechanisms such as increasing nutrient uptake, inducing resistance against pathogens, and increasing plant tolerance to salinity, low temperature, heavy metals, contaminated chemicals, and other abiotic factors (Oukala et al., 2021). Studies have also shown that the host plant shapes endophytic communities (Christian et al., 2016). Therefore, our findings reinforce the fact that host immune response plays an important role in shaping endophytes which further provides beneficial effects to the host. Kithan-Lundquist has also shown that apple fruit endophytes are derived mostly from the host tree through the flowers, but the fruit epiphytes are most influenced by the environment. Collectively, this evidence suggests that recruitment of endophytes is influenced by the host immunity.

*Fusarium* was one of the fungal taxa that was found at higher relative abundant in the treated samples than in the control samples in the earlier times points. The genus *Fusarium* is

one of the most abundant endophytic fungal genera, comprising approximately 70 species with a wide range of hosts. They are also a good source of secondary metabolites with structural and chemical diversity (Ahmed et al., 2023). *Fusarium solani* strain K has been shown to induce defense response in tomato plants and protect against spider mite (Garantonakis et al., 2018; Pappas et al., 2018). However, some genera belonging to *Fusarium* also cause disease in plants and fruits (Ma et al., 2013). Therefore, further study is required to investigate the role of endophytic *Fusarium* in priming defense response in healthy apples. Interestingly, fungal genera belonging to *Naganishia* were found to be relatively abundant in control but were absent from treated samples in earlier timepoints. It is plausible that there is also a microbe-microbe interaction where an increase in one or more specific species leads to a decrease in other species. Another fungal endophyte that needs investigation is *Erythrobasidium*, which was found in substantial relative abundance in treated samples in Day 0 and Day 7 but was not observed in control samples. To date, the effects of *Erythrobasidium* on fruit health has not been reported and warrant future investigation. Future studies should also focus on assessing the role of the immune response on the endophytic microbiome and the direct or indirect effects of those microbes with the host immune response and their influence in the health of the fruit.

Among bacteria, despite dissimilarity shown in β-diversity, there were no noticeable differences in relative abundance of the prevalent taxa between the control and treated samples with an exception on Day 25 with an increase in relative abundance of *Pseudomonas* in both tissues. Hence, the differences in the β-diversity could be attributed to the presence of transient and rare taxa. This primary survey study provides first-hand essential knowledge on the impact of preharvest ASM applications on the overall microbial ecology at post-harvest. In the future, this could lead to improved fruit microbiome-based orchard management and preventative

strategies for post-harvest loss. In summary, our results indicate that induction of host fruit immune responses have a clear impact on the endophytic community that potentially promotes a longer shelf life. Future studies could specifically focus on an in-depth analysis of the differential abundant microbes found between control and treated samples by isolating them and characterizing their role in the overall health of the fruit.

## MATERIALS AND METHODS

### Sampling Procedures

Healthy apple fruits were collected from the cultivar Gala at MSU Plant Pathology Farm. Eight trees (not adjacent to each other) were selected at random and 40 fruits per tree were sampled from around the circumference of the tree; each tree consisting of one replicate for a total of eight replicates. For treated samples, the same sample collection protocol was followed, however, ASM was applied a week before harvest, at a concentration of 50 mg/L. The fruits from each tree were maintained separately in clean plastic bins stored at room temperature in the laboratory. At each sampling point, five fruits were randomly selected from each replicate and pooled to make one biological replicate for a total of eight biological replicates per time point for both control and treated. From each apple, two tissue types (skin and pulp) were sampled. Fruit pulp was excised using a sterile cork-borer and a sterile blade to peel a thin layer of the skin. Immediately, each sample tissue was homogenized separately in liquid nitrogen. The homogenized samples were stored at -80°C for subsequent DNA. Sampling of the remaining apple fruits was repeated every two weeks until the seventh week (Day 49) after harvest.

### DNA Extractions and Sequencing of 16S rRNA and ITS genes

Microbial genomic DNA samples were extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, United States) following the instructions of the manufacturer.

Extracted DNA was used as the template for amplicon PCR reactions that amplified the bacterial 16S ribosomal region and fungal internal transcribed spacer (ITS) 1 region. The V4 region of the 16S rRNA was amplified using the universal 515F/806R primer set (Caporaso et al., 2011), and the ITS1 region was amplified using the ITS1F/ITS2 primer set (Ghannoum et al., 2010; White et al., 2013). Peptide nucleic acid (PNA) clamps (Lundberg et al., 2013) were added to the 16S rRNA PCR mix (total of 20 µl) with a concentration of 2.5 µM mitochondrial PNA and 2.5 µM plastid PNA to block amplification of host plastid and mitochondrial 16S DNA. Raw sequence data preparation and data analysis was performed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) (Bolyen et al., 2019). Primer and adapter sequences were removed using cutadapt, paired reads were joined, samples were denoised with dada2, and chimeric sequences were removed using VSEARCH (Rognes et al., 2016). Operational taxonomic units (OTUs) were identified at 97% similarity using QIIME's sklearn classifier and the SILVA database v132 for bacteria (Yilmaz et al., 2014). CONSTAX2 was used with the UNITE fungal general release dataset from Nov. 29, 2022 to assign fungal taxa (Abarenkov et al., 2020) with 80% confidence threshold and recommended settings (Liber et al., 2021). Finally, sequences assigned to host mitochondria and chloroplasts were discarded.

**Data Processing and Analysis**

The OTU table, taxonomy, metadata, and phylogenetic tree were imported into the R package Phyloseq v.1.24.2 (McMurdie and Holmes, 2013). Host mitochondria and chloroplast OTUs were removed. Alpha diversity was estimated with Shannon diversity index to determine the evenness based on the presence of rare OTUs (singletons and doubletons), respectively using Phyloseq v.1.24.2 (McMurdie and Holmes, 2013). Statistical significance was calculated by Analysis of Variance (ANOVA). Beta diversity was analyzed to compare the microbiome

composition among groups, based on the Bray-Curtis dissimilarity distance matrix. The ordination was calculated by Principal coordinates analysis (PCoA). To compare the microbiome composition between time points, statistical significance was calculated with permutational multivariate analysis of variance (PERMANOVA) using the vegan package v.2.6-4 (Oksanen et al., 2022). To visualize the relative abundances of OTUs/ASVs, a bar plot was constructed using the ggplot2 package (Wickham, 2016).

Differentially abundant taxa were identified using DESeq2 v.1.38.3 to extract the main effects of time point in each cultivar and tissue type (McMurdie and Holmes, 2013; Love et al., 2014). Taxa with similar trends in abundance over time were grouped using hierarchical and K means clustering. The elbow method was used to identify the optimal number of clusters. Abundance trends were visualized using pheatmap v.1.0.12 (Kolde, 2022) and ggplot2 v.3.5.0. Indicator species analysis was performed using indicspecies v.1.7.14 to identify possible unique taxa and compositional signatures at each timepoint (De Caceres and Legendre, 2009).

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## **APPENDIX A:** SUPPLEMENTARY FIGURES

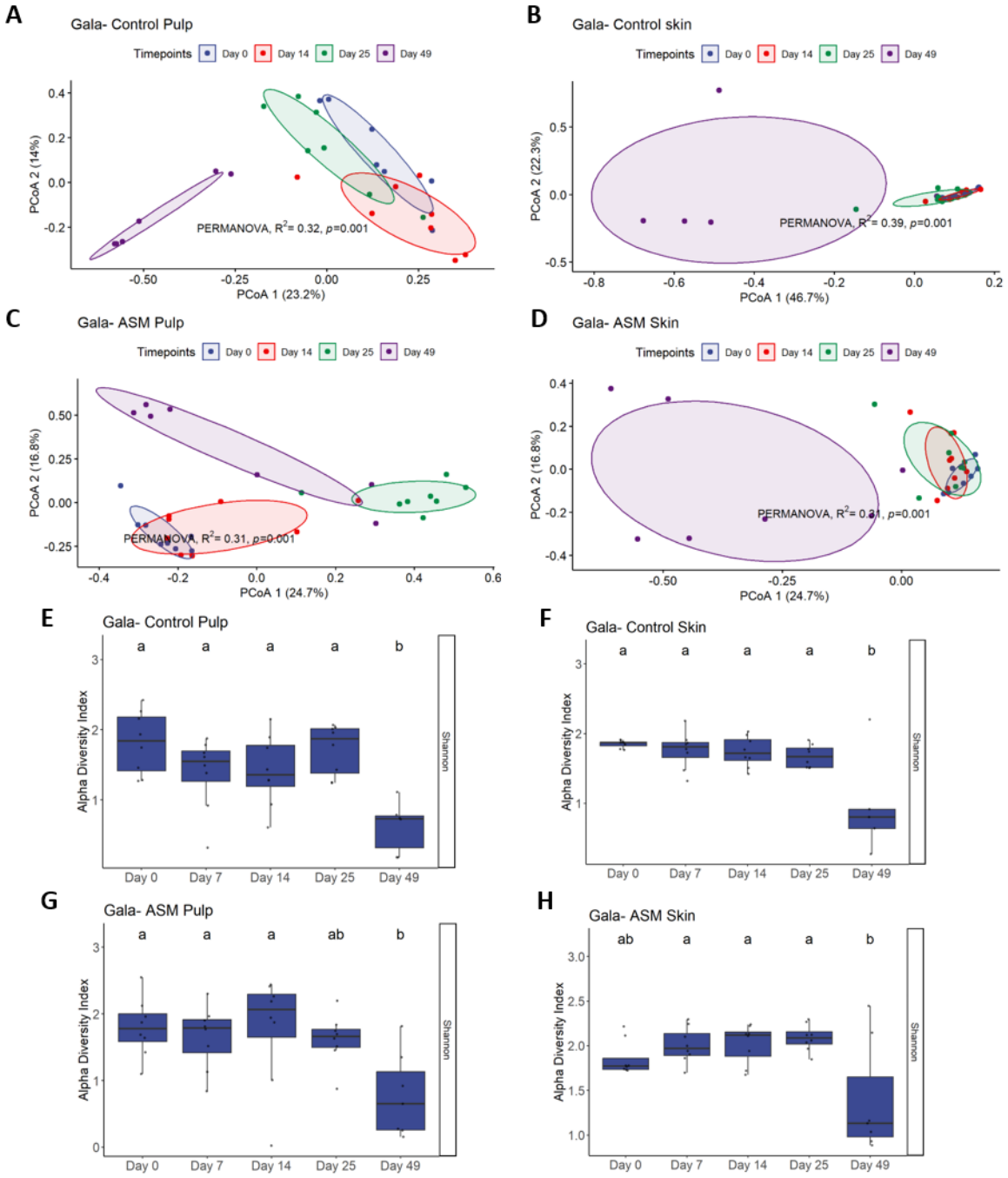


**Figure S4.1.** Principle coordinates analysis (PCoA) plot based on Bray-Curtis dissimilarity metrics comparing fungal communities across timepoints in control pulp, control skin, treated pulp and treated skin (**A, B, C & D**), respectively. Box plots showing fungal α-diversity comparison between timepoints based on the Shannon index in control pulp, control skin, treated pulp and treated skin (**E, F, G & H**), respectively. The different letters above each bar indicate statistically significant differences at $P_{adj} < 0.05$ as calculated by Tukey's HSD test.

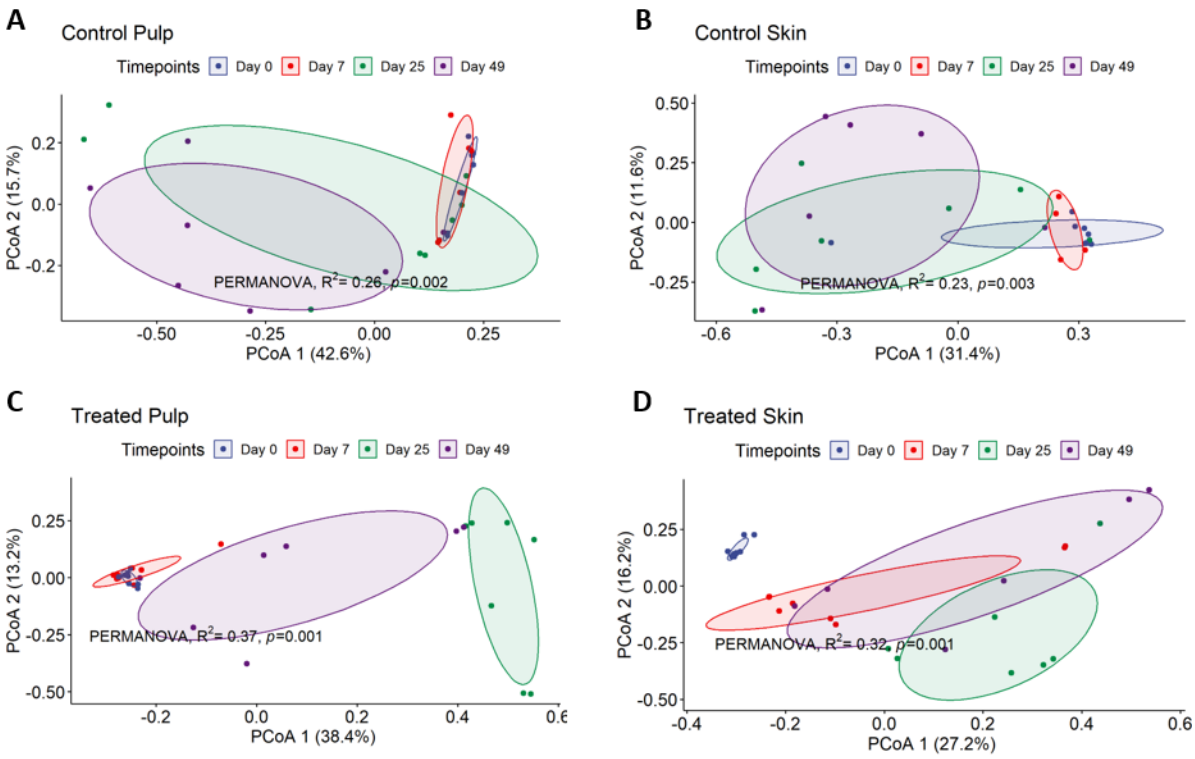


**Figure S4.2.** Principle coordinates analysis (PCoA) plot based on Bray-Curtis dissimilarity metrics comparing bacterial communities across timepoints in control pulp, control skin, treated pulp and treated skin (**A, B, C & D**), respectively.

# CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Post-harvest loss is an enormous issue worldwide. Although food production has increased substantially in recent decades, it is estimated that approximately one-third of the food – and 45-55% of all fruits and vegetables – that are produced are either lost or wasted before reaching the consumer (Gustavsson et al., 2011). Food loss corresponds to about 1.2 to 2 billion tons every year (Porat et al., 2018). Therefore, to compensate for these losses, minimizing post-harvest food losses is more sustainable than increasing production to feed a steadily growing population (Kader, 2005).

Post-harvest fungal decay results mainly from pre-harvest latent infection or wound infection that can occur either before or after harvest (Wenneker and Thomma, 2020). Despite the enormous issue with post-harvest loss of fruit by rotting microorganisms, especially during storage, very little information is known about the relationship between the host, pathogen and residing microbiota in the process of fruit rot. Developing a comprehensive understanding of the interaction between the microbiota and the host to protect against pathogens could be the key for establishing the next green revolution. Identification of: i) specific microorganisms (or a network of microbes) within a particular host microbiota that is involved in initiating the rot leading to decay, and ii) those beneficial microbes that have antagonistic activity towards rotting microbes will be critical to developing effective microbiome-based controls. Thus, the overarching goal of this dissertation is to develop an understanding of the relationship between the host, particularly its immune response, and the microbiota, as well as the impact of this relationship on post-harvest fruit susceptibility to pathogens. Towards this goal, I have pursued three distinct research objectives described in Chapters 2-4 of this dissertation. Below, I summarize the efforts and outcomes of each of these objectives with an eye towards remaining questions and possible future directions.

In Chapter 2 I investigated the microbial community succession and dynamics from flowering to fruit harvest. I also examined the epiphytic and the endophytic microbiota composition over the full developmental time course of apple fruit. Previous studies have shown a clear successional pattern of microbes across different stages of flower development (Shade et al., 2013) and fruit development (Zhimo et al., 2022). However, these studies did not distinguish endophyte and epiphyte composition, either focusing specifically on epiphytes during fruit development or pooling epiphytic and endophytic tissues prior to the analysis during flower development. Furthermore, neither study spanned the full developmental progression from flower bud to fully mature fruit. This has limited our understanding of microbial succession patterns throughout the full season and the source of microbial communities of the harvested apple fruit.

I have demonstrated that distinct community compositions in the tissue types occurred as early as the flowering stage even before the hypanthium had developed into a fruitlet. I also showed that there is a dynamic shift in both epiphytic and endophytic communities starting from full flower bloom to fruit maturation. Despite a significant shift in composition and diversity between full bloom and fruit maturation stage, the endophytic community reverts back to a very similar composition in ripe fruit to that seen in the initial stages around full bloom. This suggests that most endophytic communities may be derived from the flower. The epiphytic community, in contrast, was more dynamic, especially in the early fruit development stages, suggesting a transient imbalance in the microbiota that might have been directly related to external changes in temperature, light, UV radiation, or nutrient availability. Another noteworthy observation was that the abundances of a specific suite of OTUs become relatively constant as the fruit matures, indicating that the epiphytic community reaches a stable level. Together my data suggest that the

host plant has a substantial level of control on the endophytes whereas the epiphytes are predominantly impacted by the environmental factors. My work also showed that antibiotics and fungicides likely have a short-term impact on some non-target prevalent taxa, but that the levels of these taxa are restored by the fruit development stage, indicating a remarkable level of resilience in the microbiota. This suggests a considerable level of control over the microbiome by the host plant potentially mediated, at least in part, by the host immune response.

Some of the prevalent fungal taxa that were consistently identified at every developmental stage but with differing relative abundance, are *Alternaria, Aureobasidium, Cladosporium, Sporobolomyces,* and *Vishniacozyma.* In bacteria, *Pseudomonas, Sphingomonas, Mythylobacterium,* and *Massilia* are seen at every development stage. These taxa were also found to be present in apple fruit across different geographical areas in a study conducted by Abdelfattah, (2021), indicating these taxa may represent a core microbiome of apple fruit, i.e., a set of microbes consistently present over time on a specific host. Future studies will be essential to understand the role of these taxa on fruit development, their antagonistic activity towards pre- and post-harvest pathogens and their disease development at post-harvest. A core or stable microbiome is likely to be critical to host development, health and functioning (Berg et al., 2020). Identification of the core microbiome will enable us to filter out transient taxa and focus on stable taxa that hold a greater likelihood of influencing host phenotype (Berg et al., 2020). Therefore, defining the core taxa is essential in exploring the potential for pre- or pro-biotic treatments that support host health. Additionally, it will be necessary to understand the effects of antibiotics and fungicides on these prevalent taxa, and how the endophytes, but not the epiphytes, were able to restore to pre-treatment levels.

Another question that needs investigation is to explore the factors that contribute to the

stability among the epiphytes during the fruit-development stages. Understanding the succession of a microbial consortia and identification of a stable microbiota within a particular host can provide fundamental supporting the rational manipulation of the microbiome in pre- or post-harvest fruits for resistance against biotic and abiotic stress. My microbiota succession study has allowed an in-depth look into the dynamics of the microbiota composition spanning the flower-to-fruit transition and during apple fruit development, representing a significant step towards this goal.

In Chapter 3 I sought to investigate the temporal dynamics of the fruit microbiota and fruit defense gene expression during post-harvest storage of apple fruits. Other studies have shown that microbial communities of apple fruit undergo changes during cold storage over the course of months (Zhimo et al., 2022), however, these changes necessarily miss the temporal dynamics of the microbiota and the host defense responses under natural progression. Therefore, for my study, the fruits were stored at room-temperature in the lab to relate these dynamics of the microbiota and immune response to fruit decay. Through my research, I have demonstrated that there is a dynamic shift in microbiota composition during storage. I observed an increase in relative abundance of putative spoilage microorganisms with a decrease in putative beneficial microorganisms over time during storage. The fact that I observed beneficial microbes in healthy fruit, and conversely pathogens in unhealthy fruit, is consistent with the notion that the fruit microbiota plays a significant role in dictating fruit health.

PTI is an important component that acts as a host barrier to control the level of commensal microbes and opportunistic microbes from excessive proliferation in the host tissue for optimal plant health (Chen et al., 2020; Song et al., 2023; Pfeilmeier et al., 2024). Interestingly, my work also demonstrated that PTI was highly expressed in healthy apple fruits

but that, over time, the formerly robust PTI declined precipitously in the fruit immune response (as measured by *MdFLS2* and *MdBAK1* expression). The decline in immune response coincides with the timing of the emergence of potential pathogenic microbes. Thus, there seems to be an inverse relationship between PTI gene expression and microbiota dysbiosis, supporting my hypothesized relationship between host immune response and the microbiome. Additionally, induction of the immune response in harvested apple fruit by application of the flg22 peptide, an immune response inducer, showed a delay in the onset of fungal rot in apple fruit suggesting the role of PTI in pathogen suppression and microbial activity.

It will be important to isolate some of the identified putative beneficial strains such as *Sporobolomyces*, *Cuprividus* and *Caluabacter* to understand the underlying factors that account for these strains' contribution to healthy fruit and disease suppression. Some of the most important fungi such as *Neofabraea* spp., *Colletotrichum* spp., and *Alternaria* spp. cause post-harvest apple decay by latent infections (Kim and Xiao, 2008; Wenneker and Thomma, 2020). Some taxa belonging to these genera were seen in high relative abundance in rotting samples indicating the likely cause of rot. However, little is known about the initiation of rot by bacterial spoilage microorganisms such as *Gluconobacter* and *Acidobacter*. Therefore, there is a need for future research to study the role of bacteria in rot initiation. It is also essential to determine if some of these OTUs detected in our study at different time points (healthy vs. unhealthy) act as keystone taxa that have cascading effects in changing apple fruit microbial community dynamics (Shade et al., 2013; Jousset et al., 2017).

Induction of harvested fruit immune responses with the flg22 peptide lead to a delay in the onset of fungal rot in apple fruit, however, there is no knowledge on the impact of flg22 treatment on microbiota composition. Future research is needed to determine if this protection is

associated with composition changes of the microbiota. To my knowledge, this study represents the first exploration of apple fruit microbiota conducted during the post-harvest period and the patterns in apple fruit PTI gene expression patterns under the natural progression of fruit spoilage. My results have implications for the development of strategies to enhance fruit defense for increased fruit quality and prolonged shelf life and may motivate future studies to examine if the relationship observed in apple fruit immunity and microbiome dynamics is generally applicable to other post-harvest fruits and vegetables.

In Chapter 4 I tested the influence of host immunity on microbiome composition by applying an analog of salicylic acid, ASM, which induces host PTI. These results demonstrated a clear shift in microbial composition if pre-treated with an application of ASM, particularly among the endophytes. As the endophytes are expected to hold a profound impact on fruit rot, these results point to a mechanism by which ASM works to delay fruit rot. Significantly, these results also support my hypothesized relationship between host immunity and the microbiome composition, indicating that host immune response plays an important role in microbiome assembly and structure.

Despite ASM treatment, the distinct microbiota compositions between tissue types and post-harvest timepoints were maintained. However, by day 49 after harvest, differences in microbial community composition between pulp and skin were not observed. Additionally, I could no longer observe the dissimilar microbiota composition between ASM-treated and control tissue at day 49, suggesting dominance by opportunistic microbes. This aligns with the collapse of the PTI immune response and the rise of spoilage microbes like *Alternaria*, *Gluconobacter*, and *Acetobacter* around day 49.

It is remarkable that the ASM application had a clearer impact on the endophytes than the epiphytes since the epiphytes on the fruit surface are directly exposed to the ASM unlike the endophytes embedded in the fruit pulp. This provides further support that it is the host immune system induced by the ASM that is responsible for driving the shift in microbial composition, rather than the ASM directly impacting microbial growth. These findings underscore the importance of the host immune response in recruiting beneficial endophytes which could have significant implications for post-harvest treatment strategies to extend the shelf-life of apple fruits.

There are alternatives to ASM for inducing immune responses in apple trees. These alternatives include a variety of chemical and biological stimulators known as plant resistance inducers (PRIs) or elicitors that can activate plant defenses through exogenous application and are considered potential options to reduce the use of pesticides (Marolleau et al., 2017). Some PRIs act as non-self determinants, mimicking microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs), including the flg22 peptide described above, and which are perceived by pattern recognition receptors (PRRs) present at the cell surface. Others may mimic plant downstream signaling molecules such as phytohormone analogs or derivatives (Marolleau et al., 2017). For instance, potassium phosphites have been used as trunk-injected plant resistance inducers and have shown significant induction of pathogenesis-related protein genes in apple leaves, indicating the induction of systemic acquired resistance (SAR) under field conditions (Acimovic et al., 2015). In light of the results of my own studies, I predict that these alternative inducers can similarly impact the microbiota through induction of host immunity. It may prove insightful to test various combinations of inducers to identify the taxa that are impacted by induction of immune response and to explore their role on the impacted taxa

in post-harvest disease and health during storage. This would also assist in the identification of an optimal combination and concentration of inducers to delay fruit rot in apples and other fresh produce.

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