HortScience 60(9):1462–1468. 2025. https://doi.org/10.21273/HORTSCI18722-25

# Packing Line Processes Reshape Apple Microbiomes: Differential Effects of Chlorine and Waxing on Bacterial and Fungal Communities

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*Keywords.* food safety, *Malus* ×*domestica* Borkh., metagenomics, methane, *Penicillium*, postharvest

***Abstract.*** **Modern postharvest apple processing facilities include steps to ensure the safety and storage of fresh-market fruit. Packing lines typically include a chlorine sterilization process before fruit waxing to maximize postharvest fruit quality. We investigated the role of chlorine dump tank sanitation and waxing in shaping microbiome community assembly on apples, which is relevant for postharvest pathogen colonization and infection. We hypothesized that each step of postharvest processing would indicate further shifts in fruit microbiome composition, notable at the prechlorine and postchlorine dump tank stages, followed by the fruit-waxing step. We found that the packing line process affected bacterial composition, but there were minimal effects on fungal composition and only at specific sampling times. In addition, adding wax to fruit increased bacterial diversity, but there was no effect with chlorine sanitation. Bacterial shifts were strongest after the waxing step, with more than half of all genera increasing in relative abundance. Using PICRUSt2, we predicted metagenomic profiles and observed that taxonomic shifts corresponded with a variety of metabolic pathways increasing in abundance, including an unexpected increase in pathways associated with methanogenesis. Although PICRUSt2 predictions on their own are prone to false positives, this finding coincides with the presence of known methanogens of the class Methanobacteria in waxed samples. The results suggest that commercial packing line processes shape the apple microbiome, uncovering potentially novel functions such as methane regulation in postharvest waxed fruit, with implications for fruit preservation and quality.**

The microbial communities on fresh produce are comprised of multifaceted connections that potentially affect the preservation and quality of fruit (McLaren and Callahan 2020; Wisniewski and Droby 2019). Since the advent of next-generation sequencing, many studies have been conducted to characterize the assembly of the fruit microbiome in an effort to decipher how microbiome composition and function influence fruit development and storage. The factors that shape microbiome composition that are well understood include cold storage (Abdelfattah et al. 2020; Al Riachy et al. 2024; Biasi et al. 2021; Bösch et al. 2021; Lane et al. 2023, 2024a, 2024b; Shen et al. 2018; Wassermann et al. 2019a; Zhimo et al. 2022); management regime such as conventional vs. organic (Abdelfattah et al. 2016a, 2021; Bartuv et al. 2023; Leff and Fierer 2013; McLaughlin et al. 2023; Schiavon et al. 2023; Shen et al. 2022; Vepštaitė-Monstavičė et al. 2018; Wassermann et al. 2019b; Wicaksono et al. 2023); postharvest treatments such as waxing, hot water, and cold plasma (Abdelfattah et al. 2020; Bösch et al. 2021; Fang et al. 2023; Shen et al. 2018; Wassermann et al. 2019a; Wicaksono et al. 2022); chemical applications such as fungicides and ethylene inhibitors (Abdelfattah et al. 2016b; Lane et al. 2023, 2024a, 2024b; McLaughlin et al. 2024; Perazzolli et al. 2014); and the use of biocontrol agents (Biasi et al. 2021; Duan et al. 2024; Zhao et al. 2023a, 2023b; Zhimo et al. 2021).

Although there are many studies looking at fruit microbiomes at harvest and throughout storage, less is known about microbiome shifts during poststorage packing. For market distribution, stored apples are graded and packed, with modern packing lines using hydrohandling, a process in which bins are submerged in water fumes to float the fruit gently, for efficient emptying. The fruit are then floated through chlorinated water flumes onto a conveyor belt and sorted, with undersized, defective, damaged, or decayed fruit removed. The fruit are then sprayed with a food-grade wax (carnauba, candelilla, or shellac) to improve appearance, reduce water loss and respiration rates, and extend the shelf life of the fruit (Bai et al. 2003; Hasan et al. 2024). Washing and waxing with an edible coating was shown to shift microbiomes, with waxing increasing the relative abundance of *Pseudomonas* and washing decreasing the abundance of *Aureobasidium* (Abdelfattah et al. 2020); however, the work by Abdelfattah et al. (2020) was carried out in a laboratory setting, and therefore different from commercial packing lines. Although packhouse treatments affect mandarin oranges (Kumar et al. 2021), the absence of key apple microbiome members such as *Curtobacterium* and *Aureobasidium* (Lane et al. 2023, 2024a, 2024b) limits the transferability of these findings to apple systems.

In our study, we investigated the effect of fruit packing line processes, such as hydraulic unloading using dump tanks with chlorinated flumes, followed by waxing, on the fruit surface microbiome. We hypothesized that the chlorinated dump tank method would affect bacteria and fungi sensitive to the antimicrobial sanitizer, whereas the wax addition would shift microbiomes that are responsive to modifications of the fruit surface that affect fruit respiration and moisture loss.

## Materials and Methods

*Experimental design and sampling.* 'Honeycrisp' apples were sampled from the packing line of a commercial orchard in western New York, USA, Oct 2023, Mar 2024, and Jul 2024. Samples were collected from apples at three stages of the packing line—1) in the bin before hydraulic unloading into the dump tank, 2) in the dump tank containing chlorine (oxidation reduction potential = 750 mV; Accu-tab, Westlake Water Solutions, Houston, TX, USA), and 3) after the waxing step (AP531 FD; Pace International, Wapato, WA, USA)—and before being dried with hot air and packaged. Samples were also taken after wax application and drying in March and July. These samples showed minimal to no differences from the predried waxed samples, so they were excluded from the main analysis to keep the experimental design balanced. Eight replicates of four apples each were taken for each packing line step during every sampling month, resulting in a total of 72 samples (3 packing line steps × 3 months × 8 replicates).

For microbiome sampling, each sample of four apples was placed in a zippered plastic bag with a 6.5-pH 0.5-M phosphate buffer solution with 0.1% Polysorbate 80. Each sample was sonicated for 20 min, then underwent rotary shaking at 120 rpm for 20 min before

Received for publication 12 May 2025. Accepted for publication 16 Jun 2025.
Published online 5 Aug 2025.
This research was supported in part by the intramural research program of the US Department of Agriculture, National Institute of Food and Agriculture (Hatch no. 2021-22-186). Additional support was provided by a Cornell Diversity Fellowship and Pomology Endowment funds to C.L.
We thank Austin Fowler (Fowler Farms, Wolcott, NY, USA) for access to the packing line and fruit.
The findings and conclusions in this preliminary publication have not been formally disseminated by the US Department of Agriculture and should not be construed to represent any agency determination or policy.
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being filtered through 0.22-μL filter paper to capture microbes. After DNA extraction, samples were sent to Novogene Corp. (Sacramento, CA, USA) for quality control, amplification, and sequencing of the 16S V3 + 4 region for bacteria and the internal transcribed spacer (ITS2) region for fungi with the Illumina Novoseq 6000 platform (Illumina, San Diego, CA, USA), followed by initial filtering and merging of paired end sequences. Further information on equipment used and sequencing procedures can be found in Lane et al. (2023).

*Sequence processing.* Sequences were processed in QIIME2 version 2021.11 (Bolyen et al. 2019). First, sequences were denoised and chimeric sequences were removed with DADA2 using default parameters (Callahan et al. 2016). The resulting amplicon sequencing variants (ASVs) underwent additional chimera removal using uchime (Edgar et al. 2011). ASVs were then assigned taxonomy based on a naive Bayes classifier trained on the UNITE v. 10 database for ITS and the Greengenes2 2022 database for 16S (Abarenkov et al. 2024; McDonald et al. 2024). Chloroplast and mitochondrial sequences were then removed from the 16S dataset. ASVs were then run through the PICRUSt2 pipeline, using taxonomy to obtain predicted counts of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologies (KOs) that act as precise units of predicted molecular function (Douglas et al. 2020; Kanehisa and Goto 2000). Last, ASV abundance, KO, and taxonomy tables were exported along with representative sequences for subsequent analysis in R v. 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria). To maximize the data obtained from each sample and avoid discarding valid data, rarefication was not performed (McMurdie and Holmes 2014).

For the ITS fungal samples, a total of 2147 ASVs were obtained, with a minimum final filtered read count in a sample of 16,163 and a maximum count of 76,379. For the 16S bacterial samples, a total of 5874 ASVs were obtained, with a minimum final filtered read count in a sample of 37,081 and a maximum count of 59,958.

*Statistical analysis.* The associations between ASV composition and factors of sampling month and packing line step were assessed by performing a permutational multivariate analysis of variance (PERMANOVA) using 999 permutations (Anderson 2017), with $P < 0.05$ being used as the cutoff for significance. Pairwise PERMANOVAs were used to compare different packing line steps and months using the Holm method for multiple test correction. For interactions between the two, pairwise PERMANOVAs were performed only on differences between packing line steps within the same month. These tests were performed on a Bray–Curtis distance matrix (obtained using the vegan package) of the ASV abundance table (Beals 1984; Dixon 2003). The distance matrix was also visualized with a principal coordinate analysis. Shannon diversity was calculated from the same ASV table and compared across treatment and sampling using an analysis of variance, with a post hoc Tukey test (honestly significant difference test, $P < 0.05$ to determine significance) used to compare packing line steps and months. Tukey tests were also performed for each month to establish significance at different time points.

The R package phyloseq (R Foundation for Statistical Computing) was used to create a genera table that was then assessed by the Maaslin2 package to determine genera and predicted KEGG KOs that were significantly different between packing line steps (Mallick et al. 2021; McMurdie and Holmes 2013). Because the greatest differences were observed after waxing, the two groups compared were the postdump unwaxed apples and the waxed apples. A false discovery rate cutoff of 0.05 was used to determine significance using the Benjamini–Hochberg correction for this exploratory dataset (Benjamini and Hochberg 1995), and all other Maaslin2 parameters were set to default. KOs were then matched to all associated pathways and modules using the KEGG application programming interface to determine which ones have many KOs associated with waxing.

Table 1. $P$ and $r^2$ values for bacterial and fungal composition and Shannon diversity, derived from amplicon sequencing variant tables.

| $P$ and $r^2$ values[i] | Packing line step | Sampling time | Packing line step × sampling time |
|---|---|---|---|
| Bacterial composition $P$ value | 0.001 | 0.001 | 0.001 |
| Bacterial composition $r^2$ value | 0.183 | 0.107 | 0.0832 |
| Bacterial diversity $P$ value | $2.03 \times 10^{-8}$ | 0.0103 | 0.185 |
| Fungal composition $P$ value | 0.158 | 0.001 | 0.034 |
| Fungal composition $r^2$ value | — | 0.395 | 0.0574 |
| Fungal diversity | 0.0410 | $1.51 \times 10^{-6}$ | 0.154 |

[i] For composition, $P$ and $r^2$ values are determined from a permutational multivariate analysis of variance with 999 permutations on a Bray–Curtis dissimilarity matrix. For diversity, $P$ values are determined by an analysis of variance.

## Results

*Microbiome composition and diversity.* Bacterial composition was affected by packing line step, sampling time, and their interactions, whereas fungal composition was unaffected by the packing line step (Table 1). For bacteria, all packing line steps harbored different microbiomes (Table 2), although the waxed apple microbiome was distinctly different from unwaxed apples (Fig. 1A). The observation was reinforced further by the interaction between packing line step and sampling time (Table 1), where the differences between the pre- and postdump unwaxed apples were inconsistent between sampling month (Table 3). Fungal composition also showed this interaction effect, where predump and waxed apples had different microbiomes in March. In fungi, samples also clustered by sampling time, with October and March samples showing the greatest dissimilarity (Fig. 1B). Overall, apple waxing showed consistent effects on bacterial composition and modest effects on the fungal microbiome, depending on the sampling time.

Bacterial diversity increased after waxing across all samples taken together (Table 2), and in the specific sampling time months of October and July (Fig. 2A). No changes in diversity were observed between pre- and postdump unwaxed apples. In addition, although sampling month was a predictor of bacterial diversity (Table 1), no pairwise differences were found between months (Table 2). Fungal diversity was more strongly associated with sampling time, with October having the lowest diversity (Table 2, Fig. 2B). Packing line step was also a predictor, with no overall pairwise differences being observed (Table 2), but waxed apples had higher diversity than postdump unwaxed apples in March (Fig. 2B). These results largely line up with composition, with waxing having mostly consistent effects on bacteria and some context-dependent effects on fungi.

*Microbiome taxonomy and predicted metagenome function.* The makeup of bacterial and fungal genera at each packing line step in different months is shown in Fig. 3. In bacteria, the most abundant overall genera were *Curtobacterium* (22.52%), *Pseudomonas* (20.41%), and *Sphingomonas* (6.11%), whereas in fungi the most abundant genera were *Aureobasidium* (33.55%), *Cladosporium* (19.61%), and *Sarocladium* (5.04%). In bacteria, 128 of 257 genera increased in relative abundance from the postdump to the waxing step, and 11 decreased in abundance. For bacterial genera with greater than 1% abundance, *Acinetobacter* and *Microbacterium* increased in abundance after waxing, whereas *Curtobacterium, Massilia,* and *Pseudomonas* decreased in abundance

Table 2. Significance letters for bacterial and fungal composition and diversity at different packing line steps and sampling month.[i]

| Variable | Predump | Postdump | Waxed | October | March | July |
|---|---|---|---|---|---|---|
| Bacterial composition | a | b | c | A | B | C |
| Bacterial diversity | a | a | b | — | — | — |
| Fungal composition | — | — | — | A | B | C |
| Fungal diversity | — | — | — | A | B | B |

[i] For composition, pairwise permutational multivariate analyses of variance adjusted with the Holm method were used, whereas for diversity, post hoc Tukey tests were used. Treatments/time points with non-overlapping significance letters show significant differences at $P < 0.05$.

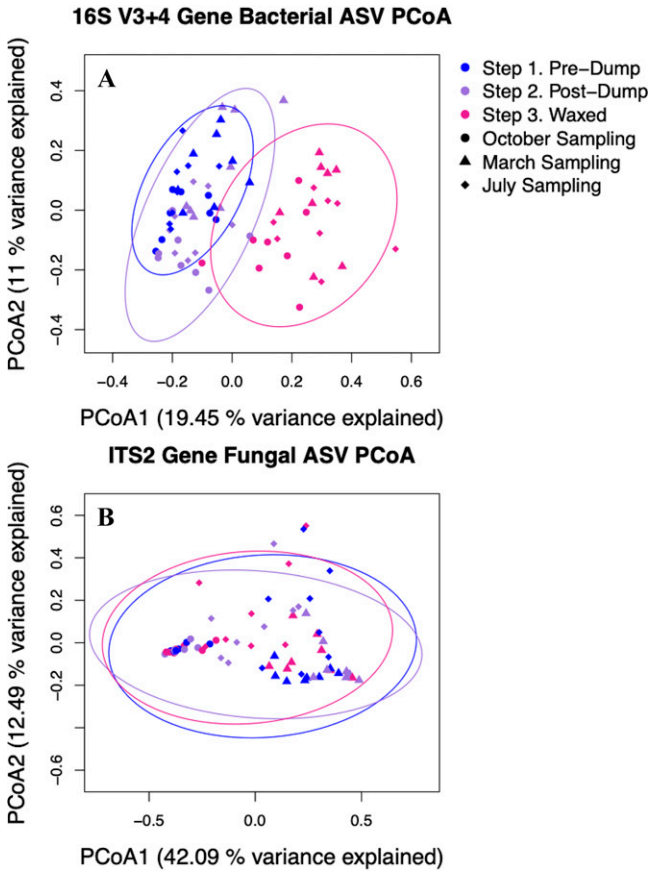


Fig. 1. Principal coordinate analysis (PCoA) using Bray–Curtis dissimilarity of bacterial (**A**) and fungal (**B**) amplicon sequencing variants (ASVs) colored by packing line step and with different symbols indicating sampling month. Ellipses represent 95% confidence intervals for each packing line step.

after waxing. In addition, bacterial genera remained relatively stable across different sampling times despite the overall microbiome shifting with sampling time (Fig. 3A), whereas fungal genus-level composition shifted drastically (Fig. 3B). For instance, *Aureobasidium* was the dominant fungal genus in October; but, in March, *Cladosporium* had higher abundance.

Taxonomic shifts resulting from waxing were associated with changes in predicted metabolic function of bacteria as well, with predicted methanogenesis increasing after waxing (Fig. 4A). We found that 32.37% of KEGG KOs associated with metabolic pathways increased in abundance and 7.19% decreased in abundance after waxing. Although not perfectly aligned with genus-level shifts, where 49.81% of genera increased in abundance and 4.28% decreased in abundance, these results indicate that the observed taxonomic changes are predicted to result in differences in functional metagenomic makeup as well. Of the KOs within specific categories, 53.38% of KOs associated with methane metabolism pathways increased in abundance, along with 73.68% of KOs associated with methanogenesis modules. Of the common methanogenic taxa, the only class present in more than one sample was Methanobacteria, which is absent from unwaxed apples but is found in two thirds (16 of 24) of waxed apple samples. The overall abundance of Methanobacteria was low and appears to vary based on sample time, but was present in waxed samples at all time points (Fig. 4B). Therefore, the stable presence of Methanobacteria points toward methanogenesis, with predicted metagenome function suggesting that this shift can be picked out at the whole microbiome scale.

## Discussion

Consistent with prior apple studies, we observed microbiome shifts after waxing; however, the specific nature of these shifts differed from previous findings. For example, some researchers found that *Pseudomonas* increased in relative abundance immediately after waxing (Abdelfattah et al. 2020), whereas we found that *Pseudomonas* decreased in relative abundance after waxing, which was most clearly observed in the March and July sampling times (Fig. 3A). In addition to differences between laboratory-scale and commercial-scale packing lines between the studies, this may be the result of sampling differences. The previous study sampled endophytes in ground tissue whereas our work focused on epiphytes sampled using a wash method.

Many other factors may have shaped our findings as well, as microbiomes showed substantial differences depending on sampling month. Given the large body of research describing microbial shifts throughout extended cold storage (Abdelfattah et al. 2020; Al Riachy et al. 2024; Biasi et al. 2021; Bösch et al. 2021; Lane et al. 2023, 2024a, 2024b; Shen et al. 2018; Wassermann et al. 2019a; Zhimo et al. 2022), it is likely that these differences in microbiome composition result in different responses to packing line steps. An additional factor is that each sampling time used a different lot, which could have influenced results. However, given that apples have a core set of microbiota found in all measured contexts (Abdelfattah et al. 2021), it is unsurprising that there is some consistency in microbiome responses, which makes our results more generalizable. Even when the microbial communities are different, such as those seen in a previous study in mandarin fruit with minimal overlap in high-abundance microbial genera with our results (Kumar et al. 2021), shifts were observed as a result of waxing. Therefore, although the specifics of microbiome shifts resulting from packing line drenching and waxing may depend on the context of the preexisting microbial community, the presence of these shifts is consistent for bacteria.

Our study also highlights the mixed evidence for microbial diversity responses to packhouse treatments. Our results show that waxing increases diversity in bacteria (Table 2), whereas previous work found that washing and waxing decrease diversity (Abdelfattah et al. 2020), or that waxing increases diversity in fungi but not in bacteria (Kumar et al. 2021). In our study, the increased bacterial diversity can likely be attributed to a decrease in the abundance of the dominant *Curtobacterium* and *Pseudomonas* (Fig. 3A). *Pseudomonas* is of particular interest because of its capability to act as a plant pathogen or to defend plants from pathogens, depending on the species, with the difference not able to be detected by amplicon sequencing (Melnyk et al. 2019). Therefore, disrupting the bacterial community through waxing may have either positive or negative effects on the apple fruit, depending on context.

The most novel result from our study is the consistent increases in abundance of KEGG KOs associated with methane dynamics after waxing, which coincides with an

Table 3. Significance letters for bacterial and fungal composition at different packing line steps within each month.[i]

| Variable | Predump | Postdump | Waxed |
|---|---|---|---|
| Bacterial composition October | a | b | c |
| Bacterial composition March | a | a | b |
| Bacterial composition July | a | b | c |
| Fungal composition March | a | ab | b |

[i] Pairwise permutational multivariate analyses of variance were used, adjusting all within-month comparisons using the Holm method. $P < 0.05$ was used as a threshold for significance, with non-overlapping significance letters indicating treatment differences and only months containing significant differences shown.

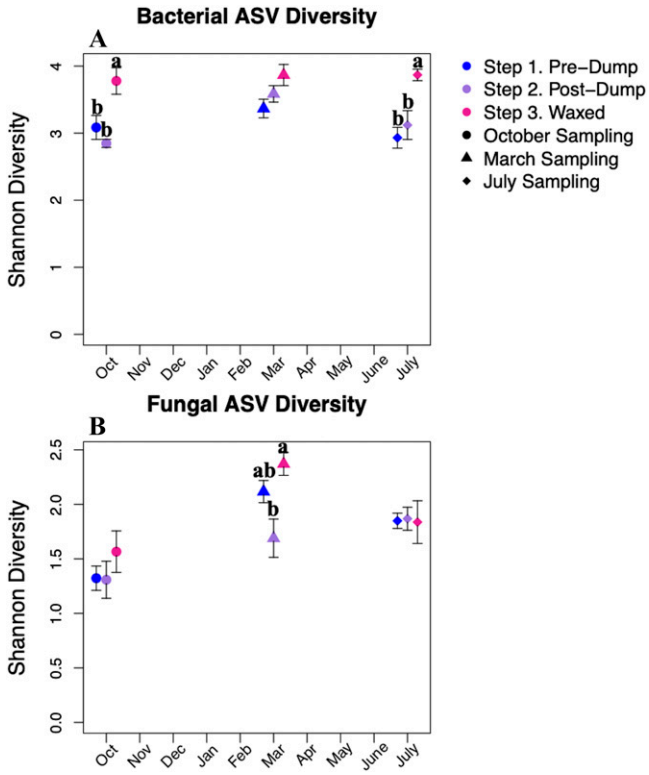


Fig. 2. Shannon diversity of bacterial (**A**) and fungal (**B**) amplicon sequencing variants (ASVs) by sampling month and packing line step. Post hoc Tukey tests at each sampling month were used to generate significance letters, where treatments with no overlapping letters are different at $P < 0.05$.

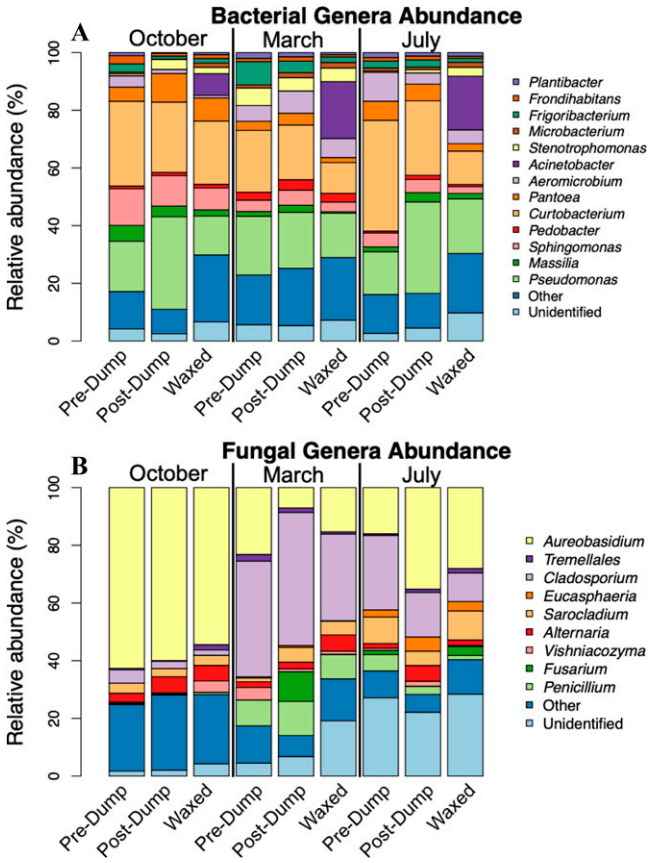


Fig. 3. Relative abundance of bacterial (**A**) and fungal (**B**) genera at each packing line step within each month. Genera with less than 1% abundance are grouped under "Other."

increased abundance of the archaean methanogen class Methanobacteria (Fig. 4B). Because metagenomes were predicted from taxonomic data using PICRUSt2 (Douglas et al. 2020) rather than directly sequenced using shotgun metagenomics, these assessments of metagenomic function bring high risk of false positives. In addition, we see that many metabolic pathways increased in abundance after waxing, and that the percentage of KEGG KOs associated with methane dynamics was modestly, but not drastically, higher than the overall percentage of metabolic KOs that increased in abundance (Fig. 4A). However, in this case, the importance of methanogenesis after waxing was also reflected in the presence of known methanogens, so conclusions around methane do not solely rely on PICRUSt2's metagenome prediction. On the other hand, amplicon sequencing risks picking up relic DNA from nonliving organisms, so the presence of Methanobacteria in low abundance is not guaranteed to reflect real methanogenesis. Nevertheless, taxonomic data show the presence of methanogens after waxing, and functional prediction suggests that this shift is more consistent than other categories of KOs despite the low abundance of methanogens.

The presence of methanogens may indicate that anaerobic microbes are able to exist on the apple surface after waxing. Anaerobic agricultural environments such as rice paddies have long been known to promote methanogenesis (Holzapfel-Pschorn et al. 1985; Liesack et al. 2000). Although edible coatings similarly limit respiration by reducing gas exchange and creating an oxygen-limited environment, they are designed to avoid depleting oxygen to less than 1% to 3% to prevent fermentation (Bai et al. 2003). Methanogens are obligate anaerobes and cannot survive in this environment (Buan 2018). As a result, previous work on microbial responses to low oxygen controlled-atmosphere (CA) storage found no evidence of increased anaerobic metabolism or methanogenesis (Lane et al. 2023). However, with wax, it is likely that there are anaerobic pockets within the wax where methanogens can persist. Although the abundance of methanogens is low (Fig. 4B), a predicted shift toward methanogenesis was the most consistent change in predicted metagenomic makeup (Fig. 4A). Beyond methanogens, many bacterial genera increased in abundance after waxing, with few decreasing, which could result from new niches or the decrease in the abundance of *Curtobacterium* and *Pseudomonas*, resulting in higher relative abundance of many rare genera.

Beyond the ecology of methanogens, the finding of predicted methane-related pathways and modules increasing in abundance after waxing may be important for postharvest physiology. Methane affects many aspects of plant physiology, from tolerance against abiotic stresses to interactions with signaling molecules (Li et al. 2020). In particular, methane has been shown to inhibit polyphenol oxidase activity (Hu et al. 2018), which is responsible for browning in apples (Murata et al. 1995). Although the archaeal methanogen class Methanobacteria had low relative

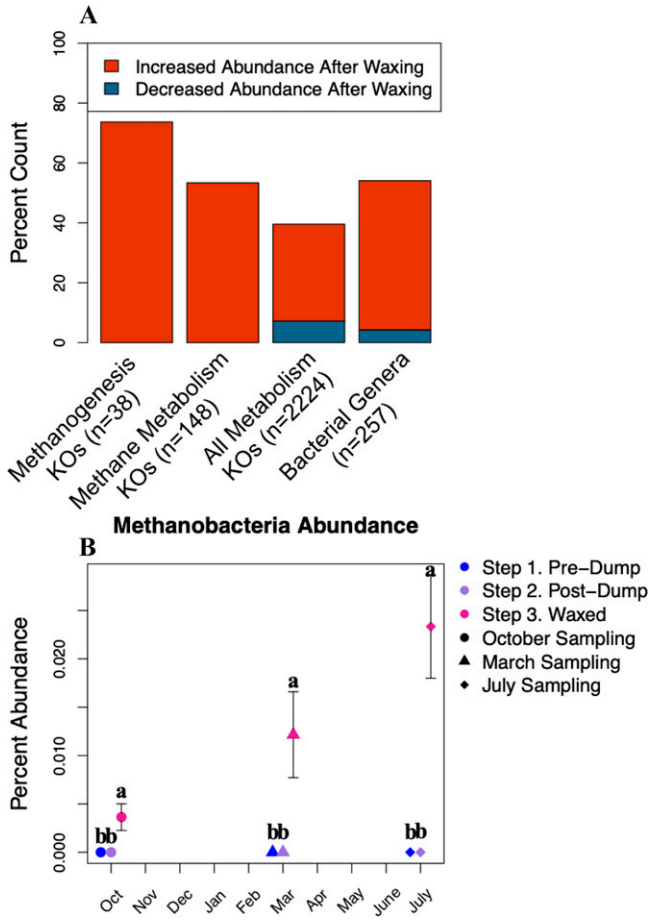


Fig. 4. (**A**) The percentage of Kyoto Encyclopedia of Genes and Genomes orthologies (KOs) associated with methanogenesis modules, methane metabolism pathways, and all metabolism pathways that increased or decreased in abundance in waxed apples, as well as bacterial genera. Significance in abundance changes were corrected using the Benjamini–Hochberg method. (**B**) The percentage of abundance of bacteria in the Methanobacteria class by sampling month and packing line step. Significance letters, where treatments with no overlapping letters are different at $P < 0.05$, were determined by testing all bacterial classes at each sampling month and correcting with the Benjamini–Hochberg method.

abundance even when present in our samples (Fig. 4B), this is also true for methanogenic archaea present in rice paddies, which account for substantial anthropogenic methane production (Lee et al. 2015). Therefore, our findings that predicted KOs related to methanogenesis increase in abundance after waxing may indicate a shift in methane production and fruit physiology. These results highlight how predictive genomic tools can be used to explore plant–microbe interactions on the whole microbiome level.

Although bacteria shifted with packing line treatment, we did not find any evidence of fungi doing so. This may be further indirect evidence for the importance of waxing reducing available oxygen on the peel surface, as fungi are obligate aerobes and therefore would not have the same ability as bacteria to shift to accommodate low-oxygen or anaerobic conditions. This has been shown in previous work that found low-oxygen CA conditions shaping microbiome composition in bacteria, but not fungi, during extended storage (Lane et al. 2023). This is contrasted by previous work that found strong effects of waxing on the fungal community (Abdelfattah et al. 2020; Kumar et al. 2021), although waxing in these studies only occurred at one time point. Therefore, our work contributes to a body of mixed evidence about waxing affecting fungi on a whole microbiome scale.

Where fungi did show larger shifts was in sampling time. Unexpectedly, early-storage October samples showed greater dissimilarity from mid-storage March samples than they did from late-storage July samples (Fig. 1B). This contrasts with previous work that showed that fungal communities tend to shift gradually throughout cold storage, becoming more differentiated over time (Abdelfattah et al. 2020; Biasi et al. 2021; Lane et al. 2024b). One possible explanation for this is that, although the commercial packing line in this study used apples from the same orchard, the fruit at the three time points used likely came from different orchard blocks. Therefore, the large differences in fungal microbiomes at the October and March time points may result from qualities of those specific orchard blocks in addition to storage time length. Yet, if this is true, it further highlights the consistency of observed bacterial dynamics across different orchard blocks.

We did not find evidence of the packing line process decreasing the abundance of postharvest pathogens such as *Penicillium*, which was present at noticeable abundances (Fig. 3B). This is similar to findings on sanitizing agents in mandarin fruit, where *Penicillium* was abundant across all steps (Kumar et al. 2021). Several other studies on apple microbiomes report on *Penicillium* at an overall > 1% abundance across healthy fruit (Abdelfattah et al. 2016a; Lane et al. 2023, 2024a; McLaughlin et al. 2023; Shi et al. 2022; Wassermann et al. 2019a), with some showing increased abundance after long-term cold storage (Abdelfattah et al. 2020; Shen et al. 2018), that aligns with high abundances in March and July in our study. In addition, multiple studies on fungicides show that application does not decrease abundance of target pathogens (Karlsson et al. 2014; Lane et al. 2024a). Therefore, it is likely that *Penicillium* may be common in apple microbiomes even without infection. However, this does not mean that sanitization is ineffective. Culturing assays have been used to show that chlorine sanitization reduces the viability of *Penicillium expansum* on stored apples (Chen et al. 2004; Salomão et al. 2008). This represents a limitation in the ability of microbiome sequencing to convey postharvest pathogen dynamics fully, suggesting that future studies could benefit from pairing amplicon sequencing with culture-dependent methods.

## Conclusion

We investigated the effects of packing line dump sanitation and waxing on the 'Honeycrisp' apple microbiome at different times during the storage year. Even though fruit were obtained from different orchard lots, the packing line treatments shifted bacterial microbiomes. The largest shift occurred at the waxing step, which affected bacterial and fungal diversity, with waxed apples having the greatest diversity. These dynamics were influenced by the context of variable preexisting microbiomes at different sampling times, yet responses were generally consistent. Bacterial taxonomic shifts were widespread, with many genera increasing in abundance. This corresponded to a diverse array of metabolic functions in the predicted metagenome increasing in abundance, the most notable of which was methanogenesis. These results indicate that commercial packing line treatments affect microbial communities on apple surfaces in a variety of ways, highlighting the interconnected nature between the microbiology of postharvest storage and the microbiology of food handling.

### References Cited

Abarenkov K, Nilsson RH, Larsson KH, Taylor AFS, May TW, Frøslev TG, Pawlowska J, Lindahl B, Põldmaa K, Truong C, Vu D, Hosoya T, Niskanen T, Piirmann T, Ivanov F, Zirk A, Peterson M, Cheeke TE, Ishigami Y, Jansson AT, Jeppesen TS, Kristiansson E, Mikryukov V, Miller JT, Oono R, Ossandon FJ, Paupério J, Saar I, Schigel D, Suija A, Tedersoo L,

Kõljalg U. 2024. The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: Sequences, taxa and classifications reconsidered. Nucl Acids Res. 52(D1):D791–D797. https://doi.org/10.1093/nar/gkad1039.

Abdelfattah A, Freilich S, Bartuv R, Zhimo VY, Kumar A, Biasi A, Salim S, Feygenberg O, Burchard E, Dardick C, Liu J, Khan A, Ellouze W, Ali S, Spadaro D, Torres R, Teixido N, Ozkaya O, Buehlmann A, Vero S, Mondino P, Berg G, Wisniewski M, Droby S. 2021. Global analysis of the apple fruit microbiome: Are all apples the same? Environ Microbiol. 23(10): 6038–6055. https://doi.org/10.1111/1462-2920.15469.

Abdelfattah A, Whitehead SR, Macarisin D, Liu J, Burchard E, Freilich S, Dardick C, Droby S, Wisniewski M. 2020. Effect of washing, waxing and low-temperature storage on the postharvest microbiome of apple. Microorganisms. 8(6):944–921. https://doi.org/10.3390/microorganisms8060944.

Abdelfattah A, Wisniewski M, Droby S, Schena L. 2016a. Spatial and compositional variation in the fungal communities of organic and conventionally grown apple fruit at the consumer point-of-purchase. Hortic Res. 3:16047. https://doi.org/10.1038/hortres.2016.47.

Abdelfattah A, Wisniewski M, Li Destri Nicosia MG, Cacciola SO, Schena L. 2016b. Metagenomic analysis of fungal diversity on strawberry plants and the effect of management practices on the fungal community structure of aerial organs. PLoS One. 11(8):e0160470. https://doi.org/10.1371/journal.pone.0160470.

Al Riachy R, Strub C, Durand N, Chochois V, Lopez-Lauri F, Fontana A, Schorr-Galindo S. 2024. The influence of long-term storage on the epiphytic microbiome of postharvest apples and on *Penicillium expansum* occurrence and patulin accumulation. Toxins (Basel). 16(2):102. https://doi.org/10.3390/toxins16020102.

Anderson MJ. 2017. Permutational Multivariate Analysis of Variance (PERMANOVA), p 1–15. Wiley StatsRef: Statistics Reference Online. https://doi.org/10.1002/9781118445112.stat07841.

Bai J, Hagenmaier RD, Baldwin EA. 2003. Coating selection for 'Delicious' and other apples. Postharvest Biol Technol. 28(3):381–390. https://doi.org/10.1016/S0925-5214(02)00201-6.

Bartuv R, Berihu M, Medina S, Salim S, Feygenberg O, Faigenboim-Doron A, Zhimo VY, Abdelfattah A, Piombo E, Wisniewski M, Freilich S, Droby S. 2023. Functional analysis of the apple fruit microbiome based on shotgun metagenomic sequencing of conventional and organic orchard samples. Environ Microbiol. 25(9): 1728–1746. https://doi.org/10.1111/1462-2920.16353.

Beals EW. 1984. Bray–Curtis ordination: An effective strategy for analysis of multivariate ecological data. Adv Ecol Res. 14:1–55. https://doi.org/10.1016/S0065-2504(08)60168-3.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc Ser B. 57(1):289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

Biasi A, Zhimo VY, Kumar A, Abdelfattah A, Salim S, Feygenberg O, Wisniewski M, Droby S. 2021. Changes in the fungal community assembly of apple fruit following postharvest application of the yeast biocontrol agent *Metschnikowia fructicola*. Horticulturae. 7(10):360. https://doi.org/10.3390/horticulturae7100360.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu Y-X, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 37(8):852–857. https://doi.org/10.1038/s41587-019-0209-9.

Bösch Y, Britt E, Perren S, Naef A, Frey JE, Bühlmann A. 2021. Dynamics of the apple fruit microbiome after harvest and implications for fruit quality. Microorganisms. 9(2):272–215. https://doi.org/10.3390/microorganisms9020272.

Buan NR. 2018. Methanogens: Pushing the boundaries of biology. Emerg Top Life Sci. 2(4): 629–646. https://doi.org/10.1042/ETLS20180031.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 13(7):581–583. https://doi.org/10.1038/nmeth.3869.

Chen L, Ingham BH, Ingham SC. 2004. Survival of *Penicillium expansum* and patulin production on stored apples after wash treatments. J Food Sci. 69(8):C669–C675. https://doi.org/10.1111/j.1750-3841.2004.tb18016.x.

Dixon P. 2003. VEGAN, a package of R functions for community ecology. J Veg Sci. 14(6): 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x.

Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, Huttenhower C, Langille MGI. 2020. PICRUSt2 for prediction of metagenome functions. Nat Biotechnol. 38(6): 685–688. https://doi.org/10.1038/s41587-020-0548-6.

Duan Z, Song H, Shi H, Gao Z, Mao J, Cao Y, Huo H, Li J, Wang X, Lin M. 2024. Postharvest treatment with *Bacillus velezensis* LX mitigates disease incidence and alters the microbiome on kiwifruit surface. Postharvest Biol Technol. 211(1):112843. https://doi.org/10.1016/j.postharvbio.2024.112843.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 27(16):2194–2200. https://doi.org/10.1093/bioinformatics/btr381.

Fang Q, Cao J, Zhong C. 2023. Effect of cold plasma on microbial community of 'Golden Delicious' apples in cold storage. Food Sci. 44(11):143–151. https://doi.org/10.7506/spkx1002-6630-20220509-111.

Hasan MU, Singh Z, Shah HMS, Kaur J, Woodward A. 2024. Water loss: A postharvest quality marker in apple storage. Food Bioprocess Technol. 17(8): 2155–2180. https://doi.org/10.1007/s11947-023-03305-9.

Holzapfel-Pschorn A, Conrad R, Seiler W. 1985. Production, oxidation and emission of methane in rice paddies. FEMS Microbiol Ecol. 31(6): 343–351. https://doi.org/10.1111/j.1574-6968.1985.tb01170.x.

Hu H, Liu D, Li P. 2018. Methane delays the senescence and browning in daylily buds by re-established redox homeostasis. J Sci Food Agric. 98(5):1977–1987. https://doi.org/10.1002/jsfa.8681.

Kanehisa M, Goto S. 2000. KEGG: Kyoto encyclopedia of genes and genomes. Nucl Acids Res. 28(1):27–30. https://doi.org/10.1093/nar/28.1.27.

Karlsson I, Friberg H, Steinberg C, Persson P. 2014. Fungicide effects on fungal community composition in the wheat phyllosphere. PLoS One. 9(11):e111786. https://doi.org/10.1371/journal.pone.0111786.

Kumar A, Zhimo VY, Biasi A, Feygenberg O, Salim S, Wisniewski M, Droby S. 2021. Impact of packhouse treatments on the peel microbiome of mandarin fruit (cv. Orr). Postharvest Biol Technol. 176:111519. https://doi.org/10.1016/j.postharvbio.2021.111519.

Lane C, Al Shoffe Y, Kao-Kniffin J, Watkins CB. 2023. Aminoethoxyvinylglycine and low oxygen controlled atmosphere storage shift functional microbiomes of 'Gala' apples. Postharvest Biol Technol. 206:112546. https://doi.org/10.1016/j.postharvbio.2023.112546.

Lane C, Al Shoffe Y, Kao-Kniffin J, Watkins CB. 2024a. Organic fungicides and diphenylamine shift microbiomes of 'Fuji' apples during storage. Postharvest Biol Technol. 218:113196. https://doi.org/10.1016/j.postharvbio.2024.113196.

Lane C, Al Shoffe Y, Schafran P, Li FW, Kao-Kniffin J, Watkins CB. 2024b. Amplicon sequencing and shotgun metagenomics reveal functional impacts of aminoethoxyvinylglycine-mediated ripening and cold storage on the microbiome of 'NY1' apples. Postharvest Biol Technol. 213:112969. https://doi.org/10.1016/j.postharvbio.2024.112969.

Lee HJ, Jeong SE, Kim PJ, Madsen EL, Jeon CO. 2015. High resolution depth distribution of bacteria, archaea, methanotrophs, and methanogens in the bulk and rhizosphere soils of a flooded rice paddy. Front Microbiol. 6:639. https://doi.org/10.3389/fmicb.2015.00639.

Leff JW, Fierer N. 2013. Bacterial communities associated with the surfaces of fresh fruits and vegetables. PLoS One. 8(3):e59310. https://doi.org/10.1371/journal.pone.0059310.

Li L, Wei S, Shen W. 2020. The role of methane in plant physiology: A review. Plant Cell Rep. 39(2):171–179. https://doi.org/10.1007/s00299-019-02478-y.

Liesack W, Schnell S, Revsbech NP. 2000. Microbiology of flooded rice paddies. FEMS Microbiol Rev. 24(5):625–645. https://doi.org/10.1016/S0168-6445(00)00050-4.

Mallick H, Rahnavard A, McIver LJ, Ma S, Zhang Y, Nguyen LH, Tickle TL, Weingart G, Ren B, Schwager EH, Chatterjee S, Thompson KN, Wilkinson JE, Subramanian A, Lu Y, Waldron L, Paulson JN, Franzosa EA, Bravo HC, Huttenhower C. 2021. Multivariable association discovery in population-scale meta-omics studies. PLoS Comput Biol. 17(11):e1009442. https://doi.org/10.1371/journal.pcbi.1009442.

McDonald D, Jiang Y, Balaban M, Cantrell K, Zhu Q, Gonzalez A, Morton JT, Nicolaou G, Parks DH, Karst SM, Albertsen M, Hugenholtz P, DeSantis T, Song SJ, Bartko A, Havulinna AS, Jousilahti P, Cheng S, Inouye M, Niiranen T, Jain M, Salomaa V, Lahti L, Mirarab S, Knight R. 2024. Greengenes2 unifies microbial data in a single reference tree. Nat Biotechnol. 42(5):715–718. https://doi.org/10.1038/s41587-023-01845-1.

McLaren MR, Callahan BJ. 2020. Pathogen resistance may be the principal evolutionary advantage provided by the microbiome. Phil Trans R Soc Lond B Biol Sci. 375(1808):20190592. https://doi.org/10.1098/rstb.2019.0592.

McLaughlin MS, Yurgel SN, Abbasi PA, Ali S. 2024. The effects of chemical fungicides and salicylic acid on the apple microbiome and fungal disease incidence under changing environmental conditions. Front Microbiol. 15(2): 1342407. https://doi.org/10.3389/fmicb.2024.1342407.

McLaughlin MS, Yurgel SN, Abbasi PA, Prithiviraj B, Ali S. 2023. Impacts of abiotic factors on the fungal communities of 'Honeycrisp' apples in Canada. Microb Biotechnol. 16(8):1639–1656. https://doi.org/10.1111/1751-7915.14207.

McMurdie PJ, Holmes S. 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 8(4):e61217. https://doi.org/10.1371/journal.pone.0061217.

McMurdie PJ, Holmes S. 2014. Waste not, want not: Why rarefying microbiome data is inadmissible. PLoS Comput Biol. 10(4):e1003531. https://doi.org/10.1371/journal.pcbi.1003531.

Melnyk RA, Hossain SS, Haney CH. 2019. Convergent gain and loss of genomic islands drive lifestyle changes in plant-associated *Pseudomonas*. ISME J. 13(6):1575–1588. https://doi.org/10.1038/s41396-019-0372-5.

Murata M, Tsurutani M, Tomita M, Homma S, Kaneko K. 1995. Relationship between apple ripening and browning: Changes in polyphenol content and polyphenol oxidase. J Agric Food Chem. 43(5):1115–1121. https://doi.org/10.1021/jf00053a001.

Perazzolli M, Antonielli L, Storari M, Puopolo G, Pancher M, Giovannini O, Pindo M, Pertot I. 2014. Resilience of the natural phyllosphere microbiota of the grapevine to chemical and biological pesticides. Appl Environ Microbiol. 80(12):3585–3596. https://doi.org/10.1128/AEM.00415-14.

Salomão BCM, Aragão GMF, Churey JJ, Worobo RW. 2008. Efficacy of sanitizing treatments against *Penicillium expansum* inoculated on six varieties of apples. J Food Prot. 71(3):643–647. https://doi.org/10.4315/0362-028x-71.3.643.

Schiavon G, Garello M, Prencipe S, Meloni GR, Buonsenso F, Spadaro D. 2023. Essential oils reduce grey mould rot of apples and modify the fruit microbiome during postharvest storage. J Fungi. 9(1):22. https://doi.org/10.3390/jof9010022.

Shen Y, Nie J, Dong Y, Kuang L, Li Y, Zhang J. 2018. Compositional shifts in the surface fungal communities of apple fruits during cold storage. Postharvest Biol Technol. 144(98):55–62. https://doi.org/10.1016/j.postharvbio.2018.05.005.

Shen Y, Zhang J, Nie J, Zhang H, Bacha SAS. 2022. Apple microbial communities and differences between two main Chinese producing regions. Food Qual Saf. 6:1–11. https://doi.org/10.1093/fqsafe/fyab033.

Shi Y, Yang Q, Zhao Q, Dhanasekaran S, Ahima J, Zhang X, Zhou S, Droby S, Zhang H. 2022. *Aureobasidium pullulans* S-2 reduced the disease incidence of tomato by influencing the postharvest microbiome during storage. Postharvest Biol Technol. 185:111809. https://doi.org/10.1016/j.postharvbio.2021.111809.

Vepštaitė-Monstavičė I, Lukša J, Stanevičienė R, Strazdaitė-Žielienė Ž, Yurchenko V, Serva S, Servienė E. 2018. Distribution of apple and blackcurrant microbiota in Lithuania and the Czech Republic. Microbiol Res. 206:1–8. https://doi.org/10.1016/j.micres.2017.09.004.

Wassermann B, Kusstatscher P, Berg G. 2019a. Microbiome response to hot water treatment and potential synergy with biological control on stored apples. Front Microbiol. 10:2502. https://doi.org/10.3389/fmicb.2019.02502.

Wassermann B, Müller H, Berg G. 2019b. An apple a day: Which bacteria do we eat with organic and conventional apples? Front Microbiol. 10(7): 1629–1613. https://doi.org/10.3389/fmicb.2019.01629.

Wicaksono WA, Buko A, Kusstatscher P, Cernava T, Sinkkonen A, Laitinen OH, Virtanen SM, Hyöty H, Berg G. 2023. Impact of cultivation and origin on the fruit microbiome of apples and blueberries and implications for the exposome. Microb Ecol. 86(2):973–984. https://doi.org/10.1007/s00248-022-02157-8.

Wicaksono WA, Buko A, Kusstatscher P, Sinkkonen A, Laitinen OH, Cernava T, Berg G, Virtanen SM, Hy H. 2022. Modulation of the food microbiome by apple fruit processing. Food Microbiol. 108:104103. https://doi.org/10.1016/j.fm.2022.104103.

Wisniewski M, Droby S. 2019. The postharvest microbiome: The other half of sustainability. Biol Control. 137:104025. https://doi.org/10.1016/j.biocontrol.2019.104025.

Zhao Q, Shi Y, Legrand Ngolong Ngea G, Zhang X, Yang Q, Zhang Q, Xu X, Zhang H. 2023a. Changes of the microbial community in kiwifruit during storage after postharvest application of *Wickerhamomyces anomalus*. Food Chem. 404(Pt A):134593. https://doi.org/10.1016/j.foodchem.2022.134593.

Zhao L, Zhou Y, Liang L, Godana EA, Zhang X, Yang X, Wu M, Song Y, Zhang H. 2023b. Changes in quality and microbiome composition of strawberry fruits following postharvest application of *Debaryomyces hansenii*, a yeast biocontrol agent. Postharvest Biol Technol. 202:112379. https://doi.org/10.1016/j.postharvbio.2023.112379.

Zhimo VY, Kumar A, Biasi A, Abdelfattah A, Sharma VK, Salim S, Feygenberg O, Bartuv R, Freilich S, Whitehead SR, Wisniewski M, Droby S. 2022. Assembly and dynamics of the apple carposphere microbiome during fruit development and storage. Front Microbiol. 13:928888. https://doi.org/10.3389/fmicb.2022.928888.

Zhimo VY, Kumar A, Biasi A, Salim S, Feygenberg O, Toamy MA, Abdelfattaah A, Medina S, Freilich S, Wisniewski M, Droby S. 2021. Compositional shifts in the strawberry fruit microbiome in response to near-harvest application of *Metschnikowia fructicola*, a yeast biocontrol agent. Postharvest Biol Technol. 175:111469. https://doi.org/10.1016/j.postharvbio.2021.111469.