BIOAg Project Report

Report Type: **Progress**

Title: **Integrative analyses of apple fruit associated microbiota for decay prevention and health benefits**

Principal Investigator(s) and Cooperator(s):

Principal Investigator: Franck Carbonero, Assistant Professor, WSU Department of Nutrition and Exercise Physiology

Co-PIs: Achour Amiri, Assistant Professor, WSU Plant Pathology
Charles Edwards, Professor, WSU School of Food Sciences

Abstract:

Microbiota associated with fruits are strongly affected by agricultural practices, but there is only sparse data available on fungal and bacterial diversity in biologically intensive, organic, and conventional apples. Increased knowledge on apple-associated microbiota is necessary to (1) better manage postharvest pathogens of fresh apple fruit, (2) identify apple-associated microbes with potential for spontaneous or semi-controlled cider production, as well as potential probiotic and health properties. This project aims at determining fungal and bacterial microbiota of Washington apples grown in different regions and different agricultural practices. Resulting data will (1) provide useful knowledge for to study microbial interactions that drive decay development and to enhance management strategies and (2) help explore the potential of natural or semi-controlled fermentation for potential prebiotic or probiotic properties.

Project Description:

Outputs

- Overview of Work Completed and in Progress:

Sampling was conducted in organic and conventional orchards over the growing season and post-harvest stages in 2021. Table 1 describes the type and origin of the 360 samples collected pre (flowering and immature fruits) and post-harvest (mature, 1 and 3-month storage). In addition, a partial sampling (in some cases, spraying occurred without notification for timely sampling) was conducted to assess the impact of the fungicide applications.

Table1: Samples type

		Fungicide		Total # samples	
Developmental Stage	Tissue	Before	After	Conventional	Organic
Flowering	Flower	10	10	20	20
Immature Fruit	Calyx	10	0	10	10

	Stem bowl	10	0	10	10
	Peel	10	0	10	10
	Flesh	10	0	10	10
Harvest (mature)	Calyx	0	10	10	10
	Stem Bowl	0	10	10	10
	Peel	0	10	10	10
	Flesh	0	10	10	10
1-month Storage	Calyx	10		10	10
	Stem Bowl	10		10	10
	Peel	10		10	10
	Flesh	10		10	10
3-Month storage	Calyx	10		10	10
	Stem Bowl	10		10	10
	Peel	10		10	10
	Flesh	10		10	10
				180	**180**

- Methods, Results,

Apple samples were stored at low temperature until processing. When enough samples were available, microbial DNA extraction were performed using the CTAB DNA extraction protocol. DNA quality and quantity was confirmed on electrophoresis gel and Nano Drop, and high concentration (>50 ng) measured for all extracts with the expected absorbance ratio quality indicator.

DNA samples will now be subjected to dual-indexed approaches for bacterial and fungal microbiome analyses. Genomic DNA will be quantified with a Qubit Fluorometer. A polymerase chain reaction (PCR) will be set up in a 96 well plate for confirmation of the bacterial DNA quality with 8F and 1541R primers (universal primer: bacterial DNA); and the quality checked with samples randomly selected with a gel electrophoresis.

A second polymerase chain reaction (PCR) will be performed to amplify the V4 region of the 16S rRNA gene (bacteria) or the ITS (fungi) using dual-indexed Illumina primers using a proofreading high fidelity Taq enzyme kit. Amplicons will be purified and normalized using the Invitrogen SequalPrep Kit, and subsequently pooled together as libraries. Libraries will undergo quality controls before sequencing on an Illumina MiSeq. Sequencing files will be stored both on the MiSeq computer and on BaseSpace (cloud space with 1TB per user).

The bacterial amplicon reads will be analyzed using MOTHUR software following the Illumina Standard Operating Procedures (SOP), while fungal sequences will be analyzed with PIPITS. Briefly, sequences will be screened and aligned to the Silva database for the 16S RNA gene sequences. Subsequently, operational taxonomic units (OTUs) and Amplicon Sequence Variants will be picked and assigned to taxonomic groups.

The results of the microbiota counts will be analyzed by the Kruskal–Wallis and Mann–Whitney pairwise tests, and by non-metric multidimensional scaling (NMDS) based on count-distance metrics (Bray–Curtis similarity index) considered to show significant similarities between groups using Past3 software. Each sample's richness and diversity will be evaluated using the Chao1 and the Shannon indices, respectively. To evaluate the effect of different environmental and management factors on microbiota composition, multivariate mixed regression models will be fitted to the normalized counts using the GLMM or GLMIMX (i.e., combination of general linear and mixed modes) approach. To test the association of the composition of microbial community with these covariates of interest, we will use the Permutational Multivariate Analysis of Variance (PERMANOVA).

- Publications, Handouts, Other Text & Web Products: N/A
- Outreach & Education Activities: N/A

Impacts

- Short-Term: Growers in conventional and organic orchards have been informally educated about our research goals and how a holistic perspective can have benefits rather than focusing on the "usual suspects" apple pathogens
- Intermediate-Term: We are getting ready to start experiments of simulated gut microbiome fermentation and intend to begin assessing the potential differential responses with organic and conventional fruits.
- Long-Term: We expect that a better understanding of the interactions and dynamics between known and unknown apple bacterial and fungal microbiota will lead to improved agricultural practices. "Spontaneous fermentation" is somewhat popular for cider production, but often rely on trial-and-error strategies. It is very likely that "yet-to-be cultivated" species can be interesting alternatives to *Saccharomyces* and their isolation can be facilitated by our diversity surveys.

<u>Additional funding applied for/secured:</u>

Application to the Washington Tree Fruit Research Commission submitted in December 2021
Microbiomes of apples and their impact on decay development

Primary PI: Dr. Achour Amiri
Co-PI : Dr. Franck Carbonero

Project Duration: 3 Year
Total Project Request for Year 1 Funding: $73,106
Total Project Request for Year 2 Funding: $81,524
Total Project Request for Year 3 Funding: $86,034

Graduate students funded:
We were not able to identify students with interests and need for financial support related to this project in the time constraints of the grant. Instead, two postdocs have and will be involved with

sample processing and data analyses. Of note, a prospective NEP PhD student has years of experience if food safety and would be a good fit for follow-up research.

Recommendations for future research:
Using the microbiota surveys and gut microbiota experiments as “preliminary data”, we will aim at proposing proposals with mechanistic goals. More applied proposals will focus on understanding the interrelationships between non-pathogens and pathogens and devising preventative strategies. Identification and isolation of species of interest for fermentation will be another avenue. Finally, more fundamental research on the gut microbiome modulation by apple products will also be considered, with possible benefits for marketing for the industry.