SOUR ROT ON GRAPES:
UNDERSTANDING THE ETIOLOGY AND DEVELOPING MANAGEMENT STRATEGIES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Plant Pathology and Plant-Microbe Biology

by

Megan Edina Pond Hall

May 2018

SOUR ROT ON GRAPES: UNDERSTANDING THE ETIOLOGY AND DEVELOPING MANAGEMENT STRATEGIES

Megan Edina Pond Hall, Ph. D.

Cornell University 2018

Sour rot, a disease affecting grapes in viticultural regions worldwide has never been clearly defined. Symptoms of the disease include browning of the berry skin, oozing of the berry pulp and the smell of acetic acid, all in the presence of *Drosophila* spp. We established a method of diagnosing sour rot that includes (i) a rating scale for characterizing visual symptoms of sour rot, which includes the defining characteristic of loss of berry integrity, and (ii) a quantitative measurement of acetic acid content within the berry. Through the isolation of microbes associated with sour rot, and inoculation experiments, we identified several yeast (*Metschnikowia* spp., *Pichia* spp., *Saccharomyces* sp.) and acetic acid bacteria (*Acetobacter* sp. and *Gluconobacter* spp.) that successfully cause sour rot symptoms, when in the presence of *Drosophila* fruit flies. We conducted three years of replicated field trials on the *Vitis* interspecific hybrid cv. Vignoles, in which we targeted these organisms through pre-harvest applications of various antimicrobial agents and an insecticide both alone and in combination. In a separate set of experiments, the use of Illumina sequencing allowed us to characterize the microbial changes on the grape berry surface at five key phenological stages: pea-sized, bunch closure, Veraison, 15° Brix and harvest in 2014 through 2016 in the Finger Lakes, New York, and 2016 in Tasmania, Australia. The

results of this study suggest that terroir is dynamic at the microbial scale, varying significantly not just between regions but also within a region and among years. Finally, grape endophytic microbes were isolated on media conducive to fungi or bacteria and subsequently identified by Illumina sequencing. Species of the yeast genera *Metschnikowia, Pichia,* and *Hanseniaspora* were recovered from every set of samples, as were species of the bacterial genera *Acinetobacter, Burkholderia* and *Bacillus*; species of the bacterial genera *Acetobacter* and *Gluconobacter* also were recovered from vineyard samples from New York and Tasmania and from supermarket-purchased grapes. The endophytic presence of these microbes within grape berries has implications with respect not only to the potential development of sour rot but also to the broader concept of microbial terroir.

BIOGRAPHICAL SKETCH

Megan was born in Toronto, Canada and raised in Portland, Oregon. She returned to Toronto to attend the University of Toronto, where she completed her Bachelor of Arts degree in East Asian Studies, and York University, where she completed her Masters of Arts in Socio-Legal Studies. She then moved to New York City where she worked in campaign politics. In 2011, she returned to her home state of Oregon where she discovered her love of viticulture. She enrolled in the Vineyard Management program at Chemeketa Community College and then as a Post-Baccalaureate student at Portland State University in Biochemistry. While working in the wine industry in the Willamette Valley, she learned the importance of understanding and managing grape diseases. In May 2013, she began her Ph.D. at Cornell University in the Section of Plant Pathology and Plant-Microbe Biology within the School of Integrative Plant Sciences in Dr. Wayne Wilcox's lab, focusing her research on sour rot of grapes. During her Ph.D., she received scholarships from the American Wine Society and the American Society of Enology and Viticulture, both National and Eastern Section, including awards for Best Viticulture Presentation at the 2015 ASEV National and Eastern Section Conferences and the President's Award for Scholarship in Viticulture in 2017. She spent six months at the University of Tasmania thanks to the Fredrick Dreer Award that she received in 2015. She defended her Ph.D. dissertation in December 2017. She is currently an Assistant Research Professor of Viticulture at the University of Missouri in Columbia, where she lives with her husband Alex and daughter Magnolia, who was born in August 2017.

DEDICATION

This dissertation is dedicated to my beautiful and smart daughter, Magnolia Wren. In her short life, she has already taught me to be patient and focus, and has brought joy and laughter into each and every day. Her smile lights up a room, and her curiosity inspires me.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor, Dr. Wayne Wilcox, who is an excellent teacher and has become a great friend. He took a chance on me when he brought me on as his graduate student, and I have worked hard to become a good scientist, with his help. It was a pleasure to be his last student before he entered into retirement.

A great deal of this research would not have been possible without the technical assistance of David Combs, Pamela Raes and Gabrielle Brind'Amour. Their willingness to help, even under tight time constraints, was invaluable. Thank you to my special committee members, Gregory Loeb, Justine Vanden Heuvel, Lance Cadle-Davidson and Kerik Cox, all of whom provided me with great advice and asked tough questions throughout my graduate career.

The community of graduate students, post-docs and researchers in Geneva deserve so many thanks. The long conversations and shared bottles of wine built the foundation for lifelong friendships. Among those that I met in Geneva, I extend a special thank you to Fred Gouker, Jason Londo, Camila Tahim, Luann Preston-Wilsey, Libby Cienewicz, and of course, my husband Alex Fredrickson. Alex's encouragement, support, input, advice and love are unparalleled. He is the partner I always hoped I would find. Life with him is exciting every day.

Last, but certainly not least, I would like to thank my parents, Marilou and Edward, who have supported me through changes in major throughout my undergraduate degree, countless career changes, highs and lows when I doubted my abilities, and their continued excitement through all of my pursuits. They are the parents I aspire to be for my daughter.

TABLE OF CONTENTS

CHAPTER 1

Grape Sour Rot: A Four-Way Interaction Involving the Host, Yeast, Acetic Acid Bacteria, and Insects

Abstract

Sour rot, a disease affecting grapes in viticultural regions worldwide has never been clearly defined. Symptoms of the disease include browning of the berry skin, oozing of the berry pulp and the smell of acetic acid, all in the presence of *Drosophila* spp. We established a method of diagnosing sour rot that includes (i) a rating scale for characterizing visual symptoms of sour rot, which includes the defining characteristic of loss of berry integrity, and (ii) a quantitative measurement of acetic acid content within the berry. This diagnostic evaluation was based on a definition of sour rot as the discoloration and loss of integrity of the grape berry partnered with the leaking of liquefied pulp containing a minimum of 0.83 g/l of acetic acid. Through the isolation of microbes associated with sour rot, and inoculation experiments, we have identified several yeast (*Metschnikowia* spp., *Pichia* spp., *Saccharomyces* sp.) and acetic acid bacteria (*Acetobacter* sp. and *Gluconobacter* spp.) that successfully cause sour rot symptoms when in the presence of *Drosophila* fruit flies. Inoculations when flies are not present do not successfully cause symptoms. We additionally determined that since sour rot symptoms developed in the presence of axenic fruit flies, *Drosophila* likely make a non-microbial contribution.

Introduction

The etiology of sour rot, a disease affecting grapes in temperate viticultural regions, is poorly understood. Disease symptoms are characterized by oxidation of the grape skin in which fruit of both red and white varieties turn brown, with the pulp oozing from

the berries and smelling of acetic acid (and/or, in some reports, ethyl acetate). Numerous fruit flies (or vinegar flies, *Drosophila* spp.) are typically associated with the diseased fruit. In vineyards with susceptible cultivars, the development of sour rot symptoms can be extremely detrimental to the grape crop. Infected clusters are often not harvested or are removed during postharvest sorting due to their unacceptability for fresh use and the risks they present in the winery, as wine made from diseased grapes has been shown to have significantly higher levels of total and volatile acidity (Barata et al. 2011).

Sour rot was originally thought to be the final stage of gray mold, caused by *Botrytis cinerea* (Bisiach et al. 1982; Bisiach et al. 1986), and while it is now accepted as a separate disease, the term is still often applied to a general decay syndrome, which may involve filamentous fungi (Rooney-Latham et al. 2008, McFadden-Smith and Gubler 2015). Several researchers have claimed that yeasts play an essential role in the development of sour rot (Barata et al. 2012a, Bisiach et al. 1982, Guerzoni and Marchetti 1987). Many also have noted the common association of acetic acid-producing bacteria (AAB) with the disease, such as species of *Gluconobacter* and *Acetobacter*, whereas Barata et al. (2012a) concluded that AAB should be considered the etiological agents of sour rot. Guerzoni and Marchetti (1987) investigated the abundance of yeast associated with the disease while also noting the invariable presence of *Drosophila* spp. on rotten grapes. Bisiach et al. (1986) concluded that controlling the disease could only be accomplished by managing *Drosophila* or reducing berry wounds, believing that the insects served as necessary vectors of the causal organisms to these wounds. Barata et al. (2012a) showed that wounded berries

did not develop sour rot when they were physically separated from *Drosophila* spp., and emphasized both the presumed role of these insects as disease vectors in addition to the inability of berries to otherwise naturally heal wound sites when the flies are present, thereby remaining in a susceptible state.

Sour rot has never been clearly defined, leading to disagreement within the published literature over its causal organisms and, consequently, appropriate control practices. Thus, the purpose of our research was to better define the symptoms, mechanism of symptom development, and etiology of this disease.

Materials and Methods

Field samples. In 2013, we collected 16 clusters exhibiting visual and olfactory sour rot symptoms from 12 vineyards in the Finger Lakes region of New York. Each cluster was individually bagged in a low-density polyethylene bag (Fisher Scientific, Pittsburgh, PA) and transported to the laboratory where the entire cluster was macerated in the plastic bag, the juice was extracted into a 50-ml Falcon tube and centrifuged at 4000 x rcf for 10 min. One milliliter of the supernatant from each sample was transferred into a 1.5-ml collection tube, centrifuged at 10,000 x rcf for 10 min, and diluted twofold in water prior to filtration through a 0.2-µm polyethersulfone (PES) membrane (Krackeler Scientific, Inc., Albany, New York) and immediate HPLC analysis. In 2014 and 2015, similarly affected clusters were collected from each of seven and nine vineyard blocks, respectively, in the Finger Lakes region. Whole clusters were bagged individually and transported to the lab, where three symptomatic berries from each of four clusters per vineyard were cut above the pedicel with

surface-sterilized scissors: one from the tip of the cluster and one each from the anterior and posterior sides near the middle of the cluster. The three-berry samples were macerated in the plastic bags and the juice was extracted into 1.5-ml collection tubes. The juice was then homogenized for 30 s using a vortex mixer and centrifuged at 10,000 x rcf for 10 min. The supernatant was transferred into clean collection tubes and stored at -4°C until use, when the clarified juice samples were thawed at room temperature for 30 min and diluted twofold in water prior to filtration through a 0.2-µm PES membrane and immediate HPLC analysis.

An additional four clusters from each of the vineyards sampled in 2014 and 2015 were used to identify sour rot-associated organisms. Three infected berries were selected from each cluster as described above. Each berry was placed into a 50 ml Falcon tube with 5 ml of distilled water, vortexed for 20 s, and 100 µl of the rinsate was then plated onto both Yeast Peptone Dextrose (YPD) medium (2% peptone, 1% yeast extract, 2% glucose, 2% agar) and Yeast Peptone Mannitol (YPM) medium (0.3% peptone, 0.5% yeast extract, 2.5% mannitol, 1.5% agar). The berry was then macerated, and 100 µl of the expressed juice was plated on additional YPD and YPM agar plates. Plates were incubated at 24° C for 3 days, or until distinct colonies developed. One colony was transferred to a 50 ml falcon tube containing 10 ml of sterile distilled water, and was vortexed for 5 s.

Endophytic microbes. To investigate the potential presence of endophytic microbes within healthy grape berries, three such berries from each of three clusters in a population not known to be associated with sour symptoms were cut in half using a sterile razor blade, and each half was placed on either YPD or YPM agar. After three

days, 400 µl of sterile distilled water was pipetted onto the plate and cells of the resulting colonies were disrupted using a disposable plastic spreader. A 400 µl-aliquot of the suspension was then pipetted from the petri dish into a 1.5-ml collection tube and placed into a -4°C freezer.

Ethanol and acetic acid analysis. Acetic acid and ethanol concentrations were quantified using modifications to the method previously described by Castellari (2001). A 20 µl-aliquot of each sample was injected onto a Rezex ROA-Organic Acid H+ ion-exclusion column (Phenomenex, Torrance, CA) at 45°C. Analytes were resolved isocratically using a mobile phase consisting of 6% (v/v) acetonitrile and 0.005N sulfuric acid in water. Both analytes were quantified using external standard curves (run in triplicate, $R^2 > 0.9999$). The acetic acid was quantified using a photodiode array detector monitoring 210 nm and the ethanol was quantified using a refractive index detector set in positive polarity mode and thermostated to 45°C. All samples were analyzed using a Prominence HPLC System (Shimadzu, Kyoto, Japan) with an inline degasser, binary pumps, autoinjector, thermostated column compartment, diode array and refractive index detectors. Data analysis was performed using LCsolution version 1.25 (Shimadzu, Kyoto, Japan).

Microbial determinations. For DNA extraction, one colony of an individual isolate, juice from three macerated grape berries, or 400 µl of thawed microbial suspension was pipetted into a test tube containing 5 ml of TE buffer (10mM Tris-HCl+1mM EDTA, ph 8.0) and 0.05 g NaCl and vortexed for 15 s. Then, 500 µl of 10% SDS was added to the suspension, vortexed for 5 s and left at room temperature for 15 min. A freeze-thaw sequence consisting of 30 min in a -80°C freezer and 5 min in a 60°C

water bath was repeated three times to lyse the fungal and bacterial cells, and 750 µl of the solution was transferred to a centrifuge tube along with 750 µl ice-cold isopropanol. The suspension was centrifuged for 10 min at 9600xg. The supernatant was carefully discarded from the tube, 500 µl of ice-cold 95% ethanol was added, and the tube was again centrifuged at 9600xg for 1 min before discarding the supernatant. The pellet was re-suspended in 100 µl TE buffer and this DNA sample was then stored at 4°C for subsequent amplification and sequencing.

DNA Sequencing. In all DNA sequencing, two primer sets were used. To amplify the V4 domain of bacterial 16s rRNA genes, primers F515 (5′-GTGTGCCAGCMGCCGCGGTAA–3′) and R806 (5′–GGACTACHVGGGTWTCTAAT–3′) were used. Fungal internal transcribed spacer (ITS) 1 loci were amplified using primers BITS (5′–CTACCTGCGGARGGATCA–3′) and B58S3 (5′–GAGATCCRTTGYTRAAAGTT–3′) (Bokulich et al. 2013).

For Sanger sequencing, two polymerase chain reactions (PCR) were performed in 25-µl reaction volumes containing GoTaq® G2 Green Master Mix (Promega Corporation, Madison, WI), 10 mM of each primer and approximately 10 ng genomic DNA. Reaction conditions used to amplify the bacterial amplicons consisted of an initial 94°C for 3 min; followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; followed by a final extension of 72°C for 10 min (Bokulich 2013). Reactions conditions used to amplify the fungal amplicons consisted of an initial 95°C for 2 min; followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s; and a final extension of 72 °C for 5 min. (Bokulich 2013). All amplifications were performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA). PCR

products were separated on a 1.5% agarose gel (Bio-Rad Laboratories Inc.) stained with ethidium bromide in 1× Tris-acetate EDTA buffer at 100 V for 1 h. Photographs of the gel were taken on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY). Amplicons were sequenced at Cornell University's Biotechnology Resource Center in Ithaca, NY.

For Illumina sequencing, Genomic DNA was sent to the Cornell University DNA Sequencing facility in Ithaca, NY for 250-bp-paired-end sequencing on the Illumina MiSeq machine. For each sample, two separate runs were performed. Both forward primers were modified to contain a unique 8-bp barcode. Quality filtering, read processing, and OTU assignment was conducted in Qiime 1.9.1 (Caporaso et al. 2010a). Sequences were trimmed once there were three consecutive bases with PHRED scores less than 20. Sequences less than 100nt were discarded. Open and closed reference OTU-picking methods used uclust and a pairwise identity of 97% (Edgar 2010). Alignment to greengenes 13_5 was done using PyNAST and alignment to UNITE 7_97 was conducted using the BLAST alignment method (Altschul et al. 1990, DeSantis et al. 2006, Caporaso et al. 2010b, Kõljalg et al. 2013). OTUs with than 0.0001% of the total abundance of the biom file were filtered out. Analysis was done in STAMP v2.1.3 and unclassified reads were not included in the analysis but they were kept to calculate abundance frequencies (Parks et al. 2014).

The two most abundant fungal field isolates were also re-submitted for sequencing to obtain more precise identification on YPD media to the Fungus Testing Laboratory at the University of Texas in San Antonio, TX. Identification was conducted by combined phenotypic characterization and DNA sequencing of the ITS region and

D1/D2 region of the large subunit of the 28S ribosomal RNA gene.

Inoculations and disease assessment. Berries were surface sterilized in a 70% ethanol solution for five min and then rinsed in sterile distilled water prior to inoculation. For each experimental unit, three berries were first wounded using a sterile toothpick inserted into the center of the berry and rotated three times, then 50 µl of a microbial suspension was pipetted into the wound.

All three inoculated berries were then placed on a 20-mm filter paper disc moistened with sterile distilled water in a 137-ml polypropylene specimen container (Fisher Scientific, Pittsburgh, PA), the lid was fastened on, and the treatments were incubated at 24°C with 12-h light/dark photocycles for 5 or eight days, depending on the experiment. Unless otherwise noted, there were four replicate experimental units per treatment, and each experiment was repeated. Following incubation, the presence of sour rot was assessed on the basis of both (i) a qualitative rating of visual symptoms on a 0 to 4 scale, where 0 = berry still appears healthy and completely intact; 1 = berry is completely intact, with some discoloration of the skin only around the wound site; 2 = berry is entirely intact, but with obvious discoloration of the skin extending beyond the wound site; 3 = berry has lost turgor and the majority of its skin discolored (early stage of sour rot); and 4 = berry is no longer intact, the inner pulp is liquefied and leaking, and the skin is completely discolored (characteristic sour rot symptoms); and (ii) a quantitative measurement of acetic acid, obtained by subsequently macerating each three-berry sample and subjecting the expressed juice to HPLC analysis, as described above. The ethanol content of each sample was similarly determined.

In preliminary studies utilizing this inoculation technique, we did not obtain typical

sour rot symptoms as seen in the field. Therefore, because of the ubiquity of *Drosophila* associated with sour rot in the field and the previous research in which Drosopholids were determined to play a role in disease development, we included *Drosophila* as a variable in all subsequent inoculation experiments.

Inclusion of *Drosophila melanogaster*. A line of colony-raised *Drosophila melanogaster* was reared for 10 to 14 days on Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply, Burlington, NC) in an incubator at 24°C on a 13-h light / 11-h dark photoperiod at 50 to 60% relative humidity. Flies were released into a 24- x 24- x 24-cm sleeve cage (Bioquip, Rancho Dominguez, CA) that was disinfested with 70% ethanol prior to use. A plastic aspirator, which was also disinfested with 70% ethanol prior to use, was utilized to collect and then release 10 flies at one time into each designated specimen container. All specimen containers, regardless of whether or not they contained flies, were placed on a shelf at room temperature with 13 h light / 11 h dark photoperiod to ensure that the flies remained active.

Drosophila spp. are known to vector microorganisms on their bodies and in their guts, particularly yeast and AAB (Chandler et al. 2011, Wong et al. 2011, Broderick et al. 2014, Staubach et al. 2013, Koyle et al. 2016). Thus, following our initial inoculation experiments with the lab colony of *D. melanogaster*, where it became clear that these insects were exerting a significant effect, we prepared axenic (lacking gut or surface microbiota) flies for subsequent use, following the methods of Koyle et al. (2016) with minor modifications. Approximately 300 colony-reared *D. melanogaster* were released into a sleeve cage containing grape-juice agar plates (10 g torula yeast [no.

1720, Bio-Serve, Flemington, NJ]; 10 g glucose; 1 g agar [no. 7060, Bio-Serve]; 10 g frozen grape juice concentrate in 100 mL water) with yeast paste (1 g active dry yeast [Red Star, Milwaukee, WI] in 15 ml water) smeared on the agar, and left to lay eggs overnight in a 24°C incubation chamber. In a biosafety cabinet, eggs were then collected by rinsing the agar plate with distilled water and pushing the slurry over a sieve made out of nylon mesh in plastic bushing; then, the bushing was placed in a 120-ml specimen container and the eggs were dechorionated by immersing them in three, 90-ml washes of 0.6% sodium hypochlorite of 2.5 min each followed by three rinses of sterile water. Thirty of these eggs at a time were then transferred, using a surface-sterilized paintbrush, to a 50-ml Falcon tube containing 7.5 ml sterile yeast-glucose diet (50g brewer's yeast, 50g glucose and 6g agar in 500 ml distilled water, autoclaved at 121°C). Eggs were then placed in an incubator at 24°C on a 13-h light and 11-h dark photoperiod. When adults developed, 10 axenic flies and 10 lab colony flies (positive control) were placed separately on each of two plates containing YPD and YPM agar at 24°C for 2 days. If no microbial colonies had developed, the axenic flies were then utilized in selected inoculation treatments according to the protocol described above for the lab colony flies.

Evolution of ethanol and acetic acid. In a 2 x 2 factorial design, berries of *V. vinifera* cv. Red Globe were either (i) inoculated with a combination of *S. cerevisiae* and *A. aceti* or not, and (ii) exposed to lab colony flies or not, using the procedures described previously. At the end of each of the 5 subsequent days following inoculation, the berries from each designated specimen cup were macerated and the expressed juice was transferred to a 2-ml tube and frozen for subsequent HPLC

analysis for acetic acid and ethanol content. There were three replicate cups for each treatment x timing combination, each containing four berries. We conducted two replicates of this experiment.

Pathogenicity experiments. *Inoculation in conjunction with wild-type flies.* Using individual isolates of species of filamentous fungi, yeast, and bacteria associated with sour rot in the literature, all obtained from the American Type Culture Collection (ATCC), we conducted inoculations of *V. vinifera* cv. Red Globe berries, purchased from a supermarket. Individual berries were removed from the clusters above the pedicel to avoid wounding them, using surface sterilized scissors. *S. cerevisiae* (ATCC 204508), *P. kluyveri* (ATCC 24209), and *H. uvarum* (ATCC 32369) were cultured on YPD*, A. aceti* (ATCC 15973), *G. oxydans* (ATCC 33448) on YPM, and *A. niger* (ATCC 16888) on potato dextrose agar (PDA). All isolates were incubated at 24°C for three to seven days. One colony from each isolate was transferred to a 50 ml Falcon tube containing 5 ml of sterile distilled water. The spore concentration of every suspension was determined with a hemacytometer and adjusted to 9.0×10^6 cells/ml with sterile distilled water. Inoculations were performed according to the method detailed previously. Inoculation treatments were either exposed to 10 lab-colony *D. melanogaster* adults or not exposed to flies. After five days of incubation, each three-berry sample was rated for sour rot symptoms on the 0-to-4 scale and prepared for ethanol and acetic acid analysis, as described above. We subsequently repeated this experiment.

Inoculation with ATCC cultures in conjunction with axenic flies. Berries of *V. vinifera* cv. Red Globe were inoculated with yeast and bacteria isolates obtained from the

ATCC, and the inoculated berries were either exposed to 10 axenic *D. melanogaster* adults or not exposed to flies. These were the same isolates used in the previously described experiment, but with the addition of axenic as opposed to wild-type flies. After eight days of incubation, each three-berry sample was rated for sour rot symptoms on the 0-to-4 scale and prepared for ethanol and acetic acid analysis.

*Pathogenicity of isolates recovered from field samples***.** 839 isolates from 2014 and 407 isolates from 2015 were sorted morphologically, which resulted in nine distinct morphological groups, the majority of which were in just six of those nine. We then determined how many vineyard sites were represented within each of the morphological categories, to determine which were associated with the sour rot-affected clusters at every site. Four groups were associated with ≥89% of the diseased clusters at every vineyard site and these isolates were used for inoculation of supermarket-purchased Red Globe berries both individually and in various combinations, according to our standard procedures. Treatments were either exposed to 10 axenic *D. melanogaster* adults or not exposed to flies. Disease symptoms and ethanol/acetic acid content were evaluated after eight days of incubation. This experiment was repeated.

Three isolates from each of the six most abundant (represented in >50% of the clusters) morphological groups were sequenced via Sanger sequencing, as described previously. Two isolates from each of the remaining four morphological categories, which were represented in less than 50% of the clusters, were sequenced via Illumina sequencing, as described previously.

Microbial contribution of lab-colony D. melanogaster. To document the contribution

of the resident *D. melanogaster* microbiota within the disease complex, 10 live wild-type adults from our lab colony were released onto plates of YPD medium, which was incubated for 3 days at 24°C on a 13-h light and 11-h dark photoperiod. Then, distilled water was poured onto the plate to create a slurry, and the resulting suspension was pipetted into a 1.5-ml collection tube and used to inoculate supermarket-purchased Red Globe berries. Inoculated and uninoculated berries were exposed to either axenic flies or no flies; a third group of uninoculated berries was exposed to lab-colony flies. Disease symptoms and ethanol/acetic acid content were evaluated after eight days of incubation. We conducted two replicates of this experiment. A 400 µl-aliquot of the inoculum was transferred to a 1.5-ml collection tube and the DNA was extracted and sequenced by the Illumina sequencing methods described above.

Botrytis x sour rot interaction. To investigate the potential competition between the development of Botrytis bunch rot and sour rot on grape clusters, we collected (i) healthy, symptomless clusters; (ii) Botrytis-affected clusters; and (iii) sour rot-affected clusters from two commercial vineyards of *V. vinifera* cv. Riesling in southern Tasmania, Australia and one research vineyard of *Vitis* interspecific hybrid cv. Vignoles in Geneva, NY. All symptomatic clusters were visually determined to have approximately 50% disease severity of either Botrytis bunch rot or sour rot, but appeared free of the other disease. Clusters were cut from the vine using pruning shears and bagged individually in low-density polyethylene bags. In independent experiments examining these two groups, five clusters per experimental unit were arranged on a flat surface in the laboratory within a 3.8-liter sealable plastic bag: four in a square with their edges 1 cm apart and one in the center of this square, with its

edges 1 cm from the edges of the surrounding clusters on all sides. The treatments consisted of four Botrytis-affected clusters surrounding one sour rot-affected cluster; four Botrytis-affected clusters surrounding one healthy cluster; four healthy clusters surrounding one sour rot-affected cluster; and a control containing five healthy clusters. All treatments were incubated for 10 days at room temperature with a 12 h light / 12 h dark photoperiod, at the end of which they were visually rated for the severity of each disease (percent cluster area symptomatic). There were 4 replicate bags per treatment.

Results

Ethanol and acetic acid content, field samples. The mean ethanol and acetic acid concentrations of the whole-cluster samples in 2013 were 2.65 ± 0.42 and 2.41 ± 0.37 g/liter, respectively. In subsequent years, when samples were confined to three symptomatic berries from pre-determined regions of the cluster, the mean values for ethanol were 1.12 ± 0.096 and 1.16 ± 0.16 g/liter in 2014 and 2015, respectively, whereas the mean for acetic acid was 0.95 ± 0.12 and 2.20 ± 0.30 g/liter in 2014 and 2015, respectively. Because we considered the latter measurements to be more precise than the whole-cluster measurements of 2013, as confirmed by their lower standard errors, we established a quantitative acetic acid threshold of 0.83 g/liter as a criterion for assessing the presence of sour rot in our inoculation experiments, which was determined by taking the mean of the 2014 field samples (i.e., the lower of the two years) minus the standard error of that sample set.

Accumulation of ethanol and acetic acid. Ethanol levels across all treatments measured a mean of 1.1 g/liter on the first day following inoculation. This

concentration had tripled by 3 days after inoculation (DAI) when inoculated berries were not exposed to flies, increasing to eight- and 16-fold greater than on the first day at 4 and 5 DAI, respectively. Ethanol accumulated similarly over the first 3 days when inoculated berries were exposed to flies but the rate of accumulation slowed thereafter, with the concentration by 5 DAI only half as great as for the inoculated berries not exposed to flies. Whereas no acetic acid was detected after 3 days of incubation in either of the inoculated treatments, >1 g/liter was detected at 4 DAI when inoculated berries were exposed to flies and this value quadrupled over the next day. In contrast, little acetic acid developed within the inoculated berries that were not exposed to flies, with the final value at 5 DAI only about 10% as great as when similar berries were exposed to the flies (Fig. 1). Modest levels of ethanol developed within uninoculated berries, both with and without exposure to *D. melanogaster*; however, a modest level of acetic acid developed within uninoculated berries only when they also were exposed to the flies. This acetic acid evolution, which occurred during the final day of incubation, was concurrent with a divergence of ethanol accumulation in the two uninoculated treatments, i.e., the ethanol concentrations were not significantly different between the two when assayed at 4 DAI whereas the concentration was significantly higher at 5 DAI in berries not exposed to flies ($P = 0.02$, t-test).

Endophytic microbes. Because we consistently detected ethanol (and, sporadically, acetic acid) in the above and other experiments in treatments where wounded berries were not exposed to a source of microbes and standard sterile techniques had been employed, we investigated the possibility that microorganisms responsible for their production are commonly present as endophytes within healthy berries. To do so,

microbial isolations were attempted from supermarket-purchased 'Red Globe' berries and those of other cultivars representing *V. vinifera*, and *Vitis* interspecific hybrids obtained from six commercial and experimental vineyards in New York, Washington and Tasmania, Australia. The techniques used for microbial isolation, purification, and identification via Illumina DNA sequencing were as described previously. Various yeast genera, primarily *Metschnikowia* and *Pichia* were consistently isolated from berries at every location, as were bacteria in the genera *Bacillus, Pseudomonas, Streptococcus, Acetobacter* and *Gluconobacter* (Hall and Wilcox, *submitted*).

Identification of microbes from field samples. DNA from two replicates of each of the four isolates resulted in two 251-bp bacterial amplicons and two 86-bp fungal amplicons. The two 251-bp fragments were amplified, and a BLAST analysis found one to have 98% (212/216) identity with *Gluconobacter cerinus* and the second to have 97% sequence similarity to many members of Enterobacteriaceae, primarily *Rahnella sp., Yersinia ruckeri* and *Hafnia* sp., with the best match being *Rahnella sp. UIWRF0013* (accession KR189951.1). The fungal fragments had a 95% (54/57) identity with several yeast species, including *Saccharomyces spp.,* and so further sequencing was undertaken to obtain more precise IDs of these two organisms. These results showed that both isolates were members of the genus *Metschnikowia*. Isolates in the groups whose representatives were identified as *Gluconobacter cerinus,* the two species of *Metschnikowia*, and *Rahnella* sp. were recovered from ≥89% of the diseased clusters. Those identified as *Acetobacter pasteurianis* were recovered from 57% of the diseased clusters in 2014 but only 8.3% in 2015, and *Pichia occidentalis* was found in 68% of the diseased clusters in 2014 and 22% in 2015.

Isolates recovered from less than 50% of the clusters were identified as members of *Candida inconspicua* (14.3% in 2014; 26.9% in 2015)*, Pichia membranifaciens* (33.3% in 2014; 48.1% in 2015*), Pichia fermentans* (47.6% in 2014; 22.2% in 2015), or as uncultured bacteria (19% in 2014; 18.5% in 2015) in BLAST searches of the Illumina sequencing data.

Pathogenicity experiments. *Inoculation in conjunction with wild-type D. melanogaster.* Exposure to wild-type flies during the incubation period significantly increased the accumulation of acetic acid in Red Globe berries ($P < 0.001$); little to no acetic acid accumulated in most treatments incubated without exposure to the flies, and concentrations in these treatments never exceeded the 0.83 g/liter threshold established as a criterion for sour rot development (Fig. 2). Ethanol accumulations were typically below 2.0 g/liter and exceeded 5.0 g/liter only in inoculations that included the yeast species *Hanseniaspora uvarum*, *Pichia kluyveri*, or *Saccharomyces cerevisiae.* In three of these four instances, the ethanol concentrations were demonstrably higher when inoculated berries were not exposed to *D. melanogaster* than when they were, whereas in all four the converse was true for acetic acid concentrations. Because we measured substantial acetic acid levels in numerous fly-exposed treatments, we partnered our quantitative acetic acid threshold with a mean qualitative disease rating of >3 (indicating that at least one sample in the set had surpassed the discoloration stage, and the berry had lost integrity) in order to establish an integrated criterion for determining which berries had developed sour rot. Only those inoculations also exposed to *D. melanogaster* developed sour rot symptoms with a mean rating >3 (Fig. 3). Of those meeting both the quantitative and qualitative

criteria, only three treatments caused sour rot symptoms: *Pichia kluyveri* x *G. oxydans*, *S. cerevisiae* x *A. aceti* and *S. cerevisiae x G. oxydans*, all in the presence of *Drosophila*.

Inoculation with ATCC cultures in conjunction with axenic D. melanogaster. Exposure of the inoculated berries to axenic flies affected the accumulation of both ethanol *(P*=0.048) and acetic acid *(P*=0.069) (Fig. 4). Ethanol levels trended modestly higher in the presence of flies. Exposure to axenic flies increased final acetic acid concentrations in six of the 11 inoculation treatments plus the uninoculated control, with the concentration (mean + standard error) exceeding the 0.83 g/liter threshold when berries were inoculated with either *S. cerevisiae* or *P. kluyveri* alone or in combination with either *A. aceti* or *G. oxydans*. Whereas acetic acid was undetectable in five of the 11 inoculation treatments that were not exposed to flies, its concentration exceeded 0.83 g/liter in the absence of flies when berries were inoculated with *S. cerevisiae,* alone or in combination with either *A. aceti* or *G. oxydans*. Seven treatments had a mean disease rating of ≥ 3, when averaged across all samples within a treatment, six of which included axenic flies (Fig. 5). When considering both the acetic acid and disease rating in concert, the only treatments that met both the quantitative and qualitative criteria for causing sour rot, were *S. cerevisiae* x *A. aceti,, S. cerevisiae x G. oxydans, P. kluyveri x A. aceti, P. kluyveri x G. oxydans,* and only with exposure to axenic flies.

*Pathogenicity of isolates recovered from field samples***.** When averaged across all treatments including the uninoculated control, exposure to axenic *D. melanogaster* significantly increased the acetic acid concentration after 8 days incubation, to 6.98

g/liter versus 1.72 g/liter when no flies were present *(P* <0.0001) (Fig. 6). In contrast, the mean ethanol content at the end of the 8-day incubation period decreased with exposure to axenic *D. melanogaster,* from 6.81 to 2.75 g/liter *(P* <0.0001) without and with inclusion of the flies, respectively, (Fig. 6). Only those treatments that included flies had a combination of acetic acid levels ≥ 0.83 g/liter and mean disease ratings > 3 when averaged across all samples within a treatment, and all such treatments except the *Rahnella* sp. produced sour rot symptoms (Fig. 7). All treatments in which either yeast was included or in which *Gluconobacter* sp. was the only source of inoculum were successful in causing symptoms.

After eight days, all berries were macerated and juice from each treatment was plated out on YPD and YPM. After four days of incubation at 24°C, two replicates of each morphologically unique colony was sequenced via Sanger sequencing. We successfully identified each of the organisms included in the inoculations, but also previously documented endogenous *Pichia* spp., *Candida* spp., and *Acetobacter* spp.

Microbial contribution of wild-type D. melanogaster. Sequencing results of the inoculum isolated from the wild-type flies showed that 80% of the bacterial OTUs from the Enterobacteriales, 5.3% from Bacillales*,* 3.2% Burkholderiales*,* and approximately 1% each of Actinomycetales, Aeromondales, Lactobacillales, Pseudomonales, Rhizobiales and Streptophyta. Nearly all (98.5%) of the fungal OTUs were from the Saccharomycetale*s,* the majority of which were *Metschnikowia* spp*.,* with the remaining 1.5% from the Tremellales. Wounded grapes that were not inoculated generated modest quantities of acetic acid, either with or without exposure to axenic flies (mean of 0.27 and 0.17 g/liter, respectively) (Fig. 8), and did not

develop qualitative symptoms consistent with sour rot (mean disease ratings of 0.5 and 1.5, respectively) (Fig. 23). There was significantly more ethanol in the uninoculated control treatment, both with and without exposure to axenic flies, than in the inoculated treatments exposed to axenic flies or in the uninoculated berries exposed to wild-type flies, all of which generated acetic acid concentrations > 5.9 g/liter (Fig. 8). Inoculated berries generated significant acetic acid with or without exposure to axenic flies. However, significant visual symptoms did not develop in berries not exposed to these flies, whereas the vast majority of berries that were exposed developed severe disease symptoms. Uninoculated berries exposed to wild-type flies generated significant acetic acid but had a mean disease rating of only 2.0 (Fig. 9).

Botrytis and sour rot interaction. The control treatment in which only clusters without visual disease symptoms were included developed moderate Botrytis and sour rot after 10 days incubation, but the placement of diseased clusters in the center of the arrangement significantly increased these levels. Botrytis bunch rot severity increased by 48.5% on previously affected clusters when exposed to a healthy cluster, but when exposed to a sour rot-affected cluster, Botrytis severity on the surrounding clusters did not increase, and instead remained at 50%, while sour rot severity on the sour rot-affected center cluster increased by 21.7% (Fig. 10). Botrytis severity only increased from 0 to 3%, less than the increase of Botrytis on healthy clusters surrounded by other healthy clusters (Fig. 11). When healthy clusters surrounded a sour rot-affected cluster, however, sour rot severity increased from naught to 53.4%, and on the sour rot-affected middle cluster, sour rot severity increased by 29.2% (Fig. 10). The center clusters were also significantly affected by the status of the clusters surrounding them.

Healthy clusters surrounded by healthy clusters developed modest levels of Botrytis, which increased nearly fourfold when they were surrounded by Botrytis-affected clusters; little and no Botrytis developed on sour-rotted clusters surrounded by sour rot-affected and healthy clusters, respectively.

Discussion

Our results indicate that sour rot is caused by an interaction involving yeast, acetic acid bacteria, and *Drosophila* fruit flies, and that all three components must be present for typical symptoms to develop. It appears that multiple yeast genera are capable of producing the ethanol precursor of acetic acid within wounded berries: both we and others (Guerzoni and Marchetti 1987, Bisiach et al. 1986, Barata et al. 2008, 2012a and 2012b), associated many different yeast with diseased samples in the field; our inoculation studies with selected species invariably resulted in ethanol production; and ethanol is a typical product of yeast metabolism. However, inoculations with yeast alone did not produce acetic acid in our experiments, consistent with the results of Barata et al (2012a). Indeed, as postulated by these authors, we did not obtain significant acetic production unless berries were co-inoculated with both yeast and acetic acid bacteria. By assaying both ethanol and acetic acid production on a daily basis following such a co-inoculation we demonstrated that acetic acid was not produced within berries until ethanol was first produced (presumably, by the yeast) and that this final step (presumably, the result of bacterial activity) appeared to be catalyzed by the presence of *Drosophila* flies. In several preliminary experiments where we compared the contribution of *D. melanogaste* and *D. suzukii* (spotted wing Drosophila) in inoculation experiments, we saw no difference between these two

species in their influence on sour rot development (*data not shown*). Thus, because *D. melanogaster* is by far the more common of the two associated with diseased samples in the field (Hall and Wilcox, in press), we focused our studies on the former species and are making the assumption that these results are applicable to drosphilids in general.

We have defined sour rot as a syndrome consisting of both the production of acetic acid within necrotic berries and the loss of berry integrity resulting in the release of liquefied pulp, the latter characteristic also having been described as a defining one for sour rot by others (Bisiach et al. 1986, Guerzoni & Marchetti 1987, Barata et al. 2008). Previous researchers determined that yeast and acetic acid bacteria were associated with sour rot (Bisiach et al. 1982 and 1986, Guerzoni & Marchetti 1987, Barata et al. 2008, 2012a and 2012b), and whereas some combinations of yeast and acetic acid bacteria successfully produced acetic acid in grape berries that we inoculated and resulted in their discoloration without exposure to *Drosophila*, a significant loss of berry integrity was not observed unless these insects were present. Loss of berry integrity, or turgor as Bisiach et al. (1986) termed it, is a crucial component in the final stage of sour rot. The associated leaking of microbe- and acetic acid-filled pulp makes these microbes readily available for vectoring to new clusters by the adult drosophilids present while also liberating acetic acid and ethanol volatiles that attract them (Bisiach et al. 1986, Barata et al. 2012a). Thus, drosophilids appear to be an integral component in both the development and spread of sour rot, as the disease has been defined here and by others (Bisiach et al. 1986, Barata et al. 2012a).

The disease “sour rot” is sometimes used as a synonym for a syndrome also termed

"summer bunch rot", caused by various filamentous fungi including *Aspergillus niger, Alternaria carbonarius, Alternaria tenuis, Botrytis cinerea, Cladosporium herbarum, Rhizopus arrhizus, Penicillium* sp. (Haviland et al. 2017). However, even though some of these organisms have been shown to be capable of decaying inoculated berries (e.g., Rooney-Latham et al. 2008), these same studies have not demonstrated the production of acetic acid in tandem with such decay. It seems possible that yeast and bacteria responsible for acetic acid production become active in berries infected with these fungi after they begin to decay, subsequently producing the vinegar aroma noted in the field where these rots occur. If so, they might be considered predisposing agents under such circumstances, although this is purely speculative in the absence of supporting experimental data. Whereas we did not focus our attention on the potential role of filamentous fungi in sour rot development, our one set of experiments in which we inoculated only with *Aspergillus niger* produced no acetic acid or significant berry discoloration without the concurrent presence of wild-type *D. melanogaster*, which we showed harbor a suite organisms on and in their bodies that are capable of producing such symptoms themselves. Thus, we conclude that filamentous fungi are of secondary importance in this complex as we have studied it, as did Bisiach et al. (1982).

Barata et al. (2012a) concluded that the role of *Drosophila* spp. in sour rot development extends beyond that of a vector of microorganisms, specifically, in that the insects prevent the grapes from healing wounds because their proliferation is faster than the ability of the berry to heal. While we did not investigate this specific phenomenon, through our use of axenic *D. melanogaster* we did conclusively

demonstrate that the flies play a critical non-microbial role in sour rot development, both by catalyzing the conversion of ethanol to acetic acid and through their role in promoting the loss of berry integrity. While we did not examine the mechanisms underlying these phenomena, we believe it could involve, at least in part, enzymes released by the larval stage of in order to facilitate consumption of the pulp (Gregg et al. 1990, Sakaguchi and Suzuki 2013). The precise mechanistic role of *Drosophila* involvement in sour rot development appears to provide a rich target for further research.

The ubiquitous presence of several genera of yeast and/or acetic acid bacteria (e.g., *Saccharomyces, Pichia, Hanseniaspora, Metschnikowia, Acetobacter, Gluconobacter)* within healthy grape berries complicates the interpretation of the results from our pathogenicity experiments, and accounts for the presence of ethanol and acetic acid within many of our control treatments. Although we tried several methods of sterilizing healthy berry pulp prior to inoculation (e.g., heat treatment), all efforts resulted in the disintegration of the berries, which then were not usable. Future research in which the interior of the grape is successfully sterilized or pasteurized without compromising the physical integrity of the cells (perhaps, through irradiation) would obviate this confounding factor. To the best of our knowledge, the presence of these microorganisms as endophytes within healthy berries has not been reported previously, and has implications beyond those pertaining to sour rot (e.g., winemaking). Nevertheless, these results suggest that the primary inoculum necessary for sour rot development may in some cases be present endophytically. Relatedly, the often-noted requirement for wounding (e.g., McFadden-Smith and Gubler 2015) may

relate to processes beyond promoting the access of fruit flies and the microorganisms they carry to the berry interior, e.g., the release of cellular contents that provide substrates from which the appropriate endophytes can produce ethanol and acetic acid and the ingress of oxygen required for acetic acid production.

Bisiach et al. 1982 examined the relationship between Botrytis bunch rot and sour rot, concluding that existing Botrytis infections were halted in the presence of sour rot and that sour rot prevented the advancement of further fungal infection. Our experiments support this conclusion, as we found that Botrytis infections did not advance in the presence of a sour rot-infected cluster, whereas sour rot infections continued to advance but at a significantly slower rate than they would have if in the presence of healthy clusters. Thus, it appears *B. cinerea* competes with the microbes responsible for causing sour rot, rather than predisposing infected grape clusters to their subsequent colonization.

Literature Cited

1. Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. J Mol Biol 215(3):403–410.

2. Barata, A., Campo, E., Malfeito-Ferreira, M., Loureiro, V., Cacho, J., Ferreira, V. 2011. Analytical and sensorial characterization of the aroma of wines produced with sour rotten grapes using GC-O and GC-MS: identification of key aroma compounds. J Agric Food Chem 59:2543–2553

3. Barata, A., Correia Santos, S., Malfeito-Ferreira, M. and Loureiro, V. 2012a. New Insights into the Ecological Interaction Between Grape Berry Microorganisms and Drosophila Flies During the Development of Sour Rot. Microb Ecol 64:416–430.

4. Barata, A., Malfeito-Ferreira, M. and Loureiro, V. 2012b. Changes in sour rotten grape berry microbiota during ripening and wine fermentation. Int J Food Microbiol. 154:152–161. Available at: http://dx.doi.org/10.1016/j.ijfoodmicro.2011.12.029.

5. Bisiach, M., Minervini, G. and Salomone, M.C. 1982. Recherches expérimentales sur la pourriture acide de la grappe et sur ses rapports avec la pourriture grise 1. EPPO Bull. 12:15–27. Available at: http://doi.wiley.com/10.1111/j.1365-2338.1982.tb01675.x.

6. Bisiach, M., Minervini, G. and Zerbetto, F. 1986. Possible integrated control of grapevine sour rot. Vitis 25:118–128. Available at: e024052.pdf.

7. Broderick, N. A., Buchon, N., and Lemaitre, B. 2014. Microbiota-induced changes in drosophila melanogaster host gene expression and gut morphology. MBio. 5 (3).

8. Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. 2010a. QIIME allows analysis of high-throughput community sequencing data. Nat Meth 7(5):335–336.

9. Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R. 2010b. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26(2):266–267.

10. Castellari, M., Versari, A., Spinabelli, U., Galassi, S., Amati, A. 2000. An Improved HPLC Method for the Analysis of Organic Acids, Carbohydrates, and Alcohols in Grape Musts and Wines. J. Liq. Chrom. & Rel. Technol., 23(13), 2047–2056.

11. Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A., & Kopp, A. 2011. Bacterial communities of diverse Drosophila species: ecological context of a host-microbe model system. PLoS Genet. 7 (9).

12. Chervin, C., Aked, J. and Crisosto, C. H. 2012. Grapes. pp. 187-211 *in*: Crop Post-Harvest: Science and Technology. D. Rees, G. Farrell and J. Orchard eds. Wiley-Blackwell, Oxford, UK.

13. DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L. 2006. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl Environ Microbiol 72(7):5069–5072.

14. Edgar, R.C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19):2460–2461.

15. Gregg, T. G., McCrate, A., Reveal, G., Hall, S., and Rypstra, A. L. (1990). Insectivory and social digestion in Drosophila. Biochem. Genet. 28, 197–207. doi: 10.1007/ BF00561337

16. Guerzoni, E. and Marchetti, R. 1987. Analysis of yeast flora associated with grape sour rot and of the chemical disease markers. Appl Environ Microbiol 53:571–576.

17. Hall, M.E., Loeb, G.M., and Wilcox, W.F. 2014. Ethanol and Acetic Acid Content of Sour-Rotted Wine grapes and Disease Control with Antimicrobial and Insecticide Sprays. (Abstr.) 65th ASEV National Conference, Technical Abstracts: 164

18. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2015a. Improving Our Understanding of Sour Rot Etiology and Management Techniques. (Abstr.) 66th ASEV National Conference, Technical Abstracts: 70

19. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2015b. Etiology and Management Strategies for Sour Rot on Grapes. (Abstr.) Phytopathology 105 (Suppl. 4): S4.56. http://dx.doi.org/10.1094/PHYTO-105-11-S4.1

20. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2016a. Etiology and Management of Sour Rot in Grapes. (Abstr.) Phytopathology 106 (Suppl. 4): S4.64. http://dx.doi.org/10.1094/PHYTO-106-12-S4.1

21. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2016b. Etiology and Management of Sour Rot in Grapes. (Abstr.) Phytopathology 107:S2.7. http://dx.doi.org/10.1094/PHYTO-107-2-S2.5

22. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2016c. Further Understanding the Cause and Management of Sour Rot. (Abstr.) 67th ASEV National Conference, Technical Abstracts: 52

23. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2017. Causal role of *Drosophila* spp. in Sour Rot Development. (Abstr.) 68th ASEV National Conference, Technical Abstracts: 64

24. Haviland, D.R., Bettiga, L.J., Varela, L.G., Roncoroni, J.A., Smith, R.J., Westerdahl, B.B., Bentley, W.J., Daane, K.M., Ferris, H., Gubler, W.D., Hembree, K.J., Ingels, C.A., Zalom, F.G., Zasada, I. 2017. UC IPM Pest Management Guidelines Grape. UC ANR Publication 3448. Oakland, CA.

25. Ioriatti, C., Walton, V., Dalton, D., Anfora, G., Grassi, A., Maistri, S. and Mazzoni, V. 2015. *Drosophila suzukii* (Diptera: Drosophilidae) and its potential impact to wine grapes during harvest in two cool climate wine grape production regions. J Econ Entomol 108(3):1148-55

26. Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., Bates, S.T., Bruns, T.D., Bengtsson-Palme, J., Callaghan, T.M., Douglas, B., Drenkhan, T., Eberhardt, U., Dueñas, M., Grebenc, T., Griffith, G.W., Hartmann, M., Kirk, P.M., Kohout, P., Larsson, E., Lindahl, B.D., Lücking, R., Martín, M.P., Matheny, P.B., Nguyen, N.H., Niskanen, T., Oja, J., Peay, K.G., Peintner, U., Peterson, M., Põldmaa, K., Saag, L., Saar, I., Schüßler, A., Scott, J.A., Senés, C., Smith, M.E., Suija, A., Taylor, D.L., Telleria, M.T., Weiss, M., Larsson, K-H. 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 22(21):5271–5277.

27. Koyle, M. L., Veloz, M., Judd, A. M., Wong, A. C., Newell, P. D., Douglas, A. E., Chaston, J. M. 2016. Rearing the Fruit Fly *Drosophila melanogaster* Under Axenic and Gnotobiotic Conditions. J. Vis. Exp. 113.

28. McFadden-Smith, W. and Gubler, W.D. 2015. Sour Rot. pp. 87-90 *in:* Compendium of Grape Diseases, Pests, and Disorders, 2nd Ed. W.F. Wilcox, W.D. Gubler and J.K. Uyemoto, eds. APS Press, St. Paul, MN. 232 pp.

29. Parks, D.H., Tyson, G.W., Hugenholtz, P., Beiko, R.G. 2014. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics 30(21):3123–3124.

30. Rooney-Latham, S., Janousek, C. N., Eskalen, A., Gubler, W. D. 2008. First report of Aspergillus carbonarius Causing Sour Rot of Table Grapes (Vitis vinifera) in California. Plant Disease 92 (4): 651.

31. Sakaguchi, H. and Suzuki, M. G. 2013. *Drosophila melanogaster* larvae control amylase secretion according to the hardness of food. Front. Physiol. 4:200. doi: 10.3389/fphys.2013.00200

32. Staubach, F., Baines, J. F., Kunzel, S., Bik, E. M., & Petrov, D. A. 2013. Host species and environmental effects on bacterial communities associated with Drosophila in the laboratory and in the natural environment. PLoS One. 8 (8).

33. Wong, C. N., Ng, P., & Douglas, A. E. 2011. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ Microbiol. 13 (7), 1889-1900.

Figure 1. Mean daily ethanol (A) and acetic acid (B) accumulation (g/L) over 5 days in *V. vinifera* cv. Red Globe berries with and without inoculation with a suspension of *S. cerevisiae* plus *A. aceti* and exposure to wild-type D. melanogaster.

Figure 2. Mean ethanol (A) and acetic acid (B) contents (g/L) in *V. vinifera* cv. Red Globe grapes 5 days after inoculation with various microbes and combinations thereof, with and without exposure to wild-type (WT) Drosophila melanogaster fruit flies during the incubation period. An asterisk (*) above a bar denotes a statistically significant difference relative to the uninoculated treatment not exposed to D. melanogaster, as determined by Dunnett's Method of Comparisons: * = P = 0.05, ** = P = 0.01, *** = P = <0.001.

Figure 3. Frequency of disease ratings (0-4 scale) applied to individual V. vinifera cv. Red Globe grapes (n = 8) inoculated with isolates of six fungal or bacterial species obtained from the American Type Culture Collection, either alone or in various combinations, and either not exposed (A) or exposed (B) to wild-type Drosophila melanogaster fruit flies during a 5-day incubation period.

Figure 4. Mean ethanol (A) and acetic acid (B) contents in *V. vinifera* cv. Red Globe grapes exposed or not to axenic Drosophila melanogaster, after 8 days incubation. Grapes were inoculated with single isolates of five yeast or bacterial species obtained from the American Type Culture Collection, either alone or in various combinations. An asterisk (*) above a bar denotes a significant difference relative to uninoculated berries not exposed to axenic D. melanogaster, as determined by Dunnett's Method of Comparisons. * = significant at P = 0.05.

Figure 5. Frequency of disease ratings (0-4 scale) applied to individual *V. vinifera* cv. Red Globe grapes (n = 8) inoculated with isolates of six fungal or bacterial species obtained from the American Type Culture Collection, either alone or in various combinations, and either not exposed (A) or exposed (B) to axenic Drosophila melanogaster fruit flies during a 8-day incubation period.

Figure 6. Mean ethanol (A) and acetic acid (B) content in inoculated *V. vinifera* cv. Red Globe grapes exposed to axenic Drosophila melanogaster (fruit flies). Grapes were inoculated with four isolates recovered from sour rot-affected berries in the Finger Lakes AVA, either alone or in combination. The isolates were identified as two species of Metchnikowia, Gluconobacter cerinus, and Rahnella sp. An

asterisk (*) above a bar denotes a significant difference relative to uninoculated berries not exposed to axenic D. melanogaster, as determined by Dunnett's Method of Comparisons. * = significant at P = 0.05.

Figure 7. Frequency of disease ratings (0-4 scale) applied to individual *V. vinifera* cv. Red Globe grapes (n = 8) inoculated with four yeast and bacterial species recovered from sour rot-affected grapes Finger Lakes AVA, alone and in various combinations, and either not exposed (A) or exposed (B) to axenic Drosophila melanogaster fruit flies during a 8-day incubation period.

Figure 8. Mean ethanol (A) and acetic acid (B) content (g/L) in inoculated *V. vinifera* cv. Red Globe grapes exposed to either axenic or wild-type (WT) Drosophila melanogaster. Grapes were inoculated with a suspension of organisms previously collected from media exposed to WT inoculum for 3 days (majority of organisms Enterobacteriales and Saccharomycetales). An asterisk (*) above a bar denotes a significant difference relative to the uninoculated control treatment, which was not exposed to D. melanogaster flies, as determined by Dunnett's Method of Comparisons. * = significant at P = 0.05.

Figure 9. Frequency of disease ratings (0-4 scale) applied to individual *V. vinifera* cv. Red Globe grapes (n = 8) either not inoculated or were inoculated with a suspension of organisms previously recovered from wild-type D. melanogaster fruit flies (WT), the majority of which were in the Enterobacteriales and Saccharomycetales. These treatments were either not exposed (A), exposed to axenic (B) or wild-type (C) *Drosophila melanogaster* during an 8-day incubation period.

Figure 10. Mean disease severity (% cluster area symptomatic) of Botrytis bunch rot and sour rot on four *V. vinifera* cv. Riesling clusters before and after exposure to one healthy or diseased cluster. The indicated clusters were arranged in a pattern of four (healthy or diseased, i.e., 50% Botrytis severity) forming a square around one healthy or diseased (Botrytis or sour rot) cluster in the center. All clusters had 1-2 cm of space between them.

Figure 11. Mean disease severity (% cluster area symptomatic) of Botrytis bunch rot and sour rot on one *V. vinifera* cv. Riesling cluster before and after exposure to four healthy or diseased clusters surrounding it. Clusters were arranged in a pattern with four clusters of one treatment forming a square surrounding the indicated cluster in the center. All clusters had 1-2 cm of space between them.

Collection Year	*Acetobacter pasteurianus* (KR149364.1)	*Gluconobacter cerinus (MG266178.1)*	*Metschnikowia pulcherrima*	Low level of demarcation between several Metschnikowia species spp.	*Pichia occidentalis* (KY816890.1)	*Rahnella* sp. (KR189951.1)
2014	57.1%	89.3%	100%	89.3%	67.9%	89.3%
2015	8.3%	100%	91.7%	100%	22.2%	100%

Table 1. Identities of the six most abundant microorganisms isolated from three sour rot-affected clusters collected from each of seven vineyards in 2014 and nine vineyards in 2015, and the percentage of clusters on which those microorganisms were found.

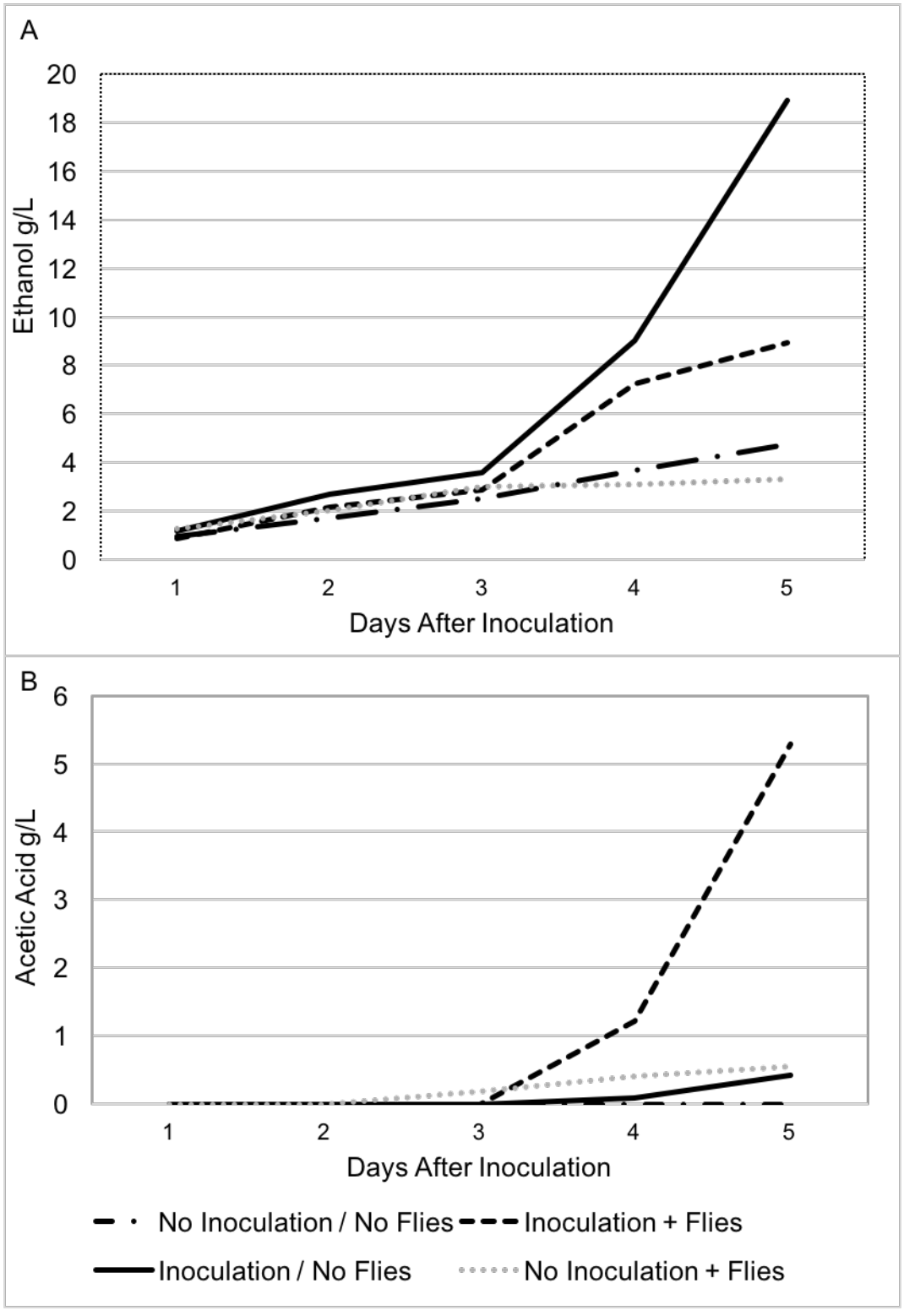

A
Ethanol g/L
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
Days After Inoculation
B
Acetic Acid g/L
6
5
4
3
2
1
0
1
2
3
4
5
Days After Inoculation
No Inoculation / No Flies
Inoculation + Flies
Inoculation / No Flies
No Inoculation + Flies

Figure 1.

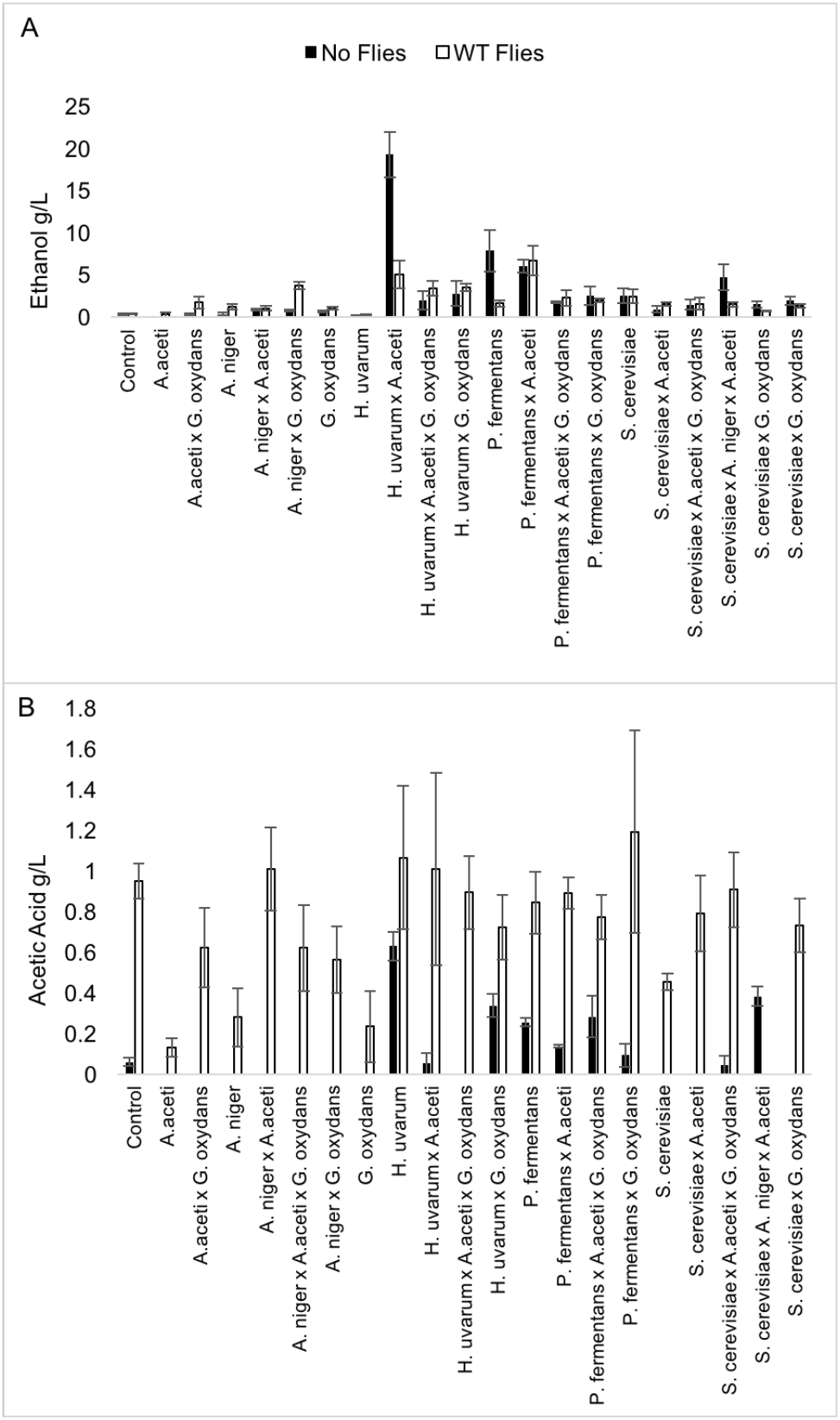

A
No Flies
WT Flies
Ethanol g/L
25
20
15
10
5
0
Control
A.aceti
A.aceti x G. oxydans
A. niger
A. niger x A.aceti
A. niger x G. oxydans
G. oxydans
H. uvarum
H. uvarum x A.aceti
H. uvarum x A.aceti x G. oxydans
H. uvarum x G. oxydans
P. fermentans
P. fermentans x A.aceti
P. fermentans x A.aceti x G. oxydans
P. fermentans x G. oxydans
S. cerevisiae
S. cerevisiae x A.aceti
S. cerevisiae x A.aceti x G. oxydans
S. cerevisiae x A. niger x A.aceti
S. cerevisiae x G. oxydans
S. cerevisiae x G. oxydans
B
Acetic Acid g/L
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
A.aceti
A.aceti x G. oxydans
A. niger
A. niger x A.aceti
A. niger x A.aceti x G. oxydans
A. niger x G. oxydans
G. oxydans
H. uvarum
H. uvarum x A.aceti
H. uvarum x A.aceti x G. oxydans
H. uvarum x G. oxydans
P. fermentans
P. fermentans x A.aceti
P. fermentans x A.aceti x G. oxydans
P. fermentans x G. oxydans
S. cerevisiae
S. cerevisiae x A.aceti
S. cerevisiae x A.aceti x G. oxydans
S. cerevisiae x A. niger x A.aceti
S. cerevisiae x G. oxydans

Figure 2.

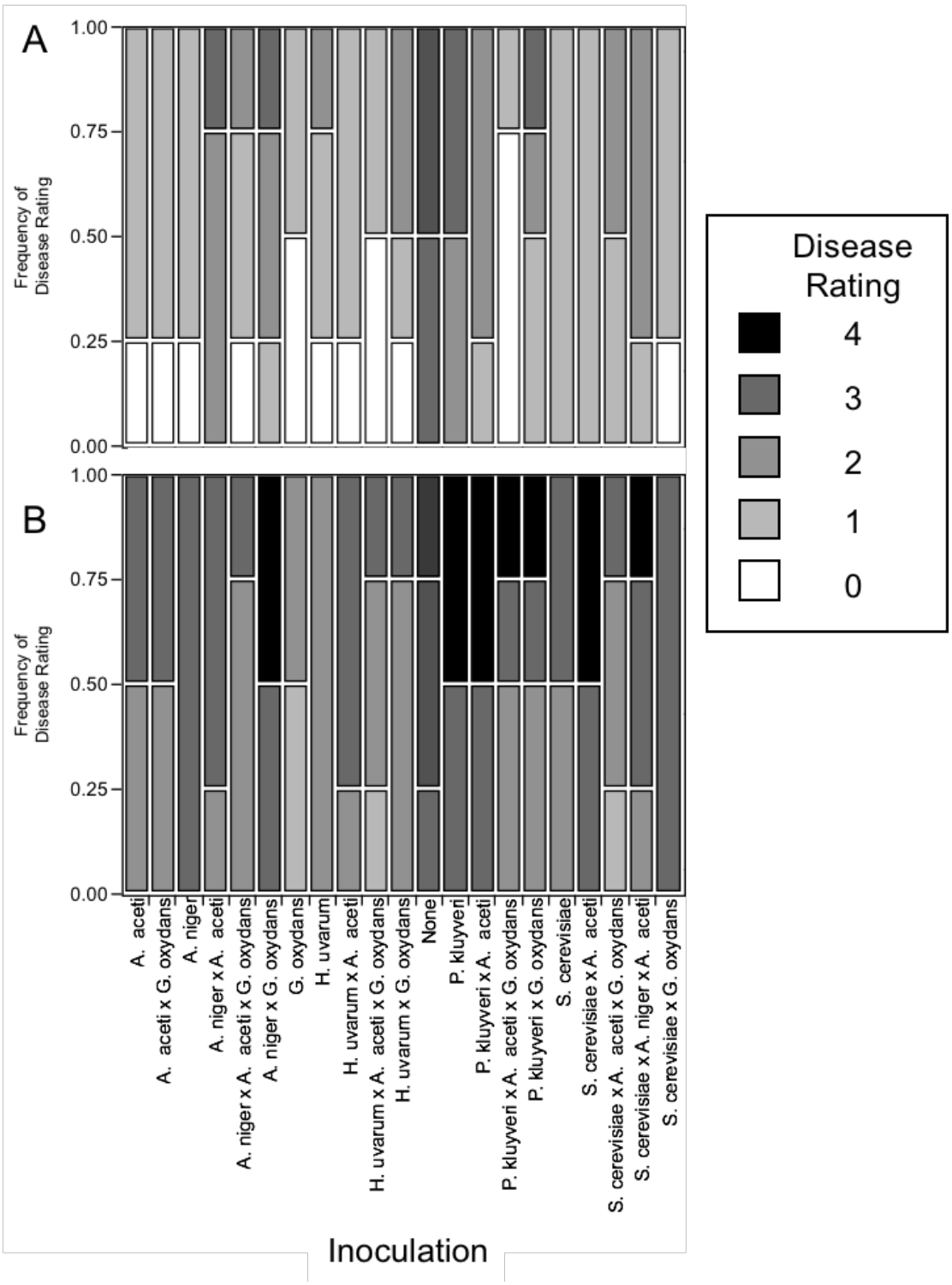

A
B
1.00
0.75
0.50
0.25
0.00
Frequency of Disease Rating
Disease Rating
4
3
2
1
0
A. aceti
A. aceti x G. oxydans
A. niger
A. niger x A. aceti
A. niger x A. aceti x G. oxydans
A. niger x G. oxydans
G. oxydans
H. uvarum
H. uvarum x A. aceti
H. uvarum x A. aceti x G. oxydans
H. uvarum x G. oxydans
None
P. kluyveri
P. kluyveri x A. aceti
P. kluyveri x A. aceti x G. oxydans
P. kluyveri x G. oxydans
S. cerevisiae
S. cerevisiae x A. aceti
S. cerevisiae x A. aceti x G. oxydans
S. cerevisiae x A. niger x A. aceti
S. cerevisiae x G. oxydans
Inoculation

Figure 3.

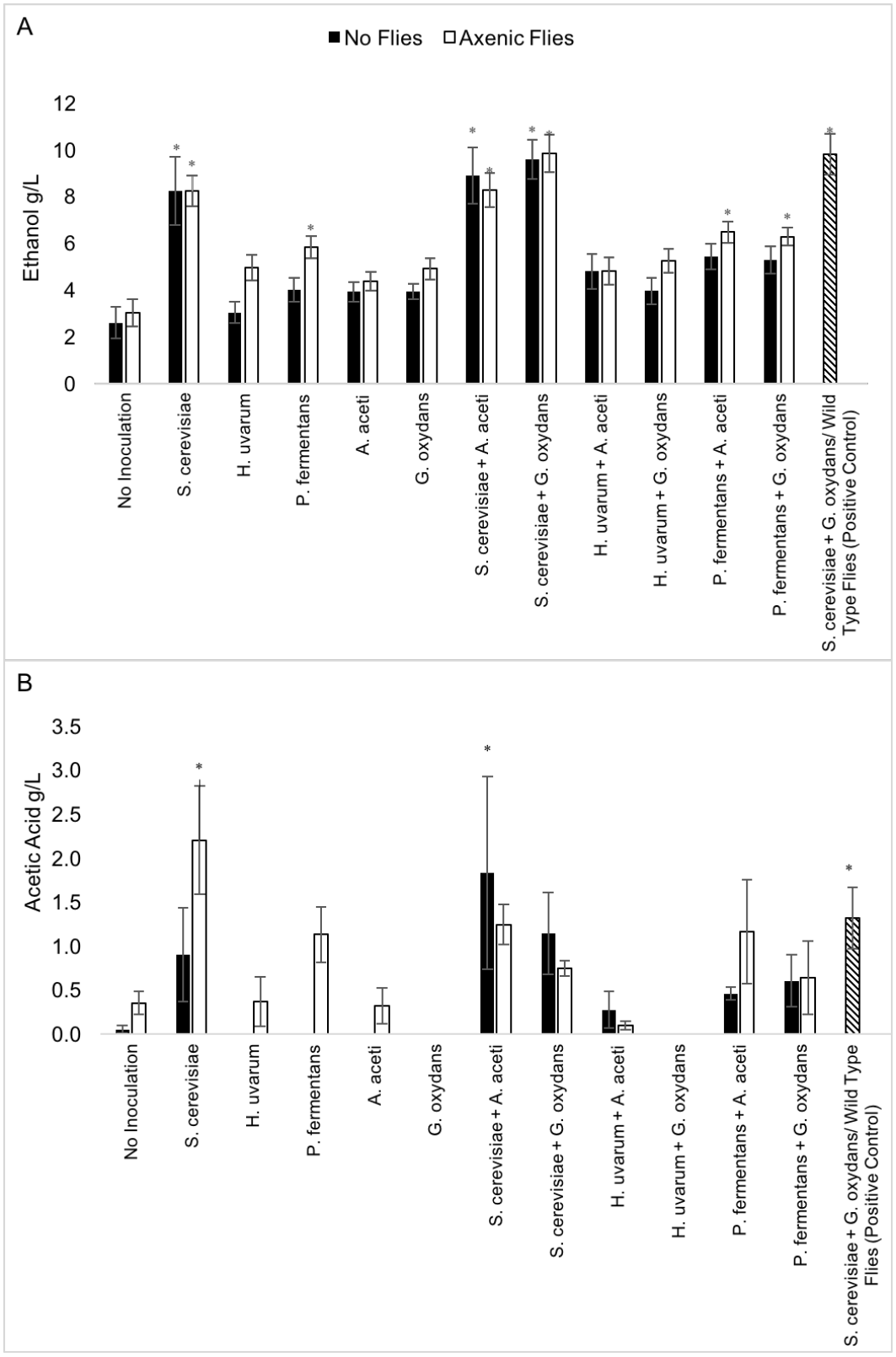

A
No Flies
Axenic Flies
Ethanol g/L
12
10
8
6
4
2
0
No Inoculation
S. cerevisiae
H. uvarum
P. fermentans
A. aceti
G. oxydans
S. cerevisiae + A. aceti
S. cerevisiae + G. oxydans
H. uvarum + A. aceti
H. uvarum + G. oxydans
P. fermentans + A. aceti
P. fermentans + G. oxydans
S. cerevisiae + G. oxydans/ Wild Type Flies (Positive Control)
B
Acetic Acid g/L
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
No Inoculation
S. cerevisiae
H. uvarum
P. fermentans
A. aceti
G. oxydans
S. cerevisiae + A. aceti
S. cerevisiae + G. oxydans
H. uvarum + A. aceti
H. uvarum + G. oxydans
P. fermentans + A. aceti
P. fermentans + G. oxydans
S. cerevisiae + G. oxydans/ Wild Type Flies (Positive Control)

Figure 4.

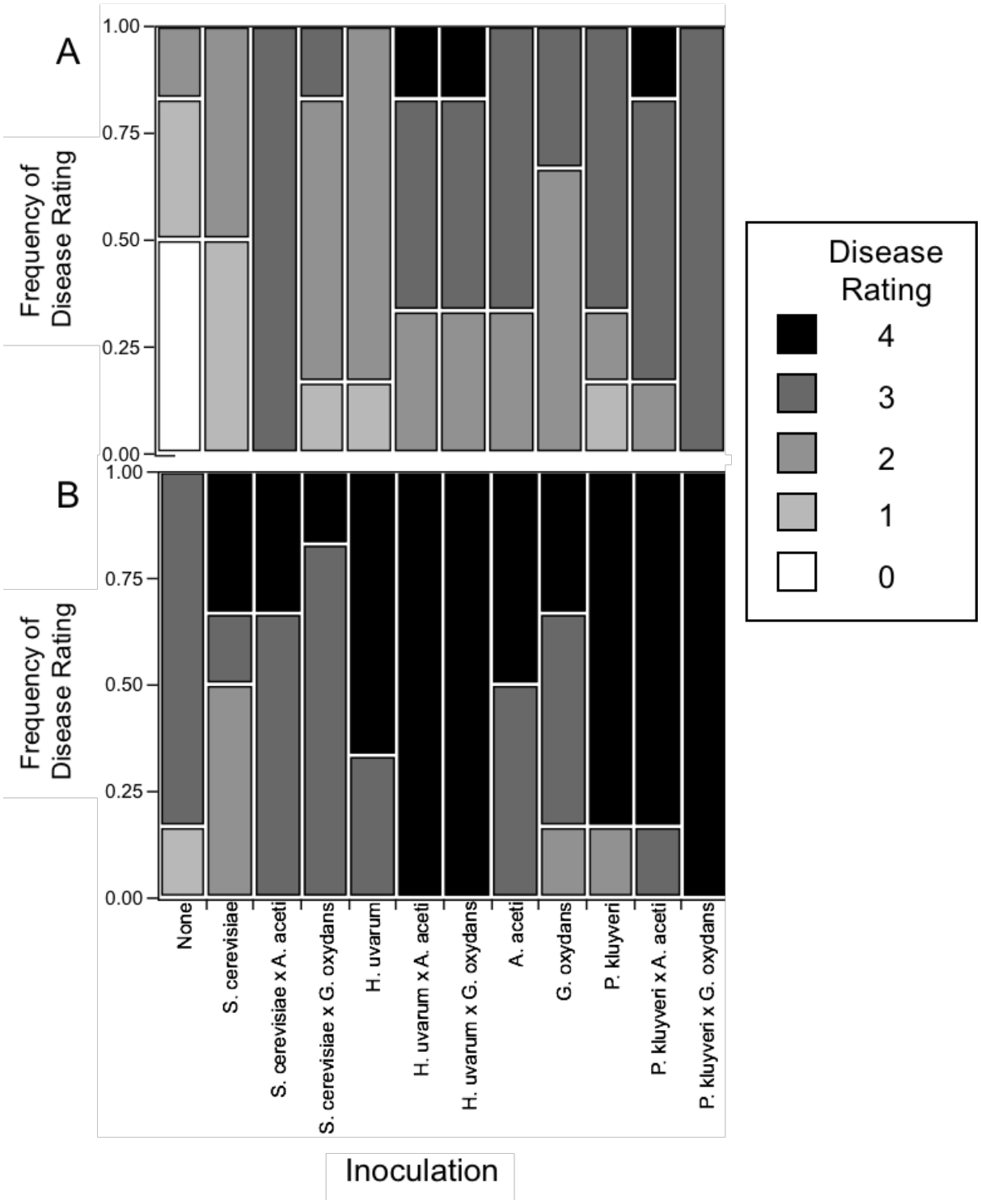

A
B
Frequency of Disease Rating
1.00
0.75
0.50
0.25
0.00
Disease Rating
4
3
2
1
0
None
S. cerevisiae
S. cerevisiae x A. aceti
S. cerevisiae x G. oxydans
H. uvarum
H. uvarum x A. aceti
H. uvarum x G. oxydans
A. aceti
G. oxydans
P. kluyveri
P. kluyveri x A. aceti
P. kluyveri x G. oxydans
Inoculation

Figure 5.

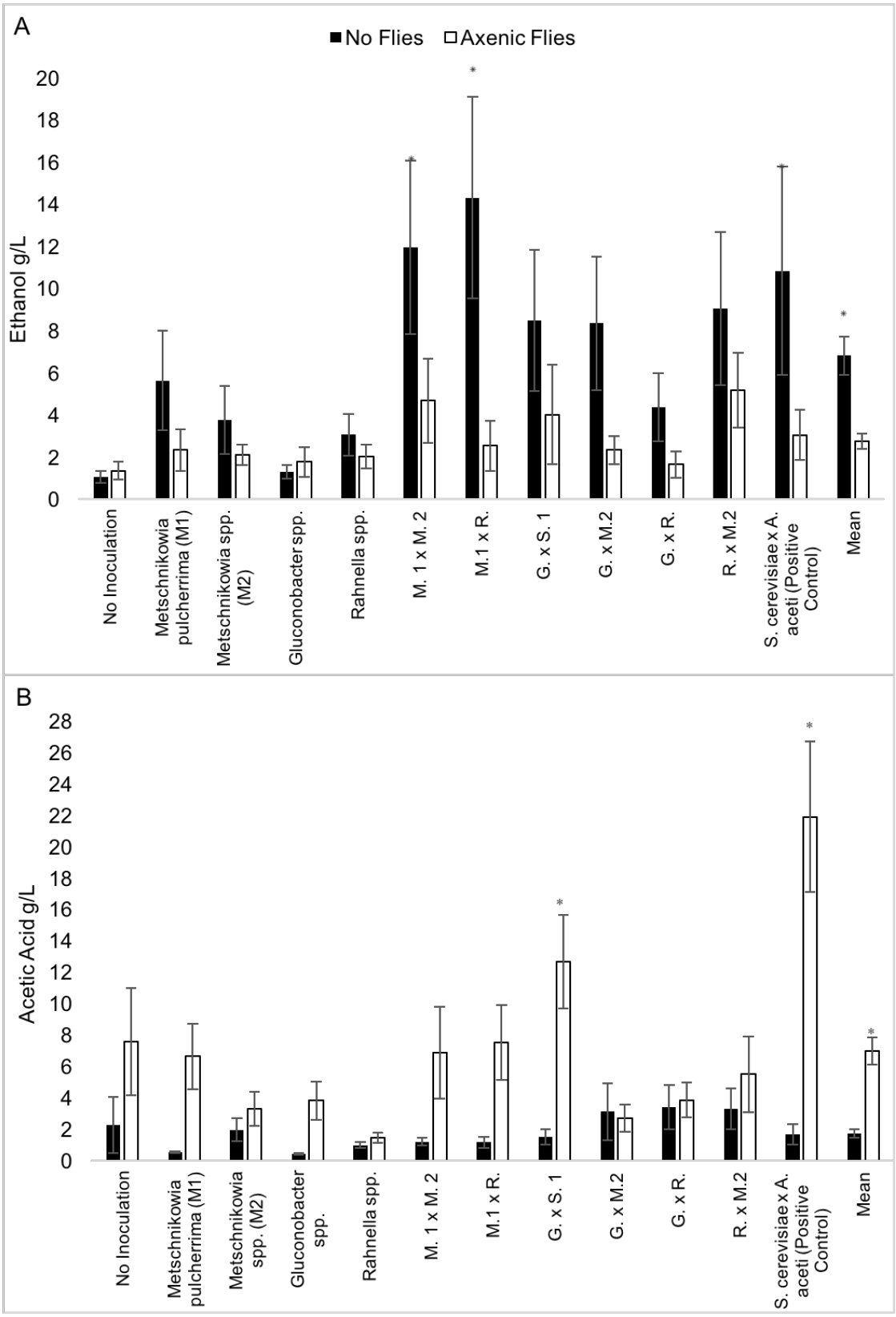

A
No Flies
Axenic Flies
Ethanol g/L
0
2
4
6
8
10
12
14
16
18
20
No Inoculation
Metschnikowia pulcherrima (M1)
Metschnikowia spp. (M2)
Gluconobacter spp.
Rahnella spp.
M. 1 x M. 2
M.1 x R.
G. x S. 1
G. x M.2
G. x R.
R. x M.2
S. cerevisiae x A. aceti (Positive Control)
Mean
B
Acetic Acid g/L
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
No Inoculation
Metschnikowia pulcherrima (M1)
Metschnikowia spp. (M2)
Gluconobacter spp.
Rahnella spp.
M. 1 x M. 2
M.1 x R.
G. x S. 1
G. x M.2
G. x R.
R. x M.2
S. cerevisiae x A. aceti (Positive Control)
Mean

Figure 6.

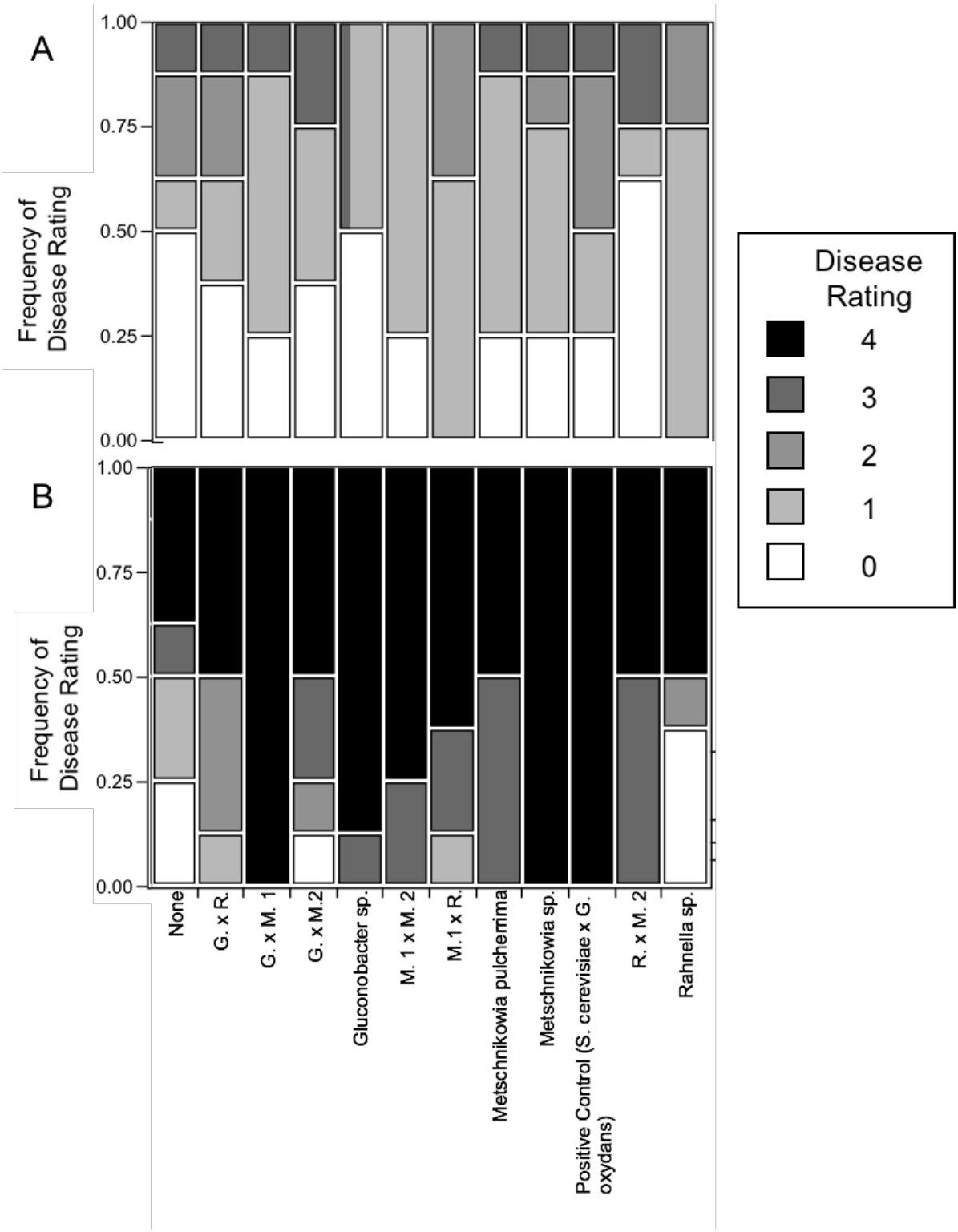

A
B
Frequency of Disease Rating
1.00
0.75
0.50
0.25
0.00
Disease Rating
4
3
2
1
0
None
G. x R.
G. x M. 1
G. x M.2
Gluconobacter sp.
M. 1 x M. 2
M.1 x R.
Metschnikowia pulchemima
Metschnikowia sp.
Positive Control (S. cerevisiae x G. oxydans)
R. x M. 2
Rahnella sp.

Figure 7.

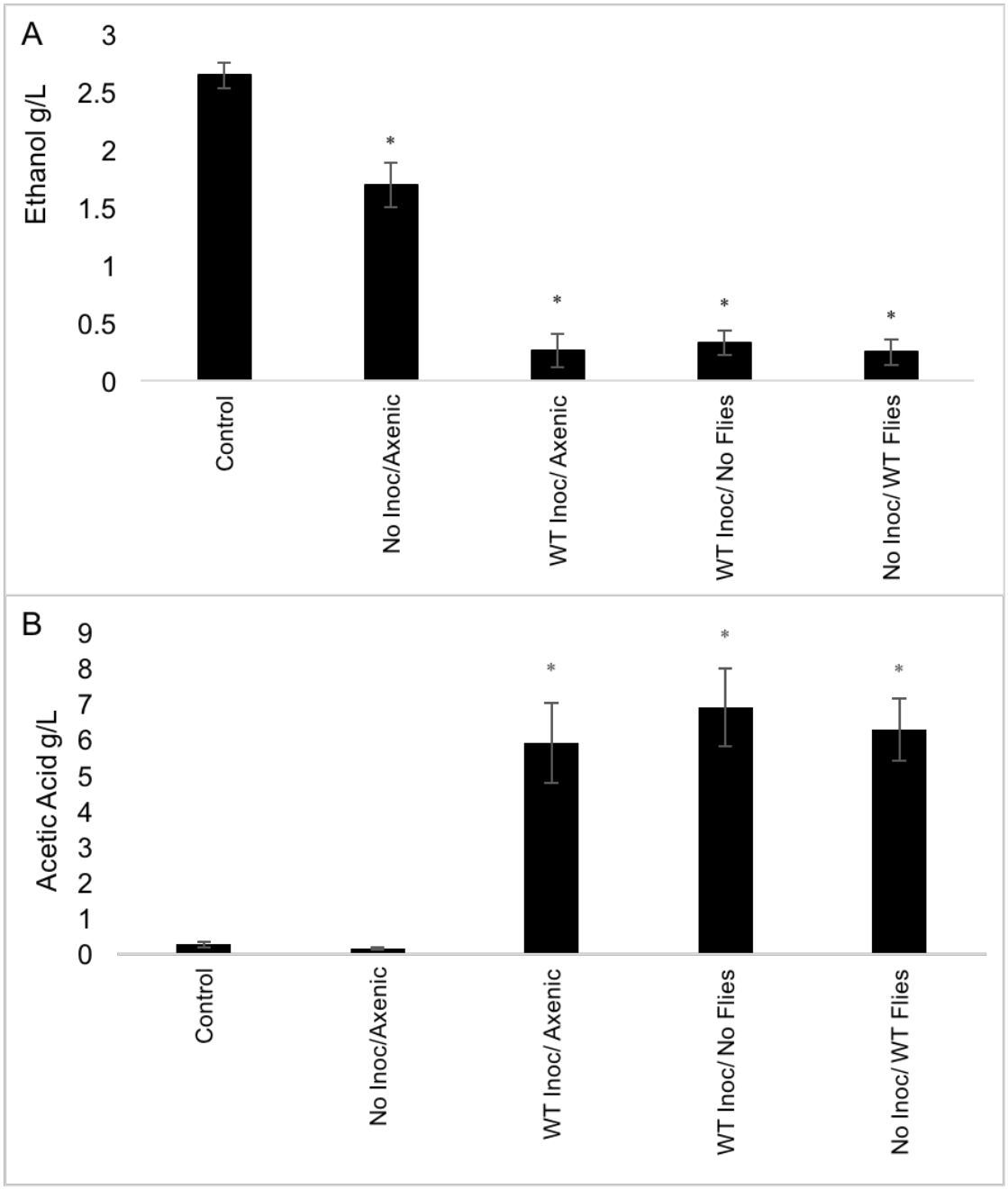

A
Ethanol g/L
3
2.5
2
1.5
1
0.5
0
*
*
*
*
Control
No Inoc/Axenic
WT Inoc/ Axenic
WT Inoc/ No Flies
No Inoc/ WT Flies
B
Acetic Acid g/L
9
8
7
6
5
4
3
2
1
0
*
*
*
Control
No Inoc/Axenic
WT Inoc/ Axenic
WT Inoc/ No Flies
No Inoc/ WT Flies

Figure 8.

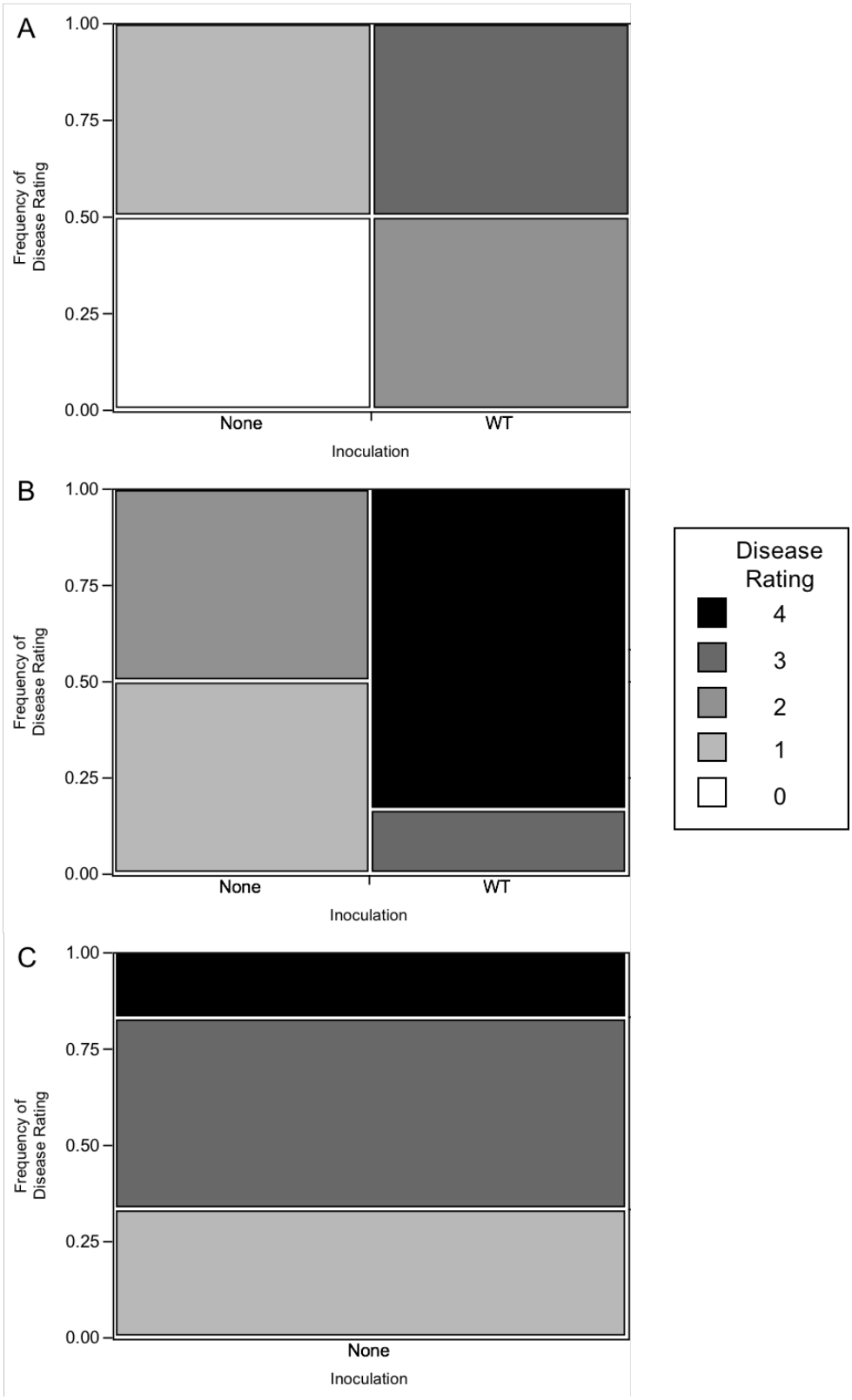

A
B
C
1.00
0.75
0.50
0.25
0.00
Frequency of Disease Rating
None
WT
Inoculation
Disease Rating
4
3
2
1
0

Figure 9.

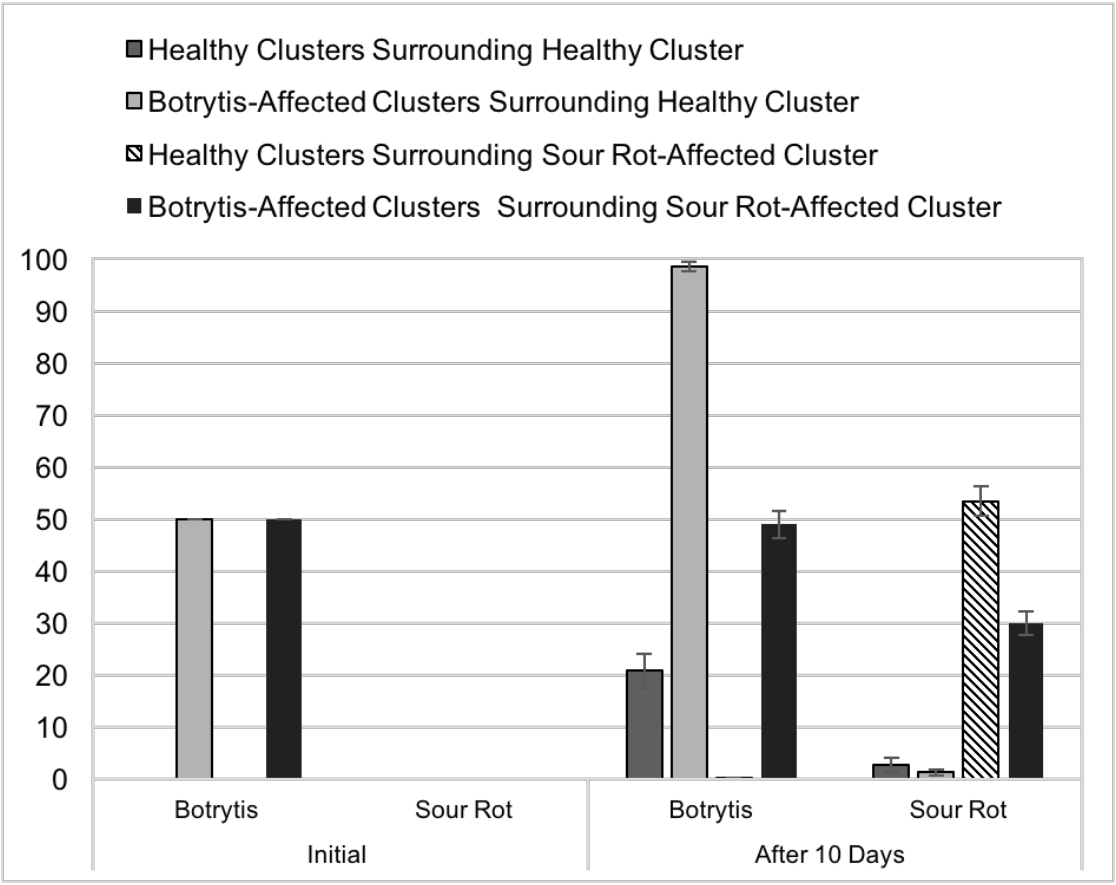

Healthy Clusters Surrounding Healthy Cluster
Botrytis-Affected Clusters Surrounding Healthy Cluster
Healthy Clusters Surrounding Sour Rot-Affected Cluster
Botrytis-Affected Clusters Surrounding Sour Rot-Affected Cluster
100
90
80
70
60
50
40
30
20
10
0
Botrytis
Sour Rot
Botrytis
Sour Rot
Initial
After 10 Days

Figure 10.

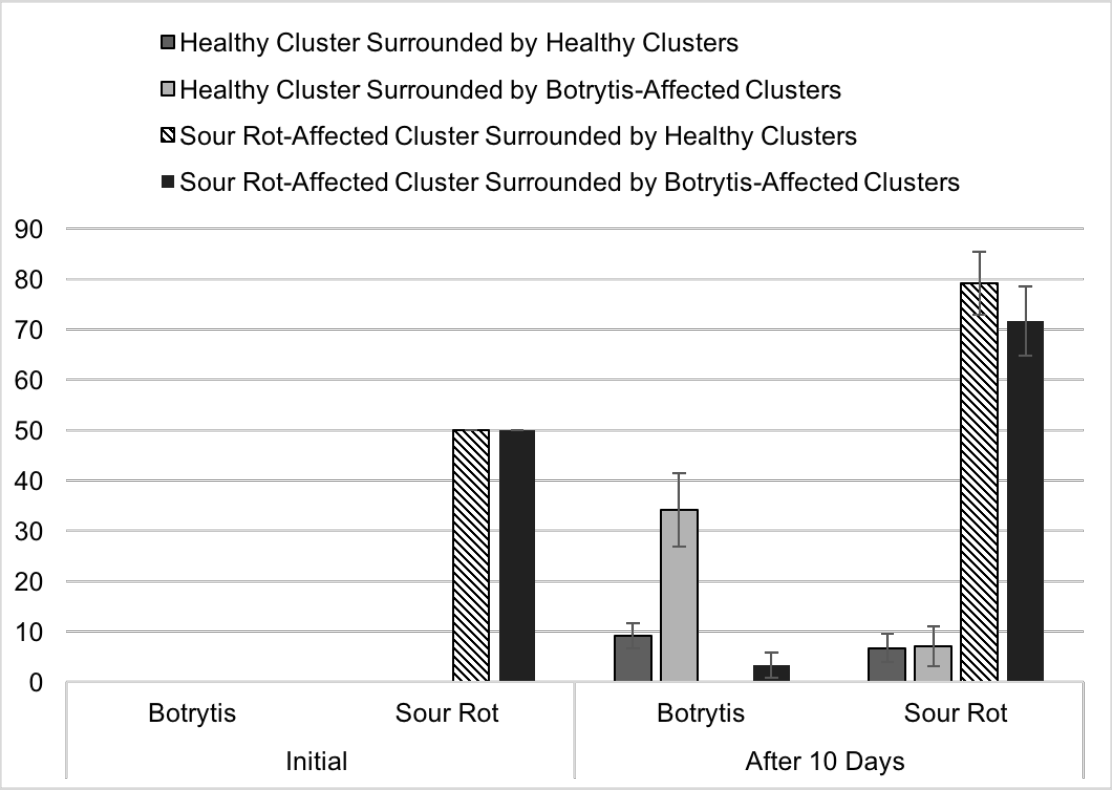
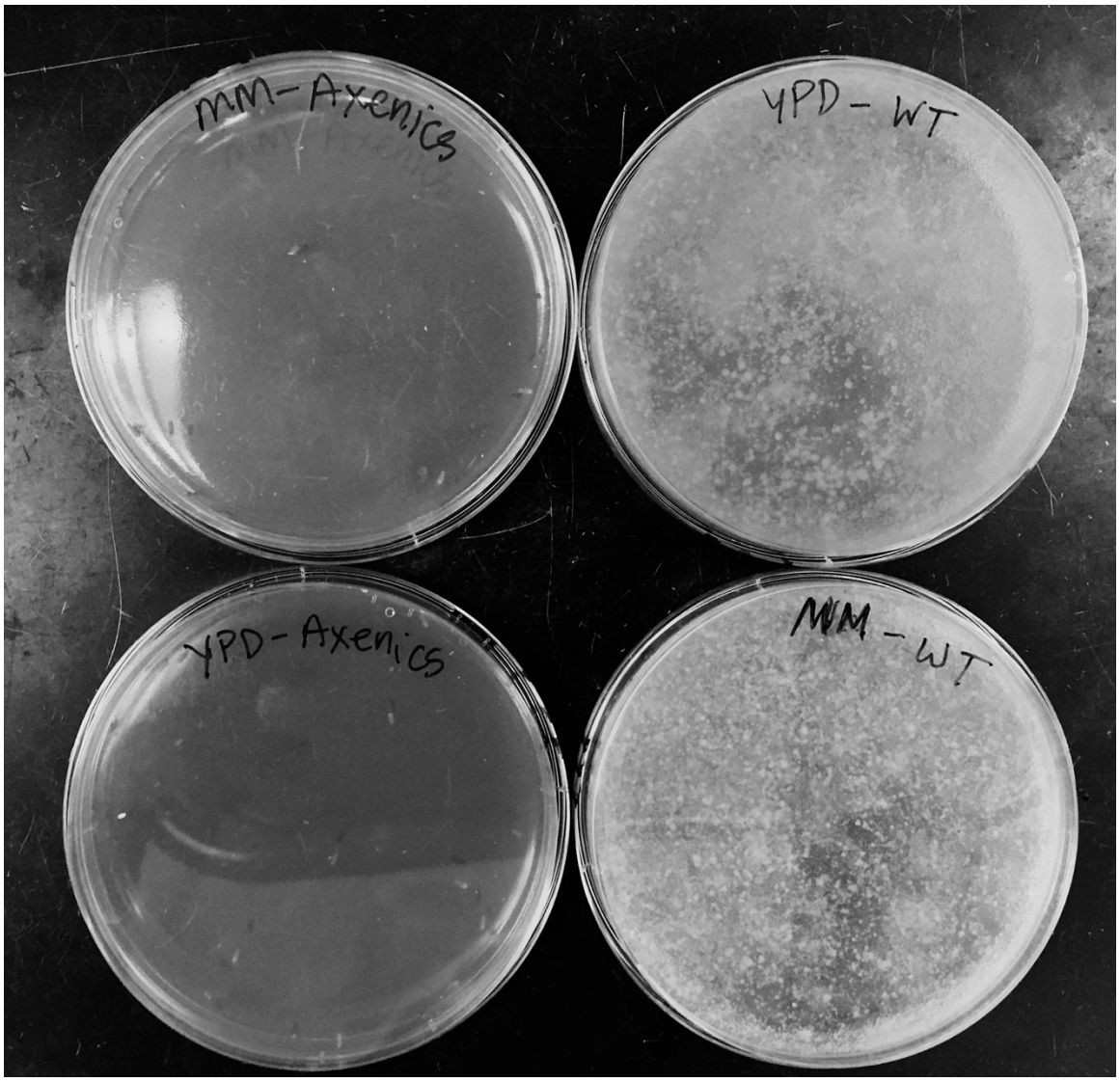

Healthy Cluster Surrounded by Healthy Clusters
Healthy Cluster Surrounded by Botrytis-Affected Clusters
Sour Rot-Affected Cluster Surrounded by Healthy Clusters
Sour Rot-Affected Cluster Surrounded by Botrytis-Affected Clusters
90
80
70
60
50
40
30
20
10
0
Botrytis
Sour Rot
Botrytis
Sour Rot
Initial
After 10 Days

Figure 11.



YPD and YPM (MM) media to which axenic and wild-type (WT) *Drosophila melanogaster* have been exposed for three days.

CHAPTER 2

Control of Sour Rot via Chemical and Canopy Management Techniques

Accepted for publication in the

American Journal of Enology and Viticulture, November 2017

Abstract

Sour rot is a disease complex characterized by rotting of the grape berry plus internal development of acetic acid, typically associated with an abundance of *Drosophila* fruit flies. Uncertainties regarding disease etiology and epidemiology have limited the development of reliable management practices. It is now known that yeast, acetic acid bacteria (AAB) and *Drosophila* spp. act together to cause the disease. Thus, we conducted three years of replicated field trials on the *Vitis* interspecific hybrid cv. Vignoles, in which we targeted these organisms through pre-harvest applications of various antimicrobial agents (potassium metabisulfite, copper hydroxide, BLAD polypeptide, and/or a mixture of hydrogen dioxide and peroxyacetic acid, depending on year) and an insecticide (spinetoram or zeta-cypermethrin, depending on year), both alone and in combination. Weekly applications of an antimicrobial plus insecticide provided an average 64% control relative to untreated vines across all three years of the trial when initiated preventively at 15°Brix, before the onset of symptoms; withholding addition of an antimicrobial to the insecticide application until symptoms appeared typically decreased the control level. Applying only an insecticide on the preventive schedule provided substantial control in two of the three years, whereas the antimicrobials were ineffective unless also applied with insecticide. Additionally, we studied disease development in a commercial vineyard of cv. Vignoles in which vines are trained to either a high wire cordon (HW) or vertical shoot position (VSP) system in groups of adjacent rows. In all three years of monitoring, disease severity was

significantly higher on vines in the HW system where drooping shoots enclosed fruit within an umbrella-like canopy, whose density between the fruiting zone and vineyard floor was greater than for VSP vines.

Key words: sour rot, yeast, acetic acid bacteria, Drosophila, trellis systems, integrated pest management

Introduction

Sour rot is a poorly-defined disease complex that is prevalent throughout temperate viticultural regions where pre-harvest rains occur. The skin of affected grapes turns a light brown color, in both red and white varieties, and then softens, releasing fermented grape pulp which smells of acetic acid (and occasionally, ethyl acetate) and drips onto other grapes within the cluster. Notably, fruit flies (*Drosophila* spp.) are commonly associated with the rotting clusters. Sour rot was originally thought to be the final and most destructive stage of gray mold, caused by *Botrytis cinerea* (Bisiach et al. 1982, 1986), and whereas this presumed scenario was later shown to be false, the term is sometimes applied to a general decay syndrome that may involve various yeasts, bacteria, and/or filamentous fungi (McFadden-Smith and Gubler 2015).

The uncertainty regarding the disease's etiology and epidemiology has severely limited the development of specific, targeted management strategies that are generally agreed upon. For example, the labels for some fungicides currently registered for use on grapes in the United States list sour rot as a target disease while ascribing its cause to filamentous fungi such as *Cladosporium* spp. and *Aspergillus* spp. Some researchers claim that yeasts play an essential role in the development of sour rot (Barata et al. 2012, Bisiach et al. 1982, Guerzoni and Marchetti 1987). Many have

noted the common association of acetic acid-producing bacteria (AAB) with the disease, such as species of *Gluconobacter* and *Acetobacter*, whereas Barata et al. (2012) concluded that AAB should be considered the etiological agents of sour rot. These latter authors also concluded that *Drosophila* spp. play a critical role as vectors of the yeasts and AAB involved in the development of sour rot, an opinion consistent with that of Bisiach et al. (1986) who similarly considered these insects to be important disease vectors.

In a separate series of experiments, we have determined that sour rot is the culmination of a process that begins with the fermentation of an injured berry's juice to ethanol by various yeasts (particularly *Saccharomyces* and *Pichia* species) and the subsequent oxidation of that ethanol to acetic acid by AAB, as proposed by Barata et al. (2012) (Hall et al. 2014, 2015, 2016, 2017). We have also found that *Drosophila* spp. play a crucial role in the development of sour rot beyond that of a vector (Hall et al. 2014, 2015, 2016, 2017). Accordingly, we initiated a series of field trials in which we examined various spray programs that employed general antimicrobial treatments likely to be effective against both yeasts and bacteria in conjunction with an insecticide treatment targeting *Drosophila* spp. Furthermore, because differential canopy management techniques have been shown to affect the development of sour rot (Zoecklein et al. 1992), we also examined the effect of two different training systems on the progress and severity of this disease.

Materials and Methods

Disease Control Trials. A series of control trials was established on different vines in each of four successive years in a vineyard of own-rooted *Vitis* interspecific hybrid 'Vignoles' in Geneva, New York (lat.: 42°52' 16", long.: -77°1' 59"), employing a

split plot design with four replications. The vineyard was planted in 2004 and trained to a vertically shoot positioned (VSP) trellis system with a 3-m row spacing and 2-m vine spacing. Whole plots consisted of single rows that were either treated with insecticide or not, with the subplots consisting of various antimicrobial treatments applied to either one or two, four-vine panels depending on row length and individual vine characteristics. Antimicrobial treatments were assigned at random within each row. The insecticide treatment was applied to alternate rows in 2013, whereas the rows receiving insecticide sprays were randomized within the trial area in 2014-16. In 2013, the insecticide used against *Drosophila* spp. was spinetoram (Delegate WG; Dow AgroSciences, Indianapolis, IN) and in the subsequent years, zeta-cypermethrin (Mustang Maxx; FMC Corp., Philadelphia, PA) was used. The antimicrobial products included potassium metabisulfite (KMS; Cellar Science, Pittsburg, CA); copper hydroxide (Kocide 3000; E. I. DuPont de Nemours & Co. Inc., Wilmington, DE); banda de Lupinus albus doce (BLAD) polypeptide (Fracture; FMC Corp., Philadelphia, PA); and a mixture of hydrogen dioxide and peroxyacetic acid (OxiDate 2.0; Biosafe Systems, Hartford, CT). Antimicrobial treatments varied among years in terms of the material applied, rate, and application timing (Table 1); a control treatment receiving no antimicrobial material was also included each year. All materials were applied with a hooded-boom sprayer delivering a volume of 935 L/ha and operating at a pressure of 2069 kPa.

Symptoms of sour rot do not appear in nearby Ontario, Canada until berries reach a sugar level of 15°Brix, and inoculated berries are not susceptible to the disease until that time (McFadden-Smith and Gubler 2015). Hence, the insecticide sprays and our basic pre-symptom antimicrobial programs were initiated shortly after a random sample of 20 berries from each of three individual rows averaged 15°Brix with a

refractometer. Antimicrobial treatments designated to begin only after symptoms appeared were applied when both visual and olfactory symptoms were detected in the vineyard. In 2016, two additional starting-point timings for antimicrobial sprays were added, based on environmental data measurements: (i) following the first rain after 15°Brix, since sour rot has long been associated with pre-harvest rains (McFadden-Smith and Gubler 2015, Oliva et al. 1999); and (ii) following a three-consecutive-day increase in maximum daily dew point (MDD), as determined by monitoring data produced daily by a weather station several hundred meters from the test site, beginning at 12°Brix. Unless otherwise noted, all treatment sprays were applied weekly upon initiation and were terminated during the final week before harvest. The Vignoles cultivar is relatively resistant to powdery mildew, downy mildew, and black rot, but mancozeb was applied three times per season to all vines (including the controls) to control these diseases and Phomopsis cane and leaf spot; Botrytis bunch rot was controlled with a rotational program utilizing fenhexamid, cyprodinil/fludioxonil, and fluopyram/tebuconazole applied at late bloom, bunch closure, veraison, and 2 weeks pre-harvest. A commercial formulation of *Bacillus thuringiensis* was applied as needed to control grape berry moth.

The harvest date for all years was determined when the fruit reached an average of 23 to 24°Brix as determined by a composite 20-berry sample collected from three rows, and was at least 2 days beyond the final spray application. At the time of harvest, 0.5 meters was measured from each post that defined the end of a treatment plot, and every cluster between those 0.5-m buffer zones was evaluated individually for sour rot severity based on a visual estimation of the percentage of the cluster showing typical necrosis and olfactory symptoms. A mixed effects model was used to analyze the mean severity ratings from each plot. The model includes the main effects of

antimicrobial treatment, insecticide, an interaction effect between treatment and insecticide, and the random effect of replicate to account for variation between replications. Each year was analyzed separately, due to differences in treatments among years. Because there was a significant main effect of insecticide in each year, the effect of insecticide within each antimicrobial treatment was analyzed via a t-test. The effect of each individual treatment in comparison to the control treatment (no antimicrobial or insecticide) was analyzed via Dunnett's Method of Comparison. The trial data in 2014 could not be utilized due to the confounding effects of a hailstorm that severely damaged the grape clusters at the time of veraison.

Training system effects. The effect of training system on sour rot development was evaluated in a commercial vineyard of *Vitis* interspecific hybrid cv. Vignoles in Branchport, NY (lat.: 42°34' 51", long.: -77°9' 45"). One block of this vineyard is divided into 14 rows of vines trained in a vertical shoot position (VSP) system, with the 14 immediately adjacent rows trained to a high wire (HW) cordon system. The top wire in the HW system is positioned at 167 cm above the vineyard floor; in the VSP system, the fruiting and catch wires are positioned 111 cm and 190 cm above the vineyard floor, respectively. A random number generator was used to select both the row number for 10 rows per training system and single vines within each row, which were marked to facilitate repeated data collection. During the pre-harvest period, disease severity was determined for all clusters on the marked vines at 3- to 4-day intervals, as described above. For statistical analysis, a Mixed Effects Model was used, examining the main effects of time and training system and the interaction of time by training system. Vine was treated as a random effect because measurements were taken over time on the same vines at every sampling point. The VSP and HW sections of the vineyard were considered as treatments within a single block. All vines

were subjected to the same fertilization and pest management program standard for this region (Weigle and Muza 2016). No summer pruning or hedging was employed.

To examine potential differences in canopy density between the HW and VSP vines, we employed two different techniques during a period approximately 2 weeks before harvest. In the first, Enhanced Point Quadrat Analysis (EPQA) (Meyers and Vanden Heuvel 2008), data were focused on the metrics of cluster exposure layer (CEL), leaf exposure layer (LEL), and occlusion leaf number (OLN). To determine these measurements, a stiff metal rod was inserted into the canopy at the fruiting zone every 20 cm over the length of the vine, positioned parallel to the ground. As the rod was inserted into the canopy, the number of leaf and cluster contacts was recorded. Using this information, the number of leaf layers within the fruiting zone could be calculated in various fashions by determining the total number of shade-producing layers (OLN), the number of shade producing layers between a cluster and the outer edge of the canopy (CEL), and the number of shade-producing layers between leaves and the outer edge of the canopy (LEL). EPQA measurements were made on the same vines used for the disease ratings. For statistical analysis, a two-sided t-test was performed to analyze the significance of the differences in mean OLN, CEL and LEL values for the vines between the two training systems, in each of the 2 years of assessment.

To further measure potential differences in canopy density, we employed methods described by Palleja and Landers (2017). Four XLMaxSonar MB7092 ultrasound sensors (MaxBotix Inc., Brainerd, MN) were mounted on a utility vehicle at heights of 60, 100, 140, and 180 cm above the vineyard floor and driven down five rows each of the HW- and VSP-trained vines (all of which had been used for the aforementioned disease and canopy density assessments) at a rate of 4.8 km/h in each direction, so as

to measure both sides of the canopy of each row. These sensors emit ultrasound wave pulses which propagate through the air, come in contact with a particular object and bounce back to the sensor, which records the returning waves (echoes). The energy and shape of these echoes, measured in volts, indicate distance from the sensor to the objects in front of it and their density. Thus, because we endeavored to keep the distance from the sensors to the outer canopy edge constant, variations among measurements for individual rows were attributable to differences in canopy density. For statistical analysis, a t-test was performed to determine the significance of the differences in these measures between the HW- and VSP-trained vines, at each ultrasound sensor height. This additional technique was employed only in the second of the 2 years of EPQA assessments.

Results

Disease control trials. In 2013, all four treatments in which both antimicrobial and insecticide treatments were applied provided highly significant ($P < 0.001$) levels of sour rot control, with disease severity reduced by 31 to 55% relative to the treatment receiving no antimicrobial or insecticide sprays. In contrast, applications of antimicrobials or insecticide (spinetoram) alone provided no significant control ($P =$ 0.27 to 0.66). In conjunction with the insecticide sprays, OxiDate 2.0 applied at a rate of 1% (v/v) provided comparable control whether it was applied pre- or post-symptom (five and three sequential sprays, respectively) (Table 2, Figure 1). T-tests showed a moderately significant ($P = 0.08$) to highly significant ($P <0.001$) difference between antimicrobial treatments applied alone versus those applied in conjunction with an insecticide (Table 3).

In 2015, disease pressure was notably higher, with the measure of sour rot severity on

untreated vines almost twice as great as in 2013 (Figures 1 and 2). Under these conditions, weekly applications of insecticide only (zeta-cypermethrin, a different material than used in 2013), beginning at 15°Brix before symptoms were visible, provided 66% control relative to the untreated vines ($P = 0.01$). When insecticide-treated vines were also sprayed concurrently with one of the three antimicrobial materials used that year, control levels were increased to 79 to 87% compared with the untreated vines, whereas the same antimicrobial treatments applied to vines that did not receive insecticide provided less control than the insecticide-only treatment. Delaying these antimicrobial treatments until symptoms were visible in the block resulted in reduced levels of control relative to the preventive approach (three versus five total applications of each antimicrobial, respectively). Similarly, Fracture applied once at 15°Brix without insecticide provided no apparent control relative to the untreated vines and when applied with insecticide, control was comparable to the insecticide-only treatment (Table 2, Figure 2). Differences in mean disease severity between antimicrobial treatments applied alone versus those applied in conjunction with an insecticide were modestly to highly significant ($P = 0.14$ to <0.01) when the antimicrobial treatments began pre-symptom but were insignificant ($P = 0.42$ to 0.91) when antimicrobial sprays were not initiated until symptoms developed (Table 3).

In 2016, sour rot severity on untreated vines was similar to that in 2015 (Figures 2 and 3). Weekly applications of zeta-cypermethrin alone beginning at 15°Brix (pre-symptom) reduced disease severity by about half ($P = 0.02$), whereas applying these in conjunction with any of the three different antimicrobials reduced disease severity by approximately two-thirds relative to untreated vines ($P <0.01$ to <0.001). In the insecticide-treated plots, post-symptom applications of OxiDate 2.0 and Fracture were modestly less effective than the preventive approach with these materials, whereas

delaying the initiation of OxiDate 2.0 sprays until either of the two climatic criteria had been satisfied did not improve control beyond that attained with insecticide sprays alone. With the exception of OxiDate 2.0 applied only after MDD increased over 3 consecutive days (the first three-consecutive-day increase in MDD occurred at 18°Brix, resulting in 2 weekly applications thereafter), none of the other six antimicrobial treatments provided significant control of sour rot in plots not also treated with the insecticide ($P = 0.13$ to 0.97) (Table 2, Figure 3). Direct comparisons showed that control was increased significantly ($P = 0.03$) when vines receiving sprays of OxiDate 2.0 beginning either pre- or post-symptom were also treated with insecticide, as was also the case with sprays of KMS initiated pre-symptom ($P = 0.002$). There was relatively little statistical significance ($P = 0.16$ to 0.78) to the effect of insecticide applications on disease severity in the pre- and post-symptom Fracture treatments or the two OxiDate 2.0 treatments initiated according to climatic criteria (Table 3).

Across all three years of control trials, treatments combining applications of an insecticide and an antimicrobial provided a weighted average of 64% control of disease severity relative to untreated vines when initiated at 15°Brix before symptoms appeared, and the difference for each of the nine total treatments relative to the appropriate untreated check was statistically significant ($P = 0.03$) to highly significant ($P < 0.001$). When applications of a subset of these antimicrobials to insecticide-treated vines were delayed until sour rot symptoms developed, control was occasionally comparable to the pre-symptom regimen for the same material but typically decreased to a varying extent among the six individual treatment x year combinations in this category ($P < 0.01$ to $P = 0.34$ in comparisons with the untreated check). In contrast, for the full range of antimicrobial treatments applied to vines that

had not also been treated with insecticide, control averaged only 23 and 28% for pre- and post-symptom programs, respectively, across the three years with typically low degrees of statistical significance in comparisons with the untreated check (Table 2). These general observations are supported by the analysis of variance showing highly significant P-values for the main effect of insecticide in all three years of the trial, but for antimicrobials only in 2013. There also was a highly significant insecticide x antimicrobial interaction in both 2013 and 2015 (Table 4).

Training system effects. Over the final 7 days before harvest in 2014, sour rot severity increased in the HW system from 20.6 to 35.4% and in the VSP system from 12.7 to 18.1%. At all three data collection points, disease severity was significantly (P =0.05) higher in the HW versus the VSP system (Fig. 4). In 2015, sour rot severity was again significantly (P =0.05) greater in the HW- versus VSP-trained vines at each of the five assessment dates over the final 12 days before harvest. Six days before harvest, the vineyard owner applied a combination of KMS (10 g/L) and zeta-cypermethrin across the entire block, after which further disease development stopped in both training systems (Fig. 5), in stark contrast with the disease progress observed in both 2014 and 2016 (Fig. 6) when no treatment was applied for sour rot control and disease severity increased continuously up to the day of harvest. In 2016, severity ratings made 10 days prior to harvest were not significantly different (P = 0.05) in the two training systems yet they were by harvest, with severity in the HW-trained vines nearly 50% greater than those trained to the VSP system. The generally rapid pre-harvest increase in disease severity was reflected by the highly significant effect of sampling time provided by the mixed-effects model in all three years (Table 5). The main effect of training system also was highly significant in 2014 and 2015 (P <0.0001). In 2016, the main effect of training system was insignificant (P=0.69),

reflecting the minor differences between the two during the first three evaluations; in contrast, the interaction between time and training system was highly significant (P=0.004), reflecting the substantial differences that had developed by harvest (Table 5).

Measures of OLN, CEL, and LEL, the EPQA parameters used to assess potential differences in canopy density, were virtually identical for vines in the two training systems in 2015 (*data not shown*). In 2016, measures for OLN and CEL were modestly but significantly ($P = 0.05$) higher for the HW-trained vines, indicating a denser fruit-zone canopy within this system, although the LEL values were once again virtually identical (Fig. 7). In 2016, the ultrasound sensor data indicated significant ($P = 0.05$) differences in canopy density between training systems at each sensor height, with VSP vines more dense at the two highest sensor levels and HW vines more dense at the two lowest levels. The VSP vines appeared least dense at the sensor level closest to the vineyard floor (60 cm height) and most dense at the 140-cm height, whereas the HW vines were least dense at the highest (180 cm) sensor level with density increasing progressively at each 40-cm increment toward the vineyard floor (Fig. 8).

Discussion

In all three years of the disease management trials, significant and consistent control was provided by applying antimicrobial and insecticide sprays in conjunction prior to the onset of sour rot symptoms, reducing disease severity by close to 70% over the untreated check. Insecticide sprays alone also provided significant control in the two years in which zeta-cypermethrin was utilized, whereas they did not in the one year in which spinetoram was the insecticide applied. However, we did not conduct a trial to

compare these two materials directly, so cannot determine whether such differential control is likely due to differential efficacies of the two insecticides or to some other factor(s) that varied among the trial seasons. In contrast, with the exception of a single treatment in a single season, antimicrobial sprays alone never provided a statistically significant level of control. Nevertheless, applications of an antimicrobial in conjunction with an insecticide typically increased the level of control relative to the insecticide alone when the antimicrobial applications were initiated before disease was observed, but not after. Although these results might suggest that a preventive spray program is substantially more efficacious than one triggered by the first detection of disease, the potential for interplot interference in our trials must be recognized. That is, our sprayed plots represented a mere fraction of the vines within a 0.6-ha block of the same cultivar, which otherwise were not treated with products likely to affect yeasts, AAB, or *Drosophila* spp., and these unsprayed rows may have provided a continuous source of both insects and microbes as the disease progressed unimpeded within them. Thus, it is possible that the degree of sour rot control provided by a post-symptom spray program could be more substantial in a vineyard in which an entire block is treated rather than just a few individual panels, and therefore, such an approach could be more effective than our trials demonstrated. This suggestion is supported by our observations in the commercial vineyard in Branchport in 2016, where an active sour rot epidemic did not progress further following a single application of KMS + zeta-cypermethrin, although the lack of unsprayed control panels in the vineyard block for comparison precludes our ability to draw conclusions from this observation. Furthermore, the experimental design of our spray trials did not allow us to examine the efficacy of delaying insecticide applications until symptom development. Thus, whereas we have clearly demonstrated the general utility of a pre-harvest spray program targeting both *Drosophila* spp. and the microbes responsible for

causing sour rot, the most efficient timing for doing so while limiting chemical inputs remains to be determined. It should also be noted that although KMS is widely used as a general antimicrobial product in winemaking, it is not registered for vineyard applications in the United States; therefore, the control that it provided in our trials should be viewed as a proof of concept rather than an implied recommendation for use on vines except where allowed.

The significant control provided by insecticide sprays targeting *Drosophila* spp. in our experiments is consistent with the results of Barata et al. (2012a), who prevented sour rot development on wounded berries if they were physically excluded from these insects, and of Bisiach et al. (1986), who obtained control of the disease in some experiments with insecticide applications targeting the pests although they concluded that the importance of *Drosophila* control would require further investigation. *Drosophila* spp. carry both yeast and AAB in their guts and on their bodies (Broderick and Lemaitre 2012), so should serve as vectors of these causal organisms to and among wounded berries as suggested by both Barata et al. (2012a) and Bisiach et al. (1986), in addition to playing a critical non-microbial role in sour rot development as we have found for axenic *D. melanogaster* individuals (Hall et al. 2015a, 2015b, 2016c, 2017). Neither yeasts nor AAB are capable of infecting unwounded berries, and berry injury is typically required for sour rot development (McFadden-Smith and Gubler 2015). Thus, Bisiach et al. (1986) also emphasized the importance of controlling of wounding agents such as Botrytis, powdery mildew, and other insect larvae within an integrated program to control the disease. Therefore, we included control measures for these wounding agents in our trials, so that we could examine the effects of spray programs targeting *Drosphila* spp., yeasts, and AAB without the influence of additional confounding factors. Nevertheless, minimizing the possibility of wounds from various biotic and abiotic agents appears to be a key component of

any sour rot management program. For example, McFadden-Smith (2009) showed that clusters whose compactness was reduced following applications of prohexidione calcium had significantly less sour rot than untreated, tighter clusters. With higher cluster compactness, berries press against one another, separating from the pedicel and creating wounds that facilitate entry of the organisms that cause sour rot but are otherwise unable to penetrate intact berries. Within this context, it should be noted that whereas there has been speculation about the potential role of the spotted wing Drosophila (*D. suzukii*) in sour rot development due to its ability to oviposit through the intact berry epidermis of some thin-skinned cultivars (Ioriatti et al. 2015), this species represents a minority of the fruit flies reared from decaying berries in New York vineyards (Loeb 2014), which is consistent with results reported from Oregon (Ioriatti et al. 2015).

In addition to the factors discussed above, we found that training system also had an effect on sour rot severity. In all three years of our monitoring, sour rot severity was significantly greater in HW-trained vines of cv. Vignoles than VSP-trained vines of the same cultivar. In an effort to quantify potential differences in canopy density between the training systems, we initially utilized EPQA measurements but were largely unable to distinguish between the two with respect to densities within the fruiting zone, the only portion of the canopy subject to EPQA assessments. However, the ultrasound technique that we utilized clearly illustrated the far greater density of leaves between the fruiting zone and the vineyard floor in the HW system, which was visible to the naked eye owing to the umbrella-like canopy structure produced as the vigorous shoots first grew upwards from the top wire and then drooped down towards the vineyard floor as they increased in length. In contrast, there is no such area created in the VSP system where catch wires maintain the shoots in an upward

position, thereby concentrating the canopy above the fruiting zone (particularly on vines not hedged during the growing season), as reflected by the relative ultrasound measurements that we obtained for the two systems. Thus, it is possible that the umbrella-like canopy structure produced by the HW system may have provided an environment more favorable for sour rot development due to factors such as reduced air circulation within the cluster region, although we did not measure environmental variables within the canopies. Interestingly, Zoeklein et al. (1992) also presented data showing substantially greater cluster rot severity on *V. vinifera* cultivars in a vineyard trained with a high cordon wire and drooping shoots versus another one with a low cordon wire and upright shoots when the same cultivars were evaluated, and although they demonstrated disease reduction through fruit zone leaf removal in both vineyards they were not able to compare the two training systems directly.

Collectively, our results and those of others indicate that an integrated program for managing sour rot ideally should consist of multiple techniques to the extent that they are practical and likely to be necessary based upon climate and individual vineyard factors, including previous history with the disease. These may include actions designed to increase sun exposure and ventilation within the fruiting zone, which also should improve the deposition of spray materials applied to protect the fruit from pests and diseases (Austin et al. 2011); reduce cluster compaction; protect against animal and microbial wounding agents; control the development of *Drosophila* spp. populations; and limit the development of the yeasts and AAB that serve as causal agents of sour rot.

Conclusions

Sour rot is a significant and challenging disease complex, caused by an interaction

between yeast, acetic acid bacteria (AAB) and *Drosophila* fruit flies, that affects grape growers worldwide. In a series of replicated trials we found that a combination of antimicrobial plus insecticide sprays targeting these organisms consistently provided significant control of the disease when applied weekly after berry soluble solids content reached 15°Brix, before symptoms appeared. Insecticide sprays appeared to provide greater control than antimicrobials, although combining the two generally was most effective. Delaying antimicrobial applications until symptoms appeared usually was less effective than initiating them before symptom development and often provided no significant benefit. In a commercial vineyard of the interspecific hybrid cv. Vignoles where different vines were trained to either a high wire cordon (HW) or vertical shoot position (VSP) system and subjected to the same grower practices, sour rot severity was significantly greater on the HW vines in three consecutive years of evaluation. Measurements of canopy density utilizing an ultrasound sensor system showed HW vines to have greater density than VSP vines between the vineyard floor and the fruiting zone whereas the VSP vines were denser above the fruiting zone; expanded point quadrat analysis, which evaluated densities only within the fruiting zone, showed little difference between the two training systems in this portion of the canopy. An integrated control program for sour rot should utilize both canopy management and spray applications that target yeasts, AAB, and *Drosophila* spp., although the most efficient protocol for timing such spray applications has yet to be determined.

Literature Cited

1. Austin CN, Meyers J, Grove GG and Wilcox WF. 2011. Powdery mildew severity as a function of canopy density: Associated impacts on sunlight penetration and spray coverage. Am J Enol Vitic 62.1:23-31.

2. Barata A, Correia Santos S, Malfeito-Ferreira M and Loureiro V. 2012a. New Insights into the Ecological Interaction Between Grape Berry Microorganisms and Drosophila Flies During the Development of Sour Rot. Microb Ecol 64:416–430.
3. Barata A, Malfeito-Ferreira M and Loureiro V. 2012b. Changes in sour rotten grape berry microbiota during ripening and wine fermentation. Int J Food Microbiol. 154:152–161. Available at: http://dx.doi.org/10.1016/j.ijfoodmicro.2011.12.029.
4. Bisiach M, Minervini G and Salomone MC. 1982. Recherches expérimentales sur la pourriture acide de la grappe et sur ses rapports avec la pourriture grise 1. EPPO Bull. 12:15–27. Available at: http://doi.wiley.com/10.1111/j.1365-2338.1982.tb01675.x.
5. Bisiach M, Minervini G. and Zerbetto F. 1986. Possible integrated control of grapevine sour rot. Vitis 25:118–128. Available at: e024052.pdf.
6. Broderick NA and Lemaitre B. 2012. Gut-associated microbes of Drosophila melanogaster. Gut Microbes. 3.4: 307-321.
7. Guerzoni E and Marchetti R. 1987. Analysis of yeast flora associated with grape sour rot and of the chemical disease markers. Appl Environ Microbiol 53:571–576.
8. Hall ME, Loeb GM, and Wilcox WF. 2014. Ethanol and Acetic Acid Content of Sour-Rotted Wine grapes and Disease Control with Antimicrobial and Insecticide Sprays. (Abstr.) 65th ASEV National Conference, Technical Abstracts: 164
9. Hall ME, Loeb GM and Wilcox WF. 2015a. Improving Our Understanding of Sour Rot Etiology and Management Techniques. (Abstr.) 66th ASEV National Conference, Technical Abstracts: 70

10. Hall ME, Loeb GM and Wilcox WF. 2015b. Etiology and Management Strategies for Sour Rot on Grapes. (Abstr.) Phytopathology 105 (Suppl. 4): S4.56. http://dx.doi.org/10.1094/PHYTO-105-11-S4.1
11. Hall ME, Loeb GM and Wilcox WF. 2016a. Etiology and Management of Sour Rot in Grapes. (Abstr.) Phytopathology 106 (Suppl. 4): S4.64. http://dx.doi.org/10.1094/PHYTO-106-12-S4.1
12. Hall ME, Loeb GM and Wilcox WF. 2016b. Etiology and Management of Sour Rot in Grapes. (Abstr.) Phytopathology 107:S2.7. http://dx.doi.org/10.1094/PHYTO-107-2-S2.5
13. Hall ME, Loeb GM and Wilcox WF. 2016c. Further Understanding the Cause and Management of Sour Rot. (Abstr.) 67th ASEV National Conference, Technical Abstracts: 52
14. Hall ME, Loeb GM and Wilcox WF. 2017. Causal role of *Drosophila* spp. in Sour Rot Development. (Abstr.) 68th ASEV National Conference, Technical Abstracts: 64
15. Ioriatti C, Walton V, Dalton D, Anfora G, Grassi A, Maistri S and Mazzoni V. 2015. *Drosophila suzukii* (Diptera: Drosophilidae) and its potential impact to wine grapes during harvest in two cool climate wine grape production regions. J Econ Entomol 108(3):1148-55
16. Loeb G. Grape insect and mite pests – 2014 field season. Lake Erie Regional Grape Program Vineyard Notes. May 13, 2014: 2-24.
17. McFadden-Smith W. 2009. Efficacy of treatments for control of Botrytis bunch rot and sour rot of grapevine, 2008. Plant Dis. Manag. Rep. 3:SMF003. Online publication. DOI:10.1094/PDMR03

18. McFadden-Smith W. and Gubler WD. 2015. Sour Rot. pp. 87-90 *in:* Compendium of Grape Diseases, Pests, and Disorders, 2nd Ed. WF Wilcox, WD Gubler and JK Uyemoto, eds. APS Press, St. Paul, MN. 232 pp.
19. Meyers JM and Vanden Heuvel JE. 2008. Enhancing the precision and spatial acuity of point quadrat analyses via calibrated exposure mapping. Am J Enol Vitic. 59.4: 425-431.
20. Oliva J, Navarro S, Navarro G, Cámara MA and Barba A. 1999. Integrated control of grape berry moth (*Lobesia botrana*), powdery mildew (*Uncinula necator*), downy mildew (*Plasmopara viticola*) and grapevine sour rot (*Acetobacter spp.*). Crop Prot. 18:581–587.
21. Palleja T and Landers AJ. 2017. Real time canopy density validation using ultrasonic envelope signals and point quadrat analysis. Computers and Electronics in Agriculture 134: 43-50
22. Weigle TH and Muza AJ, eds. 2016. New York and Pennsylvania Pest Management Guidelines for Grapes. Cornell University, Ithaca, NY. 144 pp.
23. Zoecklein BW, Wolf TK, Duncan NW, Judge JM. and Cook MK. 1992. Effects of Fruit Zone Leaf Removal on Yield, Fruit Composition, and Fruit Rot Incidence of Chardonnay and White Riesling. Am J Enol Vitic. 43:139–148

Figure 1. Sour rot severity in a vineyard block of *Vitis* interspecific hybrid cv. Vignoles in Geneva, NY in 2013 as a function of antimicrobial and insecticide treatments. Data represent the mean values across four replicate one- or two-panel plots per treatment, in which all clusters were rated. Asterisks (*) above a bar denote a significant difference relative to the treatment receiving no antimicrobial or insecticide sprays as determined by Dunnett's Method of Comparisons. *** = significant at P= 0.001.

Figure 2. Sour rot severity in a vineyard block of *Vitis* interspecific hybrid cv. Vignoles in Geneva, NY in 2015 as a function of antimicrobial and insecticide treatments. Data represent the mean values across four replicate one- or two-panel plots per treatment, in which all clusters were rated. An asterisk (*) above a bar denotes a significant difference relative to the treatment receiving no antimicrobial or insecticide sprays as determined by Dunnett's Method of Comparisons. * = significant at P = 0.05.

Figure 3. Sour rot severity in a vineyard block of *Vitis* interspecific hybrid cv. Vignoles in Geneva, NY in 2016 as a function of antimicrobial and insecticide treatments. Data represent the mean values across four replicate one- or two-panel plots per treatment, in which all clusters were rated. An asterisk (*) above a bar denotes a significant difference relative to the treatment receiving no antimicrobial or insecticide sprays as determined by Dunnett's Method of Comparisons. * = significant at P = 0.05, ** = significant at P = 0.01, *** = significant at P = 0.001.

Figure 4. Progressions of sour rot severity in a commercial vineyard block of *Vitis* interspecific hybrid cv. Vignoles in Branchport, NY over the final 7 days before

harvest in 2014 as a function of two training systems, High Wire Cordon (HW) and Vertical Shoot Positioning (VSP). Values represent the mean disease severities determined for all clusters on 10 vines in each of the two training systems. For each assessment date, means not labeled with a common letter are significantly different according to the Tukey-Kramer HSD test (p = 0.05).

Figure 5. Progression of sour rot severity in a commercial vineyard block of *Vitis* interspecific hybrid cv. Vignoles in Branchport, NY over the final 12 days before harvest in 2015, as a function of two training systems, High Wire Cordon (HW) and Vertical Shoot Positioning (VSP). Following the 7-day-preharvest assessment, the growers applied a spray consisting of potassium metabisulfite and zeta-cypermethrin to all vines (arrow). Values represent the mean disease severities determined for all clusters on 10 vines in each of the two training systems. For each assessment date, means not labeled with a common letter are significantly different according to the Tukey-Kramer HSD test (p = 0.05).

Figure 6. Progression of sour rot severity in a commercial vineyard block of *Vitis* interspecific hybrid cv. Vignoles in Branchport, NY over the final 10 days before harvest in 2016 as a function of two training systems, High Wire Cordon (HW) and Vertical Shoot Positioning (VSP). Values represent the mean disease severities determined for all clusters on 10 vines in each of the two training systems. For each assessment date, means not labeled with a common letter are significantly different according to the Tukey-Kramer HSD test (p = 0.05).

Figure 7. Enhanced Point Quadrat Analysis (EPQA) assessed in 2016 on vines of *Vitis* interspecific hybrid cv. Vignoles trained to two different systems, High Wire Cordon (HW) and Vertical Shoot Positioning (VSP), in a commercial vineyard in Branchport, NY. Occlusion layer number (OLN) represents the number of shade-producing contacts (leaves and clusters) per insertion; Cluster exposure layer (CEL) represents the number of shade layers between clusters and the nearest canopy boundary; Leaf exposure layer (LEL) represents the number of shading layers between leaves and the nearest canopy boundary. Values represent the mean assessments determined on 10 vines in each of the two training systems. For each parameter, means not labeled with a common letter are significantly different according to the Tukey-Kramer HSD test ($p = 0.05$).

Figure 8. Ultrasound sensor canopy density measurements collected in late summer 2016 from vines of *Vitis* interspecific hybrid cv. Vignoles trained to two different systems, High Wire Cordon (HW) and Vertical Shoot Positioning (VSP), in a commercial vineyard in Branchport, NY. Four sensors were mounted on a utility vehicle at heights ranging from 60 to 180 cm above the vineyard floor and were driven down both sides of each of five rows of vines in each training system, for a total of 10 passes per training system. The data collected, expressed in volts, indicate the relative canopy densities at each given height, and are presented as mean values for the 10 replicate measures per training system at each height. For each height, means not followed by a common letter are significantly different according to the Tukey-Kramer HSD test ($p = 0.05$).

Table 1. Antimicrobial treatments applied in disease control trials

Treatment, rate per L and timing[a]	Years applied
Untreated control	2013, 2015, 2016
KMS[b] 5 g, pre-symptoms	2013
KMS[b] 10 g, pre-symptoms	2013, 2015, 2016
Copper hydroxide 1.0 g, pre-symptoms	2013
KMS[b] 10 g, post-symptoms	2013, 2015
OxiDate 2.0[c] 10 mL, pre-symptoms	2015, 2016
Fracture[d] 2.5 mL, pre-symptoms	2015, 2016
Fracture[d] 2.5 mL, once at 15°Brix	2015
Fracture[d] 2.5 mL, post-symptoms	2015, 2016
OxiDate 2.0[c] 10 mL, post-symptoms	2015, 2016
OxiDate 2.0[c] 10 mL, following first rain after 15°Brix	2016
OxiDate 2.0[b] 10 mL, following 3-consecutive-day increase in maximum daily dew point after 15°Brix	2016

[a]Unless otherwise noted, all sprays were applied at weekly intervals upon initiation: pre-symptom sprays once a 20-berry sample indicated a soluble solids content of 15°Brix, post-symptom sprays once disease was observed in the trial plot, in a volume of 935 L/ha.
[b]KMS = potassium metabisulfite.
[c]OxiDate 2.0 = a commercial formulation consisting of 27% hydrogen dioxide + 2% peroxyacetic acid.
[d]Fracture = a commercial formulation containing 20% banda de Lupinus alba doce (BLAD) polypeptide.

Table 2. Mean percent disease control relative to the untreated check for each treatment in individual years and cumulatively across all years in which that treatment was administered

Antimicrobial treatment, rate/L and timing[a]	**Insecticide[e]**	**2013**		**2015**		**2016**		**Cumulative**
		% Disease control	p[f]	% Disease control	*p*	% Disease control	*p*	
None	No	--	--	--	--	--	--	--
	Yes	10.3	0.556	66.3	0.121	48.2	0.046	41.6
KMS[b] 5 g, pre-symptoms	No	12.3	0.362					12.3
	Yes	31.4	<0.001					31.4
KMS[b] 10 g, pre-symptoms	No	9.2	0.664	14.6	0.999	23.1	0.760	15.6
	Yes	47.1	<0.001	81.4	0.030	70.6	<0.001	66.4
Copper hydroxide 1.0 g, pre-symptoms	No	11.9	0.399					11.9
	Yes	55.2	<0.001					54.3
OxiDate[c] 2.0 10 mL, pre-symptoms	No			45.7	0.511	15.9	0.973	30.8
	Yes			87.0	0.017	61.1	<0.01	74.1
Fracture[d] 2.5 mL, pre-symptoms	No			32.2	0.873	41.1	0.128	36.7
	Yes			79	0.038	58.9	<0.01	69.5
KMS[b] 10 g, post-symptoms	No		0.269	47.6	0.453			30.6
	Yes		<0.001	57.6	0.239			55.2
OxiDate[c] 10 mL, post-symptoms	No			49.7	0.405	3.3	1	26.5
	Yes			52.4	0.341	59.7	0.006	56.1
Fracture[d] 2.5 mL, post-symptoms	No			45.8	0.505	23.6	0.601	39.7
	Yes			65.4	0.131	50.1	0.034	57.8
Fracture[d] 2.5 mL, once at 15°Brix	No			30.1	0.914			30.1
	Yes			66.7	0.116			66.7
OxiDate[c] 2.0 10 mL, following first rain after 15°Brix	No					36.8	0.223	36.8
	Yes					41.8	0.116	41.8
OxiDate[c] 2.0 10 mL, following 3-consecutive-day increase in maximum daily dew point after 15°Brix	No					54.5	0.017	54.5
	Yes					41.6	0.119	41.6

[a]Unless otherwise noted, all sprays were applied at weekly intervals upon initiation: pre-symptom sprays once a 20-berry sample indicated a soluble solids content of 15°Brix, post-symptom sprays once disease was observed in the trial plot. The spray volume was 935 L/ha for all applications.
[b]KMS = potassium metabisulfite.
[c]OxiDate 2.0 = a commercial formulation consisting of 27% hydrogen dioxide + 2% peroxyacetic acid

[d]Fracture = a commercial formulation containing 20% banda de Lupinus alba doce (BLAD) polypeptide.
[e]Insecticide sprays (spinetoram, 0.075 g/L in 2013; zeta-cypermethrin, 0.027 g/L in 2015 and 2016) were applied at weekly intervals once a 20-berry sample indicated a soluble solids content of 15°Brix, on the same days as relevant antimicrobial sprays were applied.
[f]P-values as calculated by Dunnett's test comparing percent disease severity of each treatment to the untreated control.

Table 3. Statistical significance of antimicrobial and insecticide treatments and their interaction with respect to sour rot severity in the three years of control trials, as determined by analysis of variance with the mixed-effects model

	Antimicrobial	**Insecticide**	**Antimicrobial*Insecticide**
	Disease severity[a]	**Disease severity[a]**	**Disease severity[a]**
	p	***p***	***p***
2013	<0.001	<0.001	0.008
2015	0.817	0.010	0.541
2016	0.214	0.016	0.017

[a] Mean % cluster area showing symptoms of sour rot, determined for all clusters per plot at the time of harvest for four replicate plots per treatment.

Table 4. Statistical significance[a] of the differences in mean sour rot severity between plots treated versus not treated with insecticide, for each of the antimicrobial treatments applied in each year of the trial

Antimicrobial treatment[b]	***2013***	***2015***	***2016***
None	0.205	0.020	0.042
KMS[c] 5 g, pre-symptoms	0.078		
KMS[c] 10 g, pre-symptoms	<0.001	0.125	0.002
Copper hydroxide 1.0 g, pre-symptoms	0.003		
KMS[c] 10 g, post-symptoms	0.001	0.776	
OxiDate[d] 2.0 10 mL, pre-symptoms		0.145	0.033
Fracture[e] 2.5 mL, pre-symptoms		0.007	0.514
Fracture[e] 2.5 mL, once at 15°Brix		0.349	
Fracture[e] 2.5 mL, post-symptoms		0.426	0.164
OxiDate[d] 10 mL, post-symptoms		0.912	0.0326*
OxiDate[d] 2.0 10 mL, following first rain after 15°Brix			0.7825
OxiDate[d] 2.0 10 mL, following 3-consecutive-day increase in maximum daily dew point after 15°Brix			0.4063

[a] P-values as calculated by two-sided t-tests.
[b]Unless otherwise noted, all sprays were applied at weekly intervals upon initiation: pre-symptom sprays once a 20-berry sample indicated a soluble solids content of 15°Brix, post-symptom sprays once disease was observed in the trial plot. The spray volume was 935 L/ha for all applications.
[c]KMS = potassium metabisulfite.
[d]OxiDate 2.0 = a commercial formulation consisting of 27% hydrogen dioxide + 2% peroxyacetic acid
[e]Fracture = a commercial formulation containing 20% banda de Lupinus alba doce (BLAD) polypeptide.

Table 5. Statistical significance of sampling time points, training system and their interaction with respect to sour rot severity in a commercial vineyard block of *Vitis* interspecific hybrid Vignoles, as determined by a mixed-effects model

	Sampling Time	**Training System**	**Sampling Time*Training System**
	Disease severity[a]	**Disease severity[a]**	**Disease severity[a]**
	p	***p***	***p***
2014	0.002	<0.001	0.15
2015	<0.001	<0.001	0.61
2016	<0.001	0.69	0.004

[a] Mean % cluster area showing symptoms of sour rot, determined for all clusters per plot at the sampling time.

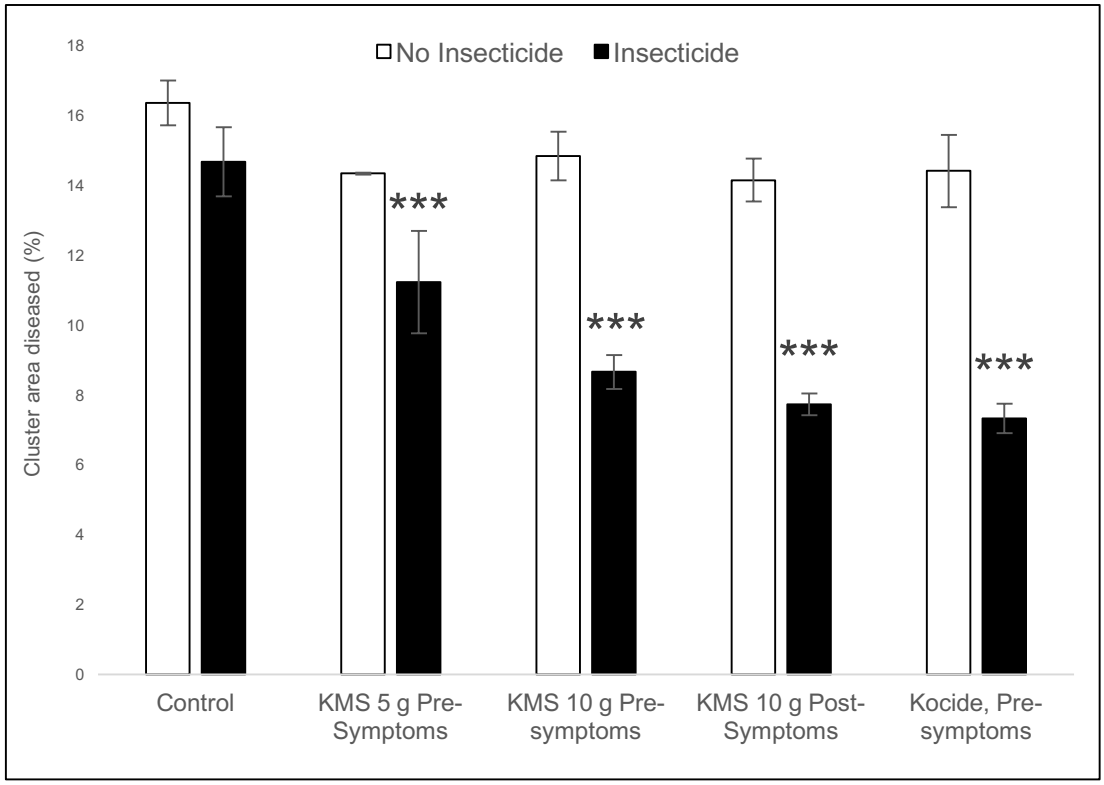

No Insecticide
Insecticide
Cluster area diseased (%)
18
16
14
12
10
8
6
4
2
0
***
***
***
***
Control
KMS 5 g Pre-Symptoms
KMS 10 g Pre-symptoms
KMS 10 g Post-Symptoms
Kocide, Pre-symptoms

Figure 1.

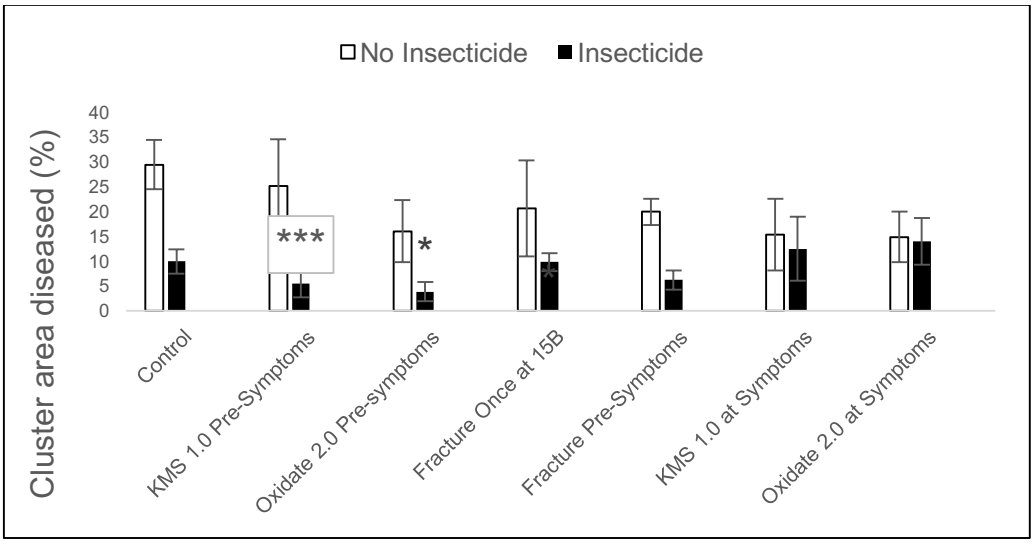


Figure 2.

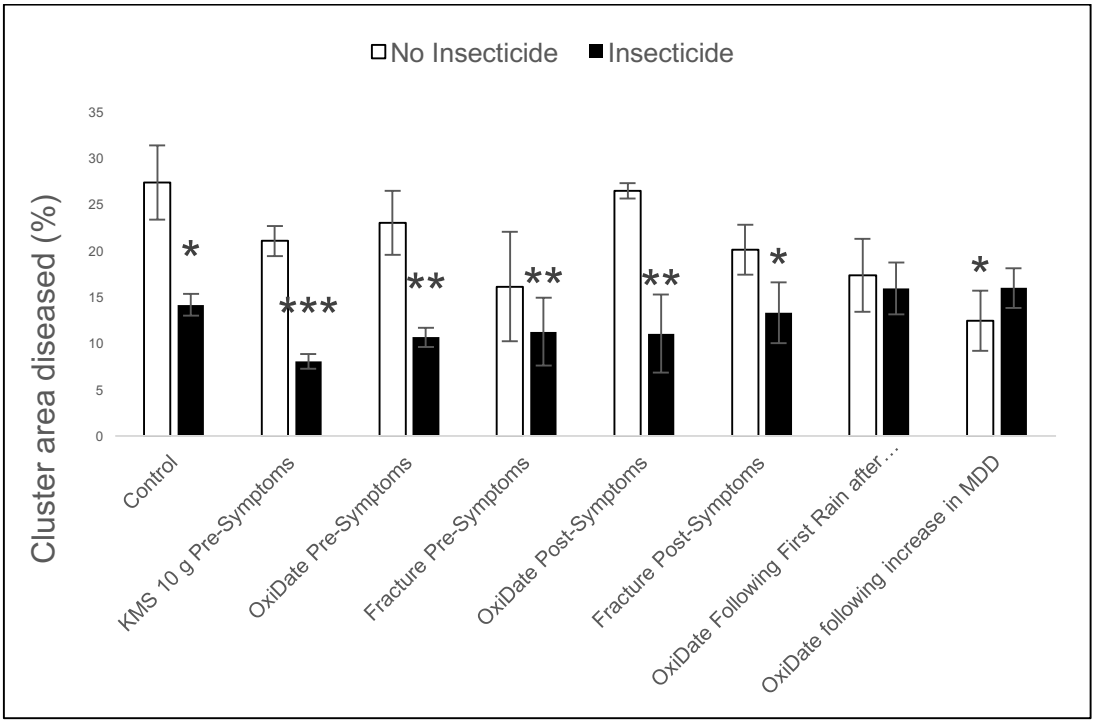


Figure 3.

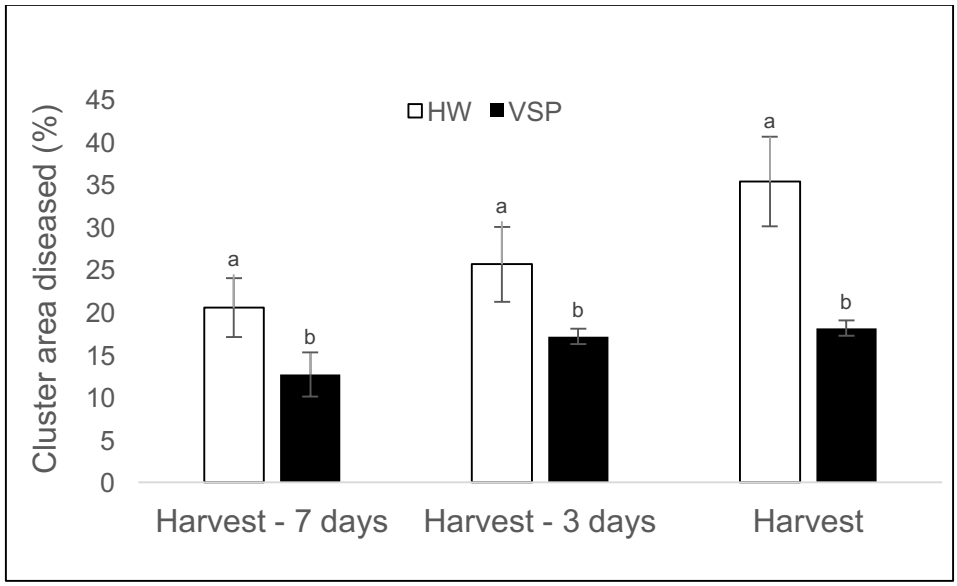

Cluster area diseased (%)
45
40
35
30
25
20
15
10
5
0
HW
VSP
a
b
a
b
a
b
Harvest - 7 days
Harvest - 3 days
Harvest

Figure 4.

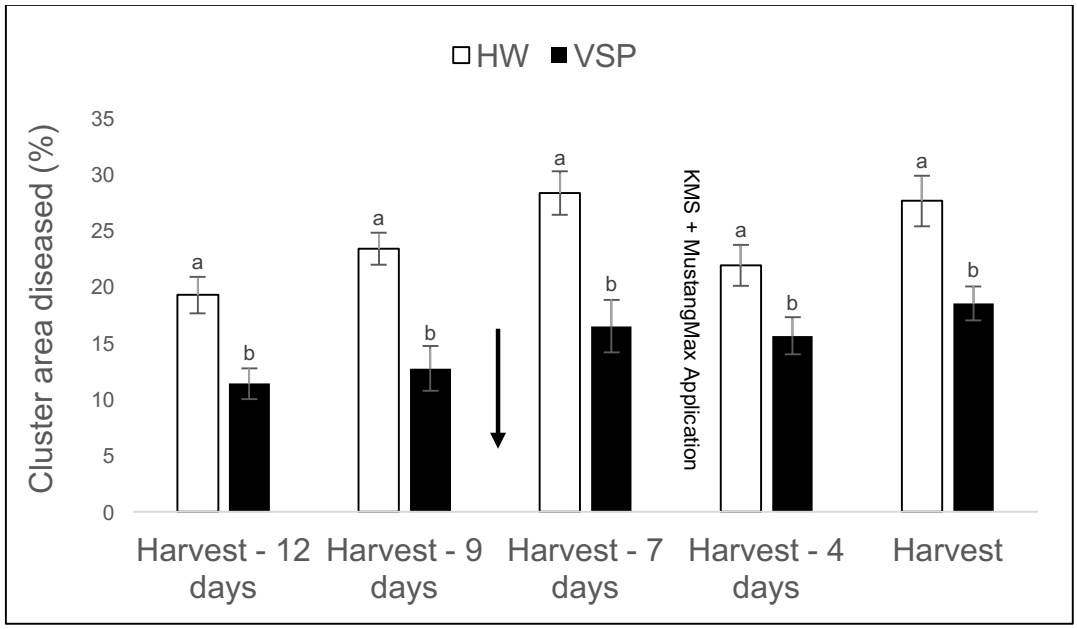

HW
VSP
Cluster area diseased (%)
35
30
25
20
15
10
5
0
a
b
a
b
a
b
a
b
a
b
KMS + MustangMax Application
Harvest - 12 days
Harvest - 9 days
Harvest - 7 days
Harvest - 4 days
Harvest

Figure 5.

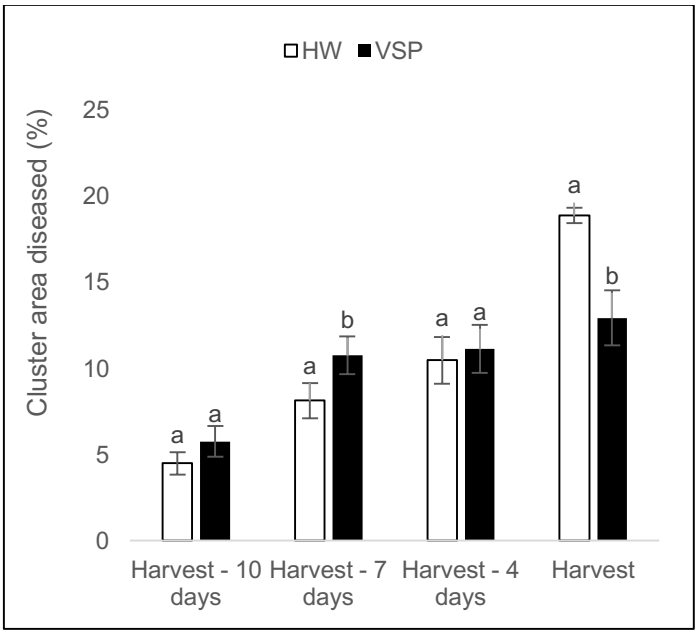


Figure 6.

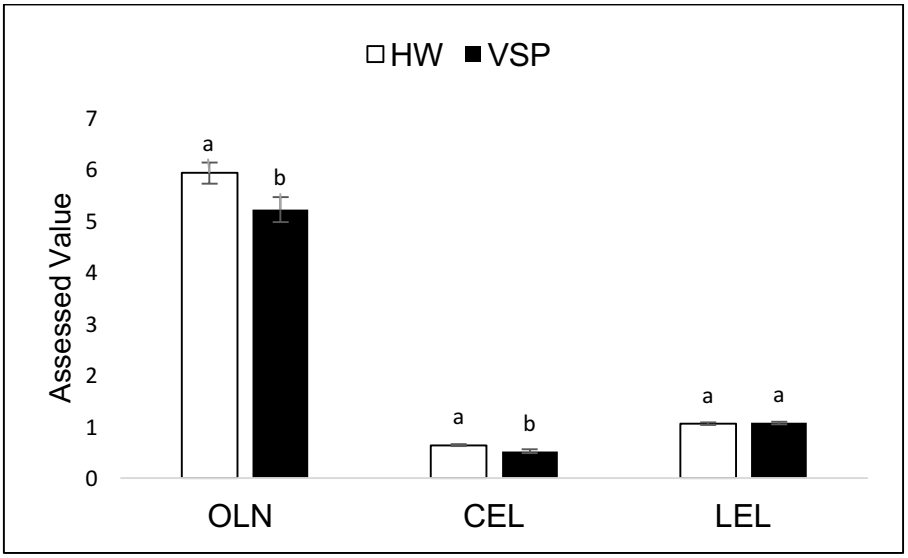


Figure 7.

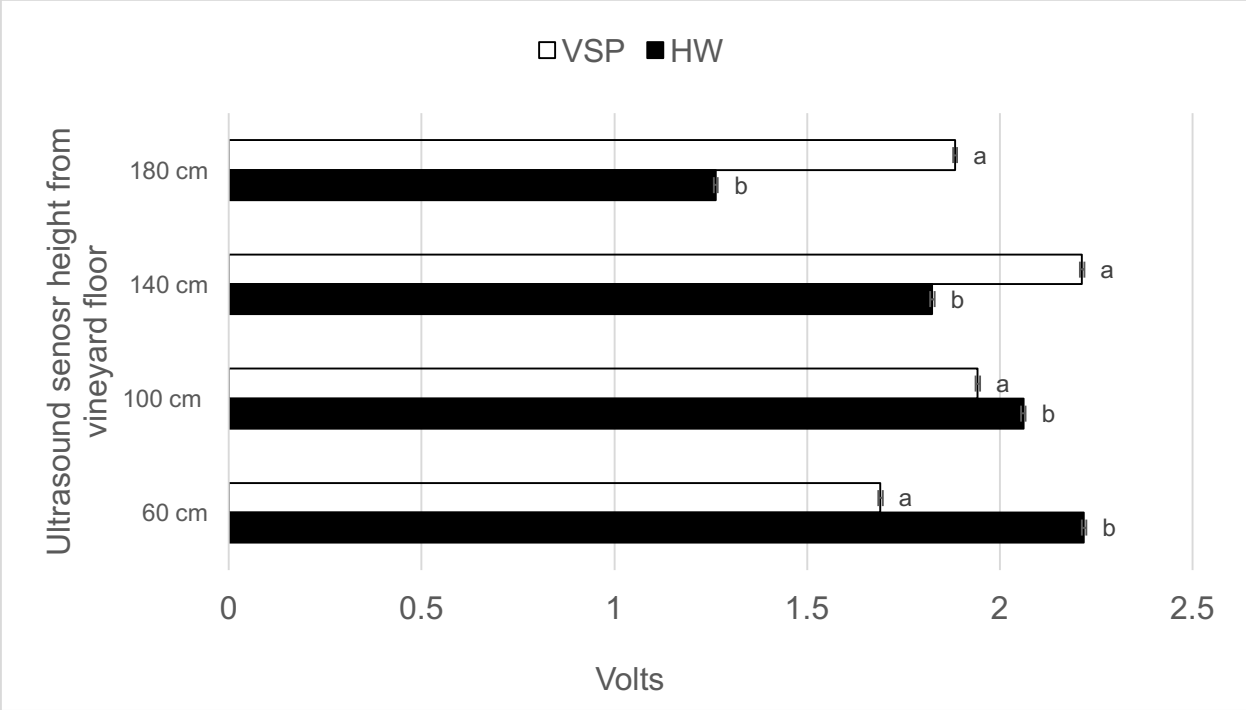

VSP
HW
Ultrasound senosr height from vineyard floor
180 cm
140 cm
100 cm
60 cm
a
b
a
b
a
b
a
b
0
0.5
1
1.5
2
2.5
Volts

Figure 8.

CHAPTER 3

Microbial Ecology of Sour Rot-Infected Grapes

Abstract

Yeast and bacteria are consistently found on healthy grape berries worldwide. High-throughput sequencing of these communities showed Pichia and Saccharomyces species, and many representatives of the Enterobacteraceae and Bacillaceae families on healthy grape at harvest regardless of region or variety. The ways in which these populations change when the berries are no longer healthy has not been explored on a large geographic or temporal scale. Sour rot is a disease complex involving the interaction between yeast, acetic acid bacteria and Drosophila fruit flies inside the grape berry. To better understand whether sour rot microbial populations differ by region, we characterized the phytobiome of sour rot-infected grapes from four diverse geographical areas across two years. In 2015 and 2016, both healthy and sour-rot affected berries were collected from a research vineyard in Geneva, NY and commercial vineyards in Tasmania, AUS, and in 2016, sour-rot infected grapes were collected from vineyards in Fredonia, NY, and Modesto, CA. We found the same predominate organisms that many researchers have pointed to previously, and those same organisms only increased in abundance when associated with sour rot symptoms. The shifts that occurred were primarily in the increased abundance of Pichia and Acetobacter species, which indicates that no new colonizers are necessary to initiate sour rot symptoms; instead it appears that disease symptoms could be caused by the endogenous yeast and bacteria, with the addition of Drosophila flies.

Introduction

The surface of a healthy grape berry is the site of abundant populations of yeast and bacteria. While the microbial composition of these dynamic communities vary by grape cultivar, site and sampling time (Bokulich et. al. 2013, Pinto et. al. 2015, Setati et. al. 2015, Mezzasalma et. al. 2017), there are also significant consistencies across regions. Grape-associated microbes have been studied after crushing grape samples (Pinto et. al. 2015, Zarraonaindia et. al. 2015) or sampling grape musts after harvest (Bokulich et. al. 2013 and 2016, Setati et. al. 2015), and the research has repeatedly shown the presence of various yeast species, and members of the bacterial orders Bacillales, Enterobacteriales, Pseudomonadales and Rhodospirillales.

While the ecology of the healthy grape at harvest has been well researched in recent years, changes in these populations due to disease are under-characterized, especially in relation to sour rot. Recent research indicated that development of grape sour rot requires the involvement of yeast, bacteria and Drosophila fruit flies (Hall et al. 2014, 2015, 2016, 2017). The yeast ferment the sugars in the grape pulp, producing ethanol, and after wounding, the newly aerobic environment allows the bacteria to oxidize the ethanol into acetic acid, generating the symptomatic sour aroma. However, it is not known how microbial populations on the surface of diseased grapes differ from those on the surface of healthy grapes at harvest. Several yeast species have been shown to cause sour rot symptoms when co-inoculated with acetic acid bacteria in the presence of Drosophila (Hall et al. 2014, 2015, 2016, 2017). However, little is known about whether the species involved differ by region or vary in abundance between the surface of symptomatic and asymptomatic grape berries. Examining whether there are certain microbes associated with the presence of sour rot besides the causal organisms will contribute to our understanding of the sour rot disease complex.

Metagenomic analysis of grape berry surfaces is complicated by the small epiphytic

biomass, which may be tightly linked with the waxy berry cuticle. Most grape metagenomic studies used homogenized tissues from the entire berry prior to DNA isolation, including those described above. Therefore, our objectives here were to sample epiphytic DNA to identify taxa associated with sour rot symptoms, to begin to understand how sour rot is related to the epiphytic phytobiome in diverse geographical regions and how different epiphytic phytobiomes affect sour rot.

Materials and Methods

Grape Sampling. Shortly before harvest in 2015, grapes were collected from three commercial vineyards and one research vineyard in the Finger Lakes region of New York (Table 1). Shortly before harvest in 2016, grapes were collected from four commercial vineyard blocks in Tasmania, Australia (Table 1). In every vineyard block, 12 panels were randomly selected, and one cluster that exhibited sour rot symptoms was selected from each of the panels. To sample across spatial variability within a cluster, three asymptomatic berries located at the (i) tip of the cluster, (ii) anterior side (toward exterior of canopy) and (iii) posterior side (toward interior of canopy) were cut from each cluster above the pedicel using scissors that were immersed in 95% ethanol between samples, and dropped directly into 50 mL Falcon tubes containing 5 ml of a TE buffer (10mM Tris-HCl+1mM EDTA, ph 8.0) solution with 10% w/v NaCl. The same procedure was used with three symptomatic berries from the same clusters. Tubes were immediately sealed and placed in a Styrofoam cooler containing an ice pack for transport to the laboratory for DNA extraction. Sour rot-affected clusters from Fredonia, NY and Modesto, CA were randomly selected from six vines of Vitis interspecific hybrids at each location (cvs. Brianna, Valiant, Frontenac, Fredonia, LaCrosse and Marquis in Fredonia, NY; and unnamed breeding lines in Modesto, CA). The infected clusters from each cultivar were placed

in polyethylene bags, put in a cooler containing an ice pack and transported to the laboratory in Geneva, NY. In the laboratory, three asymptomatic berries (representing the tip and two opposite sides) were removed from the cluster above the pedicel using surface-sterilized scissors, as described above. The berries were macerated in polyethylene sample bags, and 100 µl of juice was pipetted onto three plates each of Yeast Peptone Dextrose (YPD) and Mannitol agars. The plates were incubated at 24°C for 3 days. After 3 days of growth, 1 ml of sterile distilled water was pipetted onto each plate, and the cells were disrupted using a sterile L-shaped cell spreader (Fisher Scientific, Pittsburgh, PA). This suspension was then pipetted into a 50-ml Falcon tube containing 5 ml of TE buffer with 10% NaCl and frozen at -4°C until further processing.

DNA extraction. To each sample in the TE-NaCl solution, 500 µl of 10% SDS was added, vortexed for 5 s and left at room temperature for 15 min. A freeze-thaw sequence consisting of 30 min in a -80°C freezer and 5 min in a 60°C water bath was repeated three times to lyse the fungal and bacterial cells. A 750 µl aliquot of the solution was transferred to a 2 ml microfuge tube, along with 750 µl ice-cold isopropanol. The solution was centrifuged for 10 min at 9600xg. The supernatant was carefully transferred to a new microfuge tube, 500 µl ice-cold 95% ethanol was added, and centrifuged at 9600xg for 1 min. After removing the supernatant by pipet, the pellet was re-suspended in 100 µl TE buffer. The DNA was then stored at 4°C until further use.

Amplification and Sequencing. Genomic DNA was sent to the Cornell University Sequencing facility in Ithaca, NY for sequencing library preparation and 2x250bp paired-end sequencing on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). Dual-barcoded Nextera library preparation followed AmpSeq protocols (Yang et al. 2016) but with singleplex PCR. The V4 domain of bacterial 16S rRNA was

amplified using primers (all sequences shown 5′ to 3′): F515 (GTGTGCCAGCMGCCGCGGTAA) and R806 (GGACTACHVGGGTWTCTAAT). Fungal internal transcribed spacer (ITS) 1 loci were amplified using primers BITS (CTACCTGCGGARGGATCA) and B58S3 (GAGATCCRTTGYTRAAAGTT). To enable sample barcoding, AmpSeq linkers were added to the 5′ end of each locus-specific primer. As detailed in Yang et al., 2016, the linker to accommodate S5xx barcodes for each forward primer is: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG. The linker to accommodate N7xx barcodes for each reverse primer is: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG. Raw sequencing reads have been deposited in the SRA of NCBI.

Bioinformatic analysis. For pre-processing barcode-sorted raw read data in QIIME (Caporaso et al., 2010a), multiple_extract_barcodes was executed on two folders, containing R1 and R2 reads, file names were changed to allow QIIME to correctly identify the specifiers (_barcode, _map, _R1, _R2), and mapping files were created and formatted according to standard protocols in QIIME . To combine the demultiplexed files into one file, multiple_split_libraries_fastq was executed using two directories containing all R1 or all R2 fastq files and their corresponding mapping and barcode files with the following parameters: mapping extension set to txt, the demultiplexing method was mapping_barcode_files, and the read, barcode, sample ID, and mapping indicators were _R[1/2].fastq, _barcodes.fastq, '.' , and _map.txt, respectively. Multiple_split_libraries_fastq calls split_libraries_fastq, which was given the following parameters: barcode type was 17, phred offset was 33, phred quality threshold was 20, maximum bad run length was 300, and minimum per read length fraction was .01.

To assign the bacterial sequences to OTUs, pick_closed_reference_otus (Edgar, 2010)

was executed the seqs.fastq file with assign taxonomy and reverse strand match enabled, and RDP maximum memory set to 60000. For reference sequences, Greengenes 13_8 97_otu_taxonomy.txt and 97_otus.fasta files were used (Caporaso et al., 2010b; DeSantis et al., 2006). The otu_table_mc2.biom files from the R1 and R2 reads were then merged using QIIME's merge_otu_tables. To determine fungal taxonomies, pick_open_reference_otus was executed with the reference file path, the template file path, and reference sequence file path were all set to the UNITE 97% file sh_refs_QIIME_ver7_97_28.06.2017.fasta and the ID to taxonomy file path was set to the UNITE file called sh_taxonomy_QIIME_ver7_97_28.06.2017.txt. Reverse strand match and suppress lane mask filter were set to true, the assignment method was set to blast, RDP maximum memory set to 60000, and the entropy threshold was set to 0.10. otu_table_mc2_w_tax.biom files from the R1 and R2 reads were then merged using QIIME's merge_otu_tables.

Rare OTUs were removed by filtering if they had less than 0.0001% of the total abundance from within that biom file. Biom files were converted into spf files using the biom_to_stamp.py script provided by STAMP. The original mapping file and the spf file were read into STAMP, and an ANOVA test was done using the Tukey-Kramer method set to 0.95 and a p-value filter of 0.05. The percentage of each taxa in each sample was calculated. Then the average of the percentages for each taxa within each treatment was calculated and plotted in R. All organisms that could not be identified to the Family level were not included in the analysis.

Results

More than 90% of the field samples collected Tasmania recovered fungal OTUs, and over 50% recovered bacterial OTUs. Of those samples cultured in the lab before being sent for sequencing, both the Modesto, CA and Fredonia, NY sample sets returned

100% of the samples (Table 1).

Finger Lakes grape berries collected in 2015 had similar bacterial and fungal microbiota, regardless of the presence of sour rot symptoms (Fig. 1). However, more bacterial genera were detected on asymptomatic berries (20) than on symptomatic berries (12). While the differences in relative mean frequency were non-significant for most genera represented, *Acetobacter* was 24-fold enriched on symptomatic versus asymptomatic berries ($P<0.001$; Fig. 1). For fungi, the only significant difference between the asymptomatic and symptomatic samples was the presence of the filamentous ascomycete *Taloromyces marneffei,* which was 3.7-fold enriched on symptomatic versus asymptomatic berries ($p<0.01$; Fig. 2). *Saccharomyces cerevisiae* represented approximately 1% of the OTUs in both asymptomatic and symptomatic samples. While no other species represented more than 1% of the OTUs on asymptomatic samples, two additional species were common in symptomatic samples (Fig. 2).

As with the 2015 Finger Lakes samples, Tasmania grape berries collected in 2016 had similar bacterial and fungal microbiota, regardless of the presence of sour rot symptoms (Fig. 3). Again, more bacterial genera were detected on asymptomatic berries (10) than on symptomatic berries (8). While the relative mean frequency was non-significant for most OTUs represented, *Bacillus cereus* was 17-fold enriched on symptomatic versus asymptomatic berries ($P=0.03$; Fig. 3). The family Acetobacteraceae was common on both asymptomatic (20.4% of the OTUs) and symptomatic (32.5%) berries. More specifically, *Acetobacter*, which was highly enriched on Finger Lakes sour rot berries, was common in Tasmania on both asymptomatic (6.9% of the OTUs) and symptomatic (10.4%) berries ($p=0.22$). For fungal OTUs, *Pichia kluyveri* and *P. membranifaciens* composed the majority of the OTUs (Fig. 4). *Pichia kluyveri* was 2-fold enriched on symptomatic versus

asymptomatic berries ($P<0.01$). Similar to the 2015 Finger Lakes samples, more OTUs were common on asymptomatic berries (five) than on symptomatic berries (three) (Fig. 4). The presence of yeast and *Acetobacter* was consistent across the asymptomatic and symptomatic berries collected in both 2015 and 2016, albeit with different frequencies, as was the abundance of species.

On symptomatic berries collected in Modesto, California, 22 fungal and bacterial groups were represented, with the majority of the reads from Bacillales (63.7% of bacterial OTUs) and Saccharomycetales (52% of fungal OTUs) (Table 1). On symptomatic berries collected in Fredonia, New York, 19 groups were represented and the majority of the reads came from *Pseudomonas* spp. (54.5% of bacterial OTUs), Acetobacteraceae (29.6% of bacterial OTUs), and Saccharomycetales (64.5% of fungal OTUs) (Table 2). Organisms in the Families Acetobacteraceae and Enterobacteraceae, along with *Aureobasidium pullulans, Metschnikowia* spp. and *Pichia* spp. were expressed in both Modesto and Fredonia sample sets at a rate of more than 1%.

Discussion

Recent research into sour rot causal organisms has shown that the involvement of yeast, bacteria and Drosophila are necessary for the development of sour rot symptoms (Hall et. al. 2017 submitted), yet the dynamics of the microbial system that brings about those symptoms are still unknown. There is an abundance of yeast and bacteria on healthy grapes, but we sought to understand how those microbial populations changed when sour rot symptoms developed.

The changes in yeast and bacterial populations that we documented in four different regions illustrates the dynamics of the grape surface microbiota associated with sour rot development. Many yeast species in the presence of acetic acid bacteria can cause sour rot symptoms (Hall et al., 2017). This present comparison of the microbiota of

healthy and sour rot-affected samples from multiple regions also demonstrates that a range of yeast are present on the grape surface, but become more abundant when symptoms develop, including, *P. kluyveri, P. membranifaciens S. cerevisiae, M. chrysoperlae* and *M. pulcherrima*. The ubiquity of acetic acid bacteria genera, either *Acetobacter* or *Gluconobacter*, is also consistent with our research into causal organisms referenced above, as we found that either genus, when combined with yeast and Drosophila, was successful in causing symptoms. The increase in abundance of bacterial genera such as *Pseudomonas* in the Fredonia infected samples and *Bacillus* in the Modesto infected samples, could possibly be the result of secondary colonizers benefiting from necrosis of the grape berries and leakage of their contents. A similar effect could be occurring with the increased abundance of *Talaromyces marneffei* in the 2015 diseased samples.

Another consideration is that these measurements were taken at just one moment in time; they do not represent the microbial changes that occur during the disease progression. If we were to examine the surface microbiota throughout symptom development, we may see the yeast populations change as ethanol accumulates within the grape berries, changing from higher abundance of *Pichia* species to higher populations of *Saccharomyces* species. A similar situation may develop for bacterial genera, as acetic acid accumulates.

Extracting DNA from the grape berry surface presented us with a challenge due to the low amount of DNA on the grape surface as well as the difficulty of extracting it from the surface because of the berries' waxy cuticle. While some researchers have used commercial kits to extract this low quantity of DNA off the grape surface in the laboratory (Zarraonaindia et. al. 2015), we sought to maximize the amount of DNA while limiting contamination by cutting berries directly into a high-salt buffer solution in the field, which would become the first step of the DNA extraction process. While

the amount of DNA that we successfully extracted was sometimes low, we represented our results through frequency of various organisms within the samples. This has certain drawbacks, as we could examine only those organisms that were identified, omitting those that were not successfully matched. However, despite its shortcomings, this DNA extraction process allowed us to compare these two unique microbial communities.

The yeast and bacteria species found in these samples did not differ significantly by region or season. We found a consistent presence of yeast species, acetic acid bacteria and members of the Enterobacteriaceae family, as other researchers have previously (Bokulich et. al. 2013, Pinto et. al. 2015, Setati et. al. 2015, Mezzasalma et. al. 2017), but the shifts that occurred within these populations after sour rot developed demonstrate that the same organisms present on the surface of healthy berries are the ones also associated with disease symptoms. This presents an interesting question about how controlling sour rot-associated microbes in the field could affect the microbial identity, or terroir, of the resulting wines. Our understanding of the sour rot complex is still evolving, but comparing the dynamics of the microbial communities on healthy and diseased grapes demonstrates that there are no responsible organisms that are not already part of the ecology of the healthy grape.

Literature Cited

1. Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2013). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc. Natl. Acad. Sci. U.S.A. 11, E139–E148.
2. Bokulich, N. A., Collins, T. S., Masarweh, C., Allen, G., Heymann, H., Ebeler, S. E. and Mills, D. A. (2016). Associations among Wine Grape Microbiome,

Metabolome, and Fermentation Behavior Suggest Microbial Contribution to Regional Wine Characteristics. mBio 7:3. e00631-16.

3. Fleet, G. (2008). Wine yeasts for the future. FEMS Yeast Res. 8, 979–995.
4. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2014. Ethanol and Acetic Acid Content of Sour-Rotted Wine grapes and Disease Control with Antimicrobial and Insecticide Sprays. (Abstr.) 65th ASEV National Conference, Technical Abstracts: 164
5. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2015a. Improving Our Understanding of Sour Rot Etiology and Management Techniques. (Abstr.) 66th ASEV National Conference, Technical Abstracts: 70
6. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2015b. Etiology and Management Strategies for Sour Rot on Grapes. (Abstr.) Phytopathology 105 (Suppl. 4): S4.56. http://dx.doi.org/10.1094/PHYTO-105-11-S4.1
7. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2016a. Etiology and Management of Sour Rot in Grapes. (Abstr.) Phytopathology 106 (Suppl. 4): S4.64. http://dx.doi.org/10.1094/PHYTO-106-12-S4.1
8. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2016b. Etiology and Management of Sour Rot in Grapes. (Abstr.) Phytopathology 107:S2.7. http://dx.doi.org/10.1094/PHYTO-107-2-S2.5
9. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2016c. Further Understanding the Cause and Management of Sour Rot. (Abstr.) 67th ASEV National Conference, Technical Abstracts: 52
10. Hall, M.E., Loeb, G.M. and Wilcox, W.F. 2017. Causal role of *Drosophila* spp. in Sour Rot Development. (Abstr.) 68th ASEV National Conference, Technical Abstracts: 64

11. Mezzasalma V, Sandionigi A, Bruni I, Bruno A, Lovicu G, Casiraghi M, et al. (2017) Grape microbiome as a reliable and persistent signature of field origin and environmental conditions in Cannonau wine production. PLoS ONE12(9): e0184615.
12. Pinto C., Pinho D., Cardoso R., Custódio V., Fernandes J., Sousa S., Pinheiro M., Egas C. and Gomes A. C. (2015) Wine fermentation microbiome: a landscape from different Portuguese wine appellations. Front. Microbiol. 6:905.
13. Setati, M. E., Jacobson, D., Bauer, F. F. (2015). Sequence-based Analysis of the Vitis vinifera L. cv Cabernet Sauvignon Grape Must Mycobiome in Three South African Vineyards Employing Distinct Agronomic Systems. Front. Microbiol. 6: 1358.
14. Yang, S., Fresnedo-Ramirez, J., Wang, M., Cote, L., Schweitzer, P., Barba, P., Takacs, E. M., Clark, M., Luby, J., Manns, D. C., Sacks, G., Mansfield, A. K., Londo, J., Fennell, A., Gadoury, D., Reisch, B., Cadle-Davidson, L., Sun, Q. 2016. Horticulture Research 3: 16002.
15. Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., Bokulich, N. A., Mills, D. A., Martin, G., Taghavi, S., van der Lelie, D. and Gilbert, J. A. (2015). The Soil Microbiome Influences Grapevine-Associated Microbiota. mBio 6:2. e02527-14.

Figure 1. The relative mean frequency (%) and standard error of bacterial OTUs represented in 18 asymptomatic and 21 symptomatic sour rot samples from two commercial vineyards of *Vitis vinifera* cv. Riesling and Pinot Gris and one research vineyard of *Vitis* interspecific hybrid cv. Vignoles in the Finger Lakes region of New York in 2015. Differing letters indicate significance to p=0.05, as determined by a

two-sided t-test.

Figure 2. The relative mean frequency (%) and standard error of fungal OTUs represented in 22 asymptomatic and 29 symptomatic sour rot samples from two commercial vineyards of *Vitis vinifera* cv. Riesling and Pinot Gris and one research vineyard of *Vitis* interspecific hybrid cv. Vignoles in the Finger Lakes region of New York in 2015. Differing letters indicate significance to p=0.05, as determined by a two-sided t-test.

Figure 3. The relative mean frequency (%) and standard error of bacterial OTUs represented in 41 asymptomatic and 34 symptomatic sour rot samples from two commercial vinyards of *V. vinifera* cv. Riesling and two commercial vineyards of *V. vinifera* cv. Pinot Noir in Tasmania, Australia in 2016. Differing letters indicate significance to p=0.05, as determined by a two-sided t-test.

Figure 4. The relative mean frequency (%) and standard error of fungal OTUs represented in 86 asymptomatic and 44 symptomatic sour rot samples from two commercial vinyards of *V. vinifera* cv. Riesling and two commercial vineyards of *V. vinifera* cv. Pinot Noir in Tasmania, Australia in 2016. Differing letters indicate significance to p=0.05, as determined by a two-sided t-test.

Table 1. Number of samples, percent passing quality filtering and OTU assignment by phenology, year, and Kingdom and total OTU abundance for all samples collected.

	2015 Finger Lakes		2016 Tasmania		Modesto, CA		Fredonia, NY	
n	144		144		54		36	
	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria
Filtered	51	39	130	75	54	54	36	36

number								
Percent of total	35.4	27.1	90.3	52.1	100	100.0	100	100
Total OTUs	890896	229669	24558184	5012688	4109430	1433568	3401823	819748

Table 2. The relative mean frequency (%) of bacterial and fungal OTUs represented in 54 sour rot-infected samples from six Vitis interspecific unnamed hybrid crosses in Modesto, California in 2016.

OTU	
Bacteria	
Acetobacteraceae (Unclassified Genus)	3.5
Bacillaceae (Unclassified Genus)	16.5
Bacillales (Unclassified Family)	3.6
Bacillus	33.7
Bacillus cereus	1.7
Bacillus flexus	8.2
Brachybacterium	4.4
Brachybacterium conglomeratum	5.0
Burkholderiaceae (Unclassified Genus)	6.4
Enterobacteriaceae (Unclassified Genus)	7.2
Gluconobacter	3.9
Serratia	5.9
Fungi	
Aureobasidium pullulans	4.0
Penicillium polonicum	4.9
Penicillium vanderhammenii	6.4
Metschnikowia chrysoperlae	16.2
Metschnikowia pulcherrima	15.8
Pichia kluyveri	10.9
Pichia membranifaciens	2.6
Saccharomyces cerevisiae	6.6
Rhodosporidiobolus colostri	23.0
Tsuchiyaea wingfieldii	9.7

Table 3. The relative mean frequency (%) of bacterial

and fungal OTUs represented in 36 sour rot-infected samples from *Vitis* interspecific hybrid cvs. Brianna, Valiant, Frontenac, Fredonia, LaCrosse and Marquis in Fredonia, NY.

OTU	
Bacteria	
Acetobacter	3.2
Acetobacteraceae (Unclassified Genus)	4.1
Enterobacteriaceae (Unclassified Genus)	5.5
Gluconobacter	22.3
Leuconostoc	1.3
Leuconostocaceae (Unclassified Genus)	1.1
Pseudomonas viridiflava	3.3
Pseudomonas	51.2
Serratia	3.8
Stenotrophomonas	1.2
Xanthomonadaceae (Unclassified Genus)	2.9
Fungi	
Aureobasidium pullulans	4.4
Botrytis caroliniana	18.0
Metschnikowia chrysoperlae	4.8
Metschnikowia pulcherrima	4.1
Pichia kluyveri	3.7
Pichia membranifaciens	48.0
Pichia terricola	3.9
Papiliotrema flavescens	13.1

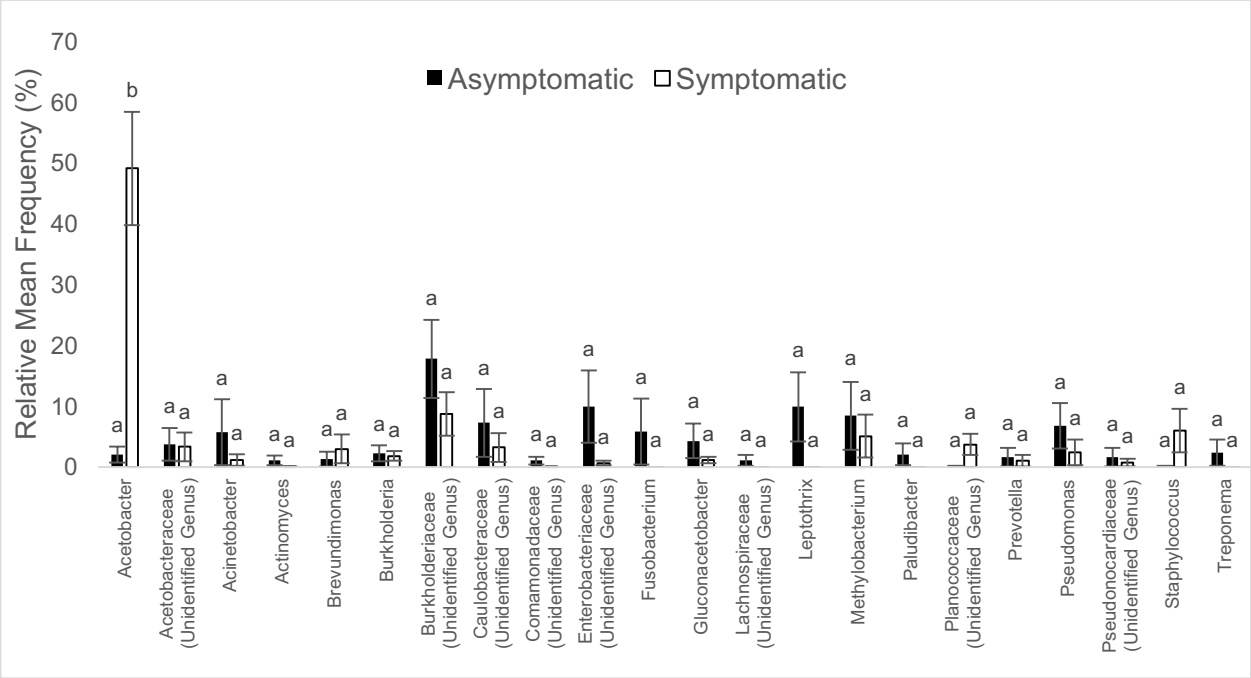

Relative Mean Frequency (%)
70
60
50
40
30
20
10
0
Asymptomatic
Symptomatic
Acetobacter
Acetobacteraceae (Unidentified Genus)
Acinetobacter
Actinomyces
Brevundimonas
Burkholderia
Burkholderiaceae (Unidentified Genus)
Caulobacteraceae (Unidentified Genus)
Comamonadaceae (Unidentified Genus)
Enterobacteriaceae (Unidentified Genus)
Fusobacterium
Gluconacetobacter
Lachnospiraceae (Unidentified Genus)
Leptothrix
Methylobacterium
Paludibacter
Planococcaceae (Unidentified Genus)
Prevotella
Pseudomonas
Pseudonocardiaceae (Unidentified Genus)
Staphylococcus
Treponema

Figure 1.

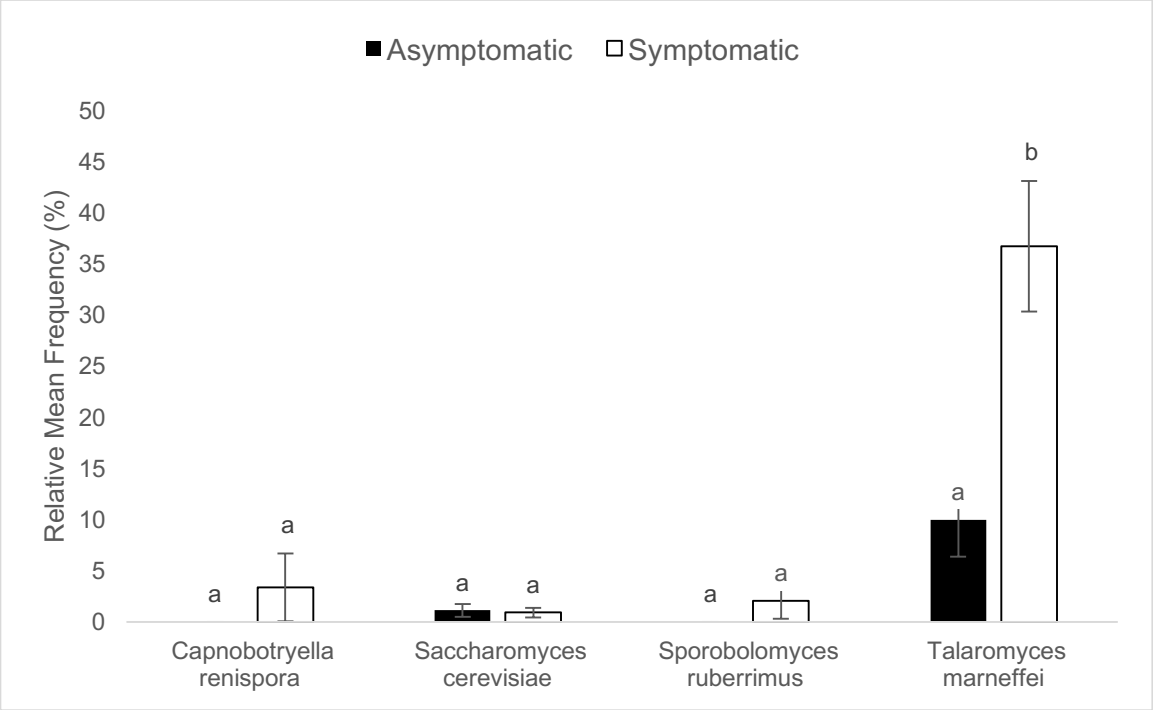

Asymptomatic
Symptomatic
Relative Mean Frequency (%)
50
45
40
35
30
25
20
15
10
5
0
a
a
a
a
a
a
a
b
Capnobotryella renispora
Saccharomyces cerevisiae
Sporobolomyces ruberrimus
Talaromyces marneffei

Figure 2.

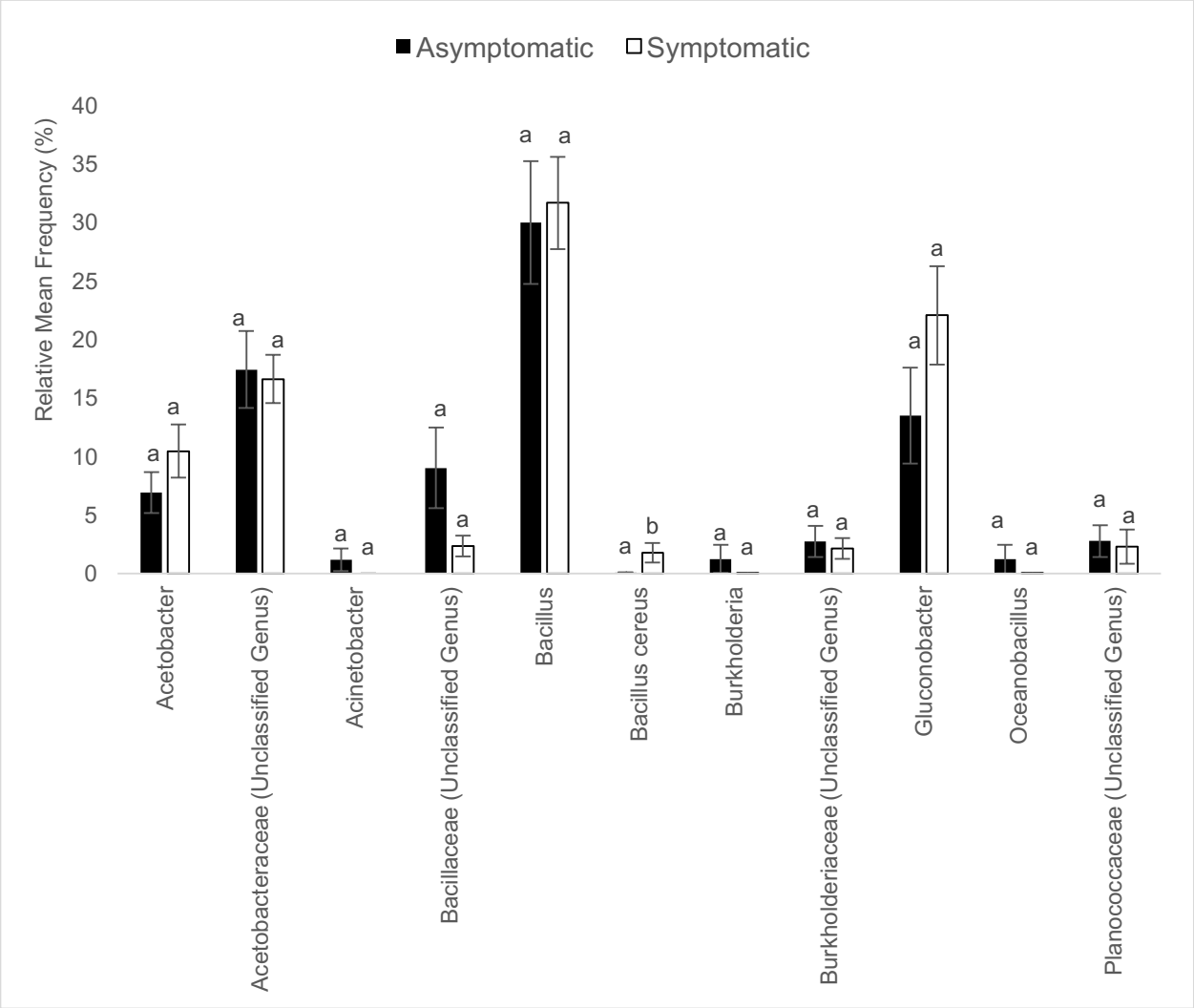

Asymptomatic
Symptomatic
Relative Mean Frequency (%)
40
35
30
25
20
15
10
5
0
a a
a a
a a
a a
a a
a b
a a
a a
a a
a a
a a
Acetobacter
Acetobacteraceae (Unclassified Genus)
Acinetobacter
Bacillaceae (Unclassified Genus)
Bacillus
Bacillus cereus
Burkholderia
Burkholderiaceae (Unclassified Genus)
Gluconobacter
Oceanobacillus
Planococcaceae (Unclassified Genus)

Figure 3.

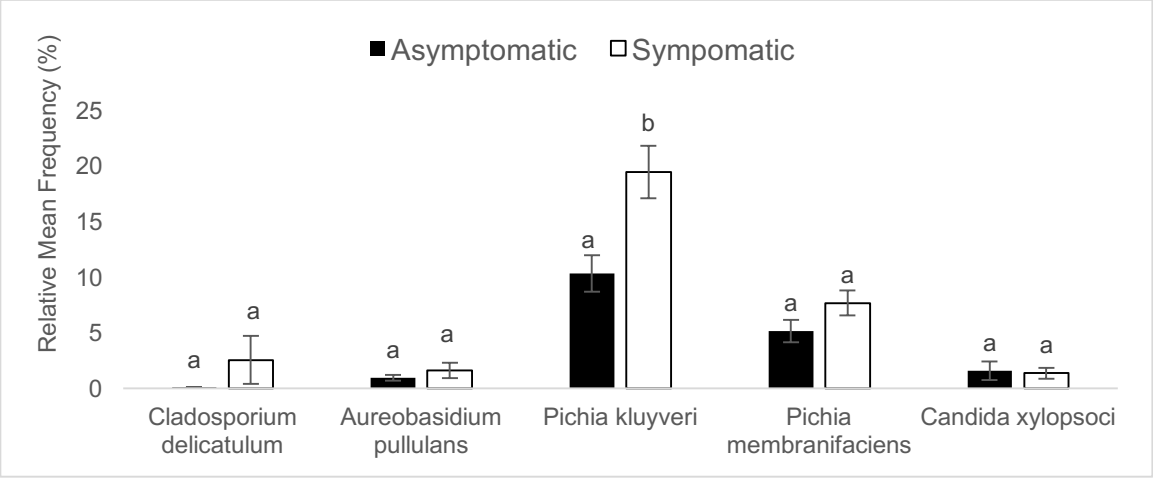

Asymptomatic
Sympomatic
Relative Mean Frequency (%)
25
20
15
10
5
0
a
a
a
a
a
b
a
a
a
a
Cladosporium delicatulum
Aureobasidium pullulans
Pichia kluyveri
Pichia membranifaciens
Candida xylopsoci

Figure 4.

CHAPTER 4

Temporal and Regional Shifts in the Surface Microbiota of Grapes within the Growing Season

Abstract

Extensive research into the microbial ecology of grapes near harvest, with a primary focus on yeasts, has established an improved understanding of some components of variation that influence grapevine terroir. Metagenomic tools such as Illumina sequencing now enable a broader exploration of the phytobiome and components of variability due to such factors as year, location, management, and phenological stage. In 2014, to characterize the microbial changes over the course of the growing season in the Finger Lakes, New York, we extracted DNA from the surface of grape berries at five key phenological stages: pea-sized, bunch closure, Veraison, 15° Brix and harvest. This experiment was repeated in two subsequent years (2015 and 2016), once again in the Finger Lakes, New York, and once in Tasmania, Australia, to examine variability of regional terroir. Both 2015 and 2016 were years with high severity of sour rot, and yeasts and acetic acid bacteria associated with the disease were detected on the berry surface in moderate to high frequency in both years, well in advance of disease symptoms. In contrast, 2014 lacked significant sour rot and yeast and acetic acid bacteria were also less common on the grape surface. Another fungal genus associated with grape disease, *Botrytis*, was increasingly prevalent through berry development in all environments. This study suggests that terroir is dynamic at the microbial scale, varying significantly not just between regions as previously shown,

but also within a region, through development and among years.

Importance

This study investigates the dynamic system of microbial terroir, in which we examined the surface microbiota of wine grapes in 2014 and 2015 in the Finger Lakes region of New York and 2016 in Tasmania, Australia at five key developmental stages: pea-sized, bunch closure, Veraison, 15° Brix and harvest. We found significant shifts in taxa presence and relative taxa abundance across the season, and determined that the terroir differed significantly not just between regions but also within a single region from one year to the next. These findings call into question how we define terroir, as the phytobiome is dynamically responding to its environment, within and between years and locations. This knowledge provides a foundation for how we might manage the berry phytobiome, potentially affecting disease management and vinification decisions.

Introduction

Recent research into the microbiota of grapes examined the microbial communities constituting a particular microbial terroir through sampling of grape at harvest or in the grape must after harvest. Microbial sampling has been examined in vineyards determined to have the same terroir (1), and native microbial populations examined across regions (2), but the changes in one region across an entire season and between two regions in multiple years has not been explored. While the microbial populations on grapes immediately before harvest has been extensively investigated (3–15) and

while some researchers have investigated changes in microbial populations for the last few weeks before harvest (8, 16), fluctuations of microbial populations from the very beginning of berry development until harvest has not been investigated. Investigating the dynamic microbiota of the grape surface from the beginning of the growing season through until harvest could provide insight into which microbial populations develop and persist until harvest, and how the interactions between microbes at various time points influence the microbial population at harvest. Understanding these fluctuations can bolster our understanding of how microbial terroir changes within a single season, and how it changes from year to year. The microbial communities that are brought into the winery after harvest are never static, and the dynamics of the system could inform how we manage disease and microbial communities in the vineyard, affecting fermentation in the winery.

Results

The sampling strategy focused on isolation of DNA from the epiphytic microfauna of three grape berries per sample. The ability to detect taxa from this small biomass increased over the course of the growing season, from % at pea-sized to % at harvest (Table 1).

The diversity of the fungal and bacterial communities varied significantly among developmental stages, location, and year. In 2014, *Mucor* spp. represented 18% of the OTUs found at the pea-sized berry stage, 33% of the OTUs found at the bunch closure stage, 52% of those found at Veraison, but only 12% at 15°Brix and 19% at harvest (Table 2). For *Erisyphe necator,* the only detection occurred at 15° Brix. The percent

of *Botrytis caroliniana* reads increased significantly ($p<0.001$) from pea-sized berries through until harvest in both 2014 and 2015, comprising 73% of the OTUs found at harvest in 2014 and 81.6% found at harvest in 2015 (Fig. 1). Within the OTUs of genus *Aspergillus*, the percent of eight species fluctuated depending on phenological stage, with 29% of the reads coming from *A. piperis* at pea-sized berries, 12.2% at bunch closure and then between 1 and 2% from Veraison to harvest. *A. flavus* represented 11.5% of reads at Veraison, 19% at 15° Brix and 9.9% at Harvest, while A. subversicolor represented between 2 and 6% of the reads at bunch closure, 15° Brix and harvest (Fig. 2). The presence of *Penicillium* was erratic, with a very low percentage of the OTUs found at pea-sized berries and Veraison, with the highest percentage of *Penicillium* OTUs and the highest diversity of *Penicillium* species found at 15° Brix (Fig. 3). Within the order *Saccharomycetales,* the highest diversity of species was found at Veraison and harvest, yet the highest percentage of reads was at bunch closure (57.6%), all from the species *Candida viswantathii* (Fig. 4). Within the bacterial reads, *Acinetobacter rhizosphaerae* represented 85% of the reads at pea-sized berries, and approximately 30% of the total OTUs for the rest of the growing season (Table 3). Fluctuations within the genus *Pseudomonas* also occurred in 2014, beginning with 14% of reads at pea-sized berries, to 61% at bunch closure and Veraison, dropping to 31% at 15° Brix and 28% at harvest (Table 3). No members of the order *Rhodospirillales* were found in any part of the 2014 season.

The 2015 season was significantly different from the 2014 season in that many of the fungal OTUs at every phenological stage represented less than 1% of the total reads.

At pea-sized berries no single OTU represented more than 18% of the reads (Table 4). Within order *Saccharomycetales,* the highest percent of OTUs was at harvest, represented entirely by *Metschnikowia pulcherrima* (45.1%), which was previously only found at Veraison with only 2% of the reads (Fig. 5). *Pichia kluyveri* had 5.7% of the total reads at pea-sized berries, 0.5% of reads at bunch closure, 47% at Veraison, only 5% at 15° Brix, and no reads at harvest (Table 4). At harvest, three species dominated as *Botrytis caroliniana* represented 26.5% of the reads, *Coriolopsis gallica* represented 46% and *Metchnikowia pulcherrima* represented 26.5% (Table 4). Like the fungal reads, many of the bacterial OTUs throughout the 2015 growing season represented less than 1% of the total reads. Members of the family *Burkholderiaceae* represented 39% of reads at pea-sized berries, 3% at bunch closure, 73% at Veraison, 86% at 15° Brix, and 65% at harvest. At harvest, *Acetobacteraceae* represented 5% of reads, *Gluconobacter* 4%, and *Gluconacetobacter* 4% (Table 5). Of those OTUs within *Rhodospirillales*, the highest percentages were represented at Veraison and Harvest, with 12.4% identified as *Gluconobacter* at Veraison and 18.6% at harvest, and 15.4% identified as *Acetobacteraceae* at Veraison and 25.7% at harvest, and 21.3% *Gluconacetobacter* at harvest, which was not previously seen at other time points (Fig. 6).

Members of order *Saccharomycetales* were the most abundant OTUs in the 2016 data set, collected in Tasmania, Australia. At pea-sized berries, *Pichia* spp. represented 80% of the total reads, while *C. xylopsoci* represented only 1%. At bunch closure, *P. kluyveri, P. membranifaciens* and *P. terricola* represented 42% of the total reads and

Candida xylopsoci, 47% of the total reads. *Pichia* spp. represented 73% of the total OTUs at Veraison, with *Candida* spp. and *Hanseniaspora* spp. representing 6% and 8% respectively. At 15° Brix, *Pichia* species represented 70% of the total reads, and 77% of the total reads at harvest (Table 6). At pea-sized berries, *P. kluyveri* represented the highest percentage of any one species within *Saccharomycetales* (19.5%), and at bunch closure here was the greatest amount of diversity within the *Saccharomycetales* OTUs (Fig. 7). For bacterial OTUs, members of *Rhodospirillales* dominated every time point. *Gluconobacter* represented 23% of reads at pea-sized berries, 43% at bunch closure, 50% at Veraison, 24% at 15° Brix and 16% at harvest (Table 7), and it also represented a significant proportion of reads from order *Aceteobacteraceae,* along with *Acetobacter* and a percentage of reads that were of an unidentified genus (Fig. 8). Members of the order *Bacillaceae* represented 39% of the OTUs at pea-sized berries, 10% at bunch closure, 17% at Veraison, 30% at 15° Brix and 46% at harvest (Table 7).

Discussion

The grape microbiota has become a popular subject of research in recent years, particularly with widespread adoption of high-throughput sequencing and metagenomics tools. While previous research focused primarily on microbial populations at harvest and in the grape must, our investigation explored the epiphytic population dynamics of grape microbiota season-long at key phenological stages in three years and two distinct grape growing regions.

On July 31, 2014, between bunch closure and Veraison, the Finger Lakes region

suffered a major hail storm which severely impacted grape development, and it is after this event that we see a large and temporary spike in *Burkholderiaceae, Pichia kluyveri* and *Dissoconium proteae* and significant reduction in *Pseudomonas* spp., *Cladosporium delicatulum* and *Bullera globospora*. In 2015 in the Finger Lakes, there was a significant amount of sour rot near harvest (17) and in Tasmania in 2016, the season was very dry but with significant sour rot infections near harvest (Hall and Wilcox, *unpublished*). The data from 2015 and 2016 has a larger representation of organisms at every time point than those data from 2014, along with a significantly higher percentage of yeast and acetic acid bacteria in the samples from 2015 and 2016, even as early as pea-sized berries. It is unknown whether the increased diversity had an impact on disease development or whether they are unrelated, because those microorganisms that play a role in the sour rot disease complex are also ubiquitous yeast and bacteria on the grape surface. The notable lack of those organisms in the 2014 data may be an indication of why sour rot infections were not prevalent that year, however.

There is a notable similarity between those data collected in 2015 and 2016, primarily in the increased diversity of microbial species, in comparison to the 2014 samples, and in the prevalence of yeast and acetic acid bacteria. Also significant are the differences between the 2014 and 2015 data. Since the data are from the same region, we expected to see similarities in the microbial populations but they were substantially different. We recognize that in combining the results from many vineyards, we are not focusing on the microbial terroir of a single vineyard and how it changed from one year to the next, but examining the microbial terroir of a region allowed us to look at patterns

among multiple sites. However, because there are limited similarities between the microbial communities of 2014 and 2015 within the same sites, yet significant similarities between 2015 and 2016, despite being from different continents, it leads us to a much larger question how we describe microbial terroir. Researchers have examined microbial changes from different regions (18), and as our research indicates that the microbial terroir may in fact change dramatically from one year to the next. The ebb and flow of organisms as the season progresses are an indication of how the environment may be impacting the growth of the grapes, or even how the microorganisms are responding to conventional sprays in the vineyard. Because we did not collect spray records for every vineyard from which we sampled, we cannot relate this data back to those specific applications. However, it is possible that the spike of *E. necator* reads in 2014 at 15° Brix is a possible example of how the population of that pathogen was controlled with a fungicide application. While these data gives us a broad look at the dynamics of the microbial system, further studies that relate microbial community data with fungicide applications would provide insight into which microbes are being controlled with each application, and which ones proliferate as a result of that population being controlled.

Grapes harbor a unique microbial community but our understanding of the dynamics of the surface microbial community across the growing season are not well understood. These microbial communities influence the development of disease symptoms in the vineyard and the downstream processing of grapes, especially as it relates to native fermentations. These microbial populations are influenced by environmental changes and the rise and fall of certain microbial members within the

population. Researchers have focused on those microbes present at harvest, but these communities are changing and being influenced from the very start of the growing season. Through understanding how the dynamics of these microbial communities change over the course of the growing season, we can better understand how we arrive at the microbial communities that we encounter at harvest, and in the resulting grape must. Moreover, in the case of sour rot, we can see that organisms that are able to cause sour rot symptoms are present on the grape at the start of the growing season, meaning that efforts to control microbial populations might not necessarily have to occur right before the onset of symptoms, but could potentially happen at a much earlier time point. While it is unclear how controlling for certain yeast or bacteria could influence the microbial community, it is also possible that counterbalancing the prevalence of certain organisms with those that are not pathogenic, could reduce the risk of disease symptoms development.

Materials and Methods

In 2014 and 2015, grapes were collected from two commercial vineyards, one of *Vitis vinifera* cv. Riesling and one of cv. Pinot Gris and one research vineyard of *Vitis* interspecific hybrid cv. Vignoles, all in the Finger Lakes region of New York. One additional commercial vineyard was added in 2015 with a planting of cv. Vignoles, also in the Finger Lakes region. In 2016, grapes were collected from five commercial vineyard blocks, one of *V. vinifera* cv. Sauvignon Blanc, and four *V. vinifera* cv. Riesling in Tasmania, Australia. To address fluctuations in microbial populations both within a vineyard and on an individual cluster, as articulated by Barata et al. (18,19), we sampled individual berries, as opposed to whole clusters, and at varying locations

in the vineyard. In every vineyard block, 12 panels were randomly selected and one cluster was randomly selected at the following phenological time points: pea-sized berries, bunch closure, Veraison, 15° Brix and harvest. The first three sampling points were determined by visually assessing the clusters in the 12 panels, and harvesting samples when 50% of the berries on a randomly selected cluster were determined to be at that particular phenological stage. For the sampling point of 15° Brix, 20 berries were selected randomly from each of three individual rows, and samples were collected when the juice averaged 15° Brix by refractometer. The harvest date for all years was determined when the fruit reached an average of 23 to 24°Brix. 20 berries were selected randomly from each of three individual rows, and samples were collected when the juice averaged 23-24° Brix by refractometer. Each randomly selected cluster was marked with flagging tape so as not to be sampled again at a future sampling point, which ensured that any changes to the cluster architecture or surface microbiota caused by sampling would not influence other samples. Three randomly selected berries, located at the tip of the cluster, the anterior side and posterior side, were cut from each cluster above the pedicel using scissors that were immersed in 95% ethanol between samples, and dropped directly into 50 mL Falcon tubes containing 5 mL of 10% w/v NaCl in TE buffer (10mM Tris-HCl+1mM EDTA, ph 8.0). The caps were screwed back on each tube immediately, and were placed in a Styrofoam cooler containing an ice pack until they were transported to the laboratory.

DNA extraction. In the laboratory, 500 µl of 10% SDS was added to the Falcon tube containing the grape berry and TE-NaCl solution, vortexed for 5 seconds and left at room temperature for 15 minutes. A freeze-thaw sequence consisting of 30 minutes in

a -80°C freezer and 5 minutes in 60°C water bath was repeated three times to lyse the fungal and bacterial cells. 750 µl of the solution was transferred to a centrifuge tube, along with 750 µl ice-cold isopropanol. The solution was centrifuged for 10 minutes at 9600xg. The supernatant was carefully removed from the tube, 500 µl ice-cold 95% ethanol was added, and the tube was again centrifuged at 9600xg for 1 minute. The pellet was re-suspended in 100 µl TE buffer. The DNA was then stored at 4°C until further use.

Amplification and Sequencing. Genomic DNA was sent to the Cornell University DNA Sequencing facility in Ithaca, NY for Illumina 250-bp-paired-end sequencing on the Illumina MiSeq machine. For each sample, two separate runs were performed. To amplify the V4 domain of bacterial 16S rRNA genes, primers F515 (5′*NNNNNNNN*GTGTGCCAGCMGCCGCGGTAA–3′) and R806 (5′–GGACTACHVGGGTWTCTAAT–3′) and for fungal internal transcribed spacer (ITS) 1 loci were amplified using primers BITS (5′-*NNNNNNNN*CTACCTGCGGARGGATCA–3′) and B58S3 (5′–GAGATCCRTTGYTRAAAGTT–3′) (19). Both forward primers were modified to contain a unique 8-bp barcode, highlighted in the italicized N-sections above.

Data Analysis. Quality filtering, read processing, and OTU assignment was conducted in Qiime 1.9.1 (20). Sequences were trimmed once there were three consecutive bases with PHRED scores less than 20. Sequences less than 100nt were discarded. Open and closed reference OTU-picking methods used uclust and a pairwise identity of 97% (21). Alignment to greengenes 13_5 was done using PyNAST and alignment to UNITE 7_97 was conducted using the BLAST alignment method (22–25). OTUs with

than 0.0001% of the total abundance of the biom file were filtered out. Analysis was done in STAMP v2.1.3 and unclassified reads were not included in the analysis but they were kept to calculate abundance frequencies and Plots were made in R v.3.3.2 (26). Data was summarized by region rather than by vineyard, and all organisms that could not be identified to the Family level were not included in the analysis.

Acknowledgments

The authors thank Isabelle O'Bryon and Michael Osier for bioinformatics support. This work was supported the NY State Dept. of Agriculture and Markets, NY Wine and Grape Foundation, Specialty Crops Research Initiative and the Dyson Fund.

Literature Cited

1. Setati ME, Jacobson D, Andong U-C, Bauer F (2012) The Vineyard Yeast Microbiome, a Mixed Model Microbial Map. PLoS One 7(12):e52609.
2. Martiny JBH, Bohannan BJM, Brown JH, Colwell RK, Fuhrman JA, Green JL, Horner-Devine MC, Kane M, Krumins JA, Kuske CR, Morin PJ, Naeem S, Øvreås L, Reysenbach A-L, Smith VH, Staley JT (2006) Microbial biogeography: putting microorganisms on the map. Nat Rev Microbiol 4:102.
3. Rosini G, Federici F, Martini A (1982) Yeast flora of grape berries during ripening. Microb Ecol 8(1):83–89.
4. Parish ME, Carroll DE (1985) Indigenous Yeasts Associated with Muscadine (Vitis rotundifolia) Grapes and Musts. Am J Enol Vitic 36(2):165 LP-169.
5. Yanagida F, Ichinose F, Shinohara T, Goto S (1992) Distribution of Wild

Yeasts in the White Grape Varieties at Central Japan. J Gen Appl Microbiol 38(5):501–504.

6. Martini A, Ciani M, Scorzetti G (1996) Direct Enumeration and Isolation of Wine Yeasts from Grape Surfaces. Am J Enol Vitic 47(4):435 LP-440.
7. Sabate J, Cano J, Esteve-Zarzoso B, Guillamón JM (2002) Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. Microbiol Res 157(4):267–274.
8. Garijo P, López R, Santamaría P, Ocón E, Olarte C, Sanz S, Gutiérrez AR (2011) Presence of enological microorganisms in the grapes and the air of a vineyard during the ripening period. Eur Food Res Technol 233(2):359–365.
9. Combina M, Elía A, Mercado L, Catania C, Ganga A, Martinez C (2005) Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. Int J Food Microbiol 99(3):237–243.
10. Raspor P, Milek DM, Polanc J, Smole Možina S, Čadež N (2006) Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. Int J Food Microbiol 109(1):97–102.
11. Brysch-Herzberg M, Seidel M (2015) Yeast diversity on grapes in two German wine growing regions. Int J Food Microbiol 214(Supplement C):137–144.
12. Drożdż I, Makarewicz M, Sroka P, Satora P, Jankowski P (2015) Comparison of the yeast microbiota of different varieties of cool-climate grapes by PCR-RAPD. Potravin Slovak J Food Sci Vol 9, No 1 Potravin. Available at: http://www.potravinarstvo.com/journal1/index.php/potravinarstvo/article/view/484 %3C/div%3E.

13. Setati ME, Jacobson D, Bauer FF (2015) Sequence-based analysis of the Vitis vinifera L. cv cabernet sauvignon grape must mycobiome in three South African vineyards employing distinct agronomic systems. Front Microbiol 6(NOV):1–12.

14. Garofalo C, Tristezza M, Grieco F, Spano G, Capozzi V (2016) From grape berries to wine: population dynamics of cultivable yeasts associated to ``Nero di Troia'' autochthonous grape cultivar. World J Microbiol Biotechnol 32(4):59.

15. Jara C, Laurie VF, Mas A, Romero J (2016) Microbial Terroir in Chilean Valleys: Diversity of Non-conventional Yeast. Front Microbiol 7:663.

16. Renouf V, Lonvaud-Funel A (2007) Development of an enrichment medium to detect Dekkera/Brettanomyces bruxellensis, a spoilage wine yeast, on the surface of grape berries. Microbiol Res 162(2):154–167.

17. Hall ME, Wilcox WF (2017) No Title. Am J Enol Vitic.

18. Barata A, Santos SC, Malfeito-Ferreira M, Loureiro V (2012) New Insights into the Ecological Interaction Between Grape Berry Microorganisms and Drosophila Flies During the Development of Sour Rot. Microb Ecol 64(2):416–430.

19. Bokulich N a, Thorngate JH, Richardson PM, Mills D a (2014) Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc Natl Acad Sci U S A 111(1):E139-48.

20. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD,

Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Meth 7(5):335–336.

21. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19):2460–2461.

22. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410.

23. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL (2006) Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl Environ Microbiol 72(7):5069–5072.

24. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R (2010) PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26(2):266–267.

25. Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Douglas B, Drenkhan T, Eberhardt U, Dueñas M, Grebenc T, Griffith GW, Hartmann M, Kirk PM, Kohout P, Larsson E, Lindahl BD, Lücking R, Martín MP, Matheny PB, Nguyen NH, Niskanen T, Oja J, Peay KG, Peintner U, Peterson M, Põldmaa K, Saag L, Saar I, Schüßler A, Scott JA, Senés C, Smith ME, Suija A, Taylor DL, Telleria MT, Weiss M, Larsson K-H (2013) Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 22(21):5271–5277.

26. Parks DH, Tyson GW, Hugenholtz P, Beiko RG (2014) STAMP: statistical

analysis of taxonomic and functional profiles. Bioinformatics 30(21):3123–3124.

Figure 1. Increasing prevalence of *Botrytis caroliniana* through berry development in three Finger Lakes vineyards in 2014 and 2015. The mean percent reads representing *Botrytis caroliniana* relative to all fungal reads in 150 samples collected in 2014 and 103 samples collected in 2015.

Figure 2. The mean percent reads for each *Aspergillus spp.* relative to all *Aspergillus* reads across five phenological stages in 150 samples collected in 2014.

Figure 3. The mean percent of reads representing *Penicillium* spp. in 150 samples collected from two commercial vineyards of *Vitis vinifera* cv. Riesling and Pinot Gris and one research vineyard of Vitis interspecific hybrid cv. Vignoles in the Finger Lakes region of New York in 2014.

Figure 4. The mean percent of reads representing order *Saccharomycetales* in 150 samples collected from two commercial vineyards of *Vitis vinifera* cv. Riesling and Pinot Gris and one research vineyard of Vitis interspecific hybrid cv. Vignoles in the Finger Lakes region of New York in 2014.

Figure 5. The mean percent of reads representing order *Saccharomycetales* in 103 samples collected at harvest from two commercial vineyards of *Vitis vinifera* cv. Riesling and Pinot Gris and one research vineyard and one commercial vineyard of Vitis interspecific hybrid cv. Vignoles in the Finger Lakes region of New York in 2015.

Figure 6. The mean percent of reads representing order *Rhodospirillales* in 91 samples collected from two commercial vineyards of *Vitis vinifera* cv. Riesling and Pinot Gris and one research vineyard of Vitis interspecific hybrid cv. Vignoles in the Finger Lakes region of New York in 2015.

Figure 7. The mean percent of reads representing order *Saccharomycetales* in 306 samples collected at harvest from five commercial vineyard blocks, one of *V. vinifera*

cv. Sauvignon Blanc, and four *V. vinifera* cv. Riesling in Tasmania, Australia in 2016.

Figure 8. The mean percent of reads representing family *Acetobacteraceae* in 178 samples collected at harvest from five commercial vineyard blocks, one of *V. vinifera* cv. Sauvignon Blanc, and four *V. vinifera* cv. Riesling in Tasmania, Australia in 2016.

Table 1. Number of samples (percent) passing quality filtering and OTU assignment by phenology, year, and Kingdom.

	2014		2015		2016	
Sampling Time	Fungal	Bacterial	Fungal	Bacterial	Fungal	Bacterial
	n=108	n=108	n=144	n=144	n=180	n=180
Pea-Sized	6 (5.6)	4 (3.7)	10 (6.9)	10 (6.9)	100 (55.6)	87 (48.3)
Bunch Closure	7 (6.5)	7 (6.5)	12 (8.3)	12 (8.3)	40 (22.2)	9 (5)
Veraison	25 (23.1)	23 (21.3)	39 (27.1)	39 (27.1)	40 (22.2)	11 (6.1)
15° Brix	58 (53.7)	52 (48.1)	25 (17.4)	23 (16)	40 (22.2)	30 (16.7)
Harvest	54 (50)	45 (41.7)	17 (11.8)	7 (4.9)	86 (47.8)	41 (22.8)

Table 2. In 2014 Finger Lakes, New York, the relative mean frequency (%) of reads for each fungal OTU across three vineyards at five phenological stages. Sample numbers per stage are presented in Table 0. [for all tables use one decimal as significant fig.]

OTU	Pea-Sized Berries	Bunch Closure	Veraison	15° Brix	Harvest
Aspergillus flavus			6.2	12.7	6.9
Aspergillus piperis	8.5	8.1			
Aureobasidium pullulans			15.6		

Botrytis caroliniana			6.1	8.9	49.1
Candida viswanathii		20.7		8.3	
Cladosporium delicatulum	17.2		13.4	15.1	
Cystofilobasidium capitatum		6.4			
Didymella calidophila	3.5				
Mortierella reticulata	3.1		6.3		
Mucor circinelloides	17.7		7.2	12.3	
Mucor nidicola		33.3	45.1		19.0
Mycosphaerella tassiana	7.8				
Penicillium citrinum		24.2		18.8	18.1
Penicillium levitum				5.2	
Penicillium lividum		7.3			
Penicillium melinii				6.2	
Talaromyces marneffei				12.5	
Trametes versicolor					6.9
Vishniacozyma heimaeyensis	4.4				
Vishniacozyma victoriae	37.8				

Table 3. In 2014 Finger Lakes, New York, the relative mean frequency (%) of reads for each bacterial OTU across three vineyards at five phenological stages. Sample numbers per stage are presented in Table 0.

OTU	**Pea-sized Berries**	**Bunch Closure**	**Veraison**	**15° Brix**	**Harvest**
Acanthamoeba castellanii					5.4
Acinetobacter rhizosphaerae	84.7	28.9	27.3	33.4	30.8
Alicyclobacillus acidocaldarius				3.5	7.1
Anoxybacillus kestanbolensis	7.3	4.13	5.3	11.7	5.6
Bacillus coagulans			0.61	2.8	
Brachybacterium conglomeratum					10.8
Enterococcus casseliflavus				4.8	
Halomonas campisalis		6.32	1.0	1.7	7.0
Janthinobacterium lividum					3.4
Lactobacillus iners			3.2		
Methylobacterium adhaesivum				10.8	2.1

Nevskia ramosa			0.7		
Pseudomonas balearica	12.6	54.4	61.1	25.4	20.4
Pseudomonas viridiflava	0.94	6.32		5.9	7.3
Stenotrophomonas acidaminiphila			0.76		

Table 4. In 2015 Finger Lakes, New York, the relative mean frequency (%) of reads for each fungal OTU across three vineyards at five phenological stages. Sample numbers per stage are presented in Table 0.

OTU	**Pea-sized berries**	**Bunch Closure**	**Veraison**	**15° Brix**	**Harvest**
Alternaria kulundii	1.13	1.65			
Aureobasidium microstictum	0.14	1.19	0.00		
Aureobasidium pullulans	2.71	3.37	4.64	1.31	
Botryosphaeria corticis		0.00	3.70	0.28	
Botrytis caroliniana	1.69	2.31	3.01	2.57	26.49
Bullera globospora	4.19	12.93	0.41	0.00	
Bullera unica	3.61	3.76	0.19		
Candida athensensis	1.31	0.01			
Capnobotryella renispora	1.30	0.00	1.05		
Cladosporium delicatulum	16.74	12.83	1.95	0.00	
Coriolopsis gallica					45.88
Dioszegia hungarica	0.29	3.20	0.27		
Diplodia allocellula		0.00	6.96	0.14	
Dissoconium proteae	17.61	5.61	18.05	1.13	
Keissleriella quadriseptata	0.56	0.30			
Leptospora rubella	0.29	0.66			
Mastigosporium album	0.56	1.18	0.16		
Metschnikowia chrysoperlae		0.01	2.47	0.65	
Metschnikowia pulcherrima		0.00	2.76	0.36	26.49
Monographella nivalis		0.31			
Mycosphaerella tassiana	1.13	1.42	0.05		
Neoascochyta exitialis	3.91	6.47	0.05		
Neoascochyta paspali	1.33	0.42			
Neodevriesia poagena	1.73	0.17			
Neopestalotiopsis foedans	0.66	0.16			
Papiliotrema aurea	0.92	2.76			
Papiliotrema flavescens	0.73	4.78	0.00		
Papiliotrema fuscus	0.56	4.34	0.27		

Pichia kluyveri	5.64	0.50	47.27	5.36	
Pilidium concavum	3.45	1.02	0.11	0.00	
Ramularia pratensis	14.76	10.61	5.32	0.71	
Rhodotorula nothofagi		0.33			
Sarocladium strictum		0.87	0.18		
Sphaerulina tirolensis	0.56	0.62			
Sporobolomyces oryzicola	1.71	1.13	0.00		
Sporobolomyces roseus	0.80	1.73	0.05		
Sporobolomyces ruberrimus	5.35	6.69	0.73	87.32	1.15
Stagonospora uniseptata	1.15	1.01	0.05		
Taphrina carpini	0.58	0.34			
Tilletiopsis washingtonensis	1.13	0.60	0.16		
Torulaspora delbrueckii	0.43		0.04		
Vishniacozyma heimaeyensis		0.26			
Vishniacozyma victoriae		1.72	0.00		
Zymoseptoria brevis	1.33	0.12			

Table 5. In 2015 Finger Lakes, New York, the relative mean frequency (%) of reads for each bacterial OTU across three vineyards at five phenological stages. Sample numbers per stage are presented in Table 0.

OTU	Pea-sized Berries	Bunch Closure	Veraison	15° Brix	Harvest
Acetobacteraceae	0.8		3.5	0.4	5.3
Acinetobacter lwoffii	1.2		1.0		
Acinetobacter	2.6	0.2	2.7	0.5	2.4
Aeromonadaceae	3.6	5.6		0.5	
Aggregatibacter	0.2				
Agrobacterium	2.2	5.7			
Aurantimonadaceae		0.5			
Burkholderia	6.3	0.6	13.3	11.7	13.8
Burkholderiaceae	32.6	2.4	69.0	74.9	50.9
Caulobacteraceae	0.3				
Chryseobacterium	0.3				
Cloacibacterium	1.0	0.2			
Comamonadaceae	0.7	1.6	2.3	0.2	1.3
Corynebacterium		0.3	0.6		1.8
Curtobacterium	0.2	0.4			
Enhydrobacter		0.2			
Enterobacteriaceae	1.9	3.6		0.9	
Erwinia	4.4	3.6		0.3	
Fusobacterium				0.6	
Gemellaceae	0.8				
Gluconacetobacter					4.4
Gluconobacter	0.8		2.8	0.2	3.8
Haemophilus	0.2				
Hymenobacter		9.6			
Kineococcus		0.9			
Lactobacillus iners			0.2		
Lactococcus		0.6			
Methylobacterium adhaesivum	0.7	0.4		0.1	
Methylobacterium organophilum	1.8				
Methylobacterium	2.9	1.6		0.2	1.8
Microbacteriaceae	1.7	4.9	0.7		2.4

Micrococcus		3.6			
Neisseria		0.2			
Neisseriaceae		0.3			
Oxalobacteraceae		0.3			
Paenibacillus	0.8	0.9			
Pedobacter	0.6	0.2	0.8	0.3	
Planococcaceae	0.3				
Polaromonas		1.4			
Pseudomonadaceae	4.9	0.3	0.9	5.8	0.9
Pseudomonas	4.9	6.2	0.7	1.9	
Pseudomonas viridiflava	6.0	11.4		0.5	0.1
Ralstonia					1.2
Rhizobiaceae	2.4	10.8		0.2	
Rothia dentocariosa					0.3
Rothia					1.8
Sinobacteraceae	0.5				0.9
Sphingobacteriaceae	0.6	6.0			
Sphingobium					
Sphingomonadaceae	1.0	0.5		0.2	
Sphingomonas	9.1	1.7	0.8	0.6	
Spirosoma	1.0				
Sporichthya				0.1	
Staphylococcus	0.4				
Streptococcus	1.4	12.9			1.8
Xanthomonadaceae	0.7	0.8			0.1

Table 6. In 2016 Tasmania, Australia, the relative mean frequency (%) of reads for each fungal OTU across three vineyards at five phenological stages. Sample numbers per stage are presented in Table 0.

OTU	**Pea-sized berries**	**Bunch Closure**	**Veraison**	**15° Brix**	**Harvest**
Aureobasidium microstictum	5.2	6.0	5.0	4.0	2.9
Aureobasidium pullulans	3.9	2.3	6.6	24.6	4.7
Blumeria graminis	2.1			0.1	0.3
Botrytis caroliniana					0.5
Bullera unica	0.5				
Candida sake	0.1				

Candida stellata	0.0				
Candida xylopsoci	1.0	46.7	6.20		7.77
Cinereomyces lindbladii		0.4			
Cladosporium delicatulum	0.6	0.0		0.50	0.47
Cuniculitrema polymorpha		2.7	0.93		0.39
Debaryomyces mycophilus	0.1				0.17
Didymella exigua					0.09
Hannaella coprosmae	0.3			0.53	0.01
Hanseniaspora valbyensis			8.5		
Lentinus squarrosulus		0.15		0.02	0.25
Malassezia globosa	1.0				
Malassezia restricta	2.0	0.03		0.33	0.46
Metschnikowia chrysoperlae	0.0	0.00		0.00	0.00
Metschnikowia pulcherrima					0.03
Mycosphaerella tassiana	0.5			0.03	0.20
Neoascochyta desmazieri	0.1				
Phlebia radiata	2.2			0.06	0.77
Phyllozyma subbrunnea		0.01			0.22
Pichia kluyveri	52.3	28.58	67.3	47.39	51.07
Pichia membranifaciens	26.6	4.81	5.5	22.39	25.50
Pichia terricola		8.14			0.03
Rhodotorula nothofagi				0.01	1.63
Saccharomycopsis crataegensis		0.22			
Schwanniomyces occidentalis	0.4				
Schwanniomyces yamadae	0.1				
Sphaerulina tirolensis	0.3				0.39
Sporobolomyces ruberrimus	0.5				
Vishniacozyma victoriae					1.82
Wickerhamomyces anomalus	0.1				0.32
Zymoseptoria brevis				0.12	

Table 7. In 2016 Tasmania, Australia, the relative mean frequency (%) of reads for each bacterial OTU across three vineyards at five phenological stages. Sample numbers per stage are presented in Table 0.

OTU	**Pea-sized berries**	**Bunch Closure**	**Veraison**	**15° Brix**	**Harvest**
Cloacibacterium	0.05		0.51		
Chryseobacterium	0.25				
Gluconobacter	21.98	41.79	49.32	23.24	15.33
Acetobacteraceae	17.72	18.38	10.31	21.91	19.80

Acetobacter	6.66	4.39	7.85	6.50	7.83
Facklamia	0.10				
Aeromonadaceae	0.42				0.00
Bacillus cereus	1.25	0.00	0.17	0.69	0.09
Bacillus	32.17	10.01	17.16	27.77	34.08
Bacillaceae	4.59	0.07	3.17	7.59	10.25
Oceanobacillus	0.09	0.00			1.39
Anoxybacillus kestanbolensis	0.14	0.00			0.00
Bacteroides	0.06				
Bradyrhizobiaceae	0.00	0.11		0.01	0.01
Burkholderiaceae	3.46	0.81	0.33	0.46	3.09
Burkholderia	0.07	0.05			1.41
Granulicatella	0.05	0.09			0.00
Mycoplana	0.00	0.12			0.00
Caulobacteraceae	0.01	0.17		0.02	0.02
Comamonadaceae	0.17			0.01	0.04
Corynebacterium	0.31	0.04	0.34		0.49
Corynebacterium durum	0.10				0.00
Enterobacteriaceae	0.23				0.00
Erwinia	0.36				
Flavobacterium	0.08				
Lactobacillus	0.00	0.16			0.02
Methylobacteriaceae	0.02	8.60			0.01
Curtobacterium	0.33				0.00
Microbacteriaceae	0.88				0.00
Rothia mucilaginosa	0.31			0.06	0.00
Micrococcus	0.28	0.00			0.00
Kocuria rhizophila	0.10				
Rothia	0.20	0.06	0.11		0.00
Enhydrobacter	0.19				
Acinetobacter	0.35	0.15		1.45	1.32
Acinetobacter	0.08				
Acinetobacter johnsonii	0.18	0.00			
Neisseria	0.11				
Neisseriaceae	0.13	0.06		0.03	0.00
Cupriavidus	0.07	0.04			0.01
Ralstonia	0.09			0.00	
Oxalobacteraceae	0.08	0.15			0.02

Brevibacillus	0.14	6.39			0.12
Paenibacillus	0.81	4.05	10.00		0.12
Haemophilus parainfluenzae	0.09	3.73		0.02	0.00
Planococcaceae	0.70	0.09	0.40	1.82	3.15
Pseudomonas viridiflava	1.07				0.00
Pseudomonas	1.42			4.25	0.00
Agrobacterium	0.01	0.12			
Sphingomonas yabuuchiae	0.07	0.03			0.01
Sphingomonas	0.12	0.05		0.04	0.12
Sphingobium	0.07	0.01			0.00
Staphylococcus	0.19	0.14	0.23	0.00	0.00
Streptococcus	0.95	0.14	0.11	0.51	0.01
Lactococcus	0.14				
Veillonella parvula				0.61	
Xanthomonadaceae	0.06				

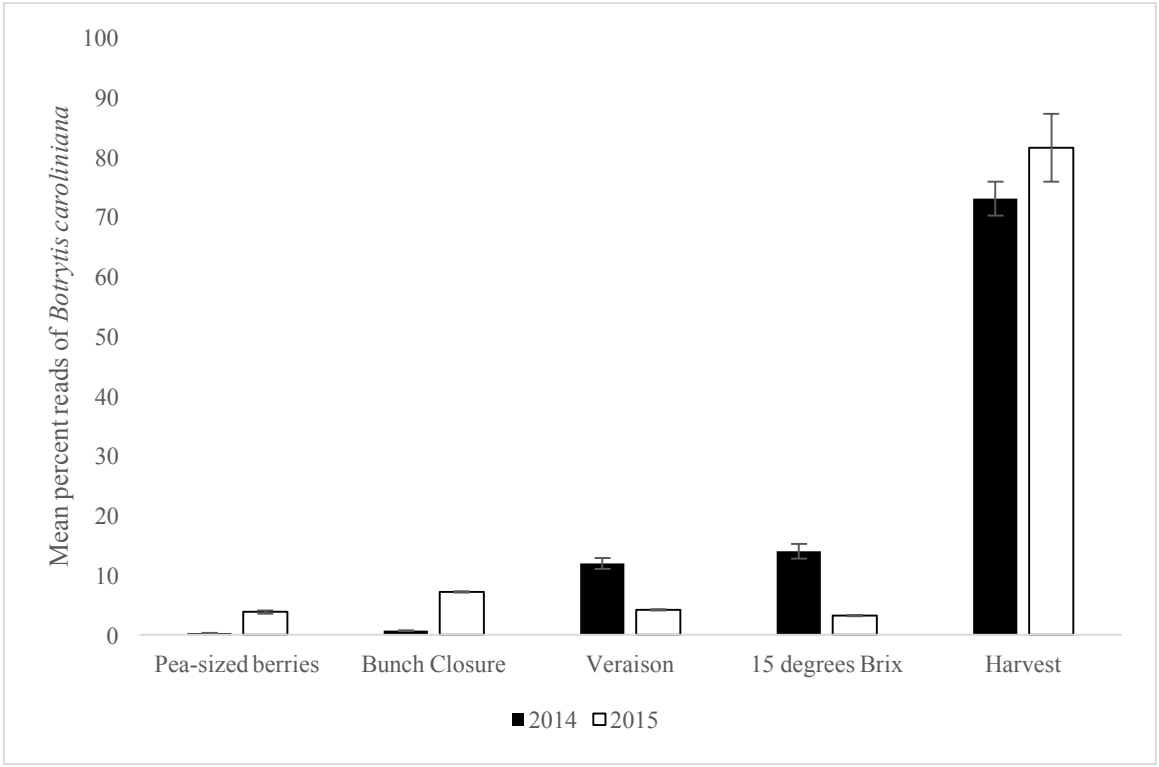


Figure 1.

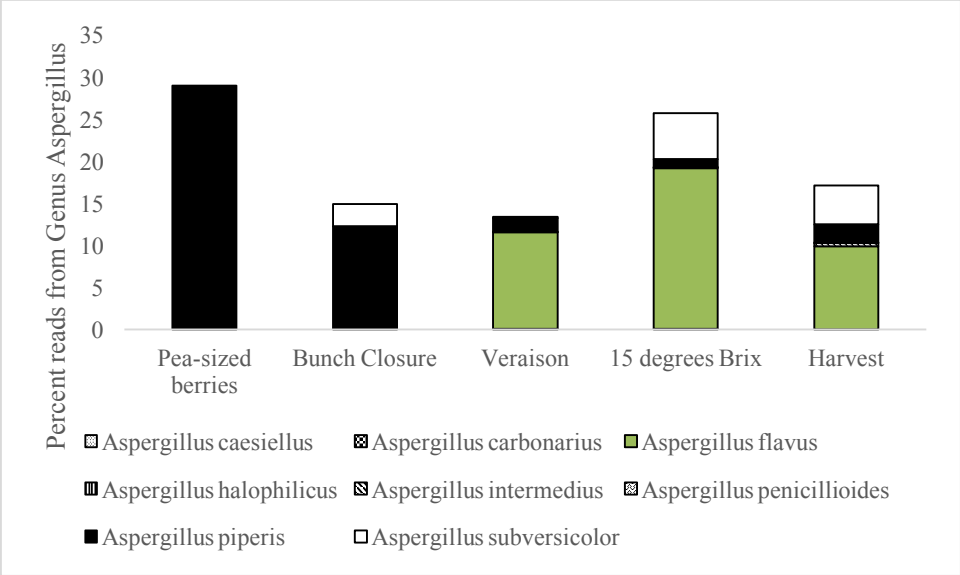

Percent reads from Genus Aspergillus
35
30
25
20
15
10
5
0
Pea-sized berries
Bunch Closure
Veraison
15 degrees Brix
Harvest
Aspergillus caesiellus
Aspergillus carbonarius
Aspergillus flavus
Aspergillus halophilicus
Aspergillus intermedius
Aspergillus penicillioides
Aspergillus piperis
Aspergillus subversicolor

Figure 2.

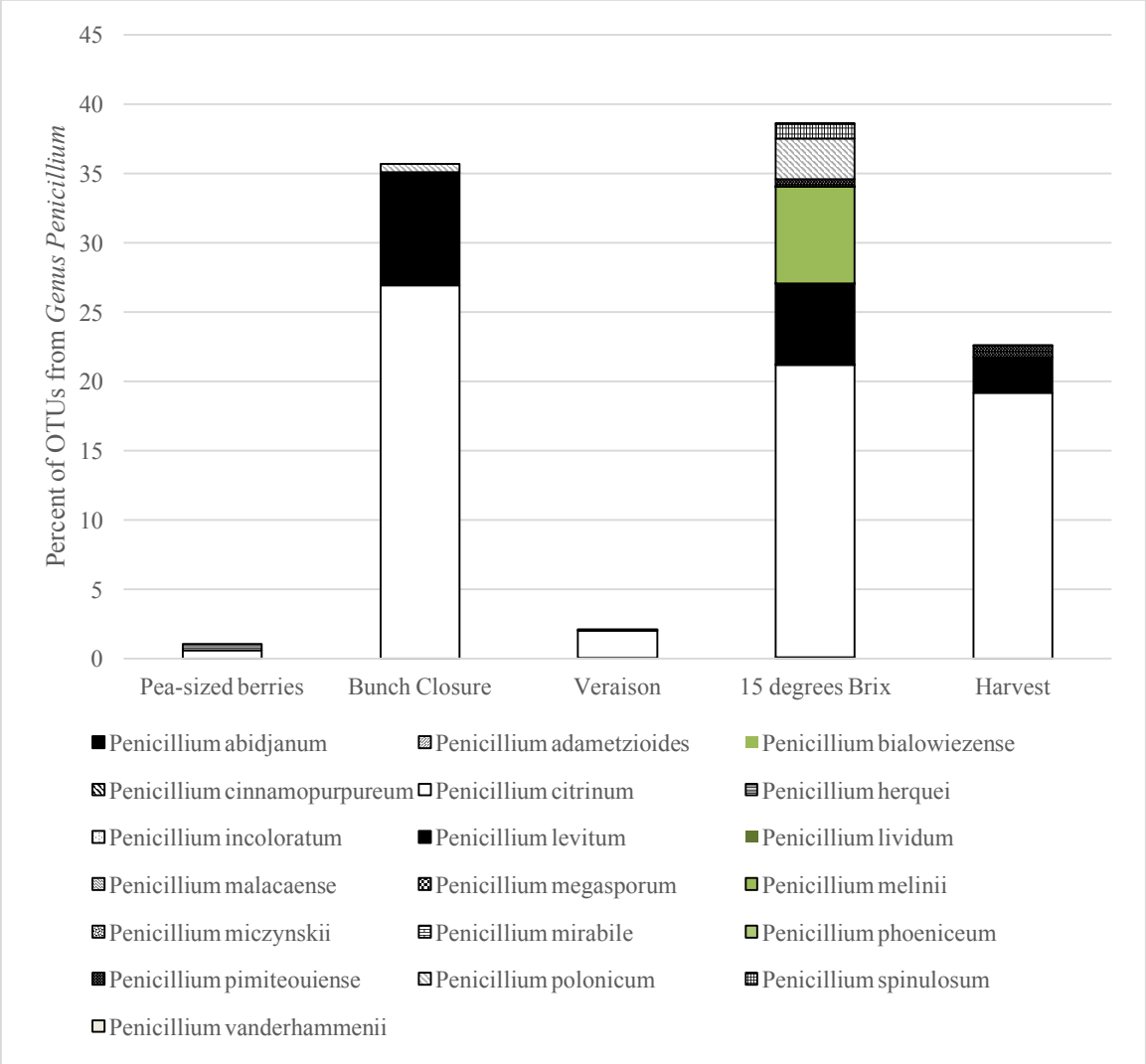

45
40
35
30
25
20
15
10
5
0
Percent of OTUs from Genus Penicillium
Pea-sized berries
Bunch Closure
Veraison
15 degrees Brix
Harvest
Penicillium abidjanum
Penicillium adametzioides
Penicillium bialowiezense
Penicillium cinnamopurpureum
Penicillium citrinum
Penicillium herquei
Penicillium incoloratum
Penicillium levitum
Penicillium lividum
Penicillium malacaense
Penicillium megasporum
Penicillium melinii
Penicillium miczynskii
Penicillium mirabile
Penicillium phoeniceum
Penicillium pimiteouiense
Penicillium polonicum
Penicillium spinulosum
Penicillium vanderhammenii

Figure 3.

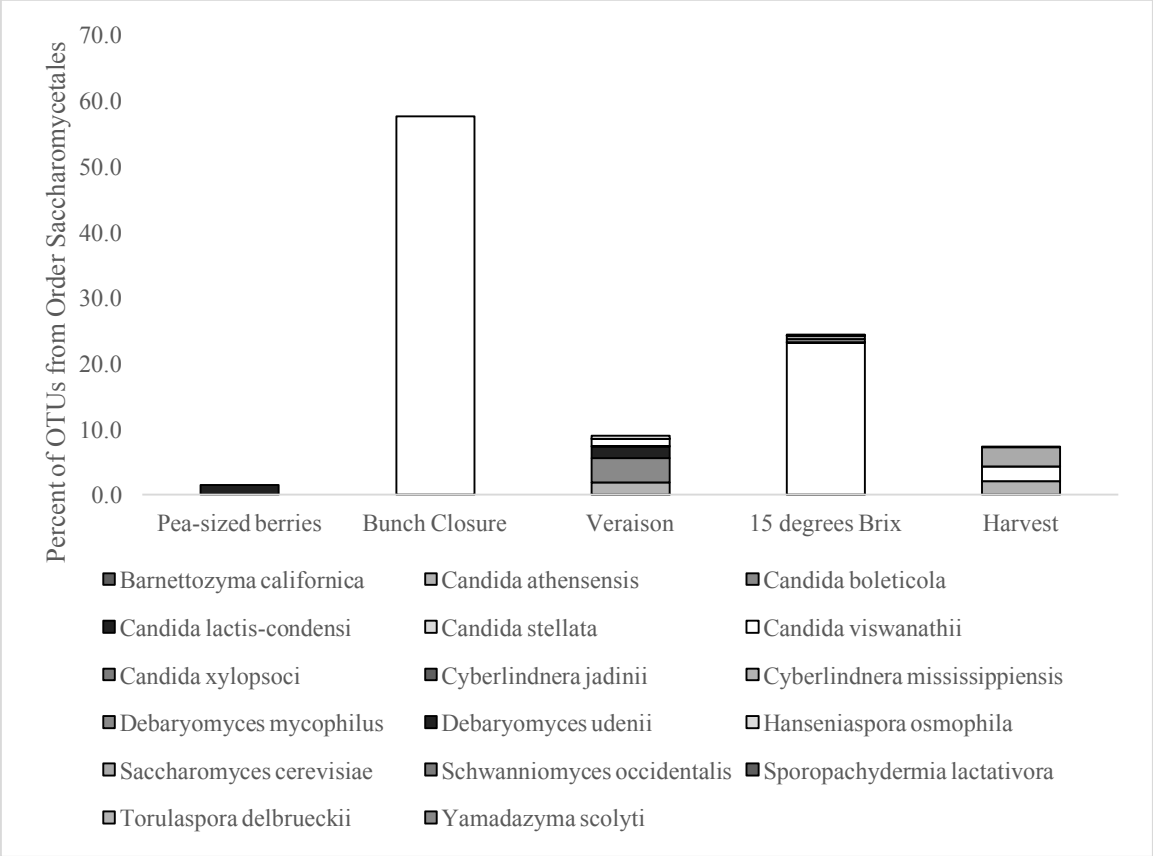

Percent of OTUs from Order Saccharomycetales
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Pea-sized berries
Bunch Closure
Veraison
15 degrees Brix
Harvest
Barnettozyma californica
Candida athensensis
Candida boleticola
Candida lactis-condensi
Candida stellata
Candida viswanathii
Candida xylopsoci
Cyberlindnera jadinii
Cyberlindnera mississippiensis
Debaryomyces mycophilus
Debaryomyces udenii
Hanseniaspora osmophila
Saccharomyces cerevisiae
Schwanniomyces occidentalis
Sporopachydermia lactativora
Torulaspora delbrueckii
Yamadazyma scolyti

Figure 4.

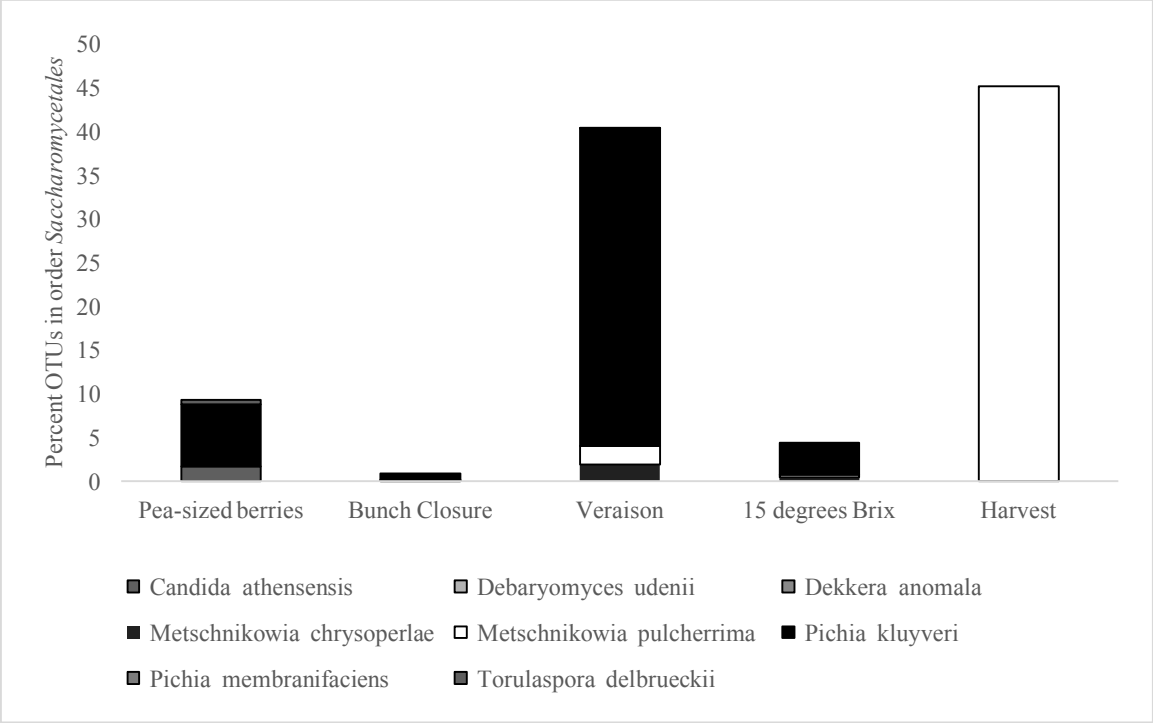


Figure 5.

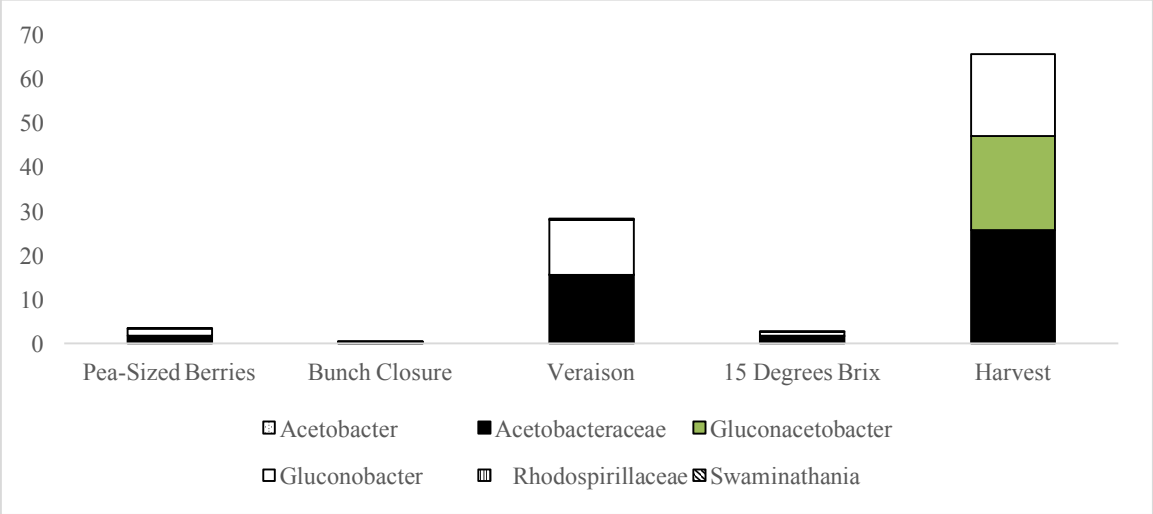


Figure 6.

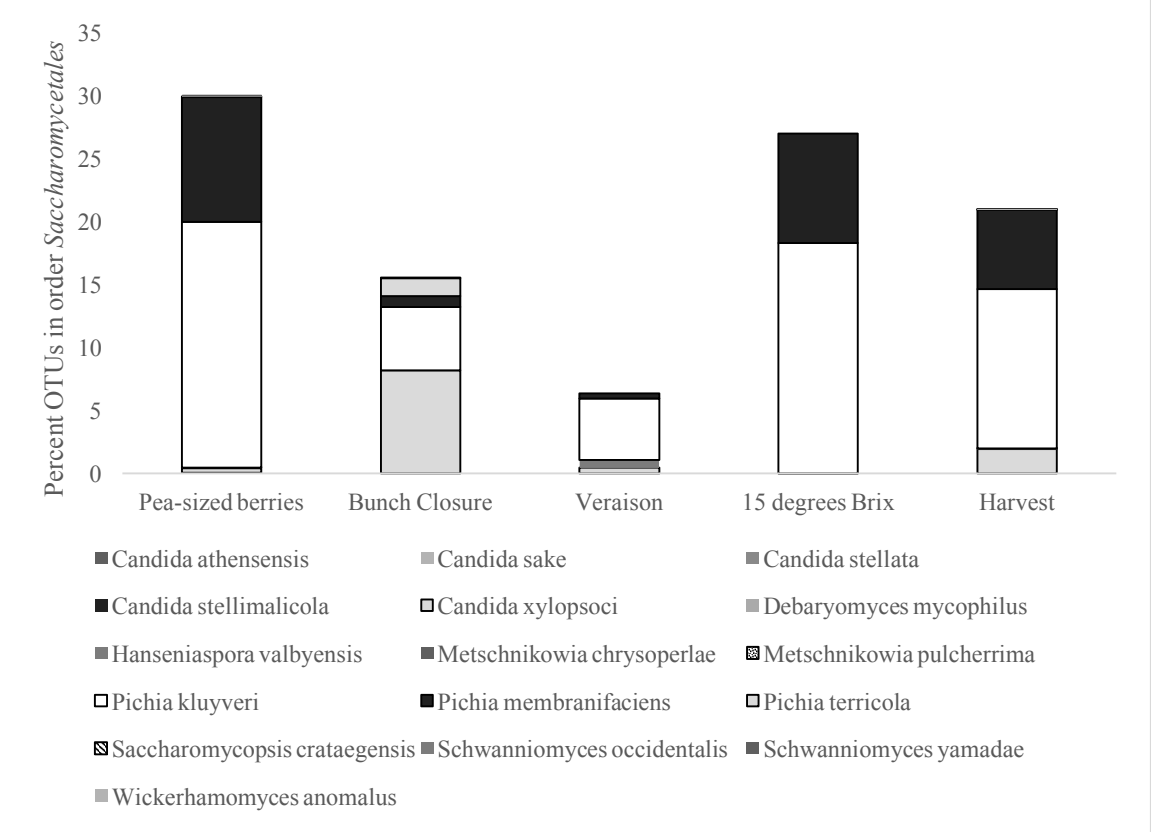


Figure 7.

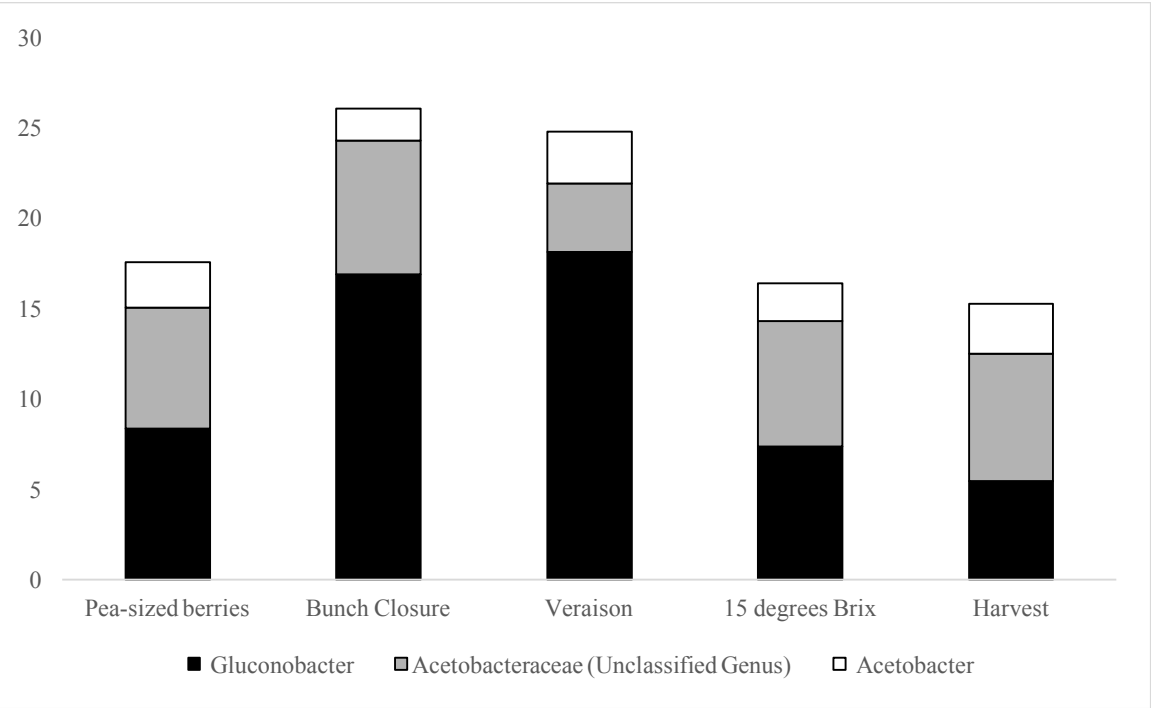


Figure 8.

CHAPTER 5

Identification and Frequencies Endophytic Microbes within Grape Berries

Abstract

Intact, healthy grape berries were sampled from vineyards in the states of Washington and New York and Tasmania, Australia, and from bunches purchased on two occasions in a supermarket. Endophytic microbes were isolated on media conducive to fungi or bacteria and subsequently identified by Illumina sequencing of their DNA. Species of the yeast genera *Metschnikowia, Pichia,* and *Hanseniaspora* were recovered from every set of samples, as were species of the bacterial genera *Acinetobacter, Burkholderia* and *Bacillus*; species of the bacterial genera *Acetobacter* and *Gluconobacter* also were recovered from vineyard samples from New York and Tasmania and from supermarket-purchased grapes. Multiple other fungal and bacterial species were recovered less often. When quantified for the Washington samples and one set from the supermarket, non-*Saccharomyces* yeast species represented the vast majority of fungal identifications, whereas the distribution of various bacterial species varied widely between and within the two sources. The endophytic presence of these microbes within grape berries has implications with respect not only to the potential development of sour rot but also to the broader concept of microbial terroir.

Introduction

The importance of epiphytic microbes on the grape surface has been researched extensively in recent years, with studies focusing on grapes sampled in the days leading up to harvest (Brysch-Herzberg and Seidel 2015; Combina et al. 2005; Drożdż

et al. 2015; Garofalo et al. 2016; Jara et al. 2016; Martini, Ciani, and Scorzetti 1996; Parish and Carroll 1985; Raspor et al. 2006; Rosini, Federici, and Martini 1982; Sabate et al. 2002; Setati et al. 2012; Yanagida et al. 1992). However, there is a paucity of data on even the mere presence, of endophytic microbes inhabiting the pulp of healthy grape berries, although individual species and groups of such organisms could potentially have a significant impact (e.g., as pathogens or in subsequent enological processes) under certain conditions. Similarly, whereas many researchers have sought to explore the microbial communities within grape musts after crushing, there has been no effort to determine whether these organisms originated on the surface of the harvested clusters or within the pulp.

In a study examining potential causes of the disease sour rot, we wounded intact healthy berries, inoculated them with various candidate microbes, and measured the evolution of ethanol and acetic acid after 5 to 8 days of incubation. In repeated experiments, we routinely found detectable levels of ethanol (and less often, acetic acid) in wounded control fruit, which had been handled aseptically but not inoculated with any microbes (Chapter 1). Because these results suggested the possible endophytic presence of yeast (and less often, acetic acid bacteria) within the berries, we undertook the following study to investigate both the ubiquity and diversity of microbes present within the pulp of healthy grapes from different geographical locations.

Materials and Methods

Isolation of endophytic microbes. Grape clusters were sampled from three vineyards

in Tasmania, Australia; three blocks of a single vineyard in Kennewick, WA; two vineyards in Geneva, NY; and from a supermarket on two separate occasions (Table 1). All grapes examined were cultivars of *Vitis vinifera* with the exception of *Vitis* x *labruscana* 'Concord'. All vineyard samples were obtained from vines exhibiting no overt symptoms of disease; clusters were intact and uninjured, at a maturity stage corresponding to approximately 1 to 5 days before harvest. Berries purchased from the supermarket were uninjured and at a commercial stage of ripeness for table grapes. From each sampled vineyard or vineyard block we selected three berries from each of three clusters chosen at random, from which we attempted to isolate potential endophytic microbes.

For the Tasmanian vineyards, each of the individual four-berry samples was macerated in a polyethylene bag, and 100 µL of the juice was plated onto both Yeast Peptone Dextrose (YPD) medium (2% peptone, 1% yeast extract, 2% glucose, 2% agar) and Yeast Peptone Mannitol (YPM) medium (0.3% peptone, 0.5% yeast extract, 2.5% mannitol, 1.5% agar), which favor growth of fungi and bacteria, respectively. After 3 to 6 days (whenever distinct colonies formed), 400 µL of sterile distilled water was pipetted onto the plate and cells of the resulting colonies were disrupted using a disposable plastic spreader. A 400-µL aliquot of the suspension was then pipetted from the petri dish into a 50 mL Falcon tube containing 5 mL of TE buffer (10mM Tris-HCl+1mM EDTA, ph 8.0) and 0.05 g NaCl and vortexed for 15 s.

For all other samples, each individual berry was cut in half using a sterile razor blade, and submerged in 70% ethanol for 5 minutes and then rinsed in sterile distilled water. Each half was then placed onto either YPD or YPM agar, with the pulp side of the

berry face-down on the media. Plates were incubated at 24°C for 6 days or until distinct colonies developed. 400 µL of sterile distilled water was pipetted onto the plate and cells of the resulting colonies were disrupted using a disposable plastic spreader. A 400 µL-aliquot of the suspension was then pipetted from the petri dish into a 50 mL Falcon tube collection tube containing 5 mL of TE buffer and 0.05 g NaCl and vortexed for 15 s.

DNA Extraction. A 500 µL-aliquot of 10% SDS was added to the suspension in the Falcon tube, vortexed for 5 s and left at room temperature for 15 min. A freeze-thaw sequence consisting of 30 min in a -80°C freezer and 5 min in a 60°C water bath was repeated three times to lyse the fungal and bacterial cells, and 750 µL of the solution was transferred to a centrifuge tube along with 750 µL ice-cold isopropanol. The suspension was centrifuged for 10 min at 9600xg. The supernatant was carefully discarded from the tube, 500 µL of ice-cold 95% ethanol was added, and the tube was again centrifuged at 9600xg for 1 min before discarding the supernatant. The pellet was re-suspended in 100 µL TE buffer and the DNA sample was then stored at -4°C for subsequent amplification and sequencing.

DNA sequencing and analysis. In all DNA sequencing, two primer sets were used. To amplify the V4 domain of bacterial 16s rRNA genes, primers F515 (5′-GTGTGCCAGCMGCCGCGGTAA–3′) and R806 (5′–GGACTACHVGGGTWTCTAAT–3′) were used. Fungal internal transcribed spacer (ITS) 1 loci were amplified using primers BITS (5′–CTACCTGCGGARGGATCA–3′) and B58S3 (5′–GAGATCCRTTGYTRAAAGTT–3′) (Bokulich et al. 2014). Genomic DNA was sent to the Cornell University DNA Sequencing facility in Ithaca, NY for

250-bp-paired-end sequencing on the Illumina MiSeq machine. For each sample, two separate runs were performed. Both forward primers were modified to contain a unique 8-bp barcode.

For all samples sets, the data was analyzed using Qiime 1.9.1 (Caporaso, Kuczynski, et al. 2010) for quality filtering, read processing, and OTU assignment. Sequences were trimmed once there were three consecutive bases with PHRED scores less than 20. Sequences less than 100nt were discarded. Open and closed reference OTU-picking methods used uclust and a pairwise identity of 97% (Edgar 2010). Alignment to greengenes 13_5 was done using PyNAST and alignment to UNITE 7_97 was conducted using the BLAST alignment method (Altschul et al. 1990; Caporaso, Bittinger, et al. 2010; DeSantis et al. 2006; Kõljalg et al. 2013).

To examine abundance in those samples collected in Washington and the second set of Red Globe grapes purchased at the supermarket, OTUs with than 0.0001% of the total abundance of the biom file were filtered out. Analysis was done in STAMP v2.1.3 and unclassified reads were not included in the analysis but they were kept to calculate abundance frequencies (Parks et al. 2014).

Results

Species in the yeast genera *Candida*, *Hanseniaspora*, and *Pichia* were identified in sample sets from all three vineyards in Tasmania, Australia; the yeast genera *Cryptococcus*, *Kloekera,* and *Rhodoturula, and Saccharomycetes* were identified in samples from two of the three, as were species of the filamentous fungi *Ambispora* and *Davidiella.* Seventeen additional fungal genera were identified in the macerates

of berries from a single vineyard (Table 2). The bacterial genera *Acetobacter*, *Bacillus*, and *Burkholderia* were also detected in samples from all three Tasmanian vineyards; species of *Acinetobacter*, Gluconobacter, and Serratia were also identified in two of the three sample sets. Nine additional bacterial genera were identified in the macerate of berries sampled from one of the three vineyards (Table 3). A similar set of organisms was identified from isolations performed on the first set of supermarket-purchased berries and those collected from vineyards in Geneva, NY. Species within the yeast genera *Candida*, *Hanseniaspora*, and *Pichia* were again present in every sample group, whereas those of *Metschnikowia*.were present in both of the purchased table grape cultivars and the field-collected Chardonnay berries (Table 4). Species in the acetic acid-producing bacterial genera *Acetobacter* and *Gluconobacter* were recovered from all field-collected varieties and from one of the two purchased table grape cultivars. All varieties also contained *Acinetobacter* spp.*,* and *Bacillus* spp. (Table 5).

In Red Globe grapes purchased from the supermarket, 89% of the fungal reads were *Metschnikowia pulcherrima*,, with *Pichia* spp., and *Saccharomyces cerevisiae* accounting for another 5.4 and 2.9%, respectively. Fourteen additional species were detected at frequencies of 0.01 to 0.77%. The majority of the bacterial reads were *Serratia marcascens,* and *Rothia* spp. (80.2 and 12.8%, respectively). Fifteen additional species were detected at frequencies of 0.01 to 2.7%. A similar set of fungal organisms was recovered from samples collected in Washington. Two *Metschnikowia* spp. represented 97.1 to 99.9% of the fungal reads in four of the five varieties sampled; in the other, they accounted for 77% of the reads, with *Botrytis*

caroliniana accounting for another 18%. An additional 15 yeast and filamentous fungal species accounted for the remainder of the reads in one or more of the sampled varieties (Table 6). Bacterial reads from the Washington samples represented a different distribution of species relative to the Red Globe berries, with *Serratia marcascens* and *Rothia* spp. detected only infrequently. The distribution of individual species also varied considerably among the individual cultivars sampled, e.g., *Acinetobacter* spp. represented 69 and 52% of the reads from Cabernet Sauvignon and Merlot berries but <2% of those from Rousanne, Chardonnay, and Gewürtztraminer. Similarly, *Actinobacillus porcinus* represented 58% of the reads from Chardonnay berries but was not detected at all in those of three other cultivars and was found only infrequently in the remaining two. Every set of samples had some representation of *Acinetobacter* spp., *Aggregatibacter* sp., *Anoxybacillus* sp., *Bacillus* spp., *Prevotella* spp., *Pseudomonas* spp., and *Rhodococcus* sp. (Table 7).

Discussion

To the best of our knowledge, this is the first study to report the presence of endophytic microbes within the pulp of intact, healthy grape berries and to document their specific identities and relative abundance. Although our study was somewhat limited in scope, the findings were generally consistent across a wide representation of grapes, including those of three different table grape cultivars from Chile and a range of wine/processing cultivars of two different species sampled from vineyards in the eastern and western United States and Australia. Multiple non-*Saccharomyces* yeasts (and, occasionally, *S. cerevisiae*) were detected in all sample populations, and these represented the vast majority of the fungi identified in those samples where the

frequencies of the individual recovered species were quantified. We also regularly identified the endophytic presence of acetic acid bacteria across the various samples, although these species generally represented a substantially smaller proportion of the total bacterial community than did yeast species within the fungal community. Other researchers have documented several of these same yeast and bacterial species as epiphytes on grape berry skins (Brysch-Herzberg and Seidel 2015; Combina et al. 2005; Drożdż et al. 2015; Garofalo et al. 2016; Jara et al. 2016; Martini et al. 1996; Parish and Carroll 1985; Raspor et al. 2006; Rosini et al. 1982; Sabate et al. 2002; Setati et al. 2012; Yanagida et al. 1992).

We undertook this study as a portion of a broader investigation of the etiology and management of the disease sour rot, which we have shown is caused by an interaction between (i) any one of multiple species of yeast, which initially produce ethanol within berries that become diseased; (ii) various acetic acid bacteria, which subsequently oxidize the ethanol to acetic acid; and (iii) *Drosophila* fruit flies, which appear to play roles both inclusive and exclusive of their direct association with the preceding microbes (Chapter 1). The origin of the causal yeast and bacteria prior to the initiation of a sour rot epidemic (i.e., the primary inoculum) has not been determined in the literature, although some researchers have suggested that they are initially delivered to healthy grapes by fruit flies (Bisiach et al. 1986; Barata et al. 2012). Although *Drosophila* spp. can undoubtedly spread the causal microbes both passively on their bodies and through their regurgitation during feeding (Chandler et al. 2011, Wong et al. 2011, Broderick et al. 2014, Staubach et al. 2013, Koyle et al. 2016), our research indicates that multiple microorganisms capable of causing disease

symptoms are already present within grape berries before the disease begins to develop. These endogenous fungal and bacterial populations have significant implications for both grape growing and winemaking, as the microbes within the berries cannot be targeted by pesticide applications and those microorganisms can bring about the fermentation and oxidation of the sugars within the grape berry before entering the winery.

The ubiquity of yeast and bacteria within berries across a wide range of grape cultivars and geographical regions has implications for our understanding of microbial terroir and how the microbial diversity within grapes might contribute to the uniqueness of the wines that they produce. Future studies into grape and wine terroir should take into account the role of endophytic microbes and their byproducts, which might be either positive or negative depending on the specific microbes present and the conditions under which they are functioning both in the field and in the winery. Clearly, there are multiple potential avenues of further research available within this realm.

Literature Cited

1. Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J. Lipman. 1990. “Basic Local Alignment Search Tool.” *Journal of Molecular Biology* 215(3):403–10. Retrieved (http://www.sciencedirect.com/science/article/pii/S0022283605803602).
2. Barata, André, Manuel Malfeito-Ferreira, and Virgílio Loureiro. 2012. “Changes in Sour Rotten Grape Berry Microbiota during Ripening and Wine Fermentation.” *International Journal of Food Microbiology* 154(3):152–61. Retrieved

(http://dx.doi.org/10.1016/j.ijfoodmicro.2011.12.029).

3. Bisiach, M., G. Minervini, and Zerbetto. 1986. “Possible Integrated Control of Grapevine Sour Rot.” *Vitis* 25:118–28. Retrieved (e024052.pdf).
4. Bokulich, Nicholas a, John H. Thorngate, Paul M. Richardson, and David a Mills. 2014. “Microbial Biogeography of Wine Grapes Is Conditioned by Cultivar, Vintage, and Climate.” *Proceedings of the National Academy of Sciences of the United States of America* 111(1):E139-48. Retrieved (http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3890796&tool=pmcentrez&rendertype=abstract).
5. Broderick, N. A., Buchon, N., and Lemaitre, B. 2014. Microbiota-induced changes in drosophila melanogaster host gene expression and gut morphology. MBio. 5 (3).
6. Brysch-Herzberg, Michael and Martin Seidel. 2015. “Yeast Diversity on Grapes in Two German Wine Growing Regions.” *International Journal of Food Microbiology* 214(Supplement C):137–44. Retrieved (http://www.sciencedirect.com/science/article/pii/S016816051530088X).
7. Caporaso, J.Gregory, Kyle Bittinger, et al. 2010. “PyNAST: A Flexible Tool for Aligning Sequences to a Template Alignment.” *Bioinformatics* 26(2):266–67. Retrieved (http://dx.doi.org/10.1093/bioinformatics/btp636).
8. Caporaso, J.Gregory, Justin Kuczynski, et al. 2010. “QIIME Allows Analysis of High-Throughput Community Sequencing Data.” *Nat Meth* 7(5):335–36. Retrieved (http://dx.doi.org/10.1038/nmeth.f.303).

9. Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A., & Kopp, A. 2011. Bacterial communities of diverse Drosophila species: ecological context of a host-microbe model system. PLoS Genet. 7 (9).

10. Combina, M. et al. 2005. "Dynamics of Indigenous Yeast Populations during Spontaneous Fermentation of Wines from Mendoza, Argentina." *International Journal of Food Microbiology* 99(3):237–43. Retrieved (http://www.sciencedirect.com/science/article/pii/S0168160504004362).

11. DeSantis, T. Z. et al. 2006. "Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB." *Applied and Environmental Microbiology* 72(7):5069–72. Retrieved (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1489311/).

12. Drożdż, Iwona, Małgorzata Makarewicz, Paweł Sroka, Paweł Satora, and Paweł Jankowski. 2015. "Comparison of the Yeast Microbiota of Different Varieties of Cool-Climate Grapes by PCR-RAPD." *Potravinarstvo Slovak Journal of Food Sciences; Vol 9, No 1 (2015): Potravinarstvo*. Retrieved (http://www.potravinarstvo.com/journal1/index.php/potravinarstvo/article/view/484 %3C/div%3E).

13. Edgar, Robert C. 2010. "Search and Clustering Orders of Magnitude Faster than BLAST." *Bioinformatics* 26(19):2460–61. Retrieved (http://dx.doi.org/10.1093/bioinformatics/btq461).

14. Garofalo, Carmela, Mariana Tristezza, Francesco Grieco, Giuseppe Spano, and Vittorio Capozzi. 2016. "From Grape Berries to Wine: Population Dynamics of Cultivable Yeasts Associated to ``Nero Di Troia'' Autochthonous Grape

Cultivar." *World Journal of Microbiology and Biotechnology* 32(4):59. Retrieved (https://doi.org/10.1007/s11274-016-2017-4).

15. Jara, Carla, V.Felipe Laurie, Albert Mas, and Jaime Romero. 2016. "Microbial Terroir in Chilean Valleys: Diversity of Non-Conventional Yeast." *Frontiers in Microbiology* 7:663. Retrieved (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4868835/).
16. Kõljalg, Urmas et al. 2013. "Towards a Unified Paradigm for Sequence-Based Identification of Fungi." *Molecular Ecology* 22(21):5271–77. Retrieved November 13, 2017 (http://doi.wiley.com/10.1111/mec.12481).
17. Koyle, M. L., Veloz, M., Judd, A. M., Wong, A. C., Newell, P. D., Douglas, A. E., Chaston, J. M. 2016. Rearing the Fruit Fly *Drosophila melanogaster* Under Axenic and Gnotobiotic Conditions. J. Vis. Exp. 113.
18. Martini, Alessandro, Maurizio Ciani, and Gloria Scorzetti. 1996. "Direct Enumeration and Isolation of Wine Yeasts from Grape Surfaces." *American Journal of Enology and Viticulture* 47(4):435 LP-440. Retrieved (http://www.ajevonline.org/content/47/4/435.abstract).
19. Parish, M. E. and D. E. Carroll. 1985. "Indigenous Yeasts Associated with Muscadine (Vitis Rotundifolia) Grapes and Musts." *American Journal of Enology and Viticulture* 36(2):165 LP-169. Retrieved (http://www.ajevonline.org/content/36/2/165.abstract).
20. Parks, Donovan H., Gene W. Tyson, Philip Hugenholtz, and Robert G. Beiko. 2014. "STAMP: Statistical Analysis of Taxonomic and Functional Profiles." *Bioinformatics* 30(21):3123–24. Retrieved

(http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4609014/).

21. Raspor, Peter, Damjana Miklič Milek, Julijana Polanc, Sonja Smole Možina, and Neža Čadež. 2006. "Yeasts Isolated from Three Varieties of Grapes Cultivated in Different Locations of the Dolenjska Vine-Growing Region, Slovenia." *International Journal of Food Microbiology* 109(1):97–102. Retrieved (http://www.sciencedirect.com/science/article/pii/S0168160506000687).
22. Rosini, Gianfranco, Federico Federici, and Alessandro Martini. 1982. "Yeast Flora of Grape Berries during Ripening." *Microbial Ecology* 8(1):83–89. Retrieved (https://doi.org/10.1007/BF02011464).
23. Sabate, Josepa, Josep Cano, Braulio Esteve-Zarzoso, and Josè M. Guillamón. 2002. "Isolation and Identification of Yeasts Associated with Vineyard and Winery by RFLP Analysis of Ribosomal Genes and Mitochondrial DNA." *Microbiological Research* 157(4):267–74. Retrieved (http://www.sciencedirect.com/science/article/pii/S0944501304700865).
24. Setati, Mathabatha Evodia, Daniel Jacobson, Ursula-Claire Andong, and Florian Bauer. 2012. "The Vineyard Yeast Microbiome, a Mixed Model Microbial Map." *PLOS ONE* 7(12):e52609. Retrieved (https://doi.org/10.1371/journal.pone.0052609).
25. Staubach, F., Baines, J. F., Kunzel, S., Bik, E. M., & Petrov, D. A. 2013. Host species and environmental effects on bacterial communities associated with Drosophila in the laboratory and in the natural environment. PLoS One. 8 (8).

26. Wong, C. N., Ng, P., & Douglas, A. E. 2011. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ Microbiol. 13 (7), 1889-1900.

27. Yanagida, Fujitoshi, Fumiko Ichinose, Takashi Shinohara, and Shoji Goto. 1992. "Distribution of Wild Yeasts in the White Grape Varieties at Central Japan." *The Journal of General and Applied Microbiology* 38(5):501–4.

Table 1. Grape cultivars, and their origins, assayed for endophytic microbes			
Varieties	**State**	**Latitude**	**Longitude**
Cabernet Sauvignon, Chardonnay, Gewurztraminer, Rousanne, Syrah	Washington, USA	46.21	-119.29
Chardonnay, Concord, Cabernet Franc	New York, USA	42.88	-77.04
Riesling	Tasmania, Australia	-42.88	147.39
Sauvignon Blanc	Tasmania, Australia	-41.50	147.20
Riesling	Tasmania, Australia	-42.81	147.42
Black Seedless, Flame Seedless	Supermarket, 1st sample		
Red Globe	Supermarket, 2nd sample		

Table 2. Fungal operational taxonomic units (OTU) identified in macerated samples from three commercial vineyards in Tasmania, Australia.

Riesling (Northern Tasmania)	**Riesling (Southern Tasmania)**	**Sauvignon Blanc**
Ambispora gerdemannii[a]	*Candida CBS989*[b]	*Ambispora appendicula*[a]
Aphanoascus durus[a]	*Candida zemplinina*[b]	*Bacidina delicata*[a]
Candida CBS989[b]	*Candida zemplinina*[b]	*Bacidina delicata*[a]
Candida zemplinina[b]	*Hanseniaspora uvarum*[b]	*Candida zemplinina*[b]
Chaenothecopsis dolichocephala[a]	*Pichia fermentans*[b]	*Cryptococcus FF011314*[a]
Cladosporium cladosporioides[a]	*Pichia kluyveri*[b]	*Hanseniaspora* sp.[b]
Cryptococcus FF011314[a]	*Pichia membranifaciens*[b]	*Hanseniaspora uvarum*[b]
Cryptococcus flavescens[a]	*Rhodotorula RhSoW01*[b]	*Hanseniaspora valbyensis*[b]
Davidiella tassiana[a]		*Kloeckera japonica*[b]
Dekkera anomala[b]		*Peltigera monticola*
Geosmithia flava[a]		*Pichia fermentans*[b]
Hanseniaspora sp.[b]		*Pichia kluyveri*[b]
Hanseniaspora uvarum[b]		*Pichia manshurica*[b]
Hanseniaspora valbyensis[b]		*Pichia membranifaciens*[b]
Kloeckera japonica[b]		*Saccharomycetes 5B12*[b]
Kloeckera lindneri[b]		*Thanatephorus cucumeris*[a]
Metschnikowia pulcherrima[b]		
Nephromopsis morrisonicola[a]		
Pichia		

fermentans [b]
Pichia
kluyveri [b]
Pichia
kudriavzevii [b]
Pichia
membranifaciens [b]
Rhodotorula
lamellibrachiae [b]
Rhodotorula
RhSoW01 [b]
Saccharomycetes
5B12 [b]
Saccharomycopsis
crataegensis [b]
Torulaspora
IFO1145 [b]
Valsa
ceratosperma [a]

[a] Non-yeast species
[b] Yeast species

Table 3. Bacterial operational taxonomic units (OTU) identified in macerated samples from three commercial vineyards in Tasmania, Australia

Riesling (Northern Tasmania)	**Riesling (Southern Tasmania)**	**Sauvignon Blanc**
Ambispora gerdemannii	*Acetobacter* sp.	*Acetobacter* sp.
Acetobacter sp.	*Acinetobacter johnsonii*	*Acinetobacter johnsonii*
Acinetobacter sp.	*Bacillus cereus*	*Bacillus cereus*
Bacillus cereus	*Bacillus flexus*	*Bacillus flexus*
Bacillus flexus	*Bacillus muralis*	*Burkholderia* sp.
Bacillus muralis	*Burkholderia* sp.	*Rothia aeria*
Burkholderia bryophila	*Gluconobacter* sp.	*Rothia mucilaginosa*
Corynebacterium durum	*Streptococcus* sp.	*Serratia marcescens*
Gluconobacter sp.		*Streptococcus* sp.
Haemophilus parainfluenzae		
Kocuria palustris		
Lactobacillus sp.		
Micrococcus luteus		
Rhodococcus fascians		
Rothia mucilaginosa		
Serratia marcescens		

Table 4. Fungal operational taxonomic units (OTU) identified in the 1st supermarket-purchased samples and those collected from vineyards in Geneva, NY

Black Seedless	**Flame Seedless**	**Chardonnay**
Candida CBS989 [a]	*Ambispora granatensis* [b]	*Ambispora callosa* [b]
Hanseniaspora uvarum [a]	*Candida CBS989* [a]	*Ambispora granatensis* [b]
Metschnikowia pulcherrima [a]	*Candida zemplinina* [a]	*Aphanoascus durus* [b]
Pichia fermentans [a]	*Geosmithia eupagioceri*	*Aureobasidium pullulans* [b]
Pichia kluyveri [a]	*Hanseniaspora guilliermondii* [a]	*Bridgeoporus nobilissimus* [b]
Pichia kudriavzevii [a]	*Hanseniaspora sp.* [a]	*Candida CBS989* [a]
Pichia manshurica [a]	*Hanseniaspora uvarum* [a]	*Candida zemplinina* [a]
Pichia membranifaciens [a]	*Hanseniaspora valbyensis* [a]	*Chaenotheca furfuracea* [b]
Saccharomycetes 5B12 [a]	*Issatchenkia hanoiensis* [a]	*Coccidioides immitis* [b]
Sporotrichum roseum [b]	*Issatchenkia terricola* [a]	*Cryphonectria radicalis* [b]
<u>Concord</u>	*Metschnikowia pulcherrima* [a]	*Cryptococcus anemochorus* [b]
Candida CBS989 [a]	*Phyllobaeis imbricata* [b]	*Curvularia pallescens* [b]
Pichia kluyveri [a]	*Pichia fermentans* [a]	*Hanseniaspora guilliermondii* [a]
Pichia fermentans [a]	*Pichia kluyveri* [a]	*Hanseniaspora sp.* [a]
Hanseniaspora uvarum [a]	*Pichia kudriavzevii* [a]	*Hanseniaspora uvarum* [a]
Pichia membranifaciens [a]	*Pichia manshurica* [a]	*Hanseniaspora valbyensis* [a]
Hanseniaspora		

uvarum [a]

Cabernet Franc

Alternaria brassicae [b]
Ambispora granatensis [b]
Candida CBS989 [a]
Candida zemplinina [a]
Davidiella tassiana [b]
Hanseniaspora sp. [a]
Hanseniaspora uvarum [a]
Issatchenkia terricola [a]
Mycocalicium victoriae [b]
Pichia fermentans [a]
Pichia kluyveri [a]
Pichia kudriavzevii [a]
Pichia manshurica [a]
Pichia membranifaciens [a]
Pichia occidentalis [a]
Saccharomycetes 5B12 [a]
Torulaspora IFO1145 [a]
Xylaria curta [b]
Pichia membranifaciens [a]
Pichia occidentalis [a]
Protoparmelia montagnei [b]
Saccharomycopsis crataegensis [a]
Trichoderma viride [b]
Issatchenkia hanoiensis [a]
Issatchenkia terricola [a]
Kloeckera japonica [a]
Metschnikowia chrysoperlae [a]
Metschnikowia pulcherrima [a]
Micarea doliiformis [b]
Peltigera chionophila [b]
Pichia fermentans [a]
Pichia kluyveri [a]
Pichia kudriavzevii [a]
Pichia manshurica [a]
Pichia membranifaciens [a]
Pichia occidentalis [a]
Rhodotorula RhSoW01 [a]
Saccharomycetes 5B12 [a]
Trichoderma viride [b]

[a] Yeast species
[b] Non-yeast species

Table 5. Bacterial operational taxonomic units (OTU) identified in the 1st supermarket-purchased samples and those collected from vineyards in Geneva, NY

Black Seedless	Flame Seedless	Chardonnay	Cabernet Franc
Acetobacter sp.	*Acinetobacter johnsonii*	*Acinetobacter johnsonii*	*Acetobacter* sp.
Acinetobacter johnsonii	*Bacillus cereus*	*Bacillus cereus*	*Acinetobacter* sp.
Bacillus cereus	*Brachybacterium conglomeratum*	*Bacillus muralis*	*Bacillus cereus*
Bacillus flexus	*Burkholderia bryophila*	*Burkholderia sp.*	*Burkholderia bryophila*
Bacillus muralis	*Corynebacterium durum*	*Burkholderia bryophila*	*Corynebacterium durum*
Gluconobacter sp.	*Rothia aeria*	*Gluconobacter cerinus*	*Gluconobacter* sp.
Rothia aeria	*Rothia mucilaginosa*	*Haemophilus parainfluenzae*	*Kocuria palustris*
Rothia mucilaginosa	*Serratia marcescens*	*Kocuria palustris*	*Lactobacillus iners*
Serratia marcescens	*Streptococcus* sp.	*Rothia* sp.	*Micrococcus luteus*
Streptococcus anginosus		*Veillonella dispar*	*Serratia marcescens*
Veillonella dispar			*Streptococcus anginosus*
Concord			*Rothia aeria*
Acinetobacter johnsonii			*Rothia mucilaginosa*
Bacillus cereus			*Serratia marcescens*
Burkholderia sp.			*Streptococcus anginosus*
Gluconobacter sp.			
Rothia sp.			
Serratia marcescens			
Streptococcus anginosus			

Table 6. The mean frequencies (%) of reads for each fungal operational taxonomic unit (OTU) recovered from the pulp of six *Vitis vinifera* varieties collected in Kennewick, WA: Cabernet Sauvignon (CS), Chardonnay (CH), Gewurztraminer (GW), Malbec (ML), Rousanne (RO) and Syrah (SY)

	CS	CH	GW	ML	RO	SY
Aureobasidium pullulans	0	0	0.02	0.02	0	0
Botrytis caroliniana	0	0	18.5	0.01	0.01	0
Candida parapsilosis	0	0	0	0	0.03	0
Capnobotryella renispora	0	0	0	0.02	0	0
Cladosporium arthropodii	0.02	0	0	0	0	0
Cladosporium delicatulum	0.01	0	0.02	0	0.01	0
Guehomyces pullulans	0	0	2.13	0	0	0
Metschnikowia chrysoperlae	80.6	91.8	67.3	95.2	86.6	67.0
Metschnikowia pulcherrima	19.2	5.3	10.2	4.56	12.9	32.9
Pichia kluyveri	0.08	0.02	0.37	0.12	0.25	0.01
Pichia membranifaciens	0	2.97	0.07	0.02	0.02	0.04
Ramularia pratensis	0	0	0	0	0	0.02
Rhodosporidiobolus colostri	0.05	0	0.95	0.06	0.06	0.01
Saccharomyces cerevisiae	0.01	0	0.03	0.02	0.01	0
Sclerotinia sclerotiorum	0.01	0	0.01	0.01	0.01	0
Tsuchiyaea wingfieldii	0	0	0.13	0	0.03	0
Vishniacozyma heimaeyensis	0	0	0	0	0	0.01
Vishniacozyma victoriae	0	0	0.28	0	0	0
Wallemia sebi	0.01	0	0	0	0	0

Table 7. The mean frequencies (%) of reads for each bacterial operational taxonomic unit (OTU) recovered from the pulp of six *Vitis vinifera* varieties collected in Kennewick, WA: Cabernet Sauvignon (CS), Chardonnay (CH), Gewurztraminer (GW), Malbec (ML), Rousanne (RO) and Syrah (SY)

	CS	CH	GW	ML	RO	SY
Acinetobacter johnsonii	68.82	1.23	1.58	49.46	0.92	8.14
Acinetobacter rhizosphaerae	0.01	0	0	1.19	0	0
Actinobacillus porcinus	0	57.78	0.09	0	2.34	0
Aggregatibacter segnis	0.52	0.25	4.85	6.1	18.8	16.73
Alloiococcus otitis	0	0.19	0	0	0	0
Anoxybacillus kestanbolensis	17.18	2.84	70.46	8.65	5.58	20.37
Bacillus cereus	0.23	0	0.03	0.69	0	0.07
Bacillus firmus	5.58	4.18	3.74	1.25	15.35	21.62
Bacillus flexus	0.01	0	0.02	0.07	0.38	1.06
Bacillus muralis	0	1.18	0	0	0	0
Brachybacterium conglomeratum	0	0	1.99	0	7.43	1.3
Burkholderia bryophila	0	0	0	0	2.96	0
Cellulomonas xylanilytica	0.68	0.06	0	0.6	4.43	1.76
Corynebacterium durum	0	0	0	0.11	0	0
Corynebacterium kroppenstedtii	0	0	0	0	0	2.91
Faecalibacterium prausnitzii	0	0	0	0.05	0	0
Haemophilus parainfluenzae	0	0	0	0.05	0	0
Janthinobacterium lividum	0	0	0.01	0	0	0
Kocuria palustris	0	0	0.05	0	0	0
Kocuria rhizophila	0	0	0.01	0	0	0
Lactobacillus iners	0	0	0	0	0.72	0
Lysinibacillus boronitolerans	0	0	0	0	0.47	0
Micrococcus luteus	0	0.1	0	0	0	0
Neisseria subflava	1.85	0	0	0	0	0
Paracoccus aminovorans	0	0	0	0	1.08	0
Pasteurella multocida	0.01	0	0	0.23	0.16	0.23
Prevotella copri	0.87	0	0	0.1	0	0

Prevotella melaninogenica	1.69	1.78	4.49	12.5	17.37	9.02
Prevotella nanceiensis	0.03	0.23	1.43	0	0	0
Propionibacterium acnes	0	0	0.05	0	2.8	0.15
Pseudomonas umsongensis	0.01	0	0.06	0	0	0
Pseudomonas veronii	0	0	0	0.12	0.82	0.45
Pseudomonas viridiflava	0.29	29.14	4.06	6.07	9.26	13.42
Rhodococcus fascians	0.05	0.05	2.52	0.35	2.58	0.48
Rothia aeria	0.34	0	0.04	0.52	0	0
Rothia dentocariosa	0.08	0.05	0.09	0.71	0.37	0.14
Rothia mucilaginosa	0.05	0	0.01	0.09	0	0
Salana multivorans	0.07	0	1.29	4.33	0.09	0
Serratia marcescens	0.03	0.13	0.09	0	0.32	0.49
Sphingomonas wittichii	0	0	0	0.32	0.16	0
Staphylococcus aureus	0.01	0	0	0.69	0.16	1.3
Staphylococcus epidermidis	0	0.73	0.07	0.9	2.93	0
Streptococcus anginosus	0.01	0	0	0.05	0	0.07
Veillonella dispar	0.01	0.1	0	0	0.08	0.28
Veillonella parvula	1.57	0	0	4.81	2.4	0

CHAPTER 6

A New Method for Extracting DNA from the Grape Berry Surface, Beginning in the Vineyard

Introduction

The prevalence of metagenomic analysis has become crucial to the study of microbial diversity, but its application in natural environments has been problematic due to the need for high quality DNA obtained from less-than-ideal environmental situations. Isolating DNA from the surface of a grape berry involves aggressive and disruptive actions, due to tight adhesion of microbes to the thick berry skin and cuticle, making it difficult to wash microbes off the surface using most commercial kits (1), with some exceptions (2,3). More commonly, researchers have used plating methods (4–9) or sampled grape must (10–12) to conduct microbial ecology studies. Also, the process of DNA extraction typically begins in the laboratory after samples have been collected from the field, which increases the likelihood that microbial communities can be altered or disrupted from the time they are collected to the time they are processed. For these reasons, we developed a DNA extraction procedure that starts in the field, extracts microbes from the surface of the grape, and is both cost effective and can be made from commonly available laboratory chemicals with low toxicity. A plethora of DNA extraction methods exist already, ranging from DNA extraction kits to homemade methods. The efficacy of these methods vary. Many published methods are not useful for environmental samples which oftentimes contain

inhibitory compounds, and grapes which have a challenging surface from which to extract microbes. Published methods that contain harsh chemicals, such as phenol or chloroform, are both costly and have safety concerns, but the affordability and ease of preparation of the materials for DNA extraction allow the extraction to begin in the vineyard.

Materials & Methods

A Red Globe grape berry purchased from the supermarket was excised directly from a cluster into a 50 mL Falcon tube containing 5 mL of a TE buffer (10mM Tris-HCl+1mM EDTA, ph 8.0) solution containing 10% NaCl. The cap was screwed back onto the tube and placed in a Stryofoam cooler with an ice pack and transported to the laboratory. There, 500 µl of 10% SDS was added to the Falcon tube containing the TE-NaCl solution, vortexed for 5 seconds and left at room temperature for 15 min. A freeze-thaw sequence consisting of 30 min in a -80°C freezer and five min in 60°C water bath was repeated three times to lyse the fungal and bacterial cells. A 750 µl-aliquot of the solution was transferred to a centrifuge tube, along with 750 µl ice-cold isopropanol. The solution was centrifuged for 10 min at 9600xg. The supernatant was carefully removed from the tube, 500 µl ice-cold 95% ethanol was added, and the tube was again centrifuged at 9600xg for 1 min. The pellet was re-suspended in 100 µl TE buffer. The DNA was then stored at -4°C until further use.

To compare the efficacy of this protocol with other commonly-used methods, we repeated the extraction using *V. vinifera* cv. Red Globe berries purchased from the supermarket. The DNA from three berries was extracted using (i) the previously-described extraction method, (ii) a MoBio PowerSoil DNA Isolation kit, (iii) a CTAB

and phenol-chloroform extraction and (iv), a negative control lacking NaCl and SDS. For the PowerSoil kit, a grape berry was immersed in 5 ml TE buffer and vortexed for 15 s. The protocol was followed as described in the manual, using a 750 µl-aliquot of berry rinsate. For the CTAB extraction, we used a modified version of a previously described protocol (13). A grape berry was immersed in 5 ml TE buffer and vortexed for 15 s. A 750 µl-aliquot was pipetted into a 1 ml of CTAB buffer (Tris 0.1 M, NaCl 1.43M, EDTA 0.02M, CTAB 0.02M) and heated at 65°C for 30 min, and then centrifuged at 8000 rpm for five min. The supernatant was mixed with an equal volume of phenol:chloroform:isoamyl alcohol (50:48:2) and centrifuged at 8000 rpm for 10 min. The aqueous phase was recovered and mixed with an equal volume of chloroform and again centrifuged at 8000 rpm for 10 min. An equal volume of isopropanol was then added to the aqueous phase and centrifuged at 14000 rpm at 4°C for 30 min. The DNA pellet was then washed twice with 70% ethanol, followed both times by centrifugation at 14000 rpm for 10 min. The DNA pellet was dried under a hood for 20 min and resuspended in 1X TE buffer. For the negative control treatment, we immersed a grape berry in 5 mL TE buffer in a 50 mL Falcon tube, vortexed it for 10 seconds and followed the remainder of the previously-described protocol, from the point of the freeze-thaw sequences onwards. Thus, the primary difference was lack of NaCl and SDS.

To amplify the fungal DNA from the berry surface, fungal internal transcribed spacer (ITS) 1 loci were amplified using primers BITS (5′–CTACCTGCGGARGGATCA–3′) and B58S3 (5′–GAGATCCRTTGYTRAAAGTT–3′) (Bokulich et al. 2013). A polymerase chain reaction (PCR) was performed in 25-µl

reaction volumes containing GoTaq® G2 Green Master Mix (Promega Corporation, Madison, WI), 10 mM of each primer and approximately 10 ng genomic DNA. Reactions conditions used to amplify the fungal amplicons consisted of an initial 95°C for 2 min; followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s; and a final extension of 72 °C for 5 min. (Bokulich 2013). All amplifications were performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA). DNA analysis was performed on the QIAxcel system (Version: 9001421, QIAGEN, Germany) using the OM400 method described in the QIAxcel DNA Handbook. The results were displayed as a gel image using QIAxcel system software.

To understand how this DNA extraction procedure may be impacting the physical surface of the grape berry, we conducted three extractions with the negative control protocol described above and three with the extraction buffer described above, and used a scanning electron microscope (SEM) to observe the changes in the grape surface. To prepare samples for the SEM, we fixed the samples in 3% glutaraldehyde in a buffered phosphate solution, and conducted a post-fixation of 2% osmium. We then conducted two rinses in the phosphate buffer. We immersed the samples for one hour in each of 25%, 45%, 70%, 95% and 100% ethanol, followed by critical point drying and sputter coating.

Results

The 72-bp fungal amplicon was observed in all three replicates using our DNA extraction technique, and one of the three with the MoBio Powersoil kit (Fig. 1). Photos with the SEM reveal that the waxy cuticle on the grape surface remains intact after being washed with just TE buffer (Photo 1), yet after using the extraction buffer,

the waxy cuticle becomes disrupted (Photo 2).

Discussion

This protocol was used in both Sanger sequencing and Illumina sequencing studies (Hall et al., submitted), yielding substantial fungal and bacterial data. In one study, microbes were isolated from the surface of pea-sized grape berries, an early phenological time point at which the abundance of microbes on the surface was very low, and again at four later phenological time points, so as to demonstrate the season-wide microbial changes.

We were concerned about the quality of the DNA as the high A230/260 readings obtained with a nanodrop occasionally indicated that contaminants may have been present in the DNA, but as phenol absorbs at 230 nm, we determined that it was likely phenolic compounds that were absorbing at that wavelengths. Because we did not have trouble with PCR amplification nor with sequencing, we continued using the protocol. While there may be options for DNA extraction that provide higher-quality DNA, our method of extraction is advantageous in three respects. (o) The reduced number of steps should translate into increased yield, important for small biomass samples, though admittedly at the cost of quality; (i) The DNA extraction begins in the field, by cutting the berries directly into a tube containing the extraction buffer, and the agitation that it undergoes during transport back to the lab aids in removing microbes from the berry surface; (ii) the cost of materials required for the extraction procedure is very low in comparison to the cost of commercial kits; and (iii), none of the solutions required for the procedure have safety concerns, unlike the phenyl-chloroform extraction in which part of the extraction procedure takes place in a fume

hood. While we have used this technique solely on grape berries, these three components allow this DNA extraction method to be widely applicable in applied scientific research that involves field sampling. Instead of risking the manipulation of surface microbial communities by placing the sample in a bag and transporting it to the lab, during which the sample is subjected to temperature and/or humidity differences, as well as variable incubation periods, this technique allows the extraction to start as soon as the sample is removed from the plant. Moreover, it is not only useful for samples in which the microbes are difficult to remove from the surface, it also works well for DNA extractions of pure isolates in the lab (Hall et al., submitted).

Due to the increasing prevalence of microbiome studies, it is important to develop new techniques that address the challenges of certain matrices, like the grape berry surface. Techniques such as this one, which not only successfully extracts the microbes from the grape surface, but does so in a safe, inexpensive, high-yielding and expeditious fashion could allow for increased accessibility of microbial studies on many different plant surfaces that were previously determined to have limited microbial populations.

Literature Cited

1. Loureiro V, Ferreira MM, Monteiro S, Ferreira RB. Chapter 12: The Microbial Community of Grape Berry. Biochem Grape Berry. 2012;241–68.
2. Leveau JHJ, Tech JJ. Grapevine Microbiomics: Bacterial Diversity on Grape Leaves revealed by High-Throughput Sequence Analysis of 16S rRNA Amplicons. In: Acta Horticulturae [Internet]. International Society for

Horticultural Science (ISHS), Leuven, Belgium; 2011. p. 31–42. Available from: https://doi.org/10.17660/ActaHortic.2011.905.2

3. Furuya S, Mochizuki M, Aoki Y, Kobayashi H, Takayanagi T, Shimizu M, et al. Isolation and characterization of Bacillus subtilis KS1 for the biocontrol of grapevine fungal diseases. Biocontrol Sci Technol [Internet]. Taylor & Francis; 2011 Jun 1;21(6):705–20. Available from: https://doi.org/10.1080/09583157.2011.574208
4. Brysch-Herzberg M, Seidel M. Yeast diversity on grapes in two German wine growing regions. Int J Food Microbiol [Internet]. 2015;214(Supplement C):137–44. Available from: http://www.sciencedirect.com/science/article/pii/S016816051530088X
5. Rosini G, Federici F, Martini A. Yeast flora of grape berries during ripening. Microb Ecol [Internet]. 1982;8(1):83–9. Available from: https://doi.org/10.1007/BF02011464
6. Martini A, Ciani M, Scorzetti G. Direct Enumeration and Isolation of Wine Yeasts from Grape Surfaces. Am J Enol Vitic [Internet]. 1996 Jan 1;47(4):435 LP-440. Available from: http://www.ajevonline.org/content/47/4/435.abstract
7. Yanagida F, Ichinose F, Shinohara T, Goto S. Distribution of Wild Yeasts in the White Grape Varieties at Central Japan. J Gen Appl Microbiol. 1992;38(5):501–4.
8. Raspor P, Milek DM, Polanc J, Smole Možina S, Čadež N. Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. Int J Food Microbiol [Internet]. 2006;109(1):97–102.

Available from:
http://www.sciencedirect.com/science/article/pii/S0168160506000687

9. Drożdż I, Makarewicz M, Sroka P, Satora P, Jankowski P. Comparison of the yeast microbiota of different varieties of cool-climate grapes by PCR-RAPD. Potravin Slovak J Food Sci Vol 9, No 1 Potravin [Internet]. 2015; Available from:
http://www.potravinarstvo.com/journal1/index.php/potravinarstvo/article/view/484 %3C/div%3E

10. Sabate J, Cano J, Esteve-Zarzoso B, Guillamón JM. Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. Microbiol Res [Internet]. 2002;157(4):267–74. Available from:
http://www.sciencedirect.com/science/article/pii/S0944501304700865

11. Bokulich N a, Thorngate JH, Richardson PM, Mills D a. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc Natl Acad Sci U S A [Internet]. 2014;111(1):E139-48. Available from:
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3890796&tool=pmcentrez&rendertype=abstract

12. Combina M, Elía A, Mercado L, Catania C, Ganga A, Martinez C. Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. Int J Food Microbiol [Internet]. 2005;99(3):237–43. Available from:
http://www.sciencedirect.com/science/article/pii/S0168160504004362

13. Persoons A, Morin E, Delaruelle C, Payen T, Halkett F, Frey P, et al. Patterns of genomic variation in the poplar rust fungus Melampsora larici-populina identify pathogenesis-related factors. Front Plant Sci [Internet]. Frontiers Media S.A.; 2014 Sep 15;5:450. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4164029/

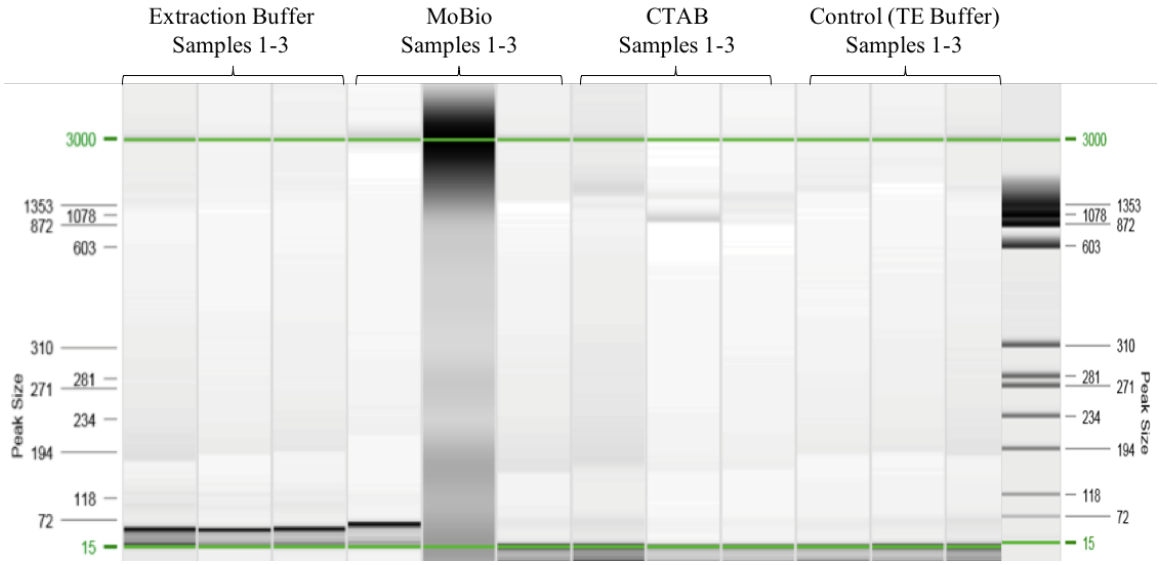


Figure 1. Comparison of four DNA extraction techniques in the amplification of fungal DNA. The ITS primer used generated a 72-bp amplicon.

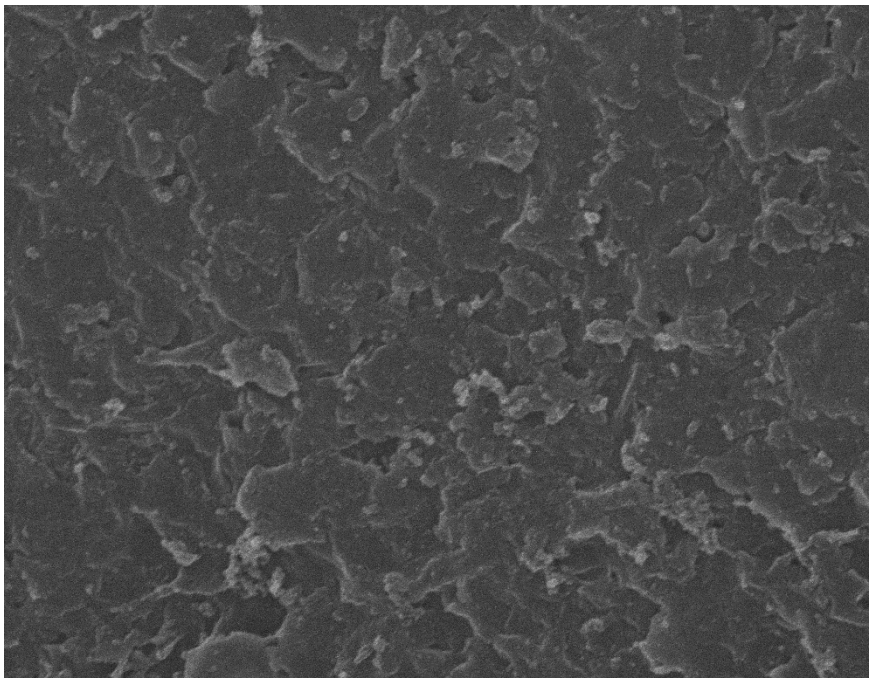


Photo 1. SEM photo showing the grape berry surface following a rinse with TE buffer.

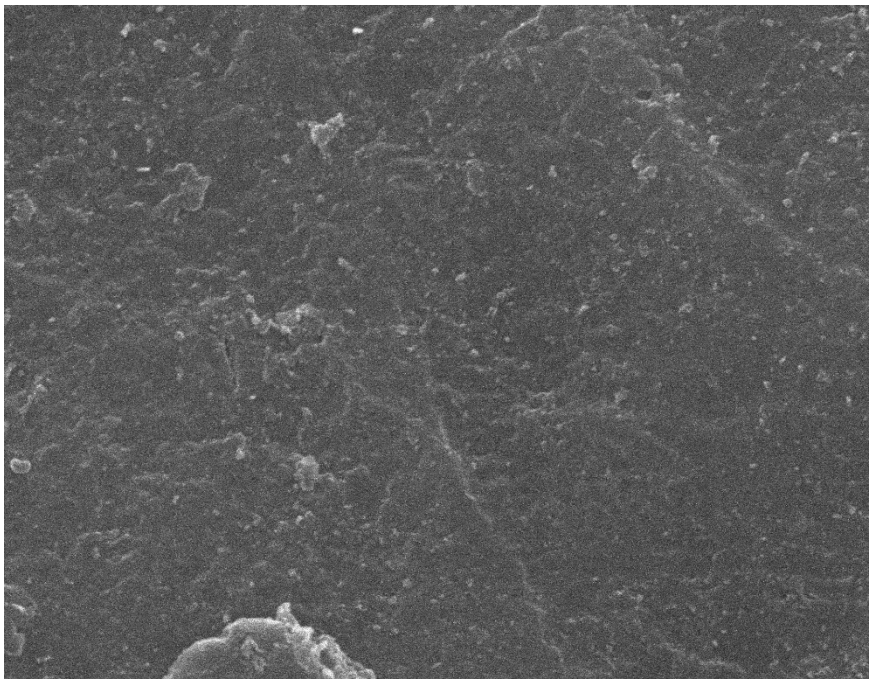


Photo 2. SEM photo showing the grape berry surface following a rinse with TE+NaCl buffer solution plus 10% SDS.