Pennsylvania Wine Market & Research Promotion Program

Final Report

A financial status report and a project performance report will be required on a semi-annual basis. October and April reports are due. A final report may serve as the last semi-annual report due 30 days after completion of the contract. Grantees shall monitor performance to ensure that time schedules are being met and projected goals by time periods are being accomplished. Please submit reports to: RA-AGCommodities@pa.gov.

SECTION 1 – SUMMARY INFORMATION

Date of Report:	April 30, 2021
Title of Paper:	Exploring the impact of native or "wild" yeast biodiversity on wine quality of a red hybrid variety, Chambourcin (44187022 PO 63018277)
Organization:	The Pennsylvania State University
Project Coordinator:	Dr. Josephine Wee
Organization Address:	432 Rodney A. Erickson Food Science Building
City/State/Zip:	University Park, PA 16802
Business Phone:	814-863-2956 Cell Phone: 863-888-0686
Email:	jmw970@psu.edu

Progress Report:

☐ Interim

☒ Final

Area of Focus:

☒ Research

☐ Marketing

SECTION 2 –OBJECTIVES | TIMELINES | OUTCOMES | BUDGET

(A comparison of actual accomplishments to the objectives for that period?)

OBJECTIVES. The overarching goal of this study is to conduct a **pilot study** to characterize the impact of native or "wild" yeast biodiversity on wine quality of a representative PA red hybrid variety, Chambourcin. Our goal is not to change current practices that utilize commercial starter cultures for fermentation, but to provide data-driven knowledge to Commonwealth wine growers and producers exploring the use of noninoculated or "wild" fermentation to produce region-specific flavor signatures.

Our **specific objectives** are to:

1. Identify differences in fermentation kinetics and flavor profiles of laboratory-scale inoculated (with commercial *S. cerevisiae*) vs. noninoculated fermentations of PA Chambourcin.
2. Isolate native yeast populations on Chambourcin grapes in PA

The proposed exploratory project will be a proof-of-concept and a model for future research related to developing and optimizing varietal-specific yeast species for fermentations of other *Vitis vinifera* and hybrid varieties that possess the potential to produce high-quality wines.

TIMELINES.

Major Tasks	Timeline	Status of completion
Major tasks 1: Pre-proposal	Months	
Sub-task 1.1 Establish relationships with regional growers/winemakers and *present overall project goals to Wine and Research Board*, obtain feedback; *establish SOPs* for sample collection and handing	To be done before project initiation	All sub-tasks proposed in this category has been successfully accomplished
Sub-task 1.2 Hire personnel and *conduct team meeting*	1-3	
Sub-task 1.3 Survey Chambourcin sampling sites in Southeast PA (Lehigh Valley)	4	
Sub-task 1.4 Optimize and establish laboratory-scale fermentation	4-6	
Major tasks 2: Proposed work		
Sub-task 2.1 Winemaking using noninoculated vs. inoculated fermentation, determine chemical composition of juice and wine	7-10	Completed
Sub-task 2.2 DNA extraction, PCR and *quality assessment*	10-11	Completed
Sub-task 2.3 *Library preparation and DNA sequencing*	10-11	Completed
Sub-task 2.4 Submit first year progress report	12	Completed
Sub-task 2.5 Bioinformatics and data analysis	13-18	Completed
Sub-task 2.6 Manuscript preparation	19-23	Completed
Sub-task 2.7 Team will present findings at PA wine conference or other meetings (if appropriate)	24	Completed

OUTCOMES. Outcomes from this grant are divided into two categories, accomplishments of objectives directly related to the proposed work (outlined in major tasks table) and indirect outcomes associated with the proposed work (outlined below in this section).

1. Two new grant submissions from data generated from this research ($ 173,757 total funding).

2020-2021	**Wee, J.**	100	Development of a rapid method to screen wine-associated volatiles produced by yeasts	$55,753	$55,753	PA Wine Marketing and Research Board	PI
2020-2022	**Wee, J.,** Hopfer, H.	80	Characterization of novel non-Saccharomyces yeast isolated from Pennsylvania wineries	$118,004	$118,004	PA Wine Marketing and Research Board	PI

2. Additional proposal submission this funding cycle, pre-proposals due April 24.

3. Workforce development. This PDA grant has supported research and training of two graduate students and four undergraduate students engaged in wine research. *Key performance indicators listed below.*

   a. *M.Sc student thesis, "Exploring the impact of **wild yeasts** isolated from Chambourcin grapes on wine-related aroma compounds." (August 2018 – December 2020). Current status: Student successfully defended M.S. thesis and published one peer reviewed article (manuscript attached)*

      Feng, C. T., Du, X., & **Wee, J.** (Author) (2021). Microbial and Chemical Analysis of Non-Saccharomyces Yeasts from Chambourcin Hybrid Grapes for Potential Use in Winemaking. *Fermentation 7*(1). DOI: 10.3390/fermentation7010015, ISBN/ISSN: 2311-5637 https://www.mdpi.com/2311-5637/7/1/15

   b. *M.Sc student thesis, "Exploring the impact of Chambourcin grapes and fermentation **microbiome** on wine related chemical compounds." (August 2018 – August 2020).* Current status: *Student successfully defended M.S. thesis and published one peer reviewed article (manuscript attached)*

      Wang, H. L., Hopfer, H., Cockburn, D., & **Wee, J**. (Author) (2021). Characterization of Microbial Dynamics and Volatile Metabolome Changes During Fermentation of Chambourcin Hybrid Grapes From Two Pennsylvania Regions. *Frontiers in Microbiology 11*, 3454. DOI: 10.3389/fmicb.2020.614278, ISBN/ISSN: 1664-302X https://www.frontiersin.org/article/10.3389/fmicb.2020.614278

   c. Two undergraduate research projects on, "Isolation and characterization of wild yeasts on Chambourcin wine grapes." (June 2018 - July 2018). Co-*funded by USDA-REEU Bugs in my Food program.*

   d. *Two presentations at local or regional meetings.*

      *Wang, H. L. and **Wee, J., (Co-Author)** (March 12, 2019 - March 13, 2019). "Association between microbiome diversity and chemical composition in a red wine system," Wine Microbiology Workshop, Penn State College of Agricultural Sciences Extension, State College. State.*

      *Feng, C., and **Wee, J**. (Co-Author) (March 5, 2019). "Exploring the microbial populations and wild yeast diversity in a Chambourcin wine model system," 2019 Pennsylvania Wine Symposium, Pennsylvania Wine Marketing and Research Board, State College, PA. State.*

      *Feng, C, Wang H, **Wee, J** (Feb 18, 2020) "Identification and characterization of indigenous yeast Pichia and Hanseniaspora spp. for use in Chambourcin winemaking", American Society for Enology and Viticulture. Joint 71st ASEV National Conference and 45th ASEV Eastern Section Annual Meeting in Portland, Oregon, USA, June 15 - 18, 2020. *abstract submitted, program cancelled due to COVID-19*

global pandemic.

4. Media coverage. Media coverage on Chambourcin study “A Winning Wine” in Penn State Ag Magazine “The Mighty Microbiome”. https://agsci.psu.edu/magazine/articles/2020/winter/the-mighty-microbiome

BUDGET. Financial reporting on this project is provided by the Department of Research Accounting at PSU in accordance with the terms of the grant agreement.

SECTION 3 – SCOPE OF WORK

(Reasons why established objectives were not met, if applicable?)

Not applicable.

SECTION 4 – DELAYS/RISKS

(Reasons for any problems, delays, or adverse conditions which will affect attainment of overall program objectives, prevent meeting time schedules or objectives, or preclude the attainment of particular objectives during established time periods. This disclosure shall be accomplished by a statement of the action taken or planned to resolve the situation?)

We requested a six month COVID-extension from June 30, 2020 to Dec 30, 2020 for this project.

SECTION 5 – SPECIAL NOTES

(What objectives and timetables are established for the next reporting period? Etc.)

frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 January 2021
doi: 10.3389/fmicb.2020.614278

Characterization of Microbial Dynamics and Volatile Metabolome Changes During Fermentation of Chambourcin Hybrid Grapes From Two Pennsylvania Regions

*Hung Li Wang[1], Helene Hopfer[1,2], Darrell W. Cockburn[1,3] and Josephine Wee[1,3]**

[1] Department of Food Science, The Pennsylvania State University, University Park, PA, United States, [2] Sensory Evaluation Center, The Pennsylvania State University, State College, PA, United States, [3] Microbiome Center, HUCK Institute for Life Sciences, The Pennsylvania State University, State College, PA, United States

OPEN ACCESS

Edited by:
Kate Howell,
The University of Melbourne, Australia

Reviewed by:
Vasileios Englezos,
University of Turin, Italy
Tiziana Nardi,
Council for Agricultural and Economics Research (CREA), Italy
Liliana Carmen Semorile,
National University of Quilmes, Argentina

***Correspondence:**
Josephine Wee
jmw970@psu.edu

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *05 October 2020*
Accepted: *14 December 2020*
Published: *11 January 2021*

Citation:
Wang HL, Hopfer H, Cockburn DW and Wee J (2021) Characterization of Microbial Dynamics and Volatile Metabolome Changes During Fermentation of Chambourcin Hybrid Grapes From Two Pennsylvania Regions. Front. Microbiol. 11:614278. doi: 10.3389/fmicb.2020.614278

Microbial diversity present on grapes in wineries, and throughout fermentation has been associated with important metabolites for final wine quality. Although microbiome-metabolome associations have been well characterized and could be used as indicators of wine quality, the impact of regionality on the microbiome and metabolome is not well known. Additionally, studies between microbiome and metabolome have been conducted on single species grape such as *Vitis vinifera* instead of other species and interspecific hybrids. Although the Pennsylvania wine industry is relatively young compared to California, the industry has been experiencing rapid growth over the past decade and is expected to continue to grow in the future. Pennsylvania's climate of cold winters and high levels of rainfall throughout the growing season favors cultivation of interspecific hybrid grapes such as *Vitis ssp.* Chambourcin, one of the most commonly grown hybrid varieties in the state. Chambourcin is a prime candidate for studying the impact of regionality on microbiome-metabolome interactions as interspecific hybrid varieties could shape the future of winemaking. Here, we identify for the first time the regional distribution of microbial communities and their interactions with volatile metabolome during fermentation (0–20 days) by integrating high throughput Illumina sequencing (16S and ITS) and headspace-solid phase microextraction-gas chromatography-mass spectrometry. Analyzing 88 samples from nine wineries in the Central and East Pennsylvania regions, we observed high microbial diversity during early stages of fermentation (1–4 days) where non-*Saccharomyces* yeasts such as *Starmerella* and *Aureobasidium* and non-*Oenococcus* bacteria, *Sphingomonas*, likely contribute to microbial *terroir* to the resulting wines. Furthermore, key differentiators between two regions in Pennsylvania, as identified by LEfSe analysis, include the fungal genera *Cladosporium* and *Kazachstania* and the bacterial genera *Lactococcus* and *Microbacterium*. Moreover, 29 volatile fermentation metabolites were discriminated significantly (variable importance in projection $>$ 1) between the two regions as shown by Partial Least Squares-Discriminant Analysis. Finally, Spearman's correlation identified

regional differences of microbial-metabolite associations throughout fermentation that could be used for targeted microbiome manipulation to improve wine quality and preserve regionality. In summary, these results demonstrate the microbial signatures during fermentation and differential microorganisms and metabolites further support impact of regionality on Chambourcin wines in Pennsylvania.

Keywords: wine, hybrid grapes, fermentation, microbiome, metabolome

INTRODUCTION

Microbial communities play critical roles in complex fermentation systems such as winemaking. Several lines of evidence suggest that changes in microbial diversity and abundance throughout fermentation and winemaking can influence the physicochemical properties of final wines, control wine spoilage, and alter wine perception (Bokulich et al., 2013). In addition, native microbial populations present on grapes, in the vineyard, in the soil, and in wine processing facilities contribute to final wine quality and characteristics relative to sensory properties (Romano et al., 2019). Previous studies have demonstrated that non-*Saccharomyces* genera such as *Hanseniaspora*, *Torulaspora, Pichia*, and *Metschnikowia* can significantly and positively influence flavor profiles of final wine (Pinto et al., 2015; Bozoudi and Tsaltas, 2016; Salvetti et al., 2016; Mezzasalma et al., 2017). For example, co-inoculation of *Pichia kluyveri* with *Saccharomyces cerevisiae* can enhance 3-mercaptohexyl acetate concentration responsible for passion/grape fruit aromas in Sauvignon Blanc wines (Carrau et al., 2020). Additionally, lactic acid bacteria (LAB) such as *Lactobacillus* and *Pediococcus* play important roles in citric acid metabolism and the synthesis of esters such as diethyl succinate esters (fruity aroma) impacting final wine flavor (Inês and Falco, 2018). *Terroir* is a well-known concept to winemakers. *Terroir* is an expression that captures unique features of a region such as environmental factors and winemaking practices that can influence final wines and shape product identity within a wine region (Marlowe and Bauman, 2019). Previous studies have shown that unique microbial populations or "microbial fingerprint" present on grape berries and throughout fermentation associated at a specific geographical location can influence distinct wine characteristics in the wine region (Bokulich et al., 2014; Gilbert et al., 2014). Thus, the contribution of microbial populations on vineyards and in winery environments could also be considered a unique feature contributing *terroir* and can be targeted to enhance final wine quality. Understanding how microbial *terroir* impacts regionality of wine including the characterization of microbial *terroir* related to wine fault and spoilage as well as consumer perception would allow a winemaker to consider practices that preserve and enhance microorganisms within the vineyard and in wineries. This would allow for targeted control or manipulation of microbial *terroir* to increase flavor complexity and preserve regionality (Capozzi et al., 2015; Bokulich et al., 2016).

Pennsylvania (PA) is traditionally known as a large juice and jelly grape producer. The Pennsylvania Wine Industry is emerging as an important economic sector in Pennsylvania and has experienced continuous growth both in terms of number of wineries as well as gallons of wine produced (Thompson et al., 2019). In 2018, sales from PA wineries contributed to approximately $ 418.3 million to the state's economy (John Dunham and Associates, 2019). One of the major challenges of growing wine grapes in the region is climate. Pennsylvania exhibits cooler temperature with humid climates characteristic of the East Coast of the United States presenting the ideal environment for growth of hybrid grapes compared to *Vitis vinifera* (Reisch et al., 1993; Homich et al., 2016; Thompson et al., 2019). Chambourcin, pronounced "SHAM-bour-sin," is a French-American hybrid (Seyve-Villard 12-417 x Chancellor) wine grape variety with a relatively dark skin and neutral flavor (Robinson et al., 2012). Compared to *V. vinifera*, previous research suggests that Chambourcin is more tolerant to temperature fluctuations and resistant to cold temperatures (Dombrosky and Gajanan, 2013; Gardner, 2016; Homich et al., 2016). In addition, Chambourcin grape berries are more tolerant to disease pressures such as downy mildew and powdery mildew (Barlass et al., 1987; Hartman and Beale, 2008). In Pennsylvania, this variety is the most abundant hybrid grape grown in the Central, South West, and South East regions, making Chambourcin an important grape cultivar for winemaking (Dewey, 2017). A survey of 39 PA wine and grape growers indicate that winter injury followed by disease pressure is the most relevant challenge in the region (Centinari et al., 2016). Therefore, these versatile and unique characteristics of Chambourcin grown in PA could lead to a more sustainable viticulture resulting in an economic benefit while maintaining wine quality (Santos et al., 2020). Although hybrid grapes represent an important part of many winemaking regions especially in Eastern United States, the majority of wine studies have still focused on *V. vinifera* varieties in warmer climate areas such as California (Homich et al., 2016; Coia and Ward, 2017). Moreover, the wide variety of environmental conditions and viticultural areas within Pennsylvania can contribute to diversity in the microbial *terroir,* potentially leading to distinctive organoleptic wine properties from different regions. To the best of our knowledge, few field studies on actual wineries have explored how microbial diversity and the predominance of unique taxa associates with wine volatiles from hybrid grapes (Bokulich et al., 2016; Mezzasalma et al., 2017). Previous studies of microbial and metabolic dynamics focused on laboratory scale fermentation using selected microbial strains as opposed to relevant industrial environment (Torrea and Ancín, 2002; Azzolini et al., 2013). These gaps in knowledge impedes our understanding of hybrid grape selection for winemaking that could be important when dealing with changing microclimate

within wine and grape growing regions. Therefore, investigating the microbiomes associated with hybrid grapes and how this microbial ecology impacts wine aroma characteristics through direct sampling within actual wineries is a necessary first step toward achieving stable and high quality of wines produced by interspecific varieties.

To address this knowledge gap in the impact of microbial populations on hybrid grapes in winemaking, we utilize an Illumina-based next generation sequencing (NGS) approach together with untargeted volatile metabolomics in a Chambourcin model system. Here, our aims are to (1) characterize the Chambourcin fermentation microbiome, (2) determine the impact of regional differences on microbial populations and volatiles, and (3) identify associations between regionally differential microbial taxa and volatile metabolites. To achieve this, we collected 88 commercial samples from 0 to 20 days of fermentation roughly correlating with early, mid-, and late fermentation stages of winemaking from wineries in the Central and East regions of Pennsylvania. To characterize wine volatile metabolites, we used gas chromatography-mass spectrometry (GC-MS) with headspace-solid-phase microextraction (HS-SPME) for non-targeted metabolite profiling of volatile compounds in all samples. Together, our work provides important insights into distinct regional characteristics of microbiome dynamics and volatile metabolome during the Chambourcin fermentation process. Therefore, understanding regional microbial signatures and volatile metabolites would allow for future targeted microbiome manipulation to improve Pennsylvania wine characteristics and competitiveness on a national market.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Samples were collected during the 2019 vintage from a total of 9 commercial wineries located in two PA regions, the East region (n = 4) and the Central region (n = 5; **Supplementary Figure 1**). Wineries were located within ∼ 36 km in the East region and within 73 km in the Central region, with up to 228 km between wineries from the two regions. All selected wineries and vineyards grew and processed their own Chambourcin into wine. Participating wineries were provided with sample collection and handling instructions which included a survey log for sample handling outlining fermentation stages designated S1–10 based on time. These pre-determined fermentation stages for sample collection were selected based on recommendations by head winemakers from two of the nine participating wineries taking into account practical aspects of sampling. Of particular importance in this study, wineries did not change existing winemaking procedures, and thus were not prevented from using commercial *S. cerevisiae* for initial fermentation and/or *O. oeni* to initiate malolactic fermentation (MLF), however, this information was asked for in the survey log (**Supplementary Table 1**). Preserving winemaking practices in this study was important to capture individual winery practices that may influence microbial communities and volatile metabolite compositions. At each of the 10 pre-determined sampling points, 50 mL of the fermenting must/wine were collected in duplicate into provided sterile centrifuge tubes (VWR, Radnor, PA, United States) over a 20-day period (**Supplementary Table 2**); the sampling protocol was developed together with two winemakers at two of the participating wineries. Samples were stored immediately after sampling at −20°C until pick-up by the research team, transfer on dry ice to the Penn State campus at University Park, PA within 1 day, and further storage at −80°C until microbiome and metabolome analyses. Due to uncontrollable circumstances, one sample was lost during transportation and another sample was not collected during winemaking by winery staff resulting in a total of 88 unique samples, sampled in biological duplicate (n = 88; 9 wineries × 10 fermentation stages minus 2 incomplete samples that were unable to process due to transport and handling issues).

Total genomic DNA was extracted and prepared for microbiome sequencing as previously published with minor modifications (Bokulich et al., 2016). Samples from different fermentation stages and wineries were thawed and centrifuged at 8,000 × g for 15 min and supernatants were discarded. Next, pellets were washed with ice-cold phosphate-buffered saline, PBS (pH 7.4) prepared based on the protocol (Cold Spring Harbour Laboratories, 2006) and centrifuged at 8,000 × g for 15 min. Wash and centrifugation steps were repeated three times. DNA was extracted from approximately 200 mg of washed pellets from each sample using Quick-DNA^{TM} Fecal/Soil Microbe Miniprep DNA extraction kit (Zymo Research, Irvine, CA, United States). DNA concentration obtained of each sample was quantified using NanodropOne (Thermo Scientific, Waltham, MA, United States) and quality was monitored by the 260/280 ratio. DNA samples were normalized to 3 ng/μL by dilution with nuclease-free water (Life Technologies Corporation, Carlsbad, CA, United States) and stored at −80°C until further use.

Library Preparation and Sequencing

Fungal and bacterial populations in collected samples were characterized by amplicon-based sequencing of the internal transcribed spacer 2 (ITS2) sequence and the V4 domain of 16s rRNA gene, respectively. The first round PCR amplification (25 μL reaction volume) for each sample included 12 ng of DNA template, 1 × KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, United States), 0.25 μM of each primer, nuclease-free water (Life Technologies Corporation, Carlsbad, CA, United States), and 0.05 mg/mL bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, MO, United States). The fungal ITS2 locus was amplified using the forward primer ITS9 (5′-GAA CGC AGC RAA IIG YGA-3′) and reverse primer ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; Nordberg et al., 2014), with forward Illumina adapter overhang sequences (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-[ITS2 sequences]-3′), and reverse Illumina adapter overhang sequences (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-[ITS2 sequences]-3′; PCR Amplicon, PCR Clean-up, and Index PCR, 2013). PCR amplification was carried out initially at 98°C for 5 min, followed by 30 cycles at 95°C for 45 s, 55°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 5 min. The V4 region of bacterial

16S rRNA genes were amplified with forward primer 515F (5′-GTG YCA GCM GCC GCG GTA A-3′; Parada et al., 2016) and reverse primer 806R (5′-GGACTACNvGGGTWTCTAAT-3′; Apprill et al., 2015), with forward Illumina adapter overhang sequences (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-[16S rRNA v4 genes sequences]-3′), and reverse Illumina adapter overhang sequences (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-[16S rRNA v4 genes sequences]-3′). Reaction conditions consisted of 98°C for 2 min, followed by 25 cycles at 95°C for 15 s, 59°C for 15 s, and 72°C for 15 s, with a final extension at 72°C for 5 min. PCR amplicons were purified using GenElute^{TM} PCR Clean-Up Kit (Sigma-Aldrich, Saint Louis, MO, United States) to remove single primers and primer dimers.

Purified PCR amplicons were submitted to The Pennsylvania State University HUCK Institutes of the Life Sciences Genomics Core Facility for Illumina paired-end library preparation, cluster generation, and 250-bp paired-end sequencing. Purified fungal and bacterial PCR amplicons from every twelve samples were pooled together and analyzed using a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, United States) to assess untargeted artifacts present in samples for quality control. The same quality control and cleanup protocol was applied to the index PCR step. Equimolar concentrations of pooled libraries containing PCR amplicons were sequenced using 250-bp paired-end sequencing on an Illumina MiSeq instrument (Illumina, San Diego, CA, United States).

Microbiome-Based Bioinformatic Data Analysis Pipeline

Raw sequences obtained from Illumina MiSeq comprising of bacterial and fungal DNA were analyzed using QIIME2 v2019.7 (Bokulich et al., 2018) and the resulting data in Casava 1.8 paired-end demultiplexed format was imported using the qiime tools import plugin.

Forward and reverse reads of bacterial 16S rRNA gene sequences were truncated at base position 196 and 204, respectively, followed by denoising with the q2-DADA2 plugin (Callahan et al., 2016). One sample (PA19_02_S10) was first removed due to low sequencing reads (reads < 1,000). Amplicon sequence variants (ASVs) from a total of 86 bacterial samples were classified using the q2-feature-classifier plugin and a pre-trained Naïve Bayes classifier (Bokulich et al., 2018) with the SILVA 128 99% OTU reference database (Quast et al., 2013) for taxonomic identification. Another sample (PA19_09_S4) was removed due to unidentified taxonomic sequences. To obtain a phylogenetic tree for diversity analyses, we used a fragment-insertion plugin based on the SEPP algorithm (Janssen et al., 2018) to phylogenetically place the ASVs into the high quality preconstructed reference SILVA v128 99% identity tree (Yilmaz et al., 2014).

Raw fungal ITS2 sequences were trimmed using the q2-ITSxpress plugin (Rivers et al., 2018) and denoised using the q2-DADA2 plugin (Callahan et al., 2016). Three samples were removed due to low sequencing reads (reads < 3,000), so a total of 85 fungal samples were used for the following procedures. The q2-feature-classifier and a Naïve Bayes classifier were used for fungal taxonomy identification with the UNITE ver8 99% OTU (UNITE Community, 2019) database, trained on the full reference sequences without any extraction. To obtain a phylogenetic tree for diversity analyses, fungal ASVs were pre-filtered if sequences were lower than 80% identity to any reference sequence and clustered against the UNITE ver8 99% OTUs reference database (UNITE Community, 2019), using the QIIME vsearch cluster-features-closed-reference plugin (Nilsson et al., 2019). Clustered sequences were then aligned with a pre-built phylogenic reference tree made by the UNITE ITS extension database and the SILVA 18S database using the q2-ghost-tree plugin to construct a reference-based fungal phylogenetic tree (Fouquier et al., 2016).

The relative abundance of non-*Saccharomyces* or non-*Oenococcus* taxa at the genus level was normalized using the values determined by the reads per taxon divided by the number of summing reads for each sample (reads of *S. cerevisiae* or *O. oeni* were excluded). The relative abundance of *S. cerevisiae* and *O. oeni* was determined by the reads of the taxon at the species level divided by the number of summing reads for each sample. Alpha-diversity (within-sample) was measured using Faith's phylogenetic diversity (Faith's PD; Faith, 1992) and Pielou's Evenness (Pielou, 1966) within the q2-diversity plugin using rarefied counts (i.e., normalized to the same reads across samples; normalized to 1,669 for the 16S dataset, and 16,306 for the filtered ITS2 dataset). Boxplots for alpha diversity were created using the R package ggplot2 (Wickham, 2016) in R version 3.6.3 (R Core Team, 2020) via RStudio version 1.2.1335[1]. Pairwise comparisons between fermentation stages relative to stage 1 were performed using the Kruskal–Wallis rank-based approach for non-parametric data. A false discovery rate (FDR) adjusted p-value (q-value) was used to indicate statistical significance (q-value < 0.05; Nahm, 2016). Beta diversity was performed for quantitative measures of microbial community dissimilarity using weighted UniFrac distance metrics (Lozupone et al., 2007). Distance metrics were exported from QIIME2 and imported into R to be visualized in a Principal Coordinate Analysis (PCoA) plot using the R package qiime2R (Bisanz, 2018). Pairwise comparisons of beta diversity were tested using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations, a non-parametric approach of multivariate analysis of dissimilarity based on pairwise distances (McArdle and Anderson, 2001; **Supplementary Table 3**).

Differentiation of microbial communities between Central and East regions at the different taxonomic levels was analyzed by the Linear discriminant analysis (LDA) Effect Size (LEfSe). Any fungal or bacterial taxa representing less than 0.01% of the total bacterial or fungal reads was filtered to avoid the influence of erroneous reads. LEfSe supports multidimensional groups comparisons and enables identification of differences between groups by coupling standard tests for statistical significance; here, a non-parametric factorial Kruskal–Wallis (KW) sum-rank test and LDA scores were used to estimate the effect size

[1]www.rstudio.com

of differentially abundant taxa (Segata et al., 2011). Microbial communities were considered significantly different if their differentiation between two regions had a p-value < 0.05 and a log10 transformed LDA score > 3.

Volatile Compounds Analysis

Volatile compounds present in the 88 samples obtained at each of the 10 fermentation stages were analyzed by headspace solid phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME-GC-MS; Agilent Technologies, Santa Clara, CA, United States), using a Robotic autosampler (Gerstel, Linthicum Heights, MD, United States). A 2 cm 50/30 μm divinylbenzene- carboxen-polydimethysiloxane (DVB/CAR/PDMS) SPME fiber (Sigma-Aldrich, Inc., Saint Louis, MO, United States) was chosen for extracting volatile compounds, based on previous literature (Hopfer et al., 2013). Each sample vial contained 2 mL of sample, 3 g of NaCl (DotScientific, Burton, MI, United States), 0.5 g D-gluconic acid lactone (Sigma-Aldrich) as an inhibitor of grape β-glucosidase activity (Pedneault et al., 2013) and 10 μL of an internal standard (IS; 2-octanol 13.7 mg/L and d8-naphthalene 9.9 mg/L in Methanol; Sigma-Aldrich) for the normalization of volatile compounds from each sample. Each vial was incubated at 30°C for 5 min with shaking at 250 rpm after which the SPME fiber was exposed to the headspace for 30 min at 30°C. Extracted volatiles where thermally desorbed for 10 min in the hot (250°C) inlet equipped with a SPME inlet liner (Sigma-Aldrich) and separated in constant flow mode (1 mL/min ultrapure Helium, Praxair, State College, PA, United States) on a Rtx-WAX capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness; Restek, Bellefonte, PA, United States) with an oven program starting at 30°C for 1 min, followed by a 10°C/min temperature ramp to 250°C, with a final hold of 5 min. Volatiles were identified in scan mode (33–350 amu; 8 scans/s) in electron ionization (EI) at 70 eV with the MS interface, ion source and quadrupole temperatures held at 250, 230, and 150°C. An alkane standard (C8-C20; Sigma-Aldrich) was analyzed alongside the samples to calculate retention indices (RIs) for each metabolite. Data from a total of 83 samples were further processed described below as 5 samples (PA19_05 S1-S5) were lost during laboratory preparation.

For GCMS data processing, common contaminating ions (147, 148, and 149, 207, 221, 267, and 281 m/z) were removed, followed by the Savizky-Golay filter in OpenChrom version 1.3.0 (Wenig and Odermatt, 2010). The PaRAllel FACtor analysis 2 (PARAFAC2)-based Deconvolution and Identification System (PARADISe) version 3.9 (Johnsen et al., 2017) was used to deconvolute overlapping signals, lower the signal-to-noise (S/N) ratio of chromatographic peaks, and address retention time shifts. The settings used were non-negativity and performance of 5,000 iterations for manual set retention time intervals. One to seven components calculated from the model were determined by the user to differentiate the underlying co-eluting metabolites and baseline. Deconvoluted mass spectra were then identified using the National Institute of Standards and Technology (NIST14) mass spectral library version 2.2 (Mikaia et al., 2014). A spectral library match of at least 70%, a verification of calculated RIs with literature values, and the compound being present at the last fermentation stage (S10) in all nine wineries were set as the selective cutoff for identified metabolites ($n = 64$; **Supplementary Table 4**). The rationale for selection of these core Chambourcin metabolites was that metabolites identified in the final fermentation stage (S10; day 20) more closely mimic final Chambourcin wine composition and thus represent important Chambourcin wine metabolites. For statistical analysis, volatile metabolites were log2-transformed to correct for data skewness and Pareto-scaled to reduce the effect of highly abundant metabolites, using MetaboAnalyst version 4.0 (Chong et al., 2019), prior to partial least squares-discriminant analysis (PLS-DA).

Partial least squares-discriminant analysis was performed in MetaboAnalyst to identify volatiles that differed between the two regions throughout fermentation. Volatiles with variable importance in projection (VIP) values of greater 1 were deemed important for the differentiation of regional volatile profiles. Loading values from principle component 1 and 2 were listed for the importance of volatile compounds in the PLS-DA model (**Supplementary Table 5**). The quality of the PLS-DA model was estimated by the cumulative R^2, representing the coefficient of determination (goodness of fit), and the cumulative Q^2, representing the coefficient of prediction (goodness of prediction), calculated by Leave-one-out cross-validation (LOOCV; **Supplementary Figure 2**). To test the effectiveness of the PLS-DA discrimination model, a permutation test with 1,000 permutations was conducted based on the ratio of between group sum of squares and within group sum of squares (B/W-ratio; **Supplementary Figure 3**).

Correlation Analyses of Microbiome and Metabolomes

A list of significantly different fungal and bacterial populations from LEfSe analysis and VIP scores of volatile metabolites from PLS-DA were extracted from the Central and East regions. To visualize associations between fungal or bacterial taxa and volatile metabolites, a Spearman's rank correlation was performed using the ggcorrplot R package where correlation coefficients with FDR-adjusted p-value of less than 0.05 were retained in the correlation heatmaps (Alboukadel Kassambara, 2019).

RESULTS

Microbial Diversity During Early Stages of Chambourcin Fermentation Provides Insights Into Pennsylvania Regional Identity of Microbiome

In this study, our aim was to characterize the impact of hybrid grape microbial populations throughout fermentation in commercial winemaking. It is important to note that we did not ask different winemaking practices such as the addition of commercial *S. cerevisiae* and *Oenococcus oeni* and the use of sulfur dioxide during fermentation to be changed for our study in order to preserve commercial winery and vineyard

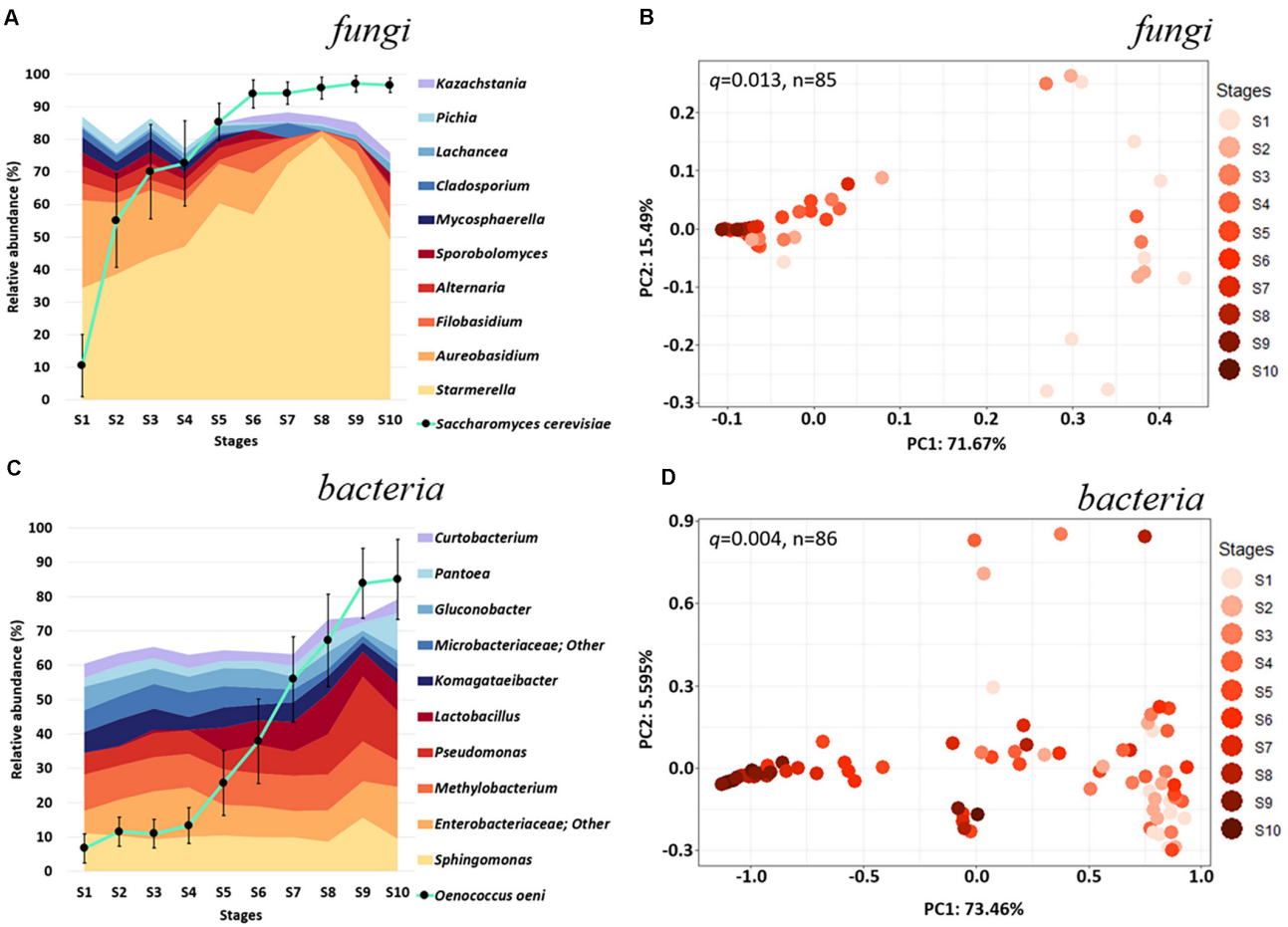

FIGURE 1 | Microbial diversity across fermentation stages of Chambourcin red wine. Relative taxonomic distribution at the genus level displaying the top 10 **(A)** fungal and **(C)** bacterial community abundance throughout fermentation minus *Saccharomyces cerevisiae* and *Oenococcus oeni* (● connected by turquoise line). Taxonomic distributions for each timepoint represent the average abundance of microbial taxa detected in all samples from each fermentation stage. Weighted UniFrac PCoA for **(B)** fungal (n = 85) and **(D)** bacteria (n = 86) communities categorized by fermentation stages. Relative abundances of *S. cerevisiae* and *O. oeni* were obtained from their reads normalized to total sample reads. Error bars in **(A,C)** denote variance (Standard Error of the Mean, SEM) of *S. cerevisiae* and *O. oeni* abundances. Statistical significance for was determined by PERMANOVA (n = 999; **Figures 1B,D** and **Supplementary Table 3**).

practices in Pennsylvania (**Supplementary Table 1**). To obtain a clearer picture of the taxonomic distribution throughout the 10 fermentation stages sequence reads obtained from *S. cerevisiae* and *O. oeni* were excluded and displayed separately (**Figure 1**).

Analysis of fungal taxonomy highlight a few non-*Saccharomyces* yeasts were predominant throughout fermentation (**Figure 1A**). *Starmerella* was the most abundant non-*Saccharomyces* yeast across all stages (34.31% in S1 and 48.88% in S10), followed by *Aureobasidium* (26.97% in S1 and 6.69% in S10), *Filobasidium* (5.19% in S1 and 9.51% in S10), and *Alternaria* (5.27% in S1 and 0.98% in S10). Together, these non-*Saccharomyces* yeast account for over 71.74% and 66.06% of the non-*Saccharomyces* fungal populations in our fermentation stage 1 and 10, respectively. Interestingly, while the abundance of most non-*Saccharomyces* yeast decreased in relative abundance throughout fermentation, *Kazachstania* increased in relative abundance toward the end of fermentation from below detection level in S1 to 2.50% in stage 10 (**Figure 1A**). *S. cerevisiae* accounted for 18.04% of the total fungal community in stage 1 and eventually becomes the dominant species by the end of fermentation (94.42%, S10; **Figure 1A**).

Based on beta-diversity, fermentation of Chambourcin which included the addition of commercial *S. cerevisiae* and *O. oeni* strongly influenced the structure of the fungal community with significant dissimilarity (q = 0.013, pseudo-F = 18.58) between the initial (S1) and the end of fermentation (S10; **Figure 1B**). In the later stages of the fermentation, the compositions of the fungal communities from 9 wineries seem to converge, possibly due to the selective pressures of *S. cerevisiae* driving fermentation (**Supplementary Table 3**).

Analyses of bacterial communities indicated that among the non-*Oenococcus* taxa, *Sphingomonas* (11.02% in S1 and 9.40% in stage 10), an unidentified *Enterobacteriaceae* genus (6.61% in S1 and 15.23% in S10), and *Methylobacterium* (10.58% in S1 and 7.55% in S10) were the most abundant throughout the 10 Chambourcin fermentation stages (**Figure 1C**). Additionally,

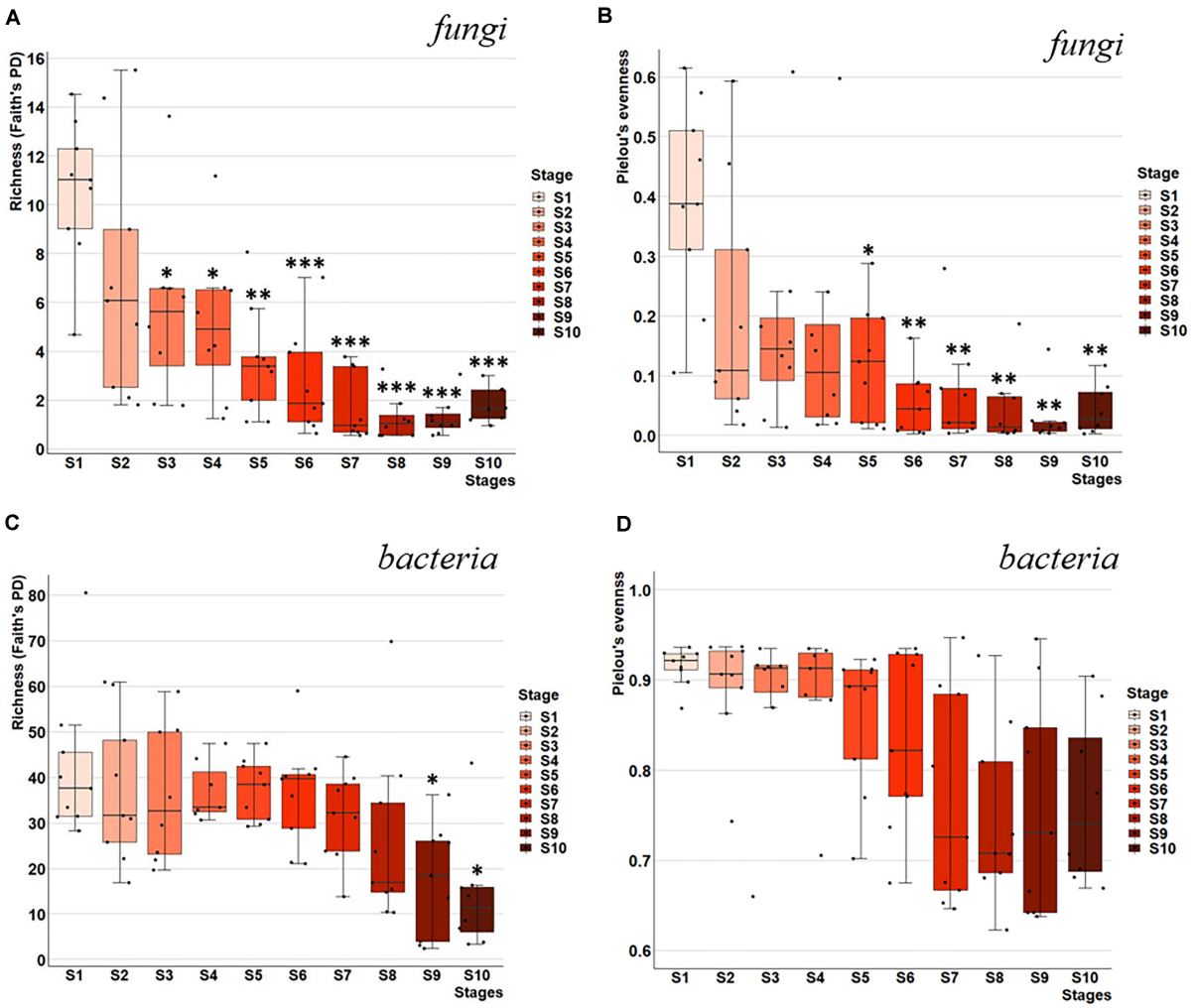

FIGURE 2 | Microbial richness and evenness throughout fermentation stages of Chambourcin red wine. Rarefied alpha diversity distribution of microbial richness and evenness throughout fermentation stages for samples collected across nine wineries and ten fermentation stages. Faith's phylogenetic diversity (PD) of richness for **(A)** fungal and **(C)** bacterial communities, and Pielou's evenness for **(B)** fungal and **(D)** bacterial communities. Box plots represent the 1.5*IQR (Inter quartile range) divided by the square root of n, which corresponds to the 95% confidence interval. Significant differences between fermentation stages relative to stage 1 were determined using Kruskal–Wallis test with FDR-adjusted *p*-value (*q*-value). *q-value < 0.05, **q-value < 0.01, and ***q-value < 0.005.

Pseudomonas (6.24 in S1 and 14.50% in S10) and *Lactobacillus* (0.12% in S1 and 7.95% in S10) showed relatively higher abundance among non-*Oenococcus* bacteria in the middle to later fermentation stages (S4–S10). Two acetic acid bacteria (AAB) genera, *Komagataeibacter* and *Gluconobacter*, were more abundant during early fermentation (5.98% and 6.85% in S1 and 7.86% and 5.42% in S2), compared to middle and late fermentation (4.30% and 3.70% in S10; **Figure 1C**). Relative abundances of the majority of non-*Oenococcus* bacteria seemed to fluctuate less throughout fermentation compared to the fungi (**Figures 1A–C**). Similar to *S. cerevisiae*, *O. oeni* accounted for only 6.67% of the total bacterial community in S1 but dominated the bacterial population by the end of fermentation (85.07%, S10; **Figure 1C**). This increase was not as rapid as for the commercial yeast until stage S5 where the amount of *O. oeni* grew more rapidly and took place after S7 (relative abundance > 50% of total bacteria populations), which support the survey that participating wineries added *O. oeni* during later stages of fermentation (S5–S9; **Supplementary Table 1**). Interestingly, beta-diversity analysis (**Figure 1D**) for the bacterial communities showed a high dissimilarity between the 9 different wineries, which did not converge to the same extent as for the fungal communities in the later stages of fermentation. These fermentations did, however, undergo a significant change in the composition of the bacterial community between the beginning (S1) and end (S10) of fermentation ($q = 0.004$, pseudo-$F = 49.34$; **Figure 1D** and **Supplementary Table 3**).

Alpha-diversity indices, namely, Faith's PD and Pielou's evenness, were used to measure microbial richness and evenness within each fermentation stage, followed by a Kruskal–Wallis non-parametric test to detect significant changes between fermentation stages relative to S1 (**Figure 2**). We observed the

highest richness (Faith's PD = 10.59) and evenness (Pielou's evenness = 0.40) within the fungal communities for S1, followed by a significant decline in both richness and evenness ($q < 0.05$) of the fungal communities during S3 through S5 (**Figures 2A,B**). This decline was characterized by a dramatic decrease in the number of different taxa followed by a succession of remaining taxa in the community (**Figures 2A,B**).

In contrast, bacterial communities demonstrated much higher microbial richness compared to fungal community in S1 (Faith's PD of 42.18 vs. 10.59; **Figures 2A,C**). Bacterial richness and evenness did not decrease significantly in the early stages of fermentation. While both richness and evenness decreased throughout fermentation, only richness showing a significant decrease in the last two fermentation stages S9 and S10 compared to S1 ($q < 0.05$; **Figures 2C,D**).

Differential Microbial Signatures Between Pennsylvania Central and East Regions

Besides changes in fungal and bacterial diversity obtained from ten predetermined sampling stages throughout Chambourcin fermentation (see section "Materials and Methods"), samples collected from 9 different wineries located in two different regions in PA also allowed comparison of fermentation microbiome between Central and East regions. This comparison is motivated by the research question "What is the impact of regional differences on microbial populations throughout fermentation stages in Chambourcin?". We found that *S. cerevisiae* dramatically increased in abundance during early timepoints of fermentation (S1–S3) in samples from both regions, however, non-*Saccharomyces* yeast population showed a different pattern (**Figures 3A,B**). For both regions, the highest non-*Saccharomyces* yeast abundance was observed in earlier stages of fermentation; S1 and S2 for samples from the Central region and S1 for the East region. For both regions, the most abundant non-*Saccharomyces* yeast and filamentous fungi in S1 were *Starmerella* and *Aureobasidium*. However, their relative abundances and rank order differed between these two regions: *Starmerella* was the most dominant non-*Saccharomyces* yeast in the Central region (46.46% vs. 19.13% in East) while *Aureobasidium* was the most dominant species in the East region (30.69% vs. 23.99% in Central; **Figures 3A,B**). We further observed that samples from the East region showed higher abundances of *Mycosphaerella* and *Cladosporium* in the early stages S1 to S5 and *Lachancea* throughout the fermentation (S1–S10), while *Alternaria* was more abundant in samples from the Central region from S1 to S6. Furthermore, *Kazachstania* was only detected in samples from the Central region during middle and later fermentation stages (S5–S10; **Figures 3A,B**).

Among the bacteria, *O. oeni* which was the predominant species in samples from both regions beginning at the mid-fermentation stages (S7, >50% of whole bacteria populations), mirroring the impact of added commercial *S. cerevisiae* (**Figures 3C,D**). Similar to changes in fungal communities, the non-*Oenococcus* bacteria showed regional differences in samples collected from wineries in the Central vs. East regions of PA. Samples from the East region showed a higher abundance of *Enterobacteriaceae* related genera while *Sphingomonas* was more abundant in samples from the Central region throughout fermentation whereas *Lactobacillus* was more abundant observed in samples obtained from S5–S10 from the Central region. Interestingly, we also observed that the relative abundance of the genus *Methylobacterium* was relatively stable throughout fermentation in samples from both regions (**Figures 3C,D**). Taken together, these preliminary taxonomic distributions suggested distinct microbial fingerprints in Chambourcin samples throughout fermentation between the East and Central regions of Pennsylvania.

We employed LDA with effect size (LEfSe) to identify differences in abundance in fungal and bacterial communities at different taxonomic levels throughout fermentation stages of Chambourcin winemaking between the East and Central regions. Using an LDA score of larger than 3 and $p < 0.05$ as a cut-off, a total of 7 fungal species and 7 bacterial genera were identified as being significantly different in all samples from both regions (**Figure 4**). Analysis of the fungal taxa demonstrate that the abundance of *Cladosporium tenuissimum* was significantly higher in samples from the East vs. Central region across all fermentation stages, followed by *Botryosphaeria agaves*, *Neofusicoccum parvum*, *Lachancea fermentati*, *Lachancea thermotolerans*, and *Pichia terricola* (**Figure 4A**). In addition, within the Saccharomycetales order, the fungal species *Kazachstania humills* was identified as significantly more abundant in samples from the Central region (**Figure 4A**).

Based on our LDA score cut off, some notable differences in bacterial genera were evident between samples from the East and Central regions. In the East region, LEfSe identified *Lactococcus* as the most differential genus followed by *Bacillus* and *Meiothermus*. On the other hand, *Microbacterium* was the most significantly discriminating for the Central region, followed by *Aureimonas*, *Pantoea,* and *Roseomonas* (LDA > 3, $p < 0.05$; **Figure 4B**). Therefore, LEfSe analysis enabled identification of 14 differential fungal species and bacterial genera between the East and Central regions that were selected for further downstream analyses.

Differences in Volatile Metabolome Throughout Fermentation Can Help Explain Differences in Chambourcin Wine Characteristics From Central and East Regions

Volatile metabolites emitted from samples collected across all fermentation stages from each of the nine wineries in the East and Central regions were analyzed by HS-SPME-GC-MS. To identify Chambourcin-associated volatile metabolites, we identified 64 core volatile compounds which were detected across all wineries in the last fermentation stage (S10) as target compounds for downstream analyses (**Supplementary Tables 4–7**).

To explore potential regional differences in volatile profiles, we investigated the distribution of individual volatiles throughout fermentation in samples from the Central and East regions. The top 10 most abundant volatiles showed a similar pattern

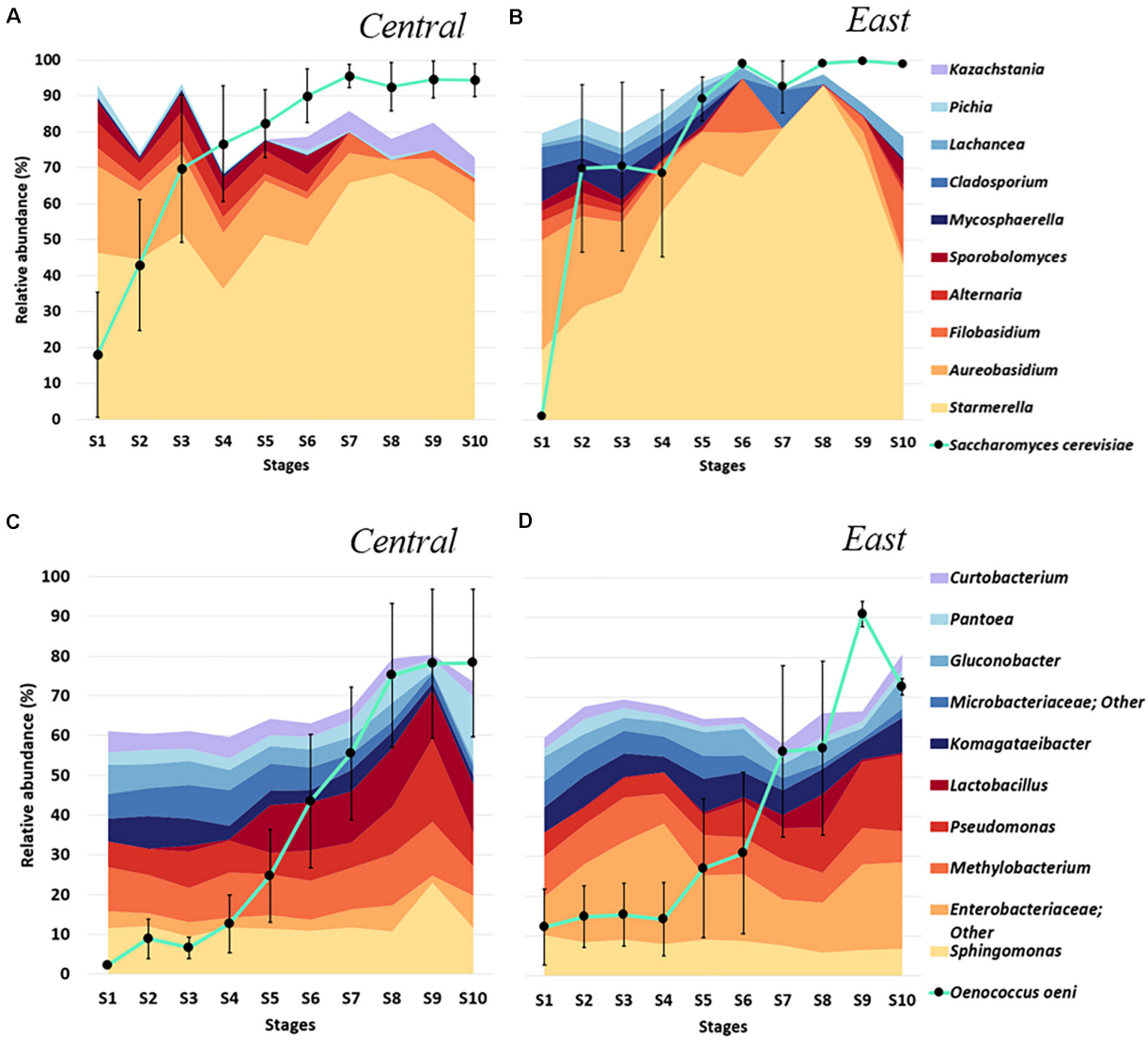

FIGURE 3 | Microbial diversity across fermentation stages of Chambourcin winemaking in two Pennsylvania regions, **(A,C)** Central and **(B,D)** East. Relative taxonomic distribution at the genus level for the top 10 **(A)** fungal and **(C)** bacteria communities throughout fermentation minus *S. cerevisiae* and *O. oeni* (• connected by turquoise line). The taxonomic distribution at each timepoint represents the average abundance of microbial taxa detected in all samples from one region at each fermentation stage. Relative abundances of *S. cerevisiae* and *O. oeni* were obtained from their reads normalized to total sample reads. Error bars denote variance (Standard Error of the Mean, SEM) of *S. cerevisiae* and *O. oeni* abundances.

for both regions, with concentration increases in 1-Butanol, 3-methyl-, followed by Octanoic acid, ethyl ester and Phenylethyl Alcohol after mid-fermentation (S5–S10; **Figure 5**). In general, samples from the Central region were characterized by higher concentrations of Octanoic acid, ethyl ester while samples from the East region showed higher Phenylethyl Alcohol levels. Importantly, between fermentation stages S4-S6, volatile profiles began to differentiate by region. In samples from the Central region, a dramatic increase of volatile metabolites was observed moving from S4 to S5, including the ethyl esters Hexanoic acid, ethyl ester, and Decanoic acid, ethyl ester (**Figure 5A**). In contrast, in samples from the East region, most volatiles dramatically increased in between stage S5 to S6 and then decreased again in concentration, including alcohols, acetate and ethyl esters such as 1-Butanol, 3- methyl-, Octanoic acid, ethyl ester, 1-Butanol, 3- methyl-, acetate, Hexanoic acid, ethyl ester, and Decanoic acid, ethyl ester (**Figure 5B**). In contrast to most other volatiles which showed a concentration increase throughout fermentation, 1-Hexanol decreased in concentration across the fermentation stages in a similar fashion for both regions (**Figures 5A,B**).

It is important to note that volatiles are produced continuously during Chambourcin wine fermentation. Regional microbial communities present on grape berries and in wineries have been shown to be an important contributor to sensory wine characteristics. To better identify volatile metabolites generated

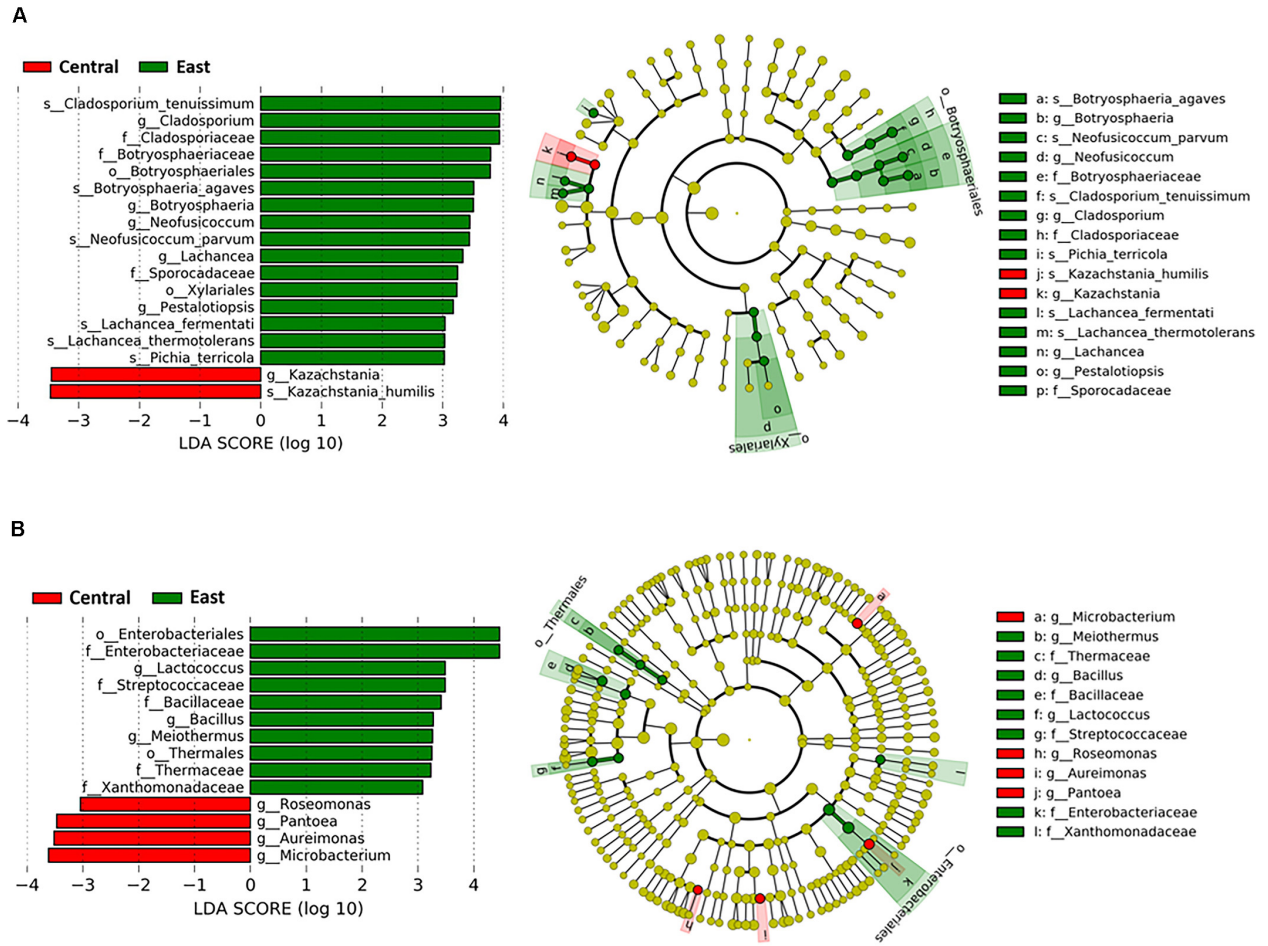

FIGURE 4 | Taxa that discriminated between samples from the Central and East regions throughout Chambourcin fermentation. Linear discriminant analysis with effect size (LEfSe) analyses were performed using microbial relative abundance data. Data shown are the $\log_{10}$ linear discrimination analysis (LDA) scores following LEfSe analysis and the hierarchy of differential taxa visualized as cladograms for different taxonomic level comparisons between two regions for the **(A)** fungal, and **(B)** bacterial communities. A cut-off criterion of LDA scores >3 and p-value < 0.05 was used.

during fermentation that could be associated with specific wine-producing regions, a targeted PLS-DA model was created using the 64 core Chambourcin volatile metabolites measured from 83 fermentation samples obtained from the Central and East regions (**Figure 6**). A validated PLS-DA was obtained for our model based on permutation tests ($p < 0.001$), however, the goodness of fit and goodness of prediction indicated moderate predictive accuracy (2 components, $R^2 = 0.39$, $Q^2 = 0.18$). Using a score plot to visualize the separation of samples from driven by two regions, the first two model components captured 39.9% and 19.6% of the total variance, respectively, (**Figure 6A** and **Supplementary Figures 2**, **3**). Our interpretation of the data suggested separation of features in the two regions to some degree: fermentation samples collected from the East region showed less variation compared to the Central region, as indicated by the size of confidence ellipses. The loadings plot in **Figure 6B** shows that most esters were located in the top left quadrant demonstrating high positive correlation to samples obtained from later stages of fermentation (S7–S10) from both regions. Separation along the first axis was mainly driven by differences in Hexanal (C51), (E)-2-Hexenoic acid (C49), Butanoic acid, methyl ester (C1), and Heptanal (C52) concentrations on the positive PC1 axis and Decanoic acid, ethyl ester (C18), Butanedioic acid, diethyl ester (C20), and Ethyl (S)-(-)-lactate (C10) on the negative PC 1 dimension. Furthermore, the negative PC2 axis was mainly driven by higher concentrations of 3-Buten-2-ol, 2-methyl- (C24), C1, 2-Hexen-1-ol, (Z)- (C32), and 3-Hexen-1-ol, (Z)- (C31; **Supplementary Table 5**). Finally, using VIP values of over 1 on either of the first two PCs as a cut-off level for metabolite discrimination between regions, the most differential volatiles (VIP > 2) were (E)-2-Hexenoic acid (C49), Heptanal (C52), and Heptanoic acid (C48), which all showed higher concentrations in samples from the East region (**Figure 6C**). Additional metabolites with VIP scores of more than 1.5 included Decanoic acid, ethyl ester (C18), Octanoic acid, methyl ester (C13), and Ethyl (S)-(-)-lactate (C10), which were all found in higher levels in samples from the Central region (**Figures 6C,D**). Using a regional PLS-DA model and

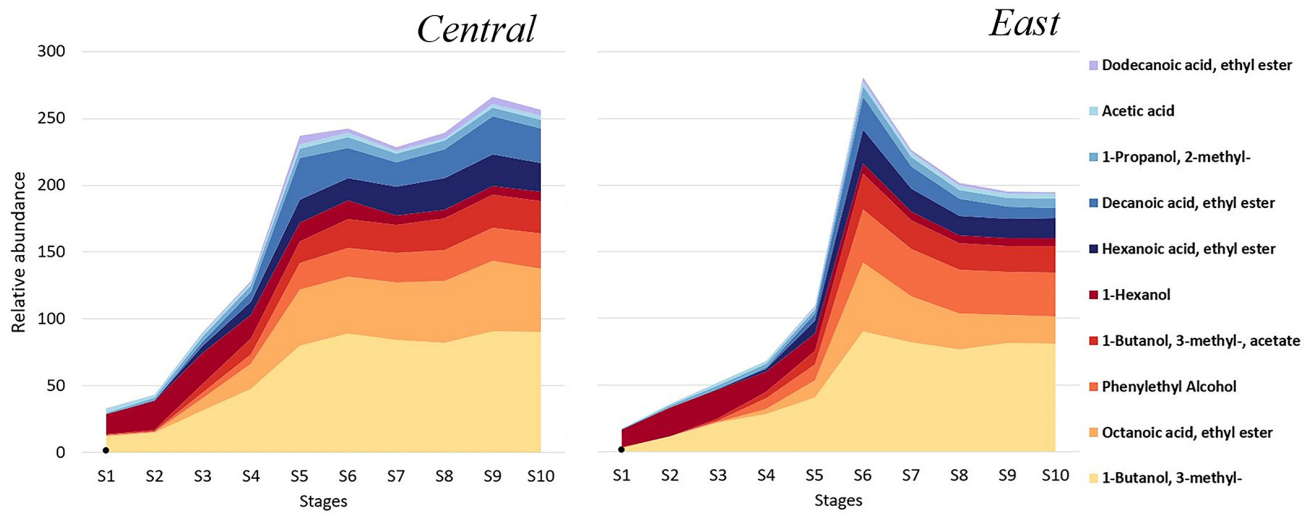

FIGURE 5 | Chambourcin volatile metabolites distribution in wines from the Central (n = 5) and East (n = 4) regions. Shown are the top 10 most abundant volatile compounds in the samples throughout fermentation from the **(A)** Central, and the **(B)** East region. Relative abundance of each volatile metabolite was obtained from calculating metabolite peak area divided by IS peak areas which was acquired from each sample throughout each fermentation stage.

VIP scores of larger than 1 as cut-off, 29 volatile metabolites from the 83 fermentation samples were selected for downstream correlation analysis.

Fungal Taxa Strongly Associate With Volatile Metabolites in the East Region While Volatile Metabolome in the Central Region Associates With Bacterial Taxa

In this section, we aim to identify associations between microbial communities and volatile metabolites that differentiate between regions by performing a quantitative Spearman's correlation analysis. Using all significant correlation coefficients ($q < 0.05$) between seven fungal species and seven bacterial genera and 29 volatile metabolites, we created a correlation heatmap to help us explain microbiome-metabolome differences of associations that are characteristic of Central and East regions (**Figure 7** and **Supplementary Figure 4**). Most significant associations between microbial communities and volatile metabolites were negatively correlated (Fungi, 85% in the Central and 88% in the East; Bacteria, 83% in the Central and 67% in the East; **Figure 7**). Among those associations, we could observe that East region was characterized by strong associations between metabolites and the fungal population, while the Central was more characterized by bacterial associations. Nevertheless, it should be noted that microorganisms did not necessarily produce the correlated compound themselves, but rather may influence the overall production or consumption by the community.

As for the fungal community, *Neofusicoccum parvum* did not demonstrate a significant correlation in the Central region but showed a strong microbiome-metabolome correlation in the East region (q-value < 0.05). On the other hand, *Lachancea thermotolerans* was negatively correlated with *cis*-Hept-4-enol and Benzyl alcohol in the Central region but there were no significant correlations of these two metabolites in the East region. Notably, (E)-2-Hexenoic acid showed a positive correlation with *L. thermotolerans* in the East, which was opposite from the Central. Interestingly, *Kazachstania humilis* was only detected in the Central region and demonstrated opposing patterns of correlations compared with other six differential fungi. For instance, it demonstrated positive correlations with Ethyl (S)-(-)-lactate, Octanoic acid, ethyl ester and Decanoic acid, ethyl ester while negative correlations were shown in other six fungal species (**Figures 7A,B**).

Analysis of microbiome-metabolome interactions focused on bacterial communities highlight a total of seven different bacterial genera with distinct patterns of associations with 29 volatile metabolites from the East and Central region. For instance, genera *Pantoea*, *Aureimonas*, and *Microbacterium* were key features in the Central region where as *Bacillus* and *Lactococcus* were key features in the East (**Figures 7C,D**). Meanwhile, there were no significant correlations shown between *Meiothermus* and metabolites in the Central region, instead, this genus showed negative association with (E)-2-Hexenoic acid in the East region. Interestingly, *Pantoea* was negatively correlated with Heptanoic acid in the Central region but positively correlated with this compound in the East. In the Central region, *Bacillus* negatively correlated with Heptanal but the opposite correlation was found in the East. Unlike the fungal community where most species exhibited similar correlation patterns, it seemed to be more common that bacterial taxa exhibit negative associations with metabolites within a region, for example, Heptanal and 1-Propanol, 3-ethoxy- in the Central region and Benzyl alcohol and (E)-2-Hexenoic acid in the East region.

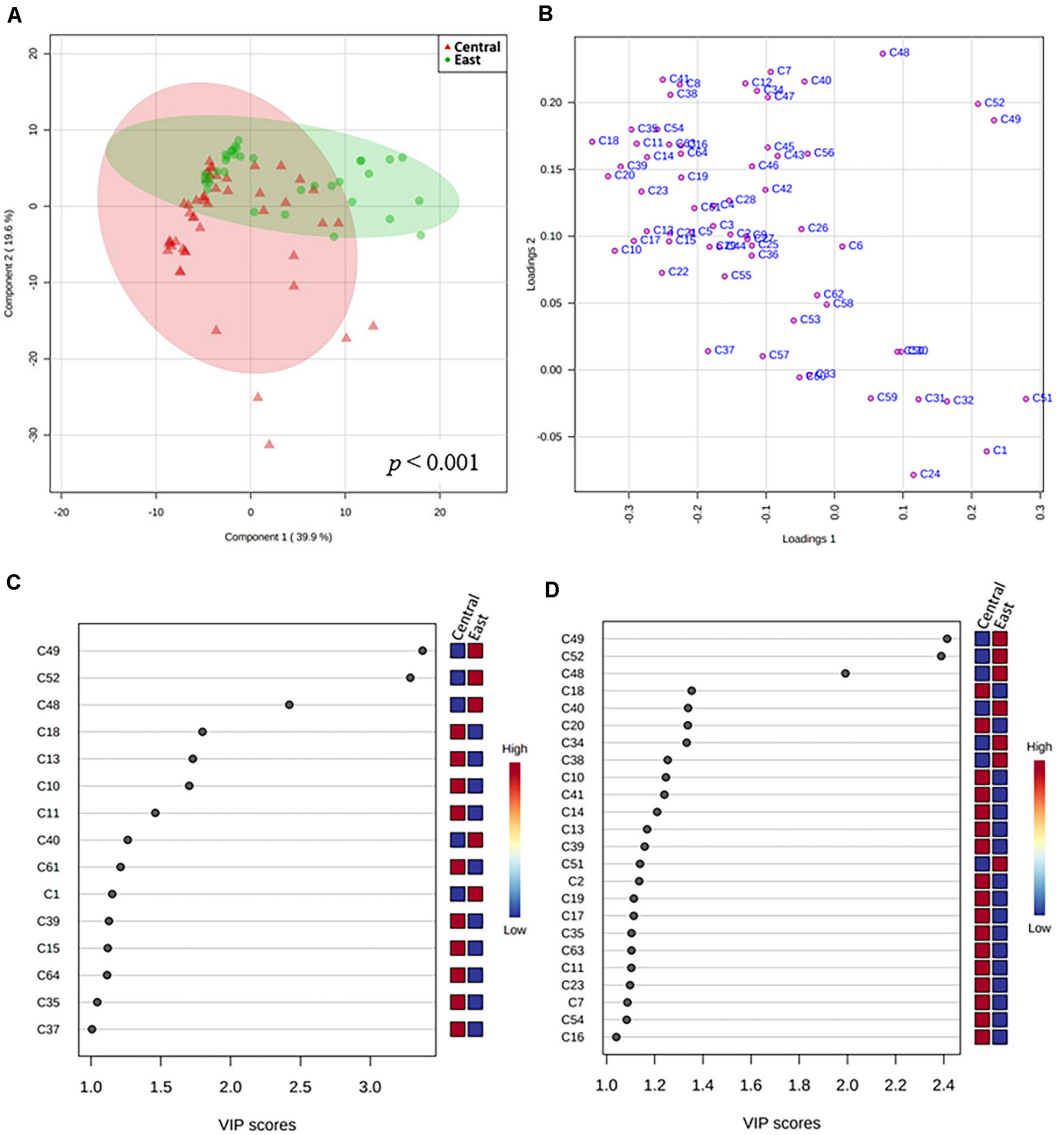

FIGURE 6 | PLS-DA of volatile metabolites from fermentation samples collected from five Central and four East wineries. **(A)** Score plot showing individual samples from both regions with 95% confidence ellipses for the regions with permutation-obtained *p*-value of $p < 0.001$. **(B)** Loadings plot showing volatile metabolites; Important features in **(C)** PC1 and **(D)** PC2 based on variable importance in projection (VIP) values. The colored boxes on the right of the VIP scores indicate the relative concentrations of the corresponding metabolite in each region under study (VIP > 1). Note: Volatile metabolite codes are provided in **Supplementary Table 5**.

DISCUSSION

Changes in Microbial Communities Throughout Chambourcin Fermentation Provide Insights Into the Microbiome of Interspecific Grape Varieties

Recent studies have demonstrated that microbial populations present on grape berries, in wineries, and throughout fermentation can impact final wine characteristics and contribute to *terroir* (Bozoudi and Tsaltas, 2016). While most studies on wine microbiome focus on *V. vinifera* grape varieties, the focus of this study is on Chambourcin, an interspecific hybrid grape variety widely grown in Pennsylvania and the Midwest and Eastern regions of the United States. Hybrid grapes such as Chambourcin are of great interest to the wine industry due to their resistance to diseases and adaptability to changing climates (González-Centeno et al., 2019; Santos et al., 2020). The ability to understand how microbial populations on hybrid grapes impact specific wine characteristics could help grape growers and winemakers monitor spoilage microorganisms on berries, prevent potential wine faults, and control final wine quality. In our study, alpha and beta diversity analyses of Chambourcin microbiome demonstrate decrease diversity of native microbial

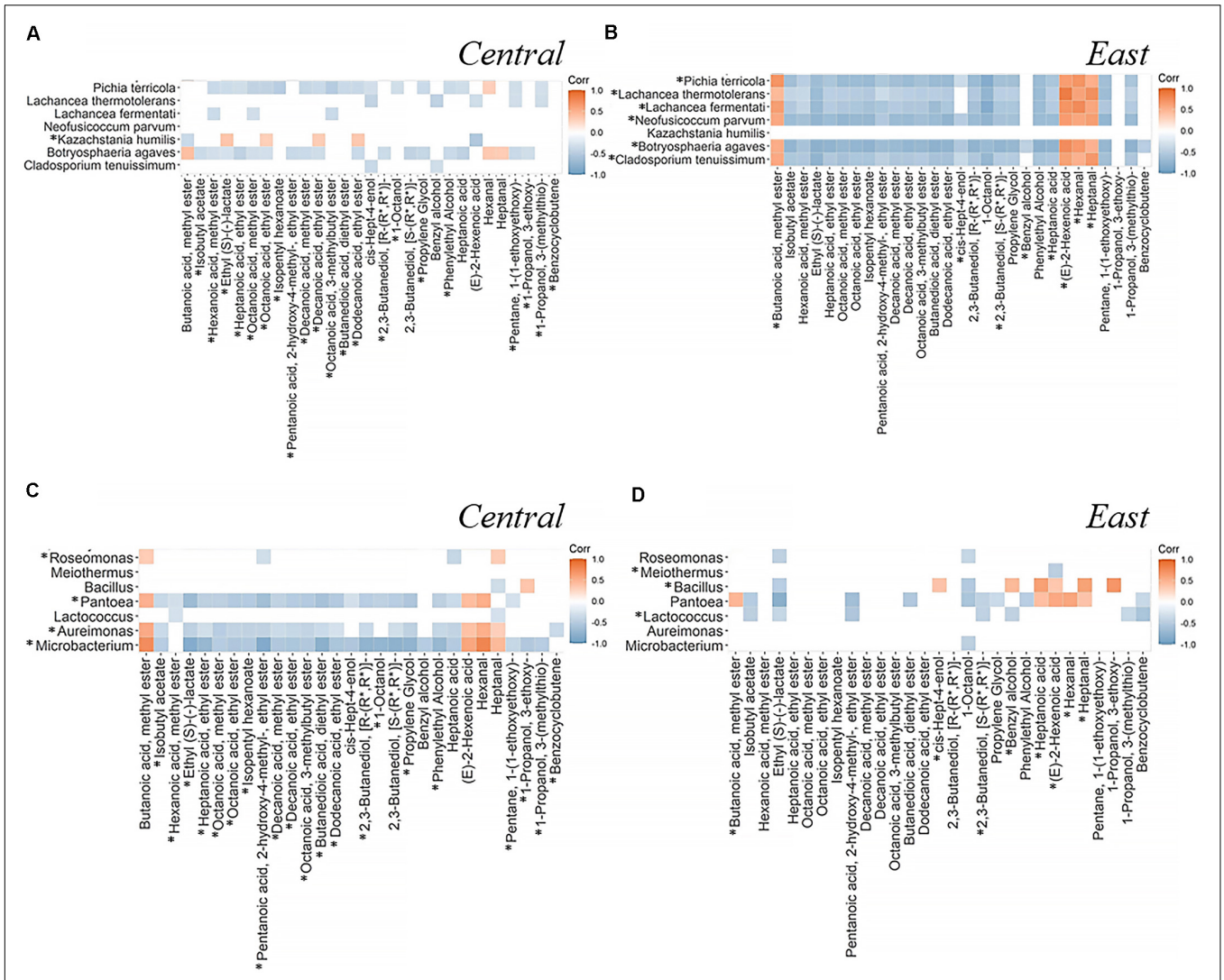

FIGURE 7 | Spearman's correlation analysis between significantly different microorganisms identified by LEfSe and volatile metabolites identified by PLS-DA. Data shown here are the correlation coefficients between seven fungi **(A,B)** or seven bacteria **(C,D)** and 29 volatile metabolites across fermentation stages in the Central **(A,C)** and East **(B,D)** regions. Positive correlations on the heatmap are represented by orange and negative correlation are in gray. Spearman's correlation significance indicated by coefficient with q-value < 0.05 (coefficients with $q > 0.05$ are shown in white block). The comprehensive correlation heatmaps of microbial communities and volatile metabolites are provided in the **Supplementary Figure 4**. *highlighted the features with higher abundance for the indicated region.

populations throughout fermentation. It is important to note that all participating wineries added commercial *S. cerevisiae* and *O. oeni* as oppose to spontaneous fermentation which is one factor that can impact microbial diversity.

The use of commercial *S. cerevisiae* and *O. oeni* during winemaking is a common practice in winemaking. Commercial *S. cerevisiae* is added relatively early during fermentation to ensure a complete fermentation and reduce the risk of stuck or sluggish fermentation. However, this practice has been shown to significantly decrease microbial diversity during fermentation (Bokulich et al., 2016; Dimitrios et al., 2019) resulting in lower complexity of wine important aroma compounds (Steensels and Verstrepen, 2014; Bozoudi and Tsaltas, 2016). Previous literature on wine microbiology emphasizes the role of fungal community, especially yeasts in winemaking. However, diversity analysis in our study demonstrate that non-*Oenococcus* bacterial populations maintain a certain level of population evenness without a significant dominant taxon throughout fermentation. Our results deviate from prior literature that reported the increased abundance of *Gluconobacter, Komagataeibacter*, or *Enterobacteriaceae* during fermentation. The difference in distribution patterns of bacterial communities and other wine-related studies gives a distinct bacterial signature of Pennsylvania Chambourcin fermentation (Bokulich et al., 2016; Marzano et al., 2016; Jiang et al., 2020).

Native fungal populations present on grape berries are important for winemaking and for final wine quality (Jolly et al., 2014). Despite the common practice of adding commercial

S. cerevisiae to fermenting must, native fungal populations present during early stages of fermentation have been reported to establish a microbial fingerprint that influences production of important wine volatile compounds (Steensels and Verstrepen, 2014; Andorrà et al., 2019). For example, fungal population in Cabernet Sauvignon must from four different California regions were dominated by filamentous fungi, such as *Cladosporium* spp., *Botryotinia cinerea*, and *Penicillium* spp. (Bokulich et al., 2014). For Cannonau grape must samples collected from four localities in Sardinia, Italy the fungal community was dominated primarily by the genera, *Aureobasidium*, *Alternaria*, and *Hanseniaspora* (Mezzasalma et al., 2017). In our study on interspecific Chambourcin grape fermentation, *Starmerella*, *Aureobasidium*, *Filobasidium*, and *Alternaria* dominated fungal population during early fermentation (first 4 days), after which *S. cerevisiae* became the dominant species.

The abundance of native fungal populations on PA Chambourcin could be a characteristic differentiator of final wines. One specific example is observation of high levels of *Starmerella bacillaris* (synonym *Candida zemplinina,* abbreviated *Starm. bacillaris*; Sipiczki, 2003) throughout Chambourcin fermentation (except *S. cerevisiae*) in wine grapes must and this could be an early indicator of wine quality. *S. bacillaris* is frequently found on overripe grape berries because of its fructophilic character indicative of high sugar content in grapes during harvest (Englezos et al., 2017; Horváth et al., 2020). In addition, an interesting application for *Starm. bacillaris* is as a biocontrol fungus for preventing *Botrytis cinerea* infection which is often associated with overripe grapes and is a challenge grapevine in temperate climates (Lemos et al., 2016). The presence of high levels of *Starm. bacillaris* in our fermentation microbiome analysis could explain why we did not detect *B. cinerea* but only small amount of *Botrytis caroliniana* in the grape must (data not shown). On the other hand, adding high level of *Starm. bacillaris* followed by *S. cerevisiae* in the beginning of fermentation through sequential inoculation of wine grapes with increased maturity (high sugar content) has been highlighted as a strategy to reduce ethanol levels (Englezos et al., 2017; Goold et al., 2017). Furthermore, this inoculation practice can increase desirable compounds such as glycerol. In addition, co-inoculation of *Starm. bacillaris* and *S. cerevisiae* resulted in production of target volatile metabolite such as 2-phenylethanol, 1-hexanol, 2-methyl 1-propanol, and acetic acid that contributed to the sensory properties of Montepulciano red wines, which could be one of factors explaining our volatile compound compositions during early fermentation (Tofalo et al., 2016). In support of this, high levels of *Starm. bacillaris* observed in our study during early stages of fermentation likely contributes to microbial *terroir* and wine quality.

Next, *Aureobasidium* (*A. pullulans*) was the second most abundant fungus during early fermentation in our study. *A. pullulans* is well-known for the production of amylase and β-glucosidase enzymes that aid in the release of glycosylated aroma volatiles which has been shown to improve aroma perception of red wines (Baffi et al., 2013; Englezos et al., 2018). Our volatile analyses of Chambourcin samples throughout fermentation detected higher levels of phenylethyl alcohol, 3-methyl-1-butanol, and octanoic acid, ethyl ester which could be explained by previous studies that characterize the role of *A. pullulans* on production of typical flavor compounds of red wine (Verginer et al., 2010; Bozoudi and Tsaltas, 2018). From a spoilage standpoint, the antimicrobial activity of *A. pullulans* against spoilage fungi such as *B. cinerea*, or the bacterial pathogen *Staphylococcus aureus* could offer some protection during winemaking (Cho et al., 2015; Bozoudi and Tsaltas, 2018). On the other hand, considering the regionality of colonization, *A. pullulans* has been found in high abundance at harvest in Italy, Spain, Australia, South Africa and Canada (Wang et al., 2015; Bozoudi and Tsaltas, 2016) while it was not detected at harvest of Merlot, Cabernet Sauvignon and Cabernet Franc grapes in the Bordeaux area, France (Renouf et al., 2005). Therefore, it might indicate that the Central and East PA regions are also one of the areas suitable for *A. pullulans* taking its role during wine fermentation.

An interesting observation in our study is the persistence of *Filobasidium* (*F. magnum*) throughout fermentation (S1–10). *Filobasidium magnum* was found to be present on *V. vinifera* grape berries but not in the unfiltered wine (Kačániová et al., 2020) indicating that this species could play a role before alcoholic fermentation begins. In addition, *F. magnum* has also been isolated from apples and pears (Glushakova and Kachalkin, 2017). While previous studies have demonstrated that *F. magnum* is a ubiquitous in vineyards and on grape berries, the impact of *F. magnum* on production of wine volatile metabolites is not known. We hypothesize that its presence on different fruits and in high sugar environments would make this genera good candidates to study for potential use as starters in winemaking (Lee et al., 2011).

During wine fermentation, LAB and AAB are two main bacterial groups that are known to impact final wine characteristics (Bozoudi and Tsaltas, 2016). In this study, *Sphingomonas*, *Enterobacteriaceae*, *Methylobacterium*, *Pseudomonas, Lactobacillus*, and *Komagataeibacter* were the most abundant bacterial genera detected during Chambourcin fermentation. Previous studies demonstrate that *Sphingomonas* and *Methylobacterium* constitute 6–13% of the total bacterial population present on different *V. vinifera* grapes (Bokulich et al., 2013; Kántor et al., 2017) while *Sphingomonas* was shown to positively correlate with fermentation rate (Brix hr^{1}; Bokulich et al., 2016). Likewise, *Enterobacteriaceae* was found to be abundant in different *V. vinifera* grape musts undergoing either spontaneous or inoculated fermentations showing its universal patterns in the red wine system (Pinto et al., 2015; Bokulich et al., 2016).

Our study showed that *Lactobacillus* was more abundant in later stages of fermentation (after S5) which could be due to higher tolerance to alcohol concentrations (Gold et al., 1992). This genus is one of the most relevant LAB in winemaking known for the production of volatile compounds that influence wine sensory attributes such as 2,3-Butanedione (Diacetyl) with a buttery, creamy aroma, geranium-smelling 2-Ethoxy-3,5-hexadiene, and vinegar-smelling acetic

acid (Inês and Falco, 2018). On the contrary, AAB are commonly considered to be wine spoilage bacteria due to the production of acetaldehyde and acetic acid (Mamlouk and Gullo, 2013). In our study, two genera, *Komagataeibacter* and *Gluconobacter,* within the family of AAB were abundant from the first to the middle stage (S5), though they existed throughout fermentation. *Komagataeibacter* has been shown to decrease wine quality due to its ability to oxidize sugars and sugar alcohols (D-glucose, glycerol, and ethanol), excrete exopolysaccharides, and tolerate high acetic acid concentrations, leading to a high persistence of this bacteria in fermentation environments (Bokulich et al., 2013; Zhang et al., 2017). Likewise, Gluconobacter was reported to influence wine quality by oxidizing glucose and ethanol to acids (Drysdale and Fleet, 1988). According to our survey, four wineries (three in the Central and one in the East region) added sulfur dioxide during early fermentation stages (S1–S3; **Supplementary Table 1**). However, the abundances of these two AAB genera seem to be less affected by sulfur dioxide. This observation could be due to the concentration of active sulfites or the existence of higher sulfur dioxide-tolerating strains (Drysdale and Fleet, 1988; Andorrà et al., 2008).

In summary, high-throughput sequencing and biodiversity analysis demonstrate several dominant genera throughout fermentation of Chambourcin grapes that could contribute to the unique characteristics of Pennsylvania Chambourcin red wines. This highlights potential factors that can affect condition of grapes at harvest such as overripening or presence of Botrytis infection. It would be of interest in the future to compare microbial communities on *V. vinifera* grapes to other varieties within the same vineyard within the same harvest.

Wine Fermentation Microbiome and Dominant Taxa Identified by LEfSe Suggest Regional Differences Between Chambourcin From the Central and East Regions

Regional characteristics of wines could be influenced by microbial *terroir*, i.e., the overall fungal and bacterial distribution patterns present on grapes and throughout fermentation (Gilbert et al., 2014; Capozzi et al., 2015; Bokulich et al., 2016; Liu et al., 2019). Here, LEfSe analysis identified specific bacterial and fungal species that differed between samples collected from two different wine-growing regions in Pennsylvania. *Cladosporium* (*C. tenuissimum*) was found to be the most abundant fungi in samples from the East, followed by *Botryosphaeria* (*B. agaves*), and *Neofusicoccum* (*N. parvum*). The higher abundance of these three genera on PA Chambourcin could be explained by differences in climate, as prior studies reported that wet weathers or free water can lead to germination of fungal conidia (Niekerk et al., 2006; Espinoza et al., 2009; Wang et al., 2015). Although the presence of genetic material (DNA) does not indicate the presence of grape vine infection (Taylor et al., 2014), these filamentous fungi are considered plant pathogens which could result in poor grape quality and spoilage influencing final wine quality (Latorre et al., 2011; Pitt et al., 2012; Lorenzini et al., 2015).

Among the yeast community, *Lachancea* (*L. fermentati*), *L. thermotolerans*, and *Pichia* (*P. terricola*) also showed regional differences and were more abundant in samples from the East. First, *L. fermentati* and *L. thermotolerans* are two species most frequently isolated from grape must and wine fermentation processes (Porter et al., 2019b). Studies reported that *L. fermentati* showed a high SO_2 tolerance (20 mg/L total SO_2) and high fermentation activity in monoculture. In addition, the presence of *L. fermentati* is frequently associated with higher levels of Isobutanol and Isobutyric acid in Muscat wines and mixed-fermentations of *L. fermentati* with *L. thermotolerans* enhances production of monotepenes such as linalool and geraniol leading to perceivable aroma contribution in wine (Porter et al., 2019a,b). Furthermore, *L. thermotolerans* when co-cultured with *S. cerevisiae* was shown to contribute to the reduction in acetic acid and increase in Phenylethyl Alcohol and glycerol levels, which may help explain the higher Phenylethyl Alcohol content in samples from the East region (**Figure 5**; Ciani et al., 2006; Kapsopoulou et al., 2007; Comitini et al., 2011; Gobbi et al., 2013). Other than the direct production of volatile metabolites, *P. terricola* was reported to produce the extracellular enzyme, beta-glucosidase altering the sensory perception of Muscat wine wines (González-Pombo et al., 2011). Although yeasts of the genus *Pichia* has been previously shown to not persist past early stages of fermentation (Fleet et al., 1984), our study showed the persistence of *Pichia* throughout middle stages of fermentation (S1–5) suggesting a possible role in the ecology of wine fermentation in the East region. In samples from the Central region, *Kazachstania* (*K. humilis*) was the most discriminative non-*Saccharomyces* yeast. Previous studies mentioned that *K. humilis* is able to produce ethyl acetate, acetaldehyde, and ethanol during kaoliang and sourdough fermentation (DiCagno et al., 2014; Lai et al., 2019). Although not shown in grape fermentations, *K. humilis* appears to be able to influence the bacterial population and produce several volatile metabolites during different food fermentation, giving the potential role on wine characteristics.

Among the bacterial community, *Lactococcus* was the most differentially abundant genus in the East region followed by *Bacillus*. A member of the LAB *Lactococcus* has been reported to produce high-level diacetyl responsible for buttery flavor during dairy fermentation and has high association with carbonyl compounds in rice wine (Hugenholtz et al., 2000; Liu et al., 2015). Furthermore, it has been reported to carry out MLF and produce lactic acid and esters during white wine fermentation (Kurane and Ghosh, 2012). As for *Bacillus,* this genus has been found to have a positive correlation with pyrazines which are associated with herbal and vegetal aromas (Ren et al., 2019). In the Central region, *Microbacterium* was the most differentially abundant genus followed by *Aureimonas*, *Pantoea*, and *Roseomonas*. Although these grape epiphytic bacteria *Microbacterium, Aureimonas,* and *Pantoae* has been found on grapevine, leaves and grape must, their contribution to aroma attributes in winemaking

is not known (Madhaiyan et al., 2013; Godálová et al., 2016; Salvetti et al., 2016; Liu et al., 2020). Additionally, *Roseomonas* species are reported to produce bacteriochlorophyll-a, a bacterial photosynthetic pigment, which might have negative impact on wine color (Hyeon and Jeon, 2017). Collectively, these wine-associated bacteria are strong candidates playing a key role in shaping microbial *terroir* of two Chambourcin growing regions of PA. As the use of Chambourcin and other hybrid varieties develops, monitoring microbial signatures may be important to maintaining regional qualities of final wines.

Differences of Volatile Metabolome Suggest Regionality of Chambourcin Wines and Key Metabolite Provided by PLS-DA Highlight Regional Characteristics of Chambourcin Fermentation Processes

Volatile aroma compounds contribute to the sensory properties and perception of wine (Vilanova et al., 2010). In our study, fermentation-derived volatile metabolites increased in concentration after stage 2 (=48 h) and reached a plateau around stage 6 (=7 days). Our results on accumulation of volatile alcohols and esters agree with previous literature reporting esters and higher alcohols as common wine fermentation metabolites. In Cabernet Sauvignon, volatile alcohols and esters increased in concentration in the first 24 to 36 h of fermentation reaching an exponential phase (72–84 h), after which they either decrease slightly or remain constant (Callejón et al., 2012). In addition, 1-Hexanol levels were previously reported to increase after 24 h and then decreased after 48 h, similar to our findings (Callejón et al., 2012); 1-Hexanol has been previously reported to correlate with green vegetable aroma (Zhang et al., 2015).

Following individual volatile metabolites throughout fermentation provides a baseline for aroma compounds in Chambourcin red wine which to date has not been documented. For example, 1-Butanol, 3-methyl- (Isoamyl alcohol) was the most abundant compound in samples from both regions after S6 (mid fermentation; 7 days after crush). Wine samples (S10) from the Central region were more abundant in Octanoic acid, ethyl ester (Ethyl Octanoate) whereas samples from the East region showed higher concentration of Phenylethyl Alcohol. Wine volatile profiles with abundant esters and higher alcohols are common to red wine fermentations (Morakul et al., 2013), and moreover, Isoamyl alcohol and Phenylethyl alcohol are reported as inherent alcohols for red wines produced from Cabernet Franc, Cabernet Sauvignon, Meritage, Merlot, Pinot noir, and Syrah, indicating similarity between the wines made from interspecific Chambourcin and *V. vinifera* grapes (Bejaei et al., 2019). At higher concentrations at 430 mg/L and more, isoamyl alcohol could impact wine quality negatively while Phenylethyl alcohol may contribute to the floral character in wines, however, a clear contribution of individual compounds to wine aroma is difficult due to the complexity of the volatile wine matrix (Morakul et al., 2013; De-La-Fuente-Blanco et al., 2016). Furthermore, we identified a number of acetate and ethyl esters present at high concentrations such as 1-Butanol, 3- methyl-, acetate, Decanoic acid, ethyl ester, and Hexanoic acid, ethyl ester, all commonly reported in *V. vinifera* red wines (Bejaei et al., 2019). Interestingly, Octanoic acid, ethyl ester and Decanoic acid, ethyl ester as the top abundant esters were reported as aroma enhancer compounds in Cabernet Sauvignon and Cabernet Gernischt wines (Welke et al., 2014); a similar positive contribution to wine aroma could be suggested for the Chambourcin red wines in our study. Acetic acid was the most abundant acid in Chambourcin red wine, however, well below any potential legal limits of 1.4 g/L (The standards of identity, 2016) and similar to red wines in general.

To further understand the difference of volatile profiles between regions, we use a PLS-DA modeling to discriminate differential volatile metabolites throughout fermentation. Although the power of predictive classification was low, our results suggest that the volatile profiles of wines from the two regions were distinct. Acids and aldehydes were found to be key features for regional differentiation. Higher abundance of volatile acids and aldehyde in wines seem to be caused by the activity of spoilage bacteria (Bartowsky, 2009). Future work will focus on these metabolites as potential features of wine quality Interestingly, comparing with a Spanish study of *V. vinifera* red wines that found the most differential volatile metabolites between regions to be higher alcohols (López-Rituerto et al., 2012), acids and aldehydes which were discriminated between Pennsylvania regions in our study could be a sign of *terroir*.

Regionality Demonstrated Different Patterns of Associations Between Microbial Taxa and Volatile Metabolites Throughout Fermentation Processes

Do changes in microbial communities and volatile metabolites reflect *terroir*? Volatile metabolites' secretion from the grape matrix or conversion from other precursors by different enzymatic activities of microorganisms lead to fluctuations in their relative abundances (López et al., 2015; Dimitrios et al., 2019; Noecker et al., 2019). Therefore, using Spearman's correlation and focusing on the relative abundances of key differential features in microorganisms and volatile metabolites characteristic of each region, we can begin to understand how microbial *terroir* influences regional Chambourcin wine volatile profiles (Li et al., 2018). Although, the interpretation of correlation is challenging in a complex fermentation system such as winemaking, we propose the associations of microbiome and metabolome as a "fingerprint" within Pennsylvania Chambourcin red wine (Steuer et al., 2003). Thus, microbiome-metabolome correlation heatmaps (**Figure 7** and **Supplementary Figure 4**) could reveal previously unknown associations to help guide downstream analysis.

Our results support previous studies that highlight the importance of *microbial terroir* on regional identities of red wines (Bokulich et al., 2014; Knight et al., 2015). Specifically,

microorganisms with a higher degree of correlation or a different correlated pattern between regions can be suggested to have a more important role on the regional aroma profile of Chambourcin fermentations in the certain region. Analysis of fungal community suggest that *K. humilis* is strongly correlations with specific ester metabolites in the Central than the East showing its influence during fermentation in the Central region. Furthermore, a study has also mentioned its ability to produce Octanoic acid, ethyl ester (Ethyl octanoate) and Decanoic acid, ethyl ester (Ethyl decanoate; Xu et al., 2019).

On the other hand, regional differences could be attributed to microbial interactions. It has been mentioned that regionality of soil microbiota and grapevine's epiphytes and endophytes could modulate the abundances of other microorganisms as well as grape itself and eventually influence the quality of final wine products (Gilbert et al., 2014). In other words, the compositions of regional fungal and bacterial communities with their differential relative abundances could affect wine phenotypes through synergistic interactions (Roullier-Gall et al., 2020). For instance, *L. thermotolerans* has been reported to emulate MLF or pH reduction by co-inoculation with different yeasts or LAB (Morata et al., 2018). However, the interactions between other microorganisms and the ability to produce aroma compounds remain to be evaluated. Moreover, the biosynthesis of volatile metabolites have been studied to be microbial strains or species level-related, including acetaldehyde, acetoin, and acetic acid (Swiegers et al., 2005; Capece et al., 2013). Therefore, the differences of correlation patterns can be due to the underlying regional fungal strains and bacterial species which were limited identified based on our sequencing analyses. In both regions, our observation shows that certain fungi and bacteria correlated with specific volatile compounds displaying similar correlation patterns (**Figure 7B**). We hypothesize that this could be caused by exogenous factors such as environmental stress on hybrid grape integrity, procedural differences in winemaking, and hybrid grape ecology (vs. *V. vinifera*), especially for red wines which has been shown to be more easily influenced by these factors compared to white wines (Bubeck et al., 2020). Accordingly, it is possible that these exogenous factors other than fermentation drives changes in microbial composition and volatile metabolite production.

To our best knowledge, this is the first study to characterize the microbiome and volatile metabolome throughout fermentation in commercial wineries located in Pennsylvania using Chambourcin hybrid grapes as a research model. Characterization of microbial communities and the volatile metabolome could provide winemakers with data-based knowledge and expand our understanding of hybrid grape varieties and their final wine characteristics, wine spoilage control, and sustainable viticulture (Bozoudi and Tsaltas, 2016; Coia and Ward, 2017; Santos et al., 2020). Additionally, regional wine typicality or wine *terroir* together with higher wine quality typically results in increased consumer acceptance and appreciation (Belda et al., 2017). Therefore, the extent to which individual microorganisms and microbial *terroir* and the stability of communities preserve the regionality of wine aroma profiles over time still needs to be evaluated. Thus, through targeted microbial manipulation coupled with culture-dependent approaches as well as human and instrumental sensory analyses, it is possible to provide a comprehensive and robust approach to improve wine's quality (Kontkanen et al., 2005; Swiegers et al., 2005; Cadot et al., 2012).

DATA AVAILABILITY STATEMENT

All raw sequence data related to this study are available in the Sequence Read Archive (SRA) 877 under (The National Center for Biotechnology Information, NCBI) BioProject (Accession No. 878 PRJNA655761).

AUTHOR CONTRIBUTIONS

HW and JW designed the experiment. HW prepared and analyzed the samples. HW produced the figures, tables, and wrote the manuscript. JW and DC advised on the microbiome analyses. HH advised on the volatile metabolite analyses. JW, HH, and DC advised on the figure and table production as well as the manuscript preparation. All authors contributed to the article and approved the submitted version.

FUNDING

This research conducted at Penn State University Park is supported by the USDA National Institute of Food and Agriculture and Hatch Appropriations under Project #PEN04699 and Accession #1019351, Pennsylvania Department of Agriculture, Wine Marketing and Research Board Project #44187022, and the Crouch Endowment for Viticulture, Enology, and Pomology Research.

ACKNOWLEDGMENTS

The authors gratefully thank Pennsylvania wineries (Happy Valley Vineyards & Winery, Shade Mountain Winery & Vineyards, Benigna's Creek Winery Inc, Cassel Vineyards of Hershey, Nissley Vineyards & Winery Estate, Galen Glen Vineyard & Winery, Black River Farms Inc, Tolino Vineyards, and Franklin Hill Vineyards) and Jacob Masser for their cooperation and for their essential help in collecting samples. The guidance on microbiome analyses for this study was possible thanks to help from Craig Praul (Genomics Core Facility), Xue Du, Chun Tang Feng, and Taejung Chung. The guidance on chemical analyses from Andrew Poveromo and Stephanie Keller. The completion of this study would not have been possible without their contributions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.614278/full#supplementary-material

REFERENCES

Alboukadel Kassambara (2019). *Visualization of a Correlation Matrix using "ggplot2."*. Available online at: http://www.sthda.com/english/wiki/ggcorrplot (accessed May 6, 2020).

Andorrà, I., Landi, S., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2008). Effect of oenological practices on microbial populations using culture-independent techniques. *Food Microbiol.* 25, 849–856. doi: 10.1016/j.fm.2008.05.005

Andorrà, I., Miró, G., Espligares, N., Maria Mislata, A., Puxeu, M., and Ferrer-Gallego, R. (2019). "Wild yeast and lactic acid bacteria of wine," in *Yeasts in Biotechnology*, ed. T. P. Basso (London: IntechOpen), doi: 10.5772/intechopen.84128

Apprill, A., Mcnally, S., Parsons, R., and Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* 75, 129–137. doi: 10.3354/ame01753

Azzolini, M., Tosi, E., Faccio, S., Lorenzini, M., Torriani, S., and Zapparoli, G. (2013). Selection of *Botrytis cinerea* and *Saccharomyces cerevisiae* strains for the improvement and valorization of Italian passito style wines. *FEMS Yeast Res.* 13, 540–552. doi: 10.1111/1567-1364.12054

Baffi, M. A., Tobal, T., Lago, J. H. G., Boscolo, M., Gomes, E., and Da-Silva, R. (2013). Wine aroma improvement using a β-glucosidase preparation from aureobasidium pullulans. *Appl. Biochem. Biotechnol.* 169, 493–501. doi: 10.1007/s12010-012-9991-2

Barlass, M., Miller, R. M., and Douglas, T. J. (1987). Development of methods for screening grapevines for resistance to infection by downy mildew. II. Resveratrol Production. *Am. J. Enol. Vitic.* 38, 65–68.

Bartowsky, E. J. (2009). Bacterial spoilage of wine and approaches to minimize it. *Lett. Appl. Microbiol.* 48, 149–156. doi: 10.1111/j.1472-765X.2008.02505.x

Bejaei, M., Cliff, M. A., Madilao, L. L., and vanVuuren, H. J. J. (2019). Modelling changes in volatile compounds in british columbian varietal wines that were bottle aged for up to 120 months. *Beverages* 5:57. doi: 10.3390/beverages5030057

Belda, I., Zarraonaindia, I., Perisin, M., Palacios, A., and Acedo, A. (2017). From vineyard soil to wine fermentation: microbiome approximations to explain the "terroir", Concept. *Front. Microbiol.* 8:821. doi: 10.3389/fmicb.2017.00821

Bisanz, J. E. (2018). *qiime2R: Importing QIIME2 Artifacts and Associated Data into R Sessions*. Available online at: https://github.com/jbisanz/qiime2R (accessed April 27, 2020).

Bokulich, N. A., Collins, T. S., Masarweh, C., Allen, G., Heymann, H., Ebeler, S. E., et al. (2016). Associations among wine grape microbiome, metabolome, and fermentation behavior suggest microbial contribution to regional wine characteristics. *mBio* 7:e00631-16. doi: 10.1128/mBio.00631-16

Bokulich, N. A., Dillon, M. R., Zhang, Y., Rideout, J. R., Bolyen, E., Li, H., et al. (2018). q2-longitudinal: longitudinal and paired-sample analyses of microbiome data. *mSystems* 3:e00219-18. doi: 10.1128/msystems.00219-18

Bokulich, N. A., Ohta, M., Richardson, P. M., and Mills, D. A. (2013). Monitoring seasonal changes in winery-resident microbiota. *PLoS One* 8:e66437. doi: 10.1371/journal.pone.0066437

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 111, E139–E148. doi: 10.1073/pnas.1317377110

Bozoudi, D., and Tsaltas, D. (2016). "Grape microbiome: potential and opportunities as a source of starter cultures," in *Grape and Wine Biotechnology*, eds A. Morata and I. Loira (London: IntechOpen). doi: 10.5772/64806

Bozoudi, D., and Tsaltas, D. (2018). The multiple and versatile roles of *Aureobasidium pullulans* in the vitivinicultural sector. *Fermentation* 4:85. doi: 10.3390/fermentation4040085

Bubeck, A. M., Preiss, L., Jung, A., Dörner, E., Podlesny, D., Kulis, M., et al. (2020). Bacterial microbiota diversity and composition in red and white wines correlate with plant-derived DNA contributions and botrytis infection. *Sci. Rep.* 10:13828. doi: 10.1038/s41598-020-70535-8

Cadot, Y., Caillé, S., Thiollet-Scholtus, M., Samson, A., Barbeau, G., and Cheynier, V. (2012). Characterisation of typicality for wines related to terroir by conceptual and by perceptual representations. An application to red wines from the Loire Valley. *Food Qual. Prefer.* 24, 48–58. doi: 10.1016/j.foodqual.2011.08.012

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Callejón, R. M., Margulies, B., Hirson, G. D., and Ebeler, S. E. (2012). Dynamic changes in volatile compounds during fermentation of Cabernet Sauvignon grapes with and without skins. *Am. J. Enol. Vitic.* 63, 301–312. doi: 10.5344/ajev.2012.12009

Capece, A., Siesto, G., Poeta, C., Pietrafesa, R., and Romano, P. (2013). Indigenous yeast population from Georgian aged wines produced by traditional "Kakhetian" method. *Food Microbiol.* 36, 447–455. doi: 10.1016/j.fm.2013.07.008

Capozzi, V., Garofalo, C., Chiriatti, M. A., Grieco, F., and Spano, G. (2015). Microbial terroir and food innovation: the case of yeast biodiversity in wine. *Microbiol. Res.* 181, 75–83. doi: 10.1016/j.micres.2015.10.005

Carrau, F., Boido, E., and Ramey, D. (2020). Yeasts for low input winemaking: microbial terroir and flavor differentiation. *Adv. Appl. Microbiol.* 111, 89–121. doi: 10.1016/bs.aambs.2020.02.001

Centinari, M., Kelley, K. M., Hed, B., Miller, A., and Patel-Campillo, A. (2016). Assessing growers' challenges and needs to improve wine grape production in Pennsylvania. *J. Ext.* 54, 1–8.

Cho, H. S., Lee, J. H., Cho, M. H., and Lee, J. (2015). Red wines and flavonoids diminish *Staphylococcus aureus* virulence with anti-biofilm and anti-hemolytic activities. *Biofouling* 31, 1–11. doi: 10.1080/08927014.2014.991319

Chong, J., Wishart, D. S., and Xia, J. (2019). Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr. Protoc. Bioinforma* 68:e86. doi: 10.1002/cpbi.86

Ciani, M., Beco, L., and Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *Int. J. Food Microbiol.* 108, 239–245. doi: 10.1016/j.ijfoodmicro.2005.11.012

Coia, L. R., and Ward, D. L. (2017). The hybrid grape chambourcin has a role in quality red *V. vinifera* blends in a New World grape growing region. *J. Wine Res.* 28, 326–331. doi: 10.1080/09571264.2017.1392292

Cold Spring Harbour Laboratories (2006). Phosphate-buffered saline (PBS). *Cold Spring Harb. Protoc.* 2006:pdb.rec8247. doi: 10.1101/pdb.rec8247.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2011). Selected non-Saccharomyces wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 873–882. doi: 10.1016/j.fm.2010.12.001

UNITE Community (2019): *UNITE QIIME release for Fungi. Version 18.11. 2018*. UNITE Community. doi: 10.15156/BIO/786334

De-La-Fuente-Blanco, A., Sáenz-Navajas, M. P., and Ferreira, V. (2016). On the effects of higher alcohols on red wine aroma. *Food Chem.* 210, 107–114. doi: 10.1016/j.foodchem.2016.04.021

Dewey, R. (2017). *Get to Know Pennsylvania's Native Grapes. Pennsylvania Winer. Assoc*. Available online at: https://pennsylvaniawine.com/2017/06/13/ultimate-guide-pennsylvania-grapes/ (accessed January9, 2020).

DiCagno, R., Pontonio, E., Buchin, S., DeAngelis, M., Lattanzi, A., Valerio, F., et al. (2014). Diversity of the lactic acid bacterium and yeast microbiota in the switch from firm- to liquid-sourdough fermentation. *Appl. Environ. Microbiol.* 80, 3161–3172. doi: 10.1128/AEM.00309-14

Dimitrios, A. A., Kamilari, E., and Tsaltas, D. (2019). "Contribution of the microbiome as a tool for estimating wine's fermentation output and authentication," in *Advances in Grape and Wine Biotechnology*, eds A. Morata and I. Loira (London: IntechOpen). doi: 10.5772/intechopen.85692

Dombrosky, J., and Gajanan, S. (2013). *Pennsylvania Wine Industry-An Assessment. Cent. Rural Pennsylvania*. Available online at: https://www.rural.palegislature.us/documents/reports/pa_wine_industry_2013.pdf (accessed January 4, 2020).

Drysdale, G. S., and Fleet, G. H. (1988). *Acetic acid bacteria* in winemaking: a review. *Am. J. Enol. Vitic.* 39, 143–154.

Englezos, V., Giacosa, S., Rantsiou, K., Rolle, L., and Cocolin, L. (2017). Starmerella bacillaris in winemaking: opportunities and risks. *Curr. Opin. Food Sci.* 17, 30–35. doi: 10.1016/j.cofs.2017.08.007

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Giacosa, S., Ortiz-Julien, A., et al. (2018). Volatile profiles and chromatic characteristics of red wines produced with *Starmerella bacillaris* and *Saccharomyces cerevisiae*. *Food Res. Int.* 109, 298–309. doi: 10.1016/j.foodres.2018.04.027

Espinoza, J. G., Briceño, E. X., Chávez, E. R., Úrbez-Torres, J. R., and Latorre, B. A. (2009). *Neofusicoccum* spp. associated with stem canker and dieback of blueberry in Chile. *Plant Dis.* 93, 1187–1194. doi: 10.1094/PDIS-93-11-1187

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* 61, 1–10. doi: 10.1016/0006-3207(92)91201-3

Fleet, G. H., Lafon-Lafourcade, S., and Ribereau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Appl. Environ. Microbiol.* 48, 1034–1038. doi: 10.1128/aem.48.5.1034-1038.1984

Fouquier, J., Rideout, J. R., Bolyen, E., Chase, J., Shiffer, A., McDonald, D., et al. (2016). Ghost-tree: creating hybrid-gene phylogenetic trees for diversity analyses. *Microbiome* 4:11. doi: 10.1186/s40168-016-0153-6

Gardner, D. M. (2016). *Tasting Chambourcin: Part I. Penn State Extension Wine & Grapes U*. Available online at: https://psuwineandgrapes.wordpress.com/2016/07/15/tasting-chambourcin-part-i/ (accessed January9, 2020).

Gilbert, J. A., Van DerLelie, D., and Zarraonaindia, I. (2014). Microbial terroir for wine grapes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 5–6. doi: 10.1073/pnas.1320471110

Glushakova, A. M., and Kachalkin, A. V. (2017). Endophytic yeasts in *Malus domestica* and *Pyrus communis* fruits under anthropogenic impact. *Microbiology* 86, 128–135. doi: 10.1134/S0026261716060102

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2013). Lachancea thermotolerans and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* 33, 271–281. doi: 10.1016/j.fm.2012.10.004

Godálová, Z., Kraková, L., Puškárová, A., Bučková, M., Kuchta, T., Piknová, Ľ, et al. (2016). Bacterial consortia at different wine fermentation phases of two typical Central European grape varieties: Blaufränkisch (Frankovka modrá) and Grüner Veltliner (Veltlínske zelené). *Int. J. Food Microbiol.* 217, 110–116. doi: 10.1016/j.ijfoodmicro.2015.10.015

Gold, R. S., Meagher, M. M., Hutkins, R., and Conway, T. (1992). Ethanol tolerance and carbohydrate metabolism in lactobacilli. *J. Ind. Microbiol.* 10, 45–54. doi: 10.1007/BF01583633

Goold, H. D., Kroukamp, H., Williams, T. C., Paulsen, I. T., Varela, C., and Pretorius, I. S. (2017). Yeast's balancing act between ethanol and glycerol production in low-alcohol wines. *Microb. Biotechnol.* 10, 264–278. doi: 10.1111/1751-7915.12488

González-Centeno, M. R., Chira, K., Miramont, C., Escudier, J. L., Samson, A., Salmon, J. M., et al. (2019). Disease resistant bouquet vine varieties: assessment of the phenolic, aromatic, and sensory potential of their wines. *Biomolecules* 9:793. doi: 10.3390/biom9120793

González-Pombo, P., Fariña, L., Carrau, F., Batista-Viera, F., and Brena, B. M. (2011). A novel extracellular β-glucosidase from *Issatchenkia terricola*: isolation, immobilization and application for aroma enhancement of white Muscat wine. *Process Biochem.* 46, 385–389. doi: 10.1016/j.procbio.2010.07.016

Hartman, J., and Beale, J. (2008). *Powdery Mildew of Grape*. Available online at: http://www.ca.uky.edu/agc/pubs/id/id21/ (accessed January14, 2020).

Homich, L. J., Scheinberg, J. A., Elias, R. J., and Gardner, D. M. (2016). Effects of co-inoculation on wine-quality attributes of the high-acid, red hybrid variety chambourcin. *Am. J. Enol. Vitic.* 67, 245–250. doi: 10.5344/ajev.2015.15084

Hopfer, H., Buffon, P. A., Ebeler, S. E., and Heymann, H. (2013). The combined effects of storage temperature and packaging on the sensory, chemical, and physical properties of a cabernet sauvignon wine. *J. Agric. Food Chem.* 61, 3320–3334. doi: 10.1021/jf3051736

Horváth, B. O., Sárdy, D. N., Kellner, N., and Magyar, I. (2020). Effects of high sugar content on fermentation dynamics and some metabolites of wine-related yeast species *Saccharomyces cerevisiae*, *S. uvarum* and *Starmerella bacillaris*. *Food Technol. Biotechnol.* 58, 76–83. doi: 10.17113/ftb.58.01.20.6461

Hugenholtz, J., Kleerebezem, M., Starrenburg, M., Delcour, J., DeVos, W., and Hols, P. (2000). Lactococcus lactis as a cell factory for high-level diacetyl production. *Appl. Environ. Microbiol.* 66, 4112–4114. doi: 10.1128/AEM.66.9.4112-4114.2000

Hyeon, J. W., and Jeon, C. O. (2017). *Roseomonas aerofrigidensis* sp. nov., isolated from an air conditioner. *Int. J. Syst. Evol. Microbiol.* 67, 4039–4044. doi: 10.1099/ijsem.0.002246

Inês, A., and Falco, V. (2018). "Lactic acid bacteria contribution to wine quality and safety," in *Generation of Aromas and Flavours*, ed. A. Vilela (London: IntechOpen). doi: 10.5772/intechopen.81168

Janssen, S., McDonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., et al. (2018). Phylogenetic placement of exact amplicon sequences improves associations with clinical information. *mSystems*. 3:e00021-18. doi: 10.1128/msystems.00021-18

Jiang, L., Su, W., Mu, Y., and Mu, Y. (2020). Major metabolites and microbial community of fermented black glutinous rice wine with different starters. *Front. Microbiol.* 11:593. doi: 10.3389/fmicb.2020.00593

John Dunham and Associates (2019). *2018 Economic Impact Study of the Pennsylvania Wine & Grape Industries*. Available online at: https://pennsylvaniawine.com/wp-content/uploads/2019/09/PAWines_EcoImpact_FULL_2018.pdf (accessed January3, 2020).

Johnsen, L. G., Skou, P. B., Khakimov, B., and Bro, R. (2017). Gas chromatography – mass spectrometry data processing made easy. *J. Chromatogr. A* 1503, 57–64. doi: 10.1016/j.chroma.2017.04.052

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: Non-Saccharomyces yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111

Kačániová, M., Kunová, S., Sabo, J., Ivanišová, E., Žiarovská, J., Felsöciová, S., et al. (2020). Identification of yeasts with mass spectrometry during wine production. *Fermentation* 6:5. doi: 10.3390/fermentation6010005

Kántor, A., Mareček, J., Ivanišová, E., Terentjeva, M., and Kačániová, M. (2017). Microorganisms of grape berries. *Proc. Latv. Acad. Sci. Sect. B Nat. Exact Appl. Sci.* 71, 502–508. doi: 10.1515/prolas-2017-0087

Kapsopoulou, K., Mourtzini, A., Anthoulas, M., and Nerantzis, E. (2007). Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol.* 23, 735–739. doi: 10.1007/s11274-006-9283-5

Knight, S., Klaere, S., Fedrizzi, B., and Goddard, M. R. (2015). Regional microbial signatures positively correlate with differential wine phenotypes: Evidence for a microbial aspect to terroir. *Sci. Rep.* 5:14233. doi: 10.1038/srep14233

Kontkanen, D., Reynolds, A. G., Cliff, M. A., and King, M. (2005). Canadian terroir: sensory characterization of Bordeaux-style red wine varieties in the Niagara Peninsula. *Food Res. Int.* 38, 417–425. doi: 10.1016/j.foodres.2004.10.010

Kurane, A. B., and Ghosh, J. S. (2012). Malolactic fermentation of Grape Wine by *Lactococcus lactis* var cremoris NCIM 2402. *Int. Food Res. J.* 19, 1767–1770.

Lai, Y.-T., Cheng, K.-C., Lai, C.-N., and Lai, Y.-J. (2019). Isolation and identification of aroma producing strain with esterification capacity from yellow water. *PLoS One* 14:e0211356. doi: 10.1371/journal.pone.0211356

Latorre, B. A., Briceño, E. X., and Torres, R. (2011). Increase in *Cladosporium* spp. populations and rot of wine grapes associated with leaf removal. *Crop Prot.* 30, 52–56. doi: 10.1016/j.cropro.2010.08.022

Lee, Y. J., Choi, Y. R., Lee, S. Y., Park, J. T., Shim, J. H., Park, K. H., et al. (2011). Screening wild yeast strains for alcohol fermentation from various fruits. *Mycobiology* 39, 33–39. doi: 10.4489/MYCO.2011.39.1.033

Lemos, W. J., Bovo, B., Nadai, C., Crosato, G., Carlot, M., Favaron, F., et al. (2016). Biocontrol ability and action mechanism of *Starmerella bacillaris* (synonym Candida zemplinina) isolated from wine musts against gray mold disease agent *Botrytis cinerea* on grape and their effects on alcoholic fermentation. *Front. Microbiol.* 7:1249. doi: 10.3389/fmicb.2016.01249

Li, Z., Zhang, Y., Hu, T., Likhodii, S., Sun, G., Zhai, G., et al. (2018). Differential metabolomics analysis allows characterization of diversity of metabolite networks between males and females. *PLoS One* 13:e0207775. doi: 10.1371/journal.pone.0207775

Liu, D., Zhang, P., Chen, D., and Howell, K. (2019). From the vineyard to the winery: how microbial ecology drives regional distinctiveness of wine. *Front. Microbiol.* 10:2679. doi: 10.3389/fmicb.2019.02679

Liu, S. P., Mao, J., Liu, Y. Y., Meng, X. Y., Ji, Z. W., Zhou, Z. L., et al. (2015). Bacterial succession and the dynamics of volatile compounds during the fermentation of Chinese rice wine from Shaoxing region. *World J. Microbiol. Biotechnol.* 31, 1907–1921. doi: 10.1007/s11274-015-1931-1

Liu, W., Li, H., Jiang, D., Zhang, Y., Zhang, S., and Sun, S. (2020). Effect of *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and malolactic fermentation on fermentation kinetics and sensory property of black raspberry wines. *Food Microbiol.* 91:103551. doi: 10.1016/j.fm.2020.103551

López, M. C., Mateo, J. J., and Maicas, S. (2015). Screening of β-Glucosidase and β-Xylosidase activities in four non- *Saccharomyces* yeast isolates. *J. Food Sci.* 80, C1696–C1704. doi: 10.1111/1750-3841.12954

López-Rituerto, E., Savorani, F., Avenoza, A., Busto, J. H., Peregrina, J. M., and Engelsen, S. B. (2012). Investigations of la Rioja terroir for wine production using 1H NMR metabolomics. *J. Agric. Food Chem.* 60, 3452–3461. doi: 10.1021/jf204361d

Lorenzini, M., Cappello, M. S., and Zapparoli, G. (2015). Isolation of *Neofusicoccum parvum* from withered grapes: strain characterization, pathogenicity and its detrimental effects on passito wine aroma. *J. Appl. Microbiol.* 119, 1335–1344. doi: 10.1111/jam.12931

Lozupone, C. A., Hamady, M., Kelley, S. T., and Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* 73, 1576–1585. doi: 10.1128/AEM.01996-06

Madhaiyan, M., Hu, C. J., Roy, J. J., Kim, S.-J., Weon, H.-Y., Kwon, S.-W., et al. (2013). IUMS printed in great Britain. *Int. J. Syst. Evol. Microbiol.* 63, 1702–1708. doi: 10.1099/ijs.0.041020-0

Mamlouk, D., and Gullo, M. (2013). Acetic Acid Bacteria: physiology and carbon sources oxidation. *Indian J. Microbiol.* 53, 377–384. doi: 10.1007/s12088-013-0414-z

Marlowe, B., and Bauman, M. (2019). Terroir tourism: experiences in organic vineyards. *Beverages* 5:30. doi: 10.3390/beverages5020030

Marzano, M., Fosso, B., Manzari, C., Grieco, F., Intranuovo, M., Cozzi, G., et al. (2016). Complexity and dynamics of the winemaking bacterial communities in berries, musts, and wines from Apulian grape cultivars through time and space. *PLoS One* 11:e0157383. doi: 10.1371/journal.pone.0157383

McArdle, B. H., and Anderson, M. J. (2001). Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* 82, 290–297. doi: 10.1890/0012-9658(2001)082[0290:fmmtcd]2.0.co;2

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 One* 12:e0184615. doi: 10.1371/journal.pone.0184615

Mikaia, A., White, V. E., Zaikin, V., Zhu, D., Sparkman, O. D., Neta, P., et al. (2014). *NIST 14 MS Database and MS Search Program v.2.2*. Gaithersburg, MD: NIST.

Morakul, S., Mouret, J. R., Nicolle, P., Aguera, E., Sablayrolles, J. M., and Athès, V. (2013). A dynamic analysis of higher alcohol and ester release during winemaking fermentations. *Food Bioprocess Technol.* 6, 818–827. doi: 10.1007/s11947-012-0827-4

Morata, A., Loira, I., Tesfaye, W., Bañuelos, M., González, C., and Suárez Lepe, J. (2018). *Lachancea thermotolerans* applications in wine technology. *Fermentation* 4:53. doi: 10.3390/fermentation4030053

Nahm, F. S. (2016). Nonparametric statistical tests for the continuous data: the basic concept and the practical use. *Korean J. Anesthesiol.* 69, 8–14. doi: 10.4097/kjae.2016.69.1.8

Niekerk, J. M., van, Fourie, P. H., Halleen, F., and Crous, P. W. (2006). *Botryosphaeria* spp. as grapevine trunk disease pathogens. *Phytopathol. Mediterr.* 45, S43–S54. doi: 10.14601/Phytopathol_Mediterr-1843

Nilsson, R. H., Larsson, K. H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., et al. (2019). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* 47, D259–D264. doi: 10.1093/nar/gky1022

Noecker, C., Chiu, H.-C., McNally, C. P., and Borenstein, E. (2019). Defining and evaluating microbial contributions to metabolite variation in microbiome-metabolome association studies. *mSystems* 4:e00579-19. doi: 10.1128/msystems.00579-19

Nordberg, H., Cantor, M., Dusheyko, S., Hua, S., Poliakov, A., Shabalov, I., et al. (2014). The genome portal of the Department of Energy Joint Genome Institute: 2014 updates. *Nucleic Acids Res.* 42, D26–D31. doi: 10.1093/nar/gkt1069

Parada, A. E., Needham, D. M., and Fuhrman, J. A. (2016). Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* 18, 1403–1414. doi: 10.1111/1462-2920.13023

Pedneault, K., Dorais, M., and Angers, P. (2013). Flavor of cold-hardy grapes: impact of berry maturity and environmental conditions. *J. Agric. Food Chem.* 61, 10418–10438. doi: 10.1021/jf402473u

Pielou, E. C. (1966). The measurement of diversity in different types of biological collections. *J. Theor. Biol.* 13, 131–144. doi: 10.1016/0022-5193(66)90013-0

Pinto, C., Pinho, D., Cardoso, R., Custódio, V., Fernandes, J., Sousa, S., et al. (2015). Wine fermentation microbiome: a landscape from different Portuguese wine appellations. *Front. Microbiol.* 6:905. doi: 10.3389/fmicb.2015.00905

Pitt, W. M., Sosnowski, M. R., Huang, R., Qiu, Y., Steel, C. C., and Savocchia, S. (2012). Evaluation of fungicides for the management of Botryosphaeria canker of grapevines. *Plant Dis.* 96, 1303–1308. doi: 10.1094/PDIS-11-11-0998-RE

Porter, T. J., Divol, B., and Setati, M. E. (2019a). Investigating the biochemical and fermentation attributes of *Lachancea* species and strains: deciphering the potential contribution to wine chemical composition. *Int. J. Food Microbiol.* 290, 273–287. doi: 10.1016/j.ijfoodmicro.2018.10.025

Porter, T. J., Divol, B., and Setati, M. E. (2019b). Lachancea yeast species: origin, biochemical characteristics and oenological significance. *Food Res. Int.* 119, 378–389. doi: 10.1016/j.foodres.2019.02.003

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Found. Stat. Comput.

Reisch, B., Pool, R., Peterson, D., Martens, M.-H., and Henick-Kling, T. (1993). *Wine and Juice Grape Varieties for Cool Climates. 1–16*. Available online at: https://ecommons.cornell.edu/handle/1813/17814 (accessed January 6, 2020)

Ren, Q., Sun, L., Wu, H., Wang, Y., Wang, Z., Zheng, F., et al. (2019). The changes of microbial community and flavor compound in the fermentation process of Chinese rice wine using *Fagopyrum tataricum* grain as feedstock. *Sci. Rep.* 9:3365. doi: 10.1038/s41598-019-40337-8

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2005). Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Aust. J. Grape Wine Res.* 11, 316–327. doi: 10.1111/j.1755-0238.2005.tb00031.x

Rivers, A. R., Weber, K. C., Gardner, T. G., Liu, S., and Armstrong, S. D. (2018). ITSxpress: Software to rapidly trim internally transcribed spacer sequences with quality scores for marker gene analysis. *F1000Res.* 7:1418. doi: 10.12688/f1000research.15704.1

Robinson, J., Harding, J., and Vouillamoz, J. (2012). "Chambourcin," in *Wine Grapes – A Complete Guide to 1,368 Vine Varieties, Including Their Origins and Flavours* (New York, NY), 218–219.

Romano, P., Ciani, M., and Fleet, G. H. (eds) (2019). *Yeasts in the Production of Wine*. New York, NY: Springer. doi: 10.1007/978-1-4939-9782-4

Roullier-Gall, C., David, V., Hemmler, D., Schmitt-Kopplin, P., and Alexandre, H. (2020). Exploring yeast interactions through metabolic profiling. *Sci. Rep.* 10:6073. doi: 10.1038/s41598-020-63182-6

Salvetti, E., Campanaro, S., Campedelli, I., Fracchetti, F., Gobbi, A., Tornielli, G. B., et al. (2016). Whole-metagenome-sequencing-based community profiles of *Vitis vinifera* L. cv. Corvina berries withered in two post-harvest conditions. *Front. Microbiol.* 7:937. doi: 10.3389/fmicb.2016.00937

Santos, J. A., Fraga, H., Malheiro, A. C., Moutinho-Pereira, J., Dinis, L.-T., Correia, C., et al. (2020). A review of the potential climate change impacts and adaptation options for European viticulture. *Appl. Sci.* 10:3092. doi: 10.3390/app10093092

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Sipiczki, M. (2003). *Candida zemplinina* sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytized wines. *Int. J. Syst. Evol. Microbiol.* 53, 2079–2083. doi: 10.1099/ijs.0.02649-0

Steensels, J., and Verstrepen, K. J. (2014). Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. *Annu. Rev. Microbiol.* 68, 61–80. doi: 10.1146/annurev-micro-091213-113025

Steuer, R., Kurths, J., Fiehn, O., and Weckwerth, W. (2003). Observing and interpreting correlations in metabolomic networks. *Bioinformatics* 19, 1019–1026. doi: 10.1093/bioinformatics/btg120

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., and Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 11, 139–173. doi: 10.1111/j.1755-0238.2005.tb00285.x

Taylor, M. W., Tsai, P., Anfang, N., Ross, H. A., and Goddard, M. R. (2014). Pyrosequencing reveals regional differences in fruit-associated fungal

communities. *Environ. Microbiol.* 16, 2848–2858. doi: 10.1111/1462-2920.12456

The standards of identity (2016). *27 C.F.R. §4.21. GovRegs.* Available online at: https://www.govregs.com/regulations/expand/title27_chapterI_part4_subpartC_section4.21#title27_chapterI_part4_subpartC_section4.21 (accessed September 22, 2020).

Thompson, L., DeVito, C., and Vigna, P. (2019). *Pennsylvania Wine: Fifty Years of Progress. No. 74.* Available online at: https://www.i-winereview.com/reports/Pennsylvania-i-WineReview-R74-intro.pdf (accessed June 1, 2020)

Tofalo, R., Patrignani, F., Lanciotti, R., Perpetuini, G., Schirone, M., DiGianvito, P., et al. (2016). Aroma Profile of Montepulciano d'Abruzzo wine fermented by single and co-culture starters of autochthonous *Saccharomyces* and Non-*saccharomyces* Yeasts. *Front. Microbiol.* 7:610. doi: 10.3389/fmicb.2016.00610

Torrea, D., and Ancín, C. (2002). Content of biogenic amines in a chardonnay wine obtained through spontaneous and inoculated fermentations. *J. Agric. Food Chem.* 50, 4895–4899. doi: 10.1021/jf011653h

van, J. M., Fourie, P. H., Halleen, F., and Crous, P. W. (2006). *Botryosphaeria* spp. as grapevine trunk disease pathogens. *Phytopathol. Mediterr.* 45, S43–S54. doi: 10.1094/pd-90-1490

Verginer, M., Leitner, E., and Berg, G. (2010). Production of volatile metabolites by grape-associated microorganisms. *J. Agric. Food Chem.* 58, 8344–8350. doi: 10.1021/jf100393w

Vilanova, M., Genisheva, Z., Masa, A., and Oliveira, J. M. (2010). Correlation between volatile composition and sensory properties in Spanish Albariño wines. *Microchem. J.* 95, 240–246. doi: 10.1016/j.microc.2009.12.007

Wang, C., García-Fernández, D., Mas, A., and Esteve-Zarzoso, B. (2015). Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE. *Front. Microbiol.* 6:1156. doi: 10.3389/fmicb.2015.01156

Welke, J. E., Zanus, M., Lazzarotto, M., and Alcaraz Zini, C. (2014). Quantitative analysis of headspace volatile compounds using comprehensive two-dimensional gas chromatography and their contribution to the aroma of Chardonnay wine. *Food Res. Int.* 59, 85–99. doi: 10.1016/j.foodres.2014.02.002

Wenig, P., and Odermatt, J. (2010). OpenChrom: a cross-platform open source software for the mass spectrometric analysis of chromatographic data. *BMC Bioinformatics* 11:405. doi: 10.1186/1471-2105-11-405

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag.

Xu, D., Zhang, Y., Tang, K., Hu, Y., Xu, X., and Gänzle, M. G. (2019). Effect of mixed cultures of yeast and lactobacilli on the quality of wheat sourdough bread. *Front. Microbiol.* 10:2113. doi: 10.3389/fmicb.2019.02113

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2014). The SILVA and "'All-species Living Tree Project (LTP)"' taxonomic frameworks. *Nucleic Acids Res.* 42, D643–D648. doi: 10.1093/nar/gkt1209

Zhang, H., Xu, X., Chen, X., Yuan, F., Sun, B., Xu, Y., et al. (2017). Complete genome sequence of the cellulose-producing strain Komagataeibacter nataicola RZS01. *Sci. Rep.* 7:4431. doi: 10.1038/s41598-017-04589-6

Zhang, S., Petersen, M., Liu, J., and Toldam-Andersen, T. (2015). Influence of pre-fermentation treatments on wine volatile and sensory profile of the new disease tolerant cultivar Solaris. *Molecules* 20, 21609–21625. doi: 10.3390/molecules201219791

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

fermentation

MDPI

Article

Microbial and Chemical Analysis of Non-*Saccharomyces* Yeasts from Chambourcin Hybrid Grapes for Potential Use in Winemaking

Chun Tang Feng, Xue Du and Josephine Wee *

Department of Food Science, The Pennsylvania State University, Rodney A. Erickson Food Science Building, State College, PA 16803, USA; czf262@psu.edu (C.T.F.); xmd5032@psu.edu (X.D.)
* Correspondence: jmw970@psu.edu; Tel.: +1-814-863-2956

Abstract: Native microorganisms present on grapes can influence final wine quality. Chambourcin is the most abundant hybrid grape grown in Pennsylvania and is more resistant to cold temperatures and fungal diseases compared to *Vitis vinifera*. Here, non-*Saccharomyces* yeasts were isolated from spontaneously fermenting Chambourcin must from three regional vineyards. Using cultured-based methods and ITS sequencing, *Hanseniaspora* and *Pichia* spp. were the most dominant genus out of 29 fungal species identified. Five strains of *Hanseniaspora uvarum*, *H. opuntiae*, *Pichia kluyveri*, *P. kudriavzevii*, and *Aureobasidium pullulans* were characterized for the ability to tolerate sulfite and ethanol. *Hanseniaspora opuntiae* PSWCC64 and *P. kudriavzevii* PSWCC102 can tolerate 8–10% ethanol and were able to utilize 60–80% sugars during fermentation. Laboratory scale fermentations of candidate strain into sterile Chambourcin juice allowed for analyzing compounds associated with wine flavor. Nine nonvolatile compounds were conserved in inoculated fermentations. In contrast, *Hanseniaspora* strains PSWCC64 and PSWCC70 were positively correlated with 2-heptanol and ionone associated to fruity and floral odor and *P. kudriazevii* PSWCC102 was positively correlated with a group of esters and acetals associated to fruity and herbaceous aroma. Microbial and chemical characterization of non-*Saccharomyces* yeasts presents an exciting approach to enhance flavor complexity and regionality of hybrid wines.

Keywords: wine; hybrid grapes; fermentation; non-*Saccharomyces* yeast

Citation: Feng, C.T.; Du, X.; Wee, J. Microbial and Chemical Analysis of Non-*Saccharomyces* Yeasts from Chambourcin Hybrid Grapes for Potential Use in Winemaking. *Fermentation* **2021**, *7*, 15. https://doi.org/fermentation7010015

Academic Editor: Luísa Seuanes Serafim
Received: 19 December 2020
Accepted: 19 January 2021
Published: 21 January 2021

1. Introduction

Saccharomyces cerevisiae has been widely applied to many fermentation processes including baking, winemaking, and brewing for thousands of years [1]. High tolerance of glucose and ethanol, and ability to convert sugars to alcohol in fermentation makes *S. cerevisiae* important for alcoholic beverages [2]. In winemaking, commercial strains of *S. cerevisiae* are selected for their efficient and reliable fermentation capabilities especially important in producing final wines that are consistent in taste and aroma. Although commercial *S. cerevisiae* yeasts are common in winemaking, there is increasing interest in using non-*Saccharomyces* yeasts (also known as native 'wild' yeasts) during early stages of wine fermentation. Previous studies demonstrate that unique physiological properties of certain non-*Saccharomyces* yeast strains can influence resulting wine such as alcohol levels and volatile metabolite profile [3–7]. For example, *Metschnikowia pulcherrima* and *Torulaspora delbrueckii* were able to produce 0.28 and 0.3 g ethanol/g sugar, respectively, compared to *S. cerevisiae* at 0.46 g ethanol/g sugar [8]. Decreased ethanol levels in wines is favorable from a regulatory and health-related perspective [9]. Coinoculation of non-*Saccharomyces* yeasts such as *Hanseniaspora uvarum* and *Starmerella bacillaris* were able to enhance aromatic profile by producing higher alcohols that correspond to floral odor (i.e., β-phenylethyl alcohol) [10–12]. β-phenylethyl alcohol is an important phenolic higher alcohol in wine and consumers have responded favorably to richer, fruitier, and more complex

styles of wine [9,13]. Thus, targeted use of non-*Saccharomyces* yeasts through sequential or coinoculation can favorably impact physicochemical properties that determine quality of final wines.

The composition, distribution, and abundance of non-*Saccharomyces* yeasts are affected by environmental factors such as weather, soil, rainfall, and winemaking practices [14]. Among diverse non-*Saccharomyces* yeasts present within vineyards and winery environments, *Hanseniaspora* spp., *Pichia* spp., and *Candida* spp. are dominant yeast populations that have been previously reported to contribute to the initial stages of fermentation and improve the organoleptic characteristics of final wine [15]. To date, most studies that characterize the diversity of non-*Saccharomyces* yeasts have been associated with *Vitis vinifera* varieties. Much is still unknown relative to how microbial populations present on hybrid grapes affect final wine quality. Hybrid grape varieties or also known as interspecific varieties have been of recent interest due to their versatile characteristics related to the ability to tolerate extreme cold weathers and increased disease resistance [16]. For example, Marquette (cross between two hybrids, MN 1094 × Ravat 262) is resistant to downy mildew and cold temperature at −34 °C [17]. Chambourcin (Joannès Seyve × Seibel 5455) is a French–American hybrid grape varietal and is the most abundant hybrid grape grown in Pennsylvania, USA. Chambourcin grapes have been shown to tolerate cold temperatures (−25 °C) and fungal diseases such as grey rot caused by *Botrytis cinerrea* [17]. Therefore, hybrid grapes could be used as an approach to decrease risks of wine faults.

During the initial and early stages of winemaking, sulfur dioxide (SO_2) and ethanol are critical intrinsic factors that can directly impact the diversity and abundance of microbial populations including non-*Saccharomyces* yeasts. The addition of SO_2 is recommended for protection against spoilage fungi or bacteria. Therefore, one important characteristic of wine yeasts is the ability to tolerate and grow in varying levels of sulfites during winemaking. On the other hand, tolerance of wine yeasts to increasing concentrations of ethanol during alcoholic fermentation is also a prerequisite for winemaking. Generally, red wines contain between 12% and 14% of ethanol and commercial strains of *S. cerevisiae* have been reported to be able to tolerate up to 13% ethanol [18]. However, depending on the yeast strain, cell growth and viability can be inhibited by high ethanol concentrations, and thus limit fermentation productivity and ethanol yield [19–22]. Therefore, physiological properties of yeast strains such as SO_2 and ethanol tolerance is important to support the use of non-*Saccharomyces* yeasts in winemaking applications.

Wine flavor is a complex interaction of nonvolatile and volatile chemical constituents that contribute to taste and smell. Certain core compounds such as ethanol, glycerol, organic acids, and residual sugars contribute to the primary taste of wine. Sensory panelists perceive these core nonvolatile compounds as a mouth-warming effect, viscosity, sourness, and sweetness. These core compounds are fundamental components of wine which an individual experiences via sense [23]. On the other hand, volatile compounds in wine are composed of hundreds of different compounds with concentrations ranging from 10^{-1} to 10^{-10} g/kg typically perceived as wine aroma [24]. Wine associated volatile metabolites are a result of three processes, (1) metabolism of grape-derived compounds into active aroma compounds, (2) biosynthesis of fermentation-derived metabolites, and (3) post-fermentation practice-derived metabolites such as barrel aging [25]. Previous literature have identified methoxypyrazines, C13-norisoprenoids, volatile sulfur compounds, and terpenes as the major contribution to primary aroma, and volatile fatty acids, higher alcohols, esters, and aldehydes as contribution to secondary aroma [26,27]. However, the balance and interaction of these chemical compounds determine the wine quality. The biosynthesis of these compounds depends on the microorganism present in winemaking. Therefore, in order to isolate and identify novel non-*Saccharomyces* yeast strains for applications in winemaking, it is critical to analyze core volatile and nonvolatile metabolites of individual strains.

The annual economic impact of the wine industry in the State of Pennsylvania is approximately USD 2.5 billion with more than 300 wineries across the State. Pennsylvania is ranked fifth for wine production in the USA [28,29]. The overall objective of this study is to

determine the potential contribution of hybrid grapes-associated non-*Saccharomyces* yeasts for use in winemaking to enhance regionality of local wines. Cultured-based methods coupled with ITS sequencing were used to isolate and identify Chambourcin-associated non-*Saccharomyces* yeasts from three regional vineyards in Pennsylvania. UHPLC-RI and GC-MS were used to measure volatile and nonvolatile compounds within inoculated fermentation using five candidate non-*Saccharomyces* yeast in a sterile Chambourcin grape juice system. Here, we focus on volatile metabolite changes that drive differentiation between candidate non-*Saccharomyces* yeasts compared to the commercial benchmark strain, *S. cerevisiae*. We hypothesize that non-*Saccharomyces* yeast isolated from hybrid grapes like Chambourcin can enhance regional characteristics of hybrid grapes and potentially increase the quality and appreciation of hybrid wines.

2. Materials and Methods

2.1. Grape Sampling and Juice Collection

Chambourcin grapes were obtained from three regional vineyards in Pennsylvania, USA. Grapes were collected and refrigerated at 4 °C until further processing (not more than 72 h). One hundred and fifty grams of grape berries were crushed to produce must and allowed to ferment in a 1000-mL sterile beaker covered with aluminum foil at 25 °C for 24 h. Previous studies demonstrate that addition of sulfite is one factor that can decrease richness and evenness of non-*Saccharomyces* yeast during fermentation, thus representative yeast selection was conducted without supplemental sulfite to maximize isolation of diverse populations.

2.2. Growth Media and Fungal Isolation

To isolate and identify non-*Saccharomyces* yeasts, a combination of microbiological culture-based approaches with molecular methods facilitates the identification of species. Fungal isolation was conducted as outlined in Raymond Eder et al. (2017) and Vaudano et al. (2019) with minor modifications related to type of selective agar and sampling timepoint [14,30]. To isolate grape-associated fungal populations, appropriate dilutions (10^{-1}∼10^{-4} dilutions) of fermented Chambourcin must at 0 and 24 h were plated on Dichloran Rose Bengal Chloramphenicol (DRBC) agar based on manufacturer's instructions (Difco, Sparks, MD, USA). DRBC agar is recommended for the enumeration and selection of yeasts and molds in food and dietary supplements. It contains peptone as a source of carbon and nitrogen, dextrose as a sugar source, and magnesium sulfate to provide trace elements [31]. Chloramphenicol is added to inhibit bacterial growth resulting in better recovery of fungal cells, and rose bengal is added to increase the selectivity of non-*Saccharomyces* yeast by suppression of rapidly growing molds such as *Neurospora* and *Rhizopus* spp. [32]. Dichloran is added to inhibit the spreading of molds by reducing colony diameters [33]. Culture plates were incubated at 25 °C for 5 days. Twenty colonies were selected based on unique colony morphology observed from 0 and 24 h samples across all three regional wineries. Five milliliter enrichments were prepared for individual isolates using liquid yeast peptone dextrose (YPD) medium (1% yeast extract (Difco, Sparks, MD, USA), 2% peptone (Difco, Sparks, MD, USA) and 2% dextrose (VWR International, Radnor, PA, USA)), and grown at 25 °C for 24 h with shaking at 200 rpm (standard laboratory conditions). Yeast Peptone Dextrose is a complete medium used for cultivation of a wide range of yeasts, including *Saccharomyces cerevisiae* [34]. Four milliliters of enrichment culture (YPD + single colony yeast isolate grown for 24 h) was centrifuged at 3000× *g* for 3 min, supernatant was discarded, and cell pellets were kept frozen at −80 °C until further use. Remaining enrichment cultures were stored at −80 °C as a yeast cryo-stock supplemented with 30% (*v*/*v*) glycerol and deposited into our laboratory's culture collection. *Saccharomyces cerevisiae* BY4742 [ATCC 4040004, YVC1] was used as a reference strain, grown in liquid YPD medium under standard laboratory conditions, and stored with 30% (*v*/*v*) glycerol at −80 °C.

2.3. Molecular Identification of Fungal Isolates

After culturing of yeast isolates, internal transcribed spacer (ITS) region consisting of the 5.8S rRNA genes has been shown to provide the highest probability of successful identification for the broadest range of fungi [35]. Thus, studies to date use a combination of culture-based methods and sequencing to identify fungal populations in complex fermentation systems such as winemaking. A total of 120 isolated strains were identified by analysis of the ribosomal internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) from three regional vineyards [30]. It is important to note that for molecular identification of Ascomycota yeast, the 28S nuclear ribosomal large subunit rRNA gene (LSU) is used in combination with the ITS region to improve accuracy of identification [35]. For the purpose of this study, ITS was chosen to support wine yeast literature as well as metagenomic HTS analyses [36]. Genomic DNA were extracted from frozen cell pellets using a MasterPure Yeast DNA Purification Kit based on manufacturer's instructions (Lucigen, Middleton, WI, USA). For isolates which resemble filamentous fungi characterized by aerial mycelium growth, isolates were rinsed with 0.1 M $MgCl_2$ and recentrifuged to obtain dry cell tissue for cell lysis and precipitation of DNA. Genomic DNA for colonies with yeast-like morphologies were extracted based on manufacturers' instructions.

The ITS region was amplified by PCR using the universal primers, ITS1 (5′- TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) [37]. PCR was carried out in a final volume of 25 µL, containing 12.5 µL of PCR Master Mix 5Prime HotMasterMix (Quantabio, Beverly, MA, USA), 1.25 µL of 10 nM forward primer and 1.25 µL of 10 nM reverse primer, and 10 µL of template DNA (10 ng/µL). PCR cycles were as follows: initial denaturation at 93 °C for 3 min and 35 cycles of denaturation at 93 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR amplicons were separated on 1.5% agarose gel in 1 x TAE buffer, stained with SYBR Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific, MA, USA) and visualized using UV transillumination. PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany). Sanger sequencing was performed using ITS4 primers mentioned above at The Pennsylvania State University's HUCK Institutes of Life Sciences (University Park, PA, USA) on an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

ITS sequences obtained from Sanger sequencing were subjected to visual quality assessment on DNA sequencing chromatogram, and then queried using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) as outlined in Brysch-Herzberg and Seidel (2015) and Raymond Eder et al. (2017) with minor modifications on criteria of alignment score for molecular identification [30,38]. The species of reference strain was assigned to an isolate when an identity score of $\geq$ 99%. Identity scores lower than 99% were assigned a genus and not further identified to species level in this study. Of important to this study, five candidate strains were subsequently aligned with GeneBank type strain sequence with the following identity score: 100% for *Hanseniaspora uvarum* PSWCC70, 99.25% for *H. opuntiae* PSWCC64, 99.74% for *Pichia kluyveri* PSWCC62, 93.85% for *P. kudriavzevii* PSWCC102, and 100% for *Aureobasidium pullulans* PSWCC82. For isolates with multiple possible genus and species identification from the National Center for Biotechnology Information (NCBI) database, MycoBank (http://www.mycobank.org/; [39]) was used for fungal synonyms searching and UNITE (https://unite.ut.ee/) was used for similarity searches against additional fungal databases to increase identification for that particular fungal isolate. ITS sequences for this study have been deposited into NCBI (#Accession MW301459-MW301578). Phylogenetic analysis was conducted using the Molecular Evolutionary Genetics Analysis (MEGA) software version 10.1.7 [40,41]. ITS sequences were aligned with Multiple Sequence Comparison by Log-Expectation (MUSCLE) and phylogenetic tree was constructed using the maximum likelihood (ML) method supported by 500 bootstrap replications [42,43].

2.4. Physiological Characterization of Non-Saccharomyces Yeasts

Candidate non-*Saccharomyces* yeasts for downstream characterization experiments were selected based on results obtained from sequencing analysis and the absence of toxin production based on previously published studies. For example, strains with highest statistical confidence based on BLAST results, de novo sequences alignment, and phylogenetic analysis were selected as candidate strains. Candidate strains *Hanseniaspora uvarum* PSWCC70, *H. opuntiae* PSWCC64, *Pichia kluyveri* PSWCC62, *P. kudriavzevii* PSWCC102, and *Aureobasidium pullulans* PSWCC82 isolated from spontaneously fermenting Chambourcin must were chosen for downstream analysis. *Saccharomyces cerevisiae* BY4742 was used as a laboratory control.

Candidate non-*Saccharomyces* yeast strains were grown under varying concentrations of sulfite and ethanol using a microplate-based method described by Tofalo et al. (2014) and Englezos et al. (2015) with minor modifications related to the concentration of substrate [44,45]. For characterization of sulfite tolerance, yeast strains were grown in liquid YPD media with final concentrations of 0, 40, 60, 80, 100 mg/L sodium metabisulfite (VWR International, Radnor, PA) at pH = 3.0. Sulfites are most effective as an antimicrobial agent in acidic conditions due to the effect from the molecular SO_2 penetrating the cell wall and disrupting the enzymatic. For characterization of ethanol tolerance, yeast strains were grown in liquid YPD media (pH = 6.5) containing 0, 8, 10, 12, and 14% (v/v) ethanol (Decon Labs, King of Prussia, PA, USA). Varying ethanol concentrations were chosen to represent 12–14% ethanol typically present in red wines.

Five candidate strains and one control strain BY4742 were streaked on YPD agar (1.5% Bacto Agar) (Difco, Sparks, MD, USA) to obtain single colonies which were transferred to 5 mL of liquid YPD media and allowed to grow for 24 h at 25 °C with shaking (200 rpm). Cell pellets were collected by centrifugation at 4000× g for 5 min and washed twice with sterile phosphate-buffered saline (PBS) and resuspended in fresh liquid YPD media to obtain a starting optical density (OD) of 0.2 at 600 nm. A starting OD_{600} of 0.2 is approximately equal to a cell density of $10^6 \sim 10^7$ cfu/mL. The resulting yeast culture (20 µL) was added to 180 µL liquid YPD media supplemented with varying concentrations of sodium metabisulfite or ethanol described above. The microplate-based assay was conducted at 25 °C and OD_{600} was measured every 30 min after orbital shaking for 10 s using the continuous measurement mode on a microplate reader for 48 h (BioTek Instruments, Winooski, VT, USA). To determine ethanol and sulfite tolerance of these isolates, the ratio (%) between growth of the isolate in YPD media with and without sodium metabisulfite or ethanol at the end of incubation time (t = 48 h) was calculated using the following equation:

$$\text{Growth ratio } (\%) = \frac{\text{Growth in substract } (OD_{600})}{\text{Growth in YPD } (OD_{600})} \times 100\%$$

Isolates with a percentage growth ratio of larger than 10% were considered tolerant [45]. Three biological experiments containing triplicate samples for each experimental condition were used for sulfite and ethanol tolerance experiments.

2.5. Laboratory Scale Fermentation

Fresh Chambourcin grapes were obtained from three regional vineyards as described above. Grapes were crushed in the lab, must obtained, juice centrifuged, and filter-sterilized through a 0.2 µm membrane filter (VWR International, Radnor, PA, USA) and stored at −20 °C until used. For the remaining of this manuscript, we use the term "sterile juice" to represent filter sterile Chambourcin juice. Five candidate strains and control *S. cerevisiae* BY4742 were inoculated into sterile juice (pH = 3.26) for laboratory scale fermentation. Inoculated fermentation of sterile juice has been previously established [4,46]. We use the term "inoculated fermentations" to represent filtered sterile juice inoculated with candidate yeast strains or control *S. cerevisiae*. Yeast cultures were prepared from enriched 5 mL of liquid YPD media with a single colony. Cell pellets were washed with PBS resuspended in

fresh liquid YPD media to obtain a starting optical density (OD) of 0.2 at 600 nm with same procedure as for the tolerance test.

Laboratory scale fermentations were carried out starting OD_{600} = 0.2 ($10^6 \sim 10^7$ cfu/mL) inoculated to 50 mL sterile juice in 250-mL glass Erlenmeyer flask fitted with an airlock closure to enable carbon dioxide release. Inoculated fermentations were performed at 25 °C in static condition, and fermentation was monitored by measuring weight loss due to carbon dioxide release as previously described [4,46,47] (see Supplementary Materials Figure S1). Fermentation was considered complete when weight loss of each sample was lower than 0.05 g in 24 h as outlined in [4]. The resulting fermented juice was collected at the end of fermentation and frozen at −20 °C for analysis of volatile and nonvolatile compounds. For volatile and nonvolatile analysis, three independent laboratory scale fermentations were conducted for each experimental condition.

2.6. Analysis of Flavor Compounds of Fermented Chambourcin Juice

2.6.1. Analysis of Nonvolatile Compounds by UHPLC

To analyze nonvolatile compounds, sterile juice and inoculated fermentations were filtered through a 0.2 µm membrane. Nine nonvolatile compound standards and mobile phase were prepared including glucose (99%, Acros Organics, Thermo Fisher Scientific, Waltham, MA, USA), fructose (99%, Alfa Aesar, Haverhill, MA, USA), glacial acetic acid (VWR International, Radnor, PA, USA), tartaric acid (99%, TCI America, Portland, OR, USA), citric acid (99.5%, VWR International, Radnor, PA, USA), malic acid (99%, Acros Organics, Thermo Fisher Scientific, Waltham, MA, USA), succinic acid (99%, Acros Organics, Thermo Fisher Scientific, Waltham, MA, USA), glycerol (VWR International, Radnor, PA, USA), ethanol (Decon Labs, King of Prussia, PA, USA), and sulfuric acid (Sigma-Aldrich, St. Louis, MO, USA) were purchased. Standards and mobile phase were dissolved in deionized water to a desired concentration and filtered through 0.2 and 0.45 µm membrane filters, respectively. Standards were prepared according to previously published concentration of compounds in juice or wine [48,49]. Five concentrations of each compound were made by a two-fold serial dilution to construct a standard curve. The highest concentration of each compound was glucose and fructose: 40 g/L; glacial acetic acid: 0.8 g/L, tartaric acid: 8 g/L, citric acid: 2 g/L, malic acid: 6 g/L, succinic acid: 2 g/L, glycerol 6 g/L, and ethanol: 7% (v/v). Serial dilution standards were injected to construct a standards curve and the retention time (RT) of each compound was recorded and validated. Equations of each standard curve had R^2 = 1.

Chromatographic separations were performed on an Ultra High-Performance Liquid Chromatography (UHPLC) system (Vanquish UHPLC Systems, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a refractive index (RI) detector (RefractoMax 521, Thermo Fisher Scientific, Waltham, MA, USA) at the HUCK CSL Behring Fermentation Facility at Penn State. Targeted compounds were separated and analyzed on an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA), protected by a Micro-Guard Cation H guard column (30 × 4.6 mm) (Bio-Rad Laboratories, Hercules, CA, USA) and kept at 60 °C. The analytical conditions used were as follows: 10 µL of injection volume, flow 0.5 mL/min, eluent 5 mM H_2SO_4. Temperatures set for autosampler and RI detector were 4 and 35 °C, respectively. A standard curve was prepared using standards to determine the relationship between concentration and the peak area of a particular compound eluted. The chromatographic peak corresponding to each compound was identified by comparing the retention time with that of standards. All standards and samples were injected in technical triplicate.

2.6.2. Analysis of Volatile Compounds by Gas Chromatography-Mass Spectrometry

Aromatic compounds were analyzed by gas chromatography-mass spectrometry (GC-MS) (7890B System, 5977B MSD, Agilent Technologies, Santa Clara, CA, USA) using the method described here with minor modifications [50]. In total, 2 mL of samples was mixed with 3 g sodium chloride (VWR International, Radnor, PA, USA), 50 µL internal

standard (including 13.7 mg/L 2-octanol and 9.9 mg/L naphthalene-d8 in methanol), and 0.5g D-gluconic acid lactone (Sigma-Aldrich, St. Louis, MO, USA), which inhibits grape β-glucosidase activity during sample preparation and analysis [51]. Samples were then vortexed and analyzed immediately. Each injection was performed in technical triplicates from three biological flasks.

Solid-phase microextraction (SPME) mixture comprised heptanal (0.452 g/L), octanal (0.484 g/L), nonanal (0.477 g/L), 1-decanol (0.394 g/L), 1-undecanol (0.392 g/L), 1-dodecanol (0.521 g/L), n-decane (0.451 g/L), n-dodecane (0.481 g/L), n-tetradecane (0.497 g/L), methanol (0.511 g/L), (-)-trans- and (-)-cis-carveol (0.778 g/L), (+)-carvone (0.584 g/L), a-pinene (0.553 g/L), b-pinene (0.472 g/L), and p-cymene (0.424 g/L) in acetone. A total of 50 µL of internal standard as a blank and 10 µL of SPME mixture as a quality control were used and placed in the first and last order of each batch of samples, respectively. Performance test of GC-system was carried by the mixture of 5 µL alkane standard solution ($C_8 - C_{20}$, ~40 mg/L each in hexane; Sigma-Aldrich, St. Louis, MO, USA) and 50 µL internal standard.

Samples were incubated at 30 °C for 5 min under agitation at 250 rpm, and then extracted using a 2 cm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber assembly (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Separation was carried out with a Rtx-Wax capillary column (25 m × 0.25 mm I.D. × 0.25 µm film thickness; Restek Corporation, Bellefonte, PA, USA) in splitless mode. The transfer line and ion source (70 eV) were maintained at 250 and 230 °C, respectively. The oven temperature was programmed as follows: hold at 30 °C for 1 min; increase to 250 °C at rate of 10 °C/min and hold for 5 min. Helium was used as the carrier gas under constant flow at 1 mL/min. Mass spectra were acquired at a rate of 33–350 amu scan.

To identify fermentation associated volatile compounds, chromatograms were stripped of common contaminating ions (147, 148, 149, 207, 221, 267, and 281 m/z) using the Denoising function in OpenChrom followed by the Savitzky–Golay smoothing filter with default settings (width = 15; order = 2) to enhance chromatographic data by reducing noise while maintaining the shape and height of waveform peaks [52–54]. The PARAFAC2 based Deconvolution and Identification System (PARADISe) computer platform (version 3.9) was used to deconvolute mass spectra with 7000 iterations and non-negativity constraint settings [55]. Retention time intervals were manually selected to increase the resolution of peak identification. Identification based on deconvoluted mass spectra were conducted using the National Institute of Standards and Technology (NIST14) mass spectral library with criteria of match factor over 700, and validated with Kovats retention indices referred to NIST14 library, PubChem (https://pubchem.ncbi.nlm.nih.gov/) or literature. Relative abundance of volatile compounds was calculated by dividing the peak area of volatile compounds by the peak area of internal standards (IS) and then subtracted by the relative abundance of such in the blank, 50 µL internal standard.

2.7. Statistical Analysis

IBM® SPSS® Statistic software version 26 was used for ANOVA and Tukey post-hoc test [56]. One-way analysis of variance (ANOVA) to calculate statistical significance of optical density (OD_{600}) data obtained from the tolerance assays and quantification of non-volatile compounds by UHPLC analysis. Significant differences were established by using the Tukey post-hoc test ($p < 0.05$) comparing the mean values of treatment groups. Partial least squares-discriminant analysis (PLS-DA) and clustered heatmap was generated using MetaboAnalyst to help visualize volatile and nonvolatile compounds driving separation between inoculated fermentations (https://www.metaboanalyst.ca/) [57]. Input data was normalized using log transformation and Pareto scaling function to remove heteroskedasticity resulting in a data set with normal distribution [58]. ANOVA was used to identify significant differences of relative abundance of volatile compounds across inoculated fermentations conducted by candidate yeast strains using false discovery rate (FDR) adjusted p-value (or q-value) of 0.05.

3. Results

3.1. Spontaneous Fermentation of Chambourcin Grape Must Provides Insights into Fungal Diversity of Red Hybrid Grapes

One hundred and twenty isolates from Chambourcin grape must were obtained from three vineyards during the 2019 vintage. Phylogenetic analysis of the fungal ITS region clustered these isolates into four main clades (Figure 1). Forty isolates were identified from each location by colony morphology and Sanger sequencing (see Section 2). *Hanseniaspora* spp. and *Pichia* spp. were the most abundant populations followed by *Sporidiobolus pararoseus*, *Starmerella bacillaris,* and *Aureobasidium pullulans* (Figure 2A). Observations from all three regional wineries suggests that *H. uvarum*, *H. meyeri*, and *H. opuntiae* were the most abundant (41.5%) in grape must. *Pichia fermentans*, *P. kluyveri*, *P. kudriavzevii*, and *P. terricola* were the second most abundant species (10.8%), as well as *S. pararoseus*. *S. bacillaris* (9%), *Aureobasidium pullulans,* and *Filobasidium floriforme* were also identified with equal relative abundance (5%). In PAV1, fungal composition was most diverse (17 fungal species identified) highlighted by diversity of *Hanseniaspora* spp. and *Pichia* spp. as well as the presence of filamentous fungi such as *Cladosporium angustisporum*, *Epicoccum sorghinum*, *Neopestalotiopsis clavispora*, and *Pestalotiopsis vismiae*. In PAV2, 11 fungal species were identified with *H. uvarum*, *S. pararoseus*, and *A. pullulans* being the most dominant species. Finally, 12 fungal species were identified in PAV3 where *H. uvarum* accounted for 50% of isolated strains and *Pichia* spp., *S. bacillaris* and *Candida* spp. accounted for the remaining fungal community.

Other yeast species isolated and identified from spontaneous fermentation include *Candida* spp., *Meyerozyma carpophila*, *Kregervanrija* sp., and *Papiliotrema* sp. *Candida* species included *C. californica* and *C. railenensis*. *Candida californica* was identified only from PAV3. One strain of *Kregervanrija* sp. and *Papiliotrema* sp. was found in PAV1 and PAV2, respectively. Using this culture-based approach, filamentous fungi were less dominant and less consistent across vineyards compared to the abundance of yeast species present on grape berries. *Leptosphaerulina chartarum* was the only species found across vineyards at PAV1 and PAV2. Other examples of filamentous fungi identified within individual vineyards include *Alternaria alternata*, *Cladosporium* spp., *Epicoccum sorghinum*, *Fusarium* sp., *Mucor nidicola*, *Neopestalotiopsis clavispora*, *Penicillium brevicompactum*, *P. spinulosum,* and *Pestalotiopsis vismiae* (Table S1). After 24 h of spontaneous fermentation, decreased fungal diversity in Chambourcin must was observed and filamentous fungi were not identified in Chambourcin grape must. *Hanseniaspora* spp. and *Pichia* spp. were the most dominant species in PAV1, and *Hanseniaspora* spp. and *S. bacillaris* were dominant in PAV2 and PAV3 after 24 h of spontaneous fermentation (Figure 2B).

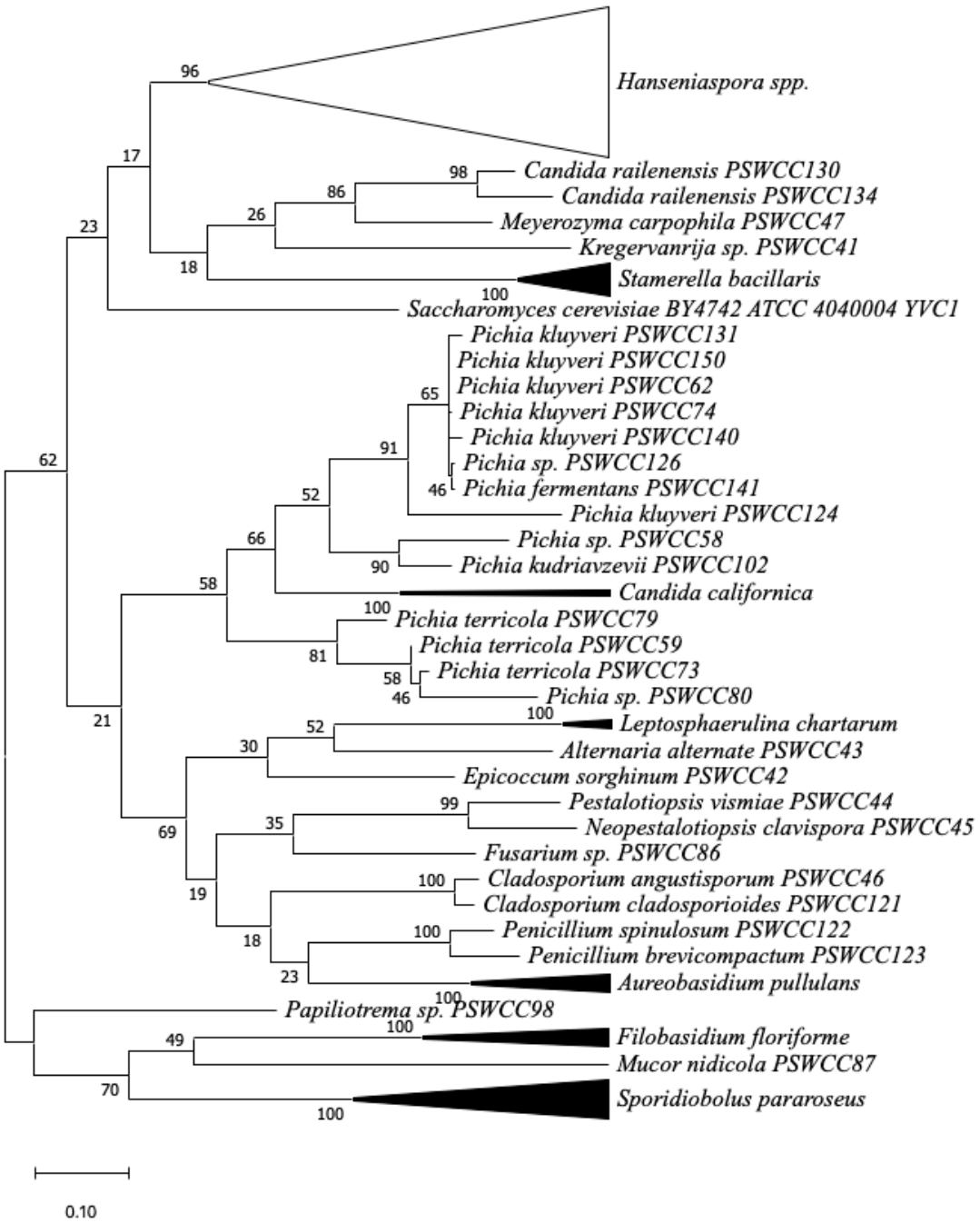

Figure 1. Phylogenetic diversity of fungal strains associated with Chambourcin hybrid grape must during spontaneous fermentation based on ribosomal internal transcribed spacer (ITS) regions and 5.8S rRNA gene sequences. Branches corresponding to partitions reproduced from less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. *Hanseniaspora* clade was suppressed into a black empty triangle including all *Hanseniaspora* species clustered with 96% bootstrap support. Subtrees including same species supported by 100% bootstrap value were suppressed and represented as solid black triangles, including *Starmerella bacillaris*, *Candida californica*, *Leptosphaerulina chartarum*, *Aureobasidium pullulans*, *Filobasidium floriforme*, and *Sporidiobolus pararoseus* strains.

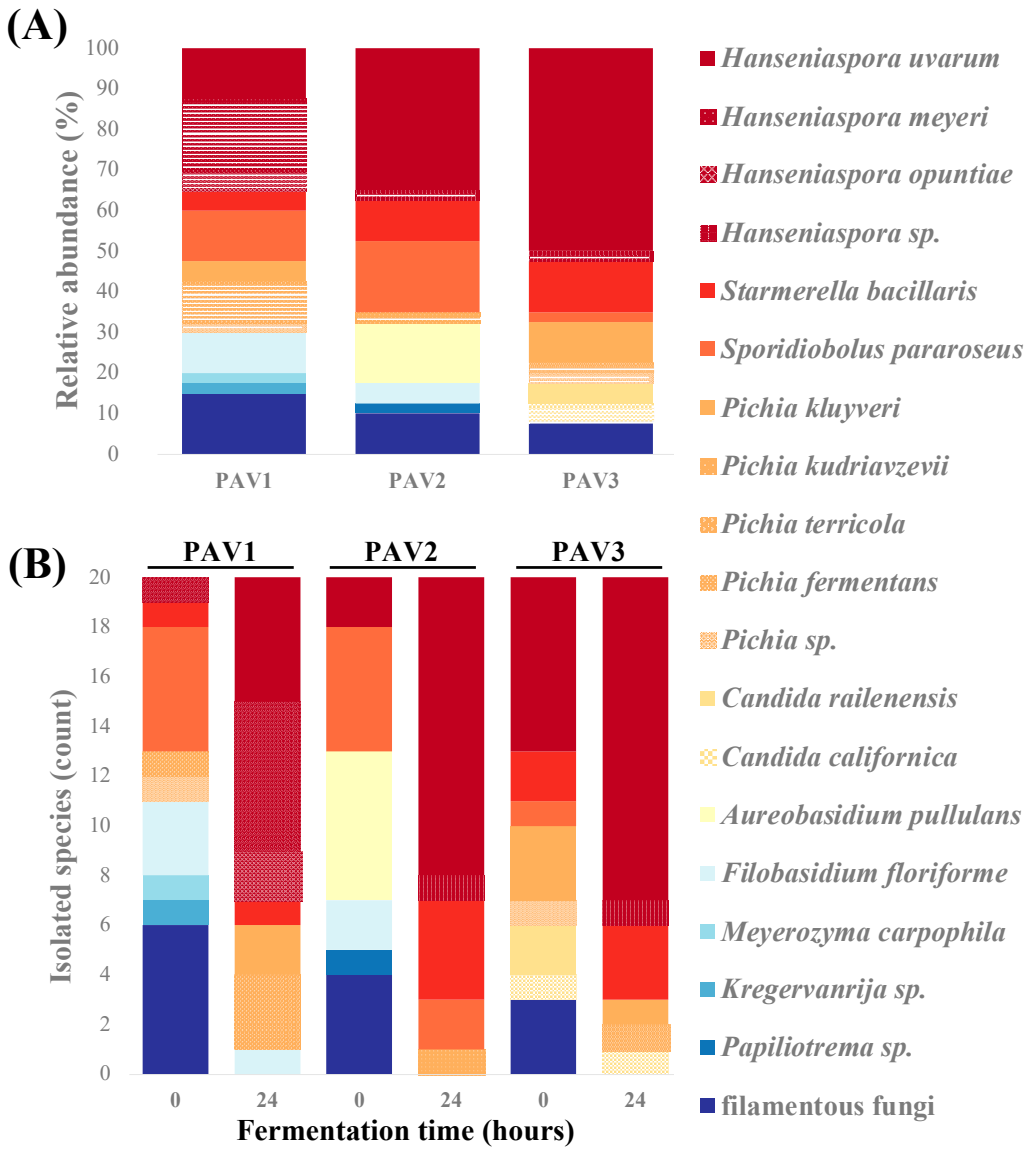

Figure 2. Main contributing fungal species during spontaneous fermentation of Chambourcin grape must. (**A**) Relative proportion of identified fungal species (%) from 120 randomly selected colonies (20 isolates/sampling time; 40 isolates from each location) obtained during spontaneous fermentation. (**B**) Fungal species identified on Dichloran Rose Bengal Chloramphenicol (DRBC) agar plates at 0 and 24 h (20 colonies/time point).

3.2. Physiological Characterization of Non-Saccharomyces Yeasts

Hanseniaspora uvarum PSWCC70, *H. opuntiae* PSWCC64, *Pichia kluyveri* PSWCC62, *P. kudriavzevii* PSWCC102, and *Aureobasidium pullulans* PSWCC82 were candidate yeasts selected for downstream microbial and chemical analysis. Previous literature suggests *Hanseniaspora* spp. and *Pichia* spp. can utilize fruit sugars for fermentation and have not been shown to produce harmful toxins whereas *Aureobasidium pullulans* is well known for the production of extracellular enzymes such as pectinases [59]. Therefore, we characterized sulfite and ethanol tolerance of these five strains compared to control *S. cerevisiae* BY4742. *Hanseniaspora opuntiae* PSWCC64 showed a tolerance at 80 mg/L sodium metabisulfite, and other candidate strains and *S. cerevisiae* BY4742 had tolerance at 100 mg/L. *Hanseniaspora uvarum* PSWCC70 and *H. opuntiae* PSWCC64 were relatively sensitive to sulfite with significantly decreased growth ratio when sodium metabisulfite concentration was higher than 80 mg/L (Figure 3A). On the other hand, all five candidate strains demonstrated lower tolerance to ethanol compared to BY4742. *Hanseniaspora uvarum* PSWCC70, *H. opuntiae* PSWCC64, and *P. kluyveri* PSWCC62 demonstrate

tolerance at 8% of ethanol while *A. pullulans* PSWCC82 was not tolerant to any concentration of ethanol tested. Interestingly, *P. kudriavzevii* PSWCC102 was the only non-*Saccharomyces* yeast candidate with comparable ethanol tolerance to BY4742 grown in 10% ethanol (Figure 3B). These results suggest that *H. opuntiae* PSWCC64 and *P. kudriavzevii* PSWCC102 could adapt to high ethanol environment better than the other candidate non-*Saccharomyces* yeasts during the alcoholic fermentation process.

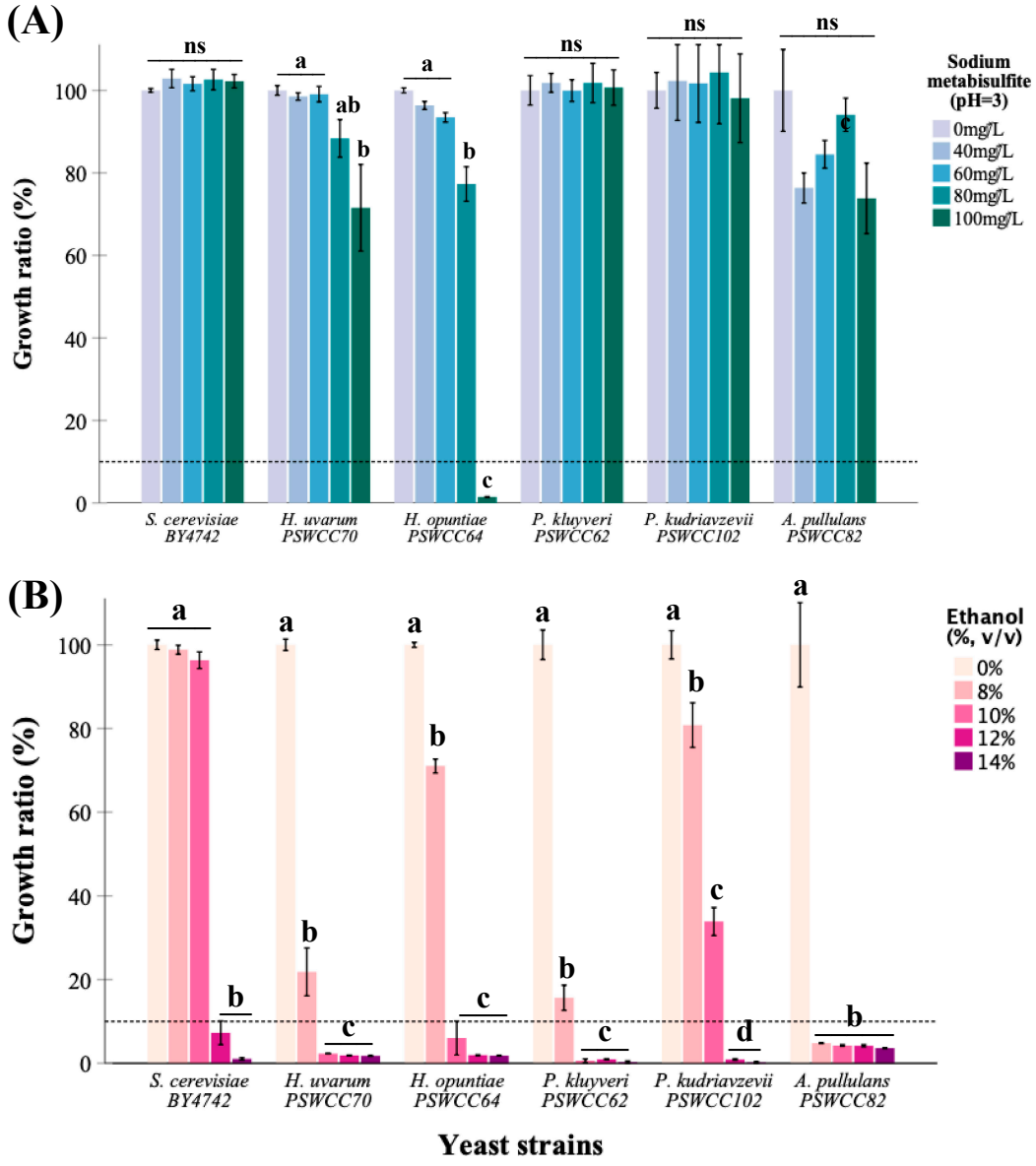

Figure 3. (**A**) Sulfite and (**B**) ethanol tolerance of candidate non-*Saccharomyces* isolates compared to laboratory control, *S. cerevisiae* BY4742. The heights of bar graphs represent tolerance which is defined as a ratio greater than 10% between growth of a strain in media supplemented with and without sulfite or ethanol. Yeast Peptone Dextrose (pH 3.0) was used as a growth medium for sulfite tolerance assay whereas YPD at pH 7.0 was used for ethanol tolerance (see Section 2). Dotted line across the y-axis represents a 10% cutoff. Strains used in this study are represented on the x-axis. Data is presented as mean ratio ± SEM with a common superscript indicating significance ($p < 0.05$).

3.3. Characterization of Core Nonvolatile Compounds Highlight Fermentative Potential of Candidate Non-Saccharomyces Yeasts

Nine nonvolatile compounds that represent fermentative potential of non-*Saccharomyces* yeasts compared to control strain, *S. cerevisiae* BY4742 were measured at the end of fermentation (Figure S1). Glucose (89.56 ± 0.78 g/L) and fructose (90.75 ± 0.56 g/L) are major sugars in Chambourcin juice. We hypothesized that candidate non-*Saccharomyces* strains can contribute to production of fermentation important metabolites. In support of this, the ability of non-*Saccharomyces* strains to convert sugars into different wine important fermentation by-products was examined. There was no significant difference in residual sugars measured in inoculated fermentations of *Pichia kudriavzevii* PSWCC102 (35.99 g/L) compared to *S. cerevisiae* BY4742 (36.88 g/L) (Figure 4A). Residual sugars are typically expressed as °Brix and are important to monitor the progress of fermentation. During inoculated fermentations, *S. cerevisiae* BY4742 and *P. kudriazevii* PSWCC102 converted about 80% of total sugars (glucose and fructose) followed by *H. opuntiae* PSWCC64 and *A. pullulans* PSWCC82 (60%), and *H. uvarum* PSWCC70 and *P. kluyveri* PSWCC62 (40%). All non-*Saccharomyces* strains produced similar levels of ethanol measured as "g EtOH/g Sugars" except *A. pullulans* PSWCC82. Interestingly, the conversion efficiency of *H. uvarum* PSWCC70 (0.54 (±0.03)) was not significantly different when compared to *S. cerevisiae* BY4742 (0.51 (±0.05)) (Figure 4B).

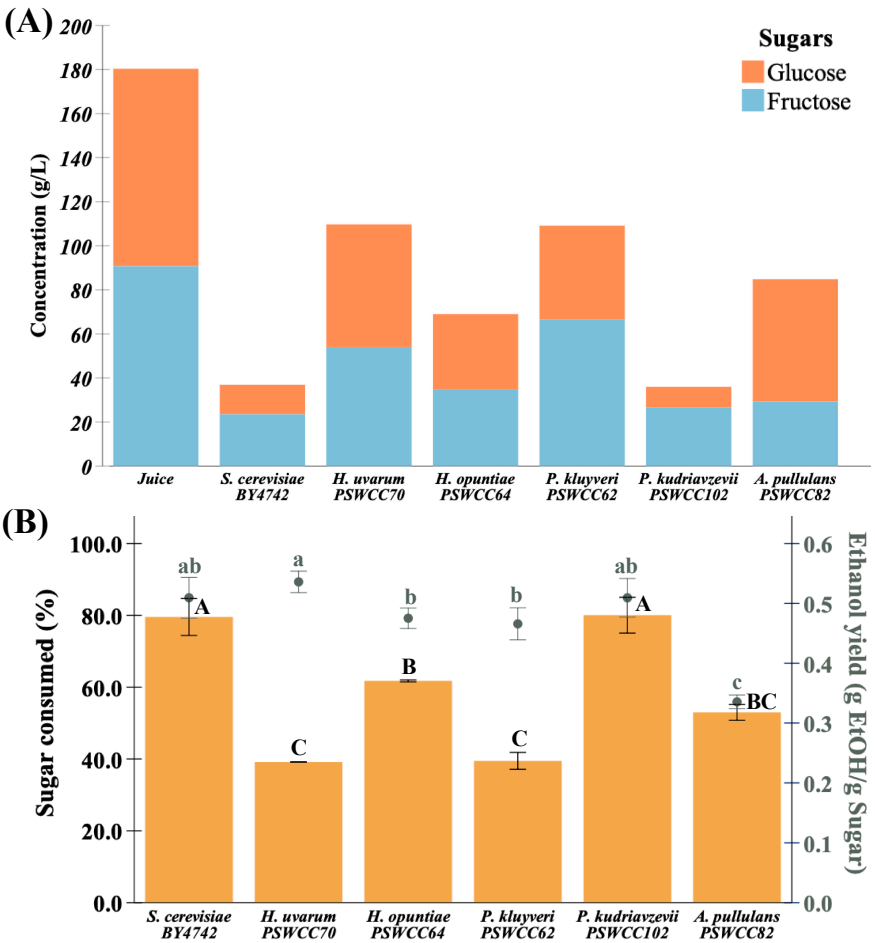

Figure 4. Composition of major sugars, sugar consumed, and ethanol yield in sterile juice compared to inoculated fermentations with candidate non-*Saccharomyces* yeast strains measured by UHPLC analysis. (**A**) Glucose (blue) and fructose (orange) represent major sugars in grape juice and wine. (**B**) Percent sugar consumed and ethanol yield during fermentation. Circles represent ethanol yield measured at the end of fermentation. Different types of yeasts used in this study are represented on the x-axis. Data presented as mean ratio ± SEM with a common superscript indicating significance ($p < 0.05$). Strains used in this study are represented on the x-axis, *S. cerevisiae* BY4742 was used as a control.

Ethanol and glycerol are two major primary metabolites produced during alcoholic fermentation of yeast and are important indicators of fermentation. The amount of ethanol produced by *P. kudriazevii* PSWCC102 (74.71 g/L) was not statistically different compared to *S. cerevisiae* BY4742 (74.29 g/L), but was significantly higher than ethanol levels produced by *H. uvarum* PSWCC70 (37.84 g/L), *H. opuntiae* PSWCC64 (52.90 g/L), *P. kluyveri* PSWCC62 (2.75 g/L), and *A. pullulans* PSWCC82 (32.11 g/L). However, there was no significant difference between glycerol production by *S. cerevisiae* BY4742, *P. kluyveri* PSWCC62, *P. kudriazevii* PSWCC102, and *A. pullulans* PSWCC82 (5.95~7.54 g/L) (Figure 5A). One aspect of wine quality relative to fermentation-derived nonvolatile metabolites are the types and levels of organic acids. Organic acids contribute to sourness, bitterness, and tartness of final wines [49]. Although present at low levels compared to ethanol, five organic acids were chosen for analysis of common acids present in inoculated fermentations. Specifically, *P. kudriazevii* PSWCC102 produced 1.90 ± 0.69 g/L of malic acid and 0.81±0.06 g/L of acetic acid with no significant difference compared to *S. cerevisiae* BY4742. Concentration of tartaric acid decreased after fermentation by most of the yeast strains (0.29~0.54 g/L) and was significantly different compared to sterile juice (0.69 ± 0.02 g/L). The control strain, BY4742 produced the highest amount of citric acid (0.20 ± 0.003 g/L) followed by *A. pullulans* PSWCC82 (0.17 ± 0.005 g/L). *Hanseniaspora opuntiae* PSWCC64 produced the highest amount of succinic acid (0.17 ± 0.02 g/L) followed by *S. cerevisiae* BY4742 with no significant difference (Figure 5B).

Partial least squares-discriminant analysis (PLS-DA) was used to identify how nine core nonvolatile compounds present in inoculated fermentations drive differentiation of candidate non-*Saccharomyces* yeast strains compared to *S. cerevisiae*. In PLS-DA score plot the first two components explained 76.5% of variance which separated experimental control (sterile juice) and inoculated fermentations (Figure 6A). Uninoculated juice was included in this analysis as a benchmark for comparison to ensure that the model system using Chambourcin sterile juice is reliable and reproducible to characterize candidate yeast strains for use in winemaking. The main compounds separating the samples analyzed in component 1 are glucose (C3) and fructose (C5) (negative loading), and malic acid (C4), succinic acid (C6), and ethanol (C9) (negative loading). In the case of PC2, tartaric acid (C2) was the major compound responsible for the separation between inoculated fermentations (Figure 6B).

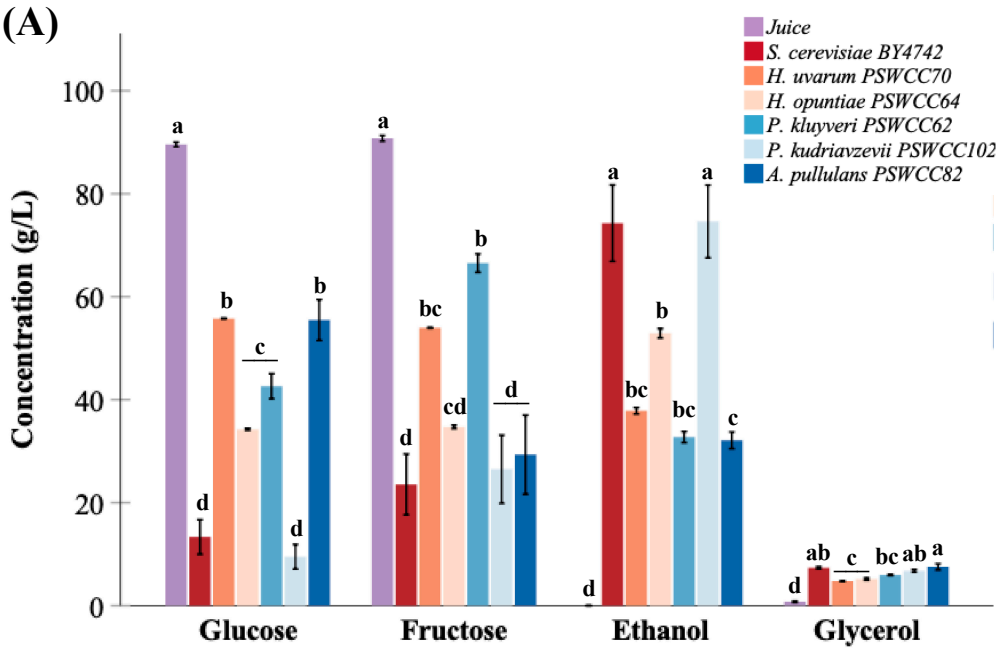

Figure 5. *Cont.*

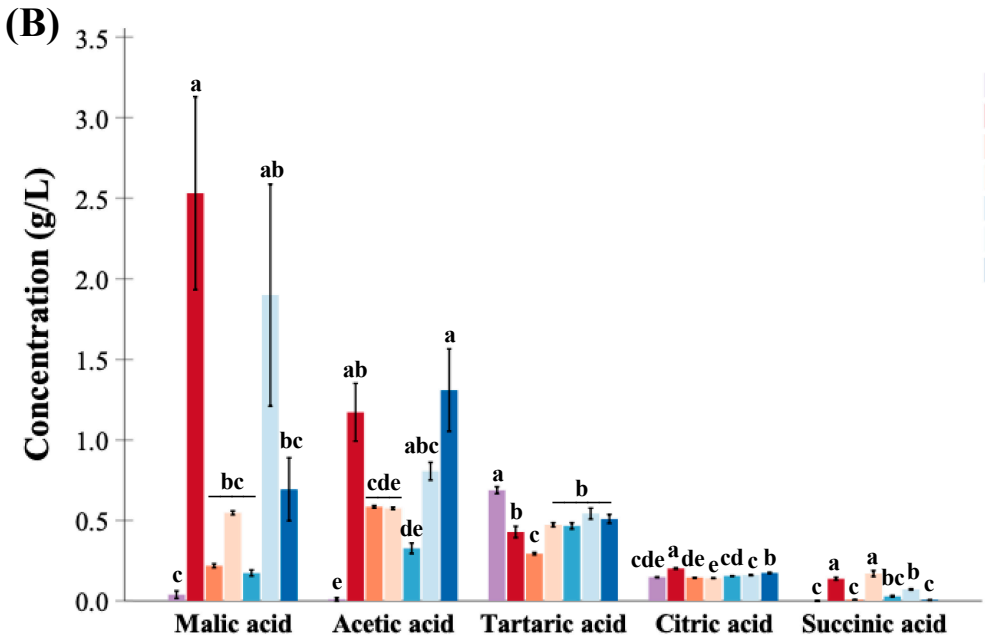

Figure 5. Nonvolatile compounds in sterile juice compared with inoculated fermentations with candidate non-*Saccharomyces* yeast strains with *S. cerevisiae* BY4742 as control. UHPLC-RI was used to measure nonvolatile compounds in inoculated fermentation, sugars (glucose and fructose), ethanol, glycerol (**A**), and acids (malic acid, acetic acid, tartaric acid, citric acid, and succinic acid) (**B**). The different types of yeasts used in this study are represented on the x-axis. *S. cerevisiae* BY4742 was used as a control. Data presented as mean ratio ± SEM with a common superscript indicating significance ($p < 0.05$).

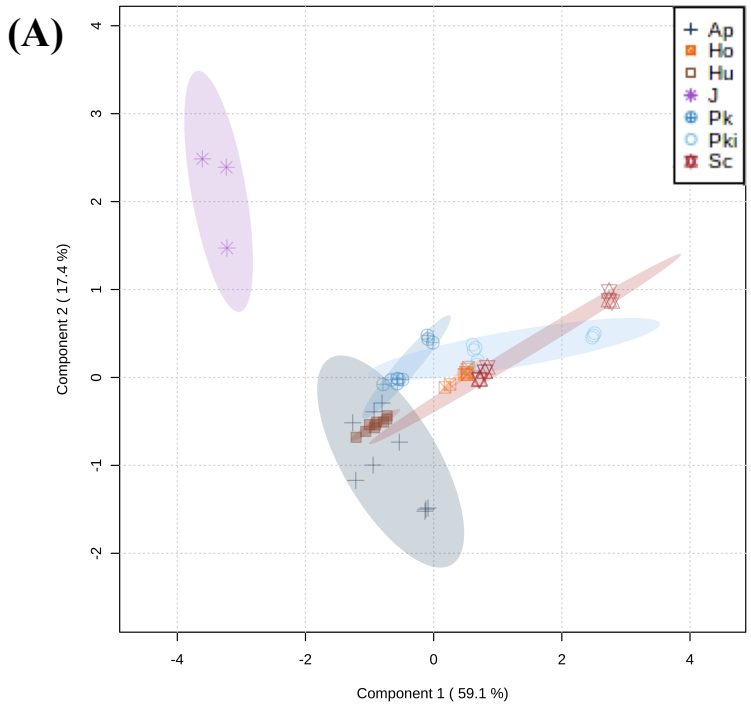

Figure 6. *Cont.*

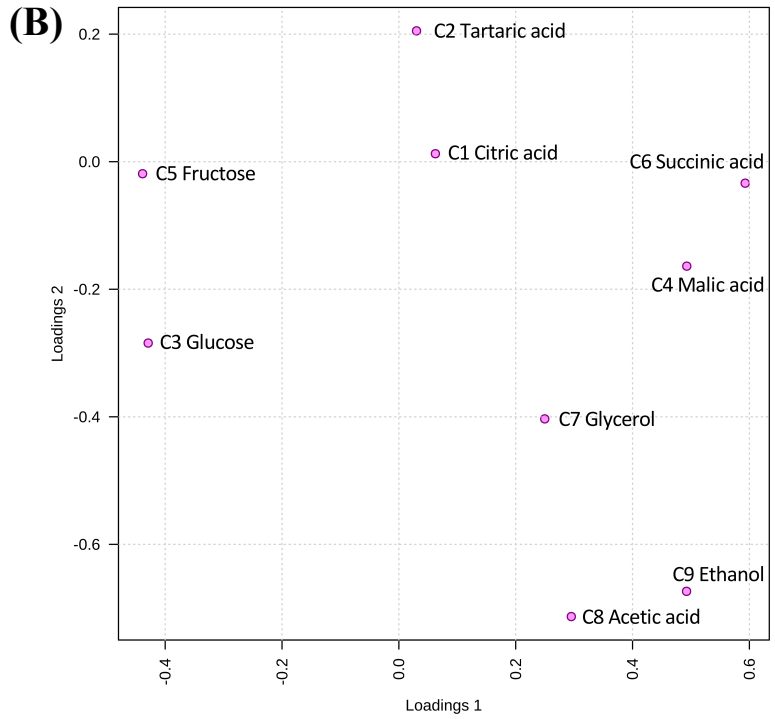

Figure 6. Nonvolatile compounds from inoculated fermentations differentiates from sterile juice. (**A**) PLS-DA scores plot and (**B**) loading plot of the variables with components 1 and 2 based on the nonvolatile composition of sterile juice (eight spoked asterisk) and inoculated fermentations with candidate yeast strains and *S. cerevisiae* BY4742. Each symbol in panel A represents a yeast strain and the ellipse around symbols represents a 95% confidence region of biological and technical triplicates.

3.4. Distinct Fermentation-Derived Volatile Metabolites Provide Insights into Unique Properties of Candidate Non-Saccharomyces Yeast from Chambourcin

One aspect of wine quality is the contribution of fermentation-derived volatile compounds which can enhance sensory characteristics of final wine. Using GC-MS, 74 volatiles were identified when comparing sterile juice and inoculated fermentations and assigned to 11 classes based on their chemical structure (Figure S2). All 74 identified compounds were validated and included for further analysis (Table S2). Statistical differences of volatile compounds in inoculated fermentations are shown in Table S3. The correlation between volatile profiles and candidate yeast strains were analyzed by PLS-DA based on the identified volatile compounds. In total, 59.9% of the experimental variance was explained by the first three components, while components 1, 2 and 3 accounted for 20.8%, 21.3%, and 17.8%, respectively (Figure 7A–C). In the PLS-DA score plot, three components separated the inoculated fermentations into distinctive groups. Fifteen identified volatile compounds with the highest variable importance in projection (VIP) scores by PLS-DA can help explain volatile metabolome features driving separation between inoculated fermentations (Figure 7D). In this context, we interpret volatile compounds with high VIP scores as the most discriminant variables in PLS-DA. Here, we are most interested in features that differentiate candidate non-*Saccharomyces* fermentations compared to *S. cerevisiae* inoculated fermentations. For example, ionone (V42); 3-(methylthio)propyl acetate (V33); 2-heptanol (V9); 1-propanol, 3-(methylthio)- (V72); hexanoic acid, methyl ester (V23) were the most abundant compounds in inoculated fermentations with *Hanseniaspora* strains PSWCC64 and PSWCC70 but had low abundance in *S. cerevisiae* control strain. In addition, linalool (V53); acetoin (V40); acetyl valeryl (V38); 2-buten-1-ol, 3-methyl-, acetate (V6); 3(2H)-Thiophenone, dihydro-2-methyl- (V67) were also important features driving the differences of inoculated fermentation present with high intensity in *A. pullulans* but low intensity in *S. cerevisiae* control strain. On the contrary, two of the other important features,

butanoic acid, 2-methyl- (V50) and octanoic acid, ethyl ester (V32), were present with high intensity in both *P. kudriazevii* and *S. cerevisiae*.

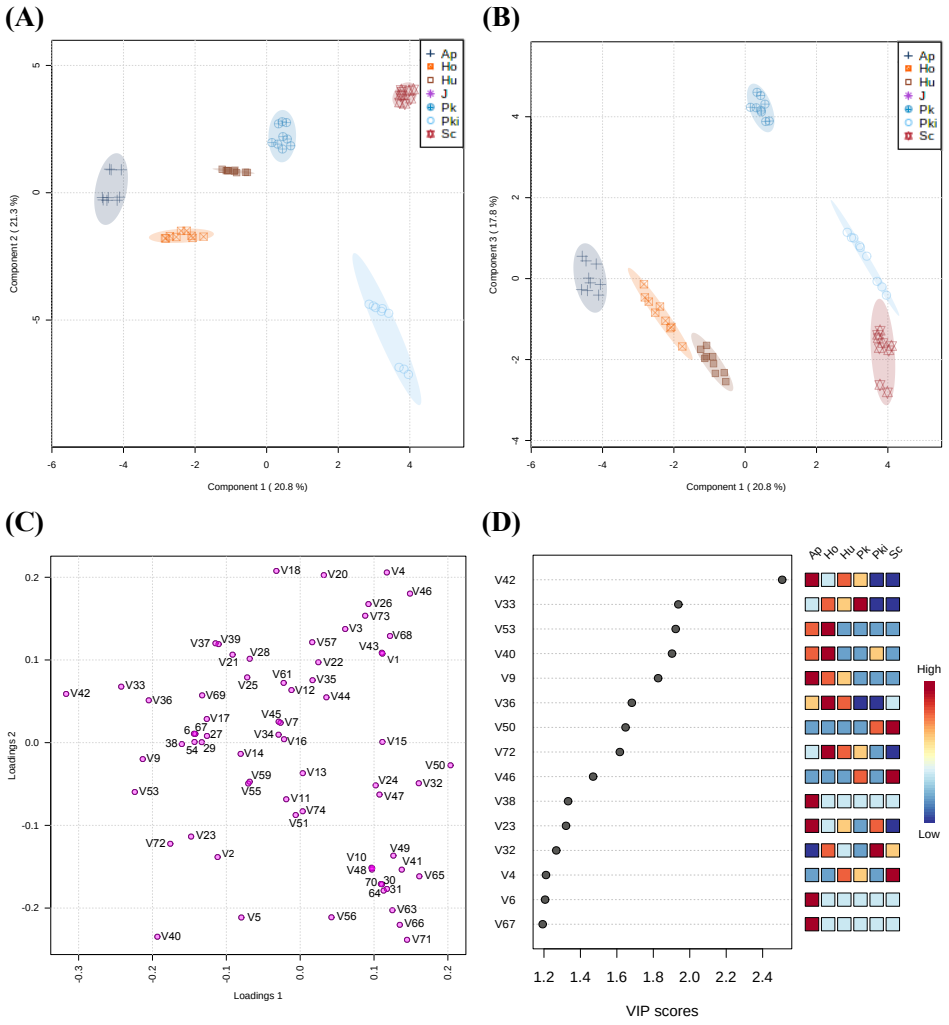

Figure 7. Partial least squares discriminant analysis (PLS-DA) performed on volatile compounds in inoculated fermentations by candidate yeast strains. (**A**,**B**) Score plots demonstrated a clear separation between inoculated fermentations and the variables could be explained with three components. Each symbol represents a yeast strain and ellipse around symbols represents at 95% confidence region of biological and technical triplicates. (**C**) Variables in loading plot of the components 1 and 2, and (**D**) selected compounds as important features based on variable important in projection (VIP) scores. The colored boxes on the right indicate the relative concentrations of the corresponding volatiles in each group with red (high) and blue (low).

Next, we asked whether important features identified using the VIP scores plot were associated with particular strains or categories of metabolites. Based on Pearson's correlation, we observed hierarchal clustering of 74 volatile metabolites against non-*Saccharomyces* strains compared to *S. cerevisiae* (Figure 8). Of particular interest is the group of metabolites associated with *P. kudriazevii* but not with other strains. Volatile metabolites associated with this cluster include 3-methyl-1-pentanol (V10); heptanoic acid, ethyl ester (V30); 2-hexenoic acid, ethyl ester (V31); propanoic acid, 2-methyl- (V47); butanoic acid (V48); butanoic acid, 3-methyl- (V49); benzaldehyde, 3-methyl- (V60); butane, 1-(1-ethoxyethoxy)- (V63); and butane, 1,1-diethoxy-3-methyl- (V64) was distinct compared to other strains. In addition, *A. pullulans* is associated with increased levels of 2-buten-1-ol, 3-methyl-, acetate (V6); 3-hexen-1-ol, acetate, (Z)- (V27); ethyl (S)-(-)-lactate (V29); acetyl valeryl (V38); L-à-terpineol (V54); 3(2H)-thiophenone, dihydro-2-methyl- (V67) in inoculated fermentations.

The other distinct group of compounds, 1-pentanol, 4-methyl- (V7); 2-buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)- (V45); benzaldehyde, 4-methyl- (V61) were positively correlated to *H. uvarum*. Pearson's correlation of candidate strains with relative abundance of volatile metabolites from inoculated fermentation could provide a unique fingerprint to further explore different combinations of non-*Saccharomyces* yeast strains in sequential or coinoculation.

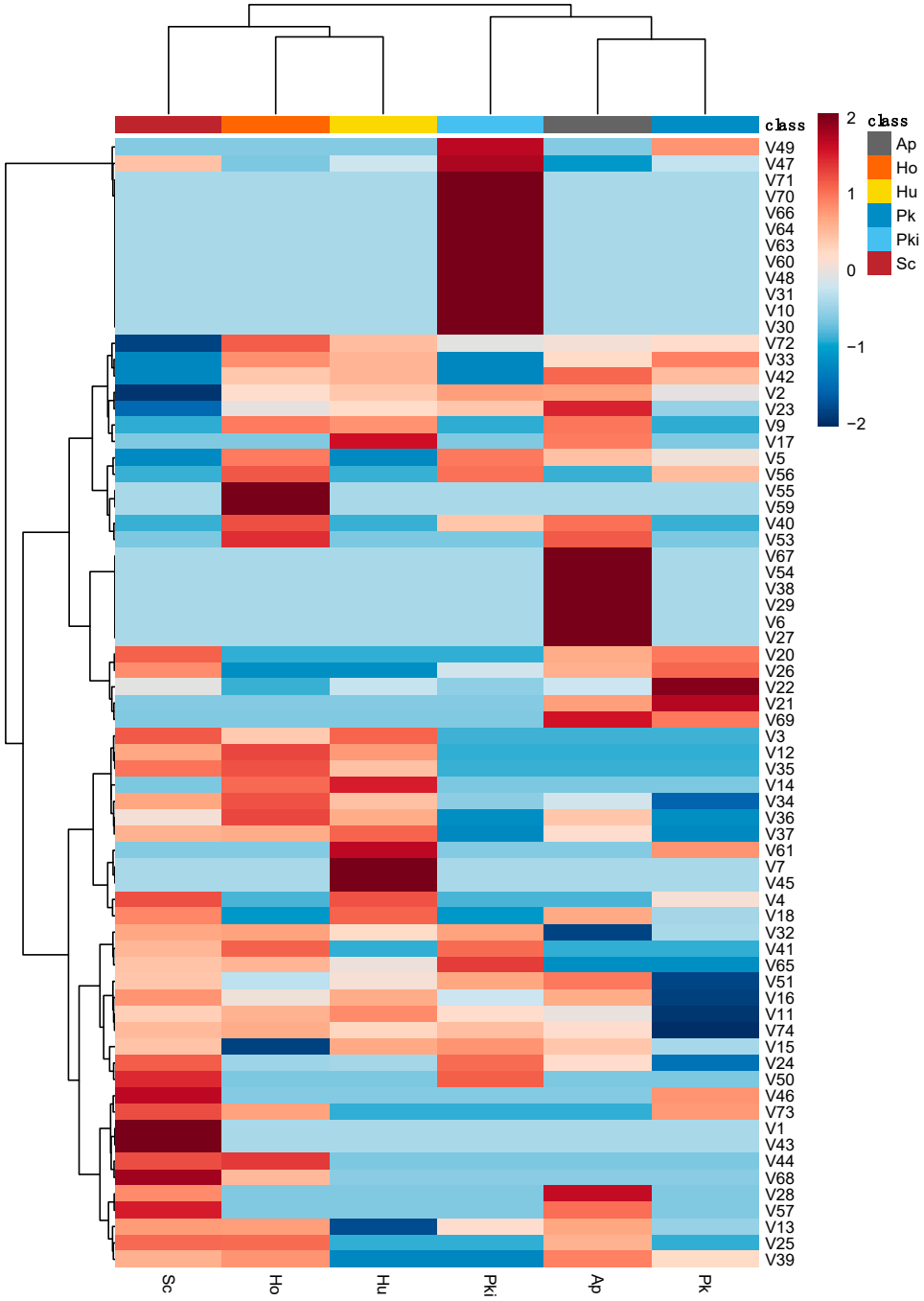

Figure 8. Clustered heatmap based on the volatile compounds in inoculated fermentations with candidate yeast strains compared to *S. cerevisiae* BY4742. Data were normalized by a pooled sample from the control group, *S. cerevisiae* BY4742. The correlation between each compound and candidate strain is illustrated with a chromatic scale (from dark blue, negative correlation, to dark red, positive correlation). Dendrogram for the hierarchical clustering was analyzed using Ward's cluster algorithm. Abbreviations: Ap, *A. pullulans* (class 1, black); Ho, *H. opuntiae* (class 2, orange); Hu, *H. uvarum* (class 3, yellow); Pk, *P. kluyveri* (class 4, dark blue); Pki, *P. kudriavzevii* (class 5, blue); Sc, *S. cerevisiae* (class 6, red).

4. Discussion

Terroir is an expression in viticulture used to describe the unique contribution of regional features such as cultivar, vintage, and climate, that define wine sensory characteristics and product identity in a particular region. Although much is known about *terroir* relative to *Vitis vinifera* varieties, much is unknown about hybrid grapes. In this work, 29 species were identified from 120 isolates and distinct patterns of microbial composition in three PA vineyards were observed. In support of a parallel study on Chambourcin microbiome, these results highlight unique differences in microbial populations even within a small geographic area (radius about 90 km) [60]. In 15 vineyards across southern Australia (5–400 km part), distinct microbial profiles were found in environmental samples collected from soil, plant, must, and across fermentation stages [61]. Previous studies also demonstrate that microbial populations can impact wine quality [62]. For example, *Starmerella bacillaris* and *Metschnikowia pulcherrima* isolated from multiple regions of Italy were found to increase production of higher alcohols such as β-phenylethyl alcohol corresponding to floral odor through coinoculation [12]. *Kazachstania aerobia* and *K. servazzii* in Shiraz grape must from southern Australia were found to have increased production of esters, such as phenylethyl acetate and isoamyl acetate associated to rose and fruity aroma [63]. These examples prompted us to ask whether microbial populations on Chambourcin hybrid grapes in PA vineyards can shape wine characteristics of the region.

Hanseniaspora spp. and *Pichia* spp. were the most dominant species identified during spontaneous fermentation of Chambourcin with relative abundances of 42% and 11% of total isolates, respectively (Figure 1 and Table S1). Previous studies have reported the dominance of *Hanseniaspora* and *Pichia* species on various grape varieties (i.e., *V. vinifera*, *V. labrusca*, and hybrid grapes), in grape must, and at early stages of fermentation [30,38,64–66]. The abundance of *Hanseniaspora* spp. and *Pichia* spp. appear as core non-*Saccharomyces* yeasts on wine grapes and could be related to nutrient availability of mature grape berries which supports fast growth of these species while suppressing other microorganisms [62]. In early stages of spontaneous fermentation, high abundance of *H. uvarum*, *P. klyuveri*, *P. novergenisis*, and *P. guilliermondii* were found on *V. vinifera* (Malbec), *V. labrusca* (Isabel and Bordeaux), and hybrid grapes (Zweigelt, cross between St. Laurent and Blaufränkisch) [65–67]. Therefore, our findings on the dominance of *Hanseniaspora* spp. and *Pichia* spp. further suggest these two species are the conserved components in microbial *terroir* of Chambourcin hybrid grapes.

Tolerance to sulfite and ethanol are two characteristics important in non-*Saccharomyces* strains for potential use in winemaking. In particular, *P. kudriavzevii* PSWCC102 can tolerate 10% (v/v) ethanol comparable to the control strain *S. cerevisiae*, whereas *Hanseniaspora* species PSWCC70 and PSWCC64 can tolerate up to 8% (v/v) ethanol. Several studies have indicated increased ethanol tolerance of up to 6% in some non-*Saccharomyces* yeasts such as *H. guilliermondii* and *C. stellate* [68]. Although there are fewer studies on mechanism of higher ethanol tolerance on non-*Saccharomyces* yeasts, increased ethanol tolerance in *S. cerevisiae* has been positively linked to the improvement of fermentation capacity and production of wine important flavor compounds [69,70]. Thus, it would be interesting in future studies to add *H. uvarum* PSWCC70, *H. opuntiae* PSWCC64, and *P. kudriavzevii* PSWCC102 in sequence or in combination with *S. cerevisiae* to assess improvement of flavor profile in wine fermentation.

Nonvolatile compounds typically contribute to production of conserved metabolites most easily perceived by sensory analysis of wines such as metabolites that contribute to the perception of sweetness, sourness, and mouthfeel (Figure 6). Previous study reported no more than 25% of tartaric acid degraded by *S. cerevisiae* and non-*Saccharomyces* species (*Kloeckera, Candida, Schizosaccharomyces,* and *Hansenula* spp.) which corresponds to our results except *H. uvarum* PSWCC70 [71]. The association between *H. uvarum* and degradation of tartaric acid could possibly be yeast strain using, for example, the carbon and energy source regardless of the presence of assimilable sugar [72]. *Hanseniaspora opuntiae* PSWCC64 producing higher concentration of succinic acid with no significant difference

with *S. cerevisiae* control strain might indicate a larger proportion of fermented sugar was used for the production of succinic acid [73]. Moreover, the trend of acetic acid production by yeast strains was similar to the glycerol production as the production of these two compounds has been linked together. According to these results, although nonvolatile profiles by candidate strains were clustered with control strain on PLS-DA score plot, different productions of nonvolatile compounds indicated that non-*Saccharomyces* yeasts utilized fermented sugar differently in the metabolic pathway to *S. cerevisiae*.

One area of interest in winemaking is strain innovation. Previous works have demonstrated that fermentation-derived metabolites from non-*Saccharomyces* yeast can contribute to unique volatile characteristics of final wines. We hypothesize that non-*Saccharomyces* isolates from local wineries can enhance and preserve regionality of final wines. To this end, we used GC-MS coupled with statistical methods that enable visualization of important volatile metabolites that drive differences between candidate strains. Notable differences in volatile wine-associated profile between candidate non-*Saccharomyces* yeasts and control strain were demonstrated by PLS-DA and Pearson's correlation. We were particularly interested in compounds that drive volatilome changes in non-*Saccharomyces* strains compared to BY4742 (Figure 7D). High VIP scores indicate important compounds positively correlated to *Hanseniaspora* strains PSWCC64 and PSWCC70 were 2-heptanol (V9) having fruity and herbaceous odor, ionone (V42) with floral and fruity odor, and 1-propanol, 3-(methylthio)-(V72) preserved odor of cauliflower and potato, which were negatively correlated to *S. cerevisiae* BY4742 [74–77]. This demonstrated the potential of candidate strains in winemaking to increase the complexity of wine aroma which *Saccharomyces* yeasts were less capable of contributing to wines. Of particular interest in this study, *P. kudriazevii* PSWCC102 positively correlates with a group of volatiles which is negatively correlated to other candidate strains. This included esters and acetals, such as heptanoic acid, ethyl ester (V30), 2-hexenoic acid, ethyl ester (V31), butane, 1-(1-ethoxyethoxy)- (V63), benzaldehyde, 3-methyl- (V60), and butane, 1,1-diethoxy-3-methyl- (V64) having pleasant fruity and herbaceous aroma [78–81]. In addition, *P. kudriavzevii* PSWCC102 demonstrates ethanol tolerance (10%) comparable to *S. cerevisiae* control strain. Therefore, *P. kudriazevii* PSWCC102 could be a potential candidate for future studies that investigate the sequential or coinoculation of *P. kudriasevii* during winemaking and production of fruity aroma compounds that are unique to the strain. Others have shown that sequential or coinoculation of non-*Saccharomyces* yeasts with *S. cerevisiae* has been demonstrated to increase complexity of wine-important volatiles and sensory qualities [82–85]. Conducting sensory analysis is also valuable to understand whether cofermentation could positively influence the sensory qualities and consumer preferences by considering the attributes of taste, flavor, mouthfeel, and color.

5. Conclusions

Our investigations on candidate strains isolated from local PA vineyards suggest the potential of non-*Saccharomyces* yeasts in winemaking based on the unique fermentation characteristics. Two candidate strains, *Hanseniaspora opuntiae* PSWCC64 and *Pichia kudriavzevii* PSWCC102 demonstrated relatively high tolerance to ethanol at 8–10% as well as the ability to produce volatile metabolites associated with flowery and fruity aroma which was negatively correlated to *S. cerevisiae* BY4742 control strain. This study provides an exciting step to incorporate non-*Saccharomyces* yeasts within winemaking of hybrid grapes to enhance regional characteristics and quality of hybrid grapes. Other isolated strains in our collection constitutes a valuable source for more microbiological, evolutionary, and ecological studies to provide more beneficial knowledge to the Pennsylvania Wine Industry and fortify the interest in the novel non-*Saccharomyces* yeasts as the wine starters.

Supplementary Materials: The following are available online at https://www.mdpi.com/2311-5637/7/1/15/s1, Figure S1: Fermentation kinetics of candidate non-*Saccharomyces* strains compared with control *S. cerevisiae* BY4742 inoculated into sterile juice. Figure S2. Volatile compounds in sterile juice and inoculated fermentations grouped based on chemical structures. Table S1. Fungal species

identified from spontaneously fermenting Chambourcin grapes isolated from three Pennsylvania vineyards at 0 and 24 h combined by number of species and relative abundance (%). Table S2. List of volatiles detected in sterile juice and inoculated fermentations with candidate non-*Saccharomyces* strains and *S. cerevisiae* BY4742 with mean retention time (RT) and Kovats retention indices (RI). Table S3. Volatile compounds with significant difference (ANOVA, FDR-adjusted p-value < 0.05) across inoculated fermentations with candidate yeast strains. Data shown here were after normalization by control group, *S. cerevisiae* BY4742.

Author Contributions: Conceptualization and methodology, C.T.F., X.D., and J.W.; validation, formal analysis, and investigation, C.T.F.; resources, J.W.; data curation and visualization, C.T.F. and J.W.; writing—original draft preparation, C.T.F.; writing—review and editing, X.D. and J.W.; supervision, project administration and funding acquisition, J.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by U.S. Department of Agriculture National Institute of Food and Agriculture and Hatch Appropriations under Project #PEN04699 and Accession #1019351 and the Crouch Endowment for Viticulture, Enology, and Pomology Research.

Institutional Review Board Statement: Not applicable to this study.

Informed Consent Statement: Not applicable to this study.

Data Availability Statement: The data presented in this study are available in article: ITS sequences for this study have been deposited into NCBI (#Accession MW301459-MW301578).

Acknowledgments: The authors acknowledge and thank the generous support and participation of Pennsylvania wineries (Happy Valley Vineyards and Winery, Shade Mountain Winery and Vineyards, and Nissley Vineyards and Winery) during experimental design, sample collection, and handling. Technical expertise and training related to UHPLC analysis was conducted with the help of Ashik Sathish (CSL Behring Fermentation Facility). The authors also acknowledge the technical expertise of Helene Hopfer and Hung Li Wang with GC-MS analysis.

Conflicts of Interest: The authors declare no conflict of interest and the funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Legras, J.-L.; Merdinoglu, D.; Cornuet, J.-M.; Karst, F. Bread, Beer and Wine: Saccharomyces Cerevisiae Diversity Reflects Human History. *Mol. Ecol.* **2007**, *16*, 2091–2102. [CrossRef] [PubMed]
2. Johnson, E.A.; Echavarri-Erasun, C. Yeast Biotechnology. In *The Yeasts*; Elsevier: Amsterdam, The Netherlands, 2011; Volume 1, pp. 21–44, ISBN 9780444521491.
3. Contreras, A.; Hidalgo, C.; Henschke, P.A.; Chambers, P.J.; Curtin, C.; Varela, C. Evaluation of Non-Saccharomyces Yeasts for the Reduction Of Alcohol Content in Wine. *Appl. Environ. Microbiol.* **2014**, *80*, 1670–1678. [CrossRef] [PubMed]
4. Lemos Junior, W.J.F.; Nadai, C.; Crepalde, L.T.; De Oliveira, V.S.; De Matos, A.D.; Giacomini, A.; Corich, V. Potential Use of Starmerella Bacillaris as Fermentation Starter for the Production of Low-Alcohol Beverages Obtained from Unripe Grapes. *Int. J. Food Microbiol.* **2019**, *303*, 1–8. [CrossRef] [PubMed]
5. Gamero, A.; Quintilla, R.; Groenewald, M.; Alkema, W.; Boekhout, T.; Hazelwood, L. High-Throughput Screening of a Large Collection of Non-Conventional Yeasts Reveals Their Potential for Aroma Formation in Food Fermentation. *Food Microbiol.* **2016**, *60*, 147–159. [CrossRef] [PubMed]
6. Escribano-Viana, R.; González-Arenzana, L.; Portu, J.; Garijo, P.; López-Alfaro, I.; López, R.; Santamaría, P.; Gutiérrez, A.R. Wine Aroma Evolution Throughout Alcoholic Fermentation Sequentially Inoculated with Non- Saccharomyces/Saccharomyces Yeasts. *Food Res. Int.* **2018**, *112*, 17–24. [CrossRef]
7. Chen, K.; Escott, C.; Loira, I.; Del Fresno, J.M.; Morata, A.; Tesfaye, W.; Calderon, F.; Suárez-Lepe, J.A.; Han, S.; Benito, S. Use of Non-Saccharomyces Yeasts and Oenological Tannin in Red Winemaking: Influence on Colour, Aroma and Sensorial Properties of Young Wines. *Food Microbiol.* **2018**, *69*, 51–63. [CrossRef]
8. Contreras, A.; Hidalgo, C.; Schmidt, S.; Henschke, P.A.; Curtin, C.; Varela, C. The Application of Non-Saccharomyces Yeast in Fermentations with Limited Aeration as a Strategy for the Production of Wine with Reduced Alcohol Content. *Int. J. Food Microbiol.* **2015**, *205*, 7–15. [CrossRef]
9. Goold, H.D.; Kroukamp, H.; Williams, T.C.; Paulsen, I.T.; Varela, C.; Pretorius, I.S. Yeast's Balancing Act Between Ethanol And Glycerol Production In Low-Alcohol Wines. *Microb. Biotechnol.* **2017**, *10*, 264–278. [CrossRef]

10. Tristezza, M.; Tufariello, M.; Capozzi, V.; Spano, G.; Mita, G.; Grieco, F. The Oenological Potential of Hanseniaspora Uvarum In Simultaneous And Sequential Co-Fermentation With Saccharomyces Cerevisiae For Industrial Wine Production. *Front. Microbiol.* **2016**, *7*, 670. [CrossRef]
11. Englezos, V.; Torchio, F.; Cravero, F.; Marengo, F.; Giacosa, S.; Gerbi, V.; Rantsiou, K.; Rolle, L.; Cocolin, L. Aroma Profile And Composition Of Barbera Wines Obtained By Mixed Fermentations Of Starmerella Bacillaris (Synonym Candida Zemplinina) And Saccharomyces Cerevisiae. *LWT Food Sci. Technol.* **2016**, *73*, 567–575. [CrossRef]
12. Binati, R.L.; Lemos Junior, W.J.F.; Luzzini, G.; Slaghenaufi, D.; Ugliano, M.; Torriani, S. Contribution Of Non-Saccharomyces Yeasts To Wine Volatile And Sensory Diversity: A Study On Lachancea Thermotolerans, Metschnikowia Spp. And Starmerella Bacillaris Strains Isolated In Italy. *Int. J. Food Microbiol.* **2020**, *318*, 108470. [CrossRef] [PubMed]
13. Jackson, R.S. Chemical Constituents of Grapes and Wine. In *Wine Science*; Elsevier: Amsterdam, The Netherlands, 2014; pp. 347–426.
14. Vaudano, E.; Quinterno, G.; Costantini, A.; Pulcini, L.; Pessione, E.; Garcia-Moruno, E. Yeast Distribution In Grignolino Grapes Growing In A New Vineyard In Piedmont And The Technological Characterization Of Indigenous *Saccharomyces* spp. Strains. *Int. J. Food Microbiol.* **2019**, *289*, 154–161. [CrossRef] [PubMed]
15. Fleet, G.H. Wine Yeasts For The Future. In Proceedings of the Fems Yeast Research; Oxford Academic: Oxford, UK, 2008; Volume 8, pp. 979–995.
16. Jones, N.K. The Influence of Recent Climate Change on Wine Regions in Quebec, Canada. *J. Wine Res.* **2012**, *23*, 103–113. [CrossRef]
17. Pedneault, K.; Provost, C. Fungus Resistant Grape Varieties as a Suitable Alternative for Organic Wine Production: Benefits, Limits, and Challenges. *Sci. Hortic. (Amst.)* **2016**, *208*, 57–77. [CrossRef]
18. Ghareib, M.; Youssef, K.A.; Khalil, A.A. Ethanol Tolerance of Saccharomyces Cerevisiae and Its Relationship to Lipid Content and Composition. *Folia Microbiol.* **1988**, *33*, 447–452. [CrossRef]
19. Cray, J.A.; Bell, A.N.W.; Bhaganna, P.; Mswaka, A.Y.; Timson, D.J.; Hallsworth, J.E. The Biology of Habitat Dominance; Can Microbes Behave As Weeds? *Microb. Biotechnol.* **2013**, *6*, 453–492. [CrossRef]
20. Brown, S.W.; Oliver, S.G.; Harrison, D.E.F.; Righelato, R.C. Ethanol Inhibition of Yeast Growth and Fermentation: Differences in the Magnitude and Complexity of the Effect. *Eur. J. Appl. Microbiol. Biotechnol.* **1981**, *11*, 151–155. [CrossRef]
21. Aguilera, F.; Peinado, R.A.; Millán, C.; Ortega, J.M.; Mauricio, J.C. Relationship Between Ethanol Tolerance, H+-Atpase Activity And The Lipid Composition Of The Plasma Membrane In Different Wine Yeast Strains. *Int. J. Food Microbiol.* **2006**, *110*, 34–42. [CrossRef]
22. Zhang, Q.; Wu, D.; Lin, Y.; Wang, X.; Kong, H.; Tanaka, S. Substrate and Product Inhibition on Yeast Performance in Ethanol Fermentation. *Energy Fuels* **2015**, *29*, 1019–1027. [CrossRef]
23. Lopez, E.F.; Gomez, E.F. Simultaneous Determination of the Major Organic Acids, Sugars, Glycerol, and Ethanol by Hplc in Grape Musts and White Wines. *J. Chromatogr. Sci.* **2013**, *34*, 254–257. [CrossRef]
24. Rapp, A.; Mandery, H. Wine Aroma. *Experientia* **1986**, *42*, 873–884. [CrossRef]
25. Styger, G.; Prior, B.; Bauer, F.F. Wine Flavor and Aroma. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 1145–1159. [CrossRef] [PubMed]
26. Padilla, B.; Gil, J.V.; Manzanares, P. Past And Future Of Non-Saccharomyces Yeasts: From Spoilage Microorganisms To Biotechnological Tools For Improving Wine Aroma Complexity. *Front. Microbiol.* **2016**, *7*, 411. [CrossRef] [PubMed]
27. Ebeler, S.E.; Thorngate, J.H. Wine Chemistry and Flavor: Looking Into the Crystal Glass. *J. Agric. Food Chem.* **2009**, *57*, 8098–8108. [CrossRef] [PubMed]
28. General Industry Stats. United States Wine and Grape Industry FAQS | Wineamerica. International Association of America Wineries. 2019. Available online: https://Wineamerica.Org/Policy/By-The-Numbers/ (accessed on 18 September 2020).
29. Pennsylvania Winery Association. About Pennsylvania Wines—Pennsylvania Wines. 2020. Available online: Https://Pennsylvaniawine.Com/About/ (accessed on 18 September 2020).
30. Raymond Eder, M.L.; Reynoso, C.; Lauret, S.C.; Rosa, A.L. Isolation and Identification of the Indigenous Yeast Population during Spontaneous Fermentation of Isabella (*Vitis Labrusca* L.) Grape Must. *Front. Microbiol.* **2017**, *8*, 532. [CrossRef]
31. Smith, N.R.; Dawson, V.T. The Bacteriostatic Action of Rose Bengal in Media Used For Plate Counts of Soil Fungi. *Soil Sci.* **1944**, *58*, 467–472. [CrossRef]
32. Salfinger, Y.; Tortorello, M.L. *Compendium of Methods for the Microbiological Examination of Foods*; American Public Health Association: Washington, DC, USA, 2013.
33. Henson, O.E. Dichloran as an Inhibitor of Mold Spreading In Fungal Plating Media: Effects on Colony Diameter and Enumeration. *Appl. Environ. Microbiol.* **1981**, *42*, 656–660. [CrossRef]
34. Dobrowolski, P. Short protocols in molecular biology. A compendium of methods from "current protocols in molecular biology". edited by Frederick, M. Ausubel et al., John Wiley & Sons, 1992., ISBN 0-471-57735-9. *Acta Biotechnologica.* **1993**, *13*, 88.
35. Schoch, C.L.; Seifert, K.A.; Huhndorf, S.; Robert, V.; Spouge, J.L.; Levesque, C.A.; Chen, W.; Consortium, F.B. Nuclear Ribosomal Internal Transcribed Spacer (Its) Region As A Universal Dna Barcode Marker For Fungi. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 6241–6246. [CrossRef]
36. Zabukovec, P.; Čadež, N.; Čuš, F. Isolation and Identification of Indigenous Wine Yeasts and Their Use in Alcoholic Fermentation. *Food Technol. Biotechnol.* **2020**, *58*, 337–347. [CrossRef]

37. White, T.; Bruns, T.; Lee, S.; Taylor, J. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Mycological Society of America: Albuquerque, NM, USA, 1990; Volume 18, pp. 315–322.
38. Brysch-Herzberg, M.; Seidel, M. Yeast Diversity on Grapes in Two German Wine Growing Regions. *Int. J. Food Microbiol.* **2015**, *214*, 137–144. [CrossRef] [PubMed]
39. Crous, P.W.; Gams, W.; Stalpers, J.A.; Robert, V.; Stegehuis, G. Mycobank: An Online Initiative to Launch Mycology into the 21st Century. *Stud. Mycol.* **2004**, *50*, 19–22.
40. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. Mega X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]
41. Stecher, G.; Tamura, K.; Kumar, S. Molecular Evolutionary Genetics Analysis (Mega) For Macos. *Mol. Biol. Evol.* **2020**, *37*, 1237–1239. [CrossRef] [PubMed]
42. Edgar, R.C. Muscle: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [CrossRef]
43. Buehler, A.J.; Evanowski, R.L.; Martin, N.H.; Boor, K.J.; Wiedmann, M. Internal Transcribed Spacer (Its) Sequencing Reveals Considerable Fungal Diversity in Dairy Products. *J. Dairy Sci.* **2017**, *100*, 8814–8825. [CrossRef]
44. Tofalo, R.; Schirone, M.; Torriani, S.; Rantsiou, K.; Cocolin, L.; Perpetuini, G.; Suzzi, G. Diversity of Candida Zemplinina Strains From Grapes And Italian Wines. *Food Microbiol.* **2012**, *29*, 18–26. [CrossRef]
45. Englezos, V.; Rantsiou, K.; Torchio, F.; Rolle, L.; Gerbi, V.; Cocolin, L. Exploitation of the Non-Saccharomyces Yeast Starmerella Bacillaris (Synonym Candida Zemplinina) In Wine Fermentation: Physiological and Molecular Characterizations. *Int. J. Food Microbiol.* **2015**, *199*, 33–40. [CrossRef]
46. Capece, A.; Siesto, G.; Romaniello, R.; Lagreca, V.M.; Pietrafesa, R.; Calabretti, A.; Romano, P. Assessment Of Competition In Wine Fermentation Among Wild Saccharomyces Cerevisiae Strains Isolated From Sangiovese Grapes In Tuscany Region. *LWT Food Sci. Technol.* **2013**, *54*, 485–492. [CrossRef]
47. Maturano, Y.P.; Mestre, M.V.; Kuchen, B.; Toro, M.E.; Mercado, L.A.; Vazquez, F.; Combina, M. Optimization Of Fermentation-Relevant Factors: A Strategy To Reduce Ethanol In Red Wine By Sequential Culture Of Native Yeasts. *Int. J. Food Microbiol.* **2019**, *289*, 40–48. [CrossRef]
48. Threlfall, R.; Main, G.; Morris, J. Effect of Freezing Grape Berries and Heating Must Samples on Extraction of Components and Composition Parameters of Red Wine Grape Varieties. *Aust. J. Grape Wine Res.* **2006**, *12*, 161–169. [CrossRef]
49. Chidi, B.S.; Rossouw, D.; Buica, A.S.; Bauer, F.F. Determining The Impact Of Industrial Wine Yeast Strains On Organic Acid Production Under White And Red Wine-Like Fermentation Conditions. *S. Afr. J. Enol. Vitic.* **2015**, *36*, 316–327. [CrossRef]
50. Pedneault, K.; Dorais, M.; Angers, P. Flavor of Cold-Hardy Grapes: Impact of Berry Maturity and Environmental Conditions. *J. Agric. Food Chem.* **2013**, *61*, 10418–10438. [CrossRef] [PubMed]
51. Fenoll, J.; Manso, A.; Hellín, P.; Ruiz, L.; Flores, P. Changes In The Aromatic Composition Of The Vitis Vinifera Grape Muscat Hamburg During Ripening. *Food Chem.* **2009**, *114*, 420–428. [CrossRef]
52. Bromba, M.U.A.; Ziegler, H. Application Hints for Savitzky-Holay Digital Smoothing Filters. *Anal. Chem.* **1981**, *53*, 1583–1586. [CrossRef]
53. Wenig, P.; Odermatt, J. *Openchrom: A Cross-Platform Open Source Software for the Mass Spectrometric Analysis of Chromatographic Data*; BMC Bioinformatics: Hamburg, Germany, 2010.
54. Schafer, R.W. What Is A Savitzky-Golay Filter? *IEEE Signal Process. Mag.* **2011**, *28*, 111–117. [CrossRef]
55. Johnsen, L.G.; Skou, P.B.; Khakimov, B.; Bro, R. Gas Chromatography—Mass Spectrometry Data Processing Made Easy. *J. Chromatogr. A* **2017**, *1503*, 57–64. [CrossRef]
56. Field, A. *Discovering Statistics Using Ibm Spss Statistics*; SAGE Publications Ltd.: Thousand Oaks, CA, USA, 2013; pp. 1–816.
57. Chong, J.; Wishart, D.S.; Xia, J. Using Metaboanalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Curr. Protoc. Bioinform.* **2019**, *68*, e86. [CrossRef]
58. Van Den Berg, R.A.; Hoefsloot, H.C.J.; Westerhuis, J.A.; Smilde, A.K.; Van Der Werf, M.J. Centering, Scaling, And Transformations: Improving The Biological Information Content Of Metabolomics Data. *Bmc Genom.* **2006**, *7*, 142. [CrossRef]
59. Manachini, P.L.; Parini, C.; Fortina, M.G. Pectic Enzymes from Aureobasidium Pullulans Lv 10. *Enzyme Microb. Technol.* **1988**, *10*, 682–685. [CrossRef]
60. Wang, H.L. Characterization of Microbial Dynamics and Volatile Metabolome Changes during Fermentation of Chambourcin Grapes in Two Pennsylvania Regions. Master Thesis, The Pennsylvania State University, The Graduate School, State College, PA, USA, 2020.
61. Liu, D.; Chen, Q.; Zhang, P.; Chen, D.; Howell, K.S. The Fungal Microbiome Is An Important Component Of Vineyard Ecosystems And Correlates With Regional Distinctiveness Of Wine. *Msphere* **2020**, *5*, e00534-20. [CrossRef] [PubMed]
62. Barata, A.; Malfeito-Ferreira, M.; Loureiro, V. The Microbial Ecology of Wine Grape Berries. *Int. J. Food Microbiol.* **2012**, *153*, 243–259. [CrossRef] [PubMed]
63. Lin, M.M.H.; Boss, P.K.; Walker, M.E.; Sumby, K.M.; Grbin, P.R.; Jiranek, V. Evaluation Of Indigenous Non-Saccharomyces Yeasts Isolated From A South Australian Vineyard For Their Potential As Wine Starter Cultures. *Int. J. Food Microbiol.* **2020**, *312*, 108373. [CrossRef] [PubMed]

64. Baffi, M.A.; Dos Santos Bezerra, C.; Arévalo-Villena, M.; Isabel Briones-Pérez, A.; Gomes, E.; Da Silva, R. Isolation And Molecular Identification Of Wine Yeasts From A Brazilian Vineyard. *Ann. Microbiol.* **2011**, *61*, 75–78. [CrossRef]
65. Bezerra-Bussoli, C.; Baffi, M.A.; Gomes, E.; Da-Silva, R. Yeast Diversity Isolated From Grape Musts during Spontaneous Fermentation from A Brazilian Winery. *Curr. Microbiol.* **2013**, *67*, 356–361. [CrossRef]
66. Eder, M.L.R.; Conti, F.; Rosa, A.L. Differences between Indigenous Yeast Populations in Spontaneously Fermenting Musts from V. Vinifera L. And V. Labrusca L. Grapes Harvested In The Same Geographic Location. *Front. Microbiol.* **2018**, *9*, 1320. [CrossRef]
67. Lopandic, K.; Tiefenbrunner, W.; Gangl, H.; Mandl, K.; Berger, S.; Leitner, G.; Abd-Ellah, G.A.; Querol, A.; Gardner, R.C.; Sterflinger, K.; et al. Molecular Profiling Of Yeasts Isolated During Spontaneous Fermentations Of Austrian Wines. *FEMS Yeast Res.* **2008**, *8*, 1063–1075. [CrossRef]
68. Pina, C.; Santos, C.; Couto, J.A.; Hogg, T. Ethanol Tolerance of Five Non-Saccharomyces Wine Yeasts In Comparison With A Strain of Saccharomyces Cerevisiae—Influence Of Different Culture Conditions. *Food Microbiol.* **2004**, *21*, 439–447. [CrossRef]
69. Kim, I.S.; Kim, Y.S.; Kim, H.; Jin, I.; Yoon, H.S. Saccharomyces Cerevisiae Knu5377 Stress Response During High-Temperature Ethanol Fermentation. *Mol. Cells* **2013**, *35*, 210–218. [CrossRef]
70. Yang, Y.; Xia, Y.; Lin, X.; Wang, G.; Zhang, H.; Xiong, Z.; Yu, H.; Yu, J.; Ai, L. Improvement Of Flavor Profiles In Chinese Rice Wine By Creating Fermenting Yeast With Superior Ethanol Tolerance And Fermentation Activity. *Food Res. Int.* **2018**, *108*, 83–92. [CrossRef]
71. Gao, C.; Fleet, G.H. Degradation Of Malic And Tartaric Acids By High Density Cell Suspensions Of Wine Yeasts. *Food Microbiol.* **1995**, *12*, 65–71. [CrossRef]
72. Hong, S.K.; Lee, H.J.; Park, H.J.; Hong, Y.A.; Rhee, I.K.; Lee, W.H.; Choi, S.W.; Lee, O.S.; Park, H.D. Degradation Of Malic Acid In Wine By Immobilized Issatchenkia Orientalis Cells With Oriental Oak Charcoal And Alginate. *Lett. Appl. Microbiol.* **2010**, *50*, 522–529. [CrossRef] [PubMed]
73. Nghiem, N.P.; Kleff, S.; Schwegmann, S. Succinic Acid: Technology Development and Commercialization. *Fermentation* **2017**, *3*, 26. [CrossRef]
74. Qian, M.C.; Wang, Y. Seasonal Variation of Volatile Composition and Odor Activity Value of "Marion" (*Rubus* spp. hyb) and "Thornless Evergreen" (R. laciniatus L.) Blackberries. *J. Food Sci.* **2005**, *70*, C13–C20. [CrossRef]
75. Plotto, A.; Barnes, K.W.; Goodner, K.L. Specific Anosmia Observed For β-Ionone, But Not For α-Ionone: Significance for Flavor Research. *J. Food Sci.* **2006**, *71*, S401–S406. [CrossRef]
76. Tripathi, J.; Chatterjee, S.; Gamre, S.; Chattopadhyay, S.; Variyar, P.S.; Sharma, A. Analysis Of Free And Bound Aroma Compounds Of Pomegranate (*Punica Granatum* L.). *LWT Food Sci. Technol.* **2014**, *59*, 461–466. [CrossRef]
77. Deed, R.C.; Hou, R.; Kinzurik, M.I.; Gardner, R.C.; Fedrizzi, B. The Role Of Yeast *Aro8, Aro9* And *Aro10* Genes In The Biosynthesis Of 3-(Methylthio)-1-Propanol From *L*-Methionine During Fermentation In Synthetic Grape Medium. *FEMS Yeast Res.* **2019**, *19*, 109. [CrossRef]
78. Schreier, P.; Paroschy, J.H. Volatile Constituents from Concord, Niagara (*Vitis Labrusca*, L.) And Elvira (*V. Labrusca*, L. × *V. Riparia*, M.) Grapes. *Can. Inst. Food Sci. Technol. J.* **1981**, *14*, 112–118. [CrossRef]
79. Zakarya, D.; Yahiaoui, M.; Fkih-Tetouani, S. Structure–Odour Relations for Bitter Almond Odorants. *J. Phys. Org. Chem.* **1993**, *6*, 627–633. [CrossRef]
80. Masino, F.; Montevecchi, G.; Riponi, C.; Antonelli, A. Composition Of Some Commercial Grappas (Grape Marc Spirit): The Anomalous Presence Of 1,1-Diethoxy-3-Methylbutane: A Case Study. *Eur. Food Res. Technol.* **2009**, *228*, 565–569. [CrossRef]
81. Niu, Y.; Yao, Z.; Xiao, Z.; Zhu, G.; Zhu, J.; Chen, J. Sensory Evaluation Of The Synergism Among Ester Odorants In Light Aroma-Type Liquor By Odor Threshold, Aroma Intensity And Flash Gc Electronic Nose. *Food Res. Int.* **2018**, *113*, 102–114. [CrossRef] [PubMed]
82. Henschke, P. Stuck Fermentation: Causes, Prevention And Cure. In *Advances in Juice Clarification and Yeast Inoculation*; Australian Society of Viticulture and Oenology: Adelaide, Australia, 1997; pp. 30–41.
83. Sadineni, V.; Kondapalli, N.; Reddy Obulam, V.S. Effect Of Co-Fermentation With Saccharomyces Cerevisiae And Torulaspora Delbrueckii Or Metschnikowia Pulcherrima On The Aroma And Sensory Properties Of Mango Wine. *Ann. Microbiol.* **2012**, *62*, 1353–1360. [CrossRef]
84. Maisonnave, P.; Sanchez, I.; Moine, V.; Dequin, S.; Galeote, V. Stuck Fermentation: Development of a Synthetic Stuck Wine and Study of a Restart Procedure. *Int. J. Food Microbiol.* **2013**, *163*, 239–247. [CrossRef] [PubMed]
85. Querol, A.; Pérez-Torrado, R.; Alonso-Del-Real, J.; Minebois, R.; Stribny, J.; Oliveira, B.M.; Barrio, E. New Trends in the Uses of Yeasts in Oenology. In *Advances in Food and Nutrition Research*; Academic Press: Cambridge, MA, USA, 2018; Volume 85, pp. 177–210, ISBN 9780128150894.