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Temporal Comparison of Microbial Community Structure in an Australian Winery

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Abstract: Most modern fermented foods and beverages are produced in fit-for-purpose facilities which are designed to ensure not only a reliable product, but also one safe for consumption. Despite careful hygiene, microorganisms can colonise these facilities and establish resident populations that can potentially contribute to the fermentation process. Although some microorganisms may not negatively affect the final product, spoilage microorganisms can be detrimental for quality, generating substantial economic losses. Here, amplicon-based phylotyping was used to map microbial communities within an Australian winery, before, during and after the 2020 vintage. Resident bacterial and yeast populations were shown to change over time, with both relative abundance and location within the winery varying according to sampling date. The bacterial family *Micrococcaceae*, and the genera *Sphingomonas* and *Brevundimonas* were the most abundant bacterial taxonomies, while *Naganishia*, *Pyrenochaeta* and *Didymella* were the most abundant fungal genera. Mapping the spatial distributions of the microbial populations identified the main locations that harboured these resident microorganisms, that include known wine spoilage yeasts and bacteria. Wine spoilage microorganisms, including the genefugura *Lactobacillus*, *Acetobacter*, *Gluconobacter* and *Brettanomyces* showed very low relative abundance and were found only in a couple of locations within the winery. Microbial populations detected in this facility were also compared to the resident microbiota identified in other fermented food facilities, revealing that microbial population structures may reflect the nature of the product created in each facility.

Keywords: metagenomics; winery; yeast; bacteria; wine

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1. Introduction

Fermentation is one of the oldest practices for food preservation. Indeed, archaeological evidence suggests that the oldest fermented beverage, which was made of grapes, hawthorn berries, honey, and rice, was produced between 7000 and 6650 BCE [1]. Nowadays, approximately 5000 different fermented foods and beverages are estimated to be produced around the world [2]. Alcoholic and non-alcoholic fermented products are made from a wide range of sources, including meat and dairy products, grains, fruits and vegetables [2,3].

Microbial activity is inherent to all fermented products, with microorganisms either introduced deliberately by direct inoculation, or unintentionally via raw materials and/or the processing environment [4]. Most modern fermented products are created in specially designed facilities and while these are devised to reduce the risk of spoilage, food facilities are not abiotic [5,6]. These facilities are susceptible to colonisation by microorganisms from various sources, including raw materials, air and humans [6].

Currently, there is an increasing interest in understanding the microbial communities that inhabit food processing facilities [6–9], as well as those involved in the fermentation of traditional and/or indigenous foods and beverages [10–12] using culture-independent

techniques. Linking bacterial and/or fungal populations to production outcomes enables mitigation of safety and spoilage risks and the delivery of consistent products with desirable qualities for consumers [8]. Resident microbial communities have been studied in breweries [13,14], cheese making facilities [4,15,16], meat processing facilities [9,17,18], a vegetable fermentation facility [8] and wineries [7,19].

During winemaking, grape bunches are transported to the winery, crushed, pressed, fermented and aged prior to bottling [19]. Numerous fungal species live on the surfaces of vines and grapes and inhabit the vineyard soil [20,21], while many others reside in the winery/cellar environment [19,22–25]. Wine fermentation is conducted by a consortium of wine yeast and bacteria that establish themselves either from the grape surface or from the winery via shared equipment or other vectors such as insects [26]. Thus, vineyard and winery microbial communities have the potential to participate during fermentation and influence wine flavour and aroma [26]. However, several microorganisms within these communities are known to have the potential to spoil wine [27–29] generating significant economic costs to reduce the risk of infection and to treat infected wines [30].

Here, amplicon-based microbial profiling (phylotyping) was used to map the microbial communities that inhabit an Australian winery. Population structure was determined at three different dates, before, during and after vintage, and compared to the microbiota found in other fermentation facilities, including two wineries.

2. Materials and Methods

2.1. Winery Sampling

Samples were collected from the Hickinbotham Roseworthy Wine Science Laboratory (HRWSL) located on the Waite campus of the University of Adelaide, Adelaide, Australia, mean annual temperature 15–22 °C. This winery and research facility has a capacity of 200 tonnes and is used for teaching, research and commercial wine production. HRWSL floors were pressure cleaned with cold water before and after vintage and no chemicals were used in this process. During vintage, excess grape material was removed by hosing down with cold water every night. Samples were taken from 40 locations spread across the concrete floor of the main HRWSL operations/fermentation area (Figure 1A), using sterile, cotton-tipped swabs moistened with NRS medium (NRSII Transwab, Rowe Scientific, Adelaide, Australia). Each swab was streaked over a 10 cm^2 area, with sampling locations set out in a 5 × 8 grid pattern that covered a total area of 622 m^2 (Figure 1B). Sampling occurred at three different dates, before vintage (December 2019), during vintage (March 2020) and after vintage (June 2020). After sampling, tubes containing NRS media were centrifuged for 5 min at 16,000× *g*, with pellets stored at −20 °C.

2.2. Determination of Microbial Populations

Total DNA was isolated from each sample using the DNeasy PowerFood Microbial kit (Qiagen, Hilden, Germany). Bead-beating was carried out using a combination of 0.1 mm and 0.5 mm zirconia/silica beads (BioSpec Products, Oklahoma, OK, USA) in a Precellys Evolution homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at 8000 RPM for 4 × 60 s. Then, 1 ng of total DNA from each sample was subjected to a three-step PCR process targeting either the V3–V4 region of the bacterial 16S rRNA [31] or the ITS2 locus from the fungal ribosomal internal transcribed spacer (ITS) region [32] while adding both custom in-line barcodes and sequences necessary for Illumina sequencing (including compatible Illumina dual-indexes) Sequencing was performed using 2 × 300 bp chemistry on an Illumina MiSeq sequencer (Ramaciotti Centre for Genomics, Sydney, Australia). Following sequencing, paired-end reads were quality trimmed (Trimmomatic v0.38) [33], adaptor trimmed (Cutadapt v1.16) [34] and merged into single synthetic reads (FLASH2 v2.2.00) [35]. Merged reads were de-replicated (USEARCH v10.0.240) [36] and clustered (Swarm v2.2.2) [37] into operational taxonomic units (OTUs) as presented previously [38]. Fungal taxonomic annotation was performed against the UNITE database (qiime_ver8_dynamic_02.02.2019) using a 98% similarity cut off and the assign_taxonomy.py

module of QIIME v1.9.1 [39], while bacterial taxonomy used the SILVA 16S database (version 132 QIIME release) as a reference. Abundances for all bacterial and fungal OTUs identified in this study are listed in Tables S1 and S2, respectively. Raw reads are available from the BioProject website (accession number PRJNA742433).

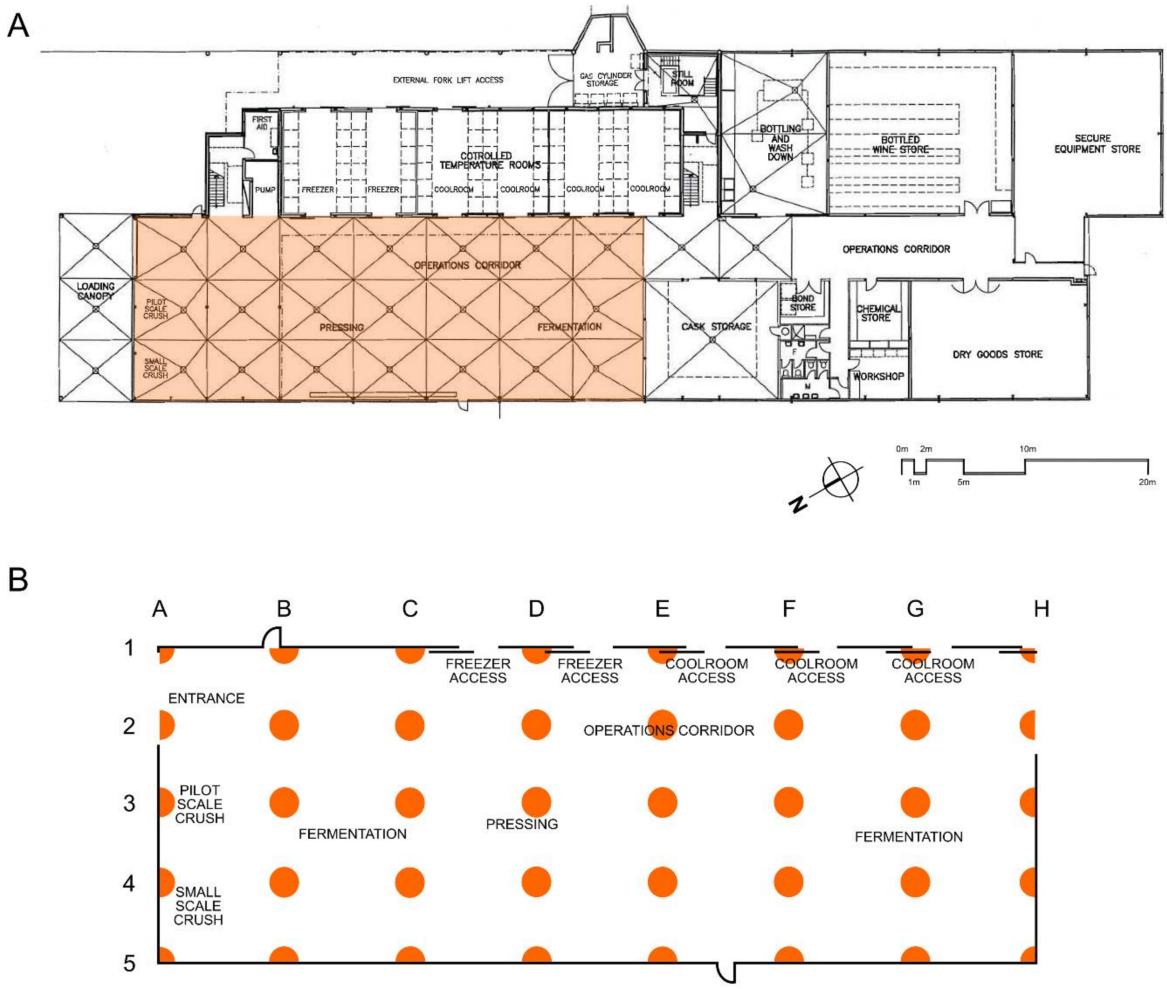

Figure 1. Sampling locations at the Hickinbotham Roseworthy Wine Science Laboratory (HRWSL). (**A**) Swab samples were taken from the floor of the main HRWSL operations/fermentation area (orange shade). (**B**) Samples were taken in a 5 × 8 grid pattern at three different time points, before vintage (December 2019), during vintage (March 2020) and after vintage (June 2020).

2.3. Data Analysis

Data analysis and graphical representation for yeast populations were performed using the R packages phyloseq [40], microbiome [41], vegan [42], microbiomeViz [43], plotly [44], ggtree [45], ggtreeExtra [46], ggpubr [47], ggplot2 [48] and viridis [49] in R version 4.0.2 [50]. Samples were filtered for read depth (1000 reads minimum) and OTUs were filtered by abundance (0.001% minimum). OTU counts were then standardized to the median sequencing depth and then proportionally normalized by dividing the genus level OTU count by the sum of all OTUs within a sample. Alpha diversity indexes were obtained with the command alpha from the microbiome package [41]. Distance-based redundancy analysis (DBRDA) was undertaken using the proportionally normalized data with location and sampling date as major factors. Analysis of variance (ANOVA) was run on the resulting DBRDA analysis using the function aov from the R package stats [50]. Mapping of microbial populations to the winery floor plan was performed using the plot_ly and add_trace commands of the package plotly [44]. Cladograms were first obtained using

the commands fix_duplicate_tax, parsePhyloseq and tree.backbone from the package microbiomeViz [43] and ggtree from the ggtree package [45], and then annotated using the commands clade.anno from the microbiomeViz package [43] and geom_fruit from the ggtreeExtra package [46].

Statistical differences for relative abundances between species according to location and sampling date were evaluated using Kruskal–Wallis tests with the command plot_taxa_boxplot from the microbiomeutilities package [51]. Phylotyping data from other studies, including a brewery [5], two cheese making facilities [4] and two wineries [7,19,52], were used to compare microbial community structure between food and beverage facilities. It was not possible to obtain the raw sequences for other studies and assign taxonomies to different OTUs using the same database. Therefore, taxonomy tables from each study were used for comparison. Taxonomy tables from different studies were combined into a single phyloseq object and then agglomerated by family or genus depending on the analysis.

3. Results

Floor swab samples were collected from the HRWSL main operations/fermentation area at three different time points, before vintage (December 2019), during vintage (March 2020) and after vintage (June 2020). This represents the main area where grapes are received, crushed and fermented and where completed ferments are pressed and transferred to tanks and oak barrels.

3.1. Microbial Populations in the Winery

An overview of the microbial populations found in the HRWSL across the three sampling dates and their taxonomies is provided in Figure 2. A total of ten different bacterial phyla were identified, with *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* accounting for over 95% of total number of bacterial genera. These phyla showed great diversity involving 83, 35, 35 and 14 different genera, respectively (Figure 2A). *Sphingomonas* was the most abundant genera for the phylum *Proteobacteria*, while the genus *Exiguobacterium* and the family *Micrococcaceae* were the most abundant taxonomies for the phyla *Firmicutes* and *Actinobacteria*, respectively. *Actinobacteria* and *Proteobacteria* were the most abundant phyla accounting for more than 80% abundance regardless of sampling date (Table S3). The phyla *Actinobacteria*, *Firmicutes* and *Patescibacteria* showed a statistically significant increase in relative abundance according to sampling date (Kruskal–Wallis test, $p < 0.05$), while *Cyanobacteria*, *Deinococcus-Thermus* and *Proteobacteria* decreased in abundance (Figure S1A, Table S3). The increase in abundance found for *Actinobacteria* was driven by a significant increase in the family *Micrococcaceae*, whereas the genus *Exiguobacterium* was mainly responsible for the increase in *Firmicutes* (Figure S1B). Similarly, the genera *Sphingomonas* and *Massilia* and the family *Enterobacteriaceae* were responsible for the decreased abundance of *Proteobacteria*. The genus *Acinetobacter* showed increased abundance during vintage, while the family *Burkholderiaceae* showed high abundance before and after vintage (Figure S1B, Table S4).

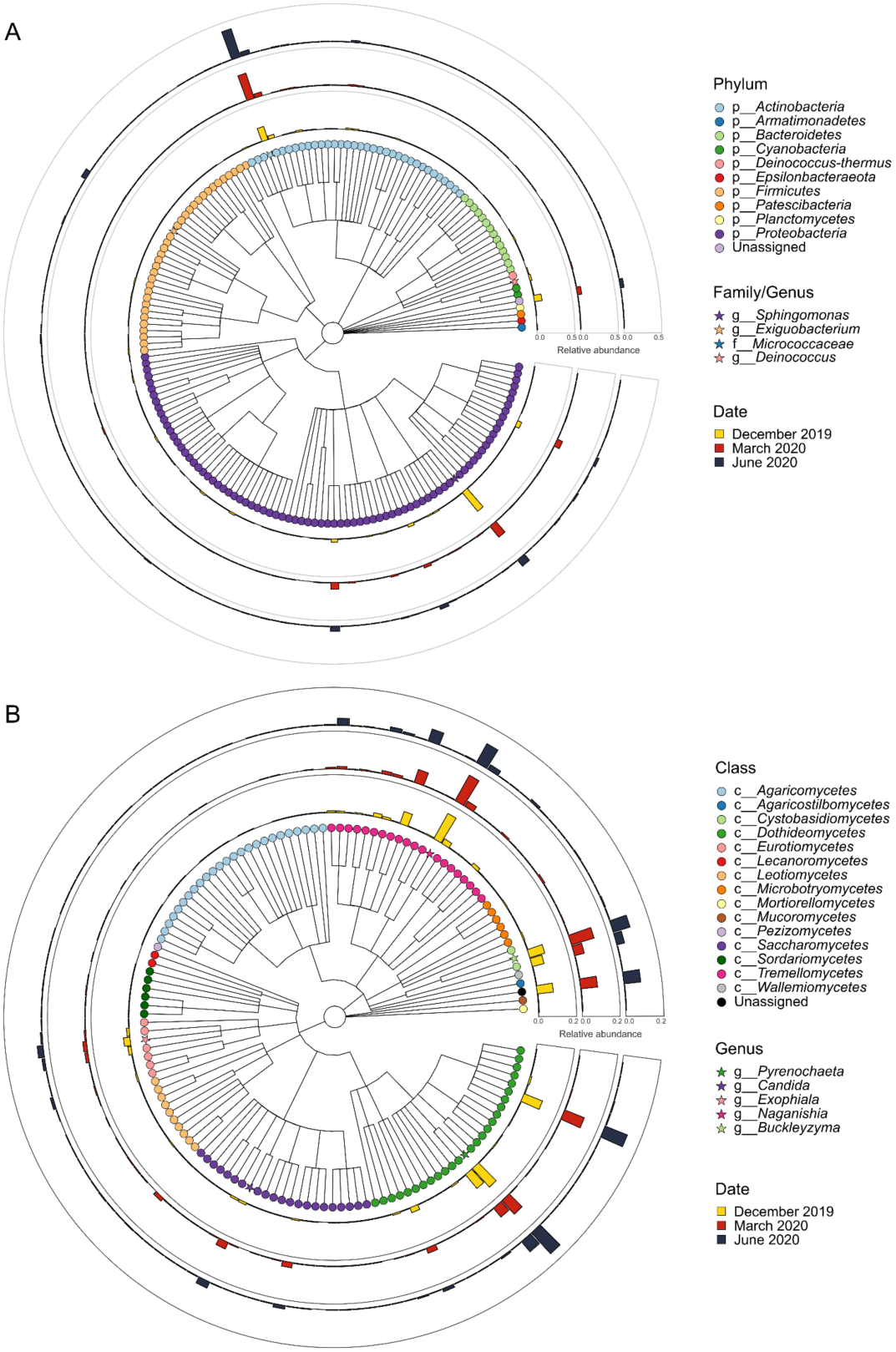

Figure 2. Microbial communities found in the Hickinbotham Roseworthy Wine Science Laboratory (HRWSL). Cladograms shows different taxonomy levels in concentric rings. From the centre, kingdom, phylum, class, order, family and genus are shown. (**A**) Bacterial communities with all identified phyla coloured, relative abundance for final taxonomies indicated in outer rings. (**B**) Fungal communities with all identified classes coloured, relative abundance for genera indicated in outer rings.

Fifteen different fungal classes were found across all sampling dates, with *Dothideomycetes*, *Agaricomycetes*, *Saccharomycetes* and *Tremellomycetes* displaying the highest number of genera per class, with 27, 25, 22 and 21 different genera, respectively. *Pyrenochaeta*, *Candida*, *Exophiala*, *Naganishia* and *Buckleyzyma* were the most abundant genera across all sampling dates in their respective classes (Figure 2B). *Dothideomycetes* and *Tremellomycetes* were the most abundant classes overall, accounting for nearly 60% of observed read counts regardless of sampling date (Table S5). *Microbotryomycetes*, *Saccharomycetes* and *Sordariomycetes* showed significant increases ($p < 0.05$) in relative abundance during vintage, whereas *Agaricomycetes* and *Eurotiomycetes* decreased in abundance at the same sampling date (Figure S2A, Table S5). The increased abundance of *Saccharomycetes*, which includes most of the yeast genera of oenological interest, is explained by a rise in the relative abundance of the genera *Candida*, *Metschnikowia* and *Saccharomyces* during vintage. In contrast, the genera *Cyphellophora*, *Guehomyces* and *Neocucurbitaria* decreased during vintage and remained low after vintage (Figure S2B, Table S6). *Hannaella* was the only genus increasing in abundance over the three sampling times (Table S6).

Sampling date also had an impact on the diversity indices for both bacterial and yeast communities (Table 1). For bacterial communities, richness, diversity and evenness were highest before vintage, whereas dominance was lowest at the same sampling date. These findings suggest a great number of bacterial species with balanced abundance before vintage. For fungal populations, only richness changed according to sampling date showing lowest values during vintage. Distance-based redundancy analysis (DBRDA) showed that both sampling date and location significantly affected the composition of bacterial and fungal communities (Table 2).

3.2. Community Structure Differs Between Food and Beverage Facilities

Phylotyping data from other studies, including bacterial 16S and fungal ITS data from a brewery [5], two cheese making facilities [4] and a winery in the US [19] and fungal ITS data from a winery in France [7,52] were used to compare microbial community structure between food and beverage facilities. These studies included samples from different types of location within each facility including floors, walls, equipment, drains etc.

Table 1. Diversity indices for bacterial and yeast communities found at the Hickinbotham Roseworthy Wine Science Laboratory according to sampling date.

	Sampling Date		
	Before Vintage December 2019	**During Vintage March 2020**	**After Vintage June 2020**
Bacterial communities			
Richness	549.90 [a]	476.05 [b]	494.03 [b]
Diversity [1]	4.04 [a]	3.50 [b]	3.33 [b]
Dominance	0.19 [a]	0.26 [b]	0.32 [b]
Evenness	0.64 [a]	0.57 [b]	0.54 [b]
Fungal communities			
Richness	322.88 [a]	292.63 [b]	358.78 [a]
Diversity [1]	2.84 [a]	2.75 [a]	2.86 [a]
Dominance	0.27 [a]	0.29 [a]	0.29 [a]
Evenness	0.49 [a]	0.49 [a]	0.49 [a]

[1] Diversity estimated by the Shannon index. Different letters indicate significative differences ($p < 0.05$).

Table 2. Distance-based redundancy analysis (DBRDA) of Bray distances for microbial communities (999 permutations) found at the Hickinbotham Roseworthy Wine Science Laboratory.

	Bacterial Communities				
	Df	**SS**	**F Model**	***p* Value**	**Significance**
Location	39	16.865	2.872	0.001	***
Sampling date	2	2.638	8.762	0.001	***
Residuals	78	11.743			
	Fungal Communities				
	Df	**SS**	**F Model**	***p* Value**	**Significance**
Location	39	14.990	4.006	0.001	***
Sampling date	2	0.608	3.169	0.001	***
Residuals	73	7.004			

Df, degrees of freedom; SS, sequential sum of squares. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Although all the facilities showed a large proportion of low abundance bacterial terminal taxonomies, food and beverage facilities showed distinct bacterial population structures (Figure 3A). The genera *Acinetobacter*, *Pseudomonas* and *Limnohabitans* were the most abundant in the brewery, whereas *Lactococcus*, *Pseudoalteromonas* and *Staphylococcus* were the most abundant genera in the cheese making facilities. Although the relative abundance for these last two genera were different between both cheese making facilities, population structure was similar (Figure 3A). Bacterial populations in both wineries were distinct, not only the main final taxonomies representing each winery, but also in their relative abundance. The family *Micrococcaceae* and the genus *Sphingomonas* were the most abundant in the Australian winery, while several taxonomies showed similar abundance in the North American winery, with the genus *Pseudomonas* and the family *Comamonadaceae* being the most abundant (Figure 3A).

Fungal population structures were also distinct in each facility, including all three wineries (Figure 3B). *Saccharomyces* was the most abundant genus in the brewery, accounting for nearly 70% of all genera. *Debaryomyces* and *Penicillium* were the most abundant genera in the cheese making facilities. All three wineries showed different proportions for the most abundant genera. The genera *Pyrenochaeta*, *Naganishia* and *Didymella* were the most abundant in the Australian winery, whereas *Cryptococcus*, *Saccharomyces* and *Aspergillus* showed the highest abundance in the North American winery. The genera *Aureobasidium*, *Penicillium* and *Alternaria* were the most abundant in the French winery. Interestingly, only the North American winery had considerable abundance for the wine related genera *Saccharomyces*, *Hanseniaspora* and *Rhodotorula* (Figure 3B).

3.3. Mapping Microbial Abundance

The relative abundance for microbial and fungal populations according to sampling date and location are shown in Figures S3 and S4, respectively. Although some changes in abundance can be easily visualised, it is not straightforward to identify all changes in population structure from bar plots, therefore relative abundance was mapped to the winery floor plan.

Mapping of the most abundant bacterial taxonomies to the winery floor plan is shown in Figure 4. The family *Micrococcaceae* was found in most locations in the winery operations area before, during and after vintage. Relative abundance increased considerably during vintage, particularly close to the entrance and on the operations corridor, and remained relatively high after vintage. The genus *Sphingomonas* was well distributed across the winery before vintage with high abundance in the pilot scale crush and pressing areas (Figure 4). *Sphingomonas* relative abundance and winery area coverage decreased during and after vintage, to be found mainly close to the winery walls. The genus *Brevundimonas* was only found in the small-scale crush area before vintage. Increased abundance was found during vintage in one of the fermentation areas, and in the pressing and on the

second fermentation areas. Relative abundance for *Brevundimonas* decreased after vintage in all these areas (Figure 4). The genus *Exigiobacterium* was only found after vintage, mainly on the right wall of the winery showing very high abundance (Figure 4).

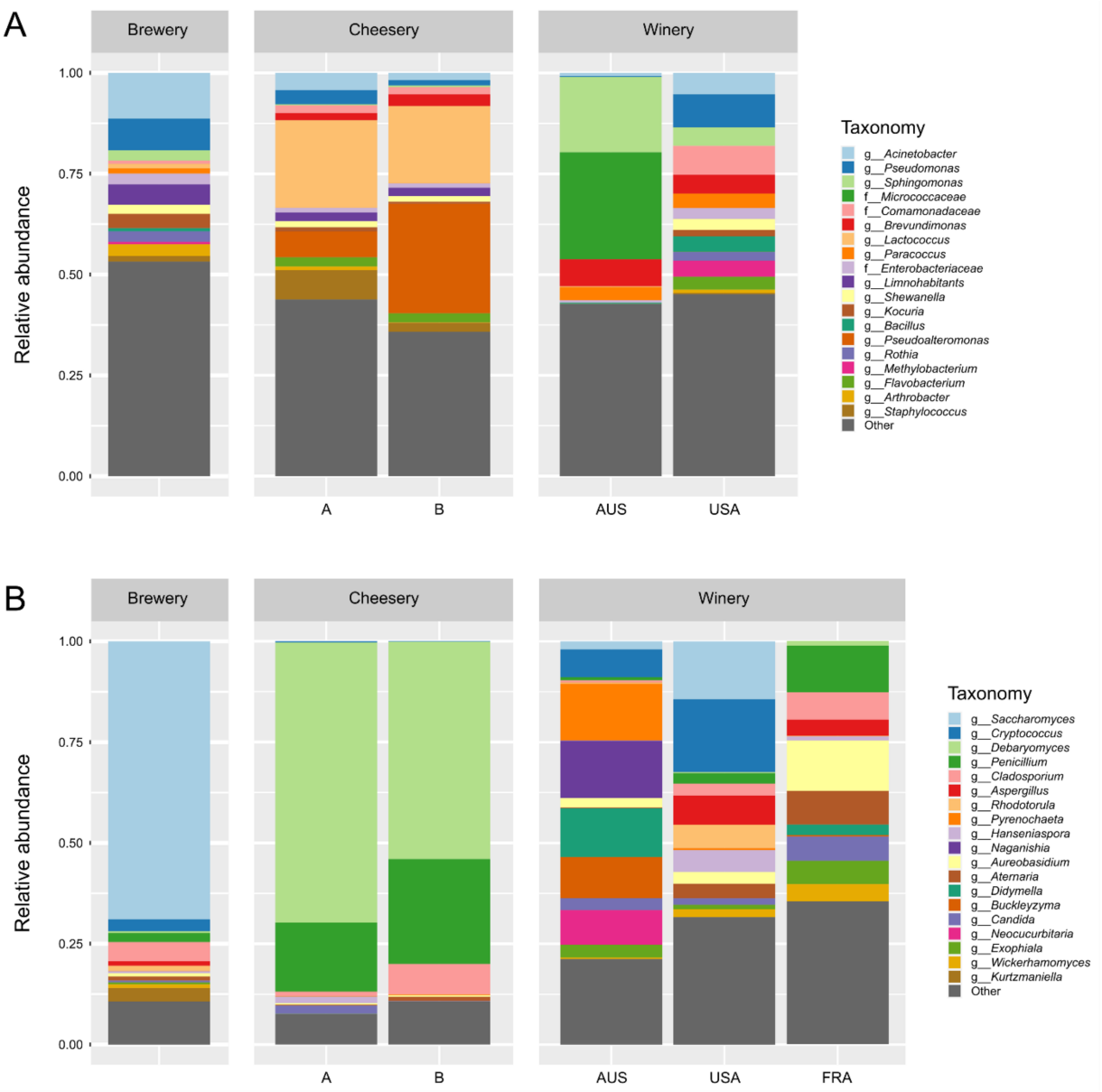

Figure 3. Microbial composition for the top 20 most abundant terminal taxonomies in different food and beverage facilities. (**A**) Bacterial community structure and (**B**) fungal community structure. Facilities include a brewery, two cheese making facilities (**A**,**B**) and three wineries located in different countries, Australia, USA and France.

The fungal genera *Naganishia* and *Pyrenochaeta* were found ubiquitously in the winery before, during and after vintage (Figure 5). Relative abundance for both genera changed over time. *Naganishia* relative abundance during vintage increased on the entrance to the winery and close to one of the cool rooms. *Pyrenochaeta* abundance increased on the operations corridor (Figure 5). The genus *Candida* showed high abundance during vintage in the small-scale crushing area, suggesting a link to grape material, abundance in this area decreased after vintage. Similarly, *Saccharomyces* showed high abundance during vintage in the exit to the operations area which could be due to wine spillage (Figure 5).

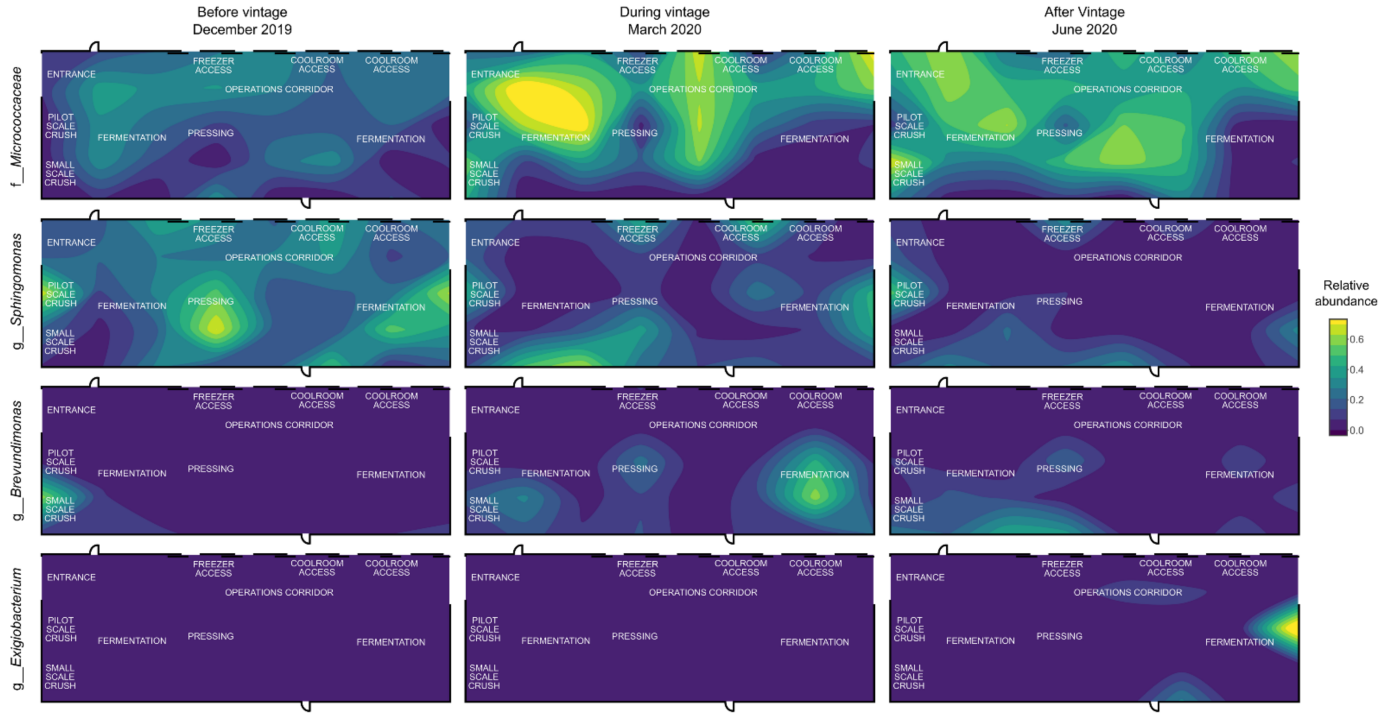

Figure 4. Mapping of the most abundant bacterial final taxonomies found at the Hickinbotham Roseworthy Wine Science Laboratory, including the family *Micrococcaceae*, and the genera *Sphingomonas*, *Brevundimonas* and *Exigiobacterium*, before, during and after vintage.

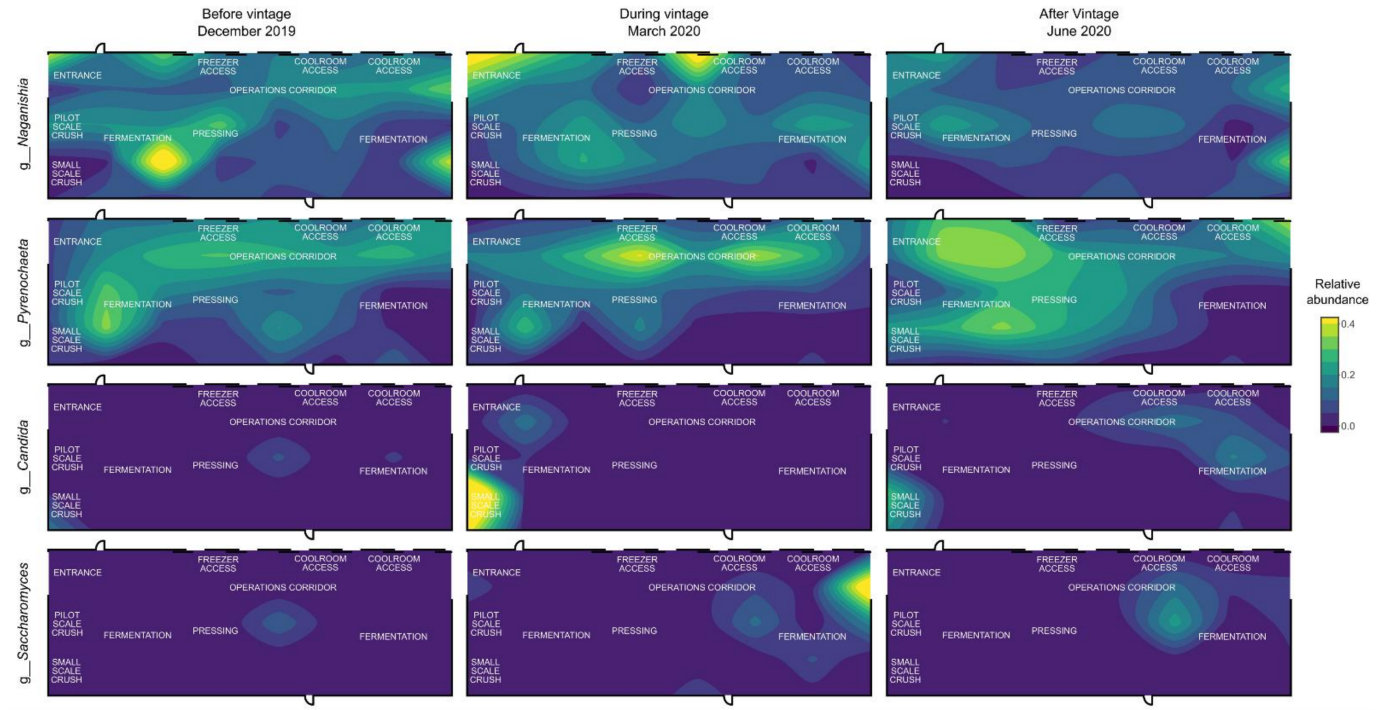

Figure 5. Mapping of the most abundant fungal genera found at the Hickinbotham Roseworthy Wine Science Laboratory, including *Naganishia*, *Pyrenochaeta*, *Candida* and *Saccharomyces*, before, during and after vintage.

The bacterial genera *Leuconostoc* and *Oenococcus*, which are associated with winemaking, showed very low abundance across all sampling dates (Figure S5). Whereas *Leuconostoc* was only found after vintage on the right wall of the winery, *Oenococcus* was associated

with the different fermentation areas depending on sampling date. Some of the fungal genera associated with winemaking also showed differences in distribution depending on sampling date (Figure S6). The genus *Metschnikowia* was mainly found during vintage in the fermentation and pressing areas. *Aureobasidium* was found in one of the fermentation areas before vintage and on the bottom wall of the winery during vintage. *Hanseniaspora* showed increased relative abundance during vintage in one of the fermentation areas, whereas *Torulaspora* was mainly found after vintage with increased abundance close to the cool rooms and in one of the fermentation areas (Figure S6).

Wine spoilage microorganisms, including the genera *Lactobacillus*, *Acetobacter*, *Gluconobacter* and *Brettanomyces* showed very low relative abundance when mapped to the winery floor plan (Figure 6). *Lactobacillus* was the only spoilage microorganism found before vintage. All three spoilage bacteria, *Lactobacillus*, *Acetobacter* and *Gluconobacter*, showed increased abundance during vintage with each microorganism associated with different areas (Figure 6). Relative abundance for all three spoilage bacteria decreased noticeably after vintage. *Brettanomyces* was only found after vintage in the top right corner of the winery (Figure 6).

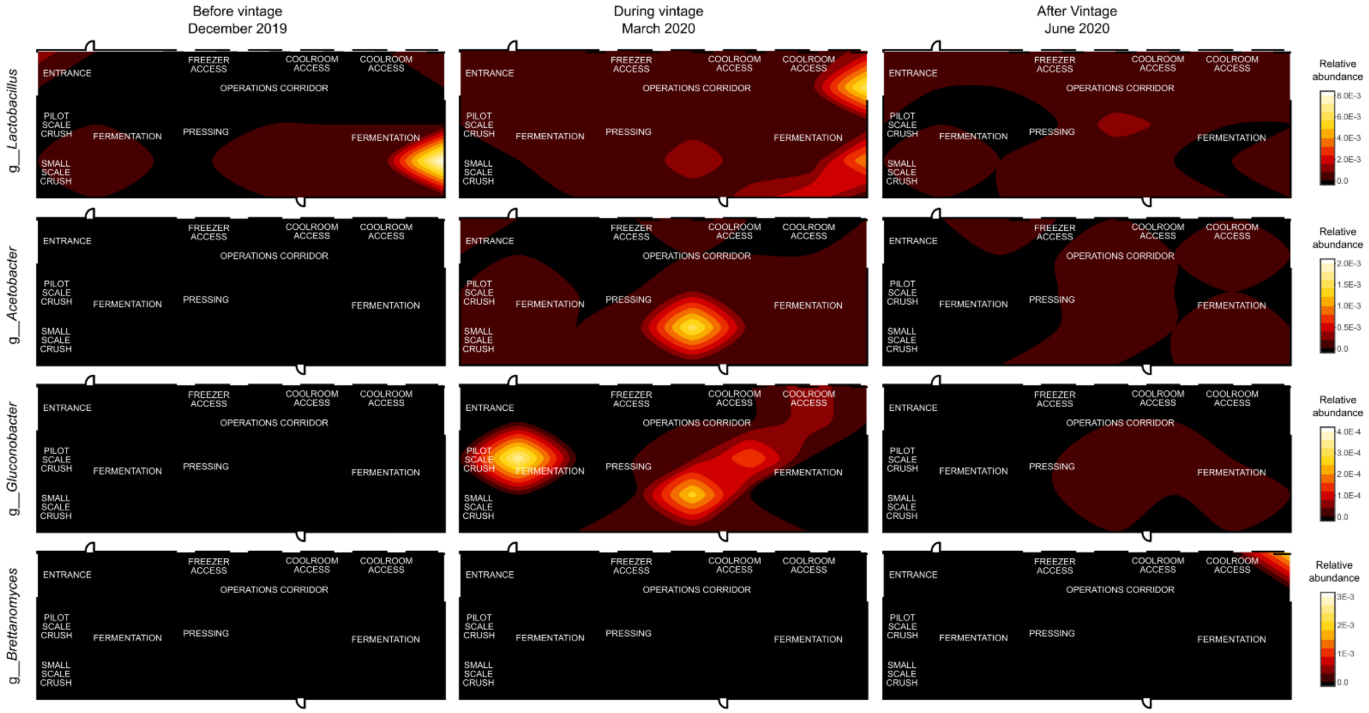

Figure 6. Mapping of the wine spoilage microorganisms, *Lactobacillus*, *Acetobacter*, *Gluconobacter* and *Brettanomyces*, before, during and after vintage found at the Hickinbotham Roseworthy Wine Science Laboratory.

4. Discussion

Food processing facilities, like any other environment, contain thriving microbial ecosystems whose population structure is niche dependent [6]. These niches are colonised when they are exposed to raw substrates or other materials, then nutrient availability and production practices shape the growth and persistence of colonising microorganisms [6]. The winery environment is no exception, grapes bring into the winery different bacterial and fungal populations, depending on variety, origin and harvesting practices [53,54], which will then come in contact with winery surfaces, including crushers, pressers, floors, walls, fermentation vessels and barrels. These different winery surfaces show clear niche specialisation, with different microbial genera dominating each niche [19].

In this work we studied the microbial populations found on one particular winery niche, the winery floor. Microbial structure changed according to sampling time and

location within the winery. Changes in the winery microbiome across time and winery equipment have been reported previously, highlighting the seasonal nature of the winemaking process [7,19]. Vintage, the period when grapes are being processed in the winery, caused most of the changes in microbial structure, particularly for bacterial populations. Thus, bacterial diversity indices and relative abundance for bacterial terminal taxonomies were both affected by vintage. Major changes in bacterial population structure during vintage have been reported previously, with aerobic bacteria, such as *Pseudomonas*, *Flavobacterium*, *Enterobacteraceae*, *Brevundimonas* and *Bacillus* dominating winery surfaces before vintage, whereas *Sphingomonas*, *Methylobacterium* and *Nakamurellaceae* dominated during vintage [19]. Although fungal communities also changed during vintage, these changes were smaller than those observed for bacterial populations. The exception being *Saccharomycetes* which showed approximately four-times higher relative abundance during vintage than prior to this period. Absolute abundance for both fungal and bacterial communities during vintage have been shown to increase significantly on all grape processing equipment compared to pre-harvest levels [19]. Although we did not determine the total bacterial or yeast cell concentrations, our results confirm that grape material is stimulating the growth of some microbial populations.

Different niches within the winery have been associated with particular microbial populations, but each ecosystem is subject to seasonal flux due to harvest disruption [19]. Thus, samples from similar surfaces, i.e., crush equipment, fermentation vessels, etc. showed comparable microbial populations, distinguishing them from other environments [7,19]. Winery floors and walls have been shown to have significant populations of *Saccharomyces*, *Candida* and *Cryptococcus*, as well as the fungi *Aspergillus*, *Penicillium* and *Exophiala* [7,19]. In this work, *Naganishia*, *Pyrenochaeta* and *Didymella* were the most abundant fungal populations found in the winery. As several *Cryptococcus* species are now classified as *Naganishia* [55], it is likely that changes in species databases explain some of the different abundances found for this genera in previous studies. *Naganishia* species are among the most resistant organisms to UV radiation, they show adaptation to low pH values and the ability to grow at temperatures below 0°C [56]. This last trait could explain the high relative abundance of this genera close to the cold room entrance. *Pyrenochaeta* species are mainly plant pathogens, including maize, tomato, eggplant and lettuce, and can survive in a broad range of pH, temperatures, and soil types [57,58]. *Didymella* species are also plant pathogens, attacking the cucurbits, including cucumber, pumpkin and zucchini [59]. Although *Pyrenochaeta* and *Didymella* have been found in other wineries [7,19], there are no reports associating either of them with grapevines, suggesting a source other than grape material. Indeed, other fungal genera which are associated with plants, soil, water and/or decaying wood material, such as *Exophiala* and *Hannaella*, can find their way into the winery [7].

Comparing the microbial communities found in this study to those observed in other fermented food and beverage facilities, revealed an association between community structure and substrate/facility. Indeed, previous studies have suggested the presence of distinct microbial niches in food facilities and highlighted the role of manufacturing practices in shaping microbial structure [4,5,15,16]. Thus, the microbiota in cheese making facilities has been shown to be dominated by microorganisms deliberately inoculated, including *Lactococcus lactis*, *Debaryomyces hansenii* and *Penicillium camemberti* [4], or when no starter is added, as is the case for Ragusano cheese, microorganisms from the wooden vat biofilm are then responsible for cheese fermentation [16]. Additionally, different bacterial genera have been found associated to different cheese types within the same facility [15]. In breweries it has been suggested that the distribution of the bacterial and fungal populations is driven by the raw materials used in the brewing process [5]. In the case of traditional lambic beer production where the wort is spontaneously fermented, the brewery environment, including the air and the wooden barrels used for the fermentation and maturation process, has been suggested as the source of the microbiota responsible for the production of this type of beer [14,60].

Although similar microbial genera were found in the wineries compared in this work, each showed distinct microbial population structures. Besides being located in different continents and having different climatic conditions, these wineries have different sizes (from approximately 600 to 2000 m^2) and source different grape varieties, including Pinot Noir and Chardonnay in France, Cabernet Sauvignon, Barbera and Zinfandel in the US, and Chardonnay, Cabernet Sauvignon and Shiraz in Australia [7,19]. Although the main source for the winery microbiota is grape material, microorganisms found in food and beverage facilities can also originate from humans or other environmental sources [6]. It is possible that all these factors play a role in shaping microbial population structure in the three wineries analysed.

Several wine spoilage microorganisms were detected and mapped to specific locations within the winery mainly during and after vintage. Food production facilities are routinely tested for the presence of pathogens and spoilage microorganisms, with specific focus on particular species or phenotypes [6]. However, standard targeted and culture-based methods can fail at detecting these organisms [6,61]. In these cases, metagenomic approaches provide crucial information to identify such microorganisms [61]. Based on the near absence of spoilage-related microorganisms before vintage, it has been suggested that winery surfaces do not contain wine spoilage organisms under normal operating conditions, and that this is the result of microbial resident communities protecting against colonization by spoilage microorganisms [19]. Indeed, competitive exclusion, the process by which species competing for identical resources are not able to coexist, has been successfully trialled in a poultry processing plant eliminating the pathogen *Listeria monocytogenes* in five of the six drains treated, which remained free of this pathogen for 13 weeks after treatment [62]. Thus, it has been suggested that surfaces within food processing facilities can be inoculated with organisms that prevent colonisation by pathogenic or spoilage microorganisms [6]. This may be particularly useful in cases when pathogens can survive the cleaning treatments implemented in the processing facility [17].

Many yeast species, including *S. cerevisiae* and non-*Saccharomyces* yeasts, in addition to the spoilage microorganisms *Brettanomyces* and *Zygosaccharomyces* have been shown to persist in the cellar over consecutive vintages [7,22,63]. Indeed, some of these yeasts can 'inoculate' fresh grape must in the winery and be part of the wine fermentation process [22,63]. Transfers of microorganisms between different surfaces can occur by direct contact, air flow and bioaerosols, which can transport whole organisms, spores, biopolymers, plant debris and decaying biomass [64]. Thus, the resident microorganisms in the winery can not only shape the microbiota during fermentation, but also alter the appearance, aroma, and flavour of wine.

In summary, we have shown that microbial communities inhabiting an Australian winery changed over time and space, and that some populations persisted over several months. Mapping microbial populations to the winery floor enabled identification of the locations where spoilage microorganisms reside. These locations should be targeted with effective cleaning procedures to reduce the risk of wine spoilage. Metagenomic techniques are therefore a powerful tool, wineries may use to evaluate their resident microbial communities and to assess their sanitation practices.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7030134/s1. Figure S1: Boxplots showing relative abundance for the most abundant bacterial phyla and for the 20 most abundant bacterial final taxonomies. Figure S2: Boxplots showing relative abundance for the most abundant fungal classes and for the 20 most abundant fungal genera. Figure S3: Relative abundance for the most abundant bacterial final taxonomies found at the Hickinbotham Roseworthy Wine Science Laboratory according to location and sampling date. Figure S4: Relative abundance for the most abundant fungal genera found at the Hickinbotham Roseworthy Wine Science Laboratory according to location and sampling date. Figure S5: Mapping of the wine-related bacterial genera *Leuconostoc* and *Oenoccocus*, before, during and after vintage. Figure S6: Mapping of the wine-related fungal genera *Metschnikowia*, *Aureobasidium*, *Hanseniaspora* and *Torulaspora*, before, during and after vintage. Table S1: Abundance for bacterial OTUs. Table S2:

Abundance for fungal OTUs. Table S3: Relative abundance (%) for bacterial phyla found at the Hickinbotham Roseworthy Wine Science Laboratory according to sampling date. Table S4: Relative abundance (%) for the top 20 bacterial terminal taxonomies found at the Hickinbotham Roseworthy Wine Science Laboratory according to sampling date. Table S5: Relative abundance (%) for fungal classes found at the Hickinbotham Roseworthy Wine Science Laboratory according to sampling date. Table S6: Relative abundance (%) for the top 20 fungal genera found at the Hickinbotham Roseworthy Wine Science Laboratory according to sampling date.

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References

1. McGovern, P.E.; Zhang, J.; Tang, J.; Zhang, Z.; Hall, G.R.; Moreau, R.; Nunez, A.; Butrym, E.D.; Richards, M.P.; Wang, C.S.; et al. Fermented beverages of pre- and proto-historic China. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17593–17598. [CrossRef] [PubMed]
2. Tamang, J.P.; Holzapfel, W.H.; Shin, D.H.; Felis, G.E. Microbiology of ethnic fermented foods and alcoholic beverages of the world. *Front. Microbiol.* **2017**, *8*, 1377. [CrossRef] [PubMed]
3. Amato, K.R.; Chaves, O.M.; Mallott, E.K.; Eppley, T.M.; Abreu, F.; Baden, A.L.; Barnett, A.A.; Bicca-Marques, J.C.; Boyle, S.A.; Campbell, C.J.; et al. Fermented food consumption in wild nonhuman primates and its ecological drivers. *Am. J. Phys. Anthropol.* **2021**, *175*, 513–530. [CrossRef]
4. Bokulich, N.A.; Mills, D.A. Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. *Appl. Environ. Microbiol.* **2013**, *79*, 5214–5223. [CrossRef]
5. Bokulich, N.A.; Bergsveinson, J.; Ziola, B.; Mills, D.A. Mapping microbial ecosystems and spoilage-gene flow in breweries highlights patterns of contamination and resistance. *elife* **2015**, *4*, e04634. [CrossRef]
6. Doyle, C.J.; O'Toole, P.W.; Cotter, P.D. Metagenome-based surveillance and diagnostic approaches to studying the microbial ecology of food production and processing environments. *Environ. Microbiol.* **2017**, *19*, 4382–4391. [CrossRef] [PubMed]
7. Abdo, H.; Catacchio, C.R.; Ventura, M.; D'Addabbo, P.; Alexandre, H.; Guilloux-Benatier, M.; Rousseaux, S. The establishment of a fungal consortium in a new winery. *Sci. Rep.* **2020**, *10*, 7962. [CrossRef]
8. Einson, J.E.; Rani, A.; You, X.M.; Rodriguez, A.A.; Randell, C.L.; Barnaba, T.; Mammel, M.K.; Kotewicz, M.L.; Elkins, C.A.; Sela, D.A. A vegetable fermentation facility hosts distinct microbiomes reflecting the production environment. *Appl. Environ. Microbiol.* **2018**, *84*, e01680-18. [CrossRef] [PubMed]
9. Zwirzitz, B.; Wetzels, S.U.; Dixon, E.D.; Stessl, B.; Zaiser, A.; Rabanser, I.; Thalguter, S.; Pinior, B.; Roch, F.F.; Strachan, C.; et al. The sources and transmission routes of microbial populations throughout a meat processing facility. *Npj Biofilms Microbiomes* **2020**, *6*, 26. [CrossRef]
10. Chacon-Vargas, K.; Torres, J.; Giles-Gomez, M.; Escalante, A.; Gibbons, J.G. Genomic profiling of bacterial and fungal communities and their predictive functionality during pulque fermentation by whole-genome shotgun sequencing. *Sci. Rep.* **2020**, *10*, 15115. [CrossRef] [PubMed]
11. Jung, M.J.; Nam, Y.D.; Roh, S.W.; Bae, J.W. Unexpected convergence of fungal and bacterial communities during fermentation of traditional Korean alcoholic beverages inoculated with various natural starters. *Food Microbiol.* **2012**, *30*, 112–123. [CrossRef]
12. Varela, C.; Sundstrom, J.; Cuijvers, K.; Jiranek, V.; Borneman, A. Discovering the indigenous microbial communities associated with the natural fermentation of sap from the cider gum *Eucalyptus gunnii*. *Sci. Rep.* **2020**, *10*, 14716. [CrossRef]
13. Bokulich, N.A.; Bamforth, C.W.; Mills, D.A. Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale. *PLoS ONE* **2012**, *7*, e35507. [CrossRef] [PubMed]
14. Spitaels, F.; Wieme, A.D.; Janssens, M.; Aerts, M.; Daniel, H.-M.; Van Landschoot, A.; De Vuyst, L.; Vandamme, P. The microbial diversity of traditional spontaneously fermented lambic beer. *PLoS ONE* **2014**, *9*, e95384. [CrossRef] [PubMed]

15. Calasso, M.; Ercolini, D.; Mancini, L.; Stellato, G.; Minervini, F.; Di Cagno, R.; De Angelis, M.; Gobbetti, M. Relationships among house, rind and core microbiotas during manufacture of traditional Italian cheeses at the same dairy plant. *Food Microbiol.* **2016**, *54*, 115–126. [CrossRef]
16. Lortal, S.; Di Blasi, A.; Madec, M.N.; Pediliggieri, C.; Tuminello, L.; Tanguy, G.; Fauquant, J.; Lecuona, Y.; Campo, P.; Carpino, S.; et al. Tina wooden vat biofilm: A safe and highly efficient lactic acid bacteria delivering system in PDO Ragusano cheese making. *Int. J. Food Microbiol.* **2009**, *132*, 1–8. [CrossRef]
17. Hultman, J.; Rahkila, R.; Ali, J.; Rousu, J.; Björkroth, K.J. Meat processing plant microbiome and contamination patterns of cold-tolerant bacteria causing food safety and spoilage risks in the manufacture of vacuum-packaged cooked sausages. *Appl. Environ. Microbiol.* **2015**, *81*, 7088–7097. [CrossRef]
18. Spescha, C.; Stephan, R.; Zweifel, C. Microbiological contamination of pig carcasses at different stages of slaughter in two European Union-approved abattoirs. *J. Food Prot.* **2006**, *69*, 2568–2575. [CrossRef]
19. Bokulich, N.A.; Ohta, M.; Richardson, P.M.; Mills, D.A. Monitoring seasonal changes in winery-resident microbiota. *PLoS ONE* **2013**, *8*, e66437. [CrossRef]
20. Morrison-Whittle, P.; Goddard, M.R. Quantifying the relative roles of selective and neutral processes in defining eukaryotic microbial communities. *ISME J.* **2015**, *9*, 2003–2011. [CrossRef] [PubMed]
21. Taylor, M.W.; Tsai, P.; Anfang, N.; Ross, H.A.; Goddard, M.R. Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environ. Microbiol.* **2014**, *16*, 2848–2858. [CrossRef]
22. Grangeteau, C.; Gerhards, D.; von Wallbrunn, C.; Alexandre, H.; Rousseaux, S.; Guilloux-Benatier, M. Persistence of two non-*Saccharomyces* yeasts (*Hanseniaspora* and *Starmerella*) in the cellar. *Front. Microbiol.* **2016**, *7*, 268. [CrossRef] [PubMed]
23. Ocon, E.; Gutierrez, A.R.; Garijo, P.; Lopez, R.; Santamaria, P. Presence of non-*Saccharomyces* yeasts in cellar equipment and grape juice during harvest time. *Food Microbiol.* **2010**, *27*, 1023–1027. [CrossRef]
24. Pérez-Martín, F.; Seseña, S.; Fernández-González, M.; Arévalo, M.; Palop, M.L. Microbial communities in air and wine of a winery at two consecutive vintages. *Int. J. Food Microbiol.* **2014**, *190*, 44–53. [CrossRef] [PubMed]
25. Sabate, J.; Cano, J.; Esteve-Zarzoso, B.; Guillamon, J.M. Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. *Microbiol. Res.* **2002**, *157*, 267–274. [CrossRef] [PubMed]
26. Varela, C.; Borneman, A.R. Yeasts found in vineyards and wineries. *Yeast* **2017**, *34*, 111–128. [CrossRef]
27. Curtin, C.; Varela, C.; Borneman, A. Harnessing improved understanding of *Brettanomyces bruxellensis* biology to mitigate the risk of wine spoilage. *Aust. J. Grape Wine Res.* **2015**, *21*, 680–692. [CrossRef]
28. Loureiro, V.; Malfeito-Ferreira, M. Spoilage yeasts in the wine industry. *Int. J. Food Microbiol.* **2003**, *86*, 23–50. [CrossRef]
29. Ribéreau-Gayón, P.; Dubourdieu Donéche, D.B.; Lonvaud, A. Red winemaking. In *Handbook of Enology*; Ribéreau-Gayón, P., Ed.; John Wiley & Sons, Ltd.: Chichester, UK, 2006.
30. Alston, J.M.; Arvik, T.; Hart, J.; Lapsley, J.T. Brettanomics I: The cost of *Brettanomyces* in California wine production. *J. Wine Econ.* **2021**, *16*, 4–31. [CrossRef]
31. Parker, M.; Onetto, C.; Hixson, J.; Bilogrevic, E.; Schueth, L.; Pisaniello, L.; Borneman, A.; Herderich, M.; de Barros Lopes, M.; Francis, L. Factors contributing to interindividual variation in retronasal odor perception from aroma glycosides: The tole of odorant sensory detection threshold, oral microbiota, and hydrolysis in saliva. *J. Agric. Food Chem.* **2019**, *68*, 10299–10309. [CrossRef]
32. Bokulich, N.A.; Mills, D.A. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Appl. Environ. Microbiol.* **2013**, *79*, 2519–2526. [CrossRef] [PubMed]
33. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120. [CrossRef]
34. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **2011**, *17*, 1. [CrossRef]
35. Magoc, T.; Salzberg, S.L. FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **2011**, *27*, 2957–2963. [CrossRef] [PubMed]
36. Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **2010**, *26*, 2460–2461. [CrossRef]
37. Mahe, F.; Rognes, T.; Quince, C.; de Vargas, C.; Dunthorn, M. Swarm: Robust and fast clustering method for amplicon-based studies. *Peer J.* **2014**, *2*, e593. [CrossRef] [PubMed]
38. Sternes, P.R.; Lee, D.; Kutyna, D.R.; Borneman, A.R. A combined meta-barcoding and shotgun metagenomic analysis of spontaneous wine fermentation. *bioRxiv* **2017**, *6*, gix040. [CrossRef]
39. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Pena, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef]
40. McMurdie, P.J.; Holmes, S. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef]
41. Lahti, L.; Shetty, S. Tools for Microbiome Analysis in R. Version 1.9.1. Available online: http://microbiome.github.com/microbiome (accessed on 30 June 2019).
42. Oksanen, J.; Blanchet, G.; Friendly, M.; Kindt, R.; Legendre, P.; McGlinn, D.; Minchin, P.; O'Hara, R.; Simpson, G.; Solymos, P.; et al. Vegan: Community Ecology Package. R Package Version 2.5.4. Available online: https://CRAN.R-project.org/package=vegan (accessed on 30 June 2019).

43. Li, C.; Yu, G.; Zhu, C. microbiomeviz—An R Package for Visualizing Microbiome Data. Available online: https://github.com/lch14forever/microbiomeViz (accessed on 30 June 2019).
44. Dash Enterprise, Collaborative Data Science. Available online: https://plot.ly (accessed on 30 June 2019).
45. Yu, G.; Smith, D.; Zhu, H.; Guan, Y.; Lam, T.T. ggtree: An R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods. Ecol. Evol.* **2017**, *8*, 28–36. [CrossRef]
46. Xu, S.; Yu, G. ggtreeExtra: An R Package to Add Geom Layers on Circular or Other Layout Tree of "ggtree". Available online: https://github.com/YuLab-SMU/ggtreeExtra/ (accessed on 30 June 2019).
47. Kassambara, A. ggpubr: 'ggplot2' Based Publication Eady Plots. R Package Version 0.2. Available online: https://CRAN.R-project.org/package=ggpubr (accessed on 30 June 2019).
48. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2009.
49. Matplotlib. Default Color Maps from 'Matplotlib'. Available online: https://github.com/sjmgarnier/viridis (accessed on 30 November 2020).
50. R Core Team. R: A Language and Environment for Statistical Computing. Available online: http://www.R-project.org/ (accessed on 30 June 2019).
51. Shetty, S.; Lahti, L. Microbiomeutilities: An R Package with Utility Functions for The Microbiome R Package. Available online: https://github.com/microsud/microbiomeutilities-shiny (accessed on 30 June 2019).
52. Abdo, H.; Catacchio, C.R.; Ventura, M.; D'Addabbo, P.; Calabrese, F.M.; Laurent, J.; David-Vaizant, V.; Alexandre, H.; Guilloux-Benatier, M.; Rousseaux, S. Colonization of wild *Saccharomyces cerevisiae* strains in a new winery. *Beverages* **2020**, *6*, 9. [CrossRef]
53. Bokulich, N.A.; Thorngate, J.H.; Richardson, P.M.; Mills, D.A. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, E139–E148. [CrossRef] [PubMed]
54. Fleet, G.H.; Heard, G.M. Yeasts: Growth during fermentation. In *Wine Microbiology and Biotechnology*; Fleet, G.H., Ed.; Harwood Academic: Chur, Switzerland, 1993; pp. 27–54.
55. Liu, X.Z.; Wang, Q.M.; Göker, M.; Groenewald, M.; Kachalkin, A.V.; Lumbsch, H.T.; Millanes, A.M.; Wedin, M.; Yurkov, A.M.; Boekhout, T.; et al. Towards an integrated phylogenetic classification of the *Tremellomycete*. *Stud. Mycol.* **2015**, *81*, 85–147. [CrossRef]
56. Schmidt, S.K.; Vimercati, L.; Darcy, J.L.; Arán, P.; Gendron, E.M.S.; Solon, A.J.; Porazinska, D.; Dorador, C.A. *Naganishia* in high places: Functioning populations or dormant cells from the atmosphere? *Mycology* **2017**, *8*, 153–163. [CrossRef]
57. Infantino, A.; Aragona, M.; Brunetti, A.; Lahoz, E.; Oliva, A.; Porta-Puglia, A. Molecular and physiological characterization of Italian isolates of *Pyrenochaeta lycopersici*. *Mycol. Res.* **2003**, *107*, 707–716. [CrossRef] [PubMed]
58. Lević, J.; Stanković, S.; Krnjaja, V.; Bočarov-Stančić, A. Simple and efficient method for detection and identification of *Pyrenochaeta terrestris* on maize root. *Crop. Prot.* **2012**, *38*, 66–71. [CrossRef]
59. Keinath, A.P. From native plants in central Europe to cultivated crops worldwide: The emergence of *Didymella bryoniae* as a cucurbit pathogen. *HortScience* **2011**, *46*, 532. [CrossRef]
60. De Roos, J.; Van der Veken, D.; De Vuyst, L. The interior surfaces of wooden barrels are an additional microbial inoculation source for lambic beer production. *Appl. Environ. Microbiol.* **2019**, *85*, e02226-18. [CrossRef]
61. Quigley, L.; O'Sullivan, D.J.; Daly, D.; O'Sullivan, O.; Burdikova, Z.; Vana, R.; Beresford, T.P.; Ross, R.P.; Fitzgerald, G.F.; McSweeney, P.L.H.; et al. *Thermus* and the pink discoloration defect in cheese. *mSystems* **2016**, *1*, e00023-16. [CrossRef]
62. Zhao, T.; Podtburg, T.C.; Zhao, P.; Chen, D.; Baker, D.A.; Cords, B.; Doyle, M.P. Reduction by competitive bacteria of *Listeria monocytogenes* in biofilms and Listeria bacteria in floor drains in a ready-to-eat poultry processing plant. *J. Food Prot.* **2013**, *76*, 601–607. [CrossRef]
63. Santamaría, P.; Garijo, P.; López, R.; Tenorio, C.; Rosa Gutiérrez, A. Analysis of yeast population during spontaneous alcoholic fermentation: Effect of the age of the cellar and the practice of inoculation. *Int J. Food Microbiol.* **2005**, *103*, 49–56. [CrossRef] [PubMed]
64. Theisinger, S.M.; de Smidt, O. Bioaerosols in the food and beverage industry. In *Ideas and Applications toward Sample Preparation for Food and Beverage Analysis*; InTech: Rijeka, Croatia, 2017.