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Investigating the Influence of Fungicide Usage and Vineyard Disease Management on the Grapevine Microbiome and Wine Quality

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Declaration of Originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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List of Abbreviations

MLF	Malolactic Fermentation
LAB	Lactic Acid Bacteria
AAB	Acetic Acid Bacteria
PBS	Phosphate Buffer Saline
PMA	Propidium Monoazide
Cu	Copper
S	Sulphur
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
vPCR	Viability Polymerase Chain Reaction
bp	Base Pairs
Kbp	Kilo-Base Pairs
ITS	Internal Transcribed Spacer
DNA	Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
HPC	High Powered Computer
RATA	Rate All That Apply
ANOVA	Analysis of Variance
PERMANOVA	Permutational Multivariate Analysis of Variance
ASV	Amplicon Sequence Variant

List of Presentations

Welsh, B.L., Philipona, R.E., Bastian, S.E.P., Kidd, S.P. *Vines, Wines, and Microbiomes: How Fungicides Shape the Grapevine Microbiome.* MicroSeq Conference, 2021. Online Presentation.

Welsh, B.L., Philipona, R.E., Bastian, S.E.P., Kidd, S.P. *Harvesting the Living Phyllosphere Microbiome.* University of Adelaide School of Biological Sciences Postgraduate Symposium, 2021, Adelaide, Australia. Poster Presentation.

Welsh, B.L., Philipona, R.E., Bastian, S.E.P., Kidd, S.P. *Vines, Wines, and Microbiomes: How Fungicides Shape the Grapevine Microbiome.* JAMS FAME, 2021, Adelaide, Australia. Presentation.

Welsh, B.L., Philipona, R.E., Bastian, S.E.P., Kidd, S.P. *The Impact of Fungicides on the Phyllosphere Microbiome of Grapevines.* Australian Wine Industry Technical Conference, 2022, Adelaide, Australia. Poster Presentation.

Welsh, B.L., Philipona, R.E., Bastian, S.E.P., Kidd, S.P. *Vines, Wines, and Microbiomes: The Impact of Fungicides on the Phyllosphere Microbiome of Grapevines.* Australian Wine Industry Technical Conference, In the Wine Light, 2022, Adelaide, Australia. Presentation.

Welsh, B.L., Philipona, R.E., Bastian, S.E.P., Kidd, S.P. *The Impact of Fungicides on the Phyllosphere Microbiome of Grapevines.* University of Adelaide School of Biological Sciences Postgraduate Symposium, 2022, Adelaide, Australia. Poster Presentation.

List of Publications

Welsh, B.L., Eisenhofer, R., Bastian, S.E.P., Kidd, S.P. (2023). “Monitoring the viable grapevine microbiome to enhance the quality of wild wines”, *Microbiol Aust* **44** (13-17)

Abstract

The grapevine microbiome has recently become recognised as an essential feature in winemaking. As we advance our understanding of the role of these microorganisms in grape growing, wine production, and wine flavour and aroma, research must be redirected to preserving beneficial microbial communities. This is highlighted by the increasing number of wine producers beginning to ferment wines using 'wild' grapevine yeasts as opposed to typical inoculations with Saccharomyces cerevisiae (Chapter IV). To effectively characterise these communities and the factors that impact them, first we must improve current microbial harvesting methods, which need more standardisation across the scientific field and second, we need greater capability in differentiating between DNA from viable and non-viable sources. We developed a novel, phyllosphere microbiome harvesting protocol to combat these issues, adopting swabs for more standardised harvesting (Chapter III). In addition, we employed the viability selection dye Propidium Monoazide (PMA), which binds to DNA from non-living sources (relic DNA), preventing it from being amplified by PCR-based methods (Chapter III). This interaction leaves only DNA from viable cells free to be amplified and analysed using PCR based methods such as quantitative PCR (qPCR) or DNA sequencing.

With this viable phyllosphere harvesting protocol, we commenced an investigation into the effect of fungicide usage on the phyllosphere microbiome of grapevines used for winemaking (Chapter V). The use of broad-target fungicides can influence both grapevine health and wine quality but is also one factor that may impact how the grapevine microbiome manifests and develops over time. Common anti-fungal sprays such as copper and sulphur do not specifically target pathogenic fungi and, therefore, have the potential to impact the structure of beneficial fungal and bacterial communities. Using the method above for sampling the phyllosphere

microbiome, combined with 16S and ITS gene amplicon sequencing, we defined the longitudinal effects that two common fungicides, Copper- and Sulphur-based, have on the structure of live communities within the grapevine microbiome in a semi-controlled environment using potted Shiraz grapevines. From these results, we found little to no effect on the microbial communities caused by the copper- or sulphur-based fungicides. The results demonstrated that despite an immediate, rapid loss of diversity, likely due to the spraying action, the diversity recovered over the course of the following weeks.

Following this initial characterisation, a broader examination of the effects of fungicide regimes in commercial vineyards was conducted (Chapter VI). Each of the three vineyards selected utilised a different type of vineyard management practice, conventional, organic, or biodynamic, and thus different disease control. Grape samples were collected from each; a subset was taken for microbial analysis before the rest was used for small-lot wine-making with identical parameters to produce completed wine samples for sensory analysis. Microbial samples were also collected at key time points throughout the fermentation process until the wines were complete. Finished wines underwent chemical and sensory analyses, and both results were compared to the microbial samples' 16S rRNA and ITS gene amplicon sequencing results. Trends were identified between the three different vineyards to understand the varying effects each vineyard disease management method had on the phyllosphere microbiome and the impact these effects have on the resulting wines. We found that the structure of the phyllosphere microbiome directly correlated to the sensory outcomes of the final wines. We also observed some distinct differences between the vineyards, particularly the organic group.

This thesis presents an in-depth investigation into the repercussions of common fungicides on the phyllosphere microbiome and its temporal dynamics, concurrently exploring the correlation between these interactions and the sensory outcomes of wines. This aspect has yet to be fully explored. This research expands our fundamental understanding of the grapevine microbiome in the context of grape cultivation and winemaking, offering potential insights into refined phyllosphere characterisation, preservation, and practical utilisation.

Chapter I

Review of the Phyllosphere Microbiome of grapevines used for winemaking and its importance to viticulture and oenology.

This chapter comprises a review of the current literature surrounding investigations into the phyllosphere microbiome and its growing importance for the global wine industry. This chapter also details the aims and objectives of the research projects contained in this thesis.

Statement of Authorship

Title of Paper	Review of the Phyllosphere Microbiome of grapevines used for winemaking and its importance to viticulture and oenology.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Brady L. Welsh		
Contribution to the Paper	Reading of relevant literature and compiling information. Writing and drafting. Research project design.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this p		
Signature		Date	13 / 11 / 2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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INTRODUCTION

The microbiome is an essential feature on and within almost all multi-cellular organisms. The microbiome encompasses the microbial communities (microbiota), their genetic material, and their environment (Marchesi & Ravel, 2015). These microorganisms have been investigated for years and are known to play crucial roles in almost all aspects of life, including agriculture (Attwood et al. 2019). The production of wine is one example of how agriculture can be affected by the microbial world. Considering the stakes the wine industry has in the socioeconomic status of Australia (AgEconPlus, 2019), maintaining a rich understanding of all aspects of the winemaking process is necessary for the future development and innovation of the industry. With recent expansions into the role of vine-borne microbes on fermentation and sensory profiles of wine, the microbial world has revealed a new level of complexity to winemaking and, thus, a new degree of intrigue. Key factors are critical in how the grapevines' microbiome manifests and develops over time. Disease control is one such factor that has an intended impact on the present microorganisms, namely pathogens. With the importance of the microbiome in the health and development of grapevines, changes in these beneficial communities through the regular use of broad-spectrum fungicides could act as a selective pressure on the grapevine's microbiome, driving changes to the winemaking process, from vine to wine.

AIM OF THE REVIEW

Microbiomes are essential to plant physiology and vital to viticultural and oenological practices. Wine production depends on many factors before and during the fermentation process, and the structure of the phyllosphere microbiome acts as one of these factors. With the phyllosphere's role in grapevine health and the early stages of fermentation, a better understanding of the role the phyllosphere microbiome plays throughout the grape growing and winemaking practises is needed. This review aims to discuss the grapevine phyllosphere microbiome, its origins, interactions, and role in winemaking and impacts on the sensory profile of wines. Here we also discuss the role of vineyard disease management methods and the current gaps in the knowledge of their impact on wine through potential off-target effects on the phyllosphere microbiome.

THE MICROBIOME

The microbiome is a diverse community of microorganisms unique to virtually all multi-cellular organisms. The microbiota comprises the microbiome's microbial communities and includes various species of bacteria, viruses, fungi, and archaea (Marchesi & Ravel, 2015). Some of these communities of microbes survive in a complex symbiosis with their host, playing critical roles in the organism's health, while others exist as commensals or pathogens (Hirsch & Fujishige, 2008). The microbiome has become far easier to observe with developments in metagenomic analysis and other culture-independent techniques, such as next-generation sequencing and PCR-based technologies. As our ability to monitor the microbiome improves, so does our understanding of its impact on many areas of our lives. This includes health, medicine, farming, agriculture, ecology, and conservation. Nearly all multi-cellular organisms are known to have their own unique microbiome, acting as a microbial fingerprint, with no two individuals sharing the same microbial diversity. Plants are no exception to this, with the plant

microbiome existing in every possible niche, each with a distinct microbial diversity that can assist the host plant with various processes, such as accessing low-abundance nutrients and resisting multiple biotic and abiotic stressors (Kishore et al. 2005; Mark et al. 2005; Beckers et al. 2017; Thapa et al. 2017; Thapa & Prasanna, 2018).

THE PHYLLOSPHERE MICROBIOME

Plant microbiomes are separated into distinct compartments, each hosting its own diversity of microorganisms. The main sub-divisions of a plant's microbiome are the Rhizosphere microbiome (the area surrounding the roots), the Endosphere microbiome (the area within the plant's tissues), and the Phyllosphere microbiome (the surface of the aerial portion of the plant) (Figure 1). The Rhizosphere has been the main focus since its coinage (Hiltner, 1904); however, current research is shifting towards the two other microbial niches, demonstrating their equal importance in plant health (Morris, 2002).

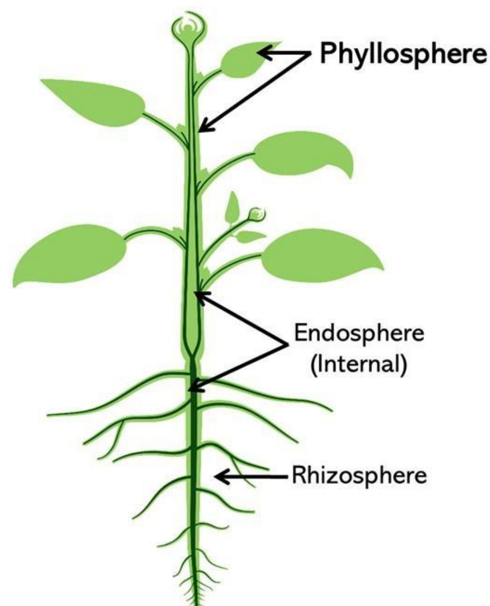

Figure 1 | The three main distinct compartments of the plant microbiome.

As the interface between the plant and its environment, the Phyllosphere microbiome has many vital roles in protecting the plant from different biotic and abiotic stressors that could impact its health (Bashir et al. 2022). This makes the Phyllosphere microbiome an important focus for industries such as agriculture (Gupta et al. 2021) and even more so for the wine industry, which can rely on these microorganisms throughout the grape growing process and downstream operations such as fermentation (Gouka et al. 2022)

Origin of the Phyllosphere Microbiome

The origins of the Phyllosphere microbiome of a plant are not as well understood as the Rhizosphere or Endosphere; however, many findings have been made for a multitude of microbial pools that act as the primary sources of Phyllosphere microorganisms. The initial colonisation of the phyllosphere by epiphytic microorganisms can occur both vertically (from the 'mother' plant) or horizontally (from the environment) (Figure 2) (Bright & Bulgheresi, 2010; Bashir *et al.* 2022). This initial colonisation occurs vertically at budburst for perennial plants, recruiting its phyllosphere microbiome from neighbouring microbial sources (Osono, 2014). This microbial colonisation of the plant surface continues throughout the plant's life cycle, with the aerial environment surrounding the plant acting as the primary source of exogenous microorganisms through water droplets, wind, and insects, amongst others (Morris, 2002; Osono, 2014). For these microorganisms to successfully be recruited to the Phyllosphere, however, they must be selected by the host plant via various selective pressures. Host plants each have their unique arsenal of pressures to select for their specific microorganisms to maintain a healthy phyllosphere microbiome while also warding off harmful diseases. These pressures can include physical barriers or stressors within the leaf microenvironment, each of

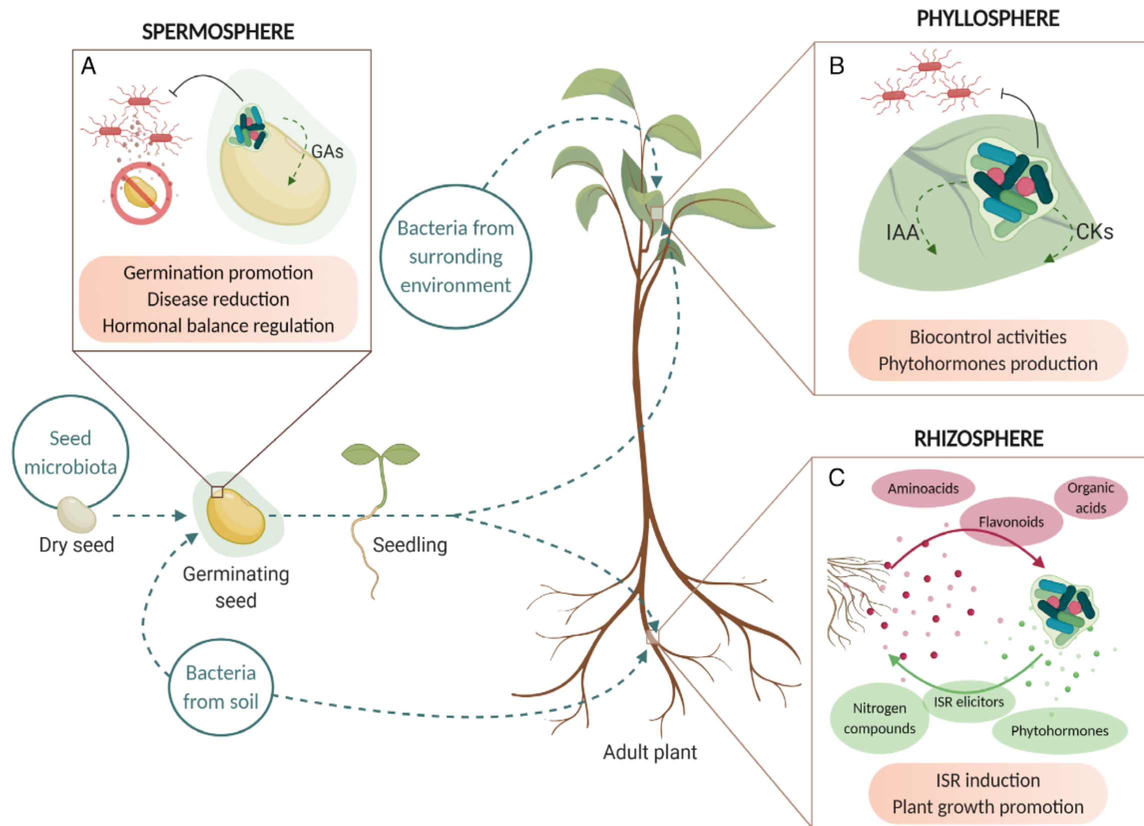

Figure 2 | Diagram showing the plant-microbiome interactions and recruitment of the microbiome in various regions of the plant throughout the plant's development. (Berlanga-Clavero et al. 2020).

which must be overcome by the potential symbiont (Morris, 2002; Bashir et al. 2022). To successfully inhabit the phyllosphere, these microorganisms must have specialised traits which allow them to survive the hostile leaf microenvironment, adhere to the leaf surface, and obtain the required nutrients, all of which lead to the colonisation of the phyllosphere by a set of specific, specialised microorganisms.

Structure of the Phyllosphere Microbiome

The microbial communities of the phyllosphere microbiome vary between each plant. Each plant selects for a specific set of adapted microorganisms. However, some phyllosphere taxa

are observed across plant species despite this variation. For bacteria, the most commonly observed taxa include Proteobacteria, Bacteroides, Firmicutes, and Actinobacteria, and for fungi, the most common genera include *Cryptococcus*, *Sporobolomyces*, *Rhodotorula*, *Acremonium*, *Aspergillus* and *Penicillium* (Bashir *et al.* 2022). Although the occurrence of each taxon is primarily host dependent as well as many other factors. Apart from the host-associated pressures, these microbial communities are also affected by various environmental stressors that shape the structure of the phyllosphere microbiome. Each taxon's frequency highly depends on the season, geographic location, water availability, UV exposure, and human intervention (Bashir et al. 2022). Controlled development of these specific communities is crucial to the host plant's development as many of the recruited species play essential roles in the host's overall health.

Roles of the Phyllosphere Microbiome

Acting as the interface between the plant and the plant's environment, the microbial species that inhabit this plant region play important roles in the interactions between the two. Each microorganism in the phyllosphere can play a different role that assists the host plant (Figure 2), including nutrient acquisition or disease resistance through competition with pathogens (Morris, 2002; Bashir *et al.*, 2022). Common bacterial epiphytes, such as the members of the Proteobacteria phylum, assist with nitrogen acquisition by the host plant via nitrogen fixation and nitrification (Fürnkranz et al. 2008), while epiphytic fungi such as *Aureobasidium pullulans* can compete with other fungal plant pathogens, making them useful biocontrol agents (Cordier et al. 2012). As well as interacting with the plant's environment, the phyllosphere organisms can signal the plant host through various hormones (Berlanga-Clavero et al. 2020) (Figure 2). These hormones, such as auxins and cytokinins, can promote host growth or signal increased resistance to some pathogens, and others can inhibit some plant systems to benefit

the host. These symbiotic relationships highlight the importance of the phyllosphere microbiome in the overall health of the host plant and the importance of maintaining or controlling the phyllosphere microbiome in agricultural practices.

The Grapevine Phyllosphere

Grapevines are perennial plants used for various agricultural practices worldwide. The phyllosphere microbiome of grapevines, specifically those used for winemaking, is an example of how the plant microbiome can be utilised within agriculture. As a diverse pool of microorganisms, the phyllosphere can directly impact the sensory profile of wine (Combina et al., 2005; Piao et al., 2015) as well as influence the yield and quality of the grape berries produced on the vine by influencing the health of the host vine (Bettenfeld *et al.,* 2022). The grapevine phyllosphere microbiome also acts as a highly diverse pool of microbes that can be utilised during the fermentation process during wine production. Current investigations into the microbiome of grapevines have focused on its involvement in the regional variation in wine characteristics (terroir). Depending on which country, region, or vineyard a grapevine is planted and grown has been found to have some influence on the microbial communities present on the surface of the plant (Renouf et al. 2006; Bokulich et al. 2016; Mezzasalma et al. 2017; Vitulo et al. 2019; Kioroglou et al. 2019). Interactions such as these have driven more research to define the grapevine microbiome further and understand its role in vine health and wine production.

ROLE OF MICROBES IN WINEMAKING

Fermentation Process

Louis Pasteur discovered that producing wine from grape juice resulted from microbial organisms in the nineteenth century. With advancements in biochemistry and microbiology,

the understanding behind this complex fermentation process has been investigated extensively. The fermentation process, generally, is completed during 'primary fermentation' by inoculated yeasts, wherein most of the alcohol content is produced and is followed by 'secondary fermentation', which occurs during optional aging processes that advance the wine sensory profile (Styger et al. 2011; Piao et al. 2015). Two categories of microorganisms: fungi (predominantly yeasts) and bacteria are recognised as the driving force of the primary and secondary fermentation processes, as well as the more recently recognised 'spontaneous fermentation' process performed by wild yeasts and bacteria, which originate from the plant's microbiome (Gutiérrez et al. 1999; Beneduce et al. 2004; Clemente-Jimenez et al. 2004; Combina et al. 2005). The end products of these fermentation processes are a variety of organic compounds, the most abundant of which is alcohol (ethanol). However, various other compounds are produced, each contributing to the wine's sensory profile.

Sensory Profiles

During the winemaking process, winemakers must consider many variables that could impact the wine outcomes, from which grapes are used to strain inoculated yeasts. Each step is essential for developing a desired sensory profile within the wine. Sensory compounds obtained from the grape berries themselves, or those produced by the microbial communities present during fermentation, dictate how the wine will present in flavour and aroma (Vilanova et al. 2010). The sensory profile of wine can be perceived either by taste or smell (Figure 3) through complex interactions with more than 1,000 volatile and non-volatile compounds (Tao and Li 2009), such as higher alcohols, organic acids, and esters. (Tao and Li 2009; Fairbairn et al. 2014). Further variations and configurations of these compounds are present in each type of wine, providing a unique sensory profile to each.

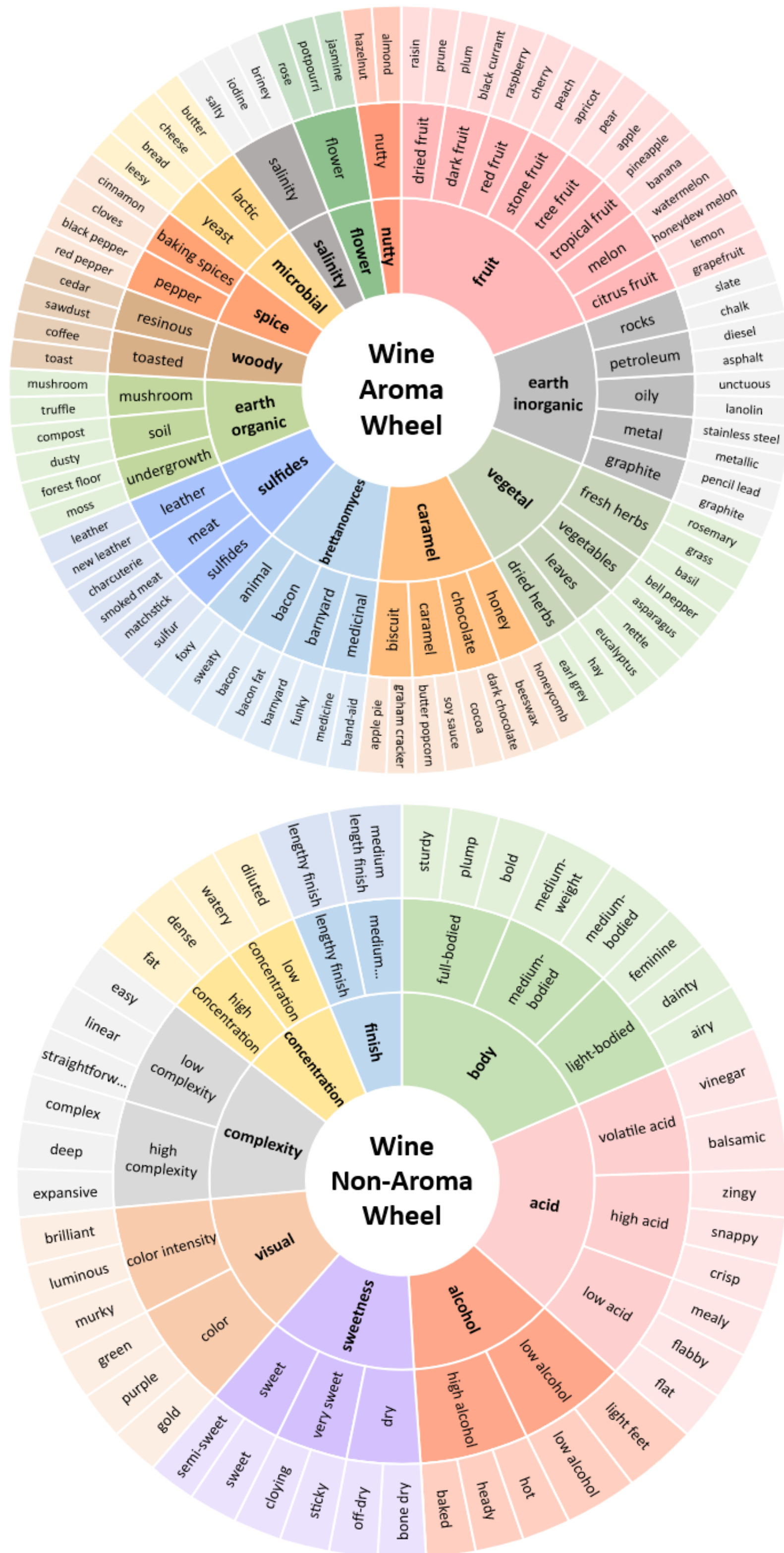

Figure 3 | Wine sensory profiles can be broken down into particular characteristics, each contributing to a wine's unique profile (Shuring 2019).

The impact of multiple factors that affect these sensory compounds has already been investigated at the plant level, showing that environmental factors such as water availability (Bonada et al. 2015) and soil type (Falcão et al. 2008; Zarraonaindia et al. 2015) play integral roles in the resulting sensory profile. From this, the grapevine's environment has been found to affect the resulting wine's outcome significantly; however, the driving force behind the development of a wine's sensory profile is predominantly driven by microbial action. Through complex biological mechanisms, microorganisms can produce many volatile and non-volatile compounds that determine a wine's sensory profile. Both yeasts and bacteria, either endogenous or exogenous to the ferment, are the main drivers of fermentation, and as such, their impact on the wine's sensory profile is well understood.

Fungi (Yeast)

As well as yeasts being the lead fermentation machinery in winemaking, other vine-borne fungal communities are responsible for much of the flavour and aroma compounds (Gil et al. 1996; Styger et al. 2011; Montevecchi et al. 2015). Both wild and inoculated yeast species provide enzymes responsible for transforming neutral grape compounds into flavour-active compounds (Stashenko et al. 1992; Styger et al. 2011). Different inoculated yeasts each uniquely impact the wine by producing different primary and secondary metabolites.

Although a multitude of yeasts are available for inoculation, the inoculated *Saccharomyces cerevisiae* or 'brewer's yeast', is almost exclusively responsible for the production of alcohol (ethanol) through fermentation of sugars available in grape juice (must), with *S. cerevisiae* being a species of domesticated yeast that can survive in high-alcohol conditions (Casey and Ingledew 1986). However, researchers have found wild yeast species to outcompete the inoculated yeasts in the early stages of fermentation. During these early stages, known as

spontaneous fermentation, the wild yeasts can produce various sensory compounds and textures within the wine before the live populations decline due to increased alcohol levels. This increase drives a selective pressure that facilitates the proliferation of *S. cerevisiae* and other alcohol-tolerant communities, triggering the primary fermentation stage (Bagheri et al. 2017).

Bacteria

Bacterial species present during fermentation are also known to play some critical roles in the winemaking process; however, the full extent of these processes is still limited. With the improved understanding of the phyllosphere and endophytic microbiomes, researchers have found that the surface and tissues of the grapevine are hosts to a high diversity of bacterial species alongside the yeast communities (Portillo et al. 2016; Morgan et al. 2017; Vitulo et al. 2019; Dong et al. 2019). Current literature has focused on a few key species present in these large and diverse communities; however, due to the interconnected nature of the microbiome, it is essential to understand how the bacterial communities affect the outcome of the fermentation process. The current understanding of bacteria's role in the winemaking process is limited to a small group of bacterial species performing malolactic fermentation (MLF) (Figure 4) driving buttery and cheesy flavours in the wine (Figure 3 & 4). These bacterial species, known as lactic acid bacteria (LAB), include *Lactobacillus*, *Pediococcus* and *Oenococcus* spp., and primarily originate from the plant microbiota (Lonvaud-Funel 1999; Bae et al. 2006). Although bacteria are commonly associated with spoilage and the production of unwanted sensory compounds, some bacteria benefit the winemaking process by contributing wine esters through esterase activities (Sumby et al. 2010; Pérez-Martín et al. 2013). These esterase reactions produce aroma-promoting ethyl esters of organic acids, fatty acids, and acetates of higher alcohols, similar to yeasts (Pérez-Martín et al. 2013).

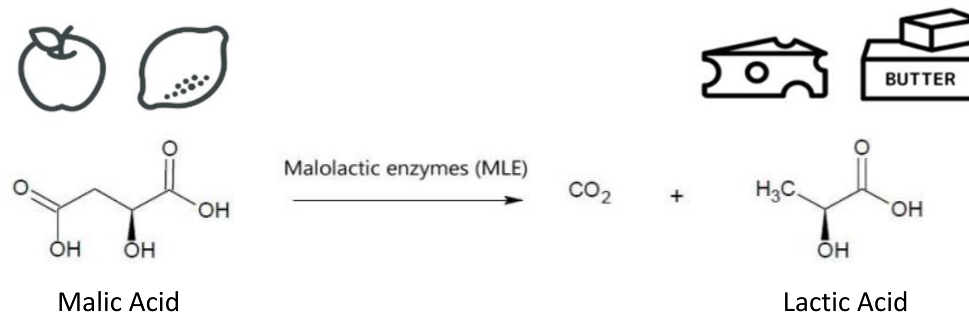

Figure 4 | The process of malolactic fermentation is the degradation of malic acid into lactic acid by malolactic enzymes produced by lactic acid bacteria (LAB). This reaction drives changes in the wine's sensory profile from sour apple/citrus aromas to soft cheese/buttery aromas.

The MLF activities also reduce acidity in wine while providing microbiological stability by reducing available nutrients in the form of malic acid (Liu 2002). Although lactic acid bacteria are known to play vital roles in the winemaking process, there have been fewer investigations to understand the complete diversity of bacteria within the grapevine microbiome and what effect 'non-LAB' species may play throughout the winemaking process.

DISEASE TREATMENTS

Disease control by human intervention is essential in protecting grapevines for winemaking. It could be one factor that determines the structure and diversity of the grapevine microbiome. Many vineyards employ antimicrobial chemical spray regimes, commonly copper or sulphur-based, to destroy harmful pathogens. Grape growers frequently use copper-based compounds for the metal's fungicidal properties, particularly against downy mildews. Although it is not entirely understood on a molecular level how copper can kill microbes, many previous studies have demonstrated the almost universal antimicrobial effects of metallic copper and copper-based compounds (Large 1945; Roca et al. 2007; Grass et al. 2011). Although extremely useful

and versatile for disease management in vineyards, the ubiquitous nature of copper and sulphur also threatens the microbiome of treated plants. With the non-targeted nature of both compounds, these and other broad-spectrum treatments likely act as an intense selective pressure on the microbial communities that make up the plants' microbiota. This could present downstream consequences on the wine's fermentation and sensory profile and the plant's health and viability.

The effects of broad-spectrum fungicides have already been presented for other plants, such as tomatoes and tobacco (Ottesen *et al.* 2014; Chen *et al.* 2021), as well as the impacts of fungicide drift on the microbiome of surrounding wild plants (Castañeda *et al.* 2018). With the total vineyard area in Australia being over 140,000 ha as of 2021 (AgEconPlus 2021), understanding the impacts of fungicide use on the plant microbiome is of growing interest, not only for improved viticultural outcomes but for the introduction of more sustainable practices in vineyards within the wine industry.

CONCLUSION

Studies have closely examined the off-target effects of pesticides to ensure that various treatments pose little to no direct threat to the plant's health. However, current studies focus little on the off-target effects of these disease treatments on the plant's microbiome. As many of these pesticides are broad-spectrum, they likely impact the microbial diversity of the plant's microbiome. Due to the interconnectedness of the plant's health with its microbiome, preserving these microbial communities is essential in producing healthy, high-yielding plants and high-quality wines. Therefore, to ensure grapevines maintain a healthy microbiome, the off-target effects of these broad-spectrum disease treatments must first be identified and understood. By furthering our understanding of these interactions, grape growers can refine

current methods for more efficient disease control with healthier grapevines, improved fruit quality, and the production of higher-quality wines.

RESEARCH QUESTIONS

Currently, investigations into the interactions between the phyllosphere microbiome and disease management in grapevines and wine production are limited. This gap includes how current disease treatments and preventative measures for grapevines affect the microbiome of the plant and the impact this has on the quality of wines produced from treated plants. This project will propose two main research questions, which I will use to investigate these interactions and provide better insight into disease management and current measurements' impact on the winemaking process.

Do current pesticide regimes adversely affect the phyllosphere microbiome diversity of grapevine plants?

Current literature investigating off-target effects of antimicrobial spray regimes used to fight and prevent disease is limited to the direct impacts on the grapevine plant. However, disruption of the phyllosphere microbiome is another potential off-target effect. Many of the widely used pesticide treatments have broad-target effects, which could affect the symbiotic and commensal communities of the microbiota. Due to the importance of the grapevine microbiome, the impacts of these fungicides on the phyllosphere microbiome could put treated vines at risk of decreased quality and viability.

How do pesticide treatment regimens affect the phyllosphere microbiota of grape berry fruit and wine ferment, and what are the downstream effects on resulting wines?

Current research is quickly demonstrating the importance of vine-borne microbes in the winemaking process, particularly during fermentation. Due to the growing interest in

preserving the diversity of these vine-borne microbes to facilitate more advanced 'spontaneous fermentation' reactions, the effect of various biotic and abiotic factors on these communities is under investigation. In addition, most pesticide regimes continue into the plant life cycle's grape development stage and directly affect the surface microbiota of developing fruit. This impact could drive selective pressures on the microbes derived from the vine to ferment, altering the wine's sensory profile outcomes.

AIMS/OBJECTIVES OF THE PROJECT

I have produced three aims to enhance the current understanding of the interactions between pesticide treatments, the phyllosphere microbiome, and the effect on the wine's sensory profile.

Aim 1: Using 10 potted *Vitis vinifera* (Shiraz) grapevine plants, microbial harvesting techniques, including whole leaf wash and swabbing, can be compared for optimal microbial yield. I will then be able to use this method of harvesting to test for effective quantification of live microbial abundance using propidium monoazide (PMAxx) photoactive viability dye and qPCR techniques (together known as vPCR). This work will act as an ancillary project of the more extensive study performed in subsequent parts of this project.

Hypothesis: **The live microbial communities can be effectively quantified using more controlled sampling techniques and PMA viability selective dye.**

Aim 2: Using 15 potted *Vitis vinifera* (Shiraz) grapevines, I aim to explore the effect of disease treatments on the microbiome. I will subject potted vines to controlled variations of common copper- and sulphur-based fungicides collecting phyllosphere samples at several post-treatment time points. The microbial diversity found on the leaf phyllosphere samples will be

compared using 16S (bacteria) and ITS (fungi) rRNA gene amplicon sequencing methods. This analysis will provide insight into how each antimicrobial treatment affects the microbial communities of the grapevine phyllosphere over time and the identification of species that demonstrate resistance or tolerance to treatments.

Hypothesis: **The non-specific antimicrobial treatments, copper and sulphur, will act as a selective pressure on the microbial communities of the phyllosphere microbiome, reducing the diversity of the microbiota to select groups of resistant species.**

Aim 3: Using *Vitis vinifera* (Shiraz) grapevines grown in established vineyards with varying pesticide treatment regimens (conventional, organic, and biodynamic); the fruit will be picked and used for small-lot winemaking. Microbial samples will be collected from the grape berry surface and small-lot ferments for 16S and ITS rRNA gene sequencing. I will then assess the resulting wines through RATA (Rate All That Apply) sensory analysis and overlay the results of the microbial analysis to observe potential correlations between the microorganisms present on the fruit and the sensory profile of the wine.

Hypothesis: **Reduced diversity of the grape berry phyllosphere microbiome will affect the development of sensory compounds produced during spontaneous fermentation, resulting in distinct changes to the wine's sensory profile between disease management techniques.**

SIGNIFICANCE/CONTRIBUTION TO THE DISCIPLINE

These analyses will develop a broad understanding of various pesticide treatments' role in shaping the phyllosphere microbiome of grapevines used for winemaking. It is likely that repeated exposure to antimicrobial sprays, particularly broad-spectrum fungicides, will act as a potent stressor on the microbiome, reducing its abundance and diversity. By understanding how the leaf and fruit phyllosphere microbiomes are affected by different pesticide treatments. Investigations can begin to maintain the grapevine's microbial diversity, thus reducing any indirect challenges the vine faces. Additionally, explorations into the adverse effects on the ferment and derived wines will allow for a better understanding of the role of wild microorganisms in the spontaneous fermentation stages. This research could be of great significance to the Australian Wine Industry due to increased interest in wines produced using only wild fermentation requiring preservation of the grapevine microbiota. This research could also point out areas of improvement for sustainable vineyard practices by encouraging more improved and targeted disease management.

As well as this, an effective viability selection protocol and improved microbial harvest protocol for use on phyllosphere microbiome samples are also significant to all plant phyllosphere studies. The development of a protocol allowing for effective analysis of live bacterial and fungal communities on the plant's surface will be an essential tool for investigating the effect of various stressors in all plant-microbiome studies and is not limited to investigations of *Vitis* species.

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Chapter II

A Methodological Review: Contamination and Controls in Phyllosphere Research.

This chapter focuses on the current standards involved in conducting phyllosphere microbiome research. In many other fields of microbiome research, there is a clear understanding of the potential dangers of microbial contamination in microbiome samples and how using various controls should be standard practice when conducting microbiome research. I have completed a systematic review of the literature observing the current standard procedure for contamination mitigation in phyllosphere microbiome research and have made suggestions on how the current standards can be improved.

Statement of Authorship

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Contribution to the Paper	Reading of relevant literature and compiling information. Primary writer and figure production. Review and editing of manuscript, conceived and designed the study. Facilitated design of methodologies. Literature search and data analysis.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Raphael Eisenhofer Philipona		
Contribution to the Paper	Reading of relevant literature and compiling information. Writing and figure production. Review and editing of manuscript, conceived and designed the study. Facilitated design of methodologies. Literature search and data analysis.		
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TITLE

"A Methodological Review: Contamination and Controls in Phyllosphere Research."

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KEYWORDS

Microbiome, microbiota, phyllosphere, epiphytic, plants, controls, contamination, standards

SUMMARY

Purpose:

Contamination is an ever-present risk in all microbiome studies, especially when low-biomass sample types are being analysed. Despite efforts to mitigate contaminating taxa, such as by wearing appropriate PPE, using specialised laboratories, and practising aseptic techniques, contaminants will be present. Alongside contamination mitigation, the collection of multiple positive and negative contamination controls is essential for reducing the impact of contamination.

Methods:

Within this analysis, we take a systematic approach to search the current literature and observe the prevalence of various positive and negative contamination controls in recent phyllosphere microbiome studies. Using tools such as 'Publish or Perish' for literature searches and 'Covidence' for study screening and data extraction, we were able to conduct a search on 450 studies, apply relevant inclusion/exclusion criteria, and observe the current standard for quality control in the phyllosphere research landscape.

Results:

In brief, our systematic approach showed that only 6.29% of studies collected a positive control, 20.98% collected a negative control, and only 2.1% of studies collected both a positive and negative control. Of these collected controls, 91.67% of positive controls were sequenced; however, worryingly, only 42.42% of negative controls were sequenced and analysed. In light of these results, we propose a set of minimum standards to be used in future phyllosphere microbiome studies.

ABSTRACT

DNA contamination can critically confound microbiome studies. Here we take a systematic approach to review the current literature and investigate the prevalence of contamination controls in phyllosphere microbiome research over the past decade. By utilising systematic review principles for this review, we were able to conduct a thorough investigation, screening 450 articles from three databases for eligibility and extracting data in a controlled and methodical manner. Worryingly, we observed a surprisingly low usage of both positive and negative contamination controls in phyllosphere research. As a result, we propose a set of minimum standards to combat the effects of contamination in future phyllosphere research.

INTRODUCTION

Microbiomes are becoming increasingly studied in many biological disciplines. These microbial communities are seemingly everywhere in the literature, and although they are of particular medical interest today, some of the first microbiomes defined were those found on plants (Whipps *et al.* 1988). Today, current literature shows us that plant microbiomes can be just as complex as our own, with each plant supporting multiple, distinct communities of microorganisms (Morris 2002; Turner *et al.* 2013). Phyllosphere microbiome research is a relatively new area of interest in plants that seeks to investigate the microbial communities that inhabit the surface of a plant's shoots (the phyllosphere) (Morris 2002; Bashir *et al.* 2022). Phyllosphere research holds much promise for further understanding plant physiology with implications in many plant-focused industries, particularly ecology and agriculture (Parasuraman *et al.* 2019; Zhu *et al.* 2022; Welsh *et al.* 2023).

To study the microbiome, an array of molecular techniques are used, such as real-time PCR and DNA sequencing technologies. As these techniques are highly sensitive, they can detect contaminant DNA (DNA external from the sample) (Eisenhofer *et al.* 2019). This contaminating DNA can originate from many sources, such as the environment during sample collection (Witt *et al.* 2009), lab surfaces and reagents (Salter *et al.* 2014), as well as cross-contamination (Ballenghien *et al.* 2017) and the researcher themselves. It has been shown that failure to account for DNA contamination can confound microbiome studies (Salter et al. 2014), and has led researchers into thinking that microbiomes exist where none are present (e.g. placenta) (Perez-Muñoz *et al.* 2017; Panzer *et al.* 2023). Despite contamination being increasingly recognised within other microbiome fields, plant microbiome research, namely phyllosphere microbiome research, lacks many of the control measures used to minimise these effects.

Here we draw focus on phyllosphere microbiome research, which has been shown to be typically low-biomass, likely due to the hostility of the phyllosphere micro-environment (Bashir *et al.* 2022). Naturally, as these samples are low-biomass, they may be especially susceptible to contamination (Eisenhofer *et al.* 2019). However, currently the scale and potential impacts of contamination on phyllosphere microbiome research is unknown. To fill this gap, we applied a systematic approach for surveying the phyllosphere literature to determine the status of this field in respect to current best practices in microbiome research.

OBJECTIVE OF THE REVIEW

A key reason for conducting this methodological review was the observation that many phyllosphere microbiome studies report bacterial genera that have been previously identified as common contaminants (e.g. *Sphingomonas, Novosphingobium, Pseudomonas,*

Bradyrhizobium) (Eisenhofer *et al.* 2019; Piro and Renard 2023). While there is some solid evidence for the presence of some of these genera on plants — *Pseudomonas syringae* is a well-studied plant pathogen — confirmation is lacking for others. If negative controls are not included, disentangling real from contaminant sequences for these genera is unfeasible, particularly given the limited taxonomic resolution of short 16S rRNA variable regions.

Here we use methods inspired by systematic review processes (Figure 1) to perform a methodological review of the literature to identify the prevalence of the contamination controls in the design of phyllosphere microbiome studies. The specific study questions we discuss are: (1) How prevalent are controls used to minimise contamination or the effects of contamination? and; (2) What standards should be introduced to phyllosphere microbiome research to reduce the effects of contamination as a minimum standard? By performing this analysis using systematic approaches we can begin to understand the approaches currently being utilised to combat contamination in phyllosphere microbiome studies. Here we employ a broad search

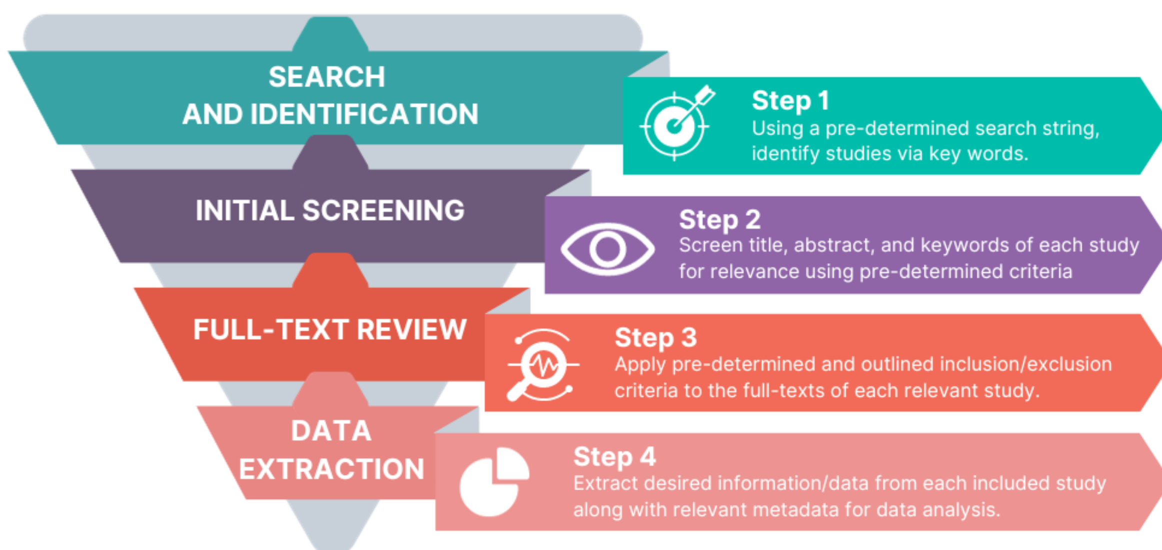

Figure 1 | Funnel pipeline representing the systematic approach to the search and screening process, involving pre-determined methodologies.

criterion on a number of databases to collect a catalogue of phyllosphere research and assess the current contamination mitigation efforts within the field to determine the rate at which various contamination control methods are collected and used.

METHODS/DESIGN

Before initial literature searches, a detailed review protocol was developed to ensure consistency across searches and detail the inclusion and exclusion criteria for determining the study set. The literature search was conducted between May and June 2023 but only included articles published between 2012 and 2022. The search was conducted using the program 'Publish or Perish' which was used to search three major databases and search engines (PubMed, Google Scholar, and Scopus). Search strings were developed using the desired keywords for the literature search. The same search string was used across all databases:

phyllosphere OR epiphytic* AND microbiome OR "microbial communities" OR "fungal communities" OR "bacterial communities" OR communities*

The first 150 results were selected from each database search which was subsequently imported into the program 'Covidence' and de-duplicated. Studies were included according to the inclusion criteria shown in Table 1.

Prior to article screening, a kappa-statistic (McHugh 2012) was also conducted to assess the interrater reliability between the two reviewers (raters). 20 papers were taken from the search results of each database (n = 60) which were each screened by both reviewers and either included or excluded. The expected agreement (60%) and observed agreement (92%) were then

Table 1 | Inclusion criteria developed prior to screening database search results for eligible original research articles.

Feature	Criterion for Inclusion
Language:	English
Article Type:	Original research article.
Relevant Subject(s):	Terrestrial plant phyllosphere microbiome.
Intervention(s) and Comparison(s):	Any
Outcomes:	All successfully published in peer-reviewed journals.
Methodologies:	16S rRNA and/or ITS gene amplicon sequencing.

calculated and used to calculate the kappa-statistic (K = 0.79) which indicates substantial agreement and high interrater reliability.

Each article then was assessed for relevance independently by the reviewers by title, abstract and keywords, using the program 'Covidence'. Following initial screening, the remaining articles were then assessed by full-text and excluded or included based on the prior established criteria. Due to the broad nature of the search applied, many sources of heterogeneity exist within this dataset. To acknowledge these sources of heterogeneity, we identified the top contributors and produced a metadata table containing each included article with relevant details extracted from them. As a result, the top contributing sources of heterogeneity were determined to be country, host organism, sample type, microbial harvesting method, extraction kit used, amplicon primer set, and sequencing platform. Information of these sources was collected for each article (Supplementary Table 1).

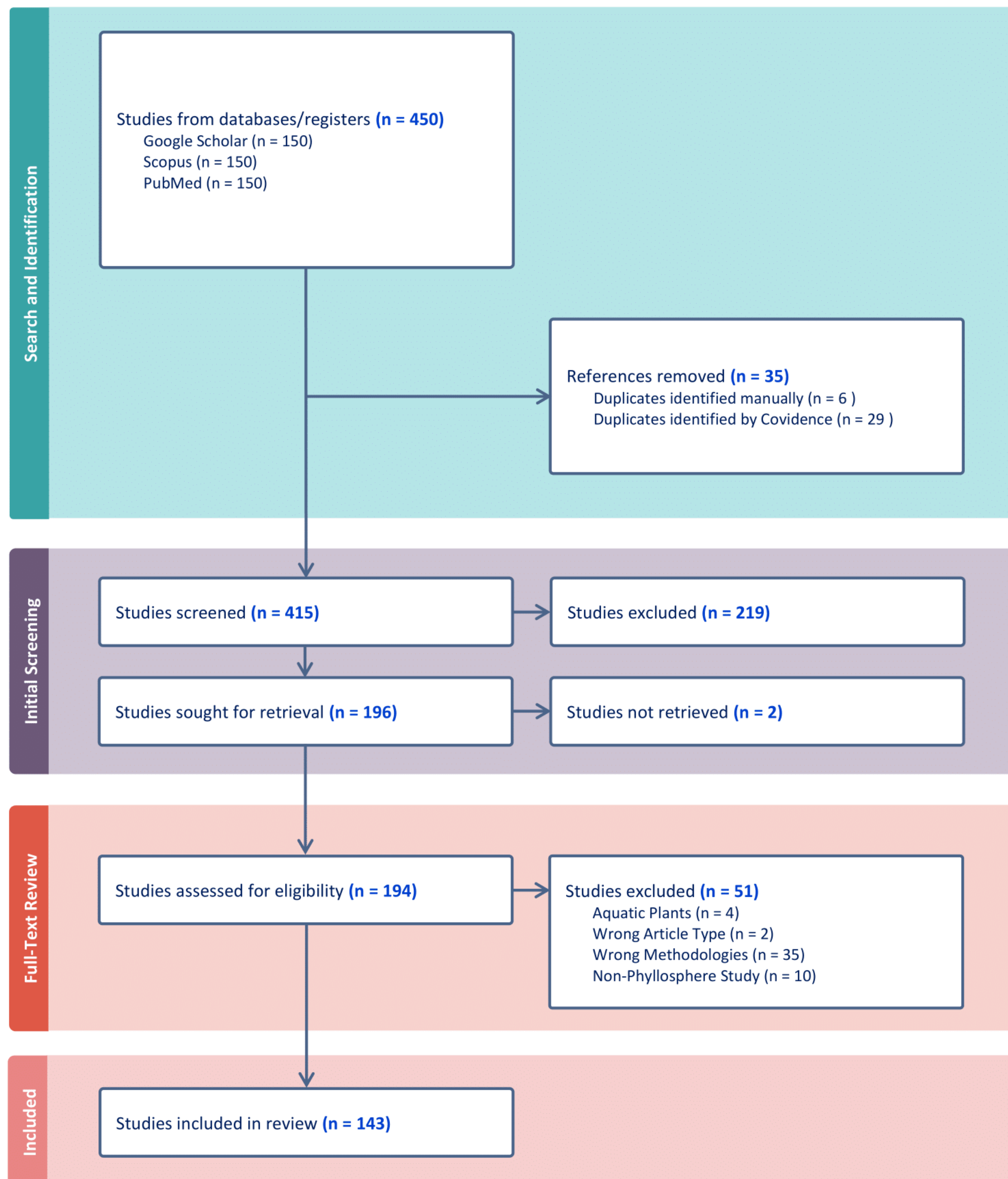

Figure 2 | A PRISMA-style flow diagram demonstrating the systematic process that was followed to include eligible papers captured by our search. From the initial search (n = 450), 31.78% passed the inclusion criteria (n = 143).

ANALYSIS AND RESULTS

From our initial search results of 450 articles, a total of 143 studies passed our inclusion/exclusion criteria (Figure 2), and their geographic distribution varied (Figure 3).

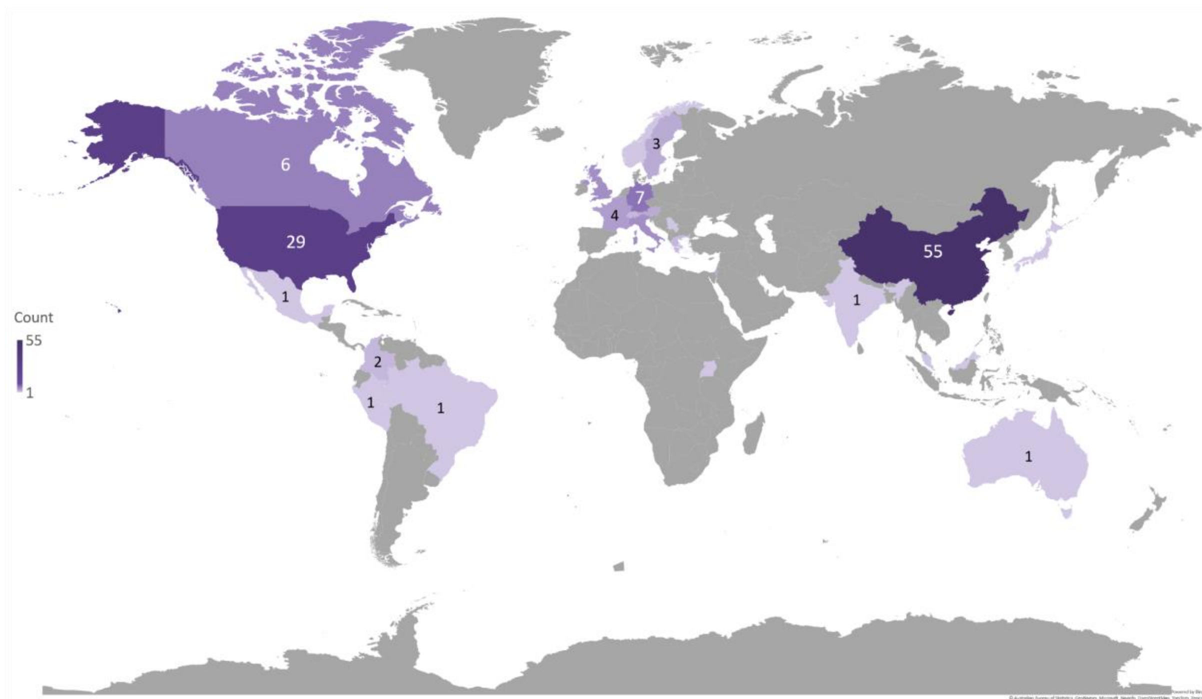

Figure 3 | Map of the world showing the geographical location of each included study.

Among the included studies, China was the most common location for the phyllosphere microbiome research, accounting for 38.5% of all studies (n = 55) with the second most prevalent country being the United States, with 20.3% of the included studies (n = 29).

Our analysis of the included studies revealed a lack of consensus regarding contamination control standards among the current phyllosphere microbiome studies. Of the included studies, only 6.3% utilised positive controls exclusively, while 21% relied solely on negative controls (Figure 4a). A smaller proportion, 2.1% of the studies, employed both positive and negative controls simultaneously (Figure 4a). Notably, these results show that, of the included studies, 70.63% did not use, or at least report on, contamination controls within their methodologies.

Regarding the specific types of controls used, PCR negative controls were the most reported type among the negative controls (Figure 4b). For positive controls, Spike-Ins were

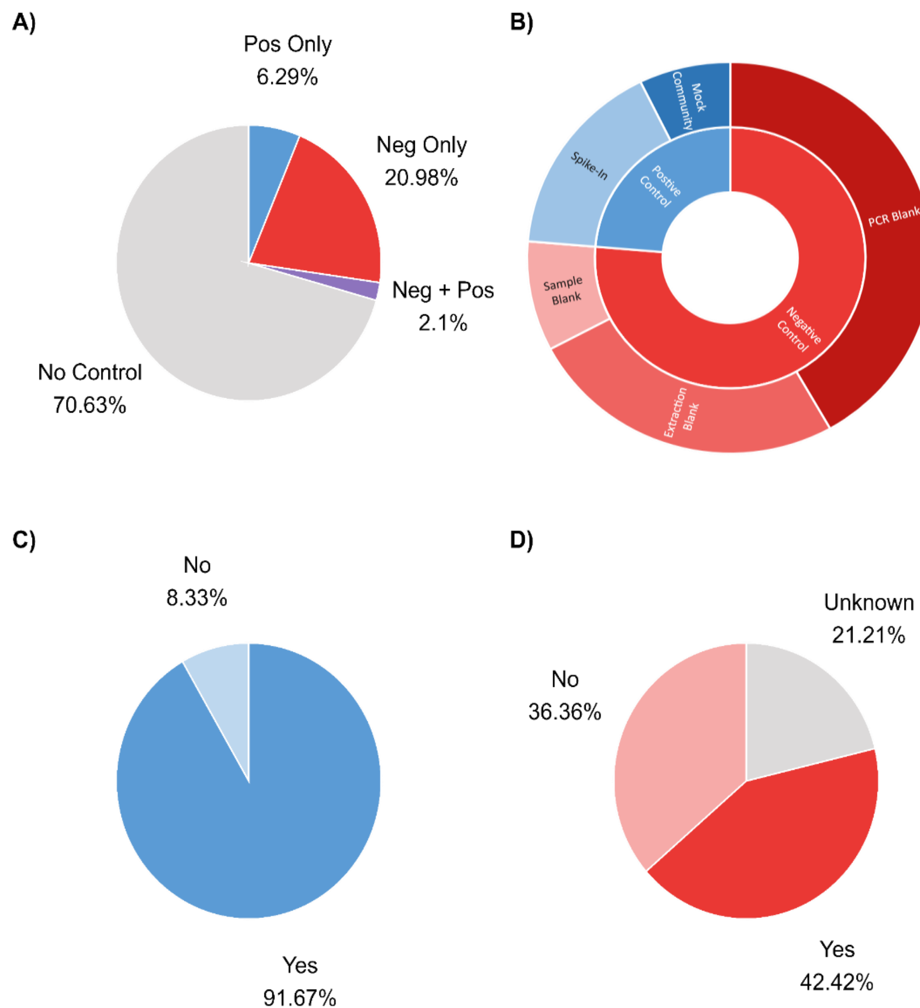

Figure 4 | Prevalence of each of the recommended positive and negative controls in phyllosphere microbiome research. A) The percentage of studies that included at least one of each control against those that did not collect a control. B) The prevalence of each collected control type. C and D) Pie charts showing the proportion of studies that sequenced and analysed their positive and negative controls respectively.

the preferred choice. Surprisingly, none of the studies incorporated all recommended controls, and only one study implemented the minimum standard we aim to suggest. Additionally, some studies deviated from using typical controls and utilised non-typical controls including fake plant negative controls and multiple extractions (Supplementary Table 2). A considerable

majority of positive controls (91.7%) were subjected to DNA sequencing (Figure 4c). In contrast, less than half (42.4%) of the negative controls were sequenced. (Figure 4d). In summary, only 9.8% (14/143) of studies had negative controls that were sequenced and analysed.

We finally sought to examine whether the usage of negative controls in phyllosphere research has increased through time as the broader microbiome field has become more aware of DNA contamination. From 2012-2022, we failed to observe a trend of increasing negative control usage and sequencing in the phyllosphere microbiome literature.

RECOMMENDATIONS FOR FUTURE PHYLLOSPHERE RESEARCH

In light of our findings that a small fraction (10%) of phyllosphere microbiome studies include and sequence negative controls, and that this is not increasing through time, we provide the following recommendations for future research in this space (Box 1). We elaborate on these recommendations below and remark on some findings relating to the diverse set of methodologies used to study plant phyllosphere microbiomes.

Box 1

- Include and sequence each negative control type.
- Determine the limit of detection.
- Analyse, compare and report on negative controls in relation to biological samples.

INCLUDE AND SEQUENCE ONE OF EACH NEGATIVE CONTROL

The use of a series of negative controls can help to identify individual instances and frequencies of contamination throughout sample collection and processing. Here we detail a set of negative contamination controls to act as a minimum standard for phyllosphere microbiome research:

Sampling Controls

During sample collection, contaminants from the environment, researchers, or reagents/tools can be identified through the collection of sampling blank controls (SBCs). These controls act to identify contaminating species which may be collected throughout the sampling process. SBCs are collected by acting out the complete process of sample collection, e.g., opening tubes and using swabs, without contacting the biological subject e.g., a leaf.

Extraction Controls

The DNA extraction process is a part of the sample processing pipeline which can act as a large source contaminating taxa or DNA. Different DNA extraction kits (and lots within a kit) can harbour their own contaminant profiles — coined the 'kitome' (Salter *et al.* 2014; Olomu *et al.* 2020). To identify the 'kitome' contaminants, and other contaminants present during the DNA extraction process, a set of extraction blank controls (EBCs) is recommended. EBCs help to identify contaminant taxa or DNA present within the DNA extraction kit, from the laboratory environment, or the researcher but may also help to identify large levels of cross-contamination between neighbouring samples. EBCs are prepared identically to the DNA extraction of biological samples without the addition of any sample before the cell-lysis step.

PCR Controls

During PCR amplification or quantification through real-time PCR, contaminating DNA can enter samples and be amplified alongside target DNA. To detect these signals, a PCR negative control 'No-Template Control' (NTC), can be used. NTCs are set up identically to biological samples, however, instead of DNA template being added to the reaction, it is replaced with additional PCR-grade water (ideally, the same water used to prepare the PCR master mix).

OPTIONAL BUT HIGHLY RECOMMENDED CONTROLS

Positive controls are far less common, and more costly, than negative contamination controls, however, can help with further quality assessments such as limit of detection, methodological bias, and detecting cross contamination where feasible.

Mock Communities

Microbiome mock communities are cultures of known quality and quantity which are prepared simultaneously to samples. These controls undergo DNA extraction, PCR amplification and DNA sequencing. Mock community controls allow for the analysis of a known sample alongside microbiome samples, which can indicate stages of failure or bias in a DNA processing pipeline.

Spike-Ins

Spike-in controls are DNA controls added to samples prior to PCR amplification and typically come in the form of synthetic target molecules, such as 16S rDNA (Gala *et al.* 2023). These controls can be used to monitor cross-contamination (Tourlouss *et al*. 2018) and quantify absolute changes in abundance between samples (Tkacz *et al*. 2018).

DETERMINE THE LIMIT OF DETECTION

Comparing the amount of DNA in biological samples to negative controls (limit of detection) is a crucial step for authenticating robust microbiome signals from samples. Briefly, quantitative PCR (qPCR) is applied to both biological samples and negative controls (and optionally to samples known to have robust biomass — e.g., mock, soil). This allows researchers to estimate the microbial biomass of their samples, and if this biomass is similar to quantities observed in negative controls, provides a warning that the biological samples have little-to-no DNA and are thus highly susceptible to contamination. For a good example of this approach being applied, we highlight the paper by Leiby *et al.* (2018).

ANALYSE, COMPARE AND REPORT ON NEGATIVE CONTROLS IN RELATION TO BIOLOGICAL SAMPLES

All collected controls are intended to be processed identically to biological samples and sequenced or quantified alongside actual samples for bioinformatic analyses. By producing these stepwise negative controls (SBC → EBC → NTC) the origin of each set of contaminants can be established and significant contamination events can be isolated and resolved for future projects. Tools such as Decontam or SCRuB (Davis et al. 2018; Austin et al. 2023), should also be used to properly identify and map contaminants present within actual samples through prevalence filtering using the negative controls. Along with the collection, sequencing, and analysis of these negative controls, the raw sequencing reads produced should also be made publicly accessible alongside samples to allow for reproducibility.

OTHER FINDINGS ON METHOD USE AND DATA AVAILABILITY

While not the main focus of this paper, we also collected information on phyllosphere sample collection, DNA extraction methods, PCR primer usage, and the availability of sequencing data

(this data is available on GitHub and can be further scrutinised). Briefly, we found a diversity of phyllosphere sample collection techniques, including the washing of leaf surfaces, pulverisation of whole leaves, and concentration of cells via membrane filtration. Within these broad categories, we also observed a wide array of differences, such as wash buffer composition, sonication vs. rocking, cell pelleting speeds, and sample input mass. DNA extraction kit choice and PCR primer usage was also varied. We also sought to determine what proportion of phyllosphere studies had raw sequencing data that was publicly accessible and found that raw sequencing data could not be accessed for 22.8% (32/140) studies. From these supplemental findings, we suggest that more work is done comparing different sample collection methods. Currently it is unclear whether these different methods are comparable to one another, and future standardisation could allow for large-scale comparisons between phyllosphere studies. Such comparisons could yield new insights, as has been demonstrated before in initiatives such as the Earth Microbiome Project (Thompson *et al.* 2017).

CONCLUSIONS

In summary, our systematic-style review demonstrated a concerning lack of consensus and adherence to modern contamination control standards in the phyllosphere microbiome literature. The limited usage of negative controls raises doubts about the reliability and validity of the reported findings. To ensure the integrity of phyllosphere microbiome research, efforts are needed to promote the adoption of contamination control measures in the field. Through the adoption of a minimum standard for contamination control we can further advance our understanding of the phyllosphere microbial communities and their implications for plant ecology and agriculture.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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SUPPLEMENTARY DATA

Supplementary Table 1 | Basic information tabulated for each included study. *Targets denoted by 'Unknown' indicate mention of ITS and/or 16S amplification, but specific target region was not detailed.

Study Reference	Bacterial DNA Target*	Fungal DNA Target*	Negative Controls?	Positive Controls?
Sun 2022	V3-V4	ITS1	No	No
Wicaksono 2023	V4-V6	ITS1	No	No
Mhuireach 2022	V3-V4		Yes	No
Zahid 2022	V3-V4		No	No
Fan 2023	V4-V5		No	No
Xiang 2022	V4	ITS1	No	No
Hu 2022	Unknown	Unknown	No	No
Scherer 2022	V3-V4		No	No
Graindorge 2022	V5-V6		No	Yes
Zhang 2022	V3-V4		No	No
Kröber 2022	Unknown		No	Yes
Sun 2022	V5-V7	ITS1	No	No
Johnston-Monje 2022	V4	ITS1	Yes	No
Yeo 2022	V3-V4		No	No
Liu 2022	V3-V4	ITS1	No	No
Dai 2022	V3-V4	ITS1-5F	No	No
Ishida 2022	V3-V4		No	No
Zheng 2022	V4-V5		Yes	No
Wang 2022	V5-V7		No	No
Tan 2022	V5-V7		No	No
Legein 2022	V4		Yes	No
Sanjenbam 2022	V3-V4		No	No
Kanukollu 2022	V3 (?)		No	No
Francioli 2022	V5-V6		No	No

Xu 2022	V3-V4	ITS1	No	No
Li 2022	V3-V4	ITS1	No	No
Schäfer 2022	V5-V7		Yes	No
Song 2022	V5-V6	ITS1	No	No
Li 2022	V3-V4		No	Yes
Olimi 2022	V4	ITS1	No	No
Liu 2022	V5-V6	ITS1	No	No
Liu 2022	V3-V4	ITS1	Yes	No
Zhang 2022	V4-V5		No	No
Li 2022	V4		No	No
Wang 2022	Unknown		No	No
Vincent 2022	V1-V4		No	No
Wang 2022	V4		No	No
Smets 2022	V4		Yes	No
Longa 2022	V5-V7	ITS3-4	No	No
Besaury 2022	V4		No	No
Song 2022	V5-V6	ITS1	No	No
Zhang 2022	V4		No	No
Wang 2021	V5-V6	ITS	No	No
Leducq 2022	V5-V6		Yes	Yes
Singer 2021	V4	ITS2	Yes	No
Runge 2023	V4-V5	ITS2	No	No
Meyer 2022	V5-V7		Yes	No
Wei 2022	V4	ITS1	No	No
Yan 2022	V3-V4		No	No
Dixit 2021	V4		No	Yes
He 2021	V1-V3	ITS1-ITS4	No	No
Debray 2022	V4	ITS2	Yes	No
Sun 2021	V3-V4		No	No
Massoni 2021	V5-V7		Yes	No
Mittelstrass 2021	V3-V4	ITS1	No	No
Palmer 2021	V4		No	No
Deng 2022	V3-V4		No	No
Lajoie 2021	V5-V6		No	No

Sauer 2021	V5-V6	ITS1	Yes	No
Mogouong 2021	V6-V8	ITS2	No	No
Du 2021	V5-V6		No	No
Ke 2021	V3-V4		No	No
Bechtold 2021	V5-V6		No	Yes
Zhou 2021	V3-V4		No	No
Dove 2021	V4	ITS2	No	Yes
Ibekwe 2021	V4		No	No
Huang 2021	V4-V5		No	No
Kusstatscher 2020	V4-V5		No	No
Zhou 2021	V4-V5		No	No
Li 2021	V3-V4		No	No
Sun 2021	V3-V4		No	No
Katsoula 2021	V4	ITS2	Yes	No
Dove 2021	V4	ITS2	No	Yes
Abdelfattah 2021	V4	ITS2	Yes	No
Taffner 2020	V4		No	No
Agler 2016	V3-V4, V5-V7	ITS1, ITS2	No	No
Bodenhausen 2013	V6-V7		No	No
Coleman-Derr 2016	V4	ITS2	No	No
Kembel 2014	V5-V6		No	No
Rastogi 2012	V5-V7		No	No
Xiong 2021	V5-V6		No	No
Ritpitakphong 2016	V5-V6		No	No
Copeland 2015	V5-V7		Yes	Yes
Zhu 2017	V4-V5		No	No
Maignien 2014	V4-V6		Yes	No
Jackson 2013	V5-V9		Yes	No
Laforest-Lapointe 2016	V5-V6		No	No
Fonseca-García 2016	V4	ITS2	No	No
Carlström 2019	V5-V7		No	No
Kim 2012	V1-V3		No	No

Berg 2018	V5		No	No
Cordier 2012		ITS1	Yes	No
Grady 2019	V4		No	No
Perazzolli 2014	V5-V9	Unknown	No	No
Williams 2013	V5-V9		Yes	No
Erlacher 2014	V4-V5		No	No
Cordier 2012		ITS1	Yes	No
Karlsson 2014		ITS2	No	No
Abdelfattah 2015	Unknown	ITS2	No	No
Yao 2019		ITS2	Yes	No
Morella 2020	V3-V4	ITS2	Yes	No
Dong 2019	V3-V4		No	No
Chen 2018	V4-V5		Yes	No
Kong 2018	V5-V6		No	No
Chen 2018	V4-V5		No	No
Tkacz 2020	V4	ITS1	Yes	No
Lopez-Velasco 2013	V4		No	No
Ottesen 2016	V4		Yes	No
Chen 2021		ITS1	Yes	No
Ruiz-Pérez 2016	V5-V6		No	No
Aydogan 2018	V5-V6		No	No
Jia 2020		ITS1	No	No
Herpell 2020	Whole 16S		Yes	No
Zhou 2021	V4-V5		No	No
Finkel 2016	V6		No	No
Ottesen 2013	V2		No	No
Wuyts 2020	V4		Yes	No
Katsoula 2020	V4	ITS2	No	No
Ashhab 2021	Whole 16S		Yes	No
Miller 2019	V4		No	No
Perazzolli 2020	V6-V8	ITS2	No	No
Espenshade 2019	V5-V7		No	No
Crombie 2018	Unknown		No	No

Kong 2020	V5-V6	ITS2	No	No
Parizadeh 2021	V5-V6		Yes	Yes
Janakiev 2020	V1-V3		No	No
Izhaki 2013	V3		No	No
Bao 2020	V5-V6		No	No
Ren 2014	V4		No	No
Aydogan 2020	V3-V4		No	No
Zhang 2019	V5-V7	ITS	No	No
Dees 2015	V6-V8		No	No
Yao 2020	V4		Yes	No
Hong 2017	V4	ITS1	No	No
Zhang 2018		ITS1	No	No
Xueliang 2020	V3-V4		No	No
Qin 2019	V4		No	No
Finkel 2012	V4-V6		No	Yes
Hough 2020	V4		No	No
Burch 2016	V5-V7		No	No
Khodadad 2020	V4	Unknown	No	Yes
Bowsher 2021		ITS2	No	No
Qian 2018		ITS2	Yes	No

Supplementary Table 2 | Non-typical controls found through data extraction of the included texts.

Study Reference	Non-Typical Negative Controls	Non-Typical Positive Controls
Mhuireach 2022	—	qPCR – though no limit of detection.
Johnston-Monje 2022	Negative controls mentioned but not detailed.	—
Meyer 2022	—	ddPCR – though no limit of detection.
Maignien 2014	Air sampling on CellTak-coated microscope slides.	qPCR – though no limit of detection.
Ottesen 2016	Plastic plants.	—
Finkel 2012	—	Multiple extractions.

Chapter III

Dead or Alive? Improving Current Phyllosphere Microbiome Harvesting Techniques and Quantification of Viable Communities.

This chapter focuses on testing the viability selection dye, Propidium Monoazide, to observe the living communities of the phyllosphere microbiome. As well as this, we investigate the use of swab techniques as an alternative and more controlled method of harvesting the phyllosphere microbiome.

Statement of Authorship

Title of Paper	Dead or Alive? Improving current phyllosphere microbiome harvesting techniques and quantification of viable communities.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript prepared for submission to the Journal of Applied and Environmental Microbiology.

Principal Author

Name of Principal Author (Candidate)	Brady L. Welsh		
Contribution to the Paper	Conceptualisation, method development, sampling, data generation, data analysis, writing and drafting.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	02 / 08 / 2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stephen P. Kidd		
Contribution to the Paper	Conceptualisation. Review and editing of manuscript.		
Signature		Date	

Name of Co-Author	Susan E. P. Bastian		
Contribution to the Paper	Conceptualisation. Review and editing of manuscript.		
Signature		Date	

Name of Co-Author	Raphael Eisenhofer		
Contribution to the Paper	Conceptualisation. Review and editing of manuscript. Methodology design.		
Signature		Date	

SUMMARY

Purpose:

Signals generated by relic DNA in quantitative PCR or DNA sequencing analyses can lead to false positives appearing within the results of microbiome datasets due to the inability to discern which signals originated from living microbes associated with the host. We have assessed the reduction in amplified relic DNA by using the viability selection dye, propidium monoazide (PMA), during the quantification of plant phyllosphere microbiome samples. PMA dye can be applied to microbiome research allowing for improved characterisation of viable, host-associated microorganisms.

Methods:

We propose using swabs for harvesting the phyllosphere microbiome, which can allow for standardised collection and efficient PMA treatment of phyllosphere microorganisms. We use quantitative PCR (qPCR) to measure yields of 16S rRNA (bacteria) and ITS (fungi) gene targets within leaf phyllosphere microbiome samples of grapevines and compare samples treated with PMA dye to those untreated to test the ability of PMA to remove relic DNA from phyllosphere samples.

Results:

This study shows that swabs are an effective alternative to harvesting the phyllosphere microbiome, which is more time efficient and standardisable than current methods, which involve lengthy washes of whole leaves which are physically heterogeneous and difficult to standardise. The results also conclude that the PMA viability dye effectively removed relic DNA from phyllosphere samples while maintaining a yield that could be effectively analysed.

TITLE

"Dead or Alive? Improving current phyllosphere harvesting techniques and quantifying the viable communities."

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KEYWORDS

Microbiome, microbiota, phyllosphere, epiphytic, sampling protocol, viability PCR

ABSTRACT

One of the most significant shortcomings of current microbiome analysis techniques is the inability to discern which organisms are viable or 'alive' at the time of sampling. We have utilised the viability selection dye propidium monoazide (PMA) to suppress relic DNA in pre-extracted samples to combat this. PMA prevents relic DNA amplification during PCR while being incapable of crossing the intact membrane of live cells, leaving DNA from living sources free for amplification. Here we demonstrate the ability of PMA to distinguish the living phyllosphere microbiome using viability PCR (vPCR) by removing phyllosphere relic DNA. Here we also propose using swabbing methods as an alternative phyllosphere microorganism harvesting technique against the current standards, which involve lengthy washes of whole leaves. With the use of swabs, greater control over the location of sampling and improved standardisation across sampling areas can be achieved, allowing for more accurate comparisons between phyllosphere samples, particularly during vPCR.

INTRODUCTION

The plant microbiome is divided into distinct regions hosting various prokaryotes, eukaryotes, and viruses. The three major regions of the plant microbiome are the rhizosphere (surrounding the roots), endosphere (within the plant tissues) and phyllosphere (surface of the aerial portion of the plant). The rhizosphere has been the main focus since its coinage (Hiltner, 1904). However, interest in the two other main microbial niches is increasing, demonstrating their importance in plant health (Andrews and Harris, 2000; Berlanga-Clavero *et al.*, 2020; Bashir *et al.*, 2022; Gouka, Raaijmakers and Cordovez, 2022). As the phyllosphere encapsulates the surface of the host plant, it acts as the interface between the host and its aerial environment, making the phyllosphere a relatively hostile and nutrient-poor niche, especially when compared

to the rhizosphere and endosphere (Morris, 2002; Bashir *et al.*, 2022). Despite this, the microorganisms making up the phyllosphere microbiome have specifically adapted to survive in these harsh conditions. As such, the phyllosphere of a leaf is recognised to be inhabited by up to 10^7 bacterial cells per cm^2 (Vorholt, 2012). Despite a lack of research having been done on the phyllosphere microbiome, its importance in plant health has been established in many roles in plant metabolism and disease resistance (Fürnkranz *et al.*, 2008; Berlanga-Clavero *et al.*, 2020), acting as the first line of defence against pathogenic microorganisms for the aerial structures through competitive interactions (Berg *et al.*, 2017; Shade, 2017; Bashir *et al.*, 2022). As well as direct benefits for host plants, industries such as the wine industry have also been able to use phyllosphere microorganisms for a diverse range of functions, such as those performed during wine fermentation (Gutiérrez *et al.*, 1999; Beneduce *et al.*, 2004; Clemente-Jimenez *et al.*, 2004; Combina *et al.*, 2005). These findings into the importance of the phyllosphere microbiome have highlighted a need for further research into its roles and structure.

To effectively research these complex communities of microorganisms which make up the phyllosphere microbiome, sophisticated DNA sequencing and quantification techniques are required. By targeting the hypervariable regions of the 16S and ITS ribosomal RNA genes, we can observe the presence of genetic material extracted from bacteria and fungi, respectively (Weisburg *et al.*, 1991; Schoch *et al.*, 2012). One caveat that comes with these PCR-based microbial identification methods is the inability to differentiate between DNA originating from viable microorganisms and DNA from non-living sources, known as relic DNA (Carini *et al.*, 2016). Relic DNA makes up the DNA from sources such as dead cells, compromised cells or the environment (eDNA). It can be amplified and sequenced alongside target DNA, leading to false positives within results (Nocker, Cheung and Camper, 2006). As such, results obtained

using these methods at their current capacity must be regarded with suspicion as to which microorganisms were truly associated with their respective host at the time of sampling.

PROPOSED PROTOCOL

We propose a harvesting protocol that can allow for the selective harvesting of viable phyllosphere microorganisms with the effective removal of relic DNA. Using the viability selection dye, propidium monoazide (PMA), relic DNA can be blocked from amplification and thereby will not be observed by using PCR or DNA sequencing methods (Nocker, Cheung and Camper, 2006; Carini *et al.*, 2016; Baymiev *et al.*, 2020). The PMA dye (Biotium, USA) can block relic DNA by covalently binding to it when exposed to light (464 nm photolysis); however, the dye is incapable of crossing the intact membrane of viable cells, leaving the DNA from viable cells unhindered and amplifiable during subsequent PCR (Figure 1) (Nocker, Cheung and Camper, 2006). Alongside the PMA dye, correlating methodology from human skin microbiome harvesting techniques (Ogai *et al.*, 2018), we propose the use of nylon swabs as a more controlled, reproducible and simple method of harvesting phyllosphere microorganisms as opposed to the current standard, which involves lengthy washes of whole leaves in large volumes of buffer solutions. Swab harvesting allows for far more control over the area of the phyllosphere being sampled, and by using stencils, specific regions of the phyllosphere can be targeted with an improved ability to standardise sample areas across biological replicates.

Using quantitative PCR (qPCR), we compared the yields of two swab harvesting techniques (direct swab extraction and pelleted swab extraction) to a whole leaf wash harvesting method adapted from several recent phyllosphere research articles (Singh, Gobbi, *et al.*, 2018; Singh, Santoni, *et al.*, 2018; Miura *et al.*, 2019; Singh *et al.*, 2019; Noble *et al.*, 2020; Al Ashhab *et*

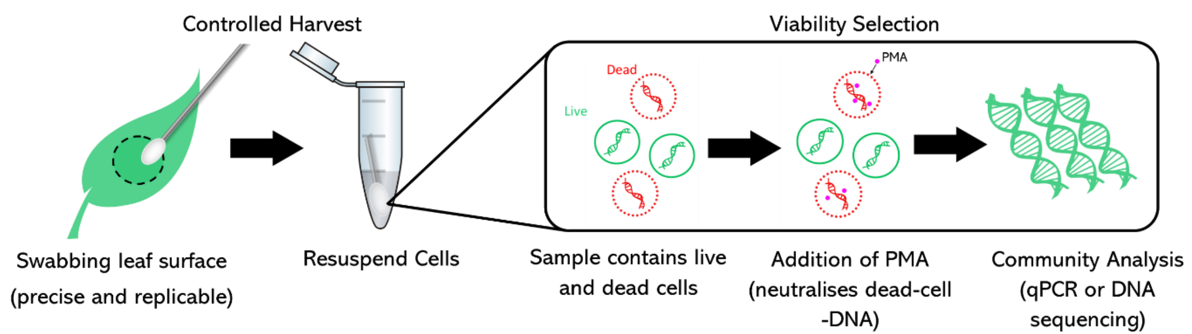


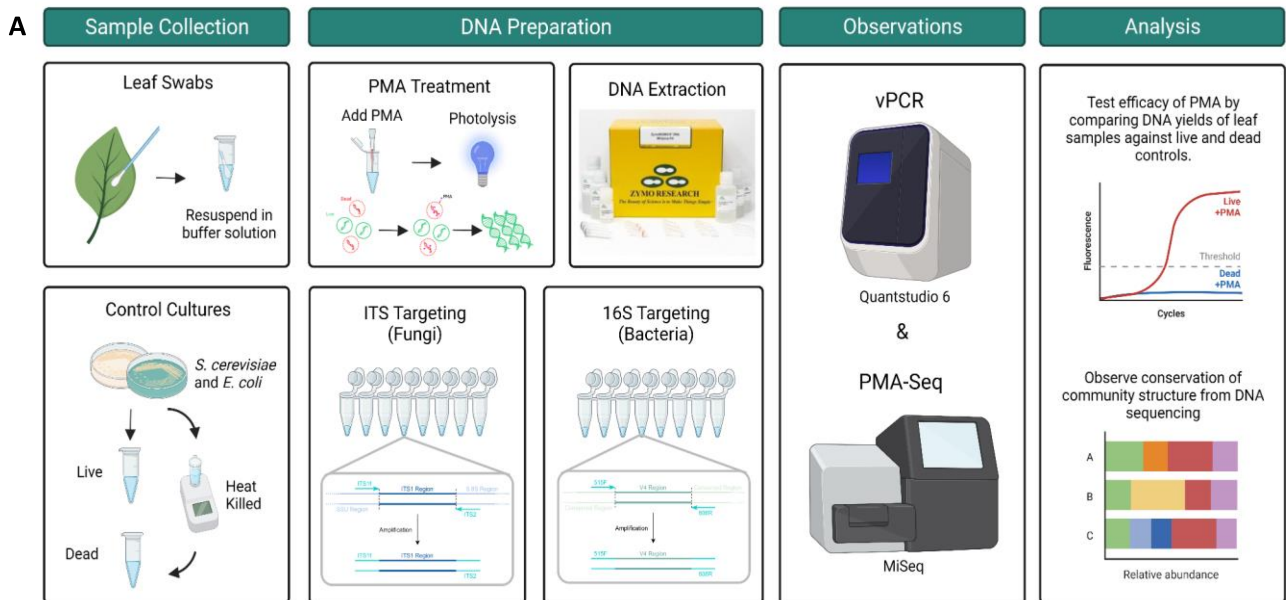
Figure 1 | Workflow diagram for proposed protocol utilising swab harvesting methods partnered with viability selection with PMA dye.

al., 2021). Using these swab harvesting methods, we tested PMA dye's efficacy on phyllosphere microbiome samples using viability real-time PCR (vPCR). Using separate controls of live and killed cultures of bacteria (*Escherichia coli*) and fungi (*Saccharomyces cerevisiae*), we assessed the ability of the PMA treatment method to remove relic DNA from cultures of a known state (heat-killed or live culture) in order to confirm the observed reduction of relic DNA in our phyllosphere microbiome swab samples.

MATERIALS AND METHODS

Grapevine Preparation:

20 *Vitis vinifera* (Shiraz 1654, own roots) grapevines were obtained from Yalumba Nursery (Yalumba, South Australia). They were immediately transplanted into 10-inch pots and placed in a temperature and light-controlled glasshouse at the University of Adelaide Waite campus (SAARDI). Grapevines were hand watered twice weekly and supplemented with seaweed liquid fertiliser and multi-nutrient slow-release pellet fertiliser at identical rates as needed. Use of pesticides was limited but recorded if necessary, and all vines were exposed to the same rate



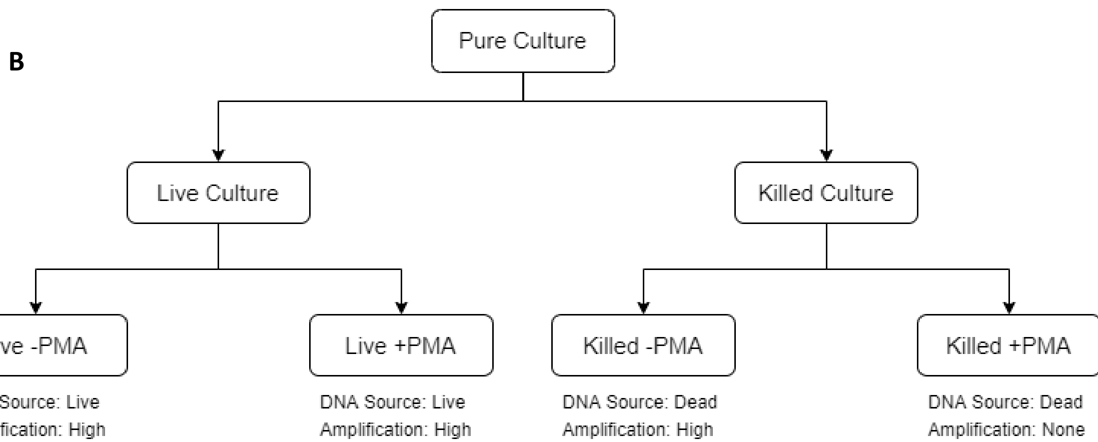


Figure 2 | (A) Methodology was used to test the efficacy of both the swab harvesting technique and the PMA viability selection. (B) Visual representation of the monoclonal culture controls used to test the PMA dye. All control samples were made from a single parent culture of either *S. cerevisiae* (fungal control) or *E. coli* (bacterial control). Predominant DNA source and amplification prediction are listed under each final control type.

and type. The grapevines were grown until fresh canes were approximately 1 m tall, from which their growing tips were removed to halt further primary growth. All samples were collected on the same day, with each sample group being collected in groups of 10 replicates.

Whole Leaf Harvest:

A whole leaf wash method was adapted from multiple recent research articles (Singh *et al.*, 2018b; Singh *et al.*, 2018a; Miura *et al.*, 2019; Singh *et al.*, 2019; Noble *et al.*, 2020; Al Ashhab *et al.*, 2021) for comparison against the proposed swab harvesting method. Healthy leaves were collected from each grapevine while wearing gloves and using secateurs sterilised with 5% sodium hypochlorite. The petiole was removed, and the leaf was gently placed into 50 mL polypropylene tubes. Leaves were then suspended in 15 mL of PBST (PBS, pH 7.4 with 0.01% Tween20). Samples were shaken at 150 rpm for 2 hrs, followed by 10 min sonication in an ultrasonic bath at 60 Hz for optimum release of microorganisms. The leaves were then removed from the wash buffer solution, which was then centrifuged at 5000 x g for 20 minutes to pellet the phyllosphere microbes. The supernatant was discarded, and pellets were moved to 1.5 mL Eppendorf tubes and resuspended in 100 µL of PBS, which was stored at -20°C until DNA extraction.

Swab Harvest and PMA Treatment:

Swab samples were collected by removing a single asymptomatic leaf from each vine while wearing gloves and using secateurs sterilised with 5% sodium hypochlorite. The leaf was placed inside a 3D-printed swabbing stencil (Supplementary Figure 1) placed over the centre vein on the adaxial side of the leaf, which was also sterilised with 5% sodium hypochlorite. Using nylon flocked swabs (COPAN, USA) wetted with PBS, the entire exposed area of the leaf was swabbed for 30 seconds with moderate pressure. This process was performed three times with the same swab for the three-leaf swab samples. Swab sample negative controls were also collected by simulating all prior steps without contacting surfaces. All swab heads were then placed into 2 mL Eppendorf tubes containing either no liquid medium (for direct DNA extraction) or 400uL of PBS or PBS + PMAxx (25µM) (for pelleting prior to DNA extraction).

Swab sample negative controls were also collected by simulating all prior steps without contacting any surfaces; blank swab heads were placed into 2mL Eppendorf tubes containing no liquid medium for direct DNA extraction. Tubes containing no liquid medium were immediately frozen at -20°C for storage until DNA extraction. Swab heads suspended in liquid medium were vortexed to release microorganisms into solution. The swab heads were then removed, and PMA-treated samples were incubated for 10 minutes in the dark before being exposed to blue light via a 3D-printed lightbox (Supplementary Figure 2) fitted with blue (~460 nm wavelength) LED lights for 15 minutes for photoactivation of the dye. All suspensions were centrifuged at 5000 x g for 20 minutes, and pellets were washed with fresh PBS twice before being resuspended in 100 µL of PBS and stored at -20ºC before DNA extraction.

Control Cultures:

Control cultures used to assess the efficacy of the PMAxx treatment method were made for both bacterial and fungal controls (16S and ITS analyses, respectively). Bacterial control cultures were made using *Escherichia coli,* and fungal control cultures were made using *Saccharomyces cerevisiae*. Both organisms were sub-cultured on TSA or WL nutrient agar plates for bacteria and fungi, respectively, to isolate single colonies. Isolates were grown overnight (18 hrs) in 5 mL of fresh nutrient broth at 37°C for *E. coli* and 5 mL of WL media broth at 30°C for *S. cerevisiae*. Both cultures were shaken at 200 rpm. Each culture was then inoculated into 10 mL of their respective fresh media and incubated with shaking until they reached early log phase (*E. coli*: OD600 = 0.2 and *S. cerevisiae*: OD600 = 0.4) (Supplementary Figure 3).

Once cultures were ready, twelve 400 µL aliquots were made for each organism. Half of each was then heat-killed in a dry bath by heating to 75°C for 10 min (Wang *et al.*, 2021). Each

group (Bacteria Live, Bacteria Dead, Yeast Live, Yeast Dead) were then treated with PMAxx dye, in triplicate, by addition of 1uL of 10 mM stock solution to make a final concentration of 25 µM PMAxx. PMA-treated samples were incubated in the dark for 10 min before being light treated in blue light for 15 min in a 3D printed light box. Cells were then pelleted by centrifugation at 5000 x g for 20 min, the supernatant was removed, and the pellets were stored at -20°C prior to DNA extraction. The controls were produced in triplicate and were as follows: *E. coli* Live, *E. coli* Dead, *E. coli* Live +PMA, *E. coli* Dead +PMA, *S. cerevisiae* Live, *S. cerevisiae* Dead, *S. cerevisiae* Live +PMA, *S. cerevisiae* Dead +PMA (Figure 2B).

DNA Extractions and qPCR:

DNA extractions were performed using the ZymoBiomics DNA Miniprep Kit according to the manufacturer's protocol (ZymoBiomics, USA). All swab heads and pellets, both treated and untreated with PMAxx, were extracted at the University of Adelaide in a specialised microbiome extraction lab. Swab heads (biological samples and blanks) stored in dry tubes were placed directly into lysis tubes for direct DNA extraction. Pellets were vortexed briefly to resuspend, and 100uL was added to lysis tubes for pellet DNA extraction. Final extracts were stored at -20°C prior to qPCR quantification. Following guidelines proposed by Eisenhofer *et al.* (2019), we also prepared four extraction blank control samples (EBCs) to act as negative controls for detecting contamination within and between samples.

Single qPCR reactions were prepared of 12.5 µL Brilliant II SYBR Green qPCR Mastermix (Agilent), 10.5 µL dH20, 1 µL of 5 µM forward and reverse primers and 1 µL of template DNA. The qPCR reactions were conducted in the SARFMEE laboratory (University of Adelaide) on a QuantStudio 6 (Applied Biosystems) using primers targeting either the V4 region of the 16S rRNA gene for bacteria (forward primer 515F: GTGCCAGCMGCCGCGGTAA, reverse

primer 806R: GGACTACHVGGGTWTCTAAT) or the ITS1 region of the ITS rRNA gene for Fungi (forward primer ITS1f: CTTGGTCATTTAGAGGAAGTAA and reverse primer ITS2_r: GCTGCGTTCTTCATCGATGC). Primers and protocols were adapted from those used by the Earth Microbiome Project (Thompson *et al.*, 2017). Single reactions were also prepared using ZymoBiomics bacterial and fungal DNA standards (ZymoBiomics, USA) to generate a standard curve for more accurate quantification of sample DNA.

PMA-Seq

Following confirmation of the single swab harvest method, new leaf swab samples were collected from 10 Vitis vinifera 'Syrah' (Shiraz 1658, own roots) grapevines in triplicate. Using the 'pelleting' methods outlined above, half of the samples were treated with PMAxx viability dye, and the other half were left untreated. DNA extractions were then performed as above using the ZymoBiomics DNA Miniprep Kit according to the manufacturer's instructions, including EBCs. For 16S libraries, barcoded V4 region 16S rRNA gene amplicons were amplified using the 515f/806R primer pair (Caporaso et al. 2011) (forward primer 515F: GTGCCAGCMGCCGCGGTAA and barcoded reverse primer 806R: GGACTACHVGGGTWTCTAAT). For ITS libraries, ITS gene amplicons were amplified using a two-step method targeting the ITS1 region using the ITS1f/ITS2 primer pair (White et al. 1990; Gardes and Bruns 1993) (ITS1f: CTTGGTCATTTAGAGGAAGTAA and ITS2_r: GCTGCGTTCTTCATCGATGC). PCR reactions were completed following PCR reaction methods adapted from the Earth Microbiome Project protocol (Thompson et al. 2017).

For all samples, 2 µL of PCR product was mixed into 198 µL Qubit working solution and quantified using Qubit 2.0 Fluorometer. Samples were then pooled and cleaned using AxyPrep (Axygen) following the manufacturer's instructions. The final pool was quantified and quality-

checked using an Agilent TapeStation (D1000 Screen Tape). DNA sequencing was performed on an Illumina MiSeq v2 (2 x 150 for 16S and 2 x 250 bp for ITS) at SAHMRI (South Australian Health and Medical Research Institute) by SAGC (South Australian Genomics Center). Processing code found in: https://github.com/brady-welsh/2023_Grapevine_FvP.

RESULTS

Swab Harvest Test

The swab yield was compared to whole leaf wash yields to evaluate the ability of swabs as an effective method of harvesting the phyllosphere microbiome. We analysed ten replicate samples collected using each method (whole leaf, 1-leaf swabs, and 3-leaf swabs) and DNA input quantity was estimated for both 16S and ITS target regions using standard curves generated by concurrent quantification of the DNA standards (Supplementary Figure 4). Resulting yields for the 16S gene targets were observed to have significantly higher yields compared to ITS gene targets, suggesting a greater quantity of bacterial inhabitants compared to fungi, which is consistent with our previous understandings of the phyllosphere microbiome (Bashir *et al.*, 2022).

For both the 16S and ITS gene targets (Figure 3), the yield from the 1-leaf swab samples was comparable to the whole leaf wash samples demonstrating no significant difference between the two harvesting types (unpaired *t-test*, p = 0.51 and 0.46 for 16S and ITS respectively). While the 1-leaf wash samples displayed effective microbial harvest, the 3-leaf swab samples for both the 16S and ITS targets displayed significantly lower yields than the 1-leaf swab samples and the whole leaf wash samples.

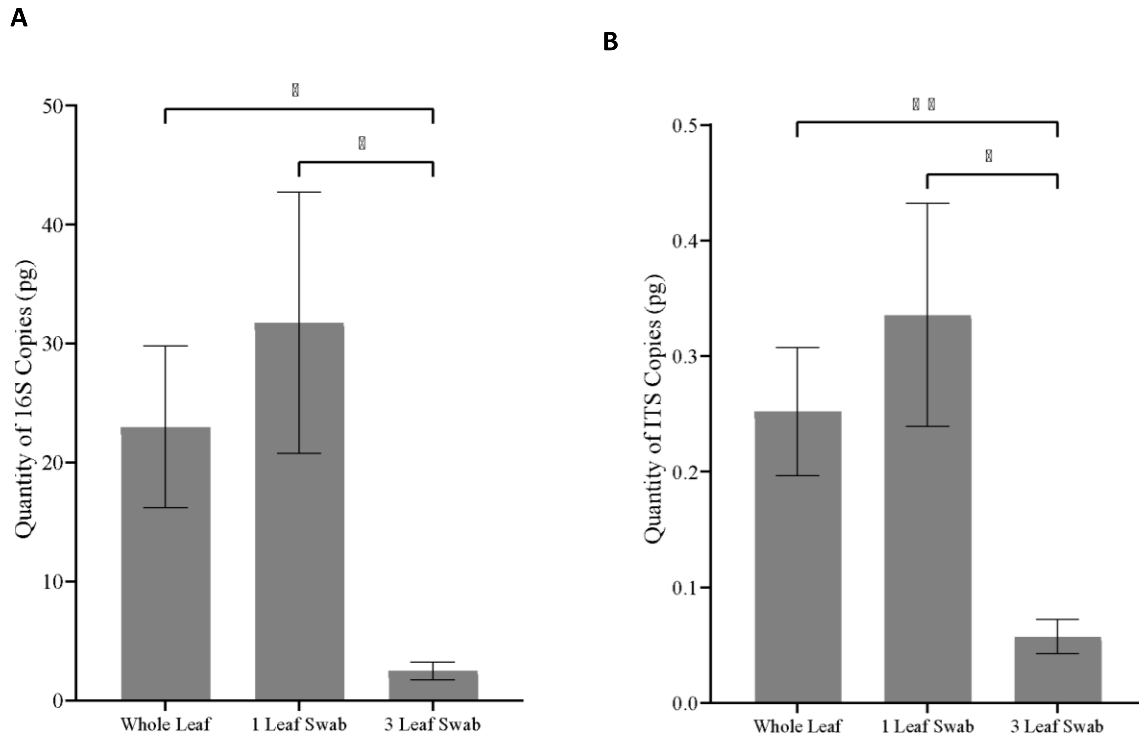
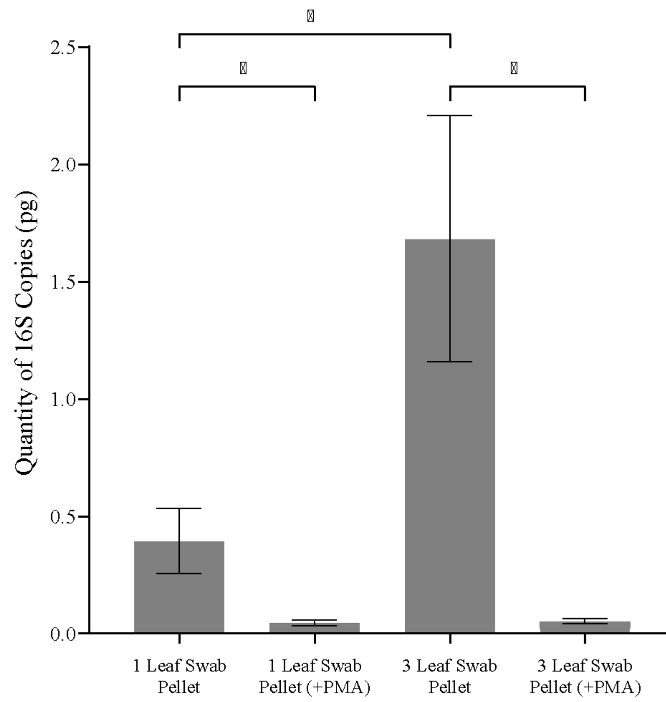


Figure 3 | The estimated quantity of input 16S (A) and ITS (B) DNA targets within each sample type. Data was analysed by One-Way ANOVA (* = $p < 0.05$, ** = $p < 0.01$).

PMA vPCR Test

To evaluate the effectiveness of PMA on the phyllosphere microbiome samples, we used vPCR (Viability Real-Time PCR), as described by the manufacturer (Biotium, USA), to measure the fold-reduction of DNA within each sample type. DNA input quantity was calculated for 16S and ITS target regions using the generated standard curves and was used as a relative estimate of the abundance of cells within each sample (Figure 4). Both PMA-treated and untreated sample types were produced. Untreated groups were used as 'whole DNA' controls, quantifying DNA from both relic and target sources, whereas PMA-treated groups were only representative of DNA extracted from viable cells. To effectively treat the swab samples with PMA and efficiently light treat the samples, the microbes were pelleted from the swabs and

A



B

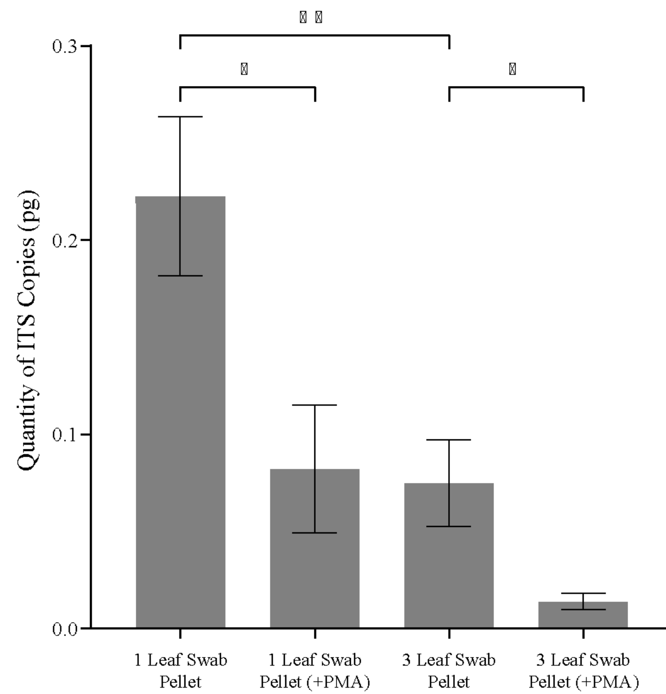


Figure 4 | Quantities of 16S (A) and ITS (B) DNA targets from pelleted samples either untreated or treated with PMA. Swab samples were either taken from a single leaf or three leaves with a single swab. Data was analysed by One-Way ANOVA (* = $p < 0.05$, ** = $p < 0.01$).

resuspended into PBS solution before treatment. This resulted in a significant loss of DNA; however, sufficient DNA was recovered to complete the analysis.

Our evaluation of the ability of PMA to reduce relic DNA from phyllosphere microbiome samples began with the comparison of treated and untreated swab pellet extractions (Figure 4). In both cases, PMA treatment resulted in a significant reduction of DNA amplification. For the 16S target, this loss was higher, with a 13-fold reduction of DNA in 1-leaf swab samples and a 15-fold reduction in 3-leaf swab samples compared to the ITS 8-fold and 10-fold reduction, respectively. For both 16S and ITS targets, this suggested the presence of significant quantities of relic DNA in the phyllosphere microbiome samples (unpaired *t-test*, $p = 0.036$ and 0.015 for 16S 1-leaf swab and 3-leaf swab, respectively, and $p = 0.017$ and 0.023 for ITS 1-leaf swab and 3-leaf swab, respectively).

The reduction of amplifiable DNA yields confirmed that PMA was successfully blocking input DNA; however, to confirm this action specifically targeted relic DNA the percentage reduction of treated and untreated samples was compared against the control cultures of *E. coli* and *S. cerevisiae*. The percentage of viable cells was calculated for the control cultures and the biological samples as described by the manufacturer (Biotium, USA) (Figure 5). The killed control cultures demonstrated that our PMA treatment method effectively removed relic DNA from non-living sources showing a 100% reduction of amplifiable DNA. The live control cultures also confirmed the effectiveness of our PMA method, with treatment resulting in a 28.3% and 21.6% reduction of the 16S and ITS targets, respectively. The observed reduction suggests the existence of some relic DNA within the live control cultures, either from non-viable or compromised cells or suggests some reduction from non-specific effects of PMA on viable cells. These results confirmed that our PMA treatment method for our phyllosphere

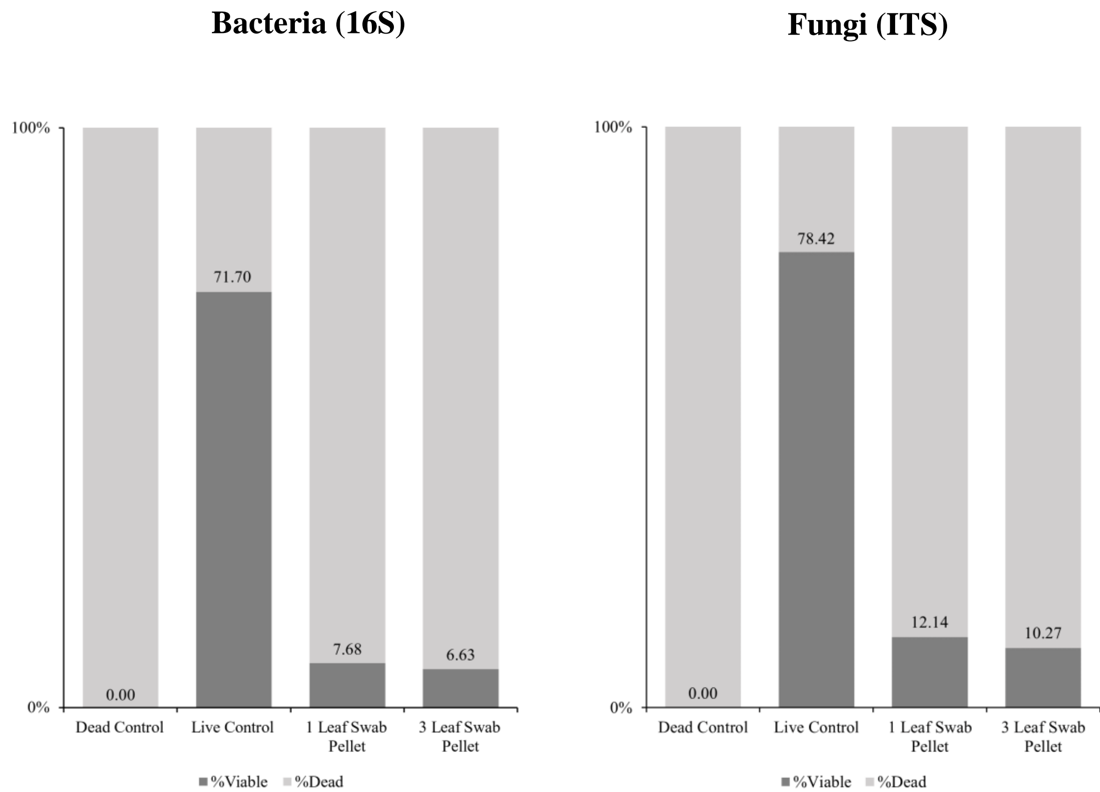


Figure 5 | Viable percentages of live and dead culture controls against the phyllosphere microbiome swab samples. Light grey bars are generated from untreated samples and represent all DNA within the samples. Dark grey bars are generated from samples treated with PMA and represent the percentage of the total DNA derived from living sources.

samples was effective. However, they revealed that our swab samples contained considerable relic DNA derived from non-viable cells or the environment which can also be seen in the preliminary PMA-Seq results (Figure 6).

DISCUSSION

Here we proposed using swab methods as an improved strategy for harvesting the phyllosphere microbiome of plants and using the viability selection dye, PMA, to effectively observe viable phyllosphere inhabitants. Using qPCR, we could quantify the yields of 16S, and ITS gene targets within swab samples and compare these to the current research standard for harvesting

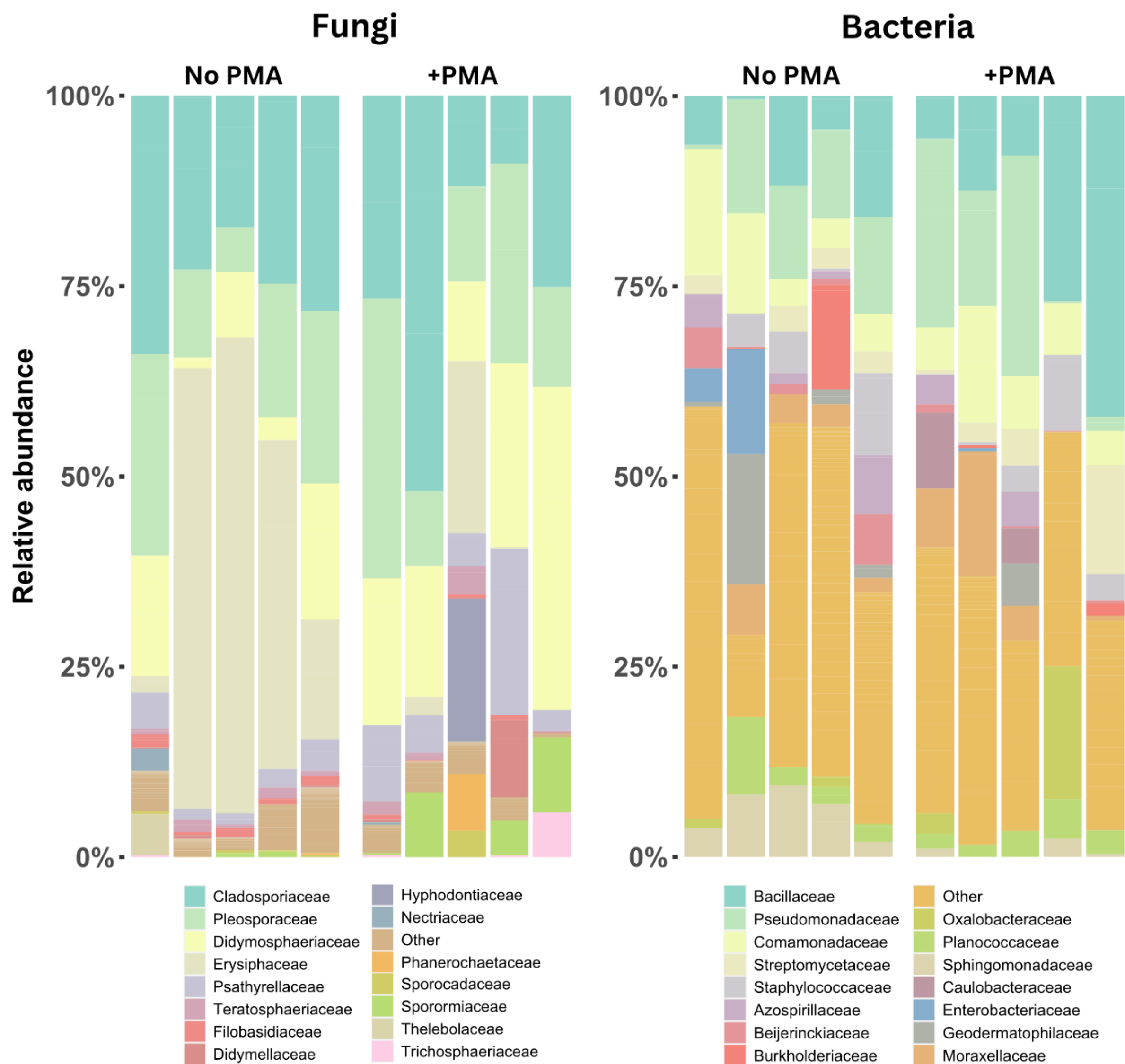


Figure 6 | Stacked bar charts showing the top 15 families of fungi and bacteria collected from leaf phyllosphere samples. Samples were either treated with PMA dye to remove relic DNA or left untreated (representative of the total DNA).

phyllosphere microorganisms. The results of these analyses revealed the effectiveness of these swabbing methods, demonstrating a comparable microbial yield to the whole-leaf wash methods.

Although these results show comparable yields, swab samples were collected from a considerably lower leaf surface area (16 cm^2 adaxial surface) than the whole leaf wash samples (entire adaxial and abaxial surfaces). This result suggested a potentially greater microbial harvesting efficiency for the swab methods; however, only in samples where one leaf was harvested per swab. The results indicate that attempting to pool multiple phyllosphere samples by swabbing multiple leaves significantly reduces microbial yield, likely due to the loss of the liquid medium (PBS) over repeated use on multiple leaves. Although more testing would be required, these results do suggest the optimal number of leaves per swab is one, and the pooling of samples should be conducted in subsequent steps of DNA processing using multiple independent swab collections.

Swabs are an advantageous alternative to the typical whole-leaf wash methods. These swabbing techniques allow for greater control during sampling, enabling researchers to harvest microorganisms from more localised regions of the phyllosphere with the ability to sample consistent surface areas across multiple leaves. Far greater standardisation can be achieved across different phyllosphere tissues, allowing for more exact quantification of the phyllosphere microbiome and more accurate relative qualitative comparisons. Our experience also demonstrated the swab methods to be a much faster alternative to the whole leaf wash methods, removing the need for lengthy washing and centrifugation steps. Swab harvesting also reduces the need for repeated opening and changing of tubes throughout the harvesting

process, minimising the risk of contamination and providing opportunities for additional sampling negative controls (e.g. swab blanks).

Confirming the use of these swab methods, we were then able to effectively sample the phyllosphere with the speed and accuracy required for PMA treatment. Using the swabbing technique, we conducted vPCR analysis on leaf phyllosphere swab samples to test microbial yields following PMA treatment. PMA treatment caused a significant reduction of DNA within all phyllosphere samples. Although this was expected, the level at which this reduction occurred was substantial. PMA treatment efficiency testing was conducted using monoclonal bacterial and fungal cultures, and this demonstrated that the protocol used was effective at neutralising non-target DNA and that the high reductions were likely due to relic DNA within the phyllosphere samples. Surprisingly, these results revealed that only 7-8% of bacterial DNA and 10-12% of fungal DNA within the leaf swab samples originated from viable sources. Carini *et al.* (2016) observed high levels of relic DNA levels in prior research, where they observed up to 55% prokaryotic and fungal relic DNA within rhizosphere samples following PMA treatment. Such levels of relic DNA within the samples indicate a high possibility of false-positive results in data when not utilising viability selection, namely in DNA sequencing data sets. Unfortunately, this study was limited by a need for more resources to perform DNA sequencing on the present set of samples. This limitation denied us the ability to test whether the swab sampling method returned a difference in microbial composition or diversity compared to the field standard. However, we obtained a secondary dataset which allowed us to test the efficacy of PMA-Seq on our swab samples. The effect of potential false positives was observed in this preliminary PMA-Seq data (Figure 6), which presented high levels of taxa connected to powdery mildew infections (Erysiphaceae) within samples not treated with PMA. These results suggested an infection in the asymptomatic leaves sampled; however, these same

samples, when treated with PMA, revealed that these sequences were likely observations of relic DNA and did not originate from viable cells.

By using viability selection protocols, such as PMA treatment, future research can utilise more scrutinous methods for phyllosphere microbiome analyses. Using these techniques, viable inhabitants can be defined, leading to a more accurate understanding of the associated microbiome, such as a more effective characterisation of a core microbiome. The utilisation of viability selection can also be advantageous to agricultural industries. With a growing interest in cultivating a healthy micro-biodiversity in agricultural settings, such as vineyards, effective monitoring of viable inhabitants could be a powerful tool to construct more tailored growing conditions to support healthy microbial diversities in crops.

CONCLUSION

The current standards for harvesting the phyllosphere microbiome are limited in that they need more standardisation and control, resulting in the collection of relic DNA alongside the target DNA. Here we proposed an alternative method of harvesting the phyllosphere microbiome through the use of swabs and demonstrated effective collection of phyllosphere microorganisms; however, further testing is required for greater optimisation, especially for the pooling of microbes across multiple leaves. Using these swab samples, we also confirmed the efficiency of PMA at removing relic DNA from samples. Using this viability dye, the phyllosphere can be observed more accurately, removing signal from non-living sources and the potential for false-positive observations. Following this harvesting protocol, greater control is possible when harvesting the phyllosphere microbiome resulting in more effective detection and characterisation of the viable microbial inhabitants of the phyllosphere.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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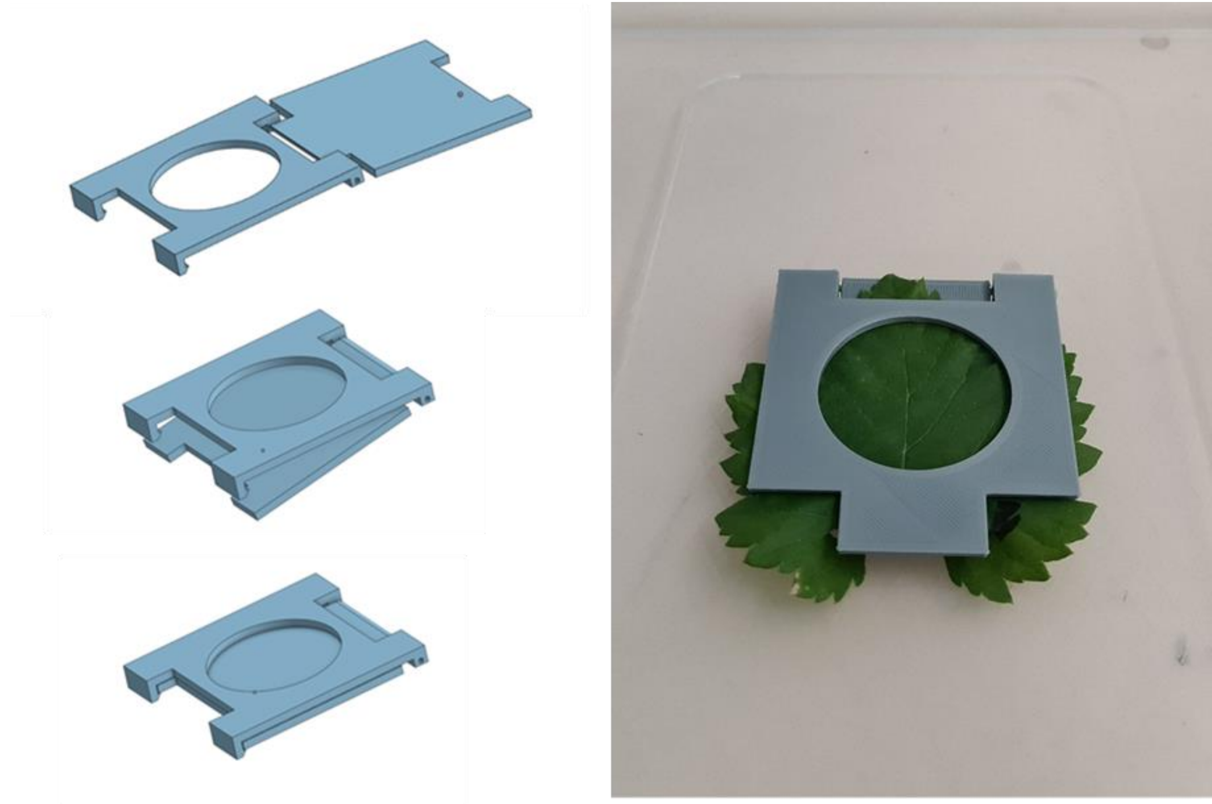
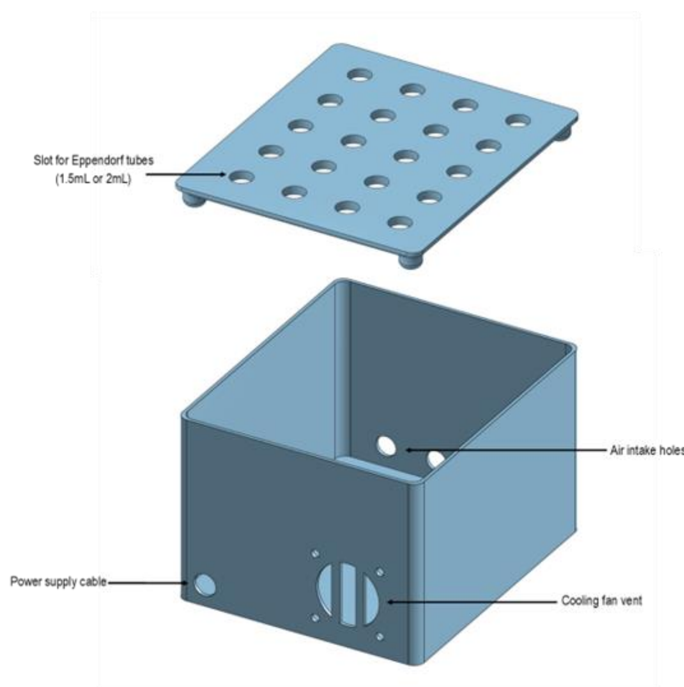
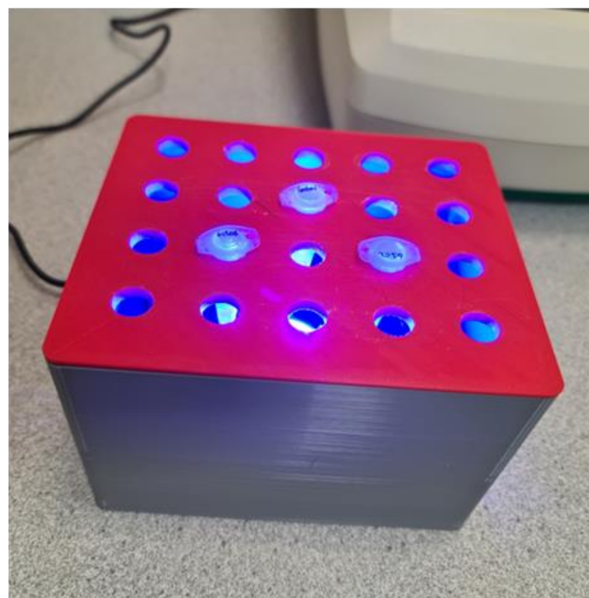
SUPPLEMENTARY DATA

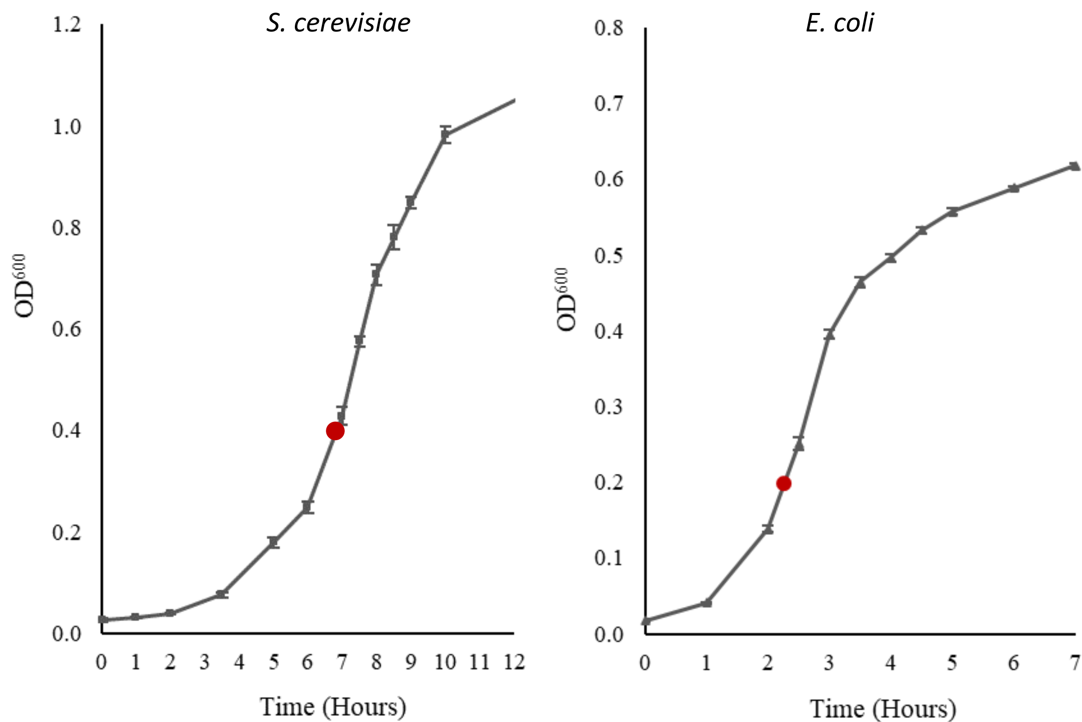


Supplementary Figure 1 | 3D model and print of the leaf swabbing stencil. The clip can secure itself around the leaf preventing the leaf from moving within the template during swabbing. The template exposes a 16cm2 leaf region which can be swabbed efficiently due to the circular shape. The design was printed using a 3D printer and used for all subsequent swab samples.

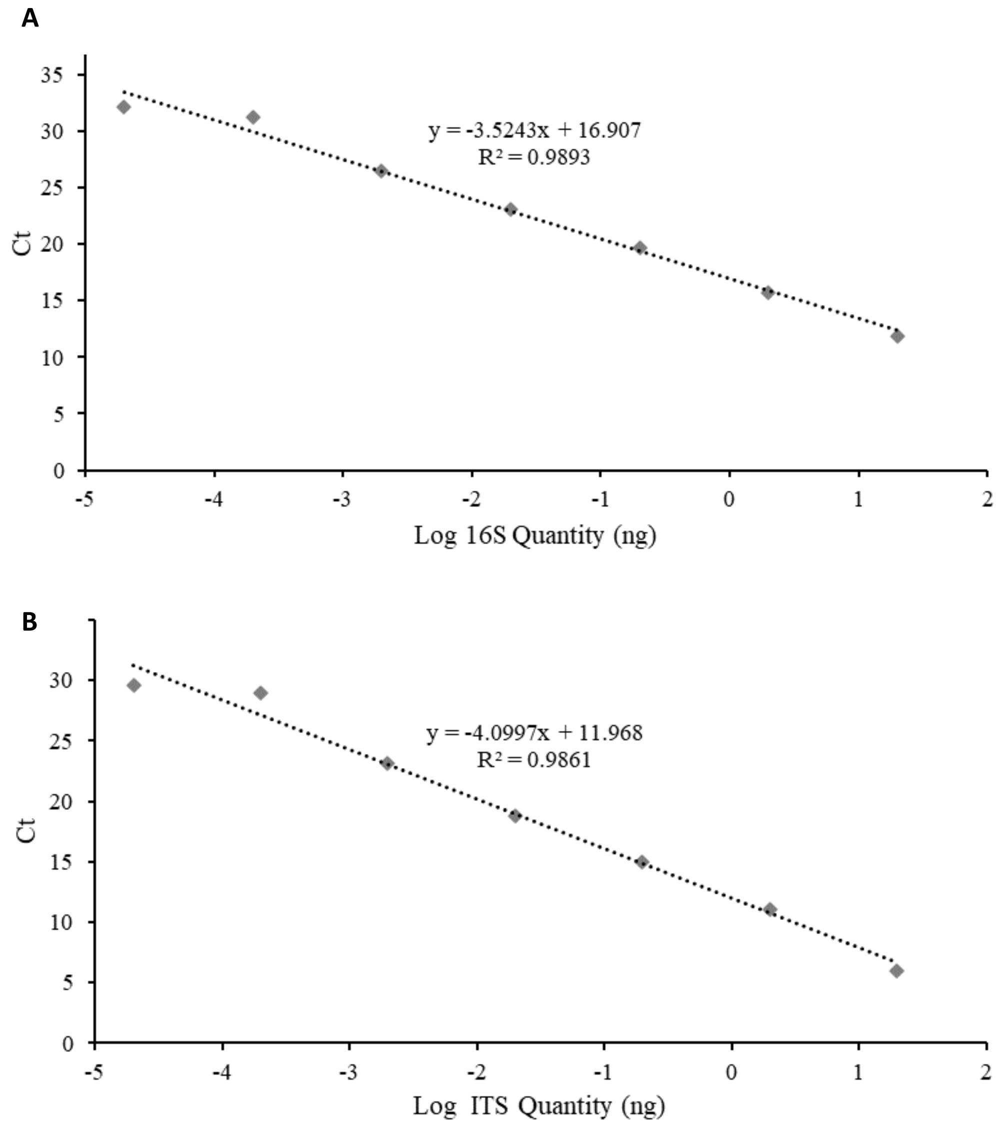




Supplementary Figure 2 | 3D model and print of the light-box used for the photolysis of PMA within samples. The box can be cheaply printed using a 3D printer and fitted with a 464nm blue LED (photo maximum wavelength for PMAxx). The box can fit 20 1.5mL or 2.0mL Eppendorf tubes, and the model can be adjusted for others before printing. The box was fitted with a cooling fan to ensure the temperature of the samples did not rise, although this was of minor concern due to the energy-efficient nature of LEDs.



Supplementary Figure 3 | Growth curves produced to identify early-log phase of control cultures of *S. cerevisiae* and *E. coli* for testing the efficacy of the viability selection dye PMAxx. For *S. cerevisiae* controls, an OD^{600} of 0.4 was determined to be indicative of early-log phase growth and for *E. coli*, an OD^{600} of 0.2 was determined to be indicative of early log-phase (denoted by red points on plots).



Supplementary Figure 4 | Standard curve plots generated for qPCR DNA quantification. Zymobiomics bacterial and fungal DNA standards were prepared in duplicate to generate the 16S (A) and ITS (B) target standard curves, respectively. The standard curve equation was then used to determine the quantity of both targets in each sample.

Chapter IV

Perspective Piece: Harnessing the Phyllosphere Microbiome for Wine Making

Here in this chapter, we discuss the need for, and improvement of, current vineyard micro-biodiversity for utilisation during wild fermentation.

Statement of Authorship

Title of Paper	Monitoring the viable grapevine microbiome to enhance the quality of wild wines.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Welsh, B. L., R. Eisenhofer, S. E. P. Bastian, and S. P. Kidd, 2023 Monitoring the viable grapevine microbiome to enhance the quality of wild wines. Microbiol. Aust. 44: 13–17.

Principal Author

Name of Principal Author (Candidate)	Brady L. Welsh		
Contribution to the Paper	Reading of relevant literature and compiling information. Primary writer and figure production. Review and editing of manuscript. Literature search and data analysis.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	01/ 02 / 2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stephen P. Kidd		
Contribution to the Paper	Review and editing of manuscript.		
Signature		Date	

Name of Co-Author	Raphael Eisenhofer Philipona		
Contribution to the Paper	Review and editing of manuscript.		
Signature		Date	

Name of Co-Author	Susan E. P. Bastian		
Contribution to the Paper	Review and editing of manuscript.		
Signature		Date	

TITLE

Monitoring the viable grapevine microbiome to enhance the quality of wild wines.

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Microbiome, Microbiota, Wild, Wine, Fermentation, Micro-Biodiversity, Metagenomics

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Monitoring the viable grapevine microbiome to enhance the quality of wild wines

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ABSTRACT

Grapevines that are used for winemaking host a diverse range of microorganisms that make up their microbiome. The microbes that inhabit the grapevine have been used by winemakers to produce wine for centuries, although modern wine producers often rely on inoculated microorganisms such as *Saccharomyces cerevisiae*. In the Australian wine industry, there is a movement towards returning to the utilisation of the microbiome for wine fermentation. With the recent increase in the understanding of the role of the grapevine microbiome in grapevine health, fermentation and subsequent wine sensory traits, the microbial world offers a new level of complexity that can be harnessed for winemaking. In order to develop and maintain a desired vineyard micro-biodiversity, extensive microbial monitoring is required. Here we discuss the utilisation of a viability selection dye in order to distinguish between microorganisms that are live and associated with the host, and relic signals generated from non-living sources.

Keywords: fermentation, metagenomics, micro-biodiversity, microbiome, microbiota, wild, wine.

Fermentation and the grapevine microbiome

The process of producing wine from grape juice was discovered to be the result of microbial organisms in the nineteenth century by Louis Pasteur. With advancements in biochemistry and microbiology, the understanding behind this complex fermentation process has been investigated extensively. As such, winemaking is one of the oldest human utilisations of microorganisms through the fine-tuned control of these complex fermentation reactions. The fermentation process is mostly completed during 'primary fermentation', typically by inoculated yeasts, wherein most of the alcohol content is produced by the alcoholic fermentation of glucose and fructose into ethanol. This is followed by 'secondary fermentation', which leads to microbial stability and advance the wine's sensory profile (its flavour and aroma) through processes such as malolactic fermentation.[1] Two categories of microorganisms, fungi (predominantly yeasts) and bacteria, are recognised as the driving force of the primary and secondary fermentation processes, as well as the more recently recognised 'spontaneous fermentation' process performed by wild yeasts and bacteria, which originate from the plant's microbiome.[2–6]

The grapevine microbiome, similar to other, complex microbiomes such as the human gut microbiome, is separated into distinct compartments with each hosting its own diversity of microbes. The main subdivisions of the grapevine microbiome are the rhizosphere microbiome (the area surrounding the roots), the endosphere microbiome (the area within the plant's tissues) and the phyllosphere microbiome (the surface of the aerial portion of the plant). Of these, the rhizosphere has been the main focus since its coinage[7]; however, current literature is beginning to recognise the importance of the phyllosphere and endosphere microbiomes because of their role in vine health as well as wine fermentation.[8] Both phyllospheric and endospheric microorganisms inhabit the grape berry tissues, collectively known as the carposphere microbiome, and as such act as diverse pools of fungal and bacterial taxa that can be utilised as wild inoculations to be utilised during spontaneous fermentation[9,10] (Table 1).

The movement towards wild wines

Currently, within the Australian wine industry, a movement is underway with some wineries adopting traditional practises for winemaking through the production of 'wild wines'. Wild wines are fermented exclusively through spontaneous fermentation, using wild

Table 1. Top 10 most abundant bacterial and fungal genera present in the grape carposphere microbiome identified across multiple regions globally.[10]

Top 10 fungi	Top 10 bacteria
Alternaria	*Bacillus*
Aureobasidium	*Blastococcus*
Botrytis	*Enterobacter*
Cladosporium	*Erwinia*
Cryptococcus	*Gaiella*
Davidiella	*Massilia*
Guehomyces	*Methylobacterium*
Penicillium	*Micrococcus*
Sporobolomyces	*Pseudomonas*
Rhodotorula	*Sphingomonas*

microorganisms derived from the grapevine microbiome to ferment wines to completion as opposed to exogenously inoculated *Saccharomyces cerevisiae* cultures (Fig. 1). By utilising the grapevine microbiome for wine fermentation, winemakers are capable of producing complex wines that have unique and diverse sensory traits.[6,11] Furthermore, many methods of maintaining optimal micro-biodiversity in vineyards, such as bio-dynamic and low-input approaches, align with the Australian wine industry's promotion of 'organic' and pesticide-free products for health-conscious target consumers and more sustainable viticultural practises.[12]

Although wild wines are becoming favourable, there is a multitude of challenges that must be overcome to successfully complete fermentation and produce wines with palatable characteristics. Two methods of producing wild wines currently exist. The first utilises vine-borne microbial isolates that can be inoculated into wine ferments to drive and support the complex fermentation reactions.[13–15] Although this method does not use specialised microorganisms such as *S. cerevisiae*, it also does not completely depend on the endogenous grapevine microbiota, and thus does not require the involved viticultural processes, such as monitoring, which come along with wild ferments. The second, and more-traditional method, utilises the vineyard's own micro-biodiversity, which is cultivated on the grapevine that makes its way into the wine ferment during the crushing process. This can produce complex sensory traits that vary between seasons and regions depending on a variety of different factors (natural, human-derived and environmental).

As with most organisms, a healthy grapevine microbiome is one that holds a richness and diversity specific to its host organism,[16] with the opposite typically being an indicator of a disease state within the host vine (e.g. one pathogenic taxon dominating the niche).[16–18] As the health of a plant can be dictated by that state of its microbiome, as well as the outcomes of wild fermentation, suitable micro-biodiversities must be maintained within vineyards for effective wine production. Although the occurrence of each taxon is largely host-dependent,[10,18] these microbial communities are also affected by various stressors from the environment that shape the structure of the grapevine microbiome. The frequency of each taxon is also dependent on season, geographic location, water availability, UV exposure and human intervention.[16] Controlled cultivation of these specific communities is crucial to the host plant's development; however, for wild winemaking, effective monitoring of the micro-biodiversity is also of great importance in order to encourage the development of beneficial species and hinder the growth of unwanted microorganisms such as those related to wine spoilage.

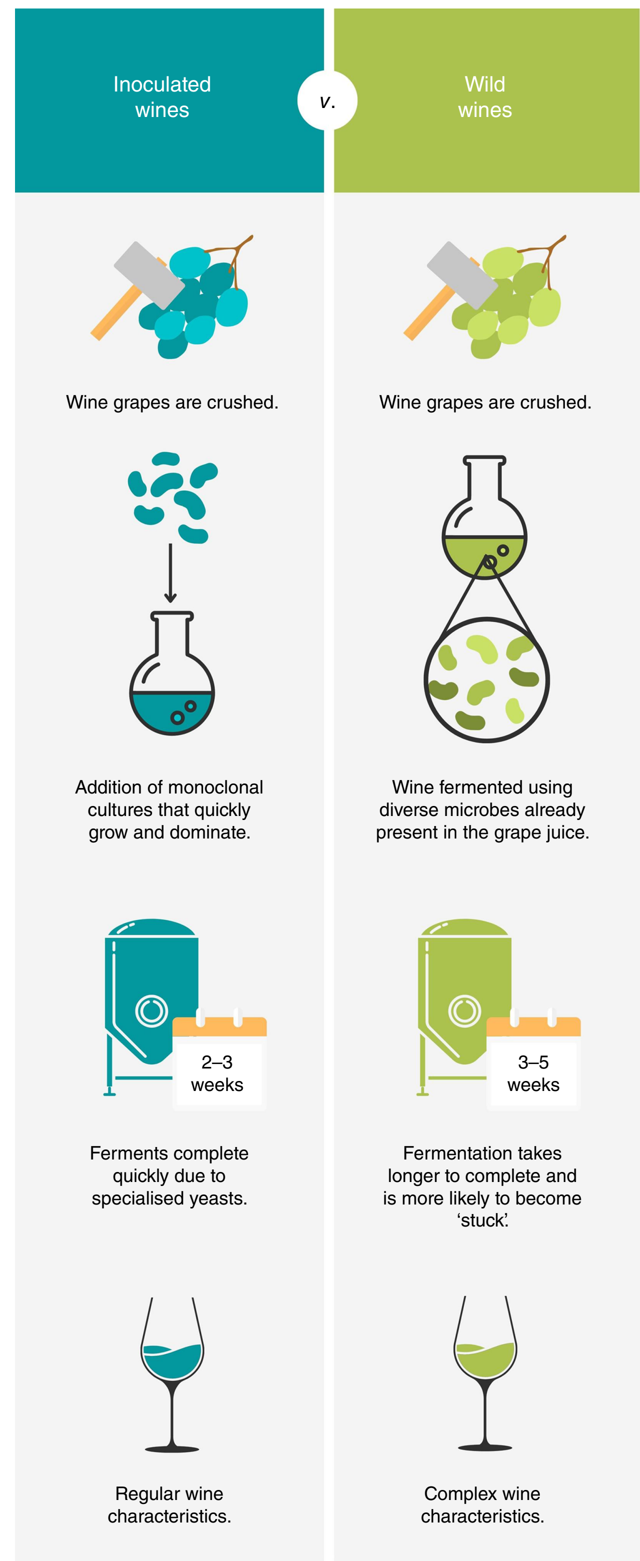

Fig. 1. Inoculated wines *v.* wild wines.

Dead or alive? Monitoring the viable grapevine microbiome

Metagenomic monitoring methods, including many of the PCR-based quantifications and DNA sequencing technologies that are typically applied to microbiome research, must be utilised to properly ensure vineyards are maintaining a healthy micro-biodiversity. The introduction of these methods would allow for incredibly efficient characterisation of the microbial richness and diversity present on a vineyard's grapevines. Understanding which microorganisms are present in the grapevine microbiomes of a vineyard will allow grape growers to understand what involvement is necessary to cultivate or preserve a healthy microbiota. However, one of the most significant downsides to current microbiome analyses is the inability to discern which organisms were 'alive', at the time of sampling.[19,20] Relic DNA makes up all DNA from non-living sources, such as dead cells, compromised cells or environmental DNA (eDNA),[21] and can be just as easily amplified and sequenced as DNA extracted from viable cells. This indiscriminate nature of PCR can provide false positives to vineyard biodiversity monitoring, which, with the wrong intervention, could lead to undesired impacts on the wild microorganisms and downstream outcomes.

Discrimination between live and dead cells is an important milestone that must be overcome to allow grape growers the ability to determine which taxa are present and associated with their host vines. To combat this, we have utilised the viability selection dye, propidium monoazide (PMA),[20–23] which is capable of covalently binding to DNA when exposed to light. The binding of the PMA dye to DNA prevents it from being amplified by PCR, and thus, cannot be observed. However, the dye cannot cross the intact membrane of live cells, allowing it to only bind to relic DNA from compromised cells or environmental sources. We tested this dye using leaf phyllosphere microbiome samples collected from *Vitis vinifera*

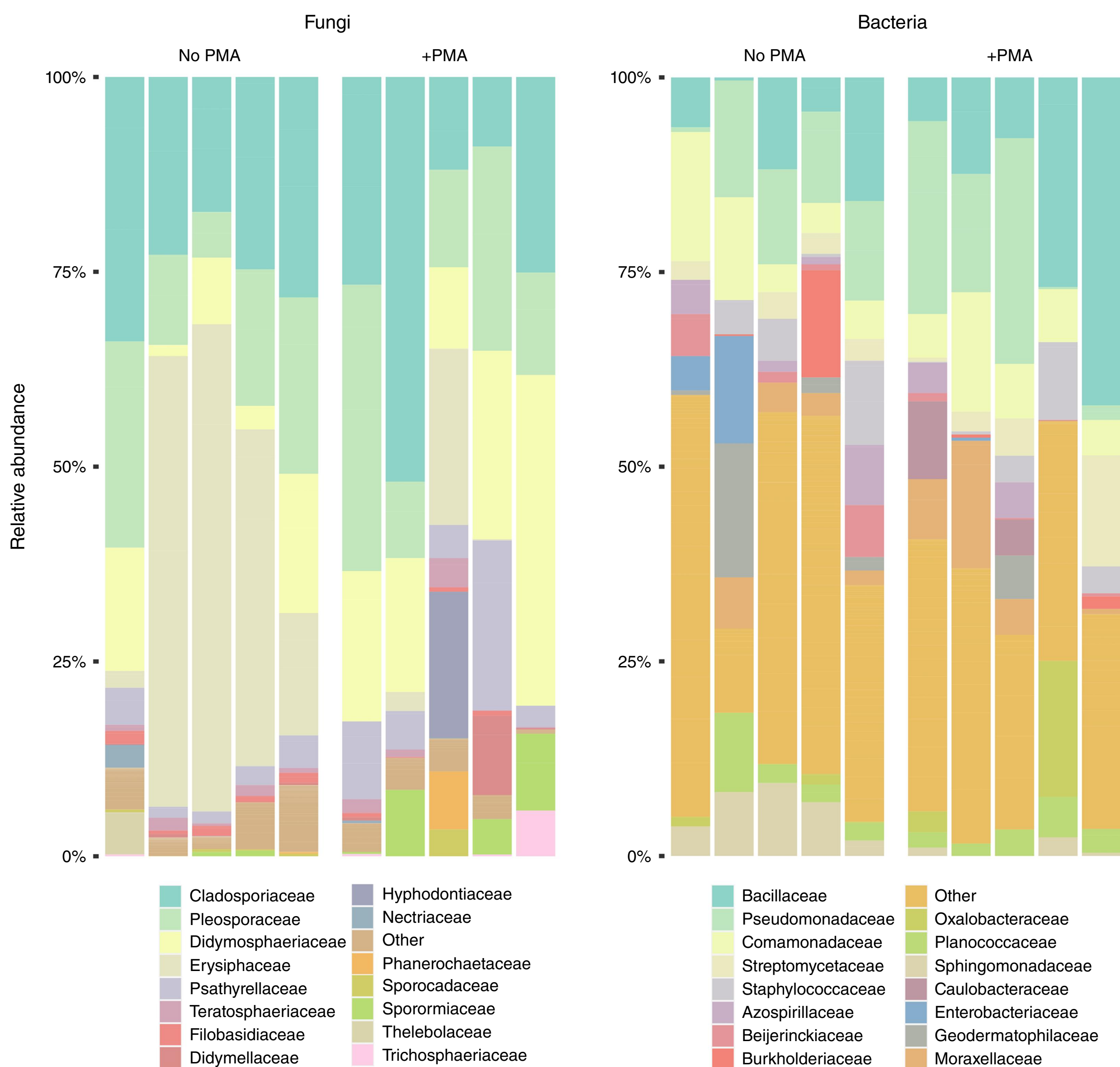

Fig. 2. Stacked bar charts showing the top 15 families of fungi and bacteria collected from leaf phyllosphere samples. Samples were either treated with PMA dye to remove relic DNA or left untreated (representative of the total DNA).

'Syrah' (Shiraz) grapevines and were able to gain sequencing data representative of the living phyllosphere microbiome.

Our PMA-Seq results utilised both *16S* and *ITS* rRNA gene amplicon sequencing to observe the bacterial and fungal communities respectively. Sequencing was conducted on two sets of grape leaf swab samples; both sets were prepared identically with one being treated with PMA (representative of the viable communities) and the other left untreated (representative of the total DNA within the sample) prior to DNA extraction and amplification (B. Welsh, unpubl. data). DNA sequencing of these samples demonstrated the PMA dye's ability to effectively remove relic DNA from grapevine microbiome samples without sacrificing the quality of the data (Fig. 2). For example, from the untreated sequencing data, we observed a high relative abundance of the fungal family Erysiphaceae, a taxon responsible for powdery mildew (a common grapevine infection); however, this abundance was lower in the samples treated with PMA. This result suggests that the observed Erysiphaceae taxa were, in fact, false positives, and the DNA responsible for those sequences likely came from non-viable sources. In a vineyard setting, if these metagenomic monitoring practices were taking place without the use of PMA viability selection, these results would have suggested a disease instance within the vines, resulting in fungicidal sprays that could affect the established structure of the grapevine microbiome. Responses to false positives could be catastrophic to the micro-biodiversity of vineyards resulting in unfavourable sensory characteristics in wild wines.

Conclusions

With the growing movement towards wild wines within the wine industry, new viticultural and oenological practices will be required. To produce grapevines with sufficient micro-biodiversity for the fermentation of wine, metagenomic analytical techniques must be adopted for sufficient monitoring of the grapevine microbiome. With the inclusion of metagenomic monitoring in vineyards, considerations must be taken to ensure these microbial communities are observed with extreme scrutiny to account for potential false positives such as those demonstrated in our results. By specifically monitoring the living microbiome, the wine industry can begin to discover and adopt practices for cultivating microbial communities which produce healthier grapevines for more sustainable practices, as well as higher quality wild wines for the current growing market.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

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Biographies

Brady Welsh is a student at The University of Adelaide undertaking a Doctor of Philosophy (PhD) in the field of grapevine microbiology investigating the interactions between the living grapevine microbiome and common vineyard fungicides.

Dr Raphael Eisenhofer is a postdoctoral researcher at the Globe Institute, University of Copenhagen, Denmark, and an adjunct assistant lecturer at the University of Adelaide. His research focus is studying the microbiomes of native Australian mammals, though he applies his metagenomic expertise to diverse study systems.

Assoc. Prof. Susan Bastian is a qualified winemaker and has been a wine educator and researcher at the University of Adelaide for more than two decades. Broadly, her research group examines the whole wine value chain, specifically grape and wine quality; viticultural and winemaking production impacts, including the influence of wine yeasts, on wine sensory and chemical composition; and wine consumer preference.

Dr Stephen Kidd is a research group leader at the University of Adelaide. His group studies the bacterial response to environmental stress, specifically the adaptation over long periods of time and the impact on the functionality of the microorganisms. This has extended to the responses of microbial communities, such as the grapevine microbiome.

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Chapter V

The Effects of Copper- and Sulphur-Based Fungicides on the Leaf Phyllosphere Microbiome of Grapevines.

We investigate the impact of two common fungicide types: copper- and sulphur-based, on the phyllosphere microbiome of grapevines. Potted *Vitis vinifera* ‘Syrah’ (Shiraz) grapevines kept in a semi-controlled, semi-natural environment were separately treated with high and low doses of the two fungicides and compared to a water spray control. PMA-seq targeting the 16S rRNA gene (V4) for bacteria, and the ITS gene (ITS1) for fungi, allowed us to observe the changes in diversity and richness of the live phyllosphere microbiome over three weeks post-treatment.

Statement of Authorship

Title of Paper	The Effects of Copper- and Sulphur-Based Fungicides on the Leaf Phyllosphere Microbiome of Grapevines.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript prepared for submission to relevant journal.

Principal Author

Name of Principal Author (Candidate)	Brady L. Welsh		
Contribution to the Paper	Conceptualisation, method development, sampling, data generation, data analysis, writing and drafting.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this pa		
Signature		Date	02 / 08 / 2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stephen P. Kidd		
Contribution to the Paper	Conceptualisation. Review and editing of manuscript.		
Signature		Date	

Name of Co-Author	Susan E. P. Bastian		
Contribution to the Paper	Conceptualisation. Review and editing of manuscript.		
Signature		Date	

Name of Co-Author	Raphael Eisenhofer		
Contribution to the Paper	Conceptualisation. Review and editing of manuscript.		
Signature		Date	

TITLE

The Effects of Copper- and Sulphur-Based Fungicides on the Viable Leaf Phyllosphere Microbiome of Grapevines.

AUTHORS

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KEYWORDS

Microbiome, microbiota, phyllosphere, viability PCR, fungicides, grapevines, vineyard

SUMMARY

Purpose

The phyllosphere microbiome plays many essential roles in viticulture and winemaking, therefore understanding the impact of various stresses on this microbial community is vital for improving the grape-growing and winemaking processes. Fungicides are one such stressor that has an intended effect on the phyllosphere microbiome, targeting pathogenic inhabitants to treat or prevent disease. However, many of the common fungicides currently used in vineyards have broad targets and could also impact the beneficial members of the phyllosphere microbiome.

Methods

Using DNA sequencing and viability selection (PMA-seq), we observe the effects of copper- and sulphur-based fungicides on the living communities of the phyllosphere microbiome. Fungicides were applied to potted grapevines, and leaf swab samples were collected over three weeks. We observed changes in the bacterial and fungal communities by sequencing the 16S rRNA and ITS targets extracted from the swab samples. With the addition of the PMA viability selection dye, we also explicitly observed changes in the living communities.

Results

Our analysis showed little change in diversity and species richness between the treatment groups and the water control. Although no trends were found for the individual fungicide groups, we did observe a consistent reduction in diversity and richness as a response to all treatments, including the water control, immediately after spraying.

ABSTRACT

Many common fungicides used in Australian vineyards have broad-spectrum, antimicrobial effects. Some of the most common fungicides, such as copper and sulphur, target cellular systems shared by many different fungal species and bacteria. As these common fungicides cannot specifically target pathogenic microorganisms, their antimicrobial effects may also impact the wild communities of the phyllosphere microbiome, which inhabits the aerial surface of the grapevine. Due to the interconnectedness of the grapevine and its microbiome, the effects of these fungicides on the beneficial microorganisms of the phyllosphere may also impact the health and productivity of the host grapevine. Using 16S rRNA and ITS gene amplicon sequencing, paired with PMA viability selection, we were able to map the impacts of both copper- and sulphur-based fungicides over time at varying concentrations with the addition of a water control to monitor the natural variation of the phyllosphere microbiome accurately.

INTRODUCTION

The phyllosphere microbiome describes the microbial environment which exists on the surface of the aerial portion of a plant (leaves, stems, flowers, fruit) (Morris 2002). This microenvironment is home to a diverse range of fungi, bacteria, archaea, and viruses that play essential roles in the health of the host plant (Fürnkranz *et al.* 2008; Cordier *et al.* 2012; Berlanga-Clavero *et al.* 2020; Bashir *et al.* 2022; Bettenfeld *et al.* 2022). The phyllosphere microbiome of grapevines is vital in many essential roles in the winemaking process, namely during the early stages of fermentation (Gutiérrez *et al.* 1999; Beneduce *et al.* 2004; Clemente-Jimenez *et al.* 2004; Combina *et al.* 2005). These wild microorganisms from the grapevine microbiome are capable of producing a wide range of volatile and non-volatile compounds

which can drive unique sensory attributes into each wine (Clemente-Jimenez *et al.* 2004; Combina *et al.* 2005; Díaz *et al.* 2013).

With the ever-growing interest in harnessing the grapevine microbiome through bio-control (Cobos *et al.* 2022), wild fermentations (Welsh *et al.* 2023) or finding new microbial isolates for domestication (bioprospecting) (Alperstein *et al.* 2020), understanding how these communities are shaped and change over time is important for the future of winemaking. Many different factors are known to be responsible for how the grapevine phyllosphere microbiome is established; as the interface between the host plant and the aerial environment, the phyllosphere microbiome is impacted by a range of biotic and abiotic factors such as water availability, UV exposure, and pest and disease prevalence (Bashir *et al.* 2022). One factor that has an intended impact on microorganisms that inhabit the phyllosphere microbiome, and is readily used in most vineyards, is fungicides.

Although the effects of fungicides on the target plant are readily investigated, these investigations typically only focus on direct negative impacts on the grapevine itself. However, due to the importance of the phyllosphere microbiome in grapevine health, including the effects of fungicides on the microbiome is equally important. Common fungicides, such as those that are copper- or sulphur-based, are used for their ability to combat various fungal infestations, such as downy and powdery mildews (Perria *et al.* 2022). Copper- and sulphur-based fungicides are proven to be highly effective at combating many common diseases and, as such, are used readily in most vineyards, being some of the only fungicides approved for use in organic-certified vineyards (Perria *et al.* 2022). Although effective at treating disease, the broad-spectrum action of these common fungicides is non-specific and targets many systems

shared between pathogenic microorganisms and the beneficial microbes that inhabit the phyllosphere microbiome (Grangeteau *et al.* 2017).

Here we use a combination of 16S rRNA and ITS gene amplicon sequencing to observe the bacterial and fungal communities, respectively, of the grapevine phyllosphere microbiome. We also employ the use of the viability selection dye propidium monoazide (PMA) to target and remove DNA from non-living sources (relic DNA) (Carini *et al.* 2016; Emerson *et al.* 2017; Baymiev *et al.* 2020). Using these PMA-Seq techniques, we observed how varying concentrations of common copper- and sulphur-based fungicides impact the living microbial communities of the phyllosphere microbiome over three weeks post-treatment. Using these methods, we were able to effectively characterise how these fungicides directly impacted the diversity of the fungal communities, as well as the lesser-focused bacterial inhabitants of the grapevine phyllosphere microbiome.

MATERIALS AND METHODS

Grapevine Preparation

15 *Vitis vinifera* 'Syrah' (Seffects658, own roots) grapevines were obtained from Yalumba Nursery (South Australia). They were immediately transplanted into 10-inch pots and placed in an outdoor shade house at the University of Adelaide Waite Campus. All vines were connected to an automatic watering system, calibrated so each grapevine received the same volume of water. Watering durations and frequency were adjusted throughout the season according to local temperatures and rain forecasts. Vines were also supplemented with seaweed liquid fertiliser and multi-nutrient slow-release pellet fertiliser at identical rates as needed. The grapevines were grown until fresh canes were approximately 1 m tall, from which their growing tips were removed to halt further primary growth.

Fungicide Sprays

Once vines were the desired height, each group was moved to an isolated position. Pre-treatment phyllosphere samples were collected immediately before spraying. Groups included copper (Amgrow, 500 g/kg copper oxychloride), sulphur (UniShield, 800 g/kg sulphur), and a water spray control. The rates of each are shown in Table 1. Only one testing spray was conducted, with no other sprays throughout sampling. Sprays were conducted with hand-held pump sprayers. Sprayers were dedicated to specific fungicides to ensure no cross-contamination between groups. Full spray coverage was maintained across all surfaces of the vine.

Table 1 | Recommended rates and mixtures prepared for each fungicide group.

Treatment		Recommended Rate	Prepared Mixture
Water Control		—	10L water
Copper	Low Rate	80g/100L	8g in 10L water
	High Rate	115g/100L	11.5g in 10L water
Sulphur	Low Rate	200g/100L	20g in 10L water
	High Rate	300g/100L	30g in 10L water

Microbial Harvest

Sampling for microbial cells (microbial harvest) was completed using a swab method with viability selection using propidium monoazide. Immediately before any fungicide sprays, leaf swab samples were collected (day 0 - 0d). Following fungicide application, leaf swabs were collected at various time points (1d, 7d, 14d, and 21d post-spray). Swab samples were collected

by removing a single asymptomatic leaf from each vine while wearing gloves and using secateurs sterilised with 5% sodium hypochlorite. The leaf's petiole was removed, and the leaf was placed inside a 3D printed swabbing stencil (16 cm^2 circular opening set over the centre vein of the leaf to standardise swab area) which was also sterilised with bleach. Using nylon flocked swabs (COPAN) wetted with PBS, the entire exposed area of the leaf was swabbed for 30 seconds with moderate pressure. Swab heads were then placed into 2 mL Eppendorf tubes containing 400 µL of PBS or PBS + PMAxx. Swab samples were then transported to the University of Adelaide North Terrace campus and vortexed to release microorganisms into the solution. The swab heads were then removed, and PMAxx-treated samples were incubated for 10 minutes in the dark before being exposed to blue light (646 nm wavelength) via a 3D printed light box for 15 minutes for photolysis of the dye. All suspensions were then centrifuged at 5000 x g for 10 minutes, and pellets were washed with fresh PBS twice before being resuspended in 100 µL of PBS and placed at -20°C before DNA extraction.

DNA Extraction and Metabarcoding

DNA extractions were performed using the ZymoBiomics DNA Miniprep Kit according to the manufacturer's protocol. All swab pellets treated and untreated with PMAxx were extracted in a specialised pre-PCR microbiome DNA extraction lab. Alongside biological samples, extraction blank controls (EBCs) were also prepared identically for the downstream detection of contaminants (Eisenhofer *et al.* 2019). For 16S libraries, barcoded V4 region 16S rRNA gene amplicons were amplified using the 515f/806R primer pair (Caporaso *et al.* 2011) (forward primer 515F: GTGCCAGCMGCCGCGGTAA and barcoded reverse primer 806R: GGACTACHVGGGTWTCTAAT). PCR reactions were prepared in the specialised microbiome extraction laboratory in a 5% sodium hypochlorite-cleaned and UV irradiated hood in the specialised microbiome extraction laboratory. Single reactions of 2.5 µL 10X HiFi

buffer, 0.1 Platinum Taq DNA Polymerase (Thermofisher), 17.7 µL dH_2O, 0.2 µL 100 mM dNTP mix, 0.5 µL of 10 µM forward and barcoded reverse primer and 2.5 µL input DNA. The DNA was amplified by initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45s, annealing at 50°C for 1 min, elongation at 68°C for 90s, with a final adenylation for 10 min at 68°C (Thompson *et al.* 2017).

For ITS libraries, ITS gene amplicons were amplified using a two-step method. First step PCRs amplified the ITS1 region using the ITS1f/ITS2 primer pair (White *et al.* 1990; Gardes and Bruns 1993) (ITS1f: CTTGGTCATTTAGAGGAAGTAA and ITS2_r: GCTGCGTTCTTCATCGATGC). Single reactions were prepared in a specialised microbiome DNA extraction laboratory of 2.5µL 10X HiFi buffer, 0.1µL Platinum Taq Polymerase (Thermofisher), 16.7µL dH_20, 0.2µL 100mM dNTP mix, 1µL of 5µM forward and reverse primer and 2.5µL input DNA. The first step PCR reaction was adapted from the Earth Microbiome Project protocol (Thompson *et al.* 2017) and was amplified by initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 52°C for 30s, elongation at 68°C for 30s, with a final adenylation for 10 min at 68°C. Second step PCRs were then used to barcode the 3' and 5' ends. Single reactions were prepared of 2.5 10X HiFi buffer, 0.1 µL Platinum Taq Polymerase (Thermofisher), 14.9 µL dH_20, 0.5 µL 100 µM dNTP mix, 2µL of forward and reverse primer pair mix, and 4 µL of step one PCR product. The second step PCR reaction was amplified by initial denaturation at 94°C for 60s, followed by 15 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, elongation at 68°C for 30s, with a final adenylation for 10 min at 68°C.

For all samples, 2 µL of PCR product was mixed into 198 µL Qubit working solution and quantified using Qubit 2.0 Fluorometer. Samples were then pooled and cleaned using AxyPrep

(Axygen) following the manufacturer's instructions. The final pool was quantified and quality-checked using an Agilent TapeStation (D1000 Screen Tape). DNA sequencing was performed on an Illumina MiSeq v2 (2 x 150 for 16S and 2 x 250 bp for ITS) at SAHMRI (South Australian Health and Medical Research Institute) by SAGC (South Australian Genomics Center).

Bioinformatic Analysis

Both QIIME2 (Bolyen *et al.* 2019) and R v4.2.2 (R Core Team 2022) were used for the bioinformatic analyses and figure production. All relevant code and output data can be found within the GitHub repository located at: https://github.com/brady-welsh/2023_Grapevine_FvP. Forward and reverse reads were merged and denoised using DADA2 (Callahan *et al.* 2016) in QIIME2. Representative sequences were then assigned taxonomy using the QIIME2 feature-classifier plugin (Bokulich *et al.* 2018) using the SILVA-138 classifier (Quast *et al.* 2013) for 16S reads, and the UNITE classifier (Nilsson *et al.* 2019) for ITS reads. Before analysis, the program Decontam (Davis *et al.* 2017) was used to identify putative-contaminating ASVs using the negative controls shown in the GitHub repository. These contaminant ASVs were then filtered from biological samples. Data was then imported into rStudio as a phyloseq (McMurdie and Holmes 2013) object using qiime2R (https://github.com/jbisanz/qiime2R) and manipulated with dplyr (https://github.com/tidyverse/dplyr), tidyr (https://github.com/tidyverse/tidyr/), and the microbiome package (Leo Lahti, Sudarshan Shetty *et al.* 2017). Samples were put through a prevalence filter (Eisenhofer *et al.* 2022) before the table was rarefied at a depth of 518 ASVs for 16S comparisons and a depth of 5,064 ASVs for ITS comparisons. Alpha diversity measures were calculated in R using phyloseq, and figures were plotted using ggplot2 (Hadley

Wickham *et al.* 2016). All subsequent mixed-effects models were then performed using GraphPad Prism v9.0.0 for Windows (GraphPad Software, USA).

Data Availability

ITS and 16S rRNA reads are available at the NCBI (16S: BioProject PRJNA1000746, ITS: Bioproject PRJNA1000758).

RESULTS

PMA TREATMENT AND COMMUNITY STRUCTURE

To successfully observe the living communities of the phyllosphere microbiome, the viability selection dye, PMA, was utilised to remove relic DNA from the samples collected. As well as the samples treated with PMA, a secondary set was collected that remained untreated. The untreated samples acted as a control group, providing genetic material from viable organisms and relic DNA. Figures 1 and 2 show stacked bar charts with the relative community structure of each set of samples (collected in triplicate) at the "Family" level. Both figures list the top 20 most abundant Families present within all samples. In Figure 1 we see the bacterial communities observed by targeting the 16S rRNA gene and classifying features using the SILVA-138 database. Figure 2 shows the fungal communities observed by targeting the ITS gene and classifying features using the UNITE database. Overall these bar charts show that, despite a significant reduction in the number of ASVs (Amplicon Sequence Variants) within samples treated with the PMA dye (Supplementary Figure 1), there is no apparent detrimental effect on the community structure other than the removal of false positives from the dataset.

The bacterial communities (Figure 1) do not appear to show any notable difference between the PMA-treated (+PMA) and untreated groups (-PMA); however, there was a surprisingly

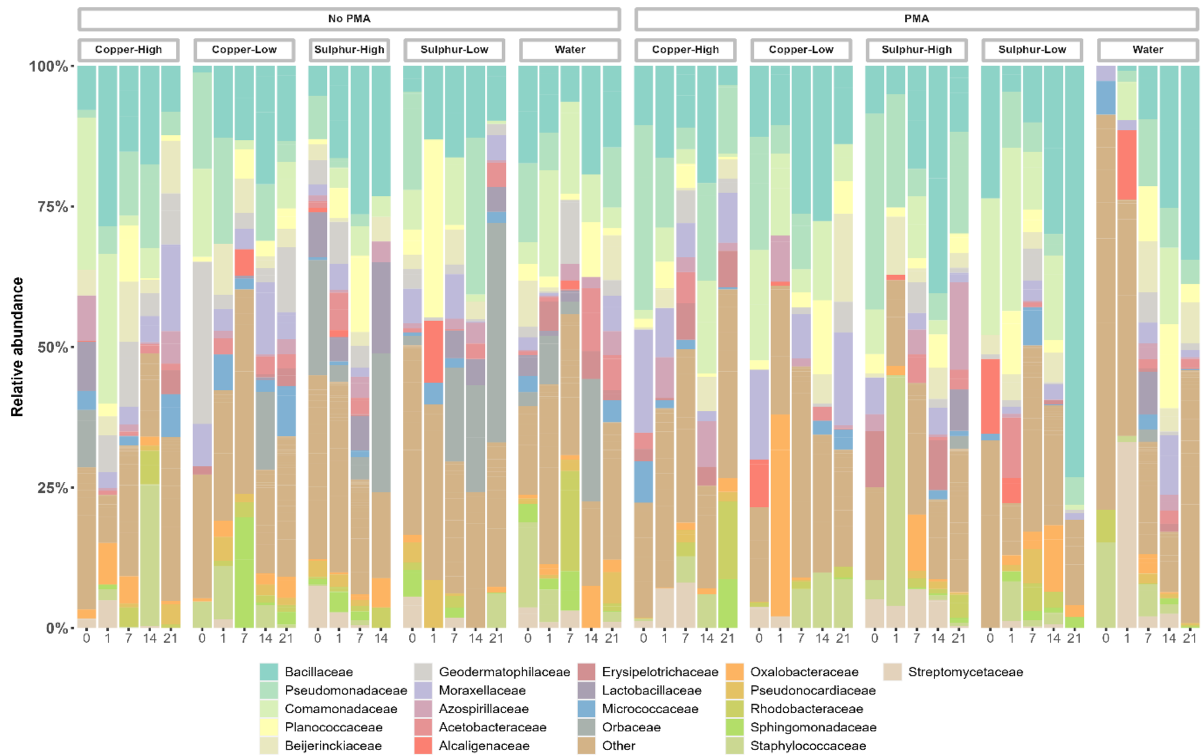

Figure 1 | Stacked bar plots showing the relative bacterial community structure within each sample group (n = 3) at the Family level.

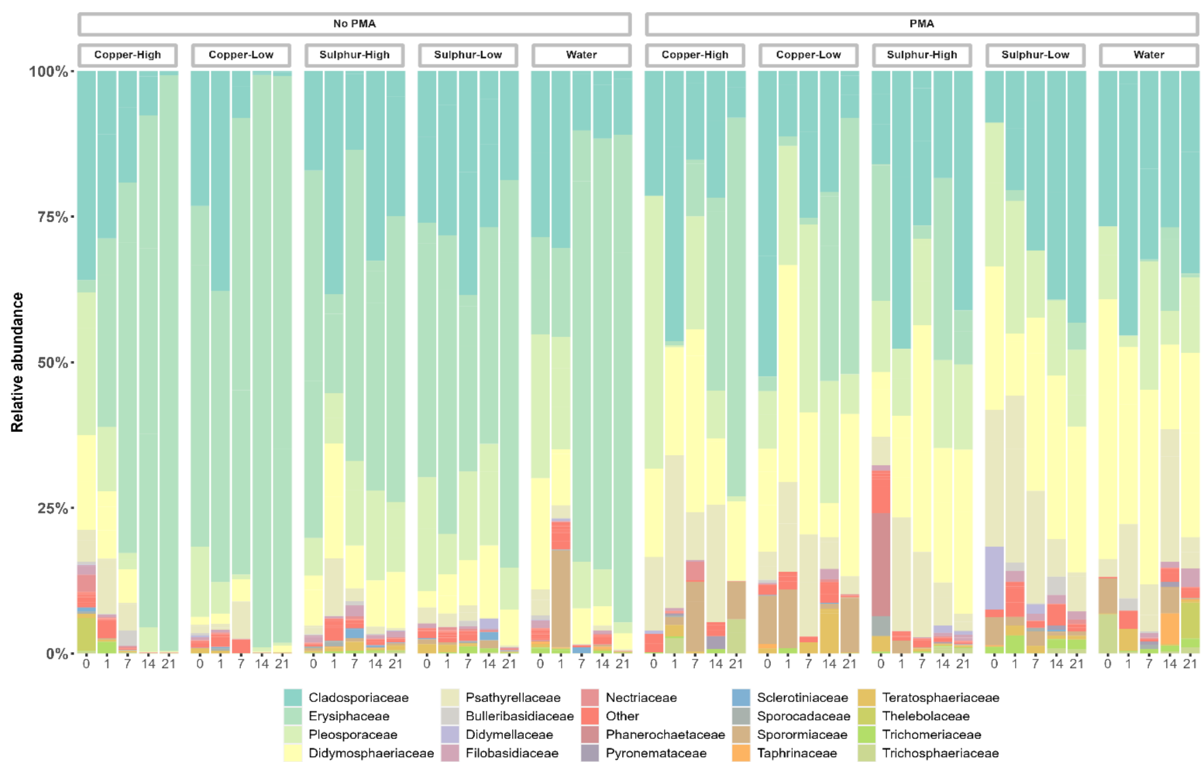

Figure 2 | Stacked bar plots showing the relative fungal community structure within each sample group (n = 3) at the Family level.

high abundance of the fungal family (Figure 2); Erysiphaceae, which includes the taxa responsible for powdery mildew infections in grapevines (*Erysiphe necator*). This taxon appears to increase over time within the -PMA samples suggesting a potential infection on all grapevines sampled. Furthermore, Feature Volatility Analysis (Figure 3) of the +PMA samples show how the relative abundance of the genus *Erysiphe* increases in all samples over the first 14 days post-treatment but continues to increase in the copper-fungicide-treated groups until 21 days post-treatment.

Time Series Analysis

Following confirmation that the PMA dye did not adversely affect the sequencing results, the +PMA group was primarily used for all subsequent analyses. Using these data, we observed the direct impacts of the two selected fungicides on the living phyllosphere microbiome over

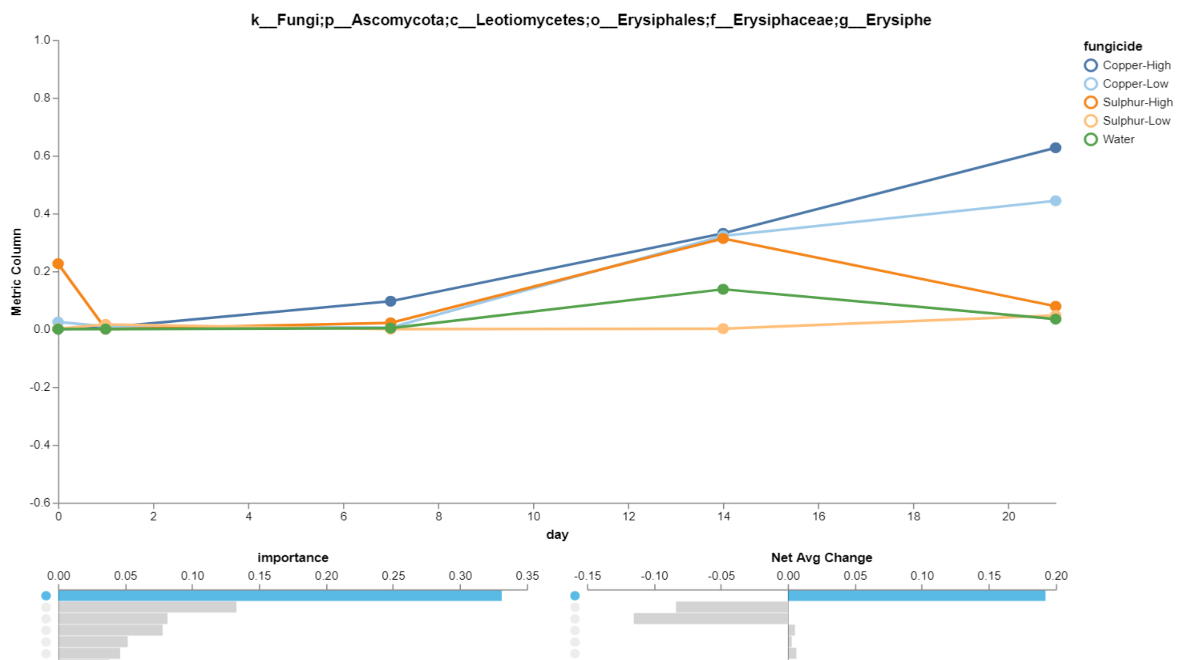

Figure 3 | Fungi (ITS) feature volatility plot generated using q2Longitudinal package in QIIME2. The plot shows changes in the presence of the genus *Erysiphe* within each treatment group. *Erysiphe* was selected as its net average change was given the highest importance score.

time. To do this, the alpha diversity metrics for diversity (Shannon diversity) and richness (ASV richness) were calculated for each group and plotted on time series 'volatility' plots for bacteria (Figure 4) and fungi (Figure 5). Each fungicide treatment was plotted against the results collected from the water spray control. A mixed-effects model was applied to test for significant changes between and within groups (supplementary tables 1–4). For all cases, there appeared to be no significant trends throughout the diversity or richness metrics of the 16S and ITS results. The effectivity of PMA was also demonstrated through the alpha diversity metrics (Supplementary Figure 2 and 3), notably the -PMA fungi volatility result, which show a consistent decline in diversity, including the water control, likely due to the increased abundance of Erysiphaceae (shown in Figure 2). Although not significant, a small ongoing decrease in bacterial diversity may be seen at 21d in the sulphur-low group (Figure 4) and an ongoing reduction in the fungal diversity for the copper-high and -low groups (Figure 5). This, however, would require a longer time series to prove ongoing, significant changes.

DISCUSSION

Although no significant trends were found between the fungicide treatments and the water control, across all groups—including the water control, there was a significant decrease in diversity and richness for all groups within the first 24 hours post-treatment. As this also consistently occurred within the water control and the fungicide treatments, the decrease was likely due to the spraying action and potentially explained by mechanical washing of the leaf surface during the application of the treatment. The results, however, also show that within the three weeks following this initial sharp reduction, the diversity and richness can return to normal levels. Figures 1 and 2 also show that the revived microbiome retains a similar microbial composition as it did before spraying. Although these results show a positive outcome for using copper- and sulphur-fungicides, especially in vineyards conscious of their

Bacteria (16S)

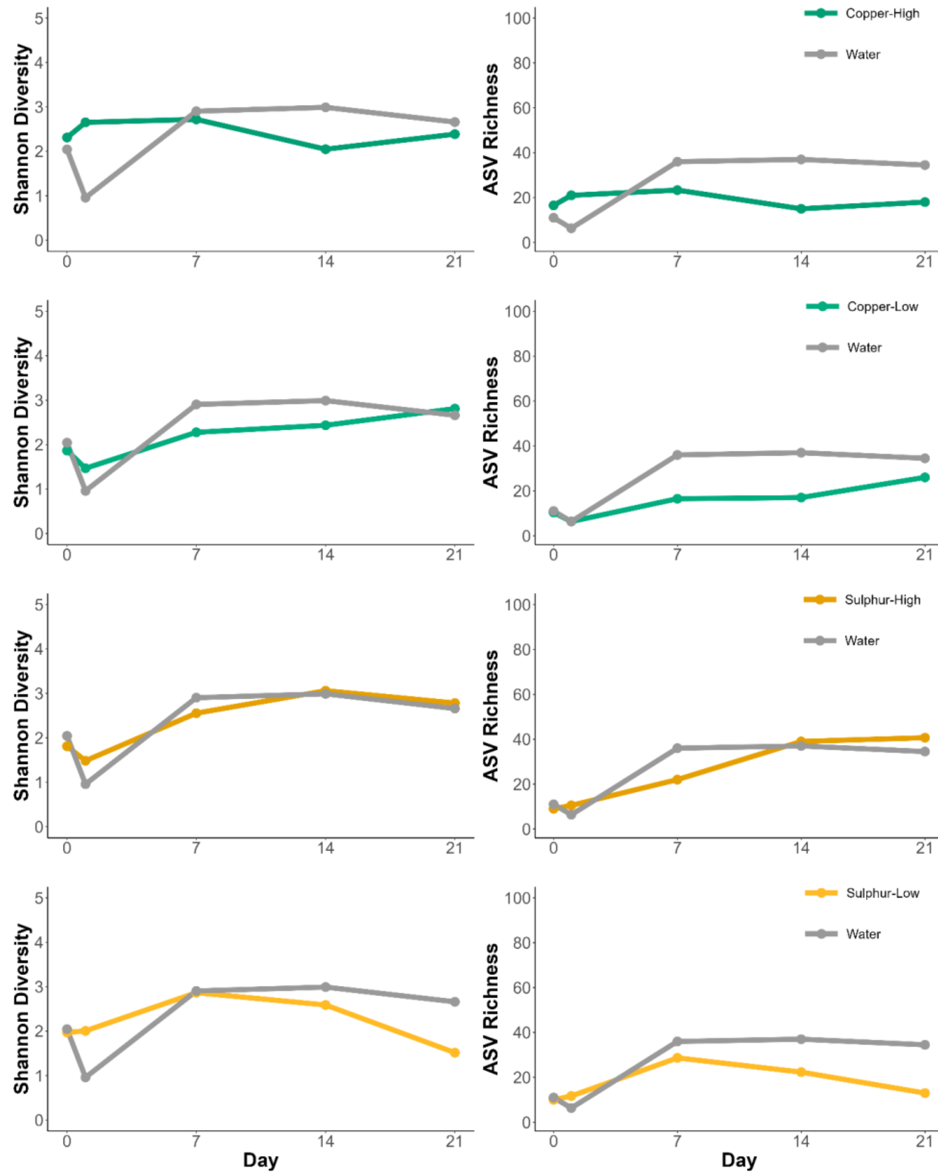

Figure 4 | Time series plots showing the changes in Shannon diversity and ASV richness of the live bacterial communities over time for each fungicide treatment. The water control group is shown in grey and overlaid on each plot for comparison. Samples shown here were all treated with PMA and are representative of the viable microorganisms present at the time of sampling.

Fungi (ITS)

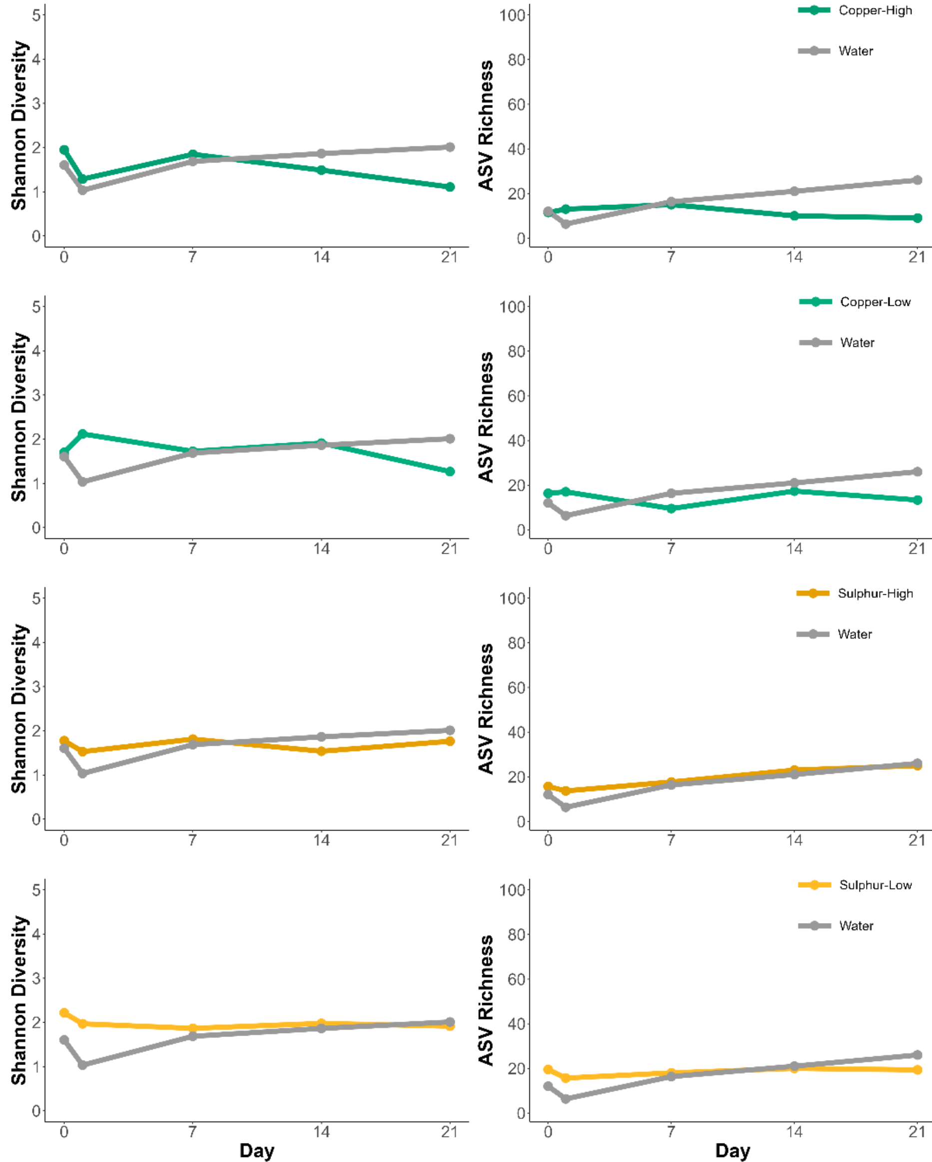

Figure 5 | Time series plots showing the changes in Shannon diversity and ASV richness of the live fungal communities over time for each fungicide treatment. The water control group is shown in grey and overlayed on each plot for comparison. Samples shown here were all treated with PMA and represent the viable microorganisms present at the time of sampling.

micro-biodiversity, this study only presents the effects from a single application. In real-world scenarios, fungicides, such as those tested, are applied multiple times, usually weekly. Given that this study shows that the spraying process leads to a sudden reduction in microbial richness and diversity, repeated applications may impact the regeneration of the microbial communities. Thus, further testing must be undertaken to understand the impact of the spraying process further.

The results of this study demonstrate that common copper- and sulphur-based fungicides at both high and low dose rates do not directly impact the composition of the phyllosphere microbiome of grapevines. With the growing interest in maintaining vineyard micro-biodiversity for either improved vineyard health and biocontrol or use in wild fermentation, the microbiome now presents itself as a new factor that can be impacted by off-target effects. Therefore, knowing which fungicides are effective at disease control while leaving the microbiota healthy and unaffected is important for utilising the microbiome in modern viticulture. Although previous studies have demonstrated that individual microbial isolates from the grapevine microbiome have shown particular susceptibilities to copper and sulphur fungicides (Grangeteau *et al.* 2017). However, as shown here, these single isolated effects observed *in vitro* may not be entirely translatable for the context of the phyllosphere microbiome as a whole.

Alongside these observations, we also detected high occurrences of *Erysiphaceae* taxa, indicating an instance of powdery mildew infection. However, over the sampling period, all vines did not appear to show any signs of infection, and this is supported by the +PMA samples, which show far lower abundances of Erysiphaceae. This suggests that the high abundance of Erysiphaceae taxa observed in the -PMA samples is likely a signal from relic DNA.

Further analysis of feature volatility also revealed increasing abundances of these taxa in copper treatments, a fungicide not used to treat powdery mildew; however, reducing abundances in sulphur treatments, a fungicide commonly used to fight powdery mildew. This suggests that the increases in Erysiphaceae in all groups may be due to an incoming infection that the application of sulphur-based fungicide may have controlled; however, it is potentially worsened by the application of copper-based fungicide.

These results act as a practical initial study highlighting the requirement for further characterisation of factors that impact the phyllosphere microbiome of grapevines used for winemaking. Further research is needed to more extensively characterise the effects of fungicides and other sprays on the live microbial communities which inhabit the phyllosphere. Further analyses, including more extended time series to observe more long-term consequences or the addition of multiple fungicide applications, will allow for characterisations that are more representative of real-world scenarios. As well, vineyards utilise a wide range of different synthetic and organic fungicides which each could have different impacts or even synergistic effects; therefore, it would be significant to properly monitor how all fungicides impact the phyllosphere microbiome and their potential effects as a possible factor in shaping the structure of a vineyard's micro-biodiversity.

CONCLUSION

Here, we observed the effects of common vineyard fungicides on the phyllosphere microbiome of *Vitis vinifera* 'Syrah' (Shiraz) grapevines using DNA sequencing. The results reveal that there appears to be no significant impact on the viable phyllosphere microbiome through the application of the common vineyard fungicide compounds; copper (copper oxychloride) and sulphur. The data does suggest a possibility that there are some impacts on the phyllosphere

microbial diversity further downstream; however, a more extended time series will be required to determine whether this change is significant when compared to untreated controls. Although the results show no effects from the fungicides, the observed reductions in diversity and richness immediately following spraying were surprising and warrant further investigation.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

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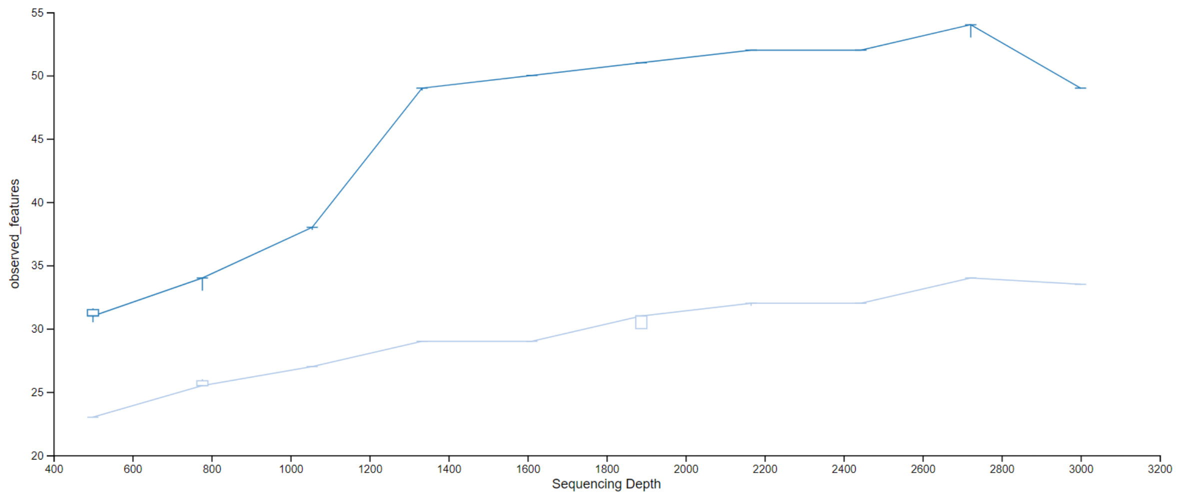
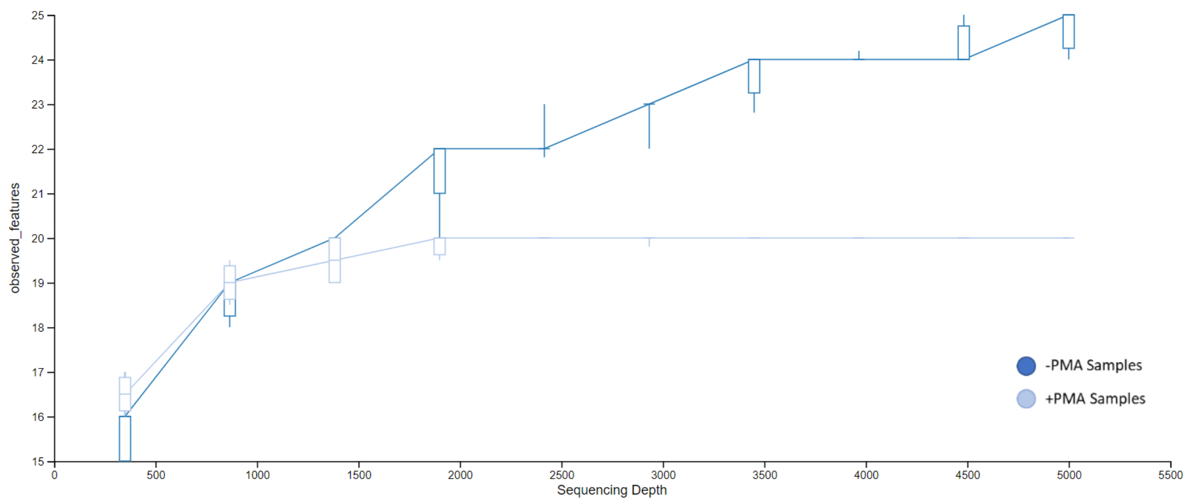
SUPPLEMENTARY DATA

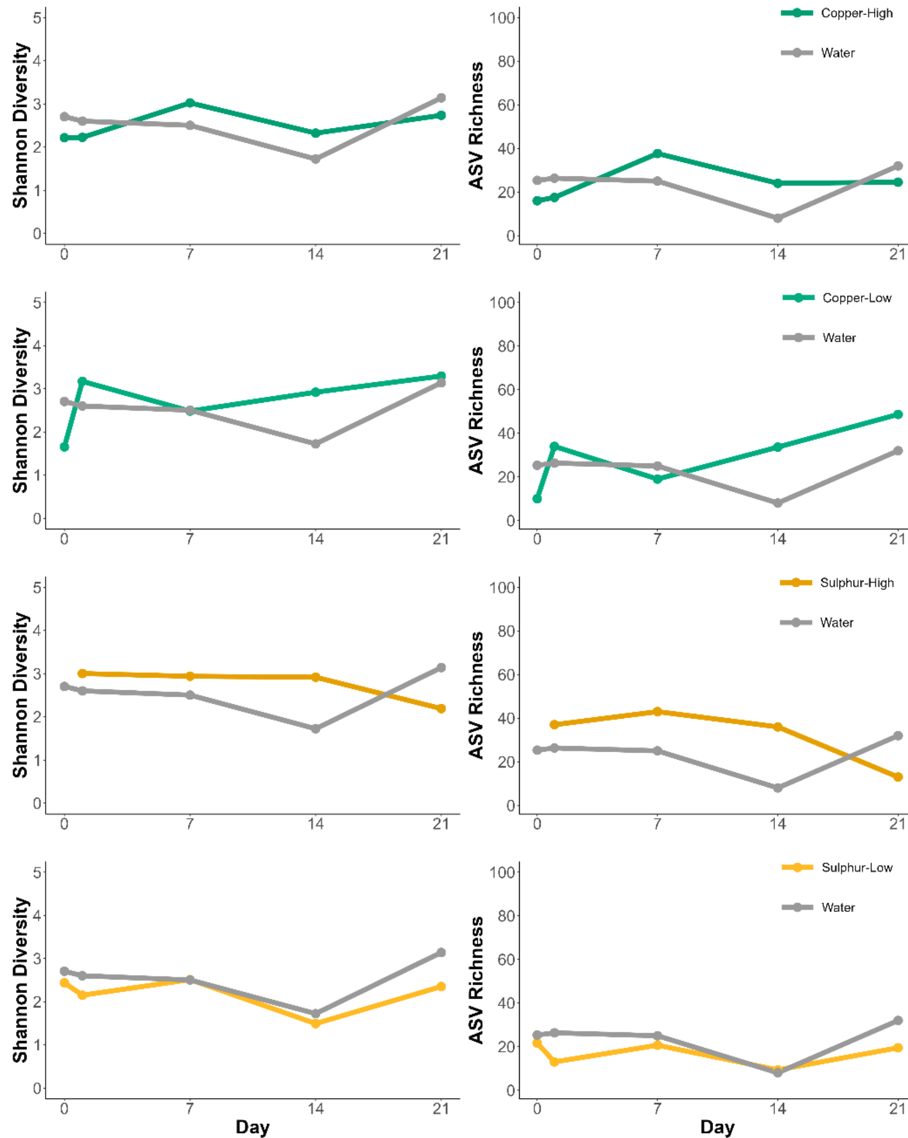
Bacteria (16S):



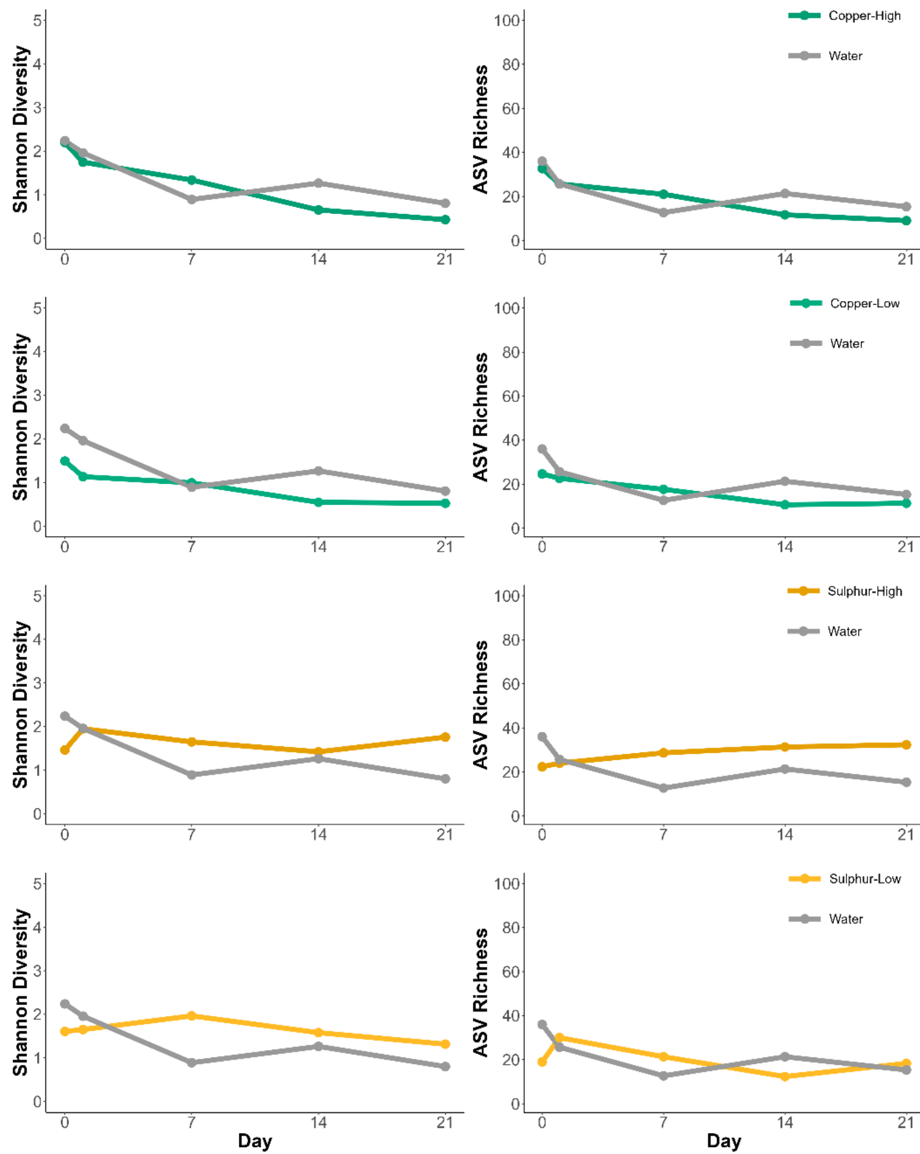
Fungi (ITS):



Supplementary Figure 1 | Plots showing the number of ASVs (observed features) across different sequencing depths. -PMA samples in both cases consistently have more ASVs than +PMA samples due to the removal of amplifiable relic DNA within the treated samples.



Supplementary Figure 2 | Time series plots showing the changes in Shannon diversity and ASV richness of the bacterial communities over time for each fungicide treatment. Shown here are all samples which were not treated with PMA and, therefore, are representative of the total DNA sampled.



Supplementary Figure 3 | Time series plots showing the changes in Shannon diversity and ASV richness of the fungal communities over time for each fungicide treatment. Shown here are all samples which were not treated with PMA and, therefore, are representative of the total DNA sampled.

Supplementary Table 1 | All significant differences for fungal (ITS) comparisons for samples not treated with PMA.

- PMA	**ITS Diversity (Within)**				**ITS Observed (Within)**			
	Group	*Comparison*	*P-Value*	*Star Code*	*Group*	*Comparison*	*P-Value*	*Star Code*
	Water	Day 0 vs. Dat 7	0.0049	**	Water	Day 0 vs. Day 7	0.0073	**
		Dat 0 vs. Day 14	0.0385	*		Day 0 vs. Day 21	0.0164	*
		Dat 0 vs. Day 21	0.0029	**				
		Day 1 vs. Day 7	0.024	*	Copper-High	Day 0 vs. Day 14	0.0148	*
		Day 1 vs. Day 21	0.0149	*		Day 0 vs. Day 21	0.0065	**
	Copper-High	Day 0 vs. Day 14	0.0015	**	Copper-Low	None	-	-
		Day 0 vs. Day 21	0.0003	***				
		Day 1 vs. Day 14	0.0207	*	Sulphur-High	None	-	-
		Day 1 vs. Day 21	0.0059	**				
					Sulphur-Low	Day 1 vs. Day 14	0.0383	*
	Copper-Low	Day 0 vs. Day 14	0.0442	*				
		Day 0 vs. Day 21	0.0396	*				
	Sulphur-High	None	-	-				
	Sulphur-Low	None	-	-				

	ITS Diverity (Between)				**ITS Observed (Between)**			
	Group	*Comparison*	*P-Value*	*Star Code*	*Group*	*Comparison*	*P-Value*	*Star Code*
	Day 0	None	-	-	Day 0	None	-	-
	Day 1	None	-	-	Day 1	None	-	-
	Day 7	None	-	-	Day 7	None	-	-
	Day 14	None	-	-	Day 14	None	-	-
	Day 21	None	-	-	Day 21	None	-	-

Supplementary Table 2 | All significant differences for fungal (ITS) comparisons for samples treated with PMA.

+ PMA	ITS Diversity (Within)				ITS Observed (Within)			
	Group	*Comparison*	*P-Value*	*Star Code*	*Group*	*Comparison*	*P-Value*	*Star Code*
	Water	Day 1 vs. Day 14	0.0275	*	Water	Day 1 vs. Day 14	0.0329	*
		Day 1 vs. Day 21	0.0223	*		Day 1 vs. Day 21	0.0114	*
	Copper-High	Day 0 vs. Day 21	0.0325	*	Copper-High	None	-	-
		Day 7 vs. Day 21	0.0479	*				
	Copper-Low	Day 1 vs. Day 21	0.0438	*	Copper-Low	None	-	-
	Sulphur-High	None	-	-	Sulphur-High	None	-	-
	Sulphur-Low	None	-	-	Sulphur-Low	None	-	-

+ PMA	ITS Diverity (Between)				ITS Observed (Between)			
	Group	*Comparison*	*P-Value*	*Star Code*	*Group*	*Comparison*	*P-Value*	*Star Code*
	Day 0	None	-	-	Day 0	None	-	-
	Day 1	none	-	-	Day 1	None	-	-
	Day 7	None	-	-	Day 7	None	-	-
	Day 14	None	-	-	Day 14	None	-	-
	Day 21	None	-	-	Day 21	None	-	-

Supplementary Table 3 | All significant differences for bacterial (16S) comparisons for samples not treated with PMA.

- PMA	16S Diversity (Within)				16S Observed (Within)			
	Group	*Comparison*	*P-Value*	*Star Code*	*Group*	*Comparison*	*P-Value*	*Star Code*
	Water	None	-	-	Water	None	-	-
	Copper-High	None	-	-	Copper-High	None	-	-
	Copper-Low	Day 0 vs. Day 21	0.0183	*	Copper-Low	Day 0 vs. Day 21	0.0263	*
	Sulphur-High	None	-	-	Sulphur-High	None	-	-
	Sulphur-Low	None	-	-	Sulphur-Low	None	-	-
	16S Diverity (Between)				**16S Observed (Between)**			
	Group	*Comparison*	*P-Value*	*Star Code*	*Group*	*Comparison*	*P-Value*	*Star Code*
	Day 0	None	-	-	Day 0	None	-	-
	Day 1	None	-	-	Day 1	None	-	-
	Day 7	None	-	-	Day 7	None	-	-
	Day 14	None	-	-	Day 14	None	-	-
	Day 21	None	-	-	Day 21	None	-	-

Supplementary Table 4 | All significant differences for bacterial (16S) comparisons for samples treated with PMA.

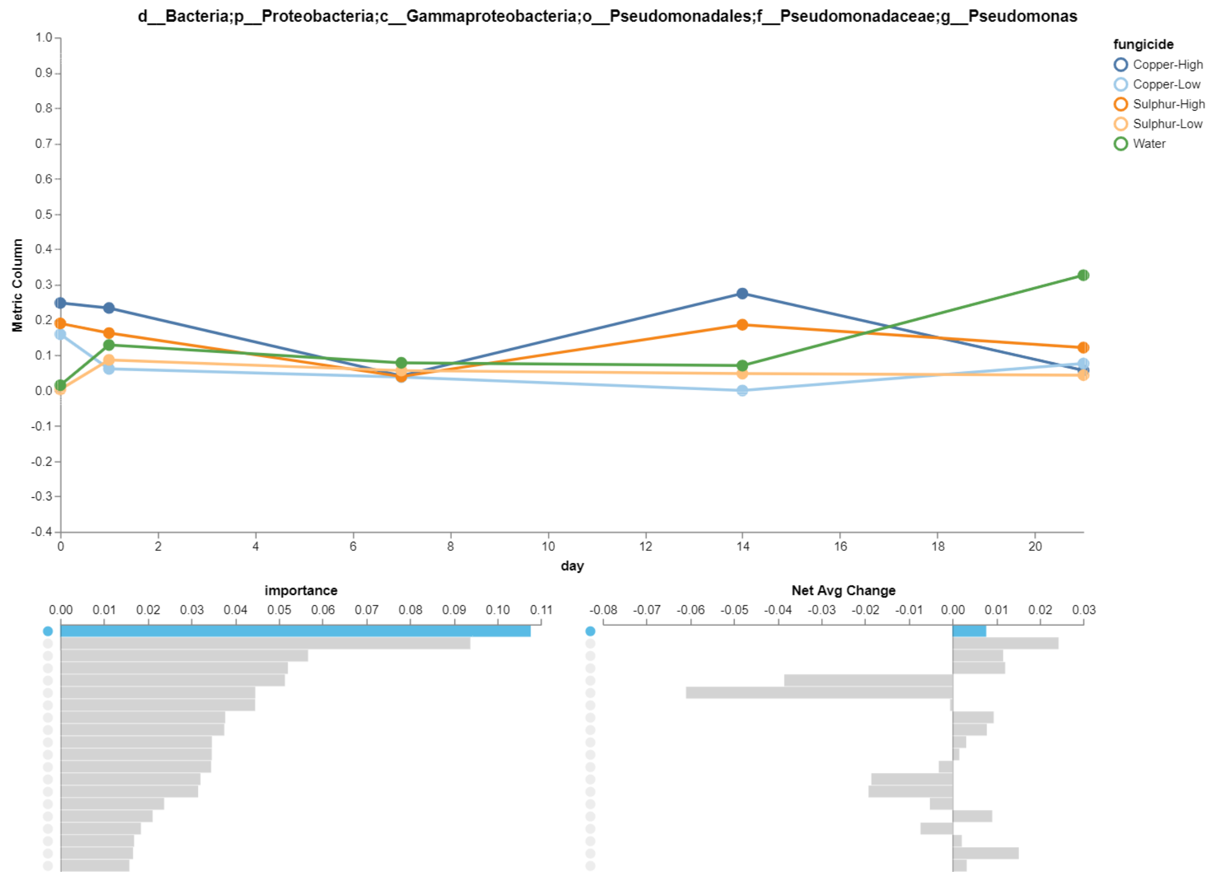
+ PMA

16S Diversity (Within)			
Group	*Comparison*	*P-Value*	*Star Code*
Water	Day 1 vs. Day 7	0.001	***
	Day 1 vs. Day 14	0.0019	**
	Day 1 vs. Day 21	0.0079	**
Copper-High	None	-	-
Copper-Low	Day 1 vs. Day 21	0.0323	*
Sulphur-High	Day 1 vs. Day 14	0.0225	*
	Day 1 vs. Day 21	0.0381	*
Sulphur-Low	Day 7 vs. Day 21	0.0315	*

16S Observed (Within)			
Group	*Comparison*	*P-Value*	*Star Code*
Water	Day 1 vs. Day 7	0.0302	*
	Day 1 vs. Day 15	0.0432	*
Copper-High	None	-	-
Copper-Low	None	-	-
Sulphur-High	Day 0 vs. Day 21	0.0376	*
	Day 1 vs. Day 21	0.047	*
Sulphur-Low	None	-	-

16S Diversity (Between)			
Group	*Comparison*	*P-Value*	*Star Code*
Day 0	None	-	-
Day 1	Water vs. Copper-H	0.0286	*
Day 7	None	-	-
Day 14	None	-	-
Day 21	None	-	-

16S Observed (Between)			
Group	*Comparison*	*P-Value*	*Star Code*
Day 0	None	-	-
Day 1	None	-	-
Day 7	None	-	-
Day 14	None	-	-
Day 21	None	-	-



Supplementary Figure 4 | Bacteria (16S) feature volatility plot generated using q2Longitudinal package in QIIME2. *Pseudomonas* was selected as its net average change was given the highest importance score. However, all net average change and importance scores were minute, and no particular feature demonstrated notable trends.

Chapter VI

Impacts of Fungicides on the Grape Berry Microbiome and the Sensory Profile of Derived Wines.

In this chapter, we characterise the differences in the phyllosphere microbiome between three vineyards using three disease management methods. We have then tracked these phyllosphere microorganisms throughout fermentation alongside the environmental changes caused by fermentation. We then partnered the microbial investigations with a sensory analysis of the resulting wines to observe correlations between the microbial and sensory profiles of wines produced from the three vineyards.

Statement of Authorship

Title of Paper	Impact of Fungicides on the Grape Berry Microbiome and the Sensory Profile of Derived Wines.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript prepared for submission to relevant journal.

Principal Author

Name of Principal Author (Candidate)	Brady L. Welsh		
Contribution to the Paper	Conceptualisation, method development, sampling, data generation, data analysis, writing and drafting.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	02 / 08 / 2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stephen P. Kidd		
Contribution to the Paper	Conceptualisation. Review and editing of manuscript.		
Signature		Date	

Name of Co-Author	Susan E. P. Bastian		
Contribution to the Paper	Conceptualisation. Method development. Review and editing of manuscript.		
Signature		Date	

Name of Co-Author	Raphael Eisenhofer		
Contribution to the Paper	Conceptualisation. Review and editing of manuscript.		
Signature		Date	

TITLE

Impact of Fungicides on the Grape Berry Microbiome and the Sensory Profile of Derived Wines.

AUTHORS

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KEYWORDS

Microbiome, phyllosphere, fermentation, grapevine, vineyard, wine, sensory

SUMMARY

Purpose:

In this study, we investigated the differences in the structure of the phyllosphere microbiome of Shiraz grapevines across three different vineyards, each using a different method of disease management: conventional, organic, and biodynamic. We observed how the fungal communities of the phyllosphere microbiome differ between the three vineyards, their changes throughout fermentation, and correlations between the microbial communities and the sensory profiles of the wines.

Methods:

Shiraz grape berries were picked from each vineyard and made into wines using small-lot winemaking in triplicate. Samples were collected at key points during fermentation for microbial analysis of the fungal phyllosphere microbiome. We produced amplicons of the ITS (Internal Transcribed Spacer) gene for DNA sequencing and fungal identification. Amplicons were sequenced using Illumina MiSeq DNA sequencing, and output reads were processed and analysed using QIIME2 and the Phyloseq package in R. For the sensory analysis, completed wines were analysed by a Rate-All-That-Apply (RATA) method, and results were analysed using by two-way ANOVA and Principle Component Analyses (PCA).

Results:

The findings show significant differences in the microbial and sensory profiles between all three vineyards. The differences between the microbiome structure and sensory outcome aligned, suggesting a correlation between the disease management methods of a vineyard and the sensory outcome of wines due to changes in the microbial system.

ABSTRACT

Fungicides play an essential role in the disease management of all Australian vineyards, ensuring the protection of grapevines from fungal infections. However, many common fungicides used in Australian vineyards cannot differentiate between fungal organisms that cause disease and the beneficial wild microorganisms that inhabit the grapevine and comprise its microbiome. As such, using fungicides that lack specificity for pathogenic fungi could lead to indirect effects on the grapevine microbiome, particularly its phyllosphere microbiome, which inhabits the plant's surface. We collected grape berry samples from Shiraz grapevines from three vineyards, utilising a conventional, organic, or biodynamic disease management method. Grape samples were used for small-lot winemaking, and microbial samples were collected throughout fermentation. Using ITS gene sequencing and the Rate-All-That-Apply sensory analysis technique on completed wines, we were able to establish correlating differences between the groups, observing higher diversities in organic microbiomes corresponding with unique sensory outcomes.

INTRODUCTION

The grapevines that are used for winemaking are inhabited by a wide range of prokaryotes, eukaryotes, and viruses which, instead of causing disease, support the plant in a symbiotic or commensal relationship (Gong and Xin 2021; Bettenfeld *et al.* 2022). The phyllosphere, which encompasses the plant's surface (Morris 2002; Singh *et al.* 2019), is inhabited by a diverse community of microorganisms. Despite being susceptible to a multitude of biotic and abiotic stressors, such as lack of nutrients or climatic changes (Bokuich *et al.* 2014; Castañeda *et al.* 2018; Bashir *et al.* 2022), the grapevine phyllosphere microbiome plays many essential roles in plant health (Fürnkranz *et al.* 2008; Zarraonaindia and Gilbert 2015; Berlanga-Clavero *et al.*

2020; Bashir *et al.* 2022). Despite the grapevine phyllosphere's importance in vineyards, as the interface between the host plant and its environment, the phyllosphere comes into direct contact with fungicide sprays; thus, the phyllosphere microorganisms may be impacted by off-target antimicrobial effects. Many types of fungicides are readily used to treat or prevent different fungal diseases, such as Powdery and Downy mildews (Perria *et al.* 2022), as well as to meet various standards for vineyard practices, such as those required for organic and biodynamic certifications. These methods both increase the scrutiny of which fungicides can be used within an organic certified vineyard, removing synthetic chemical fungicides and relying on natural compounds to manage instances of disease (Reganold and Wachter 2016). Biodynamic vineyards take this one step further by implementing the same scrutiny as organic vineyards with the addition of concepts developed by Rudolf Steiner wherein careful consideration and timing of sprays and maintenance is implemented, as well as special soil preparations (Steiner and Creeger 1993). As each of these vineyards utilises different methods for controlling infectious disease, it is likely but under investigation what impact each practice has on the grapevine microbiome also.

The importance of the grapevine microbiome is further magnified in the context of winemaking as microorganisms derived from the grapevine act as wild inoculations, which can provide a diverse range of reactions during the fermentation process resulting in unique and complex sensory characteristics (Gutiérrez *et al.* 1999; Liu *et al.* 2017; Anagnostopoulos *et al.* 2019; Welsh *et al.* 2023). Some wineries are utilising this process, producing wines fermented entirely with wild grapevine microorganisms without the inoculation of *Saccharomyces cerevisiae* or lactic acid bacteria (LAB) such as *Oenococcus oeni* (Gutiérrez *et al.* 1999; Clemente-Jimenez *et al.* 2004; Combina *et al.* 2005; Piao *et al.* 2015). Although many wild microorganisms have a positive or neutral impact on the host plant, these microbes share

similar systems to microbes that cause disease. As such, many common fungicides used in vineyards today, such as copper and sulphur fungicides, have broad-spectrum effects which may also impact the beneficial microbial communities of the grapevine microbiome through fungicidal effects targeting those shared systems and structures (Grangeteau *et al.* 2017).

We describe how different disease management regimes: conventional, organic, and biodynamic, impact the phyllosphere microbiome of grapes harvested for winemaking. Using ITS gene amplicon sequencing on grape berry and wine must samples, we were able to map the fungal communities derived from the phyllosphere microbiome. Using these methods, we investigated the effects of fungicide use on the initial community structures of fermentation and how these communities were shaped during the fermentation process. We then report how these changes impact each wine's basic chemistry and sensory outcomes using the Rate-All-That-Apply sensory analysis method (Giacalone and Hedelund 2016; Danner *et al.* 2018). By performing these analyses concurrently, we were able to map the presence of phyllosphere fungal taxa throughout the fermentation process and observe how changes in the microbial community structure on the grapevine correlate to different chemical and sensory outcomes in the same Shiraz wines.

MATERIALS AND METHODS

Vineyards And Picking:

Three vineyards (Figure 1) were selected based on their disease management plans, with each being considered either conventional, organic, or biodynamic. Each vineyard supplied the spray records for the 2021-22 season, shown in Supplementary Table 1. Each participating vineyard monitored the maturity of the allocated vines until a maturity stage of ~27 Brix (~15°Bè) was reached. Grapes were aseptically handpicked using sterilised secateurs and latex

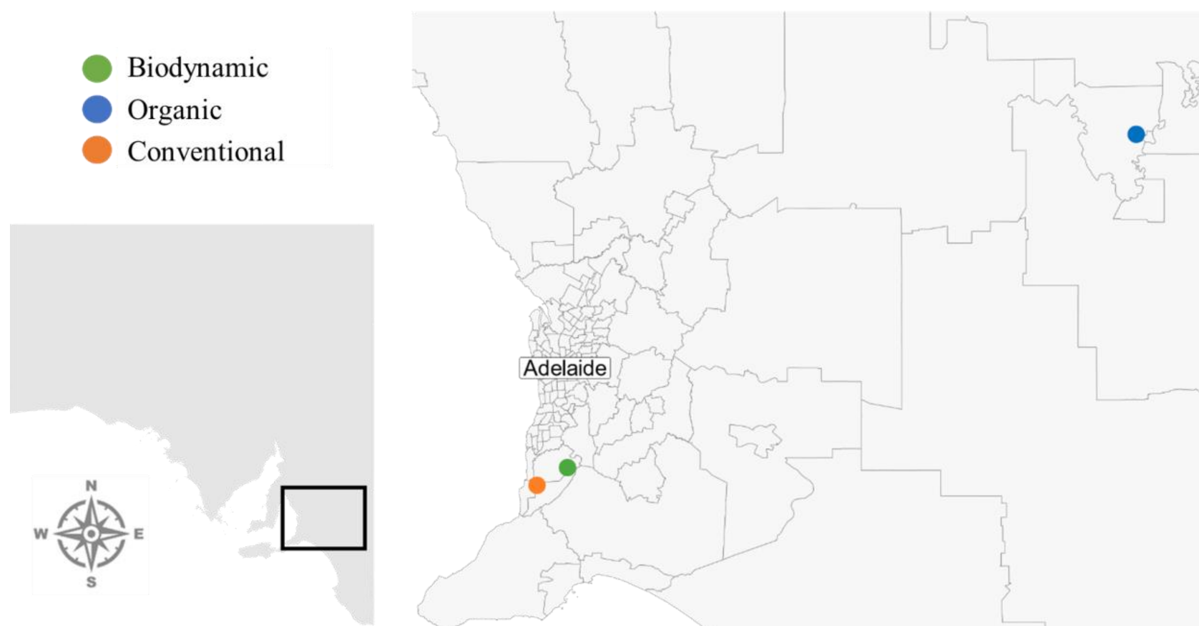


Figure 1 | Map of South Australia showing the location of the three sample vineyards.

gloves and stored in plastic-lined picking bins. 10-15kg fruit samples were collected in triplicate from different neighbouring rows per treatment and transported to the University of Adelaide, Waite Campus, and stored at 4ºC for 48hrs before crushing.

Wine Preparation:

Small-lot winemaking was conducted at Wine Innovation Central (Urrbrae, SA, Australia). Separate wines were made for each of the treatment replicates without pooling. Before crushing, ten individual berries were randomly selected from each replicate, selecting grapes from multiple locations of multiple bunches, and placed into a sterile 50mL tube and stored at 4ºC for later microbial harvest. The fruit was destemmed and crushed using an electric grape de-stemmer/crusher, and the must was placed into 30L plastic fermenters. No sulphur dioxide (SO_2) was added to preserve the wild microorganisms in all treatments. No pH adjustment was

made as further adjustment may have impacted the wild microbial communities. Ferments undergoing inoculated fermentation were inoculated immediately following crushing with sachets of *Saccharomyces cerevisiae* (Lalvin EC-1118 Wine Yeast) according to the manufacturer's instructions at a rate of 250 mg/L. All ferments were stored in a 20-22ºC fermentation room and were hand plunged once per day (3 pm daily, with ten punch downs per plunging). The sugar content of the ferments was checked every day post-plunging using a digital hydrometer (EasyDens, Anton Paar) and 5 mL of the must was taken for microbial analysis once every two days.

Once the Baumè of each ferment reached below 1.5ºBè (~27 g/L total sugar), the ferments were pressed at 1.5 bar (~21 psi) for 10 min using a water bag press (Enotecnica Pillan, Italy) and transferred to sterilised clear glass demijohns with airlocks and stored at 22ºC until dry (total residual sugars were below 2 g/L from enzymatic analysis). Sugar content in the pressed wines was monitored weekly with a ChemWell 2900 automated analyser (Awareness Technology, USA) using a Vintessential Laboratories enzyme kit (WineChek, Australia). Once fermentation was completed, 60 ppm of potassium metabisulphite (PMS) was added to halt microbial activity and stabilise wines. Wines were then stored at 0ºC for one week for cold stabilisation and racked off gross lees. Wines were bottled in 750 mL dark green bottles and stored in cold storage for one month before being used for sensory analysis. Cold storage was chosen for final storage conditions due to the risks posed by potential biological activity. As malolactic fermentation was avoided to preserve wild community structure in the ferments, energy sources remained in the form of malic acid.

Basic Wine Chemical Composition:

Chemical analyses were conducted to characterise further each wine's basic chemistry, as shown in Supplementary Table 3. All analyses were conducted in triplicate and included: pH and Titratable Acidity (TA), which were performed using an Inmotion Flex Autosampler (Mettler Toledo, Australia); Residual Sugar, Free and Total SO_2, and Volatile Acidity (VA), and Malic Acid (MA), which were performed using with a ChemWell 2900 automated analyser (Awareness Technology, USA) using Vintessential Laboratories enzyme kits (WineChek, Australia); Ethanol Content, which was performed using an Alcolyzer (Anton Parr, Austria); and colour, which was performed using the CIELAB tristimulus method (Cintra 4040, GBC Scientific Equipment, Australia).

Microbial Harvest:

Grape berry samples were transported to the University of Adelaide, Molecular Life Sciences within 12 hrs. 30 mL of PBST (PBS with 0.01% Tween20) was added to sterile tubes containing grape berries and placed on a shaker (Ratek) for 90 min at 120 rpm at room temperature. These were then sonicated using an ultrasonic bath at 60 Hz for 10 min for optimal release of microorganisms from the berry surface. The wash solution was isolated from the grape berries and centrifuged at 5,000 x g for 20 min. The supernatant was discarded, and pellets were washed with 1 mL of PBS, resuspended in 100 µL of PBS, and stored at -20ºC until DNA extraction.

Must samples (5 mL) were collected every second day during fermentation and immediately transported, on ice, to the University of Adelaide (Molecular Life Sciences laboratories for molecular analysis). 1 mL of the must was added to sterile 1.5 mL Eppendorf tubes and centrifuged at 4,000 x g for 20 min. The supernatant was removed using an automatic pipette

and discarded. Pellets were washed in 1 mL of PBS before being resuspended in 100 µL of PBS and stored at -20ºC until DNA extraction. A final wine sample was collected following pressing and transported to the University of Adelaide (Molecular Life Sciences building). 1 mL of each pressed wine sample was then processed identically to the must samples to form a pellet. Pellets were resuspended in 100 uL of PBS and stored at -20ºC until DNA extraction.

DNA Extractions and Sequencing:

DNA extractions were performed using the ZymoBiomics Miniprep DNA Extraction Kit following the manufacturer's instructions. Pellets resuspended in 100 uL of PBS were extracted, and DNA was eluted in 50 uL of DNase/RNase-free water and stored at -20ºC for analysis. Alongside biological samples, extraction blank controls (EBCs) were also prepared identically for the downstream detection of contaminants by conducting extraction blanks without adding biological material into the lysis buffer (Eisenhofer *et al.* 2019). Amplicons were amplified using a two-step method. First step PCRs amplified either the ITS1 region using the ITS1f/ITS2 primer pair (White *et al.* 1990; Gardes and Bruns 1993) (ITS1f: CTTGGTCATTTAGAGGAAGTAA and ITS2_r: GCTGCGTTCTTCATCGATGC) or the 16S V5-V6 region using the 799F/1115R primer pair (reference) (799F: 1115R:). The 16S primers were selected due to their reduced mitochondrial and plastid DNA amplification. Single reactions were prepared in a specialised microbiome DNA extraction laboratory of 2.5 µL 10X HiFi buffer, 0.1 µL Platinum Taq Polymerase (Thermofisher), 16.7 µL dH_20, 0.2 µL 100mM dNTP mix, 1 µL of 5µM forward and reverse primer and 2.5 µL input DNA. The first step PCR reaction was adapted from the Earth Microbiome Project protocol (Thompson *et al.* 2017) and was amplified by initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 52°C for 30 sec, elongation at 68°C for 30s, with a final adenylation for 10 min at 68°C. Second step PCRs were then used to barcode the 3' and

5’ ends. Single reactions were prepared of 2.5 10X HiFi buffer, 0.1 µL Platinum Taq Polymerase (Thermofisher), 14.9 µL dH_20, 0.5 µL 100 µM dNTP mix, 2 µL of forward and reverse primer pair mix, and 4 µL of step one PCR product. The second step PCR reaction was amplified by initial denaturation at 94°C for 1 min, followed by 15 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, elongation at 68°C for 30s, with a final adenylation for 10 min at 68°C.

For all samples, 2 µL of PCR product was mixed into 198 µL Qubit working solution and quantified using Qubit 2.0 Fluorometer. Samples were then pooled and cleaned using AxyPrep (Axygen) following the manufacturer’s instructions. The final pool was quantified and quality-checked using an Agilent TapeStation (D1000 Screen Tape). DNA sequencing was performed on an Illumina MiSeq v2 (2 x 250 bp) at SAHMRI (South Australian Health and Medical Research Institute) by SAGC (South Australian Genomics Center).

Bioinformatic Analysis:

Both QIIME2 (Bolyen *et al.* 2019) and R v4.2.2 (R Core Team 2022) were used for the bioinformatic analyses and figure production. All relevant code and output data are in the GitHub repository: https://github.com/brady-welsh/2023_Vineyard_FvW. Forward and reverse reads were merged and denoised using DADA2 (Callahan *et al.* 2016) in QIIME2. Amplicon Sequence Variants (ASVs) were then assigned taxonomy using the QIIME2 feature-classifier plugin (Bokulich *et al.* 2018) using the SILVA-138 classifier (Quast *et al.* 2013) for 16S reads, and the UNITE classifier (Nilsson *et al.* 2019) for ITS reads. Data was imported into rStudio as a phyloseq (McMurdie and Holmes 2013) object using qiime2R (https://github.com/jbisanz/qiime2R) and manipulated with dplyr (https://github.com/tidyverse/dplyr), tidyr (https://github.com/tidyverse/tidyr/), and the

microbiome package (Leo Lahti, Sudarshan Shetty *et al.* 2017). Samples were put through a prevalence filter (Eisenhofer *et al.* 2022) and rarefied. Alpha diversity (richness and Shannon diversity) and Beta diversity (Bray-Curtis) measures were calculated in R using phyloseq, and figures were plotted using ggplot2 (Hadley Wickham *et al.* 2016). Additionally, using the grape berry phyllosphere samples, phyllosphere organisms were identified and isolated from wine ferment samples (which also included endophytic microorganisms) using the Decontam program detailed within the GitHub repository.

Sensory Analysis:

Out of the 18 ferments, 15 groups successfully completed fermentation (dry, < 2g/L sugar). All successful wines underwent a Rate-All-That-Apply (RATA) analysis for sensory profiling (Giacalone and Hedelund 2016; Danner *et al.* 2018) and chemical composition analyses. RATA sensory analysis is a rapid and cost-effective sensory profiling method that does not require trained panellists and has been demonstrated to provide similar data to Descriptive Analysis methods (Danner *et al.* 2018). Forty-eight students studying wine production were recruited from the University of Adelaide, School of Agriculture, Food and Wine, all of whom were red-wine drinkers. Each participant undertook two 60 min RATA sessions seven days apart. Participants evaluated eight wines in Session 1 and seven in Session 2. Each of the wines was evaluated once by each of the panellists. All evaluations took place in a purpose-built sensory laboratory in individual computerised booths at 21°C, under fluorescent lighting.

During both sessions, wines were presented monadically with enforced one-minute breaks between each wine. The order in which each wines were presented to each participant was randomised and allocated in RedJade Sensory Software (RedJade Sensory Solutions LLC) using a William Squares design. Wine samples (30 mL) were served at 21°C in coded, clear

International Standards Organisation (ISO) approved 215 mL tasting glasses covered using plastic Petri dishes.

Selected sensory attributes were extracted from previous red wine RATA studies (Mezei *et al.* 2021). Selected attributes consisted of 3 attributes pertaining to colour, 26 to aroma, 3 to taste, 26 to flavour, 4 to mouthfeel, and 2 to aftertaste (Supplementary Table 2). Each attribute was measured using a 7-point rating scale to rate the intensity of only those attributes that applied to the wine. Ethics approval was provided by The University of Adelaide Committee on the Ethics of Human Experimentation under approval number H-2022-154.

Statistical Analysis

The data collected during the RATA tasting sessions were analysed using a mixed model two-way ANOVA with assessors as random and samples as fixed factor effects, with Fisher's LSD post-hoc test where significance was considered for both $P < 0.05$ and $P < 0.01$ using XLSTAT v2022.4.1 (Addinsoft SARL, France). The mean attribute ratings from the panel data were then calculated for all significant attributes and underwent Principal Component Analysis (PCA) using XLSTAT. Demographic data analyses were also performed using XLSTAT. All other statistical tests, including alpha diversity linear mixed effects, beta diversity PERMANOVAs, and the Procrustes and Mantel tests for correlation, were completed using R (v4.2.2), where all relevant code can be found in the above GitHub repository.

Data Availability

ITS and 16S reads are available at the NCBI (BioProject PRJNA1000981).

RESULTS

Preliminary Microbial Profiles

The microbial profiles and taxa were plotted based on the successful sequencing of the ITS gene (Figure 2). Unfortunately, 16S rRNA targets did not amplify effectively and therefore were not included in this analysis (Supplementary Figure 1). The top 20 families, chosen for accounting for 99.5% of reads, were identified. Notably, Saccharomycetaceae (*Saccharomyces* family) was absent in grape berry phyllosphere samples. However, Saccharomycetaceae was consistently present in all fermentation stages except in T1 wild fermentation samples. The family Saccharomycetaceae dominated the niche in conventional and biodynamic wines over time. In contrast, organic wines showed a different trend, with the Aspergilaceae family dominating fermentation from T1 onward, appearing to out-compete the Saccharomyces species. Furthermore, organic vineyards exhibited a high abundance of taxa not included in the

Figure 2 | Taxa bar plots showing the relative abundance of the topmost abundant Families across the three vineyards. Berry samples are representative of phyllosphere microorganisms only, whereas ferment samples are representative of both epiphytic, endophytic and inoculum.

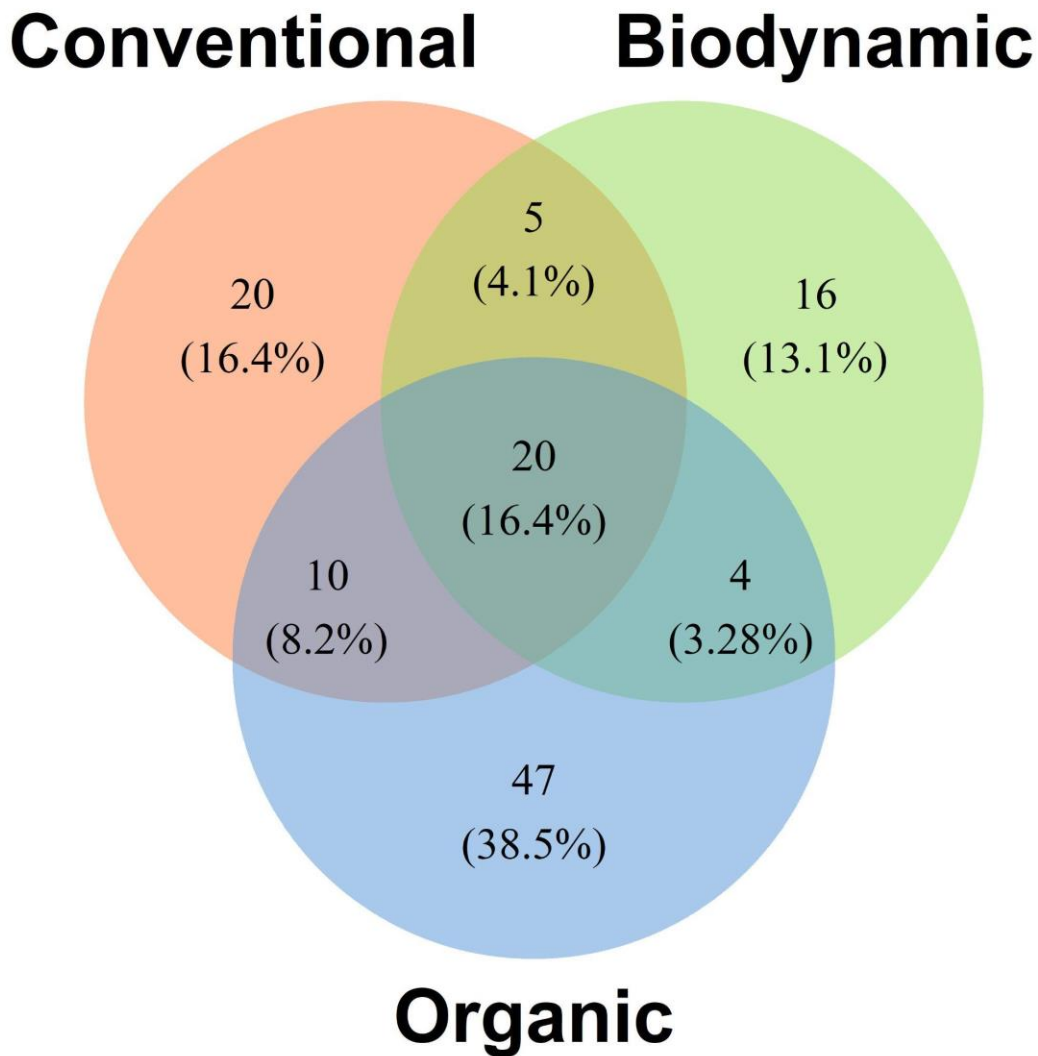


Figure 3 | Venn diagram showing ASV counts across the three vineyard groups - based on fungicide treatment protocols: Conventional, Biodynamic and Organic.

top 20 most abundant families, demonstrating a much larger microbial diversity than the other vineyards (Figure 2); this was reflected in the ASV counts from berry samples (T0) which were compared between the three vineyards (Figure 3). Organic samples contained the highest number of ASVs amongst the three vineyards, with 38.5% of observed ASVs being unique to that vineyard, indicating a large diversity of unique, low-abundant taxa. Interestingly, 16.4% of ASVs were shared between all vineyards, while the organic and conventional groups were most closely related, sharing 30 of the detected ASVs (24.6%).

The Phyllosphere Microbiome Through Fermentation

To determine the contribution of the phyllosphere microbiome to wine fermentation, we used the tool Decontam. This allowed us to track the phyllosphere microbiome throughout fermentation, and the top 20 phyllosphere families were identified, which were selected as they accounted for 99.9% of phyllosphere reads (Figure 4). The most abundant genera were also identified (Supplementary Figure 2). As a result of the phyllosphere filtering, differences between the groups during fermentation were less prominent. The dominating phyllosphere families throughout fermentation were Cladosporiaceae and Pleosporaceae, with no Saccharomycetaceae derived from the phyllosphere microbiome. Additionally, a relatively high abundance of Erysiphaceae taxa was present on organic berry samples, the family containing the taxa responsible for powdery mildew (*Erysiphe necator*). However, the abundance of Erysiphaceae during fermentation was far lower (Figure 4).

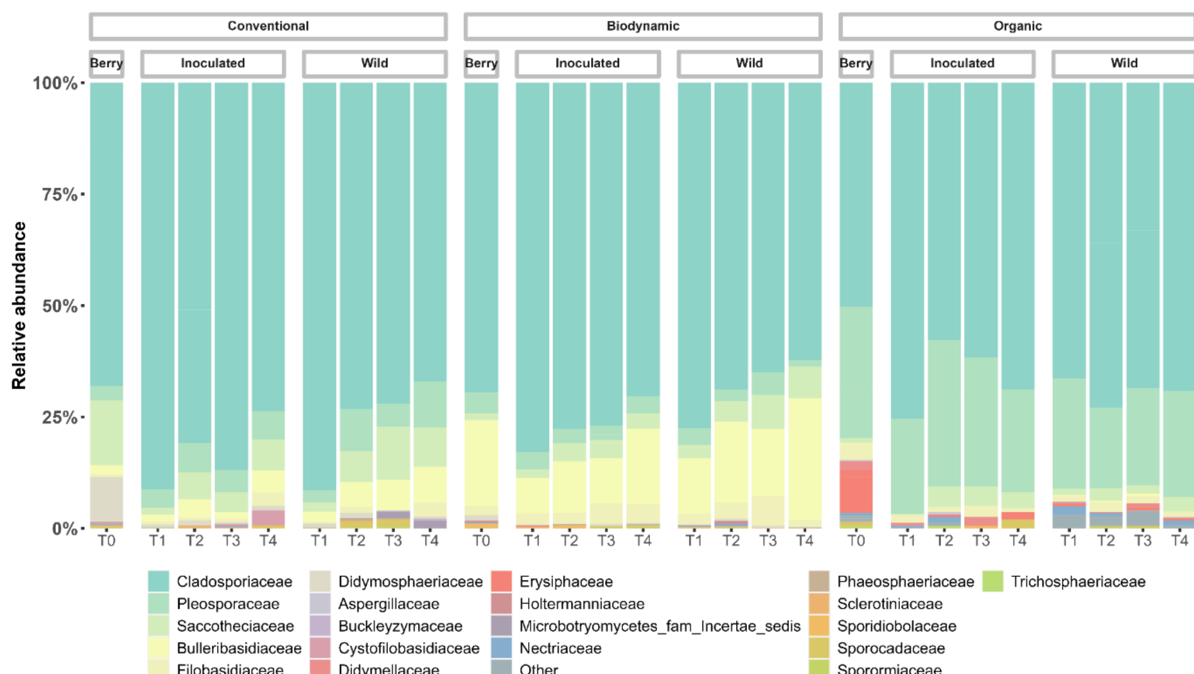


Figure 4 | Taxa bar plots showing the relative abundance of the top 20 phyllosphere Families across the three vineyards. All samples were filtered to only be representative of phyllosphere microorganisms.

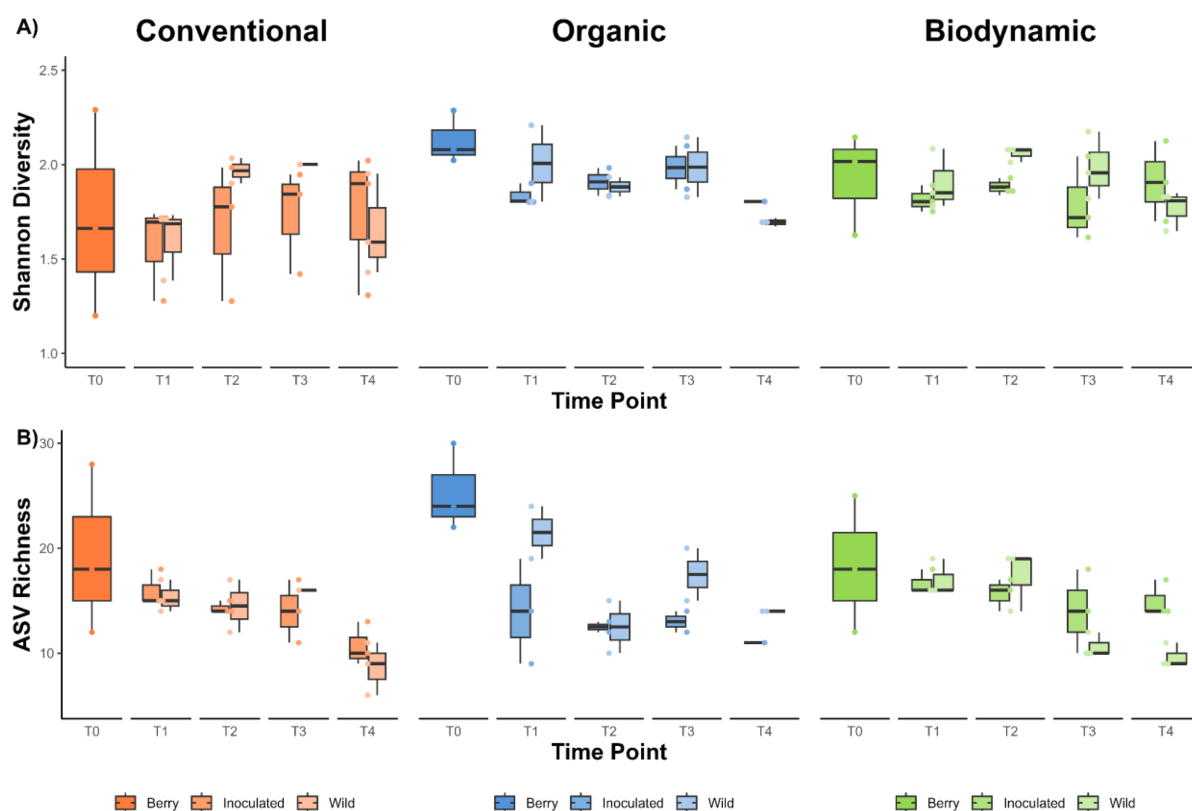


Figure 5 | Boxplots showing the phyllosphere Shannon diversity (A) and ASV richness (B) over time for the three vineyard groups. Berry samples act as T0 samples for both inoculated and wild ferments.

Using this phyllosphere filtering approach, the alpha diversity over time was calculated using phyloseq (Figure 5) and fitted against a linear mixed effects model to test for significant differences between T0 and T4 (Supplementary Tables 4 and 5). Shannon diversity appeared to remain stable throughout fermentation. However, the conventional group's data spread was more prominent (Figure 5a). For Shannon diversity, there was no significant change in diversity between T0 and T4 in all wines, except for the wild organic wines, which exhibited a significant reduction in diversity (Linear Mixed Effects Model, $p < 0.05$). ASV richness appeared to reduce over time for both the wild and inoculated samples, with the highest ASV count observed in the berry sample (T0) (Figure 5b). The fitted model showed a significant reduction

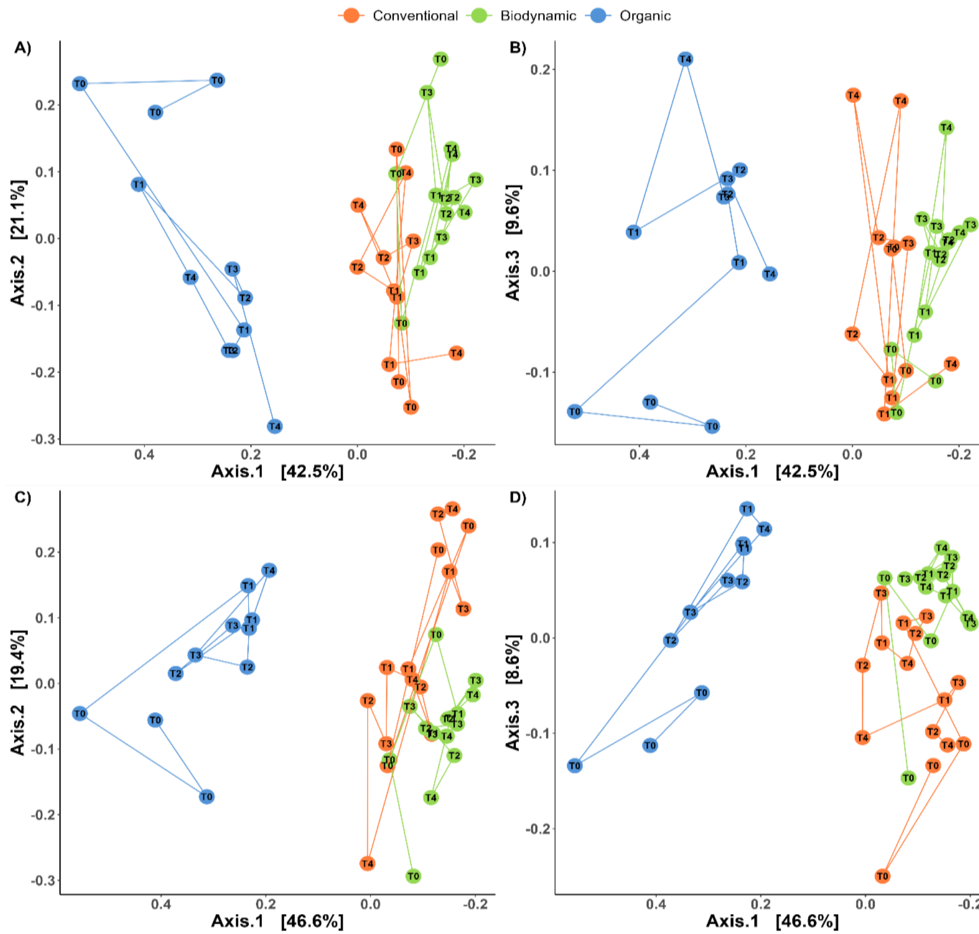


Figure 6 | PCoA plots for the Bray-Curtis Beta-diversity metric calculated for each of the three vineyards. Plots (A) and (B) show Beta-diversities for wild wine ferments. Plots (C) and (D) show Beta-diversities for inoculated wine ferments.

in richness between T0 and T4 in all wines (Linear Mixed Effects Model, $p < 0.05$), except for the inoculated biodynamic wines, which showed no significant reduction throughout fermentation.

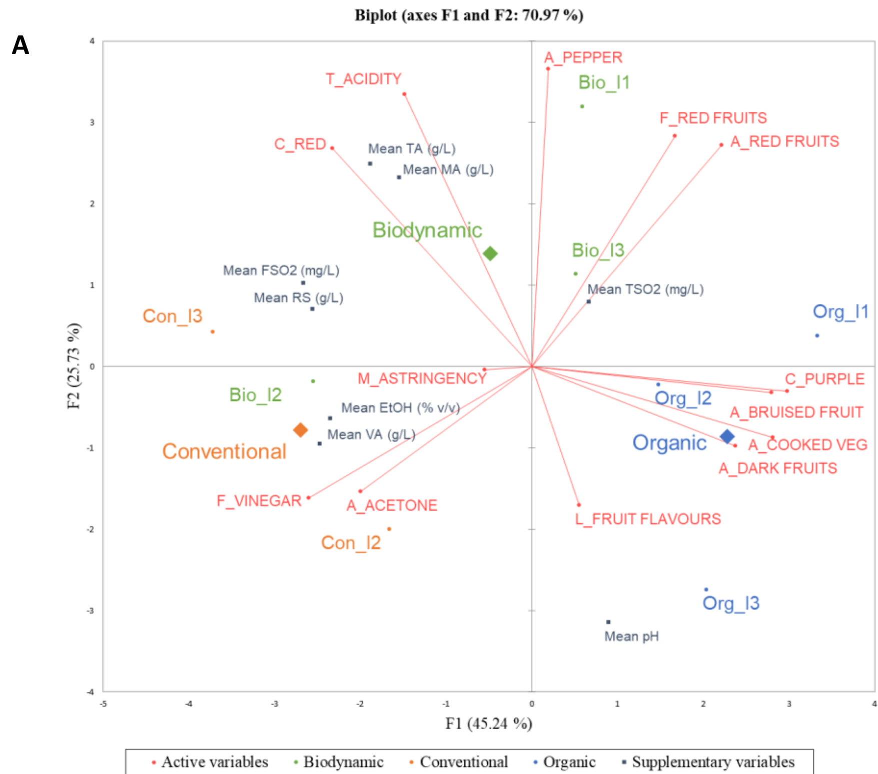
Beta diversity was calculated with the Bray-Curtis dissimilarity index using phyloseq in R (Figure 6). Vineyards appeared to group together, with the exception of the organic vineyard, which had shown higher levels of Pleosporaceae and Erysiphaceae, as well as overall higher

ASV richness and microbial diversity in earlier analyses. Interestingly, fermenting organic samples tended to cluster slightly more towards the other groups than their respective berry samples. Wild ferments exhibited significant clustering of microbial communities (Figures 6a and 6b, PERMANOVA of Bray-Curtis p-value < 0.001, $R2 = 0.445$), as did inoculated ferments (Figure 6c and 6d, PERMANOVA of Bray-Curtis p-value < 0.001, $R2 = 0.480$).

Wine Sensory Analysis

The sensory analysis identified significantly different sensory attributes between wines via two-way ANOVA. A Principal Component Analysis (PCA) was then conducted using the wine samples and the extracted significant sensory attributes. Basic chemistry measures (Supplementary Table 3) were then overlaid onto the resulting PCA plot as supplementary data (Figure 7). The PCA resulted in 70.97% of the data for inoculated wines and 66.88% for wild wines being explained in the first two dimensions.

The first principal component, F1, for the inoculated wines (45.24%) distinguished samples on the right-hand side of the plot as having bruised/dark fruit and cooked vegetable aromas to the left-hand side having more vinegar flavours and acetone-like aromas. For the wild wines, F1 (38.83%) distinguishes similar attributes (reversed direction) with the addition of acidic tastes and peppery aromas alongside the vinegar and acetone. The second principal component, F2, for the inoculated wines (25.73%) distinguished the top side as being perceived as red fruit aromas and flavours, with peppery aromas and acidic tastes. In contrast, the bottom half was perceived as having lasting fruit flavours alongside the acetone aromas and vinegar flavours. Wild F2 (28.04%) similarly distinguished the bottom half as being perceived with vinegar flavours and acetone aromas. The top half was perceived as having red/dark fruit aromas, red fruit flavours and lasting fruit flavours. F3 for the inoculated wines (15.05%) and wild wines



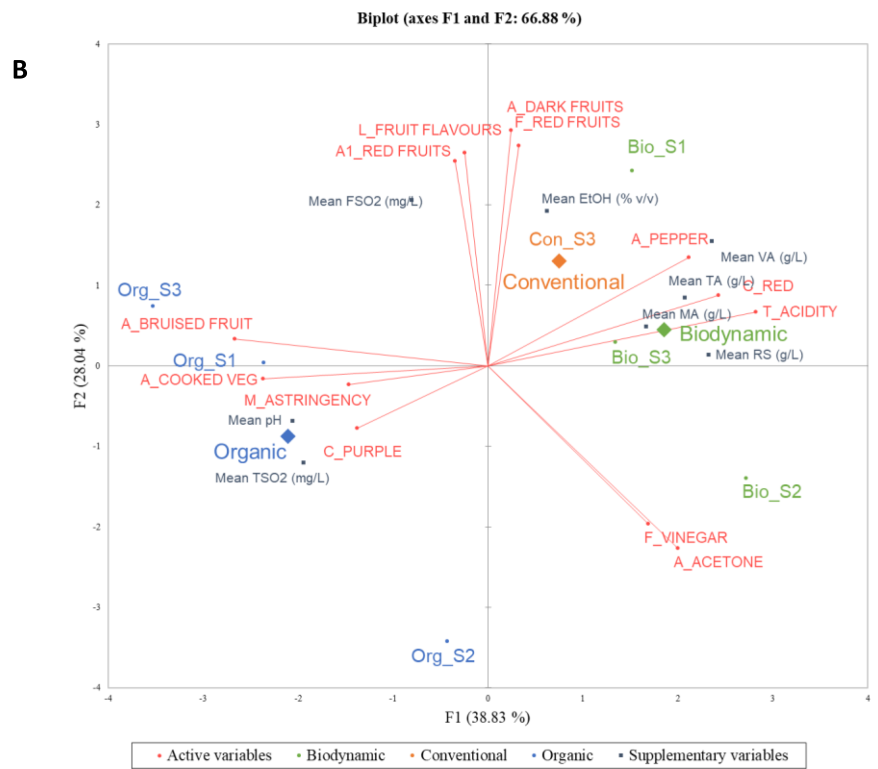


Figure 7 | PCA plots with overlayed basic chemistry analyses. Replicate values for sample groups are plotted, as well as the average position. (A) Inoculated wines and (B) Wild wines. C = Colour, A = Aroma, F = Flavour, T = Taste, L = Lasting Flavour, M = Mouthfeel.

(19.61%) appeared to explain only astringent mouthfeel and established that conventional wines tended to be more astringent than the other wines.

In both wild and inoculated wines, biodynamic and conventional wines grouped together and were opposite to the organic wines suggesting negative correlations between the organic and the other two vineyards. For organic wines, specific sensory attributes included purple colour, cooked vegetables, and bruised fruit aroma in both wild and inoculated samples. Wild organic wines also exhibited an astringent mouthfeel, while inoculated organic wines showed dark-fruit aromas and lasting fruit flavours. Biodynamic and conventional wines were associated with red colour, acidic taste, pepper aroma, and some showing vinegar flavours and acetone aromas, suggesting some oxidation and spoilage during the wine-making process.

Figure 7 further demonstrates the alignment of the basic chemistries with the sensory attributes. It can be seen within the plot that acidity measures such as pH, titratable acidity (TA) and malic acid (MA) align with acidic tastes within the wines. Similarly, Volatile acidity aligns on the plot with vinegar flavours and acetone aromas which may be due to spoilage associated with some of the wines.

Correlation Calculation and Multivariate Analysis

For the Procrustes test, the berry samples (T0) were isolated and underwent individual beta-diversity analysis using the Bray-Curtis dissimilarity index. Berry samples demonstrated significant, tight clustering (Supplementary Figure 3, PERMANOVA of Bray-Curtis p-value < 0.011, $R2 = 0.642$). As with the fermentation samples, the organic samples clustered separately from the biodynamic and conventional vineyards. This PCoA analysis was then used for the Procrustes correlation calculation with the sensory PCA analysis. The Procrustes

analysis produced a correlation coefficient of 0.619 with a p-value of 0.041, indicating a significant positive association between the diversity of the phyllosphere microbiome and the sensory outcomes of the resulting wines. Additionally, a Mantel correlation coefficient of 0.379 with a p-value of 0.044 was found, further supporting a significant positive association between these two factors.

DISCUSSION

We have undertaken a preliminary investigation of the effects of the three major viticultural management philosophy methods (Conventional, Organic, and Biodynamic) on the phyllosphere microbiome of grapevines and the downstream consequences this has on the sensory profile of the corresponding wine. Overall, these results suggest that the phyllosphere microbiome plays a critical role in the wine-making process and influences the sensory profile of the final wines. The presence of specific microbial families during fermentation, as well as the diversity and richness of the phyllosphere microbiome, are key factors influencing the characteristics of the wines produced. As such, differences in microbial composition driven by varying vineyard management methods may lead to differing sensory outcomes in the same Shiraz wines.

Interestingly, the organic vineyard exhibited distinct microbial composition compared to the conventional and biodynamic vineyards, possibly due to its relatively lower input of fungicides, primarily lower rates and concentration factors (CF) (Supplementary Table 1). These findings align with prior literature emphasising the importance of the grapevine microbiome in winemaking, with some studies showing the ability of the microbiome to be used as a fingerprinting tool for wines (Anagnostopoulos *et al.* 2019; Chen *et al.* 2023). Unexpectedly, the biodynamic and conventional vineyards clustered together, suggesting that the rate of

treatment may be more critical than the specific type of treatment, which could align with previous work conducted by Perazzoli *et al.* (2014) demonstrating the resilience of the microbiome to specific chemical and biological pesticides. Moreover, the surprising results regarding the organic group also challenge prior statements by Grangeteau *et al.* (2017), who observed generally lower microbial diversities in studies involving organic vineyards. This highlights the importance of recognising the effect of other factors on microbiome structure apart from vineyard management, such as geographic location or Shiraz clone. Despite the disease management method, Shannon diversity remained consistent during fermentation, even amongst the vineyards. At the same time, ASV richness declined in all cases, suggesting that many microorganisms present may not be well-suited for the fermentation process or that the results may be somewhat influenced by relic DNA and may benefit from future use of a viability selection process included in the methodologies (Emerson *et al.* 2017).

RELEVANCE

The implication of this research highlights the potential for optimising grape-growing practices by preserving and utilising the grapevine microbiome. This understanding of the phyllosphere microbiome, and factors that affect its structure, could lead to improved disease control and enhanced winemaking techniques through harnessing desired microbial communities, especially in wild winemaking scenarios. Moreover, our insights contribute to a better understanding of the off-target effects of fungicides and disease management methods, guiding vineyard decision-making for specific microbial outcomes and, consequently, sensory outcomes in wines. Further characterising the grapevine phyllosphere microbiome will provide valuable knowledge about its role in shaping wine characteristics.

Despite the valuable findings, there are limitations to consider, namely that of the geographical distance between the vineyards which may have led to significant differences due to climatic and edaphic variations. This research would benefit from broader vineyard participation with additional replicates to strengthen the statistical power. Nevertheless, this study serves as a framework and proof of concept for investigating the relationships between the phyllosphere microbiome and wine sensory attributes, with rigorous control of manageable factors.

In light of the results, we recommend continuous monitoring of vineyard micro-biodiversity to comprehend better the impacts of different disease management methods and their microbial outcomes. Further research should delve into specific fungicides' effects to comprehensively characterise microbial populations in vineyards. A broader-scale investigation with multiple participating vineyards would enable a more comprehensive understanding of the role of the grapevine microbiome in winemaking. Refining analyses to explore the effects of individual fungicides can provide more detailed insights into vineyard practices and microbial outcomes. By continuously expanding our knowledge of the grapevine phyllosphere microbiome, we can elevate the quality and sustainability of winemaking processes leading to higher quantities of greater quality wines.

CONCLUSION

This study further highlights the pivotal role of the phyllosphere microbiome in the fermentation process and its impact on the sensory characteristics of wines. The results demonstrate that different disease management methods—conventional, organic, and biodynamic—lead to distinct microbial compositions, influencing the final sensory profiles of Shiraz wines. While the organic vineyard showed a unique microbial composition, unexpectedly, the biodynamic and conventional vineyards exhibited similarities, suggesting

that treatment rate may play a more significant role than the specific fungicides used. This research highlights the potential for optimising grape-growing practices by harnessing desired microbial communities and understanding the off-target effects of fungicides and disease management methods to obtain desired sensory outcomes in resulting wines.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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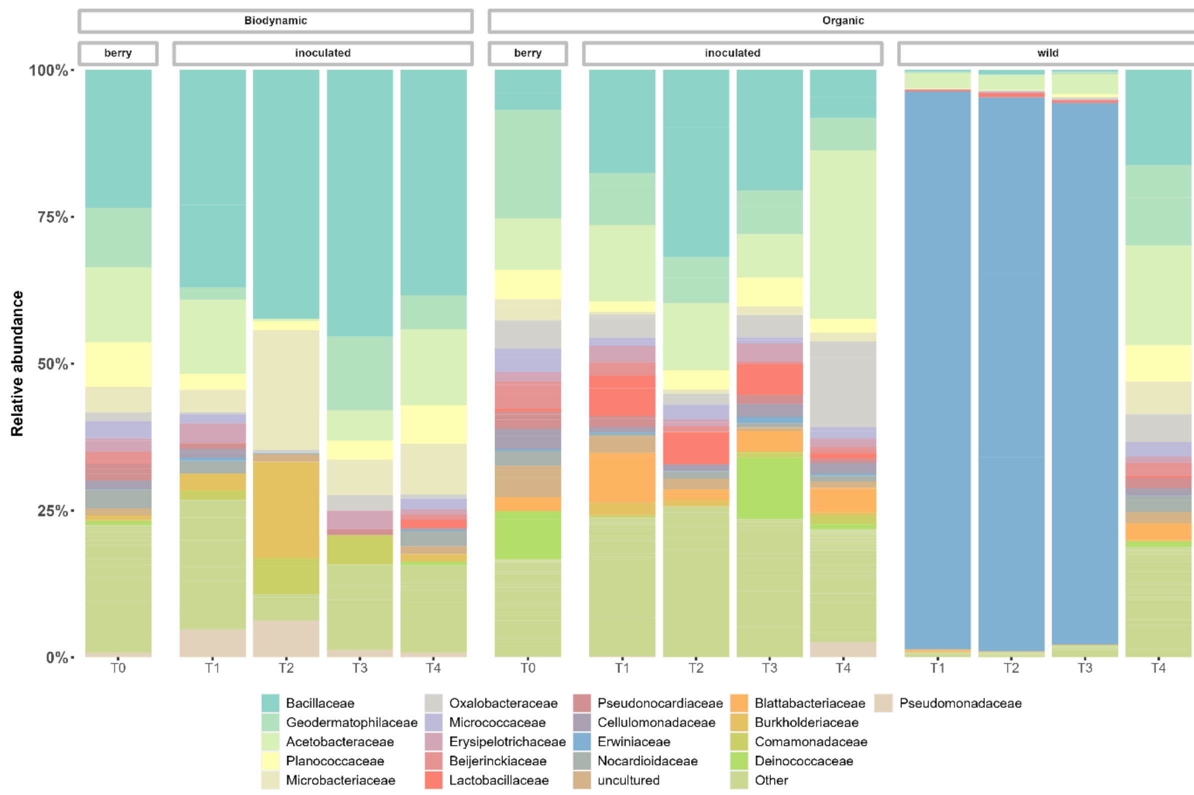
SUPPLEMENTARY DATA



Supplementary Figure 1 | Results for the few successful 16S rRNA gene target samples. No results can be concluded due to the amount of missing results.

Supplementary Figure 2 | The top most abundant fungal genera identified post-phyllosphere filtering.

Supplementary Table 1 | Fungicide applications for the three vineyard groups provided by each respective vineyard.

Conventional Vineyard					
Spray Date	Disease	Spray	Composition	Rate	CF
26/09/2021	Powdery Mildew	Unishield (UPL)	Sulphur	600 g/100L	1
	Downy Mildew	Kocide Blue Xtra (Dupont)	Copper	135 g/100L	1
9/10/2021	Powdery Mildew	Thiovit Jet (Syngenta)	Sulphur	600 g/100L	1
	Downy Mildew	Conan Sticks 720 SC (UPL)	Chlorothalonil	200 mL/100L	1
5/11/2021	Powdery Mildew	Topas 100 EC (Syngenta)	Penconazole	12.5 mL/100L	2
	Downy Mildew	Unizeb Disperss 750 DF (UPL)	Mancozeb	200 g/100L	2
16/11/2021	Powdery Mildew	Talendo (Dupont)	Proquinazid	25 mL/100L	2
	Downy Mildew	Conan Sticks 720 SC (UPL)	Chlorothalonil	200 mL/100L	2
7/12/2021	Powdery Mildew	Thiovit Jet (Syngenta)	Sulphur	240 g/100L	2
	Downy Mildew	Kocide Blue Xtra (Dupont)	Copper	175 g/100L	2
21/12/2021	Powdery Mildew	Thiovit Jet (Syngenta)	Sulphur	240 g/100L	2
	Downy Mildew	Kocide Blue Xtra (Dupont)	Copper	135 g/100L	2
15/01/2022	Powdery Mildew	Thiovit Jet (Syngenta)	Sulphur	400 g/100L	1
	Downy Mildew	Kocide Blue Xtra (Dupont)	Copper	135 g/100L	1

Biodynamic Vineyard					
Spray Date	Disease	Spray	Composition	Rate	CF
5/10/2021	Powdery Mildew	Microthiol Disperss	Sulphur	570 g/100L	2.66
	Downy Mildew	Airone WG	Copper	190 g/100L	2.66
20/10/2021	Powdery Mildew	Microthiol Disperss	Sulphur	560 g/100L	2.66
	Downy Mildew	Airone WG	Copper	190 g/100L	2.66
1/11/2021	Powdery Mildew	Microthiol Disperss	Sulphur	560 g/100L	2.66
	Downy Mildew	Airone WG	Copper	190 g/100L	2.66
16/11/2021	Powdery Mildew	Microthiol Disperss	Sulphur	380 g/100L	2
	Downy Mildew	Airone WG	Copper	130 g/100L	2
26/11/2021	Powdery Mildew	Microthiol Disperss	Sulphur	380 g/100L	2
	Downy Mildew	Champ 500WG	Copper	125 g/100L	2
9/12/2021	Powdery Mildew	Microthiol Disperss	Sulphur	380 g/100L	2
	Downy Mildew	Champ 500WG	Copper	125 g/100L	2
22/12/2021	Powdery Mildew	Microthiol Disperss	Sulphur	380 g/100L	2
18/01/2022	Powdery Mildew	Microthiol Disperss	Sulphur	600 g/100L	2
29/01/2022	Powdery Mildew	Microthiol Disperss	Sulphur	600 g/100L	2

Organic Vineyard					
Spray Date	Disease	Spray	Composition	Rate	CF
2/10/2021	Powdery Mildew	Microthiol	Sulphur	300 g/100L	1
	Downy Mildew	BlueShield	Copper	110 g/100L	1
15/10/2021	Powdery Mildew	Microthiol	Sulphur	300 g/100L	1
	Downy Mildew	BlueShield	Copper	110 g/100L	1
1/11/2021	Powdery Mildew	Microthiol	Sulphur	300 g/100L	1
	Downy Mildew	BlueShield	Copper	110 g/100L	1
19/11/2021	Powdery Mildew	Microthiol	Sulphur	300 g/100L	1
	Downy Mildew	BlueShield	Copper	110 g/100L	1
30/11/2021	Powdery Mildew	Microthiol	Sulphur	400 g/100L	1
22/12/2021	Powdery Mildew	Microthiol	Sulphur	400 g/100L	1
9/01/2022	Powdery Mildew	Microthiol	Sulphur	400 g/100L	1

CF = Concentration Factor

Supplementary Table 2 | Sensory attributes present within surveys completed during the Rate-All-That-Apply sensory analysis. Each attribute was rated in intensity from 1 to 7 if detected.

Colour	Aroma/Flavour		Taste	Mouthfeel	Aftertaste
Red	Dark Fruits	Green	Sweetness	Body	Length of Fruit Flavours
Purple	Red Fruits	Herbaceous	Acidity	Alcohol	Length of Non-Fruit Flavours
Brown	Dried Fruits	Barnyard	Bitterness	Astringency	
	Jammy	Pepper		Smoothness	
	Confectionary	Savoury			
	Chocolate	Spice			
	Vanilla	Grassy			
	Buttery	Smoky			
	Cooked Vegetable	Woody			
	Earthy	Liquorice			
	Minty	Vinegar			
	Eucalyptus	Acetone			
	Floral	Bruised Fruit			

Supplementary Table 3 | Mean measurements for each of the tested basic chemistries.

Vineyard	Fermentation	Mean EtOH (% v/v)	Mean TSO2 (mg/L)	Mean FSO2 (mg/L)	Mean VA (g/L)	Mean MA (g/L)	Mean RS (g/L)	Mean pH	Mean TA (g/L)
Organic	Inoculated	15.45	44.40	21.89	0.25	1.37	0.14	4.02	4.44
Organic	Inoculated	16.68	58.53	26.15	0.32	1.83	0.73	4.25	5.00
Organic	Inoculated	16.22	56.45	26.17	0.29	1.51	0.11	4.32	4.42
Organic	Wild	15.56	67.58	42.41	0.38	1.01	0.11	3.95	5.20
Organic	Wild	15.76	79.01	28.45	0.21	1.78	0.61	4.16	4.80
Organic	Wild	15.22	71.96	33.99	0.19	0.93	0.10	4.11	4.99
Biodynamic	Inoculated	16.31	63.62	31.97	0.37	2.41	0.43	3.88	7.81
Biodynamic	Inoculated	16.49	58.78	37.31	0.48	2.29	1.75	3.87	7.13
Biodynamic	Inoculated	16.59	58.42	35.11	0.39	2.06	0.95	3.89	6.14
Biodynamic	Wild	16.93	64.68	37.91	1.04	2.10	0.89	3.91	5.69
Biodynamic	Wild	14.57	50.28	32.75	1.10	1.17	0.76	3.71	7.08
Biodynamic	Wild	16.39	56.02	32.40	1.07	1.68	1.35	3.81	7.33
Conventional	Inoculated	17.00	53.02	32.00	0.76	1.92	0.53	4.16	6.16
Conventional	Inoculated	16.94	40.11	35.66	0.56	1.82	1.48	4.05	6.19
Conventional	Wild	16.70	49.96	34.51	1.38	1.62	0.53	3.99	7.50

Supplementary Table 4 | Linear mixed-effects model results for Shannon diversity measures.

Wild	***Organic***				
	Estimate	*Std Error*	*t*	*p*	
Intercept	2.129690906	0.09901519	21.5087	6.59E-07	***
T0 vs T1	-0.123141517	0.15655676	-0.7866	0.46147	
T0 vs T2	-0.247504334	0.15655676	-1.5809	0.16498	
T0 vs T3	-0.143076726	0.15655676	-0.9139	0.39601	
T0 vs T4	-0.434697864	0.15655676	-2.7766	0.03214	*
	Biodynamic				
	Estimate	*Std Error*	*t*	*p*	
Intercept	1.929434168	0.09763711	19.7613	2.41E-09	***
T0 vs T1	-0.02410827	0.13807973	-0.1746	0.86488	
T0 vs T2	0.127477397	0.13807973	0.92322	0.37763	
T0 vs T3	0.054174591	0.13807973	0.39234	0.70304	
T0 vs T4	-0.161054586	0.13807973	-1.1664	0.27052	
	Conventional				
	Estimate	*Std Error*	*t*	*p*	
Intercept	1.717157832	0.19780407	8.6811	5.39E-05	***
T0 vs T1	-0.115398338	0.27973719	-0.4125	0.6923	
T0 vs T2	0.250538875	0.31275569	0.80107	0.44942	
T0 vs T3	0.284466699	0.39560813	0.71906	0.4954	
T0 vs T4	-0.060089656	0.27973719	-0.2148	0.83604	

Inoc	***Organic***				
	Estimate	*Std Error*	*t*	*p*	
Intercept	2.129690906	0.0676721	31.4707	6.84E-08	***
T0 vs T1	-0.294541069	0.0957029	-3.0777	0.02173	*
T0 vs T2	-0.220891165	0.106999	-2.0644	0.08454	
T0 vs T3	-0.145218446	0.106999	-1.3572	0.22355	
T0 vs T4	-0.325237185	0.1353443	-2.403	0.05307	
	Biodynamic				
	Estimate	Std Error	t	p	
Intercept	1.929434168	0.1081067	17.8475	6.51E-09	***
T0 vs T1	-0.114163981	0.152886	-0.7467	0.47242	
T0 vs T2	-0.04638642	0.152886	-0.3034	0.7678	
T0 vs T3	-0.137190628	0.152886	-0.8973	0.39063	
T0 vs T4	-0.019077228	0.152886	-0.1248	0.90317	
	Conventional				
	Estimate	*Std Error*	*t*	*p*	
Intercept	1.717157832	0.2190372	7.83957	1.41E-05	***
T0 vs T1	-0.146645219	0.3097653	-0.4734	0.64609	
T0 vs T2	-0.0378864	0.3097653	-0.1223	0.90508	
T0 vs T3	0.019765543	0.3097653	0.06381	0.95038	
T0 vs T4	0.025720349	0.3097653	0.08303	0.93546	

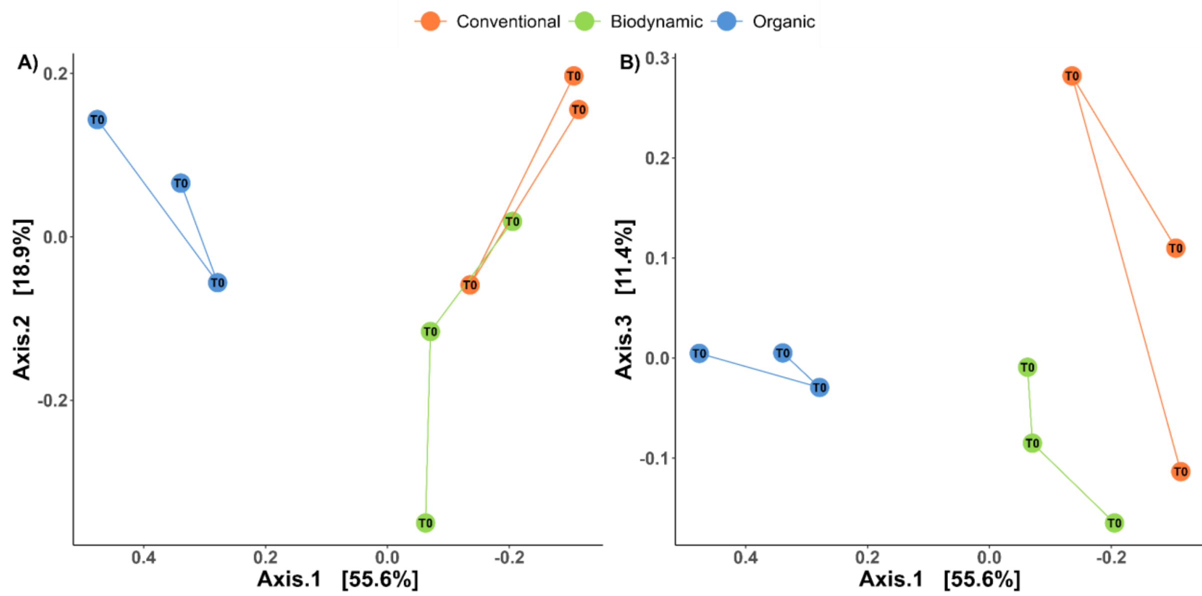
* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Supplementary Table 5 | Linear mixed-effects model results fo ASV richness measures.

Wild	***Organic***				
	Estimate	*Std Error*	*t*	*p*	
Intercept	25.33333333	2.00231348	12.652	1.49E-05	***
T0 vs T1	-3.833333333	3.16593559	-1.2108	0.27149	
T0 vs T2	-12.83333333	3.16593559	-4.0536	0.0067	**
T0 vs T3	-7.833333333	3.16593559	-2.4743	0.04818	*
T0 vs T4	-11.33333333	3.16593559	-3.5798	0.01165	*
	Biodynamic				
	Estimate	*Std Error*	*t*	*p*	
Intercept	18.33333333	1.93792558	9.46029	2.64E-06	***
T0 vs T1	-1.333333333	2.74064064	-0.4865	0.6371	
T0 vs T2	-1	2.74064064	-0.3649	0.7228	
T0 vs T3	-7.666666667	2.74064064	-2.7974	0.01888	*
T0 vs T4	-8.666666667	2.74064064	-3.1623	0.01012	*
	Conventional				
	Estimate	*Std Error*	*t*	*p*	
Intercept	19.33333333	2.76457178	6.99325	2.13E-04	***
T0 vs T1	-4	3.90969491	-1.0231	0.34031	
T0 vs T2	-4.833333333	4.3711718	-1.1057	0.30539	
T0 vs T3	-3.333333333	5.52914357	-0.6029	0.56561	
T0 vs T4	-10.66666667	3.90969491	-2.7283	0.02941	*

Inoc	***Organic***				
	Estimate	*Std Error*	*t*	*p*	
Intercept	25.33333333	2.2005891	11.5121	2.58E-05	***
T0 vs T1	-11.33333333	3.112103	-3.6417	0.01081	*
T0 vs T2	-12.83333333	3.4794369	-3.6883	0.01023	*
T0 vs T3	-12.33333333	3.4794369	-3.5446	0.01215	*
T0 vs T4	-14.33333333	4.4011783	-3.2567	0.01732	*
	Biodynamic				
	Estimate	Std Error	t	p	
Intercept	18.33333333	2.081666	8.80705	5.03E-06	***
T0 vs T1	-1.666666667	2.9439203	-0.5661	0.58378	
T0 vs T2	-2.666666667	2.9439203	-0.9058	0.38634	
T0 vs T3	-4.333333333	2.9439203	-1.472	0.17179	
T0 vs T4	-3.333333333	2.9439203	-1.1323	0.28394	
	Conventional				
	Estimate	*Std Error*	*t*	*p*	
Intercept	19.33333333	2.3380904	8.26886	8.80E-06	***
T0 vs T1	-3.333333333	3.3065591	-1.0081	0.33718	
T0 vs T2	-5	3.3065591	-1.5121	0.16144	
T0 vs T3	-5.333333333	3.3065591	-1.613	0.13783	
T0 vs T4	-8.666666667	3.3065591	-2.6211	0.02555	*

* = p < 0.05, ** = p < 0.01, *** = p < 0.001



Supplementary Figure 3 | PCoA plots for the Bray-Curtis Beta-diversity metric calculated for each of the berry samples (T0) collected. (A) Axis 1 and 2 (B) Axis 1 and 3.

Chapter VII

Summary and Conclusions

SUMMARY

With recent expansions into the understanding of the role of vine-borne microbes during fermentation, including the chemical and sensory profiles of wine, the microbial world has now revealed a new level of complexity to winemaking. Although this area of research is expanding, we still have a limited understanding of the role of the grapevine microbiome and its role in winemaking. A set of crucial factors play critical roles in how the microbiome of grapevines manifests and develops over time, such as climate, geographical location, and season. Disease control is another such factor that has an intended impact on the present microorganisms, namely pathogens. With the recognised importance of the microbiome in the health and development of grapevines, changes in these beneficial communities through the regular use of broad-spectrum fungicides could act as a selective pressure on the grapevine's microbiome, driving changes to the winemaking process, from vine to wine.

The research presented in this thesis encompassed a novel project investigating the impact of various disease management methods on the living communities of the grapevine phyllosphere microbiome. In particular, this research examined the effects of these interactions in the context of viticulture and oenology, highlighting the importance of the grapevine microbiome to the winemaking process. Herein, we discussed, in detail, the limitations of current microbiome analysis methods, novel sampling protocols to select for viable organisms, the impact of common fungicides on the grapevine phyllosphere, and the role of disease management methods on vineyard phyllosphere micro-biodiversity and concurrent wine sensory attributes.

This research demonstrated the importance of the microbiome in winemaking and the effect certain growing practices can have on shaping these communities. We highlight a growing

research area of interest within the Australian wine industry and the importance of culture-independent microbial analysis techniques in monitoring, preserving and manipulating vineyard micro-biodiversity.

DNA CONTAMINATION IN MICROBIOME STUDIES

DNA contamination from multiple sources is capable of critically confounding microbiome studies. In Chapter 2, we systematically reviewed the current literature and investigated the prevalence of contamination controls in phyllosphere microbiome research over the past decade. By utilising systematic review principles, we conducted a thorough investigation, screening 450 articles from three databases for eligibility and extracting data in a controlled and methodical manner. We observed a low usage of positive and negative contamination controls in phyllosphere research. In addition, we also observed that studies that collect negative quality controls did not sequence or analyse those samples despite collecting them.

Our systematic-style review demonstrated a concerning need for more consensus and adherence to contamination control standards in the current body of phyllosphere microbiome research. To ensure the integrity of phyllosphere microbiome research, further efforts are required to promote the adoption of standardised and comprehensive contamination control measures across the field. By adopting a minimum standard for contamination control, we could further advance the accurate understanding of the phyllosphere microbial communities, minimising the rates of errors within microbiome datasets.

THE VIABLE PHYLLOSPHERE MICROBIOME

Another source of contamination, which cannot be controlled for, is DNA from non-living sources such as compromised or 'dead' cells (non-viable), which are not associated with the host organism. Despite a large and increasing body of microbiome data, there remains a significant shortcoming in current microbiome analysis techniques in the inability to discern which organisms identified are viable or 'alive' at the time of sampling. In Chapter 3, we utilised the viability selection dye propidium monoazide (PMA) to suppress relic DNA in pre-extracted samples to combat this. PMA prevents relic DNA amplification during PCR while being incapable of crossing the intact membrane of live cells, leaving DNA from living sources accessible for amplification. We demonstrated the ability of PMA to distinguish the living phyllosphere microbiome using viability PCR (vPCR) through the removal of phyllosphere relic DNA. We also proposed using swabbing methods as an alternative phyllosphere microorganism harvesting technique as opposed to the current standard method, which involves lengthy washes of whole leaves. With the use of swabs, greater control over sampling location and standardisation across sampling areas can be achieved, allowing for more accurate comparisons within and between phyllosphere studies, particularly during vPCR.

By testing target DNA yields using qPCR and comparing them to various controls (cultures, DNA standards, mock communities, and negative controls), we demonstrated the effectiveness of our novel viable phyllosphere harvesting method. For the swabs, we observed a yield significantly higher than negative controls and comparable to current methods of phyllosphere harvesting. For the PMA testing, we confirmed our methodology using culture controls and successful reduction was observed during the vPCR reactions of our PMA-treated and untreated phyllosphere swab samples. By utilising this sampling protocol, more accurate characterisation of the phyllosphere microbiome is possible, even allowing for more accurate

estimations of a core microbiome across different hosts. The protocol may also be utilised in industry settings, allowing for effective and precise monitoring of vineyard micro-biodiversities.

One application for this viability selection method is for monitoring microbial communities on grapevines used for winemaking which host a diverse range of microorganisms that comprise their microbiome. Winemakers have used the microbes which inhabit the grapevine to produce wine for centuries, although modern wine producers often rely on inoculated microorganisms such as *Saccharomyces cerevisiae*. In the Australian wine industry, there is a movement towards returning to utilising the microbiome for wine fermentation. With the recent increase in the understanding of the role of the grapevine microbiome in grapevine health, fermentation and subsequent wine sensory traits, the microbial world offers a new level of complexity that can be harnessed for winemaking. Extensive microbial monitoring would be required to develop and maintain a desired vineyard micro-biodiversity. Using a viability selection dye (PMA, for instance) to distinguish between live microorganisms and relic signals generated from non-living sources would allow for improved monitoring capabilities and more successful outcomes. In addition to providing greater control during grape growing and winemaking, our novel sampling method can further advance the characterisation of the impact of different factors on the grapevines' microbiome, such as fungicides.

THE IMPACT OF FUNGICIDES ON THE PHYLLOSPHERE MICROBIOME

Many common fungicides used in Australian vineyards have broad-spectrum, antimicrobial effects, with some of the most common fungicides, such as copper and sulphur, targeting cellular systems shared by many different fungal species and certain bacteria. As these common

fungicides cannot specifically target pathogenic microorganisms, their antimicrobial effects may also impact the wild communities of the phyllosphere microbiome, which inhabits the aerial surface of the grapevine. Using 16S rRNA and ITS gene amplicon sequencing, paired with PMA viability selection, in Chapter 5, we were able to map the impacts of both copper- and sulphur-based fungicides over time at varying concentrations with the addition of a water control to monitor the natural variation of the phyllosphere microbiome.

Over the experimental sampling timeframe chosen, the results revealed that there appears to be no significant impact on the diversity viable phyllosphere microbiome through the application of the common vineyard fungicide compounds; copper (copper oxychloride) and sulphur over time. However, the data also suggested a possibility of some impacts on the phyllosphere microbial diversity further downstream; therefore, a more extended time series will be required to determine whether this change is significant when compared to untreated controls. Although the results show no effects from the fungicides, the observed reductions in diversity and richness immediately following spraying were surprising and warrant further investigation. Although this research provides valuable information on the immediate effects of copper- and sulphur-based fungicides, large-scale vineyards do not rely on isolated applications of singular fungicides.

Many types of fungicides are readily used to treat or prevent different fungal diseases and meet various standards for certain vineyard practices. As such, a large group of Australian vineyards are beginning to adapt their disease management methods to meet a growing market for organic and biodynamic wines. These methods both increase the scrutiny of which fungicides can be used within a certified vineyard, removing synthetic chemical fungicides and relying on

‘natural’ compounds to manage instances of disease. As each of these vineyards uses different methods for controlling infectious disease dependent upon the cultivation philosophy, it is probable that each practice has a role in shaping differences in grapevine microbiome.

The research presented in Chapter 6 described how different disease management regimes: conventional, organic, and biodynamic, impact the phyllosphere microbiome of grapes harvested for winemaking. Using 16S rRNA and ITS gene amplicon sequencing on grape berry phyllosphere, wine must and final wine samples, we were able to map the fungal communities derived from the phyllosphere microbiome. We also attempted to observe the bacterial communities; however, this was unsuccessful and will require follow-up analyses. Our research revealed that fungicide use changed the initial community structures and how these communities are shaped during fermentation. We also report how these changes impact each wine's basic chemistry and sensory outcomes using the Rate-All-That-Apply (RATA) sensory analysis method.

The results of this study further highlighted the pivotal role of the phyllosphere microbiome in the fermentation process and its impact on the sensory characteristics of wines. The results demonstrated that the different disease management methods lead to distinct microbial compositions, possibly influencing the final sensory profiles of Shiraz wines. This research shows the potential for optimising grape-growing practices by harnessing desired microbial communities and understanding any off-target effects of fungicides and disease management methods to obtain desired sensory outcomes in resulting wines.

SIGNIFICANCE AND FUTURE WORK

The research presented in this thesis holds significant importance for the wine industry in the context of grapevine health and winemaking. It sheds light on the crucial role of the phyllosphere microbiome in vineyards and its direct impact on the sensory attributes of wines. Understanding the complex interactions between vine-borne microbes, disease management practices, and the resulting wine sensory profiles is essential for producing high-quality wines consistently.

These findings demonstrate that fungicides and other viticultural practices can have far-reaching effects on the phyllosphere microbiome, influencing the microbial communities and subsequently altering the chemical and sensory characteristics of wines. This highlights the need for careful consideration and monitoring of vineyard management practices to maintain a healthy and balanced microbial ecosystem, which, in turn, contributes to the overall quality and unique characteristics of wines. Although this research advances our understanding of these interactions, it is still only a preliminary investigation with many limitations. Further improvements and advancement are still required to grasp these relationships' extent fully.

Outcomes and Improvements:

1. **Extended Time Series Analysis:** Conducting an extended time series analysis, building upon the work in Chapter 5, would provide a more comprehensive understanding of the long-term effects of fungicides on the phyllosphere microbiome. By observing changes over a more extended period, we can assess the persistence of microbial alterations and their impact on the vine's health and wine production.

2. **Testing More Fungicide Types:** Expanding the research to include testing various fungicide types, including synthetic and biological fungicides, would provide a more comprehensive understanding of their specific impacts on the grapevine microbiome and help identify potential alternatives for sustainable disease management.

3. **Investigating the Impact of Spraying as an Action:** As observed in Chapter 5, spraying fungicides, as an action, has an immediate impact on the phyllosphere microbiome. Conducting a more in-depth investigation into the effects of spraying as a mode of application would provide valuable insights into this phenomenon, potentially leading to improved application methods that minimise unintended consequences.

4. **Participation of More Vineyards:** The investigations in Chapter 6 would benefit greatly from the addition of more participating vineyards to replicate vineyard groups. The addition of replicate vineyards for each of the three management types would allow for some vineyard-specific variability to be accounted for. Ultimately, however, this research needs to be conducted within a single vineyard with the three separate management methods completed side-by-side with set vine parameters (age, clone, etc.).

5. **Adaptation of Viability Selection Method:** The viability selection method developed in Chapter 3 could be adapted to work with wine must samples during fermentation. By doing so, we can gain deeper insights into the active and living microbial species present during this critical winemaking stage, allowing for more targeted interventions to achieve desired sensory outcomes.

6. **Winemaking in Sterile Environments:** Due to the scale of which our wines had to be produced, they were made in non-sterile environments and were subjected to sources of contamination, mostly seen in the form of *S. cerevisiae*. By producing these wines in more

sterile environments, particularly the wild wines, contaminating species can be mitigates to reduce false positives within the dataset.

Future of the Field:

1**. Manipulating Microbial Communities:** With a better understanding of the phyllosphere microbiome's community structure, vineyards could employ strategies to manipulate microbial communities for desired sensory outcomes in wines. By optimising microbial composition, winemakers could craft wines with unique and consistent flavour profiles, enhancing their product range and appeal to various consumer segments demands.

2. **Phyllosphere Microbiome Spiking:** Isolating beneficial microorganisms could pave the way for grapevine microbiome spiking. This innovative approach may help vineyards develop healthy microbial ecosystems, improving vine health and resilience against diseases by introducing known beneficial phyllosphere organisms into the grapevine microbiota of their established vines.

3**. Bioprospecting Fermenting Organisms**: Continued research into identifying and isolating useful microorganisms, such as fermenting yeasts and bacteria, opens new possibilities via bioprospecting. Vineyards and yeast companies can harness these microorganisms to optimise fermentation and create distinct wine styles.

4. **Improved Characterisation of the Fermentation Environment**: Partnering metagenomic and transcriptomic research with advanced software analyses such as PICRUSt2 (Douglas *et al.* 2020) to predict the functionality of the phyllosphere microbiome during fermentation. Partnering these “-omic” technologies with robust chemical analyses such as mass spectroscopy could better characterise the fermentation environment. This comprehensive

approach can reveal the intricate actions of the grapevine microbiome during winemaking as well as other sources of microorganisms throughout the winemaking process.

5. **Observing Rhizosphere and Endosphere**: The research described in this thesis exclusively investigates the phyllosphere microbiome. Expanding this research beyond the phyllosphere to observe changes in the Rhizosphere and Endosphere simultaneously can provide a more holistic understanding of the grapevine holobiont. This will help uncover systematic and other off-target effects and comprehensively characterise the grapevine's microbiome.

CONCLUSION

The research described in this thesis contributes significantly to our understanding of the grapevine microbiome's importance in winemaking. The future implications and improvements outlined above are promising for the wine industry, with potential advancements in disease management practices, fermentation techniques, and overall wine quality. By continuing to explore and harness the potential of the grapevine microbiome, vineyards can elevate their winemaking practices and cater to evolving consumer preferences, all while promoting sustainable and environmentally friendly approaches to grape cultivation and wine production.

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