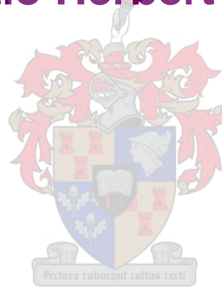
Investigating the effect of leaf removal on the grape-associated microbiome through culture-dependent and –independent approaches

by

Horatio Herbert Morgan



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Declaration

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Summary

Leaf removal is a common practice, which is often performed at either, *veraison* to increase the air circulation, light exposure, penetration of fungicides and decrease disease incidence; or at fruit-set to enhance wine colour, flavour and aroma. While there is greater understanding of how leaf removal influences the grape chemical composition and grapevine associated pathogenic fungi, most notably *Botrytis cinerea,* there is limited information on how the complete microbial community responds to these leaf removal procedures.

The current study focused on a phylogenetic survey of both the bacterial and fungal communities present in Sauvignon blanc grape must prepared from shaded (SH) and sun-exposed (EX) grapes. A combination of culture-based methods, ARISA community fingerprinting as well as direct amplicon sequencing was employed to analyse the community. The yeast community was analysed using culture-based methods for four consecutive vintages (2012 – 2015), while the bacteria were only isolated and identified for the 2015 samples. Furthermore, the two culture-independent approaches were used to profile and identify the bacterial and fungal species present in the 2014 & 2015.

A comparison of the cultivable yeast communities in the shaded and exposed grape derived must using ANOSIM revealed that there was more variation in the yeast community structures between vintages than between the treatments, thus suggesting the leaf removal only had a small effect on the yeast community (R = -0.04, p = 0.65). In contrast, ARISA data revealed a more diverse community which was different between the treatment (R = 0.5, p = 0.63) albeit with significant overlap. Our ARISA data suggested that leaf removal might have a significant influence on the filamentous fungal community and to a lesser extent on the yeast community. A more in-depth analysis of the microbiome was performed using Illumina target amplicon sequencing of the ITS-1 region, and the fungal population in the SH and EX musts exhibited significant overlap (R = -0.5, p =1) within treatments, while significant overlap between years was observed (R = 0.5, p =0.331) and the difference between the two populations was not significant. However, minor reductions in both *Botrytis* and *Penicillium* were observed in the sun-exposed derived grape must.

The cultivable bacterial community derived from the 2015 must samples demonstrated complete variation between the samples, with the absence of AAB in the exposed derived must. However, this was only based on a single vintage. Contrastingly, the B-ARISA data demonstrated that leaf removal had no influence of on the bacterial community, with the variation greatest within replicate samples (treatment) (R = -1, p = 1). Our ARISA data suggested that vintage might have a greater influence in driving bacterial communities. The in-depth analysis, was achieved by targeting the protein encoding region *rpoB* as it has been demonstrated to provide greater resolution and overcome biases associated with the 16S rRNA region. The results were similar

to that obtained in the fungal analysis (R = -0.5, p =1), with significant overlap within treatments with, some overlap between years (R = 0.25, p =0.64). However, potential plant pathogens *Stenotrophomonas* and *Xanthomonas* were reduced in the exposed treatment, together with *Achromobacter* and *Bordetella*. Interestingly concordant with the culture-dependent analysis, *Gluconobacter* was not identified in the 2015 exposed sample, but only in the shaded derived must.

Overall our data show similar trends with regards to the microbial community composition in the shaded and exposed must and whether these are largely influenced by defoliation or not. As a whole the data set suggest that the differences in the microbial community can largely be ascribed to the absence and presence of minor species and relative abundance of a few major species, that dominate the berry surface. On the surfaces leaf removal appears to have no impact on the microbial community, however on closer inspection it seems as if a few groups are impacted, ever so slightly. Therefore, future work should focus on selected microbial communities, which has been shown to be influenced by leaf removal or have the potential for disease. Moreover, the study did however only focus on a single variety, for two consecutive vintages, a greater sample set is required over several vintages and maybe two cultivars.

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Opsomming

Blaar verwydering is ŉ algemene praktyk, wat gereeld uitgevoer word tydens of deurslaan om die lug vloei, lig blootstelling, deurdringing van plaagdoder te verhoog, en die voorkoms van siektes te verlaag, of tydens vrugset om wynkleur, geur en aroma te verhoog. Alhoewel daar ŉ groter begrip is van hoe blaar verwydering die druif chemiese samestelling en die wingerd geassosieerde patogeniese swamme, veral *Botrytis cinerea*, beïnvloed, is daar beperkte inligting oor hoe die algehele mikrobiese gemeenskap reageer op hierdie blaar verwyderings praktyke.

Die huidige studie fokus op ŉ filogenetiese opname van beide die bakteriese en swam gemeenskappe teenwoordig in Sauvignon blanc druiwesap berei vanaf skadu (SH) en son-blootgestelde (EX) druiwe. ŉ Kombinasie van kultuur gebaseerde metodes, ARISA gemeenskap vingerafdruk sowel as direkte amplikon volgorde bepaling is toegepas om die gemeenskap te analiseer. Die gis gemeenskap is analiseer deur kultuur gebaseerde metodes op 4 agtereen volgende oesjare (2012-2015), terwyl die bakterieë slegs uit die 2015 oesjaar geïsoleer en identifiseer is. Verder is die twee kultuur-onafhanklike benaderings gebruik is om die bakteriese en swam spesies teenwoordig in 2014 en 2015 te identifiseer.

ŉ Vergelyking van die kultiveerbare gis gemeenskappe in die skade en die blootgestelde druiwesap met behulp van ANOSIM het aan die lig gebring dat daar meer variasie in die gis gemeenskapstrukture tussen oesjare is as tussen die behandeling, wat daarop dui dat blaar verwydering slegs ŉ klein invloed het op die gis gemeenskap (R = -0.04, p = 0.65). In teenstelling onthul ARISA data ŉ meer diverse gemeenskap wat verskil tussen behandelinge (R = 0.5, p = 0.63) al is dit met beduidende oorvleueling. Ons ARISA data dui daarop dat blaar verwydering ŉ beduidende invloed het op die filamentagtige swam gemeenskap en tot ‘n mindere mate op die gis gemeenskap. ŉ Meer in diepte analise van die mikrobioom is gedoen met Illumina teiken amplikon volgorde bepaling van die ITS-1 streek, en dit wys dat die swam bevolkings in die SH en EX druiwemos beduidend oorvleuel (R = -0.5, p = 1) tussen behandelings, terwyl beduidende oorvleueling tussen oesjare waargeneem is (R = 0.5, p = 0.331) en die verskille tussen die twee bevolkings nie beduidend was nie. Alhoewel ŉ geringe afname in beide die *Botrytis* en *Penicillium* waargeneem is in die son-blootgestelde druiwemos.

Die kultiveerbare bakteriese gemeenskap afkomstig van die 2015 mos monsters demonstreer algehele variasie tussen die monsters, met ŉ algehele afname in asynsuur bakterieë in die blootgestelde mos, maar die resultate is gebaseer op ŉ enkele oesjaar. Kontrasterend, wys die B-ARISA data dat blaar verwydering geen invloed gehad het op die bakteriese gemeenskap nie, met die grootste variasie tussen replikaat monsters (behandeling) (R = -1, p = 1). Ons ARISA data stel voor dat oesjaar ŉ groter invloed het op die bakteriese gemeenskappe. Die in-diepte analise is gedoen deur die proteïen koderende streek *rpoB* te teiken, aangesien dit ŉ groter

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resolusie vertoon en vooroordele wat verband hou met die 16S rRNA streek oorkom. Die resultate is soortgelyk aan die verkry vir die swam analise (R = -0.5, p = 1), met beduidende ooreenkomste tussen behandelings en 'n mate van oorvleueling tussen oesjare (R = 0.25, p = 0.64). Potensiële plant patogene *Stenotrophomonas* en *Xanthomonas* verminder in die blootgestelde behandeling, tesame met *Achromobacter* en *Bordetella*. Interessantheidshalwe, met die kultuur-afhanklike ontleding, is *Gluconobacter* nie geïdentifiseer in die 2015 blootgestelde monster nie, maar slegs in die skadu afgeleide mos.

Oor die algemeen wys ons data soortgelyke tendense met betrekking tot die mikrobiese gemeenskap samestelling in die skadu en son-blootgestelde mos, asook of hierdie gemeenskappe grootliks beïnvloed word deur blaar verwydering of nie. As 'n geheel toon die data stel dat die verskille in die mikrobiese gemeenskap grootliks toegeskryf kan word aan die afwesigheid en teenwoordigheid van klein spesies en die relatiewe oorvloed van 'n paar groot spesies, wat die bessie oppervlak oorheers. Op die oppervlak vertoon dit of blaar verwydering geen invloed het op die mikrobiese gemeenskap nie, maar by nadere ondersoek blyk dit dat 'n paar groepe effens beïnvloed word. Daarom moet toekomstige werk fokus op geselekteerde mikrobiese gemeenskappe, wat getoon het dat dit deur blaar verwydering beïnvloed word of wat die potensiaal het om siektes te veroorsaak. Verder het die studie net gefokus op 'n enkele kultivar vir twee opeenvolgende oesjare, 'n groter monster stel is nodig oor verskeie oesjare en meer kultivars.

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This thesis is dedicated to those who believed and supported me

Thank you very much, with you I feel as if the world is my oyster, containing a pearl yet to be discovered.

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Biographical sketch

Horatio Herbert Morgan was born on the 5 September 1990 in Secunda, Mpumalanga, South Africa. He attended Boston Primary and matriculated at The Settlers High School in 2008. In 2009 Horatio enrolled for a BSc-degree in Molecular Biology and Biotechnology at the University of Stellenbosch. He obtained he's degree in 2011, and thereafter went on a two-year sabbatical. He re-enrolled for his HonsBSc-degree in Wine Biotechnology in 2014 at the Institute for Wine Biotechnology, Stellenbosch University where he later enrolled for his MSc. He shares a passion for poetry and loves drawing. The rest is history, while he remains a Mystery.

'Do you believe I made it, I small time guy like me?
A support system of friends and family.
More than enough, Love is all anyone truly needs
Just like Martin, I started with a dream, but I ended up in science,
Fortunately ?,
And a supervisor like her, lucky me!!
We walked in the footsteps of he who left no footprints
And made a new way, the map has been re-written
Read this thesis, thereafter applaud
Because a small time guy like me, is talented after all,
I made new friends and family at campus, might have gone overboard at vensters
A window into the future, a glimpse of what I could be, that is what you, Stellenbosch, provided me, You to IWBT
A small time guy like me, completing he's Master's degree
But, is this enough, have I exploited the boundaries far enough
I have not reached my limit, Rocky balboa type spirit
I believed in myself, appreciated all your help
Me and science, more like swimming an Micheal Phelp
Go forth and Enjoy
I am no longer, a small time guy
A scientist, revolutionary, like the time phone became wireless"

"Thanks for Everything, the journey is one I am prepared for, from you, I couldn't have wished or asked of anymore. PEACE"

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of the journal *South African Journal of Enology and Viticulture*.

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Chapter 2 **Literature review**
The grapevine and wine microbiome: Insights from high-throughput amplicon sequencing

Chapter 3 **Research results**
Investigating the effect of leaf removal on the grape-associated microbiome through culture-dependent and –independent approaches

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Chapter 1

Introduction and project aims

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1. General introduction and project aims

1.1 INTRODUCTION

Leaf removal is a standard canopy management practice accomplished by removing leaves from basal portions of shoots in the fruiting zones of grapevines (*Vitis vinifera*), typically after bloom, to enhance air circulation, light exposure and the penetration of fungicides, or at fruit-set to enhance the colour, flavour and aroma of wine (Arnold & Bledsoe, 1990; Komm & Moyer, 2015; Sivilotti *et al.*, 2016; Verzera *et al.*, 2016).This decrease of leaves around the bunches, consequently alters the immediate microclimate, reducing the relative humidity within the fruiting zone (Arnold & Bledsoe, 1990; Kemp, 1994; Duncan *et al.*, 1995; Lohitnavy *et al.*, 2010). This creates an environment less conducive to disease development which consequently reduces the severity and incidence of rot caused by *Botrytis cinerea* and powdery mildew due to colonization by *Uncinula necator* (Smart *et al.*, 1990; Chellemi & Marois, 1992; Duncan *et al.*, 1995; Sabbatini & Howell, 2010; Jogaiah *et al.*, 2013; Sternad Lemut *et al.*, 2015; Mosetti *et al.*, 2016). Leaf removal also has a direct influence on other epiphytic fungi and has been shown to reduce the density of the *Penicillium* and *Aspergillus* (Duncan *et al.*, 1995). Furthermore, depending on the timing of leaf removal, other microbial populations including yeast and bacteria may be affected. Indeed, Sternad Lemut e*t al.* (2015) recently demonstrated that leaf removal pre-flowering alters the density of basidiomycetous yeast, *Aureobasidium pullulans* and acetic acid bacteria on Pinot noir grapes.

The microbial community of the grape berries are however, a diverse and complex community comprising filamentous fungi, yeasts and bacteria. These can be further divided into four groups viz. (i) residents (also referred to as oligotrophs), commonly present from berry set to harvest on undamaged grapes, (ii) adventitious microbes which are detected at any phase, as a direct result of contamination, (ii) invaders that have the capability of penetrating healthy skin tissue to obtain nutrients from the pulp, and (iv) opportunist (also known as copiotrophs) that typically colonize the berries after the skin has been damaged (Barata *et al.*, 2012; Loureiro *et al.*, 2012). Most studies that have evaluated the effect of leaf removal have mainly focused on invaders associated with bunch rot disease complexes and not on all microbial groups. These studies employed standard culture-dependent approaches, together with visual examination for determining the response of microbial populations to leaf removal. Such methods however, are laborious, time consuming and inconsistent and can only identify microbes based on their ability to grow on selective media (Andorrà *et al.*, 2008; Sun & Liu, 2014). Consequently, they only provide a limited overview

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of the diversity and dynamics of the grape-associated microbiota and how it is influenced by leaf-removal practices.

Over the last twenty years several culture-independent methods have successfully been employed to study the response of grape-associated microbiota to farming practices (Cordero-Bueso *et al.*, 2011, 2014; Martins *et al.*, 2012; Setati *et al.*, 2012) as well as the yeast diversity and dynamics during grape berry development and throughout wine fermentation (Renouf *et al.*, 2005; Pancher *et al.*, 2012; Martins *et al.*, 2014; Sun & Liu, 2014). Although these methods are generally faster and tend to enumerate higher diversity, they do have limitations. For instance, DGGE is unable to detect populations represented by less than 1% of major species in a mixed culture (Fasoli *et al.*, 2003; Andorrà *et al.*, 2008). While qPCR requires species-specific primer pairs for reliable quantification of the various yeast species investigated and T-RFLP and ARISA lead to either an over or underestimation of population sizes (Popa *et al.*, 2009; Arteau *et al.*, 2010; Sun & Liu, 2014) Recently, Next-generation sequencing methods have gained a lot of interest as tools to evaluate microbial diversity and dynamics in must and alcoholic fermentation (Bokulich *et al.*, 2014; David *et al.*, 2014; Pinto *et al.*, 2014; Setati *et al.*, 2015; Salvetti *et al.*, 2016). These high-throughput approaches are more sensitive and have been shown to detect microorganisms of relatively low abundance, and have therefore paved a path for characterizing complex microbial environments (Samarajeewa *et al.*, 2015). Consequently, their application in evaluating the influence of leaf removal practices on microbial community structures might yield novel and valuable information, especially with regard to the alteration of minor species.

1.2 PROJECT AIMS

The specific aim of this study was to carry out an in-depth assessment of the microbiome associated with Sauvignon blanc grapes under leaf removal treatments. Using a combination of three molecular techniques for the characterization of the complete fungal and bacterial community.

The specific aims of the study were as follows:

1. To determine the differences in the viable yeast and bacterial species, between the shaded and sun-exposed derived must. For yeast four consecutive years (2012-2015) were evaluated, while for bacteria only one (2015) year was evaluated;
2. To preliminary assess the entire microbial community (yeast, filamentous fungi and bacteria) from the genomic DNA extracted from the shaded sun-exposed derived must for two vintages, namely 2014 and 2015;

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3. Using high-throughput sequencing for an in-depth evaluation of the entire grape microbiome assessing the differences between the shaded and sun-exposed bunches with greater resolution and sensitivity.

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Chapter 2

Literature review

The grapevine and wine microbiome: Insights from high-throughput amplicon sequencing

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2. Literature Review

2.1 Introduction

The conversion of grape juice into wine was first confirmed to be the result of a microbial process by Louis Pasteur in the middle of the 19th century (Barnett, 2003; Jolly *et al.*, 2014; Bokulich *et al.*, 2016b). Since then, the diversity of the vineyard, grape and wine microbiota has been extensively investigated using traditional microbiological methods involving, microscopy, cultivation on different agar media and biochemical characteristics. However, the arrival of DNA-based molecular techniques such as polymerase chain reaction (PCR) and the identification of evolutionarily stable molecular marker genes such as ribosomal RNA (rRNA) genes improved our ability to identify microbial species with better resolution and reliability (Justé *et al.*, 2008; Solieri & Giudici, 2008; Cocolin *et al.*, 2013; Sun & Liu, 2014; Wang *et al.*, 2014; Abbasian *et al.*, 2015b). Consequently, for the past 3 decades, molecular techniques have been employed in conjunction with culture-dependent methodologies to identify microorganisms after isolation and growth in pure cultures (Alessandria *et al.*, 2013; Cocolin *et al.*, 2013). To date more than 40 yeast species (Jolly *et al.*, 2014), 50 bacterial species (Barata *et al.*, 2012) and approximately 70 genera of filamentous fungi (Rousseaux *et al.*, 2014) associated with grapevine and wine fermentation processes have been isolated and identified using traditional culture-based methods. These methods are, however, extremely laborious, time consuming and often inconsistent and biased (Andorrà *et al.*, 2008; Sun & Liu, 2014). In addition, only species that are able to grow on the culture media and under the cultivation conditions used can be identified, while viable but non-culturable (VBNC) cells as well as those species for which the prevailing cultivation conditions are not conducive, are often overlooked (Abbasian *et al.*, 2015b). These limitations in culture-based methods as well as the difference between culturable and *in situ* diversity increased the importance for research into culture-independent molecular approaches (Nocker *et al.*, 2007). Nevertheless, these methods remain important since the microbial species and strains retrieved in such culture-based approaches can be further exploited depending on their biochemical or genetic profiles. Indeed, the wine industry today has access to more than 100 commercial active dry yeast (ADY) strains of *Saccharomyces cerevisiae* that are used as starter cultures for controlled fermentations (Guzzon *et al.*, 2014). More recently, strains of non-*Saccharomyces* yeasts such as *Torulaspora delbrueckii, Metschnikowia pulcherrima*, *Lachancea thermotolerans* and *Pichia kluyveri* have been made available as pure starter cultures (Lu *et al.*, 2016; Pandilla *et al.*, 2016).

The introduction of the PCR further created opportunities for the development and improvement of several techniques in molecular ecology. The application of molecular

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techniques allowed researchers to study microbes not on the basis of their ability to grow on certain media types, but rather relied on the presence of nucleic acids for the detection and identification. Such methods, mostly used DNA extracted directly from the environment as a template for PCR, followed by separation and detection for microbial community profiling. Culture-independent methods are often faster, more specific, more sensitive and have a higher accuracy than culture-dependent methods (Justé *et al.*, 2008; Lv *et al.*, 2013). Additionally, they allow researchers to monitor populations that are numerically under-represented as well as those in the VBNC state (Andorrà *et al.*, 2010; Cocolin *et al.*, 2013). These methods include, single-strand conformational polymorphisms (SSCP), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphisms (T-RFLP) and automated ribosomal intergenic spacer analysis (ARISA) (Justé *et al.*, 2008; Balázs *et al.*, 2013; Cocolin *et al.*, 2013; Abbasian *et al.*, 2015b). PCR-DGGE was first applied in wine fermentation by Cocolin *et al.* (2001) to monitor the diversity and dynamics of yeast populations. Since then, it has remained the most widely used community profiling method in wine fermentation. The technique is often employed in combination with culture-dependent methods and has allowed researchers to decipher the complexity and evolution of the microbial population, during berry ripening and throughout the fermentation process (Prakitchaiwattana *et al.*, 2004; Renouf *et al.*, 2005, 2007; Di Maro *et al.*, 2007; Andorrà *et al.*, 2008). Although PCR-DGGE is typically thought to be appropriate for the analysis of less species-rich environments such as grape must, it has low sensitivity (Andorrà *et al.*, 2010) and is unable to detect populations that are present at a relative abundance of less than 1% of the population (Fasoli *et al.*, 2003; Andorrà *et al.*, 2008). More recently, SSCP (Grube *et al.*, 2011; Schmid *et al.*, 2011; Martins *et al.*, 2014), T-RFLP (Martins *et al.*, 2012; Sun & Liu, 2014) and ARISA (Brežná *et al.,* 2010; Chovanová *et al.*, 2011; Kraková *et al.*, 2012 Pancher *et al.*, 2012; Setati *et al.*, 2012; Ženišová *et al.*, 2014; Ghosh *et al.*, 2015) have been employed to profile the wine microbial diversity. These methods have allowed researchers to detect and monitor the evolution of microbial communities, and capture species that were previously not detected, or even misrepresented with culture-dependent methods (Peršoh, 2015).

Improvements in DNA sequencing, expanded the ability of researchers to study the microbial community structure and function with a higher resolution by using metagenomic approaches. Amplicon-based sequencing, often grouped under the umbrella of metagenomics, is a culture-independent approach for taxonomic, phylogenetic or functional profiling of microbial communities. This is accomplished by sequencing specific marker genes which are amplified directly from environmental DNA without prior enrichment or cultivation of the target population (Franzosa *et al.*, 2015). The innovations in high-throughput, short-amplicon sequencing are revolutionary in the way that they can describe the microbial diversity within and across

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complex biomes (Bokulich *et al*., 2013). Although high-throughput sequencing technologies have been widely used to investigate the microbial ecology of various environments (Abbasian *et al*., 2015a; Ma *et al*., 2015; Shi *et al*., 2015), their application in grapevine and wine fermentation microbial ecology is relatively recent, and their contribution to the field has not been explored. Therefore, with this review, we aim to provide an in-depth overview of the vineyard, grape and wine microbiome as unravelled through high-throughput sequencing techniques.

2.2 Next-generation sequencing

For many years, microbial community analyses relied on the isolation and identification of individual species, or cloning and sequencing of rRNA genes retrieved by PCR from environmental DNA. These methods mainly relied on first-generation DNA sequencing technology which was developed by Sanger *et al*. (1977). A few decades later, deep high-throughput, in-parallel sequencing technologies collectively referred to as Next-generation sequencing (NGS) were developed (Bleidorn, 2015). The term NGS therefore specifically refers to non-Sanger-based second and third generation sequencing techniques (Türktaş *et al*., 2015).

After Sanger introduced the chain-terminator DNA sequencing method, commercial second generation sequencing (SGS) platforms were developed. The Genome Sequencer 20 system launched in 2005 by 454 Life Sciences, was the first commercial SGS platform and was soon followed by the Genome Analyzer II launched by Solexa/Illumina in 2006. Both these platforms used a sequencing by synthesis approach. Roughly two years later, Lifetechnologies/Applied Biosystems introduced the SOLiD (Sequencing by Oligonucleotide Ligation and Detection) platform which applies fluorophore labelled oligonucleotide panels and ligation chemistry for sequencing. Subsequently, Complete Genomics developed the CGA sequencing technology which employed semiordered array of ‘DNA nanoballs” on a solid surface, while the Ion Torrent, which is regarded as the first of the “post-light sequencing” technologies, was introduced in 2010 (Reuter *et al*., 2015; Heather & Chain, 2016). The Ion Torrent’s semiconductor sequencer is thought to be a technology between second and third generation sequencing (TGS) categories, a technology capable of sequencing single molecules, negating the requirement for DNA amplification (Heather & Chain, 2016).

The majority of SGS technologies however, still have various limitations, such as errors arising from PCR (Peršoh, 2015), dephasing (Schadt *et al*., 2010) and the duration of completion “time to results” is still relatively long (Diaz-Sanchez *et al*., 2013). To overcome these drawbacks TGS or next-next generation platforms, such as Single-molecule real-time (SMRT) sequencing (Schadt *et al*., 2010; Bleidorn, 2015) and Nanopore DNA Sequencer (Diaz-

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Sanchez *et al.*, 2013), which open the possibility for single molecule sequencing were developed. These come with several advantages, (i) higher throughput, (ii) faster 'time-to-result', (iii) low cost, (iv) longer read length, (v) increased consensus accuracy enabling rare variant detection and (vi) small starting material (Schadt *et al.*, 2010; Diaz-Sanchez *et al.*, 2013; Bleidorn, 2015). However, these sequencing methodologies are still in development, and/or in the beta stage. Few commercial platforms have been evaluated, however they remain plagued by high error rates, and low output, although the technology is promising (Bleidorn, 2015). As such they cannot yet replace SGS, which remains and continue to be pivotal in microbial ecology surveys.

2.2.1 Next-generation sequencing in microbial ecology

SGS platforms have revolutionized the landscape of microbial ecology and have been the cornerstone of many phylogenetic surveys. The methods make it possible to compare and analyse the whole microbial community diversity, abundance and functional genes at far greater sequencing depths. These technologies depend on a parallel process in which each single DNA fragment is sequenced independently and separated in clonal amplicons for downstream analysis between the total sequences generated (Wooley *et al.*, 2010; Diaz-Sanchez *et al.*, 2013). With most SGS methodologies, an uninterrupted operation of a washing and scanning process is used to read tens of thousands of matching strands that are fixed to a specific location (Schadt *et al.*, 2010). The length of the fragments obtained from the analyses differs depending on the sequencing method employed (Wooley *et al.*, 2010; Bokulich *et al.*, 2016b). The Illumina and 454 pyrosequencing platforms are the most widely used for grapevine ecology surveys. Both platforms work on a sequencing-by-synthesis approach, however differ in their chemistries (Heather & Chain, 2016).

2.2.1.1 Illumina

The process of Illumina sequencing, consists of the bridge amplification of adapter-ligated DNA fragments on the surface of a glass (Pettersson *et al.*, 2009). Bases are then determined using a cyclic reversible termination technique, which sequences the template strand, a single nucleotide at a time through progressive rounds of base incorporation, washing, scanning and cleaning. In this method, labelled dNTPs are used to stop the polymerization reaction, allowing the removal of unincorporated bases. The fluorescent dye is captured to identify the bases added, and then cleaved so that the next nucleotide can be added, this is then repeated (Pettersson *et al.*, 2009; Diaz-Sanchez *et al.*, 2013; Reuter *et al.*, 2015; Heather & Chain, 2016) (**Figure 2.1**) (Goodwin *et al.*, 2016). The earlier Illumina analyser generated at least 1Gb of sequences with reads averaging 35 bp and the duration of 2-3 days. However, the introduction of HiSeq and MiSeq machines altered the duration time to approximately 4 days

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and 24-30 hours, and increased the read length to 250-300 bp, respectively with error rates of below 1%, with substitution the most occurring issue (Bleidorn, 2015; Goodwin *et al.*, 2016).

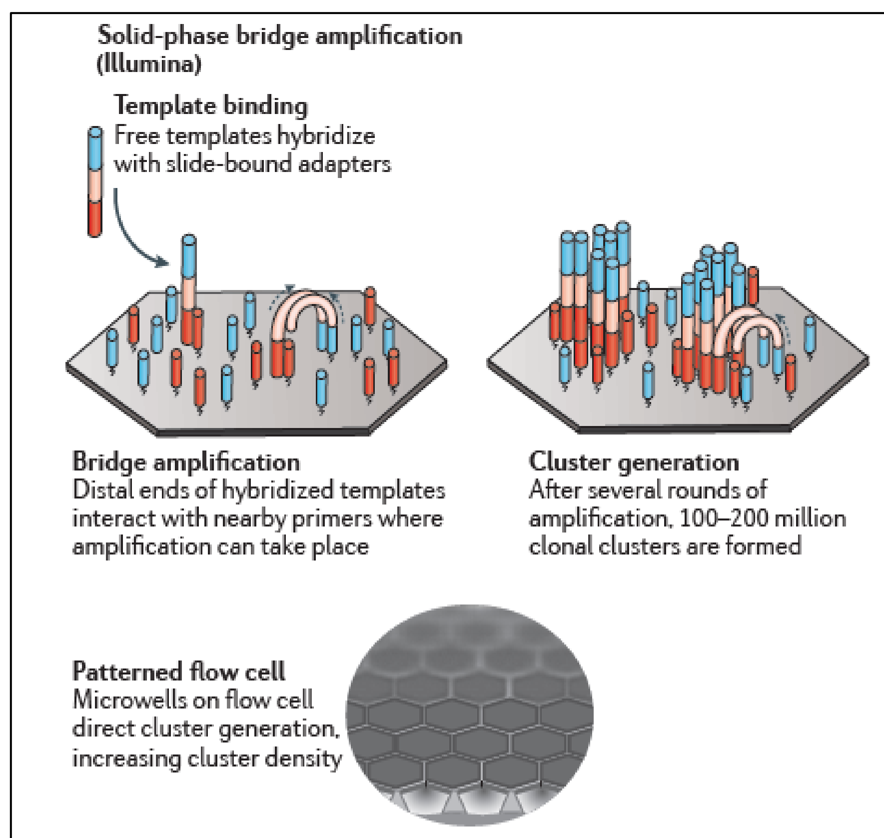

Figure 2.1 Illumina sequencing process, demonstrating bridge amplification and cluster generation (Goodwin *et al.*, 2016).

2.2.1.2 Pyrosequencing

In 454 pyrosequencing an emulsion PCR is used for bridge amplification of adapter-ligated DNA fragments on the surface of bead. The beads are thereafter distributed, where the sequencing by synthesizing occurs. After the nucleotide bases are incorporated an enzymatic luciferase coupled reaction occurs, allowing for the identification of bases, which is measured using a charged couple device (Pettersson *et al.*, 2009; Diaz-Sanchez *et al.*, 2013; Reuter *et al.*, 2015; Heather & Chain, 2016) (**Figure 2.2**) (Goodwin *et al.*, 2016). The Roche 454 FLX platform has the ability to generate 80-120 Mb of sequences averaging in 200- to 300 bp reads, for a run that averages approximately 4 hours with an error rate of below 1% (Morozova & Marra, 2008), while the FLX titanium is capable of producing read lengths of over 400 bp (Pettersson *et al.*, 2009).

The 454 pyrosequencing technique was reported in 2008, as the most published NGS platform. While, more recently Illumina has been the most successful, and considered to have

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made of the largest contribution of SGS (Huse *et al.*, 2007; Morozova & Marra, 2008; Reuter *et al.*, 2015; Heather & Chain, 2016).

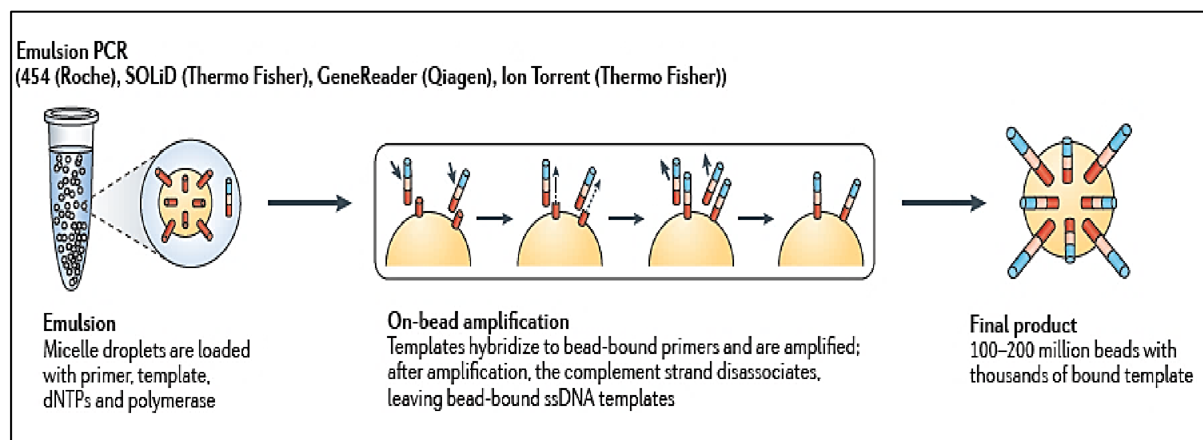

Figure 2.2 Demonstrates the SBS approach and Emulsion PCR for 454 Pyrosequencing (Goodwin *et al.*, 2016).

2.3 Application of Next-generation sequencing in deciphering the vineyard microbiome

The vineyard microbiome broadly describes the collective genomes of microorganisms present in the vineyard ecosystem, including those present in soil, grapevine, cover crops and insects associated with the plants. Furthermore, microbial transfer from nearby plants, could be transported aerially or via insects (Gilbert *et al.*, 2014). Consequently, the grape microbiome represents a reservoir of microorganisms comprising filamentous fungi, yeasts as well as bacteria. These populations are, however, variable and are influenced by various external factors, such as grape cultivar, climatic conditions, farming practices and the vineyard location (Setati *et al.*, 2012; Salvetti *et al.*, 2016). The past decade has seen a significant advancement in the manner in which researchers understand the microbial ecology of the vineyard, due to molecular profiling techniques that have further evolved, to explore microbial ecosystems (Bokulich *et al.*, 2012). Recent studies have employed SGS to decipher the grape and grapevine associated microbiome (David *et al.*, 2014; Pinto *et al.*, 2014), and to determine how viticultural practices could potentially influence these communities (Setati *et al.*, 2015; Kecskeméti *et al.*, 2016; Marzano *et al.*, 2016), their dynamics throughout grape berry development and wine fermentation (Piao *et al.*, 2015; Stefanini *et al.*, 2016) and to unravel their functional potential (Salvetti *et al.*, 2016).

For the comprehensive evaluation of the vineyard and the grape microbiome, two key questions are typically addressed. Firstly, which microorganisms are present within the environment, and secondly what each of the species contributes (Ravin *et al.*, 2015).

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Consequently, different sampling strategies are employed depending on what question the researcher seek to address.

2.3.1 Sampling strategies

The variability that is obtained from sample locations, with regards to species or taxon composition is essential when wanting to understand the spatiotemporal patterns of microbial diversity as well as the underlying mechanism controlling the composition and structure of a community (Zhou *et al.*, 2015). For the analysis of soil microbial communities, its essential to determine the type of soil sampled. Depending, on where you sample within the vineyard, the root zone and root soil are typically sampled with greater depth (deeper within the ground) directly at the stem. Three to five randomly collected samples within the replicate plot is generally obtained, the depth at which the samples are taken varies and are typically bulk soil samples of depths between 0-30 cm at varying distances from the sampled vine or stem. The replicate samples are thereafter commonly pooled and sieved through a 2 mm sieve removing any debris or plant material present (Lumini *et al.*, 2010; Burns *et al.*, 2015; Calleja-Cervantes *et al.*, 2015; Zarraonaindia *et al.*, 2015; Holland *et al.*, 2016).

Grapevine and wine associated microbial communities are investigated on diverse samples derived from root, branches, leaves and berries, with a non-standardised approach. For microbial evaluation of plant material such as roots and branches (Campisano *et al.*, 2014), grapevines of similar age and size are typically chosen, to eliminate one source of microbial variability. Only a certain area of the vine is sampled, while the material is typically peeled or crushed under aseptic conditions for further evaluation. Furthermore, other investigations have evaluated individual berries, an observation commonly seen for evaluation of the leaf microbial population, where single leaves of equal size (developmental stage) (Zarraonaindia *et al.*, 2015) are obtained from the same shoot or vine (Leveau & Tech, 2011; Pinto *et al.*, 2014). While, the evaluation of the grape microbiome throughout ripening is typically investigated at three stages according to a predetermined scale (Kecskeméti *et al.*, 2016) obtained either by washing the grape bunches in a buffer or by evaluating the grape must, typically directly after crushing, prior to pressing and the addition of a starter culture. For grape must and fermentation diversity, whole berries or freshly crushed and the fermenting must is typically analysed. For whole berries, healthy undamaged grapes are typically selected, even in the cases of grape withering (Salvetti *et al.*, 2016), except in the instances where the intention is to evaluate specific wine styles i.e. botrytized wines (Bokulich *et al.*, 2012) and/or sweet wines (Stefanini *et al.*, 2016). The sample sizes can vary from 150 to 5000 g (David *et al.*, 2014) of whole bunches collected from different vines and bunches through random sampling strategies. Additionally, these samples could be selected in a more control manner where a replicate is represented by a set number of bunches typically anything between 1 –

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24 (Marzano *et al.*, 2016) and collected in a set manner, where a given sampling area is marked, or grapes are harvested at equal distances from each other. These samples are typically collected at harvest. While, for the evaluation of fermentation dynamics samples are collected at various time points, commonly must, beginning, middle and/or end fermentation, usually based on sugar concentration (Pinto *et al.*, 2015). Other studies have investigated grape must samples collected directly from commercial wineries (Bokulich *et al.*, 2014), where the sample volumes used in these campaigns can range anywhere between 10 – 50 mL of a composite sample. Overall there doesn't seem to be a set standard in the sampling strategies used for the evaluation of microbial communities associated with the grapevine. Both the soil and grapevine samples are then stored at -80 °C until DNA extraction can be performed (Leveau & Tech, 2011; Campisano *et al.*, 2014; Perazzolli *et al.*, 2014; Pinto *et al.*, 2014, 2015; Zarraonaindia *et al.*, 2015; Setati *et al.*, 2015; Bokulich *et al.*, 2016a; Stefanini *et al.*, 2016; Kecskeméti *et al.*, 2016; Marzano *et al.*, 2016; Salvetti *et al.*, 2016).

2.3.2 Target genes

The target marker genes are universally present in all species evaluated and have the advantage of containing both highly conserved fragments that facilitate the design of PCR primers targeting all members of a community and variable regions that allow for the discrimination of different species within the community (Justé *et al.*, 2008; Cocolin *et al.*, 2013; Sun & Liu, 2014; Wang *et al.*, 2014). The bacterial small subunit ribosomal RNA gene (16S rRNA) as previously mentioned has been recognized as the gold standard for the estimation of prokaryotic diversity together with the fungal ITS1-5.8S-ITS2 region for eukaryotic diversity.

The 9 hypervariable regions (V1-V9) of bacteria have all been the target for the estimation of vineyard bacterial diversity. The V1-V3 region (Piao *et al.*, 2015), the V4 region (Bokulich *et al.*, 2015, 2016a; Burns *et al.*, 2015; Zarraonaindia *et al.*, 2015; Portillo *et al.*, 2016), V5-V6 (Marzano *et al.*, 2016), V4-V6 (Calleja-Cervantes *et al.*, 2015), V6 (Pinto *et al.*, 2015), and the V5-V9 (Leveau & Tech, 2011; Campisano *et al.*, 2014; Perazzolli *et al.*, 2014; Holland *et al.*, 2016). In a study comparing V4 and V5 region, Bokulich *et al.* (2012), found that they resulted in a similar bacterial composition with minor variation in the lower taxa although the V4 region however, provided greater taxonomic depth for certain *Proteobacteria* and LAB species. In contrast, Companaro *et al.* (2014), targeted the V3-V4 and V5-V6 regions of the 16S rRNA region and evaluated the bacterial community associated with grape marc after crushing and 30 days 'post fermentation'/storage. A total of 89 genera was identified, however only 31 of these were common in both target regions evaluated.

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The fungal ITS regions are the most commonly targeted region for fungal diversity estimation. The classification of general fungi and arbuscular mycorhizae has been accomplished by targeting the ITS region (Bokulich *et al.*, 2013, 2015, 2016a; Setati *et al.*, 2015; Bouffaud *et al.*, 2016; Holland *et al.*, 2016; Kecskeméti *et al.*, 2016; Marzano *et al.*, 2016; Stefanini *et al.*, 2016), the D1-D2 regions of the 26S rRNA (Holland *et al.*, 2014; Taylor *et al.*, 2014) and the partial 18S rRNA gene (Lumini *et al.*, 2010; David *et al.*, 2014; Holland *et al.*, 2016). A comparison of these different targets to decipher the AMF populations did provide evidence of similar genera and showed compositional differences in samples evaluated, highlighting them all as suitable target genes for AMF evaluation. Furthermore, Pinto *et al.*, (2014; 2016) targeted both the ITS2 region and D2 domain of the 26S rRNA region for fungal community analysis. The results revealed that the taxonomic depth for the 2 evaluated regions was considerably similar, however of these only a portion of the observed OTUs were shared between the two regions and that overall the ITS region provided a slightly higher coverage. Bokulich and Mills (2013) moreover, evaluated several ITS primers, and they found that targeting the ITS 1 region demonstrates higher levels of taxonomic classification accuracy (species and genus), the smallest difference between Ascomycota and Basidiomycota amplicon lengths, as well as a maximised sequence coverage. So overall the ITS 1 locus appears to be the most promising target, for a complete overview of the microbial populations in ecological studies.

2.4 Bioinformatics and Analysis

The large-scale computation, storage and analysis of growing image and sequence data sets, are informatics processes in support of DNA sequencing. The data lifecycles are managed by the introduction of bioinformatics analysis pipelines and development of novel algorithms. These can result in a reduction in error rate, shortened computational duration, efficient processes and a storage footprint which is minimized (Kriseman *et al.*, 2010). Illumina and 454 pyrosequencing generates large amounts of sequence data, and the only viable option to handle such information, is via automated approaches. There are currently several open source pipelines accessible for overseeing almost the complete analysis procedure for NGS data. These includes MOTHUR, QIIME (Kõljalg *et al.*, 2013), MG-RAST and RAMMCAP (Wooley *et al.*, 2010). These pipelines provide the tools for basic data analysis steps such as data cleaning, sequence clustering, functional annotation and taxonomic assignments (Kõljalg *et al.*, 2013).

The current section will provide brief overview in the procedures used to analyse high-throughput sequencing data in targeted amplicon sequencing for the vineyard and wine associated microbiome, followed by a brief overview of whole-metagenomics sequencing.

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2.4.1 Target/Amplicon sequencing

The analysis of amplicon sequencing data typically undergoes 3 basic steps; (i) Quality trimming and de-noising; (ii) OTU picking/clustering and (iii) taxonomic assignment. Quality-trimming is an essential step used to eradicate erroneous reads obtained through PCR, sequencing instruments and their chemistries (Bokulich *et al*., 2013). To minimize the volume of data for annotation, clustering is used. A single representative of highly similar sequences is chosen and annotated, which is then moved to the sequences of high similarity, to each cluster based on similarity (Wooley *et al*., 2010). While lastly, we assign the sequences with the representing microorganism.

The analysis data derived from pyrosequencing during quality trimming typically involves; the removal of barcodes and/or adapters, primers and denoising, which is used to correct problems associated, specifically with 454 pyrosequencer. These typically includes, the removal of sequences, with ≥ 6 homopolymers, ambiguous bases, those not meeting Phred score of (20 – 30). Furthermore, sequences of min and max length can be removed, dependent on target region and possible chimeric sequences. OTU picking commonly involves, firstly the alignment of the most abundant sequences/OTU against a reference, followed by the pair wise clustering of OTUs, with a 97% similarity threshold, commonly UCLUST. For taxonomic assignment of bacterial species, the Greengenes 16S rRNA and NCBI database is typically used whereas, for fungal assignment it is against the UNITE or SILVA database (**Figure 2.3**).

The data derived from Illumina sequencing platforms undergoes similar demultiplexing and quality trimming. Reads are typically truncated for ≥3 consecutive bases with a quality <1e-5, and removed when containing ambiguous base calls, primer/barcode errors and a phred score of <20 – 30. OTU picking or sequences clustering is at a 97% threshold using UCLUST. Additionally, in reference based picking, the process aims to remove incomplete and unannotated taxonomies, prior to taxonomic assignment typically using the Greengenes 16S rRNA or UNITE database, for bacteria and yeast respectively. Furthermore, for paired end sequencing, the reads are typically joined after quality trimming prior to OTU picking, with all sequences retained, even those not overlapping (**Figure 2.3**).

2.4.2 Shotgun metagenomics sequencing

While the goal in the analysis of the metagenomic data is to reconstruct all the genomes within the environmental sample, the computational intricacy involved makes it unfeasible. Thus, as an alternative 2 general types of analysis are performed for reconstruction; (i) assembling the reads into contigs, and performing taxonomic classification and functional assignments; (ii) read-based reconstruction of the taxonomic and functional parts of the metagenome (**Figure**

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2.3). During the assembly of sequences there are potential pitfalls e.g. (Peršoh, 2015), the memory required for algorithms to assemble reads. Moreover, issues with the population heterogeneity and variable abundances of the genomes within the sample (Scholz *et al.*, 2012; Ravin *et al.*, 2015).

Since a mixture of varying amounts of genomic fragments, from different organisms is the result of contig assembly, taxonomic classification can be complicated. Nevertheless, clustering based on the nucleotide composition and coverage carried out by different techniques could sort/bin metagenomic data based on taxonomic status. The clustering efficacy does, however, rely on various factors. Furthermore, the taxonomic status of the resulting 'bins' of contigs is obtained through the identification of phylogenetic marker genes in the bin which was analysed (Ravin *et al.*, 2015). Additional algorithms have been proposed as an alternative to the cluster based algorithms (Kriseman *et al.*, 2010).

The annotation of the metagenomic contigs can be done using various command-line pipelines and online annotations services, such as MG-RAST, IMG-M and CAMERA, which in addition to annotation, are able to conduct taxonomic and functional classification and pathway reconstruction (Wooley *et al.*, 2010; Desai *et al.*, 2012; Scholz *et al.*, 2012; Ravin *et al.*, 2015). The dependability of the taxonomic assignment and therefore the corresponding information may be decided from scores on sequence similarity and alignment coverage by quality standards or phylogenetic analyses (Peršoh, 2015).

Monitoring complex microbial communities is essential in food fermentations, in which a consortia of microbial communities are naturally involved in the processes, such as fermentation and spoilage (Bokulich *et al.*, 2016b). These technological advances, therefore represent an enormous breakthrough for microbial ecology, because metagenomics and next-generation sequencing allow for in-depth insights into not only the structure, but the function of the most complex microbial communities in their natural environments (Peršoh, 2015). The following section, will therefore focus on metagenomics and how it has been applied to study the vineyard microbial communities.

2.5 Vineyard microbial communities as derived from targeted NGS

2.5.1 Bacterial communities

Several studies have recently employed high-throughput sequencing to evaluate the bacterial community associated with the vineyard. Using NGS, research indicates that the most abundant phyla in vineyard soils include *Proteobacteria, Bacteriodetes*, *Acidobacteria* and *Firmicutes*. The genera mainly dominant amongst the *Proteobacteria* are *Bradyrhizobium*, *Steroidobacter* and *Acidobacteria*. The high-throughput analysis of the grapevine associated microbiome, demonstrated that the grapevine bacterial communities were predominated by

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Proteobacteria most commonly *Pseudomonas* spp., followed by *Firmicutes, Actinobacteria, Acidobacteria* and *Bacteroidetes*.

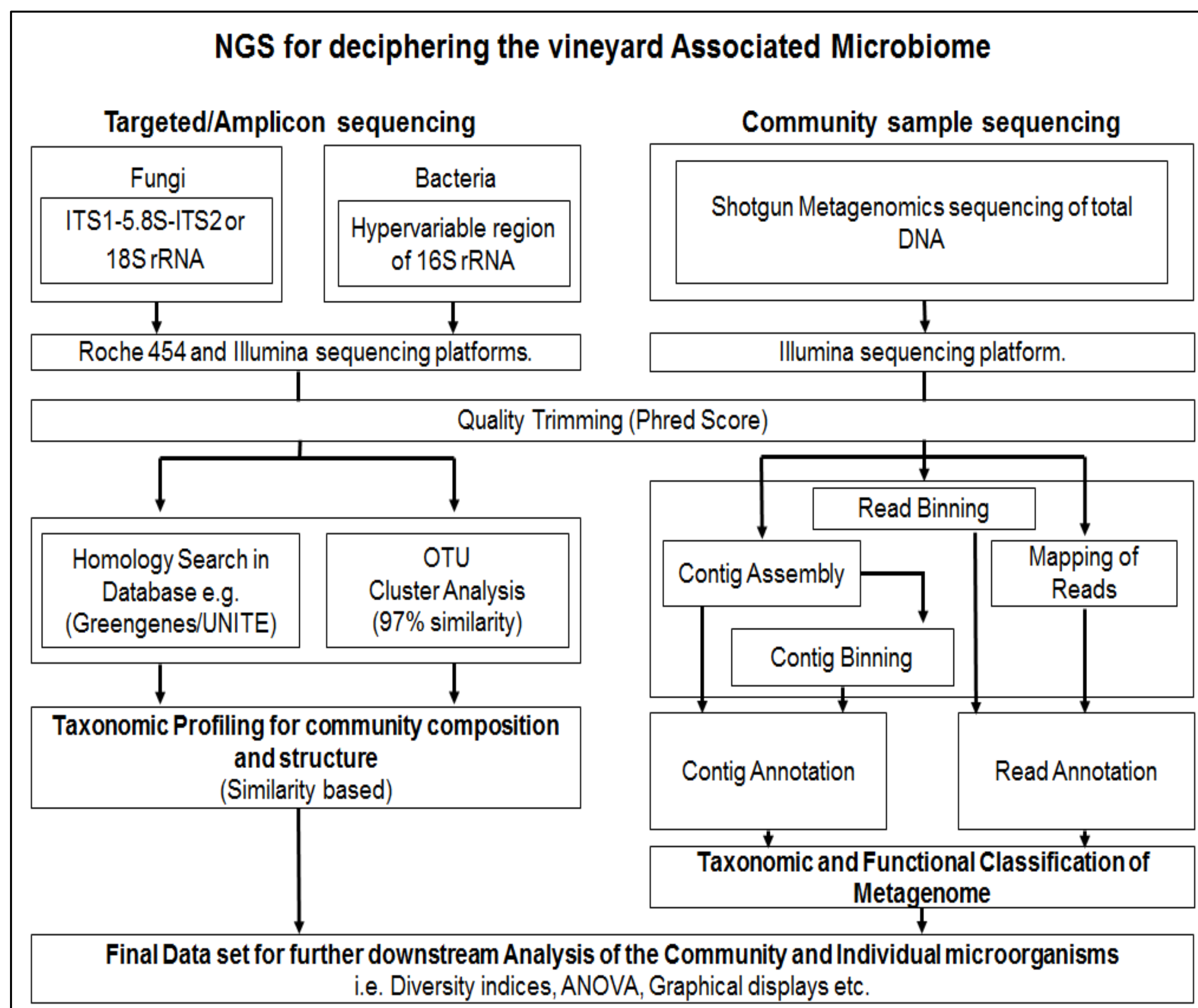

Figure 2.3 A schematic representation of the steps involved in metagenomics for either amplicon sequencing or whole genome sequencing adapted from Scholz *et al*., (2012) and Ravin *et al*., (2015).

Further comparison of the grapevine and must/wine associated bacterial communities revealed that in addition to *Pseudomonas,* the *Methylobacterium, Sphingomonas* and *Erwinia* spp. were common on the vine. The latter, however, associated with flowers, and not the leave or grape tissue, and was reported to decrease with berry ripening (Zarraonaindia *et al*., 2015; Kecskeméti *et al*., 2016). Evaluation of must derived microbial communities, reveals together with *Pseudomonas, Enterobacteriales* spp. are also dominant. Moreover, the phylum *Firmicutes* was found to be dominant in must consisting mainly of *Lactobacillales*. Furthermore, the *Firmicutes* also consisted of minor levels of *Pediococcus* and *Oenococcus*, however the latter, was not always detected, is abundant during later stages of fermentation.

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Overall NGS have made it possible to demonstrate bacterial species often overlooked in culture-based methods and community fingerprinting approaches such as DGGE. For instance, members of *Chloroflexi* and *Planctomycetes* were detected at levels of approximately 1% (Calleja-Cervantes *et al.*, 2015). Furthermore, extremely rare low abundant taxa including, *Pasteurellales, Staphylococcus, Gluconobacter* and *Streptococcus* which constituted less than 0.001% of the soil population were also observed (Zarraonaindia *et al.*, 2015), which is at abundances too low for the detection in fingerprinting and traditional methods. Furthermore, in the grapevine community several low abundant taxa were also identified such as *Chryseobacterium* and *Pediococcus* (Campanaro *et al.*, 2014). *Gluconobacter* moreover, was detected at higher levels in the must and increased with fermentation process (Bokulich *et al.*, 2015). Additionally, species and genera, such as *Gluconobacter oxydans* and *Acetobacter*, respectively which are often underestimated by culture-dependent techniques was detected and monitored with the employment of NGS (Piao *et al.*, 2015). Additionally, several novel genera believed to be associated with the wine habitat, including, *Candidatus_Liberibacter, Wolbachia, Komagateaibacter* and *Shewanella* were detected (Marzano *et al.*, 2016). Based on the data generated from NGS studies, we can conclude that there is a core bacterial community that exist within both the soil and/or grapevine. Furthermore, a population not yet reported and which seems to constitute a small proportion of the microbial community does exist, some of which might have always been there, just previously overlooked.

Bokulich *et al.* (2012) used a culture-fingerprinting based approach, specifically a targeted LAB T-RFLP to directly compare the LAB taxa obtained from these sequencing studies. The results when compared to the sequencing of V4 region proved to be more comparable in contrast to the V5 region. However, it was also found that the targeted approach and the V4 sequences provided a more similar LAB community profile and resolution for certain *Proteobacteria*. Furthermore, Campisano *et al.* (2014) investigated the impact of 2 pest management strategies on the bacterial compositions of 2 different cultivars, with the employment of community fingerprinting (ARISA) and 454 pyrosequencing, although ARISA was effective in describing differences, pyrosequencing was effective in describing more subtle differences. ARISA revealed that organic production had a higher OTU count than integrated pest management (IPM), whereas pyrosequencing depicted the opposite. Furthermore, in the assessment of grape marc, when compared to previous cultivation based methods, the presence of *Tatumella ptyseos* was shown to be at higher relative abundance than previously thought. The bacterial data generated between the two approaches, revealed that community fingerprinting approaches can be useful for preliminary assessment of microbial communities, or even effective when a specific group or species is being targeted.

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However, although such approaches show similar trends, NGS undoubtedly provides greater resolution of minor differences, as well as provides a greater diversity which includes genera previously not described.

2.5.2 Fungal communities

The fungal communities associated with the vineyard as derived from targeted metagenomics reveal that the vineyard (soil and grapevine associated) is typically dominated by the phylum *Ascomycota* followed by *Basidiomycota, Zygomycota* and then other fungi. The common species include *Botrytis cinerea*, *Cladosporium* spp., *Aureobasidium pullulans* and *Alternaria alternata* with minor amounts of *Saccharomyces, Hanseniaspora* and *Metschnikowia* detected on the grapevine associated microbiome. However, in the soil the "other fungi" are mainly represented by *Glomeromycota* or *Chytridiomycota* which was furthermore, dominated by *Penicillium* and *Cryptococcus* (Orgiazzi *et al.*, 2012). In contrast, the leaf associated microbiome is dominated by early diverging fungal lineages, while AMF specific fungi of the soil and grapevine are dominated by *Glomeromycota* (Lumini *et al.*, 2010; Bouffaud *et al.*, 2016).

Various studies have investigated the impact of agronomic systems on the grape microbiome, throughout ripening or in grape must. Kecskeméti *et al.* (2016) found no clear differences for the microbial communities for the 3 systems, with the exception of *A. alternata* for a conventional plot for one of the evaluated vintages. In another study, Setati *et al.* (2015), demonstrated that the fungal communities between the 3 systems were significantly different. With the biodynamic vineyard displaying greater diversity of fungal species than the conventional and integrated vineyard. NGS analysis of the vineyard and grape-associated microbiomes has revealed several new and rare species such as *Kabatiella microsticta* (Setati *et al.*, 2015), *Botryosphaeria dothidea* (Pinto *et al.*, 2014), and non-*Saccharomyces* spp. such as *Pichia membranifaciens*, which could be monitored throughout fermentation of sweet wine (Stefanini *et al.*, 2016).

Stefanini *et al.* (2015), compared NGS with qRT-PCR and demonstrated that NGS allows the monitoring of fermentation dynamics, and moreover, that the presence of dead cells does not interfere with the effectiveness of high throughput technologies. David *et al.* (2014) furthermore, compared the difference between DGGE and 454 pyrosequencing for monitoring the yeast biodiversity between 3 farming systems, and found that next generation sequencing of the heterogeneous samples revealed up to 7 more species than DGGE. Moreover, while monitoring changes in biodiversity in organic systems, at two thirds of alcoholic fermentation DGGE was unable to detect minor species such as *T. delbrueckii* and *M. pulcherrima*. Denaturing gradient gel electrophoresis was also unable to detect minor species at the middle

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of fermentation in ecophyto systems, highlighting the importance of NGS in deciphering the vineyard microbial consortium. For fungal community analysis, NGS demonstrated to be an effective tool to evaluate the soil and grapevine associated community with greater depth, and accuracy. They are also able to demonstrate that a core microbial community exist, within the soil and grapevine, as demonstrated by Setati *et al.* (2015). Several NGS studies demonstrated that there is a regional contribution for driving microbial communities, and microbial distinction across regions, supporting the concept of *terroir* for wine signatures (Bokulich *et al.*, 2014; Perazzolli *et al.*, 2014; Taylor *et al.*, 2014; Pinto *et al.*, 2015).

2.5.3 Whole-Metagenomic sequencing

Recently, Salvetti *et al.* (2016), employed whole genome sequencing for the first in-depth evaluation of the microbial consortium associated with Corvina berries post withering performed under two different conditions. A total of 25 bacterial phyla were detected, nine of which were common and consisted of *Acidobacteria, Actinobacteria, Cyanobacteria, Firmicutes* and *Proteobacteria*, the latter was predominant, followed by *Firmicutes, Actinobacteria* and *Bacteroidetes* as reported by Pinto *et al.* (2014) and Zarraonaindia *et al.* (2015), who both employed target metagenomics strategies. The class *Gammaproteobacteria* was dominant, were *Pseudomonadaceae* was in high abundances in the traditional approach whereas *Enterobacteriaceae* was dominant in accelerated withering. Furthermore, the genus *Carnobacterium* previously identified as grape associated by Pinto *et al.* (2015), were detected using the whole genome sequencing approach. Moreover, evaluating the eukaryotic community, they reported that *Ascomycota* was the dominant phylum, more specifically the class *Eurotiomycetes*, specifically genera belonging to *Aspergillus* and *Penicillium*, followed by *Sordariomycetes* and *Dothideomycetes*. However, common yeast such as *Aureobasidium*, *Cryptococcus, Hanseniaspora, Metschnikowia* and *Sporobolomyces* which are regularly detected in targeted strategies were not detected.

Beyond providing the inventory of the vineyard, whole metagenomic analysis provides the functional information for the evaluated microbiome. Such as providing information regarding defence, amino acid metabolism, transport, transcription and carbohydrate metabolism. Potentially allowing a greater comparison to be drawn then the assumed microbial diversity and composition, however some species might share functional properties, but these functional changes could potentially explain the observed results and allow comparison to be drawn with known species exhibiting similar characters, furthermore could also provide information of the survival mechanisms (Salvetti *et al.*, 2016; Campanaro *et al.*, 2014).

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2.6 Conclusion

The invaluable contribution of metagenomic approaches in deciphering the vineyard microbiome and its application provides great insights in the microbial community composition and structure of both bacteria and fungi, which have been highlighted by the review. The literature highlighted demonstrated the contributions of microbial populations which was once never discovered as well as that which are numerically underrepresented, in fermentations as well as the entire community. Furthermore, as research has already demonstrated the prokaryotic communities are larger and more diverse than the eukaryotic populations in the vineyard ecosystem, and so much has yet to be discovered. Interestingly, even though the microbial communities demonstrated are more complex, and diverse than previously thought and described, a large percentage of the sequence data (OTUs) remained unassigned. This large portion of anonymous/ unidentified microbes, can potentially explain current questions, and lead to future endeavours. However, this mentioned the sequence technologies still have room for improvements. The short read lengths generated by such platforms creates room for error, such as getting rid of species with short target areas during truncating and filtering, not fully re-constructing targets with larger targets, all leading to a misinterpretation of the "true" community. Moreover, a more standardised bioinformatics approach should be constructed, one that allows the most complete set of data, which in future can allow for greater accuracy when comparing research that has be completed by other researchers and institutions. Numerous studies provided taxonomic information only up to genus level and attributes this to either 'insufficient' reference databases, coverage or the novelty of the OTUs obtained. As such, a singular prokaryotic and eukaryotic database complete with sequences data across all parts, of the world of science will allow for more successful taxonomic assignment. However irrespective, of the shortcoming that platforms such as Illumina and 454 Pyrosequencing might have, it has made the largest contribution in ecological studies in the last decade.

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Chapter 3

Research results

Investigating the effect of leaf removal on the grape-associated microbiome through culture-dependent and –independent approaches

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3. Research results

3.1 Introduction

Grapevine management for the optimization of fruit yield and quality is an integral aspect in viticulture. Various proactive and remedial measures such as row and vine spacing, choice of rootstock, training and pruning practices, fertilization, shoot hedging, shoot thinning and selective leaf removal (broadly termed canopy management) are often applied in order to ensure good quality (Bledsoe *et al.*, 1988; Arnold & Bledsoe, 1990; Smart *et al.*, 1990; Hunter *et al.*, 2004; Austin & Wilcox, 2011; Terry & Kurtural, 2011; Pascual *et al.*, 2015). Canopy management strategies are particularly applied to improve cluster microclimate by altering the position or number of leaves, shoots and fruit in space (Smart *et al.,* 1990). Leaf removal or defoliation is the most popular canopy management practice used in commercial vineyards (Arnold & Bledsoe, 1990; Lohitnavy *et al.*, 2010). The method involves removal of leaves in the fruiting zones from the basal portions of shoots, typically after bloom. This decrease of leaves around the bunches, results in enhanced air circulation and light exposure (Kemp, 1994; Duncan *et al.*, 1995; Poni *et al.*, 2006; Hed *et al.*, 2014). This may influence the berry composition and yield, depending on when the leaves are removed and how much is removed (Poni *et al.*, 2006; Gregan *et al.*, 2012; Mosetti *et al.*, 2016). For instance, early season leaf removal can impact the production and degradation of methoxypyrazines, thus reducing herbaceous wine characters, in both red and white cultivars, while also increasing the concentration of skin anthocyanins, particularly in red varietals. Leaf removal furthermore, typically influences the sugar, phenolics, malic acid concentrations and titratable acidity (Hunter *et al.*, 2004; Tardaguila *et al.*, 2008; Mosetti *et al.*, 2016).

Leaf removal also improves fungicides penetration and increases temperature while reducing the relative humidity within the fruiting zone. Consequently, leaf removal in the fruiting zones has been shown to reduce epiphytic mycobiota especially species of the genera *Penicillium, Botrytis* and *Aspergillus* (Duncan *et al.*, 1995; Sternad Lemut *et al.*, 2015). Furthermore, early leaf removal was also demonstrated to be an effective means for reducing acetic acid bacteria (AAB) associated with grape berries (Sternad Lemut *et al.*, 2015). All these studies have employed traditional culture-dependent methods to evaluate the microbial communities associated with the sun-exposed and shaded clusters. These methods can however only identify yeasts and bacteria in wine and environmental samples based on their ability to grow on selective media (Kennedy & Clipson, 2003; Abbasian *et al.*, 2015) and therefore only provide a biased and limited overview of the microbial communities.

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The vineyard microbiome and the influence of farming practices on the microbial communities have been the subject of many studies that employed a combination of culture-based, community fingerprinting (Tofalo *et al.*, 2011; Setati *et al.*, 2012; Milanović *et al.*, 2013), and more recently, high-throughput sequencing. The studies include those evaluating the microbial communities in soils under tilling and no tilling (Lumini *et al.*, 2010; Orgiazzi *et al.*, 2012), irrigation frequencies (Holland *et al.*, 2014), pesticide treatment (Perazzolli *et al.*, 2014), withering practices (Salvetti *et al.*, 2016) and the differences between a vineyard and undisturbed ecosystem (Holland *et al.*, 2016). Comprehensive research has also looked at the influence of agronomic practices i.e. conventional; organic; ecophyto; biodynamic; and integrated production of wine on the microbial community (Pancher *et al.*, 2012; Campisano *et al.*, 2014; David *et al.*, 2014; Setati *et al.*, 2015). These investigations have highlighted that farming practices drives microbial community structures, within and across vineyard ecosystems. The recent upsurge in the application of high-throughput approaches in ecology studies have allowed researchers to study the microbial community structure and function with higher resolution than fingerprinting methodologies (Wang *et al.*, 2014; Franzosa *et al.*, 2015).

The current study set out to evaluate the microbial community structure associated with the Sauvignon blanc must derived from the shaded and sun-exposed berry clusters. Culture-independent techniques in conjunction with culture-dependent based methods were employed to characterize the microbial community and identify the fungal and bacterial species associated with the grape bunches under leaf removal treatments.

3.2 Methods and Materials

3.2.1 Experimental viticultural treatments

Vitis vinifera L. Sauvignon blanc grape samples were collected over 4 consecutive years 2012, 2013, 2014 and 2015 from the Morning star vineyard, situated in Elgin in the Overberg region, South Africa (GPS: E 19° 00.490' S 34° 10.159'). The grapes were collected from vines on which different leaf-removal strategies was applied. The experimental design is described in detail in (Young *et al.*, 2015). In brief for sun-exposed (EX) bunches, the leaves and lateral shoots were removed in the bunch zone of the north-east facing side of the canopy, while for the shaded bunches (SH) no treatment was applied. The treatment and sampling were performed in a checker-board layout (**Figure 3.1**).

3.2.2 Harvesting procedure and sample preparation

The grapes from both treatments for all vintages were handpicked and were kept separate for experimental reasons. For the exposed canopy treatment only the bunches which were directly exposed to sunlight were picked, whereas for the shaded treatment the entire canopy

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was harvested. The berries were harvested on the same day for both treatments. During sample preparation exposed and shaded grapes were treated identically. The grapes were crushed and the juice was homogenized. Fifty millilitre samples was then taken immediately after pressing prior to SO_2 addition and subsequently used for microbial analysis.

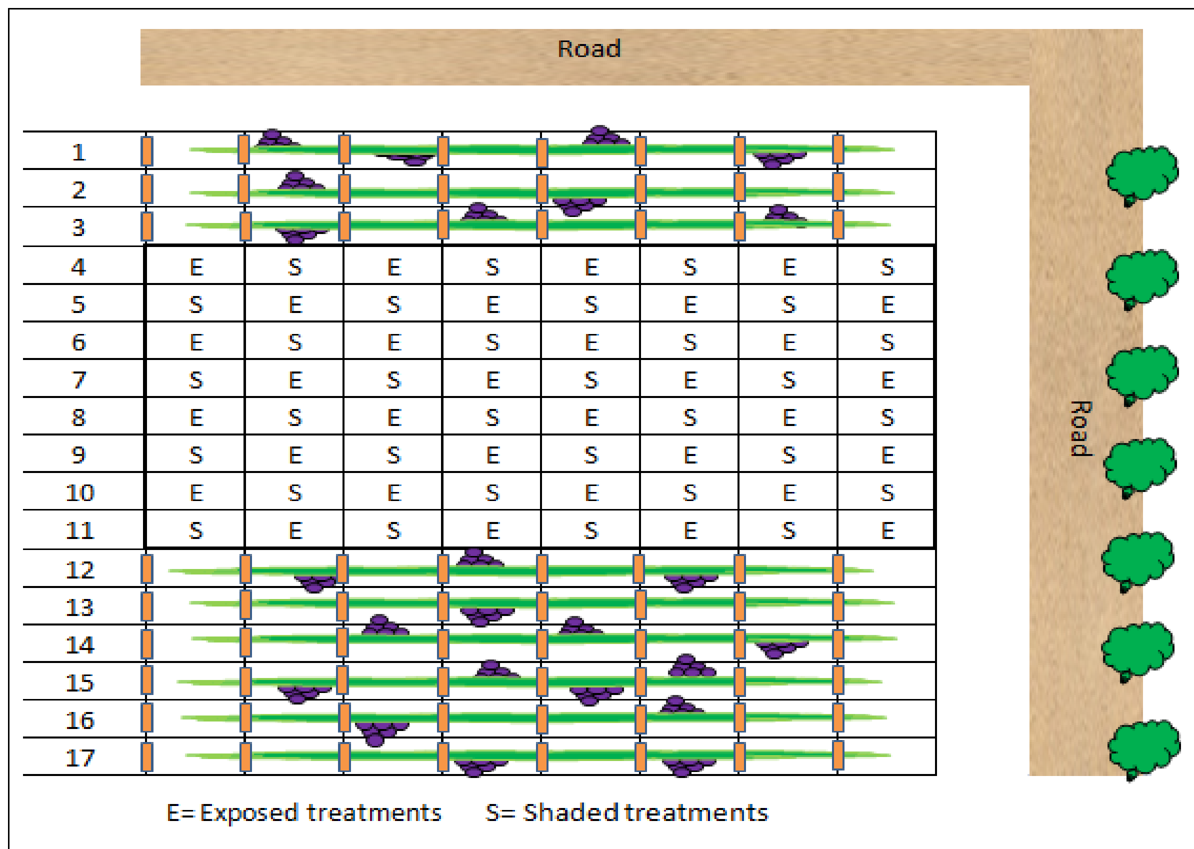

Figure 3.1 Shows the layout of the Sauvignon blanc vineyard block for 2012-2015 vintages consisting of the Exposed (EX) and Shaded (SH) canopy treatments. Rows 4-11 indicated by the dark black border represents the rows harvested.

3.2.3 Enumeration and isolation of yeast and bacterial species

Serial dilutions of the composite grape must sampled from both treatments for all years were made using sterile distilled water. One hundred micro litres of undiluted and diluted samples were spread plated on Wallerstein Laboratory Nutrient (WLN) agar (Sigma Aldrich, Steinheim, Germany) supplemented with 34 mg/L chloramphenicol (Sigma Aldrich) to suppress bacterial growth and 250 mg/L biphenyl (Riedel-de Haen AG, Seelze, Germany) to inhibit the growth of moulds. Bacteria were cultivated on de Man, Rogosa and Sharpe (MRS) media (Biolab, Merk, South Africa) supplemented with 100 mg/L Delvocid (DSM, The Netherlands) to inhibit fungal growth. All the plates were incubated at 25 °C and 30 °C for yeast and bacteria, respectively, until sufficient growth was observed and colonies were easily distinguishable. Colonies which could be distinguished morphologically from one another were selected and further streaked on their respective media to obtain pure cultures. Each of the unique pure yeast and bacteria

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isolates were grown in 5 mL YPD (Merck, Biolabs, Modderfontein, South Africa) and MRS broth, respectively, and the isolates was stored in 20% (v/v) glycerol at - 80°C.

3.2.4 Molecular identification of the yeast and bacteria isolates

Yeast genomic DNA (gDNA) extraction was performed according to the protocol of Hoffman and Winston (1987). The primer set ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990) was used to amplify the ITS1-5.8S rRNA-ITS2 region. Polymerase chain reaction (PCR) was performed with 25 µL reaction mixtures containing 2.5 µL Ex Taq buffer (10 ×, 1 Unit of Ex Taq™ polymerase (TaKaRa Bio inc., Olsu, Shiga, Japan). One microlitre of 25 mmol/L MgCl2, 4 µL of 2.5 mmol/L dNTP, 2.5 µL of 2.5 µmol/L of each primer, 1 µL of template DNA (100 ng/µL) and 11.3 µL dH2O. The PCR conditions consisted of an initial denaturation at 94°C for 3 min followed by 40 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 45 sec, and a final extension at 72°C for 10 min. The bacterial genomic DNA (gDNA) extraction was performed according to the bacterial mini-preparation procedure described by (Wilson, 1997). The primer set EubB (27F) (5′-AGAGTTTGATCMTGGCTCAG-3′) and EubA (1522R) (5′-AAGGAGGTGATCCANCCANCCRCA-3′) (Suzuki & Giovannoni, 1996) was used for the amplification of the 16S rRNA gene. The PCR was carried out in a 25 µL reaction volume as described above using similar PCR conditions except for the annealing temperature which was 55°C. The amplified PCR products for both yeast and bacteria were individually digested with restriction endonucleases *Cfo*I, *Hae*III and *Hinf*I in three separate reactions. The digested fragments were separated on a 1.5% (w/v) agarose gel stained with 0.01% (v/v) Gel Red Nucleic Acid Gel stain® (Biotium, Hayward, CA, USA). The gene ruler 100 bp plus DNA ladder (Thermo Fisher Scientific Inc., Waltham, MA USA) was used for size determination. The yeast and bacterial isolates were grouped according to similarity in banding profiles from the three enzymes. The ITS1-5.8S rRNA-ITS2 PCR amplicon of two representatives from each group was purified using the ZymocleanTM Gel DNA recovery kit (Zymo Research Corporation, Irvine, CA, USA), following the manufacture's protocol and subjected to Sanger sequencing at the Central Analytical Facility (CAF), Stellenbosch University. Both the forward and reverse strands were sequenced. The resulting sequences were aligned using Bio-edit (Informer Technologies, Inc) and the taxonomic assignment of individual isolates was performed by comparing the ITS-region and 16S rRNA sequences to the sequences available on GenBank (NCBI) (http://www.ncbi.nlm.nih.gov/pubmed) using the Basic local alignment search tool (BLAST) algorithm (Altschul *et al.*, 1997).

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3.2.5 DNA extraction from grape must

Thirty millilitres of grape must sample was collected directly after crushing and centrifuged at 5000 *g* for 10 min. The must pellet sediment was then washed three times with EDTA-PVP solution (0.15 M NaCl, 0.1 M EDTA, 2% (w/v) Polyvinyl pyrolidone) (Jara *et al.*, 2008), and thereafter three times with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The DNA was then extracted according to the protocol previously described by (Wilson, 1997), with a modification at the cell lysis step: together with the SDS and proteinase K, 200 µL of glass beads (0.65 mm diameter) and 20 µL lysozyme solution (10 mg/mL) were also added, followed by 3 min vortex and an incubation for 50 min at 37°C.

3.2.6 Automated Ribosomal Intergenic Spacer Analysis of fungal and bacterial species (F-ARISA and B-ARISA)

The fungal community composition was evaluated by amplification of the ITS-5.8S rRNA- ITS2 region of the genomic DNA using a caboxy-flourescein labelled ITS1 primer (5′-[FAM] TCCGTAGGTGAACCTGCGG-3′) together with ITS4. For the bacterial community, the ITS region situated between the 16S and 23S rRNA genes was amplified with the 6FAM-ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITS-Reub (5′-GCCAAGGCATCCACC-3′) primers (Cardinale *et al.*, 2004). The PCR amplification was performed in triplicate using the same conditions described in **3.2.4** above. The PCR amplicons were separated by capillary electrophoresis using an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Forster City, CA, USA) at the Central Analytical Facility, Stellenbosch University. Both fungal and bacterial PCR amplicons were resolved with ROX 1.1 internal size standard. The raw data were converted to electropherograms and further analysed using the ABI Gene Mapper 4.0 software (Applied Biosystems, Forster City, CA, USA). The appropriate peak sizes (between 350 bp and 900 bp for fungi and between 200 bp-1200 bp for bacteria), were selected for further downstream analyses. Using the freely accessible T-RFLP analysis Expedited (T-REX) software (http://trex.biohpc.org/) (Culman *et al.*, 2009) we further analysed the electropherogram data generated by gene mapper. Filter noise, was applied to distinguish true peaks from background noise and was measured as peak height (Abdo *et al.*, 2006). A peak height threshold of 50 fluorescence units (FU) was applied as a cut-off filter, therefore only peaks > 50 FU were considered for further analysis. Peaks were aligned using T-align algorithm (Smith *et al.* 2005) with a clustering threshold of 2 allocated to allow for errors arising from fragmental drift. Furthermore, an average of the triplicate data was calculated where any peak could only be considered if the cumulative height was greater than 150 units, more than 5 peaks were generated per analyses and the peak was detected in at least two of the three

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replicates. A data matrix representing peaks and peak heights for each sample were generated and used for diversity analyses.

3.2.7 Statistical analysis

The relative abundance of both fungal and bacterial communities was calculated as the number of occurrence of a certain species in the must, determined from colony counts and frequency of isolation. Shannon Wiener diversity index (*H′*), Menhinick's index for species richness and species Eveness (e^H/S) were calculated in Paleontological Statistics (PAST) version 3.0 (Hammer *et al.*, 2001). Analysis of similarities (ANOSIM) based on 999 permutations together with permutational multivariate analysis of variance (PERMANOVA) based on the Bray-Curtis distance matrices (Clarke, 1993) were used to statistically assess the differences in the microbial (fungal and bacterial) response between the treatments and vintage. To determine the species, or OTUs common among the treatments and year, Venn's diagrams were constructed on http://bioinformatics.psb.ugent.be/webtools/Venn/.

3.2.8 Illumina sequencing

3.2.8.1 Sequencing library construction

The fungal community was evaluated by amplifying the ITS-5.8S rRNA-ITS2 region with the primers BITS (5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACCTGCGGARGGAT CA- 3') and B58S3 (5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAGATCCRT TGYTRAAAGTT-3′) (Bokulich & Mills, 2013), with each primer containing an Illumina adapter. The PCR was performed in triplicate and carried out in the same reaction as described in **3.2.4**. The PCR conditions consisted of an initial denaturation of 95°C for 2 min followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1min with a final extension of 72°C for 5 min. The bacterial amplifications were performed by targeting the *rpoB* gene, to overcome biases associated with the 16S rRNA regions, specifically the amplification of *Vitis Vinifera* chloroplast gene sequences (Bulgari *et al.*, 2009; Leveau & Tech, 2011), using the primers rpoBF III (5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACATCGGTTTG ATCAAC- 3′) and rpoBR III (5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGTT GCATGTTGGTACCCAT-3′), with each primer containing an Illumina tag. The PCR amplification was performed in triplicate using the same reaction and PCR conditions described for the yeast amplification. The amplicons of both the yeast and bacteria were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA), following the manufactures protocol after the purification all the yeast and bacterial amplicons were pooled together into different tubes, sent to Inqaba biotechnological Industries (Pty Ltd.,

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Johannesburg, South Africa) and quantified using the NanoDrop™ 1000 spectrophotometer (Thermo Scientific). The amplicons from the triplicate PCR reactions were pooled at equal concentrations and used for Illumina preparation and sequencing. Samples were subjected to standard quality control measures (flourometric quantification and normalization). One nanogram of each amplicon pool was used in standard indexing PCR protocol for a paired-end sequencing library (Nextera) and samples were sequenced using the Illumina MiSeqV3 sequencer (2 × 300 reads).

3.2.8.2 Sequence processing and Data analysis

3.2.8.2.1 Fungal ITS amplicon analysis

Raw non-barcoded Illumina fastq files were demultiplexed and quality filtered using CLC Workbench Microbial Genomics Module 1.5.1 (QIAGEN, Aarhus, Denmark). Paired reads were merged according to the default settings and the unmerged reads were retained, sequences with a Phred score below 30 and shorter than 75 nt were discarded. Chimera detection was performed by kmer searchers. Reference-based OTU clustering was performed at 99% similarity against the UNITE v7 dynamic database (with singletons) (Kõljalg *et al.*, 2013). Classical ecology indices and Venn's diagrams as described in **3.2.7** was performed on the data following taxonomic assignment. In addition similarity percentage (SIMPER) analysis was performed to quantify the percentage contribution of individual genera to the observed dissimilarity observed between SH and EX derived must and year (Clarke, 1993).

3.2.8.2.2 Bacterial rpoB gene analysis

Raw Illumina fastq files were uploaded onto the MG-RAST server (Meyer *et al.*, 2008) and de-replicated (Gomez-Alvarez *et al.*, 2009). Sequences with a quality score of 30 Phred and below were identified with the application of dynamic trimming (Cox *et al.*, 2010) and discarded. The Fastq join script was selected to join overlapping paired-end reads. All sequences were processed for quality analysis, the resulting data sets were pre-screened with uclust (Edgar, 2010) clustering at 97% identity. Taxonomic assignment was performed in MG-RAST using the Blast Like-Alignment Tool (BLAT) search against the M5NR (Non-redundant Multi-Source Protein Annotation Database) with an *E*-value and similarity cut-off of $1e^{-8}$ and 90%, respectively. Classical ecology indices and Venn's diagrams as described in **3.2.7** was performed on the data following taxonomic assignment. In addition similarity percentage (SIMPER) analysis was performed to quantify the percentage contribution of individual genera to the observed dissimilarity observed between SH and EX derived must and year (Clarke, 1993).

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3.3 Results

The current study investigated both the fungal and bacterial community structures associated with shaded and exposed Sauvignon blanc grapes. Culture-based methods, ARISA community profiling as well as high-throughput amplicon sequencing was employed for an in-depth assessment of the community composition. The juice obtained from the SH and EX grape bunches were similar in chemical composition although the sugar levels and pH in the SH juice tended to be lower and the titratable acidity slightly higher than the EX (**Table 3.1**).

Table 3.1 Concentrations of compounds in the grape samples evaluated at harvest stage.

Date	Treatment	Total soluble solids (°brix)	Titratable Acidity	pH
2012-03-13	Exposed	24,40	6,53	3,39
2012-03-13	Shaded	23,40	7,38	3,31
2014-03-06	Exposed	21,50	7,47	3,28
2014-03-06	Shaded	20,50	8,37	3,17
2015-02-20	Exposed	22,23	11,57	3,36
2015-02-20	Shaded	21,13	12,66	3,01

3.3.1 Traditional culture-dependent approach

3.3.1.1 Yeast identification

The total yeast population concentrations for the four consecutive years were similar on both the SH and EX grapes although the SH must generally exhibited a slightly higher yeast diversity. More specifically, the viable counts in 2012, 2013, 2014 and 2015 on the EX were 1.4×10^4, 2×10^5 1.4×10^4 and 1.7×10^4 CFU/mL respectively, while for SH they were 4.5×10^4, 9×10^4, 2.4×10^5 and 2.13×10^4 CFU/mL, respectively. The yeast community composition was generally similar, with differences in the relative abundance of individual species (**Figure 3.2**).

A total of 31 different yeast species was isolated over the four-year period across both treatments. Twenty of these species were encountered in both the SH and EX must (**Figure 3.3**), albeit with varying frequencies from year to year. Amongst them only *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* were isolated in both treatments for all years, while *Starmerella bacillaris, Metschnikowia pulcherrima* and *Candida albicans,* were isolated in three years. *C. albicans* appeared to be consistently at higher levels in the EX must than the SH must. Eight minor species (each ≤ 1% of the population) such as, *Candida carvajalis, Zygoascus meyerae, Sporidiobolus ruineniae* and *Issatchenkia terricola,* were unique to the SH bunches, whereas, only three species including *Cryptococcus magnus, Torulaspora delbrueckii* and a *Candida* sp. were in the exposed bunches (**Figure 3.2**).

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However, the occurrence of these species were infrequent across the vintages (i.e. isolated in one or in certain cases two years).

Overall 2012 exhibited the highest diversity followed by 2014, 2013 and 2015, respectively. Furthermore, the exposed treatments exhibited higher species diversity (*H′* = 1.59) and evenness (e^H/S = 0.57), than the shaded must (*H′* = 1.15 and (e^H/S = 0.33) (**Table 3.2**). Analysis of similarity (ANOSIM) and PERMANOVA revealed that the cultivable yeast community in the shaded and exposed samples were not significantly different and that there was greater variation within the treatments than between (R = -0.04, F=0.39) (**Table 3.3**). Furthermore, the data suggest that the yeast community was more different between the years, albeit with significant overlap (R = 0.38, F = 5.09)

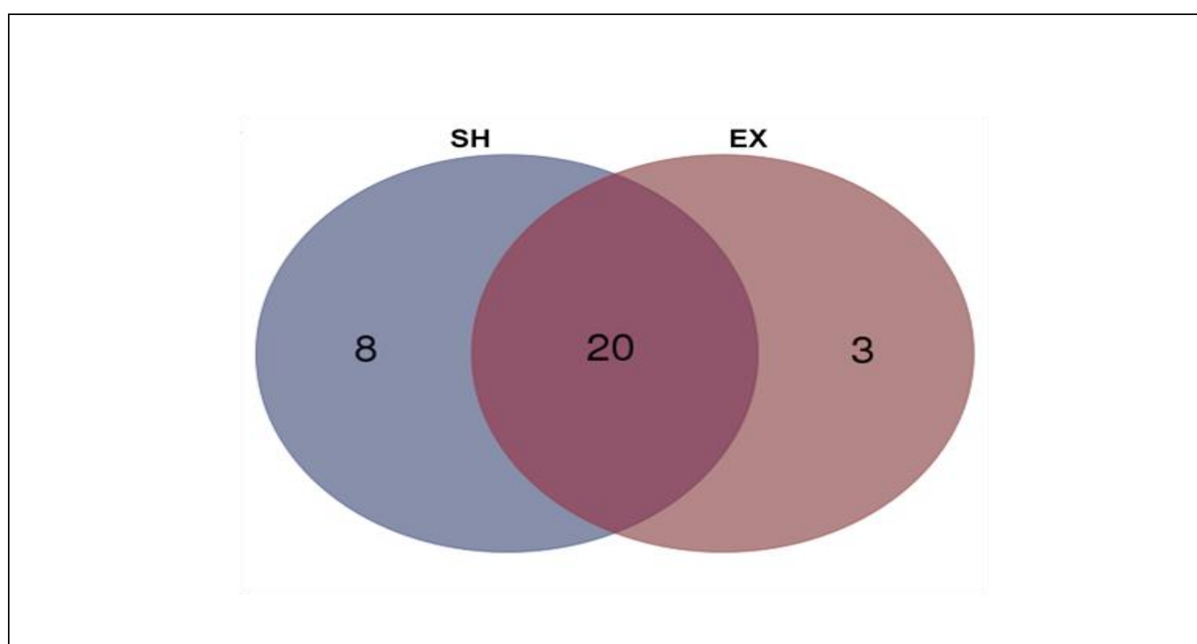

Figure 3.3 Venn diagram of the number of yeast species common in both treatments over all 4 vintages.

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Table 3.2 Diversity indices determined from using yeast isolates obtained from the culture-dependent approach, peaks generated from ARISA and fungal identification from Illumina. Years not analysed represented by the symbol (*).

Treatment	**Shaded**				**Exposed**			
Year	**2012**	**2013**	**2014**	**2015**	**2012**	**2013**	**2014**	**2015**
Culture-dependent								
Shannon diversity index	1,567	1,535	1,003	0,5158	2,181	1,626	1,533	1,013
Menhinick's index (richness)	1,4	0,9	1,3	0,7118	1,1	0,9674	1,1	0,5
Evenness	0,3424	0,5157	0,2097	0,2393	0,8048	0,5085	0,421	0,5506
ARISA								
Shannon diversity index	*	*	2,458	2,325	*	*	2,109	2,285
Menhinick's index (richness)	*	*	1,828	1,809	*	*	1,7	2,01
Evenness	*	*	0,6491	0,5682	*	*	0,4849	0,4911
Illumina								
Shannon diversity index	*	*	2,151	1,75	*	*	2,12	1,337
Menhinick's index (richness	*	*	1,6	2,3	*	*	2,2	1,3
Evenness	*	*	0,5373	2,507	*	*	0,3791	0,293

Table 3.3 ANOSIM and PERMANOVA of treatment and vintage effect on yeast and fungal microbiome associated with the grape berry for 2012-2015.

			ANOSIM		**PERMNOVA**	
Method	**Vintage**	**Factor**	**R**	***P***	**F**	***P***
Culture-dependent	All	Treatment	-0.04167	0.646	0.3874	0.636
Culture-dependent	All	Year	0.375	0.098	5.09	0.058
ARISA	2014-2015	Treatment	0.5	0.63	1.634	0.649
Illumina	2014-2015	Treatment	-0.5	1	0.481	1
Illumina	2014-2015	Year	0.5	0.331	2.235	0.332

3.3.1.2 Bacterial identification

Culture-dependent analysis of the bacterial population was only performed on the 2015 fresh must samples. The EX and SH must was found to harbour 1.4×10^3 CFU/mL and 3.9×10^2 CFU/mL, respectively. A total of seven different bacterial species was isolated, two *viz. Enterobacter* spp. and *Cedecea davisae* from the EX must and five including *Lactobacillus florum, Gluconobacter frateurii, Acetobacter persici, Gluconobacter nephelii* and *Gluconacetobacter hansenii* from the SH must (**Figure 3.4**).

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Table 3.4 Bacterial community diversity indices generated from the culture-independent approaches.

Treatment	**Shaded**		**Exposed**	
Year	**2014**	**2015**	**2014**	**2015**
B-ARISA				
Shannon diversity index	3,627	3,511	3,794	3,566
Menhinick's index (richness)	10,7	10,6	12,6	10,5
Evenness	0,3513	0,316	0,3527	0,3368
Illumina				
Shannon diversity index	2,26	2,256	2,275	2,75
Menhinick's index (richness)	2	2,7	2,3	3,4
Evenness	0,4794	0,3535	0,4228	0,4602

Table 3.5 ANOSIM and PERMANOVA of treatment and vintage effect on the bacterial microbiome associated with the grape berry for 2014 and 2015.

			ANOSIM		**PERMNOVA**	
Method	**Vintage**	**Factor**	**R**	***P***	**F**	***P***
B-ARISA	2014-2015	Treatment	-1	1	0,452	1
Illumina	2014-2015	Treatment	-0.5	1	0.855	1
Illumina	2014-2015	Year	0.25	0.638	1.154	0.31

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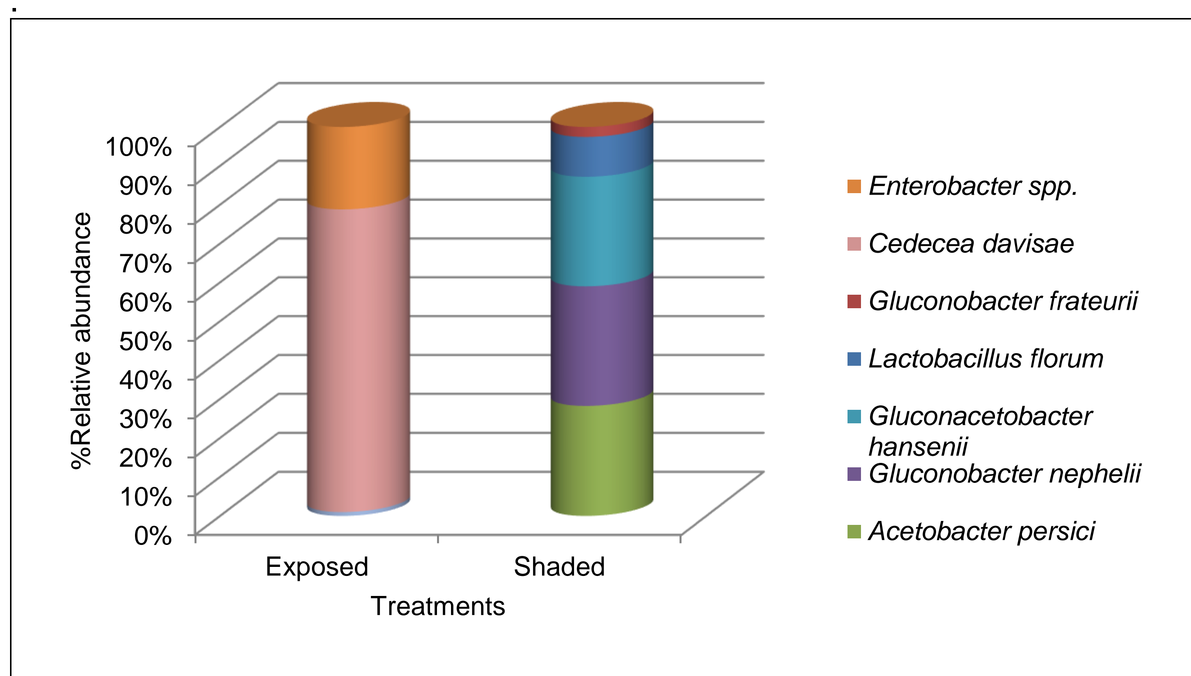

Figure 3.4 Population structure and relative abundance of bacterial species present in the grape must for the 2015 harvest.

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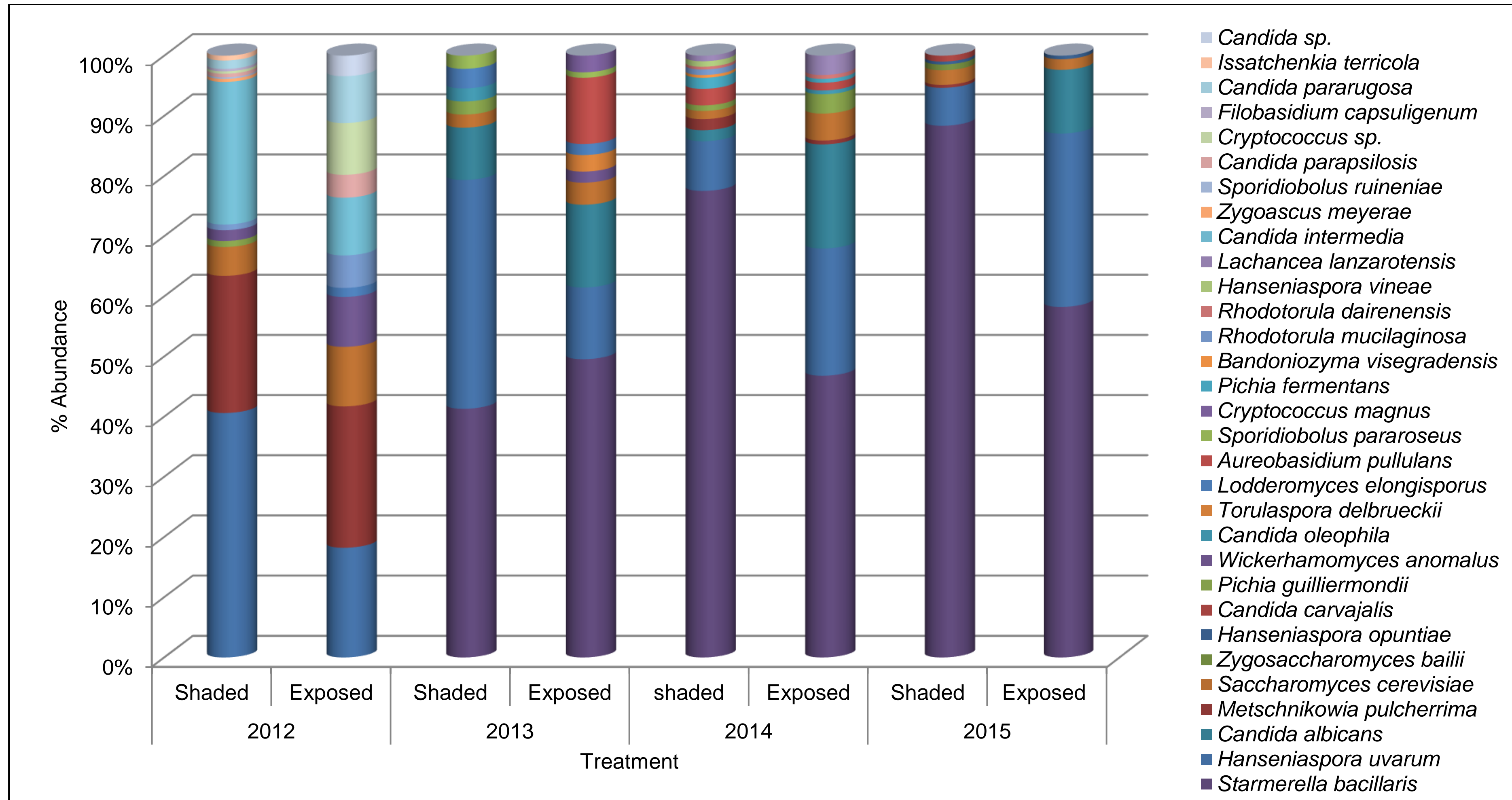

Figure 3.2 Population structure and relative abundance of yeast species present in the grape must for the years 2012-2015.

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3.3.2 Automated Ribosomal Intergenic Spacer Analysis (ARISA) fingerprinting

ARISA was employed to profile both fungal and bacterial communities in the EX and SH must for the years 2015 and 2014, using the gDNA extracted directly from the composite must sample.

3.3.2.1 Fungal ARISA (F-ARISA)

A total of 30 peaks were scored across both treatments and years, with 25 of these detected in both the SH and EX must samples at varying fluorescence intensities and frequency of occurrence. For instance, 9 peaks (437 bp, 458 bp, 539 bp, 549 bp, 576 bp, 579 bp, 606 bp, 608 bp and 750 bp) were identified in both years in the SH and EX must, while the peaks at 535 bp and 842 bp were only detected in the EX must, and 386 bp, 565 bp and 568 bp only in the SH must samples (**Figure 3.5**). Furthermore, both the 2014 and 2015 EX must were dominated by a peak of 699 bp, whereas in the SH must, peak 539 bp and 579 bp were dominant in 2014 and 2015, respectively. The remainder were either present in both the SH and EX must but in different years for instance (EX-14, EX-15, SH-14) or (EX-15, SH-14) and vice versa.

Overall the SH must displayed a slightly higher diversity and species evenness than the EX must sample (**Table 3.2**). Based on the ANOSIM and PERMANOVA, the fungal population in the SH and EX musts exhibited significant overlap (R = 0.5, F = 1.63) and the difference between the two populations was not significant (p = 0.63) as seen in **Table 3.**3 Using known fungal ITS-5.8S rRNA-ITS2 gene sizes and sequence data obtained through the culture-dependent analysis,15 peaks could be tentatively assigned to possible species (**Table 3.6**). For 2014 seven peaks were assigned to *A. pullulans, S. bacillaris, H. uvarum, M. pulcherrima, P. guilliermondii, C. albicans* and *S. cerevisiae*, while for 2015 five peaks were identified as *C. albicans, H. uvarum, S. cerevisiae, S. bacillaris* and *H. opuntiae.*

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Table 3.6 Tentative identification of ARISA peaks through possible associations with the yeast isolates ITS regions and known fungal ITS sizes in base pairs. *nd are peaks not detected in ARISA or culture-dependent analysis.

Fungal isolates	ITS-5.8S rRNA- ITS2 PCR amplicons (bp)	Peaks derived from ARISA profile (bp)
Aureobasidium pullulans	580	579
Zygosaccharomyces bailii	760	*nd
Candida azymoides	442	437
Candida oleophila	628	628
Candida parapsilosis	522	522
Candida albicans	537	535
Candida carvajalis	643	*nd
Starmerella bacillaris	460	458
Hanseniaspora opuntiae	746	746
Hanseniaspora uvarum	747	750
Hanseniaspora vineae	687	*nd
Lodderomyces elongisporus	550	553
Metschnikowia pulcherrima	377	375
Pichia guilliermondii	605	606
Pichia fermentans	444	*nd
Rhodotorula nothofagi	610	608
Rhodotorula slooffiae	585	588
Rhodotorula muciloginosa	628	628
Rhodotorula dairenensis	615	*nd
Saccharomyces cerevisiae	839	842
Wickerhamomyces anomalus	617	617
Lachancea lanzarotensis	632	*nd
Botrytis cinerea	539	539
Aspergillus fumigatus	594	594
Aspergillus niger	599	*nd
Alternaria alternata	570	571
Cladosporium cladosporiodes	551	*nd
Penicillium spinulosum	576	576

3.3.2.2 Bacterial ARISA (B-ARISA)

A total of 141 peaks were scored across both treatments and years, with each year yielding between 105-126 peaks, with 139 of these detected in both the EX and SH must samples, at varying fluorescence intensities and frequency of occurrence. For instance, 58 peaks were identified in both years in the SH and EX must, while the peak at 447 bp was only detected in the EX must and 462 bp only in the SH must samples in 2015 with a further 15 peaks unique to 2014 and 7 in 2015 (**Figures 3.6** & **3.7**). Furthermore, both the SH and EX must were dominated by a peak of 435 bp in 2015, whereas 2014 was dominated by peak 398 bp and 557 for SH and EX, respectively. The remainder of the peaks were either associated with both SH and EX must but in different years for instance (EX-14, EX-15, SH-15) representing 48 peaks or same year but different treatment (EX-15, SH-15) representing 33 peaks and vice versa. Overall the EX must displayed a slightly greater species diversity and evenness than the SH must sample (**Table 3.4**). Based on the ANOSIM and PERMANOVA, we could

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demonstrate that there was no treatment effect on the bacterial populations (*P* = 1), seen in **Table 3.5**. The statistical analysis moreover revealed that the variation of bacterial species was greater within the groups than between (R= -1, F =0.452). Our data suggest that there is more variation in the bacterial population between vintages than the treatment.

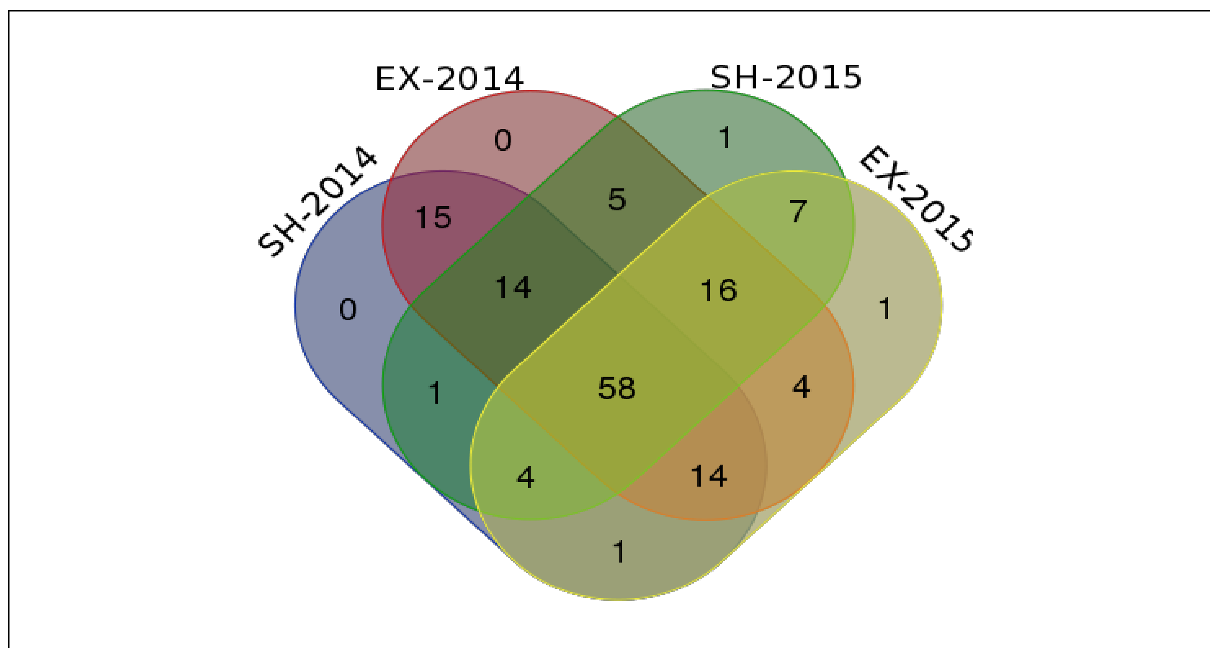

Figure 3.7 Venn diagram of the number of bacterial species common in both treatments over 2 vintages.

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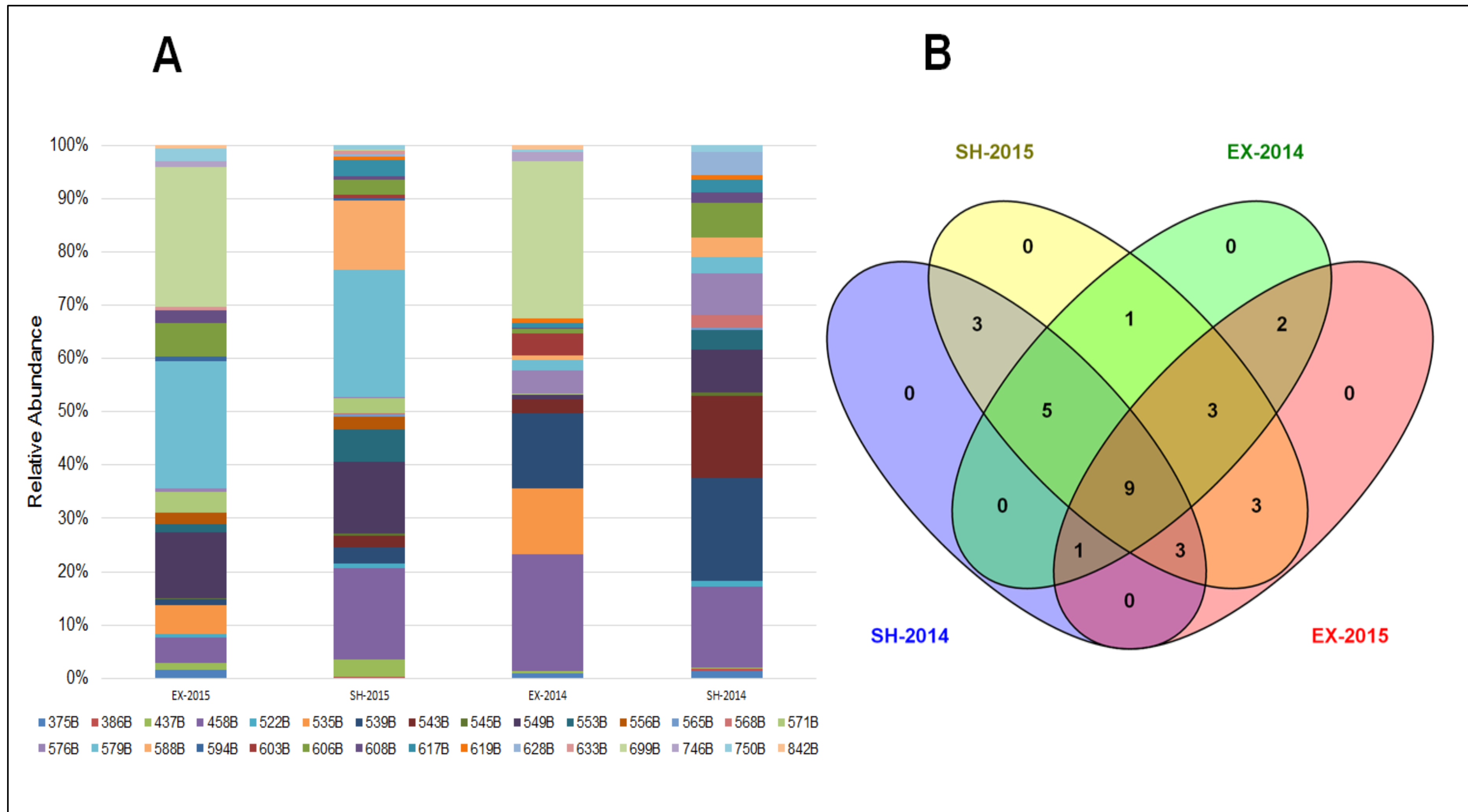

Figure 3.5 A Fungal diversity and relative abundance derived from ARISA for 2014-2015. The peak OTU and their abundance are represented as a % by the various colours which can be seen on the key below. **B** Venn illustration of number of species common between treatment and year.

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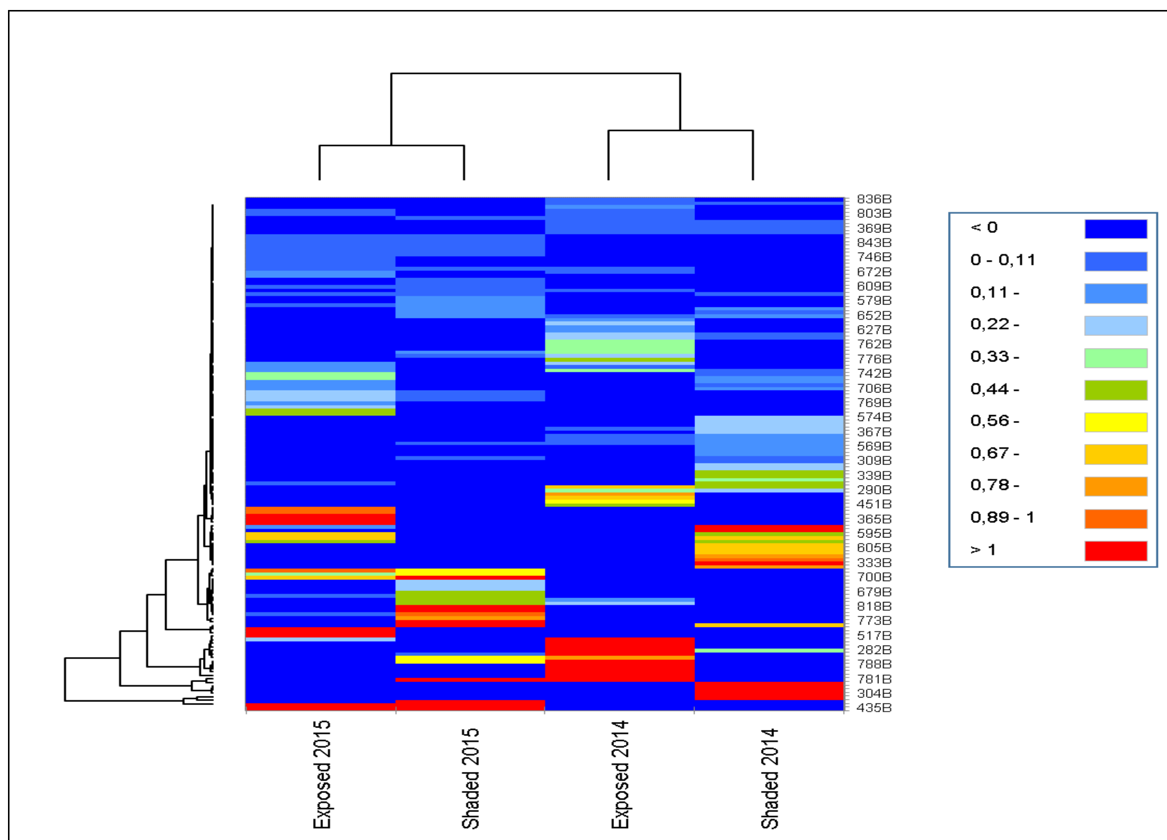

Figure 3.6 A heat map of bacterial diversity and relative abundance derived from ARISA for 2014-2015. The peak abundances are represented as colours, where the colour intensity is an indication of the relative abundance.

3.3.3 Illumina MiSeq

In the current study, Illumina paired sequencing was employed to assess the fungal (ITS1-5.8S rDNA-ITS2) and bacterial (*rpoB* gene) communities of the must derived from shaded and sun-exposed composite grape must samples.

3.3.3.1 Fungal diversity

Using the OTUs obtained after taxonomic assignment, Bray-Curtis distance matrix was employed, to measure the dissimilarity between the OTUs obtained in the both the EX and SH must for both years. A PCO analysis showed that the EX samples clustered closer to each other compared to the shaded. However, the data show that the combination of treatment and year explained 61% of the dissimilarity in the community composition, while the treatment alone explained 26% and year alone explained 13% of the dissimilarity (**Figure 3.8**).

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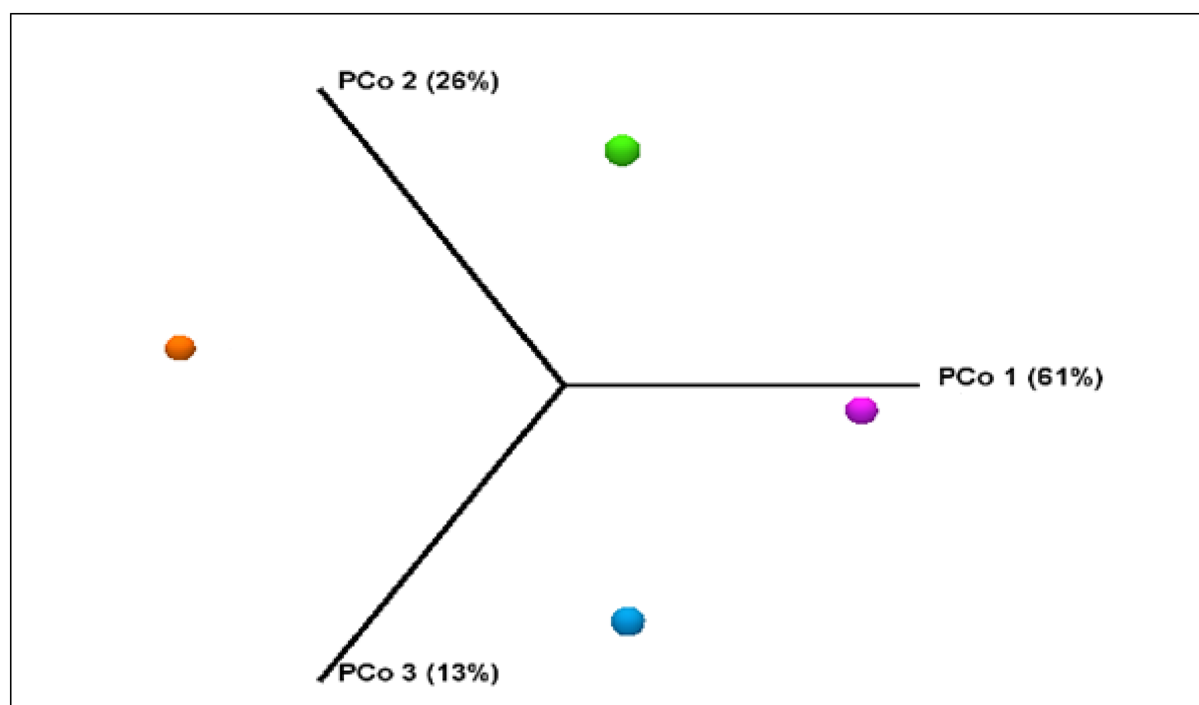

Figure 3.8 PCo Analaysis based on Bray-Curtis distance Matrix for fungal OTUs obtained from Illumina MiSeq. Orange and blue circles represents the OTUs obtained from the 2014 vintage SH and EX must, respectively. The green and purple circles represent the 2015 vintage for SH and EX must, respectively.

The phylum Ascomycota was found to be the dominant in both the SH and EX must for both years, accounting for 62 and 69% of the total fungal population, respectively. In contrast the Basidiomycota accounted for between 22 and 26%, while Zygomycota only occurred in minor incidence in 2014, only with 4.5 and 6.3 % for EX and SH must, respectively. A small proportion of fungi were unidentified in all the must evaluated. Further evaluation of the total taxa showed that the *Dothideomycetes* and *Saccharomycetes* were equally dominant in both the SH and EX must, each accounting for 26.7%. However, the *Dothideomycetes* occured more frequently in the evaluated, followed by the *Tremellomycetes*, with 10%, *Microbotryomycetes*, *Euromycetes* and *Leotiomycetes,* which accounted for 6.7% of the taxa each. The other 5 classes collectively accounted for 16.7% of the population in both the SH and EX must. A total of 30 different genera were identified across both EX and SH must and vintages, with 20 of these identified in both the EX and SH must at varying relative abundances and occurrences. Of these *Aureobasidium* was the most abundant genus in the EX must in both years and in the SH must in 2015, while *Penicillium* was dominant in the SH of 2014. Nine of the detected genera only occurred once in either SH and EX or vintage. These genera included, *Metschnikowia, Wickerhamomyces* and *Pleurostoma* found in EX must of 2014, as well as *Malassezia* and *Saccharomyces* identified in SH must of 2014, while *Zygosaccharomyces, Cryptococcus, Mycosphaerella* and *Acremonium* were detected in the

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SH must in 2015. Other genera such as *Hanseniaspora* and *Rhodotorula* were detected in both the SH and EX must but in different years (**Figures 3.9** & **3.10A**).

Based on the ANOSIM and PERMANOVA, the fungal population in the SH and EX musts exhibited significant overlap (R = -0.5, F = 0.481) within treatments, while significant overlap between years was observed (R = 0.5, F = 2.235) and the difference between the two populations was not significant (**Table 3.3.**).

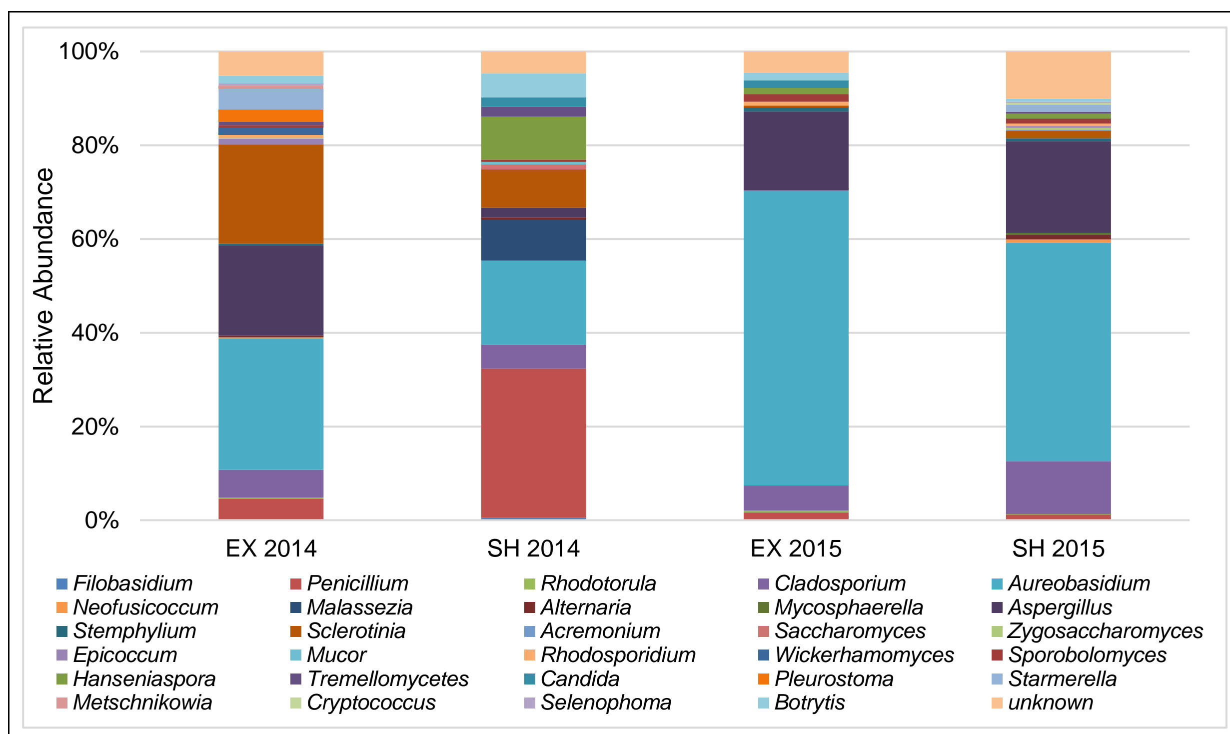

Figure 3.9 Distribution of fungal genera across the phyla Ascomycota, Basidiomycota and Zygomycota. For both SH and EX treatments and years evaluated from sequencing the ITS 1 loci region.

Figure 3.10 A Venn diagram representing a comparison of both fungi and bacteria, identified from the SH and EX treatments for 2014 and 2015. (A) Fungal community and (B) Bacterial community.

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The filamentous fungi identified in the SH and EX derived must comprised 14 genera. Five of these were detected in all evaluated samples, these included four epiphytic filamentous fungi, *Penicillium, Aspergillus, Sclerotinia* and *Botrytis,* as well as an endophytic fungus, *Cladosporium.* The fungal community was generally dominated by the epiphytic community which represented 83.33 and 89.2 % for 2014 and 76.92 and 61.5 % for 2015, for EX and SH derived must, respectively. A small proportion of fungi which was detected in the mycobiome were not previously known to be associated with grapes (e.g. members of the genera *Selenophoma* and *Pleurostoma*). Furthermore, statistical analysis revealed that there was no treatment effect on the filamentous fungi, with regard to either of the demonstrated groups individually or as a whole (data not shown). Overall these filamentous fungi accounted for more than 50% of the population in 2014, while in 2015 the accounted for 26.75 and 37.63% of the EX and SH must, respectively (**Figure 3.11**).

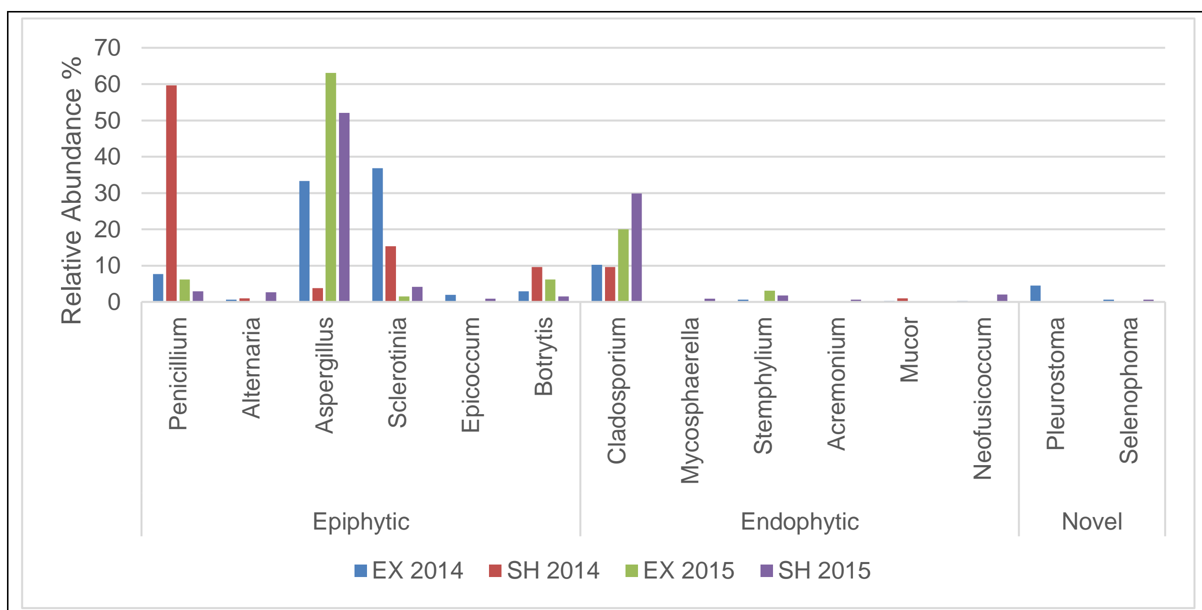

Figure 3.11 Distribution of filamentous fungi groups identified in the grape associated microbial consortium.

The yeast population, accounted for 37.2 and 42.1% of the population in the 2014 EX and SH must, respectively, while in 2015 it accounted for 68.7% in EX and 52.2% in SH derived musts. Only two species (*A. pullulans* and *Sp. roseus*) were detected throughout both years in both SH and EX derived must. The former was dominant in all samples. The oligotrophic oxidative yeasts such as *Filobasidium magnum, Rhodotorula nothofagi, Sp. roseus, Rhodosporidium babjevae, Malassezia* spp., *Cryptococcus carnescens* and *A. pullulans* represented the majority of the species identified. The strongly fermentative yeasts was comprised *Saccharomyces mikatae* and *Z. bailii,* which were only identified in SH treatments for 2014

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and 2015, respectively. *Starmerella bacillaris* was the most dominant weakly fermentative yeast in EX and SH treatments, for 2014 and 2015, respectively, while *Hanseniaspora* spp. dominated SH 2014, and was the most frequently occurring weakly fermentative yeast. *Candida* spp. on the other hand dominated EX 2015. The unknown fungi proportion accounted for approximately 5% of the total taxa in all samples, except for SH 2015 where it accounted for 10%.

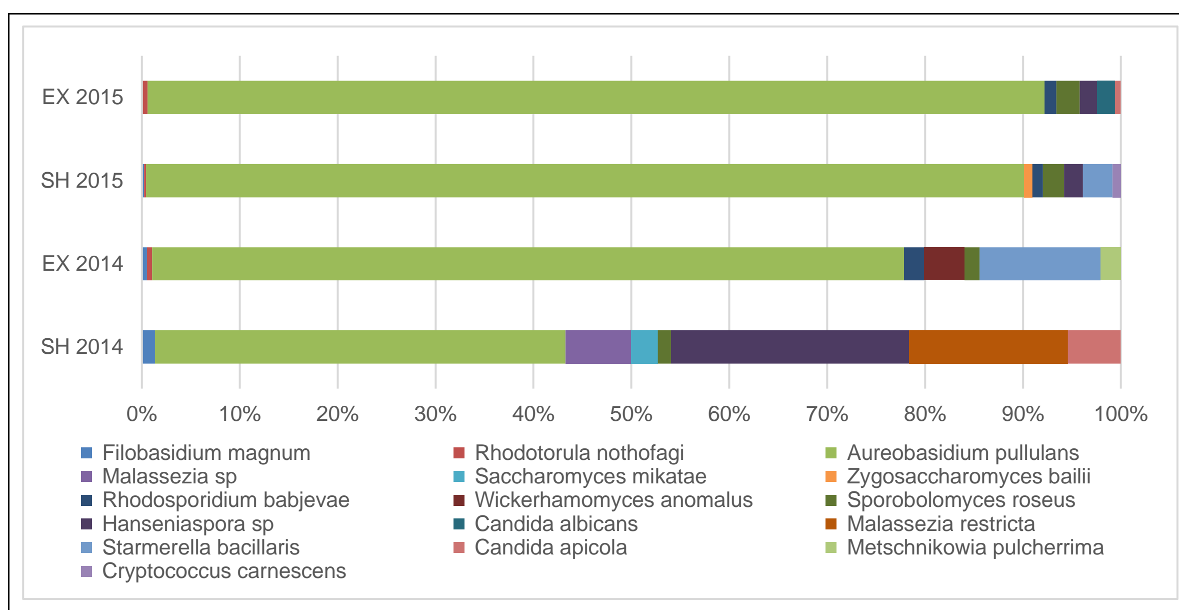

Figure 3.12 Frequency of yeast genera identified in the fungal analysis represented as relative abundance.

3.3.3.2 Bacterial Diversity

The Proteobacteria was the dominant phylum detected in the must, constituting 82.3% of the total bacterial population, followed by Firmicutes with 13.7% while both Fibrobacter and unidentified bacteria each accounted for 2% of the bacterial population. These 4 phyla were represented by 51 different genera across both SH and EX treatment and years, with 24 of these identified in both the EX and SH must at varying relative abundances and occurrences (**Figure 3.13**). Further analysis of the bacterial orders revealed that *Enterobacteriales* was the most abundant, accounting for 25.5%, followed by *Burkholderiales* with 21.6% of the taxa. Neither treatment or year had an influence on the entire group (data not shown). The remaining 53% comprised 8 orders including an unknown group, which demonstrated no response to both treatment or vintage. A total of seven genera were detected in all must samples evaluated and included, *Streptococcus, Variovorax, Citrobacter, Enterobacter, Pantoea, Tatumella* and *Xanthomonas*. The most abundant genus in the must derived from SH 2014 and EX 2015, was *Citrobacter*, while for SH 2015 and EX 2014 it was *Pseudomonas*. The abundance of three genera (*Pseudomonas, Enterobacter* and *Variovorax*) was higher in

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SH 2015 and EX 2014 derived must than SH 2014 and EX 2015, while *Streptococcus* and *Veillonella* were lower. Of the 51 bacterial genera identified, 27 only occurred once in either SH and EX, or 2014 and 2015. The remainder of the genera were either present in both the SH and EX must but in different years (**Figure 3.10B**). Further analysis into the relative abundances of the bacterial community showed that, genera of the order *Xanthomondales*, which include *Xanthomonas* and *Stenotrophomonas* were at slightly lower abundances in the EX derived must, in comparison to the SH must. Similarly, this was observed for *Achromobacter* and *Bordetella.* Interestingly these two genera were always at the exact same relative abundance in all the samples evaluated (**Figure 3.14**).

Overall SIMPER analyses based on abundance was used to demonstrate which genera contributed the most dissimilarity between samples based on both leaf removal treatment as well as year. Analysis indicates that vintage contributed the greatest level of dissimilarity with 63.6%, opposed to 59.52% through treatment. *Pseudomonas* and *Pantoea* contributed to the greatest percentage of dissimilarity in treatment, while *Pantoea* and *Tatumella* explained the largest dissimilarity in vintage. Based on the ANOSIM and PERMANOVA, we could demonstrate that there was no treatment effect on the bacterial populations (p = 1), seen in **Table 3.5**. The statistical analysis moreover revealed that the variation of bacterial species was greater within the groups than between (R= -05, F =0.855), with significant similarity within treatments. As seen with the community fingerprinting, it appears that there is greater variation with vintage (R= 0.25)

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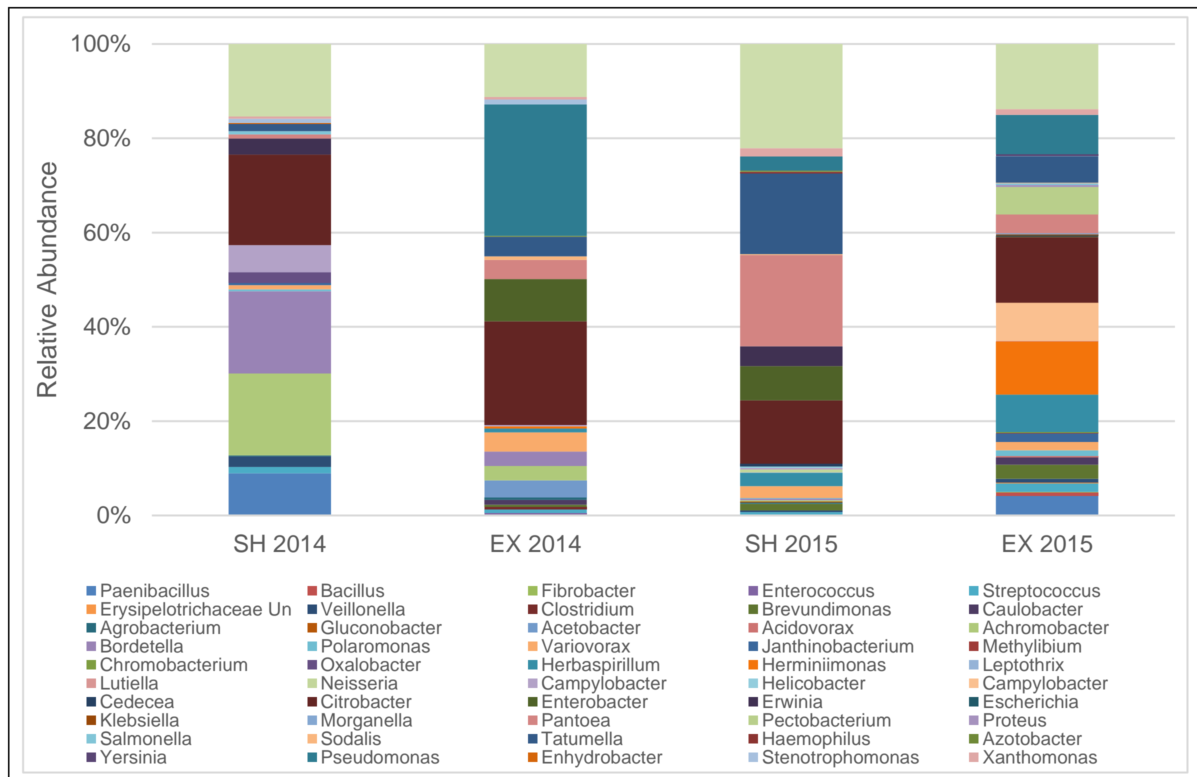

Figure 3.13 Bacterial genera diversity and relative abundance derived from the sequencing of the bacterial *rpoB* gene.

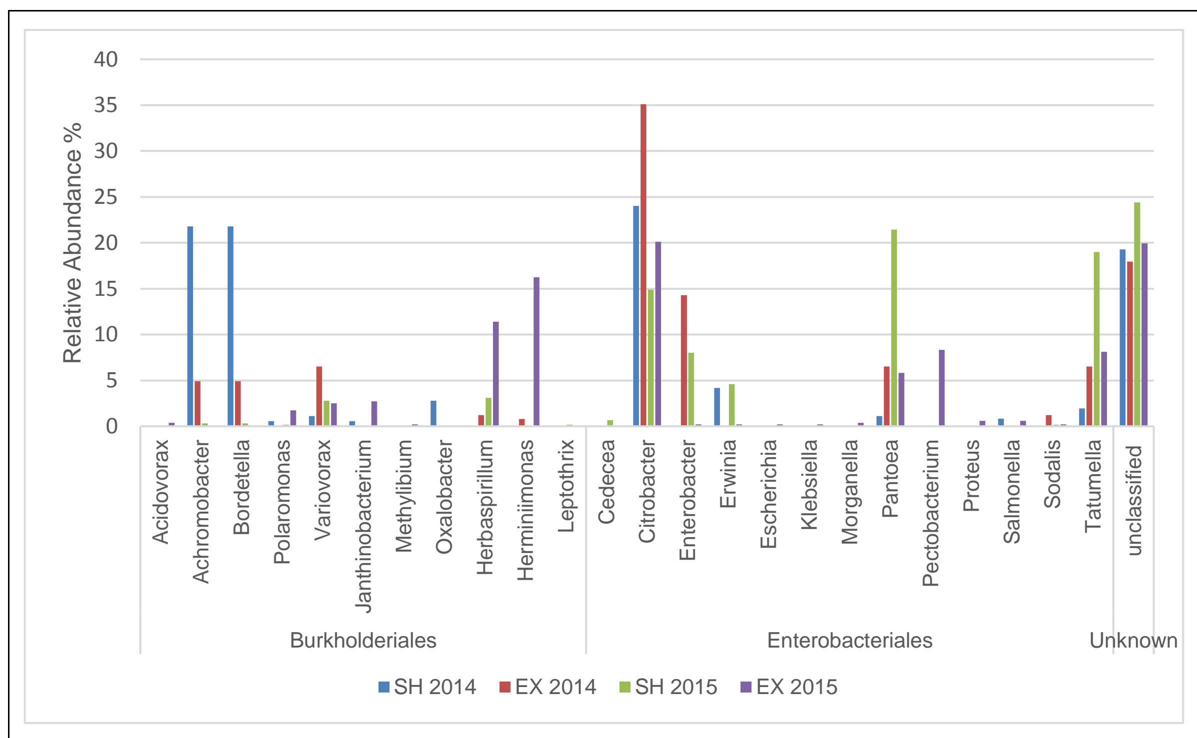

Figure 3.14 Distribution of the bacterial genera belonging to the 3 most dominant orders, including the unclassified bacterial group.

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3.4 Discussion

The current study sort to characterize the microbial community present in freshly crushed Sauvignon blanc must, obtained from shaded and sun-exposed berry bunches to assess the potential influence of leaf removal on the natural grape microbiome. The grapes were obtained from an established model vineyard on which comprehensive information relating to the macro and mesoclimatic conditions are available and in-depth microclimate data were collected throughout the season (Young *et al.,* 2015). Firstly, we employed a culture-based method, followed by community fingerprinting with the application of ARISA for a preliminary overview of a more complete fungal and bacterial community. Lastly, a high-throughput NGS was used for the in-depth characterisation of these microbial populations and their association with the berry under leaf removal.

The evaluation of the complete fungal and bacterial community, with the employment of all three molecular approaches revealed that leaf removal had no significant effect on the complete microbial populations derived from the sun-exposed or shaded must samples. This observation could firstly, partially be a result of the sampling strategy employed. For instance, while for the exposed samples only the bunches that were directly exposed to the sun were harvested, whereas for the shaded canopy all grapes were picked. This could therefore distort the true effect since the shaded grape must was therefore derived from partially sun-exposed bunches and not truly shaded bunches. However, this requires further investigation. Secondly, according to Young *et al.* (2015) the average daily canopy and bunch zone temperature was not significantly different between the treatments throughout the season even though the exposed bunches received considerably more light than the shaded bunches. Both factors which have been demonstrated to influence the fungal genera associated with grapes, including *Aspergillus* (Pardo *et al*., 2005), powdery mildew (Carroll & Wilcox, 2003) and fungal genera associated with bunch rot (Steel *et al*., 2007). Furthermore, Duncan *et al.* (1995) reported that the yeast cell concentrations were not impacted by leaf removal practices. Interestingly Manzocco *et al.* (2016) looked at UV-C treatment and the effectiveness on the microbial population of pineapple, and found that UV-C did not significantly influence the viable populations. Even though these communities did not seem to be perturbed by leaf removal treatments, with the application of ANOSIM we were able to show that although there was no differences in the wine microbial consortium in the must from the different treated bunches, greater variations were observed between vintages, even in this instance where there was significant overlap as seen in **Tables 3.3** & **3.5**. ANOSIM is a statistical tool that looks at the ranked average dissimilarity between the group and compares it to the ranked average dissimilarity within groups. Although R is scaled to lie between -1 and +1, it generally lies between 0 and +1, where an R value closer to one suggest dissimilarity between groups and

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below to zero suggest that dissimilarities are greater within groups. R substantially less than zero indicates greater dissimilarities among individual replicates within any sample. In the current study composite samples were used, therefore, the negative R values show that there are variations within the treatments from year to year. This can be tested by preparing composite biological repeats for each treatment.

A study by Sternad Lemut *et al.* (2015) reported changes in the basidiomycetous yeast and *A. pullulans* in response to leaf removal. Similarly, our study show that *Aureobasidium* was in higher numbers in the EX must than SH must, however in our case the basidiomycetous yeast were almost generally present in the minor proportion and their detection yearly was inconsistent, making it difficult to confidently attribute their differences to leaf removal.In contrast, *A. pullulans* the yeast-like fungus was found to be one of the major yeast species inhabiting the berry surface for both treatments and most vintages using both techniques. This is consistent with various studies assessing the grape microbiome of white (Comitini & Ciani, 2008; Cadez *et al.*, 2010) and red cultivars (Raspor *et al.*, 2006; Sternad Lemut *et al.*, 2015).

Leaf removal has however, been demonstrated to reduce rot such as those caused by *B. cinerea* (Sabbatini & Howell, 2010). Based on our results obtained from the culture-independent techniques, we were able to partially confirm the results. A lower abundance in the genera *Botrytis* was observed with both ARISA and Illumina form the EX derived must. Moreover, this was also observed for *Penicillium* which is reported to be associated with summer bunch rot disease complex (Molitor *et al.*, 2011; Steel *et al.*, 2013). This observation has also been demonstrated by Duncan *et al.* (1995). These observations with the employment of Illumina was however seen with the OTUs obtained, as opposed to the relative abundances. The impact on bacteria has also been investigated by Sternad Lemut *et al.* (2015) and they did report that leaf removal potentially reduced the acetic acid bacterial populations, which was observed with the absence of *Gluconobacter* in the EX derived must for 2015, as our data showed that *Gluconobacter* was indeed isolated in the SH treatments in 2015. Furthermore, a study by Tawema *et al.* (2016), demonstrated that UV-C treatment on cauliflower initially lowered the yeast and mould counts, however after time, the populations eventually increased during storage. This could explain our findings, as leaves eventually grow back and as such the bunch zones are gradually exposed to less UV radiation, resulting in the microbial populations which was once affected to start remerging. Overall the grape microbiome seems to comprise a more diverse and dominant prokaryotic community in comparison to fungi (Pinto *et al.*, 2015; Salvetti *et al.*, 2016).

A comparison of the culture-dependent and ARISA revealed, that the analysis shared similar species identification between them. In 2014 *M. pulcherrima, A. pullulans*, *S. bacillaris*,

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P. guilliermondii and *H. uvarum* were isolated in both treatments. *Candida* spp. and *S. cerevisiae* was identified in exposed treatments by both methods. The relative abundance of certain species between the two methods was similar, for instance the peak at 375 bp (likely *M. pulcherrima*) of the shaded treatment was approximately at a frequency of 2%, and at 579 bp (likely *A. pullulans*) for both treatments in 2014 at between 1-3 %. A similar observation could be made for 2015 in that *H. uvarum* and *S. bacillaris* was identified in both treatments as well as *S. cerevisiae, H. opuntiae* and *C. albicans* in the exposed treatments, with *S. cerevisiae* at similar abundance. Further comparison to the Illumina results revealed that *M. pulcherrima, S. bacillaris, H. uvarum and A. pullulans* was similarly identified in 2014 must; however, only the latter was identified in both SH and EX must. Furthermore, this similarity was also observed for *Hanseniaspora* spp. in both EX and SH must*, S. bacillaris* in SH must and *C. albicans* in EX must for 2015. Moreover, both ARISA and Illumina detected a considerably higher abundance of *A. pullulans* in 2015, while, both methods also detected *R. nothofagi* in at least 3 of the 4 evaluated samples at low abundances. Further comparison reveals that *Z. bailii* was only identified in the SH 2015 in both culture-dependent and Illumina at similar relative abundances (<1%). Despite the similarities observed, for many of the identified species there were large discrepancies in their population abundances and presence between the methodologies, which include species such as *S. bacillaris, S. cerevisiae, C. albicans* and *H. uvarum.* The differences between the methods are expected and have been previously reported (Ruan *et al.*, 2006; Brežná *et al.*, 2010; Setati *et al.*, 2015).

Despite these observations in the microbial communities, the data show similar trends with regards to the microbial community composition in the shaded and exposed must and whether these are largely influenced by defoliation or not. As a whole the data set suggest that the differences in the microbial community can largely be ascribed to the absence and presence of minor species and relative abundance of a few major species, that dominate the berry surface.

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Chapter 4

General discussion and conclusions

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4. General discussion and conclusion

4.1 CONCLUDING REMARKS AND FUTURE PROSPECTS

The current study aimed to assess the microbial communities present in must prepared from grapes derived from altered and unaltered canopies. In the altered canopies the total leaf and lateral shoot removal was performed in the bunch zones on the side facing or receiving morning sunlight. This treatment has been shown to reduce bunch rot incidence, specifically reducing epiphytic mycobiota including species of the genera *Penicillium, Botrytis* and *Aspergillus* (Duncan *et al.*, 1995; Sternad Lemut *et al.*, 2015). Early leaf removal has furthermore, also been demonstrated to be an effective means for reducing acetic acid bacteria (AAB) associated with grape berries (Sternad Lemut *et al.*, 2015).

We employed three methods to unravel the microbial community structure associated with leaf removal. Our data did not show any significant difference in the fungal and bacterial communities associated with the grapes under leaf removal and those from shaded bunches. However, some trends were evident. For instance, among the basidiomycetous yeasts, the red pigmented genera (e.g. *Rhodotorula, Rhodosporidium* and *Sporobolomyces*) tended to be higher in the exposed berries while the lighter pigmented yeasts (e.g. *Cryptococcus* and *Filobasidium*) were higher in the shaded. However, for this group of yeasts, ascription of their differences to leaf removal could not be done confidently since they were generally present at low levels and their detection was infrequent. Nevertheless, the data seem to suggest that the yeast species which have an innate photoprotective mechanisms can thrive on the sun-exposed grapes. Indeed, the red-pigmented basidiomycetous yeasts mentioned above are known to accumulate carotenoids such as torularhodin, which enhances their tolerance to UV-B and other DNA damaging stresses. Amongst the filamentous fungi, members of the genera *Penicillium*, *Botrytis* and *Alternaria* were reduced in the exposed derived must, this has previously been demonstrated, moreover, UV-B has also been shown to reduced the growth of *Alternaria* species (Fourtouni *et al.*, 1998). Analysis into the bacterial community demonstrated that potential plant pathogens *Stenotrophomonas* and *Xanthomonas* was reduced in the exposed treatment, together with *Achromobacter* and *Bordetella*. Interestingly concordant with the culture-dependent analysis, *Gluconobacter* was not identified in the 2015 exposed sample, but only in the shaded derived must.

Differences in the microbial community was evident in certain instances. Overall there was no significant effect of leaf removal on the microbial populations. This observation could be explained by the fact, while for the exposed samples only the bunches that were directly exposed to the sun were harvested, for the shaded canopy all grapes were picked. This could impact the actual effect of leaf removal since the shaded grape must was therefore derived

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from partially sun-exposed bunches and not truly shaded bunches. However, this requires further investigation. Secondly, according to Young *et al.* (2015) the average daily canopy and bunch zone temperature was not significantly different between the treatments throughout the season even though the exposed bunches received considerably more light than the shaded bunches. Furthermore, the ripening dynamics or berry physical characteristics were not influenced by leaf removal. These parameters are important in shaping the grape associated fungal diversity. Indeed, several studies have shown that the microbial diversity changes throughout berry ripening (Prakitchaiwattana *et al.*, 2004; Pinto *et al.*, 2014). Given that the bunch zone temperature as well as ripening dynamics between the berries of the shaded and exposed treated canopies was similar it is understandable that the yeast community composition would not differ significantly. While these parameters may possibly play a role in shaping the populations, it must not be overlooked that standard farming practices, specifically the application of pesticides/fungicides was not halted and was applied to both the shaded and exposed must. This therefore, could have effectively supressed the disease associated microbiota and therefore no significant impact on the microbiome was observed.

A comparison of the fungal populations derived from the techniques for 2014 and 2015, demonstrated that the high-throughput sequencing approach unveiled a more diverse population, followed by ARISA and the culture-dependent approach, respectively. This observation was assuming that each peak generated by ARISA was representative of a different species. However, it is important to note that diversity of the population derived from ARISA can be underestimated, because of identical ITS1-5.8S rRNA-ITS2 sizes, (Esteve-Zarzoso, 1999; Kovacs *et al.*, 2010; Pancher *et al.*, 2012), seen in the current study with *C. oleophila* and *Rh. muciloginosa* yeast species. Using the three approaches, proved to be beneficial and partly complimentary, especially for assigning species to genera obtained from the Illumina analysis and peaks derived from ARISA, since only partial sequences are derived from Illumina, while full ITS1-5.8S rRNA-ITS2 sequences could be obtained from culture-based isolates. Although, large discrepancies were observed between the molecular techniques, the data obtained from all three methodologies suggest that there is not impact on leaf removal on the population, For culture-dependent ($R= -0.04$, $p= 0.65$), ARISA ($R=0.5$, $p=0.63$) and Illumina ($R=-0.5$, $p=1$) analysis. The study evaluated the bacterial community in concert with the fungi, and similar to the data obtained from the fungal community, we observed that treatment had no significant impact on the grapevine derived bacterial community. The study did however reveal that time, might have a greater influence in driving the community than leaf defoliation.

This study was the first, to evaluate the influence of leaf defoliation on the natural microbial population with the application of direct high-throughput sequencing. Providing

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evidence that leaf removal impact disease associated fungi such as, *Botrytis* and *Penicillium* as well as AAB. The current study however, only evaluated a single experimental vineyard, for two vintages. Therefore, it could be worthwhile repeating the study on a greater sample set, possibly even a vineyard more susceptible to disease, with and without fungicide application, to provide irrefutable evidence of leaf removal and its influence on the grape associated microbial community,

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