Evaluating the effect of environmental parameters on the dynamics of a yeast consortium

by

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Declaration

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Summary

Wine fermentation is a complex biochemical process which is characterized by the sequential development of various weakly and strongly fermentative yeast species. Thus, in such a multi-species consortium, individual species interact with one another and with their environment. Consequently, final chemical composition of wine will be affected significantly by the contribution of individual species as well as yeast-yeast interactions present in a wine consortium. The contribution of individual strain in the wine consortium is affected by several biotic (ecological interactions, killer factors, and grape variety) as well as abiotic parameters (temperature, sulphur dioxide, oxygen and nutrient availability). However, each strain will be affected differently by the combination of these parameters. Previous studies in wine fermentations have mainly focused on mixed culture fermentations composed of two species. Thus, fundamental rules underlying the effect of these parameters in a multi-species ecosystem are not fully understood. To decipher the principles that govern the complex wine ecosystem, a simplified model consortium comprising eight species commonly encountered in South African grape microbiota was established. An Automated ribosomal intergenic spacer analysis (ARISA) method was also developed in order to monitor population dynamics of the yeast consortium. The influence of presence of *Saccharomyces cerevisiae* as a biotic stress was investigated on the dynamics of yeast consortium in synthetic must using plating method and ARISA. Furthermore, the yeast consortium was used as an inoculum in Chenin blanc grape must where the population dynamics were monitored by plating method. The results confirmed that a selective pressure applied by the keystone species, *S. cerevisiae* modified the pattern of population dynamics. Wine ecosystem was characterized by supportive and inhibitory interactions. Furthermore, in spite of the differences between the two grape matrices, a similar pattern of population dynamics was observed in both fermentations. This observation suggested broad applicability of the model consortium to study the ecological interactions in the wine fermentation. In the next step, the variation in initial cell densities of each member of the consortium was used as a tool to untangle the contribution of individual strain in the population dynamics and wine aroma. The data suggest that *S. cerevisiae* applied a selective pressure to suppress the growth of main competitor in the wine ecosystem. Moreover, the presence of individual non-*Saccharomyces* species at a higher cell density, favoured the growth of some non-*Saccharomyces* species while suppressing the growth of others in the yeast consortium. Lastly, the effect of temperature and sulphur addition on the dynamics of the yeast consortium was evaluated in the synthetic must and real grape must fermentations. The results demonstrated that ecological interactions are largely independent of the matrix, confirming that the constructed multi-species consortium is a robust model that can be used as a tool to predict microbial behavior from a simple ecosystem to the complex natural environment. Furthermore, the effect of temperature and sulphur dioxide on the growth of non-*Saccharomyces* species was species/strain dependent.

The results suggest that environmental parameters modify the pattern of population dynamics. However, ecological interactions seem to drive the wine ecosystem. The current study for the first time revealed the potential of a multi-species yeast consortium in understanding the ecological interactions in wine fermentation.

Opsomming

Wynfermentasie is ‘n komplekse biochemiese proses wat deur die opeenvolgende ontwikkeling van verskeie swak en sterk fermenterende gis spesies gekarakteriseer word. Dus, in so ‘n multi-spesie konsortium, is daar ‘n interaksie tussen die individuele spesies en tussen die spesies en hul omgewings. Gevolglik sal die finale chemise komposisie van die wyn beduidend deur die bydrae van individuele gis spesies en die gis-gis interaksies teenwoordig in die wyn beïnvloed word. Die bydrae van individuele gisrasse in die wynkonsortium word deur verskeie biotiese (ekologiese interaksies, “killer” faktore en druifvariëteit) sowel as abiotiese faktore (temperatuur, swaweldioksied, suurstof en die beskikbaarheid van voedingstowwe) beïnvloed. Elke gisras sal egter verskillend deur die kombinasie van die faktore beïnvloed word. Vorige studies wat handel oor wynfermentasie het hoofsaaklik op gemengde kultuur fermentasies met twee gis spesies gefokus. Dus is die onderliggende fundamentele reëls van die faktore in ‘n multi-spesie ekosisteem nie volledig verstaan nie.

Om die beginsels wat die komplekse wyn ekosisteem regeer te ontsyfer, is ‘n vereenvoudigde model konsortium, bestaande uit agt spesis wat algemeen voorkom in Suid-Afrikaanse druifmikrobiota, gevestig. ‘n Outomatiese ribosomale intergeniese spasiëringsanalise (ORISA) metode was ook ontwikkel om die bevolkings dinamika van die giskonsortium te monitor. Die invloed van die teenwoordigheid van *Saccharomyces cerevisiae* as ‘n biotiese stres faktor op die dinamika van die giskonsortium in ‘n sintetiese druiwemos was ondersoek deur die uitplaat metode en ORISA te gebruik. Die giskonsortium was verder gebruik as ‘n inokulum in Cenin blanc druiwe mos waar die bevolkingsdinamika deur die uitplaat metode gemonitor was. Die resultate bevestig dat die geselekteerde druk wat deur die hoeksteen spesies, *S. cerevisiae*, uitgevoer was die patroon van die bevolkingsdinamika verander het. Die wynekosisteem was gekarakteriseer deur die ondersteunende en inhiberende interaksies. Ten spyte van die verskille tussen die twee druifmatrikse is ‘n soortgelyke patroon van bevolkingsdinamika in beide fermentasies waargeneem. Hierdie observasie stel die wye toepassing van die model konsortium om die ekologiese interaksies in wynfermentasie te bestudeer voor. In die volgende stap is die variasie in aanvanklike seldigthede van elke lid van die konsortium gebruik as ‘n “tool” om die individuele bydrae van elke individuele gisras in die bevolkingsdinamika en wynaroma te bepaal. Die data stel voor dat *S. cerevisiae* ‘n selektiewe druk toegepas het om die groei van die hoof kompeteerder in die wynekosisteem te onderdruk. Die teenwoordigheid van individuele nie-*Saccharomyces* spesies teen ‘n hoër seldigtheid, het ook die groei van sommige nie-*Saccharomyces* spesies bevoordeel terwyl dit die groei van ander in die giskonsortium onderdruk het.

Laastens is die effek van temperatuur en swawel toevoegings op die dinamika van die giskonsortium geëvalueer in die sintetiese druiwe mos en werklike druiwe mos fermentasies. Die

resultate demonstreer dat ekologiese interaksies grootliks onafhanklik is van die matriks, wat bevestig dat die bewerkstelling van ‘n multi-spesies konsortium ‘n robuuste model is wat gebruik kan word as ‘n tool om die mikrobiale gedrag van ‘n eenvoudige ekosisteem tot ‘n komplekse natuurlike omgewing kan voorspel.

Daarbenewens is die effek van temperatuur en swaweldioksied op die groei van nie-*Saccharomyces* spesies afhanklik van die spesies/ras. Die resultate stel voor dat omgewings faktore die patroon van bevolkingsdinamika verander. Nietemin blyk dit dat die ekologiese interkasies die wynekosisteem dryf. Die huidige studie het vir die eerste keer die potensiaal van ‘n multi-spesies giskonsortium onthul om die ekologiese interaksies in wynfermentasie te verstaan.

This dissertation is dedicated to my parents

Biographical sketch

Bahareh Bagheri was born in 1982. She pursued a Bachelor degree in Food Science. She obtained her Master's degree in Biotechnology in 2014 from Stellenbosch University under the supervision of Dr Evodia Setati. She was accepted as PhD student under the supervision of Dr Evodia Setati and Professor Florian Bauer at Stellenbosch University.

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Preface

This dissertation is presented as a compilation of six chapters and one appendix. Each chapter is introduced separately and referencing is done according to the style of the journal Frontiers in Microbilology to which Chapter three was submitted for publication.

Chapter 1	**General Introduction and project aims**
Chapter 2	**Literature review** Microbial consortia: A tool to decipher microbial interactions and dynamics in various industrial processes
Chapter 3	**Research results I** The Impact of *Saccharomyces cerevisiae* on a wine yeast consortium in natural and inoculated fermentations
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Chapter 1

Introduction and project aims

1.1 Introduction

The main challenge in microbial ecology is to link microbial compositions to their function. This is, in particular, a difficult task in fermentation processes where the myriad of interactions may occur (Smid and Lacroix, 2013; Alonso-del-Real *et al*., 2017b). For instance, wine fermentation is typically initiated by a complex microbial community composed of several oxidative and weakly fermentative yeast of the genera *Cryptococcus*, *Rhodotorula, Issatchenkia, Hanseniaspora, Metschnikowia and Candida (*Bagheri *et al., 2015*; Eder *et al*., 2017; De Filippis *et al*., 2017). These non-*Saccharomyces* species dominate the initial stage of fermentation. However, they are sequentially replaced by the strong fermentative yeasts, mainly *Saccharomyces cerevisiae*, which proceeds the fermentation to completion (Alonso-del-Real *et al*., 2017a; Sternes *et al*., 2017; Eder et al., 2017). *S. cerevisiae* has been the focus of research for several years. However, several studies have reported that some non-*Saccharomyces* species persist throughout fermentation and contribute positively or negatively to wine aroma and flavour (Ciani *et al*., 2006; Ciani *et al*., 2010; Tristezza *et al*., 2016; Mas *et al*., 2016; Čuš *et al*., 2017). Hence, there was a shift toward understanding the role and contribution of these non-*Saccharomyces* species in wine fermentation (Fleet, 2008; Sadoudi *et al*., 2012; Gobbi *et al*., 2013; Jood *et al*., 2017).

Over the years, researchers have developed simplified co-culture fermentations to unravel the role and contribution of non-*Saccharomyces* species in wine fermentation (Boynton and Greig, 2016; Del Fresno *et al*., 2017; Shekhawat *et al*., 2017; Alonso-del-Real *et al*., 2017b). Owing to these simplified co-culture systems, it is well established that persistence of each strain in wine fermentation relies on fitness of the strain within the microbial community which is affected by many other physico-chemical and microbiological factors (Ciani *et al*., 2010; Alonso-del-Real *et al*., 2017a; Alonso-del-Real *et al*., 2017b). Investigating the effect of biotic and abiotic parameters such as temperature, sulphur addition, inoculum dosage and oxygenation, in mixed culture fermentations revealed that growth and metabolism of yeast species were affected significantly by these parameters. (Moreira *et al*., 2005; Beltran *et al*., 2006; Sadoudi *et al*., 2012; Gobbi *et al*., 2013; Shekhawat *et al*., 2017; Alonso-del-Real *et al*., 2017a). For instance, different combinations of *Saccharomyces*/non-*Saccharomyces* strains have been shown to produce wines with distinctive aromatic profiles, different from those produced by single culture of S*. cerevisiae* (Suzzi *et al*., 2012; Tofalo *et al*., 2016; Del Fresno *et al*., 2017). On the other hand, some data have indicated that a decrease in fermentation temperature and an increase in oxygenation at various stages of fermentation enhance the ethanol tolerance and persistence of non-*Saccharomyces* species such as *Hanseniaspora uvarum* and *Lachancea thermotolerans* in wine fermentation (Erten, 2002; Torija *et al*., 2003; Andorra *et al.*, 2010; Gobbi *et al*., 2013; Shekhawat *et al*., 2017). Similarly, non-*Saccharomyces* species such as *L. thermotolerans*, *S. bacillaris,* and *H. uvarum* persisted longer in wine fermentation when they were present at a higher cell density in grape musts or in early stage

of fermentations (Bagheri *et al*., 2015; Gobbi *et al*., 2013; Alonso-del-Real *et al*., 2017b). In contrast, sulphur addition was shown to enhance the fitness of *S. cerevisiae* while adversely affecting the growth of some non-*Saccharomyces* species such as *H. uvarum* and *Schizosaccharomyces pombe* in wine fermentation (Yang, 1975; Cocolin and Mills, 2003; Albertin *et al*., 2014).

Overall, using these simplified co-culture systems has generated a wealth of valuable information. Some studies have reported on antagonistic interactions between *S. cerevisiae* and *L. thermotolerans*, *S. cerevisiae* and *S. bacillaris* as well as *S. cerevisiae* and *Torulaspora delbrueckii.* In contrast, others have reported on a mutualistic interaction between *S. cerevisiae* and *M. pulcherrima* as well as a neutral interaction between *S. cerevisiae* and *Torulaspora delbrueckii* (Sadoudi *et al*., 2012; Sun *et al*., 2014; Gobbi *et al*., 2013; Taillander *et al*., 2014). However, these studies have mainly focused on small consortia composed of 2 or 3 strains whereas, natural conversion of grape sugars to wine involves a complex multi-species ecosystem. Thus, in spite of all effort devoted in the last decades, limited information is available on yeast-yeast interactions in multi-species consortia such as wine fermentations. Consequently, it is unknown how more complex wine microbial consortia are affected by biotic and abiotic parameters. Since understanding such dynamics would be an important step for better management of the fermentation process, it is essential to develop microbial consortia that serve as a representative of wine ecosystem. Such a consortium would serve as a model on which different permutations of winemaking parameters could be tested in order to simulate natural and inoculated fermentations; to decipher yeast-yeast interactions and manage population dynamics as well as fermentation tempo in order to achieve specific wine sensorial profiles.

1.2. Project aims

The aim of this project was to establish a reliable, robust model system that would serve as a predictive tool to study yeast-yeast interactions and population dynamics in wine fermentation. To achieve this aim, four objectives were set as follows:

1. Develop a model yeast consortium and reliable method to monitor yeast population dynamics.
2. To monitor population dynamics and determine possible interactions during fermentation.
3. Evaluate the effect of initial cell density on the dynamics of the yeast consortium and wine aroma in synthetic grape juice fermentation.
4. Evaluate the effect of temperature and sulphur addition on the dynamics of the yeast consortium and wine aroma in synthetic and real grape juice fermentation.

1.3. References

Albertin, W., Chasseriaud, L., Comte, G., Panfili, A., Delcamp, A., Salin, F., Bely, M. (2014). Winemaking and bioprocesses strongly shaped the genetic diversity of the ubiquitous yeast *Torulaspora delbrueckii*. *Plos one*, *9* (4). https://doi.org/10.1371/journal.pone.0094246.

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2010). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *Eur. Food Res. Technol.* 231, 215–224. doi:10.1007/s00217-010-1272-0.

Alonso-del-Real, J., Lairón-Peris, M., Barrio, E., and Querol, A. (2017a). Effect of Temperature on the Prevalence of *Saccharomyces* Non *cerevisiae* species against a *S. cerevisiae* wine strain in wine fermentation: Competition, Physiological Fitness, and Influence in Final Wine Composition. *Frontiers* in Microbiology, 8:150. doi: 10.3389/fmicb.2017.00150.

Alonso-del-Real, J., Contreras-Ruiz, A., Castiglioni, G. L., Barrio, E., and Querol, A. (2017b). The use of mixed populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to reduce ethanol content in wine: Limited Aeration, inoculum proportions, and sequential inoculation. *Front. Microbiol.* 8:150, doi:10.3389/fmicb.2017.02087.

Bagheri, B., Bauer, F. F., and Setati, M. E. (2015). The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. *S. Afr. J. Enol. Vitic.* 36, 243–251.

Beltran, G., Novo, M., Leberre, V., Sokol, S., Labourdette, D., Guillamon, J. M., et al. (2006). Integration of transcriptomic and metabolic analyses for understanding the global responses of low-temperature winemaking fermentations. *FEMS Yeast Res.* 6, 1167–1183. doi:10.1111/j.1567-1364.2006.00106.x.

Boyton, P. J., Greig, D. (2016). Species richness influences wine ecosystem function through a dominant species. *Fungal Ecol*, 22, 61-72.

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

Ciani, M., Comitini, F., Mannazzu, I., Domizio, P. (2010). Controlled mixed culture fermentation: A new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research*, *10*, 123–133. doi:10.1111/j.1567-1364.2009.00579.x

Cocolin, L., and Mills, D. A. (2003). Wine yeast inhibition by sulfur dioxide: A comparison of culture-dependent and independent methods. *Am. J. Enol. Vitic*, *54* (2), 125–130.

Cuš, F., Zabukovec, P., and Schroers, H.-J. (2017). Indigenous yeasts perform alcoholic fermentation and produce aroma compounds in wine. *Czech J. Food Sci.* 35, 339–345. doi:10.17221/398/2016-CJFS.

De Filippis, F., Parente, E., and Ercolini, D. (2017). Metagenomics insights into food fermentations. *Microb. Biotechnol.* 10, 91–102. doi:10.1111/1751-7915.12421.

Del Fresno, J. M., Morata, A., Loira, I., Bañuelos, M. A., Escott, C., Benito, S., et al. (2017). Use of non-*Saccharomyces* in single-culture, mixed and sequential fermentation to improve red wine quality. *Eur. Food Res. Technol.* 243, 2175–2185. doi:10.1007/s00217-017-2920-4.

Eder, M. L. R., Reynoso, C., Lauret, S. C., Rosa, A. L., and Rosa, A. L. (2017). Isolation and identification of the indigenous yeast population during spontaneous fermentation of isabella (*Vitis labrusca L .*) Grape Must. 8, 1–8. doi:10.3389/fmicb.2017.00532.

Erten, H. (2002). Relations between elevated temperatures and fermentation behaviour of *Kloeckera apiculata* and *Saccharomyces cerevisiae* associated with winemaking in mixed cultures. *World J.Microbiol. Biotechnol*. 18, 373–378.

Fleet, G. H. (2008). Wine yeast for the future. *FEMS Yeast* Res, 8, 979–995.

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

Jood, I., Hoff, J. W., and Setati, M. E. (2017). Evaluating fermentation characteristics of *Kazachstania* spp. and their potential influence on wine quality. *World J. Microbiol. Biotechnol.* doi:10.1007/s11274-017-2299-1.

Mas, A., Padilla, B., Esteve-Zarzoso, B., Beltran, G., Reguant, C., and Bordons, A. (2016). Taking advantage of natural biodiversity for wine making: The WILDWINE Project. *Agric. Agric. Sci. Procedia* 8, 4–9. doi:10.1016/j.aaspro.2016.02.002.

Moreira, N., Mendes, F., Hogg, T., and Vasconcelos, I. (2005). Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. *Int. J. Food Microbiol.* 103, 285–294. doi:10.1016/j.ijfoodmicro.2004.12.029.

Sadoudi, M., Tourdot-Marechal, R., Rousseaux, S., Steyer, D., Gallardo-Chacon, J. J., Ballester, J., et al (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol* 32: 243–253.

Shekhawat, K., Bauer, F. F., and Setati, M. E. (2017). Impact of oxygenation on the performance of three non-*Saccharomyces* yeasts in co-fermentation with *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-016-8001-y.

Sternes, P. R., Leea, D., Kutynaa, D. R., and Borneman, A. R. (2017). A combined meta-barcoding and shotgun metagenomic analysis of spontaneous wine fermentation. *bioRxiv* 98061, 1–30. doi:https://doi.org/10.1101/098061.

Smid, E.J., Lacroix, C. (2013). Microbe–microbe interactions in mixed culture food fermentations. *Curr. Opin. Biotechnol*. 24, 148–154.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., et al. (2012). Multistarter from organic viticulture for red wine Montepulciano d'Abruzzo production. *Front. Microbiol.* 3, 1–10. doi:10.3389/fmicb.2012.00135.

Tofalo, R., Patrignani, F., Lanciotti, R., Perpetuini, G., Schirone, M., Di Gianvito, P., et al. (2016). Aroma profile of montepulciano d'abruzzo wine fermented by single and co-culture starters of autochthonous *Saccharomyces* and non-*Saccharomyces* yeasts. *Front. Microbiol.* doi:10.3389/fmicb.2016.00610.

Torija, M. J., Rozès, N., Poblet, M., Guillamón, J. M., Mas, A. (2003). Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol*, *80* (1), 47–53. https://doi.org/10.1016/S0168-1605(02)00144-7.

Tristezza, M., Tufariello, M., Capozzi, V., Spano, G., Mita, G., and Grieco, F. (2016). The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Front. Microbiol.* 7, 1–14. doi:10.3389/fmicb.2016.00670.

Yang, H. Y., (1975). Effect of sulfur dioxide on the activity of *Schizosaccharomyces pombe*. *Am. J. Enol. Vitic*, 26, 1-4.

Chapter 2

Literature review

Microbial consortia: A tool to decipher microbial interactions and dynamics in various industrial processes

2.1. Introduction

Since the first discovery of yeast in wine fermentation, by Louis Pasteur, scientists have tried to understand the role and contribution of microorganisms in the food and beverage industries (Lin and Tanaka, 2006; Jeffries *et al*., 2006; Del Fresno *et al*., 2017). This has led to researchers isolating microorganisms from natural ecosystems in order to characterise their physiology, morphology and genetic properties in simplified single culture systems under laboratory conditions (Kolkman *et al*., 2005; Kolkman *et al*. 2006; Pham and Wright 2008). Consequently, the knowledge generated by single culture systems, has led to the commercialisation of several strains in the food and beverage industries (Laffort, 2015; Lallemand, 2015). However, single culture systems present numerous challenges and in order to meet consumer demands for diversified products, there was a shift towards the application of mixed-culture fermentations that rely on microbial consortia, mainly composed of two species (Bader *et al*., 2010; Gaikwad *et al*., 2014).

The term consortium was initially introduced by Johanes Reinke (1872a), who defined it as a system comprising two species, where the mutual relationship between the species turns them into a unit (Reinke, 1872a and, b; Reinke, 1873). Microbial consortia have been shown to be capable of performing complex tasks over short time periods by using a coordinated system, which results in a division of labour (Little *et al*., 2008; Lindemann *et al*., 2016). Furthermore, appealing properties of microbial consortia such as species richness, division of labour as well as their functional robustness, makes them a great platform to study microbial interactions (Ghosh *et al*., 2016). Thus, there is rapidly growing interest in the development of microbial consortia with specific strains to study microbial interactions in less complicated and more manageable systems. Such an approach has been shown to successfully identify several mutualistic or antagonistic interactions in fermentation processes, suggesting the potential of these systems to unravel the microbial interactions from simplified ecosystems to natural consortia (Bader *et al*., 2010; Jia *et al*., 2016).

There are several examples where microbial consortia have been successfully used in different industries including wastewater treatment, bioremediation, biofuel and food fermentations (Bernstein *et al*., 2012; Jagmann and Philipp, 2014; Jia *et al*., 2016). Evidently, the outcome of microbial consortia relies on the existing interactions between members of consortia. This review provides a summary of microbial interactions that exist between species in microbial ecosystems and highlights various industrial processes wherein microbial consortia are successfully applied. Furthermore, a comparative analysis of microbial consortia with single culture systems is presented, in order to demonstrate the benefits and drawbacks of each system.

2.2. Past and present of microbial ecology

For decades researchers have attempted to understand microbial ecosystems, using single species under laboratory condition. Monocultures are relatively simple systems, commonly used to investigate the microbial growth kinetics, the response of microorganisms to stress (biotic and abiotic), as well as cell-cell communication (Cooper *et al*., 2000; Mendes-Ferreira *et al*., 2007; Rossignol *et al*., 2009; Long *et al*., 2009; Boedicker *et al*., 2009). Furthermore, the knowledge gained from single species systems has resulted in the development of commercial starter cultures in different industries (e.g. wine and dairy industries).

Single species systems have increased our understanding of food safety and food quality (Seman *et al*., 2002; Barbosa *et al*., 2015; Quinto *et al*., 2016). For instance, modelling the growth of *Listeria monocytogenes* on processed meat has revealed that the conditions can be formulated (e.g. adding a higher amount of both sodium diacetate and potassium lactate) in such a way that growth of the pathogen (*L. monocytogenes*) decreases significantly (Seman *et al*., 2002; Barbosa *et al*., 2015; Quinto *et al*., 2016). Successful application of single species systems has also been reported in the production of food additives and preservatives such as carotenoid and nisin (Buzzini, 2001; Simova *et al*., 2004b). For instance, a wild-type *Lactococcus* strain isolated from raw milk was characterized with high nisin production rates, exhibiting strong nisin-mediated antibacterial and/or anti-*staphylococcal* activity in natural ecosystems such as skim milk and traditional Greek fresh whey cheese (Parapouli *et al*., 2013).

In spite of the knowledge gained using the single species systems, these systems have limited scope for industrial applications (Taniguchi *et al*., 1997, Lynch and Poole, 1979; Harish Kumar Reddy *et al*., 2010; Jia *et al*., 2016; Jiang *et al*., 2017). Consequently, research has shifted instead toward using microbial consortia. Microbial consortia hold appealing characteristics which makes them an attractive tool to gain insight into complex interactions present in natural ecosystems (Padilla *et al*., 2017; Brethauer and Studer, 2014).

A clear distinction between a single species system and a microbial consortium is that the consortium is capable of performing complex tasks in a limited amount of time through the division of labour (Sun and Cheng, 2002; Fu *et al*., 2009). Robustness of the microbial consortia to environmental stress or genetic perturbation is another advantage of these systems compared to single species systems (Bernstein and Carlson, 2012; Shong *et al*., 2012; Chubiz *et al*., 2015). This robustness relies on the conflict and cooperation interactions (competition, mutualism, and antagonism) occurring in the natural ecosystem (Hibbing *et al*., 2010; Blanchard and Lu, 2015).

2.3. Interactions within microbial ecosystems

Microbial ecosystems (e.g. food fermentations, wastewater, and biofilms) are characterized by complex communities in which a myriad of interactions may occur (Table 2.1). These organisms interact with their environment (microbes-environment interaction), within their population

(intraspecific interaction) or with the population of other species (interspecific interaction) (Smid and Lacroix, 2013; Cappello *et al*., 2017). Some interactions may take place as a result of the physical contacts between organisms (parasitism and symbiosis) while other interactions occur due to the changes in the physiochemical properties of the ecosystem (Verachtert *et al*., 1990; Nissen *et al*., 2003; Little *et al*., 2008). These interactions may have no effect on the fitness of members involved or may affect them positively or negatively (Verachtert *et al*., 1990; Liu *et al*., 2017). Hence, the function of the ecosystem is affected extensively by these interactions (Mounier *et al*., 2008; Boynton *et al*., 2016; Widder *et al*., 2016).

Symbiosis is a close relationship between two species in which at least one species benefits from the relationship while the second species can remain unaffected or it can be affected positively or negatively (Little *et al*., 2008). For instance, production of sake relies on symbiotic interactions between Koji molds, bacteria and yeasts (Tochikura *et al*., 2001; Furukawa *et al*., 2013). The symbiosis in sake fermentation starts with the saccharification of starch to glucose by koji molds, followed by the conversion of glucose to lactic acid and ethanol by yeasts and lactic acid bacteria (Figure 2.1A) (Furukawa, *et al*., 2013). Ethanol and lactic acid, in turn, stimulate the growth of acetic acid bacteria. Similarly, production of Bulgarian yoghurt also relies on the symbiotic interaction between *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* (Angelove *et al*., 2009). *S. thermophilus* stimulates the growth of *L. bulgaricus*, by creating anaerobic conditions whereas, *L. bulgaricus* in return generates the necessary compounds (amino-acids and peptides) for the growth of *S. thermophilus* (Angelove *et al*., 2009).

Parasitism occurs in the scenario in which one partner is benefited in the relationship at the expense of another (Little *et al*., 2008). A well-known example of parasitism was observed in a yoghurt fermentation where the fermentative capability of *S. thermophilus* was negatively affected by the presence of a bacteriophage (Figure 2.1B) (Forde and Fitzgerald, 1999; Ghosh *et al*., 2016).

Mutualism is the interaction in which both partners are positively affected by the symbiosis (Little *et al*., 2008). The mutualistic relationship was detected in kefir granules (Figure 2.1C), where *Lactobacillus kefiranofaciens* provided the carbon source (lactic acid) for *Saccharomyces cerevisiae* whereas, *S. cerevisiae* in return stimulated the growth of *L. kefiranofaciens* by utilizing the lactic acid and increasing the pH (Cheirsilp *et al*., 2003). Similar mutualistic interaction between *Zygotorulaspora florentina* and *Lactobacillus hordei* was observed in a water kefir model system (Stadie *et al*., 2013). *Z. florentina* produced the amino acid required for the growth of *L. hordei* whereas, *L. hordei* in return provided the low pH environment required for the growth of *Z. florentina* in the water kefir ecosystem (Stadie *et al*., 2013).

Commensalism takes place when one member benefits greatly from the symbiosis while the other partner remains unharmed (Dethlefsen *et al*., 2007; Ghosh *et al*., 2016). Commensalism has been observed on several occasions in fermentation processes (Dizy and Bisson, 2000; Cheirsilp *et al*.,

2003; Fleet, 2003). For instance, commensalism was reported in wine fermentations where amino acids produced by non-*Saccharomyces* species with high proteolytic activity (e.g. *Metschnikowia pulcherrima*) were used by *S. cerevisiae* (Figure 2.1D) (Dizy and Bisson, 2000; Fleet, 2003). Wine fermentation was not the only ecosystem where commensalism has been observed. This interaction has been also reported in cheese processing where substrate consumption and metabolite production were enhanced as a result of the interaction between *Geotrichum candidum* and *Penicillium camembertii* (Aziza and Amrane 2006). In this scenario, the presence of *G. candidum* enhanced the growth and metabolite production by *P. camembertii* whereas, the growth of *G. candidum* was not affected by the presence of *P. camembertii* (Aziza and Amrane 2006). Furthermore, a commensalism between *Lactobacillus Brevis* subsp *lindneri* with *S. cerevisiae* and *Lactobacillus plantarum* with *S. cerevisiae* was observed in a sourdough model system. The growth of bacteria was enhanced due to the excretion of valine and leucine by *S. cerevisiae* whereas; the growth of *S. cerevisiae* was not affected by the presence of the bacterium in the ecosystem (Gobbetti *et al*. 1994).

Antagonism happens when one partner is negatively affected by the relationship while the other partner is unharmed (Dethlefsen *et al*., 2007; Ghosh *et al*., 2016). Often the growth of one partner is inhibited by metabolites produced by the other partner (Benkerroum *et al*., 2005; Branco *et al*., 2015; Branco *et al*., 2017). There are several occasions in food fermentations where antagonistic interactions have been reported (Comitini *et al*., 2004; Hatoum *et al*., 2012). In most scenarios, growth of food pathogens or spoilage organisms has shown to be suppressed by another organism as a result of the antagonistic interactions between the two (Commitini *et al*., 2004; Dortu *et al*., 2008; Hatoum *et al*., 2012). For instance, growth of *L. monocytogenes* in fermented sausage was inhibited by bacteriocins produced by *Lactobacillus curvatus* and *Lactobacillus lactis* (Benkerroum *et al*., 2005). The antagonistic interactions were reported in wine fermentation in which growth of spoilage yeasts (*Brettanomyces*) was suppressed as a result of killer toxins produced by *Wickerhamomyces anomalus* and *Kluyveromyces wickerhamii* (Commitini *et al*., 2004). Similarly, antimicrobial peptides (AMPs) produced by *S. cerevisiae* have been shown to inhibit the growth of non-*Saccharomyces* species such as *Lachancea thermotolerans* and *Hanseniaspora guilliermondii* (Figure 2.1E) (Albergaria *et al*., 2010).

Competition happens when partners compete for the same nutrients (e.g. nitrogen and carbon source) to survive, affecting both negatively. However, the population of one member is commonly increased in comparison to the other member (Christensen *et al*., 2002; Foster and Bell, 2012; Lleixà *et al*., 2016). Numerous studies have sought evidence *of* competition between organisms in food and beverages (Mounier *et al*. *et al*., 2008; Lleixà *et al*., 2016). For instance, anti-fungal activities of *Aureobasidium pullulans* and *Pichia caribbica* derived from competition for nutrients suppressed the growth of *Penicillium expansum* and *Rhizopus stolonifera* in apple and peach,

respectively (Janisiewicz *et al*., 2000; Bencheqroun *et al*., 2007; Xu *et al*., 2013). In another scenario, the competition for nutrients was observed in a yeast-bacterium consortium where the rapid depletion of nutrients resulted in a sluggish fermentation (Figure 2.1F) (Bayrock and Ingledew, 2004; Arnink and Henick Kling, 2005).

Neutral relationship, seldom occurring in natural ecosystems, is a relationship in which none of partners are benefited or harmed (Hubbell *et al*., 2001; Sadoudi *et al*., 2012). Sadoudi *et al*., (2012), has reported a neutral interaction between *T. delbruecki and S. cerevisiae* in wine fermentation.

Table 2.1. List of studies that have reported on microbial interactions in food and beverage ecosystems.

Ecosystem	Interacting taxa	Type of interaction	Target Organism	Result of interactions	References
Sake	Molds, LAB, AAB, yeasts	Symbiosis	-	Production of Sake	Furukawa *et al*., 2013
Yoghurt	*L. bulgaricus* *S. thermophilus*	Symbiosis	-	Production of Bulgarian yoghurt	Angelove *et al*., 2009
Water Kefir	*Z. florentina*	Mutualism	*L. hordei*	Increasing biomass (yeast and LAB)	Stadie *et al*., 2013
Cheese	*G. candidum (Gc)*	Commensalism	*P. camembertii*	Higher growth rates for *Gc*	Aziza and Amrane, 2006
Sourdough	*L. brevis/ plantarum*	Commensalism	*S. cerevisiae*	Higher growth rate for LAB	Gobbetti *et al*., 1994
Meat	LAB	Antagonism	*L. monocytogenes*	Suppressing *Listeria*	Hatoum *et al*., 2012
Wine	*W. anomalus* *K. wickerhamii*	Antagonism	*Brettanomyces* (*Brett*)	Suppressing the growth of *Brett*	Comitini *et al*., 2004

The abbreviations are as follows: LAB: Lactic acid bacteria and AAB: acetic acid bacteria

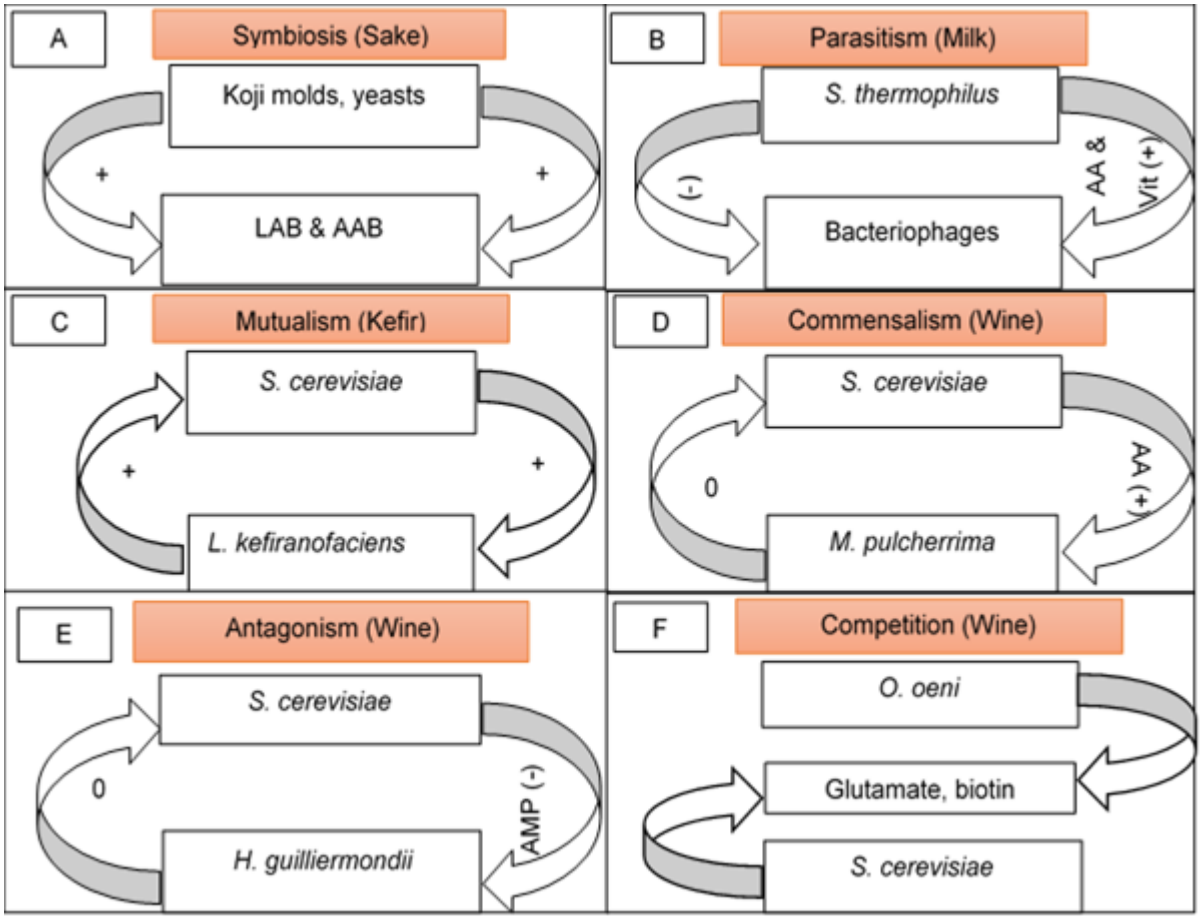

Figure 2.1. Schematic representation of microbial interactions present in food and beverage fermenting systems. The abbreviations are as follows: LAB: Lactic acid bacteria, AAB: acetic acid bacteria, AA: amino acid, Vit: vitamin, AMP: an Anti-microbial peptide. The positive and negative effect of one organism on the other organism is defined with a plus (+) and minus (-).

2.4. Microbial consortia as ecological model systems

Analysis of whole ecosystems with all of their associated diversity is a difficult task. To address the issue of complexity, scientists have used microbial consortia as simplified model systems where the interaction between organisms can be studied in a less complicated and a more manageable ecosystem (Jia *et al*., 2016; Jiang *et al*., 2017). In this context, “Model systems” refer to small groups of organisms that function as a representation of other biological systems with higher diversity and complexity (Bolker, 2009; Levy and Currie, 2015). Therefore, model systems can serve as theoretical and experimental bases, providing a platform for manipulation, observation, and comparison for hypothesis testing (Escalante-Espinosa *et al*., 2005; O’Malley, 2015; Tan *et al*., 2017).

Dallinger was one of the pioneer scientists who used the populations of protists as a model system to investigate the effect of temperature on organisms. The author demonstrated that competitive fitness of organisms decrease when cells were exposed to new temperatures (Dallinger, 1887).

After more than two decades, Woodruff, in 1914 used the protists as a model system to study density-dependent growth and succession of organisms. The result indicated that interactions between organisms are the driving force in the successive pattern of the protozoan ecosystem (Woodruff, 1914). Later on, Eddy in 1928 and Gause in 1934 constructed a multi-species consortium to investigate the mechanism underlying the microbial succession in microbial ecosystems.

The application of microbial consortia went to another era in 1961, with the groundbreaking publication of Francois Jacob and Jacques Monod, who introduced the cellular regulation by molecular networks in *Escherichia coli*. In conjunction with the work of Jacob and Monod, and in part owing to the information gleaned from molecular cloning, PCR and full genome sequencing of *S. cerevisiae* and *E.coli* in the 1970s to 1990s, researchers have developed sophisticated consortia that are used in the food, environmental, and other industrial sectors (Ghosh *et al*., 2016; Jiang *et al*., 2017). Today, microbial consortia can be classified into natural and engineered systems (Ver Berkmoes *et al*., 2009; Wang *et al*., 2013; Kato *et al*., 2014; Tan *et al*., 2017).

2.4.1. Natural microbial consortia

The classification of “natural microbial consortia (NMC)” is considered self-explanatory. Natural consortia are often multi-species communities where microbes depend on each other for production of specific metabolic substrates (Venters *et al*., 2017). In this context, defined natural consortia (DNMC) are typically designed from indigenous species which are present in high abundance in natural ecosystems such as fermented food, soil or wastewater (Ghazali *et al*., 2004; Gobbi *et al*., 2013; Shekhawat *et al*., 2017). Thus, knowing the estimates of species in a defined microbial consortium is an advantage compared to an undefined microbial consortium where a myriad of interactions can occur. Such systems can be perturbed under well-controlled conditions, providing an excellent platform to study microbial interactions in a more manageable ecosystem. However, the level of complexity (number of the species) in such systems is a pragmatic choice, depending on the objectives of specific experiments (Renault *et al*., 2015; Zomorrodi *et al*., 2014; Jia *et al*., 2016).

Defined natural consortia are commonly used to explore the microbial interactions, population dynamics as well as proteomic and transcriptomic responses of organisms under controlled conditions (Fazzini *et al*., 2010; Mehlomakulu *et al*., 2014; Shekhawat *et al*., 2017; Gobbi *et al*., 2013; Wang *et al*., 2014; Tronchoni *et al*., 2017). For instance, Prado and Kerr, (2008), demonstrated that three types of *E. coli* strains (producing, sensitive and resistant) exhibited nontransitive competitive relationships and their coexistence was favored when competition and dispersal took place. Similarly, a defined microbial consortium comprising three yeasts and six bacteria was used as a model system to investigate fungal interactions during the ripening of a

smear cheese (Mounier *et al*., 2008). Using this model consortium revealed that growth of *Geotrichum candidum* was inhibited by *Yarrowia lipolytica* whereas growth of *Debaryomyces hansenii* was inhibited by *Y. lipolytica* and *G. candidum* (Mounier *et al*., 2008).

Today, defined natural microbial consortia are being used successfully in food fermentation, wastewater treatment and biofuel industry (Bader *et al*., 2010; Ghosh *et al*., 2016; Tan *et al*., 2017; Jiang *et al*., 2017). However, recent advances in DNA sequencing, metagenomics, and metatranscriptomics have opened opportunities to study structure and function of microbial communities with a higher resolution (Maron *et al*., 2007; Wecks *et al*., 2010; Hong *et al*., 2016). Consequently, research gradually moved toward understanding function of ecosystems rather than focusing on the role of few species within ecosystems (Swenson *et al*., 2000a; Swenson *et al*., 2000b; Tan *et al*., 2017). Ecosystems such as cheese, grape must, soil or microbial biofilms that harbor a wide variety of organisms (yeast, bacteria, filamentous fungus, and algae) are therefore examples of undefined natural ecosystems (Young *et al*., 2004; Imran *et al*., 2010; Tan *et al*., 2017). Commonly, a small piece of nature composed of an unknown population is selected in order to investigate the community composition or to test their potential for performing the desired task (Handelsman *et al*., 1998, Swenson *et al*., 2000b; Watanabe *et al*., 2001; Ghosh *et al*., 2015). In this context, microcosm experiments have become a valuable tool in microbial ecology, increasing scientific understanding of natural processes (Bell and Foster, 2012). For instance, a study of growth and substrate degradation in aquatic bacterial microcosms, revealed that over the course of the experiment, there was a shift in bacterial interactions from antagonism toward a more neutral state (Rivette *et al*., 2016). Using microcosms the author confirmed that this substantial reduction in the strength of negative interactions during the bacterial succession was due to the shift in resource utilisation by the bacterial community (Rivette *et al*., 2016). Similarly, a naturally occurring planktonic bacterial community was selected out of many ecosystems, which could efficiently degrade an environmental pollutant, 3-chloroaniline (Swenson *et al*., 2000a).

Scientists have become more aware of the potential of undefined natural microbial consortia and currently, the focus has been on exploring these systems for industrial application (Bernstein, 2013; Gatti *et al*., 2014). For instance, Italian cheese such as Parmigiano Reggiano and Grana Padano exhibited distinct properties when produced using an undefined natural whey starters (Gatti *et al*., 2014). The author indicated that robustness of starter culture during cheese fermentation was directly correlated with the presence of a rich consortium of microbes (Gatti *et al*., 2014). Furthermore, an undefined natural bacterial consortium isolated from coffee mucilage was used to produce lactic acid from food waste such as peaches peel, spent coffee grounds and almond shells (Bretón-Toral *et al*., 2017).

2.4.2. Engineered microbial consortia

The root of “engineered microbial consortia” can be traced to the landmark work by Gardner who constructed the first synthetic toggle switch and repressilator in *E. coli* (Gardner *et al*., 2000). Since the discovery of Gardner, there is a growing interest in the construction of engineered microbial consortia (Jia *et al*., 2016; Ghosh *et al*., 2016). Inspired by the powerful features of natural consortia, engineered microbial consortia are developed to fulfil certain objectives by creating obligatory interactions or genetic modifications (Kirkup and Riley, 2004; Eitemann *et al*., 2008; Aydemir *et al*., 2014, Pasotti *et al*., 2017; Tan *et al*., 2017).

Engineered consortia are commonly developed with organisms of clear genetic backgrounds (e.g. *E*. *coli* and *S. cerevisiae*). Engineered single species consortia composed of different strains of the same species, aim to explore intra-species interactions, cell-cell communications or improving the function of the systems (Kirkup and Riley, 2004; Eitemann *et al*., 2008). For instance, the function of the system was improved where a consortium of two *E. coli* strains (xylose-selective mutant strain and glucose-selective *E. coli* strains), consumed sugar (xylose and glucose) 15% faster than the single strain of *E. coli* (Eitemann *et al*., 2008). It is important to stress that single species consortia have limited scope due to their relatively low complexity and may fail to adapt to the conditions present in natural ecosystems (Brenner *et al*., 2008; Jia *et al*., 2016). Hence, more attention was paid to engineer consortia composed of organisms with different genetic backgrounds in order to take advantage of diversity in order to develope systems with a higher efficiency (Xu *et al*., 2011; Bhatia *et al*., 2015; Jia *et al*., 2016).

2.4.2.1. Artificial, synthetic and semi-synthetic consortia

Engineered microbial consortia with two or more members are divided into three categories including artificial, synthetic and semi-synthetic (Bernstein and Carlson, 2012). “Artificial microbial consortia (AMC)”, aim at improving the performance of the system by developing an obligatory co-habitation among two or more ‘wild-type’ organisms which do not coexist naturally (Kim *et al*., 2008; Tang *et al*., 2012; Bernstein, 2013). For instance, the yield of ethanol production was improved in an artificial consortium of two *Clostridium* species (*C. thermocellum* and *C. thermolactricum*) compared to the respective monoculture system (Xu *et al*., 2011).

“Synthetic microbial consortia (SMC)”, aim to improve the nutrient utilization and the metabolism of systems by encouraging or enforcing interactions between two or more genetically modified organisms (Bernstein and Carlson, 2012; Bernstein, 2013; Wang *et al*., 2016). On the other hand, “semi-synthetic consortia (SsMC)”, are a hybrid system composed of one wild-type and one genetically engineered organism (Bernstein and Carlson, 2012; Bernstein, 2013; Wang *et al*., 2016). These consortia have been built based on several interactions including metabolite

exchange as well as synergistic division of resources (Minty et al., 2013; Bhatia *et al*., 2015; Wang et al., 2016).

The production of 2-keto-L-gulonic acid (the precursor of vitamin C) from D-sorbitol was achieved in one step instead of 2 steps, by creating a mutualistic interaction in a synthetic consortium between *Gluconobacter oxydans* and *Ketogulonicigenium vulgare* (Wang *et al*., 2016). Similarly, a synthetic fungus-bacterium consortium consisting of *Trichoderma reesei* and *E. coli* was successfully used to produce isobutanol from lignocellulosic biomass. The cellulases produced by *T. reesei* were used as a substrate for the growth of both species (Minty *et al*., 2013).

On the other hand, using a semi-synthetic consortium comprised of an engineered cyanobacterial species (*Synechococcus elongatus*) and a wild-type *S. cerevisiae*, the photosynthetic productivity of cyanobacteria *Synechococcus elongates* was enhanced (Ducat *et al*., 2012). Similarly, a higher amount of fatty acid methyl esters (124%) was produced in a semi-synthetic consortium composed of a mutant of *Streptomyces coelicolor* with *Ralstonia eutropha*, compared to the single culture of *S. coelicolor* (Bhatia *et al*., 2015). The potential of microbial consortia for industrial application has been briefly underlined and the summary of the studies reviewed in this section are presented in figure 2.2.

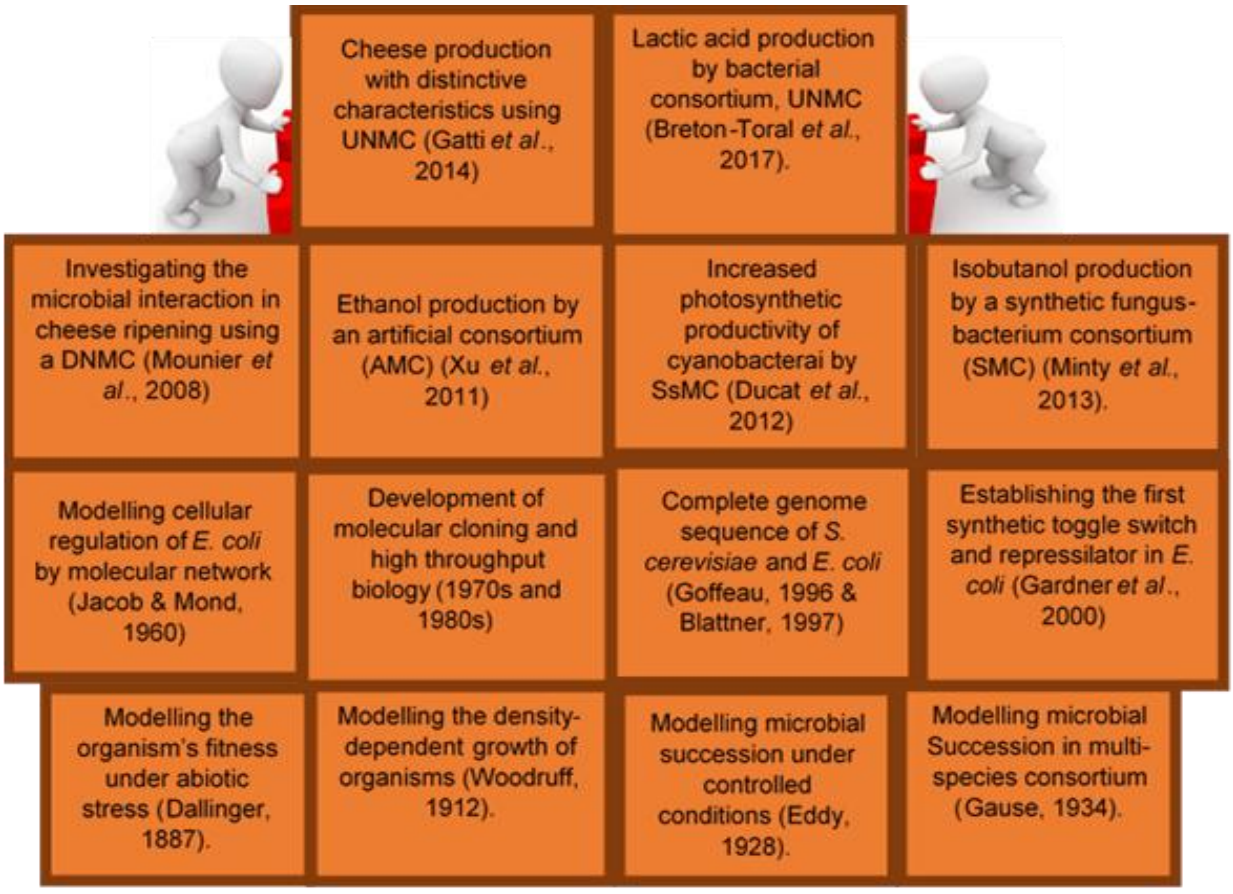

Figure 2.2. The list of discoveries in different industries, using microbial consortia.

2.5. Application of microbial consortia in different industries

There is a growing interest in the application of microbial consortia, from natural to engineered systems in different industries (Minty *et al*., 2013; Jiang *et al*., 2017). While undefined natural consortia have been used for commercial production of fermented food products such as cheese and bread for centuries, defined natural consortia (starter cultures) has already been used in the production of several fermented products such as yoghurt and wine (Siuewert *et al*., 2008; Chr, 2015; Laffort, 2015; Lallemand, 2015). On the other hand, consortia-based industrial processes have been developed in a range of applications including bioethanol production, municipal and industrial wastewater treatment as well as bioremediation (Du *et al*., 2015; Tsai *et al*., 2009; Mishra *et al*., 2015). Therefore, the application of microbial consortia in different industries will be reviewed in the following section.

2.5.1. Biofuel industry

Increased crisis regarding the price of oil and fuel as well as higher demands of renewable energy have encouraged scientists in the field of biofuels to seek solutions for more economical energy sources. Hence, great attention was paid to screen and isolate the organisms capable of producing biogas, alcohols (e.g. ethanol, propanol, and butanol), diesel and biohydrogen from different materials (Bader *et al*., 2010; Bhatia *et al*., 2015; Yen *et al*., 2015). The application of microbial consortia in ethanol and butanol production will be reviewed in the following section.

2.5.1.1. Ethanol production

Ethanol, initially produced by fermenting sugar (glucose or sucrose) present in corn and sugar cane has been considered as an environmentally friendly and economical alternative to gasoline fuel (Taniguchi, 1997; Mielenz, 2001; Chen, 2011). However, production of biofuel utilizing food as the raw material received criticisms (Jouzani and Taherzadeh, 2015). Therefore, research has focused on producing bioethanol from non-food materials, such as waste, agricultural and forestry residuals (Ezeji *et al*., 2007; Qureshi *et al*., 2010a; Qureshi *et al*., 2010b; Jouzani and Taherzadeh, 2015).

Bioethanol was traditionally produced from lignocellulosic biomass material using the pure culture of *S. cerevisiae*. However, this species on its own was not able to convert the mixture of hexoses (glucose, mannose, galactose, etc.) and pentoses (xylose, arabinose, etc.) to ethanol (Lin *et al*., 2006; Jeffries *et al*., 2006). Alternatively, applications of microbial consortia composed of an organism with efficient glucose fermentation rate (*S. cerevisiae*) in combination with another organism capable of fermenting xylose (e.*g. Pichia stipitis* and *Candida shehatae*) has gained popularity (Lebeau *et al*., 1997; De Bari *et al*., 2004; Rouhollah *et al*., 2007).

Different combinations of organisms, under batch or continuous fermentation, have been tested for this purpose (Okuda *et al*., 2008; Chen *et al*., 2011). For instance, high-quality ethanol, with the potential industrial application was produced from the wheat starch as a result of a synergistic interaction present in a yeast-bacterium consortium (*Dekkera bruxellensis* and *Lactobacillus vini)* (Passoth *et al*., 2007). Similarly, ethanol was produced from both waste house wood hydrolysate medium and mixed sugar fermentation media, using synthetic consortia of *E. coli* (genetically modified *E. coli* or recombinant *E. coli*) and *S. cerevisiae* (Beck *et al*., 1990; Okuda *et al*., 2008; Fu *et al*., 2009).

There are several articles highlighting the application of consortia composed of *S. cerevisiae* coupled with different yeast or bacteria, including *Pachysolen tannophilus*, *Zymomonas mobilis*, *Scheffersomyces stipitis* (previously known as *Pichia stipitis*), and *Candida tropicalis*, all of which have resulted in the successful production of ethanol from different sugar sources. However, in spite of the success of the microbial consortia to produce ethanol under laboratory conditions, industrial production of ethanol still faces some challenges (Taniguchi *et al*., 1997; Liu Laplace *et al*., 1993; Abate *et al*., 1996; Latif and Rajoka, 2001; De Bari *et al*., 2004; Rouhollah *et al* 2007; Okuda *et al*., 2008; Harish Kumar Reddy *et al*., 2010).

Alternatively, some attempts have been made toward the application of natural or synthetic multi-species consortia where function of the systems does not merely rely on two species. In this context, application of multi-species consortia in most cases has been shown to enhance the efficiency of the process possibly due to the contribution of different organisms with different genetic backgrounds (a division of labour) (Du *et al*., 2015; Tsai *et al*., 2009). For instance, a consortium comprising three anaerobic fungi (*Anaeromyces robustus, Neocallimastix california,* and *Piromyces finnis*) could efficiently degrade the lignocellulose using the enzyme produced by the fungi consortium (Du *et al*., 2015). Similarly, Tsai *et al*. (2009), constructed a synthetic consortium consisting of *Clostridium thermocellum, Clostridium cellulolyticum*, and *Ruminococcus flavefaciens*, capable of efficient production of ethanol, corresponding to 95% of the theoretical value. In another attempt, a multi-species consortium was developed from aerobic fungus and anaerobic ethanol producing bacteria (*Trichoderma reesei*, *S. cerevisiae* and *S. stipitis*), resulting in the efficient production of ethanol (67% yields) from wheat straw (Brethauer and Studer, 2014).

Overall, application of microbial consortia seems to be a promising path for the cost-effective production of ethanol from complex sugars, providing an alternative to food produced biofuels (Du *et al*., 2015; Tsai *et al*., 2009). However, more research needs to be conducted to solve the challenges of industrial production of ethanol. Thus, more effort needs to be applied in (1) screening and characterizing novel species for bioethanol production, (2) developing microbial consortia using novel microbial combinations and (3) investigating the metabolic interactions among members of microbial consortia (Chen, 2011).

2.5.1.2. Butanol

Butanol is an important chemical with various applications in the feedstock, chemical intermediate, production of solvents and plasticizers (Ezeji *et al*., 2007; Minty *et al*., 2013). The superior characteristics of butanol such as high energy content, good blending ability and high similarity to gasoline, makes it a suitable candidate as a next-generation liquid fuel (Dürre, 2007; Jiang *et al*., 2017). Currently, butanol is produced from the fermentation of corn or molasses; however, the production of butanol from the lignocellulosic biomass has recently gained popularity (Inui *et al*., 2008; Qureshi *et al*., 2013). Using lignocellulosic as a substrate for butanol production has a clear advantage over the other substrates since butanol, ethanol, and acetoin are produced simultaneously in one fermentation (Ezeji *et al*., 2012; Jurgens *et al*., 2012).

The first record of industrial production of butanol was reported in twentieth century, using *Clostridium acetobutylicum* (Inui *et al*., 2008). Since then, extensive research has been performed on the *Clostridium* genus, especially on the species *Clostridium acetobutylicum* and *Clostridium beijerinckii*, known to be capable of acetone, butanol, and ethanol production (ABE) (Inui *et al*., 2008; Qureshi *et al*., 2013). Despite the exclusive research done on this topic, bioethanol production from lignocellulosic biomass still faces several challenges. The strict anaerobic conditions required for the growth of *Clostridium* and the toxic effect of butanol against the cultures have been shown to decrease the efficiency of butanol production (Inui *et al*., 2008; Ezeji *et al*., 2012; Nanda *et al*., 2014). Alternatively, scientists have offered strategies such as (1) developing genetically modified strains with high butanol tolerance, (2) constructing the butanol synthetic pathway in the organisms with high butanol tolerance (e.g. *S. cerevisiae, E. coli*, *Pseudomonas putida* and *Bacillus subtilis*) and (3) application of synthetic or artificial consortia, consist of two organisms, have been applied to overcome these challenges (Tomas *et al*. 2003; Steen *et al*., 2008; Inui *et al*., 2008; Luo *et al*., 2017).

The first and second strategies could achieve up to 35% more ABE (16.91 g/L) with the genetically modified strains exhibiting up to 85% tolerance to butanol compared to the wild-type *Clostridium* strain (Tomas *et al*., 2003; Steen *et al*., 2008; Inui *et al*., 2008; Nielsen *et al*., 2009; Xiao *et al*., 2012). Recently, scientists have attempted to enhance butanol production by interrupting acetone production through a genetic knockout or replacing the acetone production with isopropanol production. The rationale behind replacing acetone with isopropanol was that the final product (isopropanol, butanol, and ethanol (IBE)), could be used as a fuel additive (Lee *et al*., 2012b; Ezeji *et al*., 2014; Sillers *et al*., 2008). The results of this approach have shown more efficiency in the production of IBE (21-27.9 g/L) compared to previous attempts. Nevertheless, most of the genetically modified strains carry plasmid DNA, and their genetic stability might be modified over a longer fermentation period (Lee *et al*., 2012b; Dusseaux *et al*., 2013; Ezeji *et al*., 2014; Sillers *et al*., 2008).

The third strategy, application of microbial consortia, has shown to be a promising path for butanol production. For instance, A co-culture consisting of *C. acetobutylicul* and *S. cerevisiae* resulted in a good yield of butanol and ABE production, 16.3 g/L, and 24.8 g/L, respectively (Luo *et al*., 2017). Similarly, a symbiotic co-culture system comprising the high ratio of *C. acetobutylicum* and a low ratio of *Bacillus cereus* produced 11.0 g/L butanol and the total of 18.1 g/L ABE under microaerobic conditions (Wu *et al*., 2016). The efficiency of this system was due to a symbiosis interaction between the two species in which depletion of oxygen by *B. cereus* provided an anaerobic environment for the growth of *Clostridium* (Wu *et al*., 2016). A similar synergistic interaction was observed in a consortium composed of *C. acetobutylicum* and *C. cellulolyticum* where the metabolic activity of *C. acetobutylicum* improved the cellulolytic activity of *C. cellulolyticum* (Salimi and Mahadevan, 2013). The constructed consortium has shown to produce 0.35 g/L butanol.

Wen *et al*., (2014a and b), constructed an artificial consortium by co-culturing a cellulolytic, anaerobic bacterium (*Clostridium cellulovorans*) and a non-cellulolytic *bacterium (Clostridium beijerinckii*). Using the artificial consortium, 2.64 g/L acetone, 8.30 g/L butanol, and 0.87 g/L ethanol were produced from a sole carbon source (extracted deshelled corn) in less than 80 hours. One of the most efficient ABE production (21.6 g/L) system was achieved in a consortium composed of *C. acetobutylicum* and *B. subtilis* in which spoilage date palm fruits were used as a substrate (Abd-Alla and El-Enany, 2012). *B. subitlis* maintained the strict anaerobic conditions by consuming the oxygen.

In conclusion, the butanol production using these co-culture systems have proven to be promising compared to using native or genetically modified single species. Furthermore, the efficiency of microbial consortia in most cases was due to the symbiotic interactions between the members of consortia. Consequently, developing microbial consortia composed of different combinations of organisms and investigating the microbial interactions in more details may provide the opportunity to develop consortia with improved efficiency.

2.5.2. Wastewater

Using microorganisms for the biodegradation of organic compounds in wastewater has a long history. Conventional biological wastewater treatment generates a huge amount of bacterial biomass that has little values. Alternatively, application of microalgae and filamentous fungi in wastewater treatment has gained attention (Abdel-Raouf *et al*., 2012; Mujtaba and Lee, 2016). The ability of microalgae to use inorganic nitrogen and phosphorus for their growth and their potential to produce valuable metabolites (e.g. sugar, fat and bioactive compounds) are some advantages of using microalgae in wastewater treatment (Abdel-Raouf *et al*., 2012; Mujtaba and Lee, 2016). Similarly, filamentous fungi have a high capacity of degrading complex carbohydrate and there were several reports in which valuable by-products such as fungal proteins, enzymes, and lactic

acid were produced when using fungus in wastewater treatment (Suntornsuk *et al*., 2002 Huang *et al*., 2003; Guest and Smith, 2002; Sankaran *et al*., 2010).

There are several reports confirming that single species have been successfully used for wastewater treatment (Table 2.2) (Bogan *et al*., 1960; shio *et al*., 1994; Stouthamer *et al*., 1997; Jin *et al*., 1998; Huang *et al*., 2003; Joo *et al*., 2007; Ruiz-Marin *et al*., 2010; Abdel-Raouf *et al*., 2012; Mujtaba and Lee, 2016). For instance, the production of fungal protein and amylase from the starch processing wastewater as well as the production of lactic acid from tomato wastewater was achieved using the single culture of *Aspergillus Oryzae* and *Rhizopus arrabizus*, respectively (Jin *et al*., 1998; Huang *et al*., 2003). Similarly, Ruiz-Marin *et al*. (2010), reported that *Scenedesmus obliquus* could remove nitrogen and phosphorus from artificial and municipal wastewater.

Despite the potential of single species systems in wastewater treatment, several reports have indicated that the mixed culture system of bacteria-algae, yeast-algae, and bacteria-yeast can improve the efficiency of the treatment process (Oswald *et al*., 1953; Hernandez *et al*., 2009; Xue *et al*., 2010; Ling *et al*., 2014). Indeed, the scientists in the field of wastewater treatment have attempted to compare the efficiency of single species systems to microbial consortia (Hirooka *et al*., 2003; Joo *et al*., 2007). For instance, a mixed culture of cyanobacterial (*Anabaena variabilis* and *Anabaena cylindrical*) was successfully used to remove 2, 4-dinitrophenol (2, 4-DNP) from industrial wastewater, without accumulating a potent mutagen, 2-amino-4-nitrophenol (2-ANP). The single culture of *A. variabilis* could also remove 2, 4-DNP. Nevertheless, 2-amino-4-nitrophenol (2-ANP) was accumulated in the system (Hirooka *et al*., 2003).

In a different study, the efficiency of ammonium removal from the wastewater in the single culture of *Alcaligenes faecalis* (wild-type and mutant) was compared to the mixed culture composed of a wild-type and a mutant of *A. faecalis* (Joo *et al., 2007)*. The result revealed that ammonium removal rate was two-fold higher in the mixed culture compared to the wild-type single culture of *A. faecalis* whereas, the denitrification was significantly higher in the mixed culture compared to the mutant single culture of *A. faecalis* (Joo *et al*., 2007). Similarly, the combined action of *Chlorella vulgaris* and *Bacillus pumilus* in an artificial consortium enhanced the efficiency of ammonium and phosphorus removal from the wastewaters (Hernandez *et al*., 2009).

Gaikwad *et al*., (2014), evaluated the degradation potential of five species individually as well as in the form of consortia. The result highlighted that the efficiency of microbial consortia (*Pseudomonas spp., Actinomycetes spp., Bacillus spp., Streptomyces spp.* and *Staphylococcus spp*.) to reduce COD and BOD (90.17% and 94.02%) was significantly higher than single species ranging from 42.11-59.76% for COD and 58.55-77.31% for BOD (L. Gaikwad *et al*., 2014). In an interesting approach, Mishra *et al*., (2015), evaluated the hydrogen production from the distillery wastewater using a single culture, a mixed culture and a multispecies consortium (*Klebsiella pneumonia*, *Citrobacter freundii* and *Bacillus coagulant*). The result revealed that hydrogen

production of the multi-species consortium was more efficient than the mixed culture and the single culture system.

Application of microbial consortia has shown to be a promising path to produce valuable products from the wastewater ecosystems (Xue *et al*. 2010; Ling *et al*., 2014). For instance, the mixed culture of algae and yeast (*Spirulina platensis*, *Rhodotorula glutinis*) has shown to increase the yield of total biomass and lipid production, from monosodium glutamate wastewater (Xue *et al*., 2010). Furthermore, the sequential inoculation of *Rhodosporidium toruloides* followed by *Chlorella pyrenoidosa* resulted in higher yield of lipid production and nutrient removal from the distillery wastewater compared to the microalgae and yeast pure cultures (Ling *et al*., 2014). Similarly, the application of mixed culture systems consisting of *Penicillium corylophilum* and *Aspergillus niger*, resulted in the maximum dry biomass production as well as the maximum reduction of COD (90%) (Alam *et al*., 2003).

To this end, we have reviewed the application of microbial consortia in wastewater treatment. In general, the results suggest that division of labour between members of microbial consortia serve to considerably enhance the efficiency of wastewater treatments. However, since wastewater ecosystems have limited nutrients, limited oxygen, high temperature and variable pH, it is challenging to select organisms that can adapt well to such a harsh environmental (Henze *et al*., 2008; Poleto *et al*., 2016). Thus, more efforts need to be applied for screening organisms capable of converting organic compounds into the desired products (Henze *et al*., 2008; Poleto *et al*., 2016).

Table 2.2. Application of single species systems and microbial consortia in wastewater treatment.

Target ecosystem	Organisms	Function of the consortium	References
Starch processing wastewater	*Aspergillus Oryzae*	Production of fungal protein and α-amylase	Jin *et al*., 1998
Wastewater	*Scenedesmus obliquus*	Efficient ammonium & phosphate removal	Ruiz-Marin *et al*., 2010
Wastewater	*Chlorella vulgaris*, *Bacillus pumilus*	Efficient ammonium and phosphorus removal	Hernandez *et al*., 2009
Distillery wastewater	*Rhodosporidium toruloides, Chlorella pyrenoidosa*	Lipid production and nutrient removal	Ling *et al*., 2014
Monosodium glutamate wastewater	*Spirulina platensis, Rhodotorula glutinis*	Total biomass and lipid production	Xue *et al*., 2010
Industrial wastewater	*Anabaena variabilis, Anabaena cylindrical*	2,4-Dinitrophenol removal	Hirooka *et al*., 2003
Municipal wastewater	*Penicillium corylophilum, Aspergillus niger*	Maximum dry biomass production and COD reduction	Alam *et al*., 2003
Distillery wastewater	*Rhodosporidium toruloides Chlorella pyrenoidosa*	High yield of lipid production and nutrient removal	Ling *et al*., 2014
Municipal wastewater	*Enterobacter cloacae, Gordonia and Pseudomonas putida*	Efficient removal TOC	Chen *et al*., 2009

Distillery wastewater	*Klebsiella pneumonia, Citrobacter freundii, and Bacillus coagulant*	Efficient hydrogen production	Mishra *et al*., 2015

2.5.3. Food and beverage industry

Fermentations can be regarded as one of the oldest bioprocesses for the production of food and beverages. Fermentation processes including cheese, sourdough, yoghurt and wine are driven by complex microbial communities. For instance, sourdough fermentation is composed of a stable association of more than 50 species of lactic acid bacteria and 20 species of yeasts (De Vuyst and Neysens, 2005). The indigenous population present in such consortia interact with one another and also with the fermentable substrate throughout the fermentation process (Smid and Lacroix, 2013). These interactions play an important role in shelf life, organoleptic properties and quality of fermented products (Smid and Lacroix, 2013). Consequently, there has been a growing interest in exploring natural food fermentation processes and more precisely attempts have been made to link the community function and population dynamics (Sieuwert *et al*., 2008; Smid and Lacroix, 2013). Since natural consortia are difficult to scrutinize, microbial consortia have been developed to facilitate our understanding of fermentation processes and microbial interactions (Dicks *et al*., 2004; Bensmira *et al*., 2010; Leite *et al*., 2012).

2.5.3.1. Dairy industry

Application of starter cultures including *L. bulgaricus* and *S. thermophiles* in the production of yoghurt is a common practice (Routray and Mishra, 2011). Thus, successful manufacture of the fermented milk products such as yoghurt, cheese, kefir, and ayran mainly relies on the microbial interactions (Routray and Mishra, 2011; Settachaimongkon *et al*., 2014; Prado *et al*., 2015). Several researchers have used microbial consortia as a tool to better understand the microbial interactions in the fermentation of the dairy products (Radke-Mitchell and Sandine, 1984; Courtin and Rul *et al*., 2004; Soomro and Masud, 2008; Bensmira *et al*., 2010; Leite *et al*., 2012; Settachaimongkon *et al*., 2014; Prado *et al*., 2015).

The effect of inoculum dosage and incubation temperature on the physical property of yoghurt was investigated using a model yoghurt consortium consisting of *S. thermophiles* and *L. delbrueckii* and a few other probiotic strains (Lee and Lucey, 2004). The results indicated that higher inoculation rate and incubation temperature negatively affected the yoghurt tangent value whereas, lower inoculation rate and higher temperature seem to negatively affect the whey separation of yoghurt (Lee and Lucey, 2004). In a similar approach, the effect of temperature on the growth of *S. thermophiles* and *L. bulgaricus* was evaluated in single culture and mixed culture fermentations. The results indicated that the growth of *L. bulgaricus* in single culture fermentations was enhanced at a higher temperature (37, 42, and 45°C) whereas optimum growth temperatures had no effect

on the growth of *L. bulgaricus* and *S. thermophiles* in the mixed culture fermentation (Radke-Mitchell and Sandine, 1984).

Microbial consortia have been used to investigate the mechanism underlying microbial interactions in fermented products (Sieuwerts *et al*., 2010). For instance, a combination of model system and transcriptomic analysis was applied to investigate molecular mechanism underlying the interaction in the yoghurt consortium composed of *S. thermophilus* and *L. bulgaricus* (Sieuwerts *et al*., 2010). The results indicated that interaction between the two species was the consequence of the exchange of metabolites in which formic acid, folic acid, and fatty acids were provided by *S. thermophiles* and the amino acid provided by *L. bulgaricus*. Furthermore, the study revealed that genes coding for exopolysaccharide production were upregulated in both organisms in mixed culture compared to monocultures (Sieuwerts *et al*., 2010).

Microbial consortia have been employed to explore the potential of the naturally occurring strains in fermented milk, yoghurt and kefir fermentations for industrial application (Soomro and Masud, 2008). It is worth adding that the starter cultures for yoghurt production are commonly selected from the indigenous population of raw milk (Figure 2.3) (Sieuwerts *et al*., 2008; Routray and Mishra, 2011). The selected strain should exhibit moderate tolerance to sugar, and resistance to phages and must produce yoghurt with a pleasant flavor and right consistency but without excessive acidity (Tramer, 1973; Soomro and Masud, 2008; Sieuwerts *et al*., 2008; Routray and Mishra, 2011; Chandrapala and Zisu, 2016). For instance, Soomro and Masud, (2008), developed several consortia from naturally occurring *L. delbrueckii* and *S. thermophiles* in yoghurt fermentations. Evaluating the important technological properties (e.g. acetaldehyde production) of different consortia, Soomro and Masud, (2008), identified two strains of *L. delbrueckii* LB5 and *S. thermophiles* ST4 that were compatible and could produce yoghurt with a pleasant aroma.

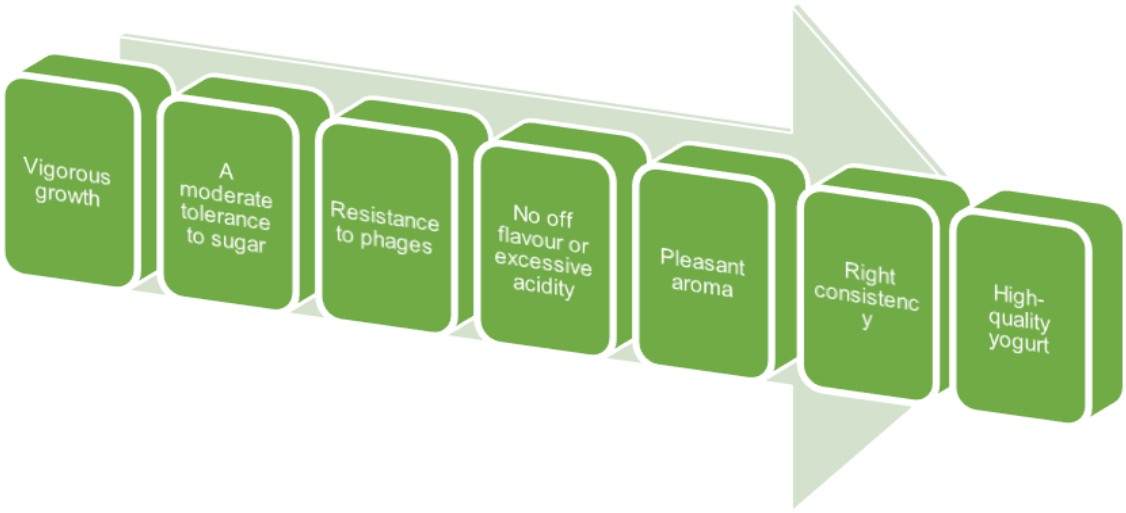

Figure 2.3. The selection criteria for the ideal starter culture for yoghurt fermentation.

Scientists attempted to explore the potential application of multi-species consortia in the production of yoghurt (Park and Lee, 2015; Ersan *et al*., 2016). In this context, the quality of soy yoghurts produced from a two species consortium (*L*. *bulgaricus* and *S*. *thermophiles*) was compared to that of a three species consortium (*Lactobacillus acidophilus, Bifidobacterium lactis,* and *S. thermophilus*). It was revealed that the yoghurt produced from the multi-species consortium contained higher organic acids and free amino acids compared to the yoghurt produced by the two-species consortium (Park and Lee, 2015). Successful application of multi-species consortia was also observed in other fermented products such as ayran and kefir (Soomro and Masud, 2008; Baruzzi *et al*., 2016). For instance, ayran produced by a multi-species consortium composed of three indigenous strains (*L. delbrueckii, Lactobacillus paracasei,* and *S. thermophiles*) exhibited extensive shelf-life of about one month (Baruzzi *et al*., 2016).

Using microbial consortia, scientists have obtained a deep understanding on the effect of various biotic and abiotic parameters on growth of microbial population (Öner *et al*., 2009; López *et al*., 2015; Palavecino Prpich *et al*., 2016). Study the microbial interactions in simplified consortia with defined organisms seem to be a useful tool to untangle the complexity of natural ecosystems. The positive outcome of such studies has resulted in commercialization of starter cultures in the dairy industry. Today, application of multi-starter cultures is a common practice for in the fermentation of some dairy products such as yoghurt and kefir (Smid and Lacroix, 2013; Prado *et al*., 2015; Sieuwerts, 2016). Hence, more effort needs to be applied to explore the potential of indigenous population in order to identify the best strain combinations for an optimal fermentation and flavour balance (Settachaimongkon *et al*., 2014).

2.5.3.2. Sausage and salami manufacturing industry

Sausage and salami are traditional Mediterranean meat products that are commonly consumed in countries such as Italy, France and Hungary. Indigenous microorganisms present in salami/sausage fermentation are known to possess typical sensory quality (Sparo *et al*., 2008; Magistà *et al*., 2016). Thus, investigating the microbial diversity present in the fermented meat opened the avenue for screening, isolating and identifying the strains that hold the potential to be used as commercial starter cultures (Frece *et al*., 2014; Magistà *et al*., 2016).

Starter culture for the salami and sausage fermentations are typically selected from the strains displaying enzymatic activities and the strains that are capable of producing bacteriocin or other anti-microbial compounds (Antara *et al*., 2004; Talon *et al*., 2007; Lopez *et al*., 2011; Santa *et al*., 2014; Magistà *et al*., 2016). The former has shown to confer typical sensory characteristics in meat production while the later may replace part of the preservation process (Antara *et al*., 2004; Lopez *et al*., 2011; Santa *et al*., 2014; Magistà *et al*., 2016).

Several studies have evaluated the potential of single culture species for sausage production. For instance, the potential of the indigenous strain of *Penicillium salami*, (isolated from salami seasoning) for small and industrial scale production of sausage was evaluated (Magistà *et al*., 2016). The strain maintained the viability and was well adapted to the fermentation process and therefore proved to be a suitable starter culture for the meat industry (Magistà *et al*., 2016). On the other hand, *L. plantarum* displayed a good growth property in the sausage fermentation and was able to show antimicrobial activities against the pathogen strains such as *L. monocytogenes*, *Staphylococcus aureus* and *E. coli* (Kargozari *et al*., 2015).

Other single culture systems such as *Pediococcus pentosaceous* or *Lactobacillus sakei*, have been used to investigate the effect of starter culture on volatile profiles of an Italian fermented sausage (Montanari *et al*., 2016). The results highlighted the considerable differences in volatile organic compounds deriving from pyruvate metabolism, using single species systems (Montanari *et al*., 2016).

Research slowly moved toward evaluating the potential of microbial consortia for sausage/ salami production. For instance, the potential of a defined natural consortium, composed of *Lactobacillus sakei* and *Staphylococcus carnosus,* for the industrial production of sausage was compared with the commercial starter culture of BITEC LS-*25 (*Gewürzmüller, GmbH, Stuttgart, Germany*)* (Frece *et al*., 2014). The results indicated that the microbial consortium maintained viability under controlled condition and could produce a sausage with better organoleptic characteristics (acceptable acidity and low rancidity) compare to the commercial starter culture (Frece *et al*., 2014).

In a different study, Barbosa *et al*. (2015), developed a two-species consortium (*L. curvatus* and *L. monocytogenes*) in which the indigenous lactic acid (*L. curvatus*) exerted anti-Listeria activity against *L. monocytogenes* throughout the salami fermentation. Similarly, performance of a microbial consortium in the production of dry sausage was evaluated in a consortium composed of autochthonous *L. sakei* and *Staphylococcus vitulinus* (Palavecino Prpich *et al*., 2016). The result indicated that the growth of undesirable organisms (*Enterobacteriaceae*), as well as lipid oxidation was decreased using the consortium (Palavecino Prpich *et al*., 2016). In a different approach, the comparative peptidomic analysis of three sausage systems composed of the single culture of *L. curvatus* and *Staphylococcus vitulinus* and a consortium composed of *L*. *curvatus* and *S. vitulinus* revealed that the consortium exhibited the greatest peptide diversity and possesses a great potential for meat protein degradation (López *et al*., 2015).

Overall, the result revealed that microbial consortia hold great potential for the production of sausage with better organoleptic properties (López *et al*., 2015; Palavecino Prpich *et al*., 2016). The results also confirmed that microbial consortia can be used as a tool to suppress the growth of

undesirable/pathogenic organisms in sausage fermentations (Kargozari *et al*., 2015; Palavecino Prpich *et al*., 2016).

2.5.3.3. Cocoa manufacturing industry

The contribution of microbes in the spontaneous fermentation of cocoa was initially reported by Preyer-Buitenzorg in 1901. Consumers demand for cocoa with complex aromatic profiles, has shifted the focus toward the isolation and application of novel starter cultures for cocoa production (Ho *et al*., 2014; Visintin *et al*., 2017). Thus, exploring the potential of novel strains for commercial application in cocoa fermentation has increased tremendously (Lefeber *et al*., 2012; Crafack *et al*., 2013; Ho *et al*., 2014; De Vuyst and Weckx, 2016; Sandhya *et al*., 2016; Visintin *et al*., 2017). According to Steinkraus, 2004 and Pereira *et al*., (2012), potential starter cultures for coca industry, must be able to tolerate osmotic pressure, pH fluctuation (3.5-6) and metabolite accumulation (e.g. ethanol, lactic acid and acetic acid).

In this context, some scientists have compared the quality of cocoa produced spontaneously to the cocoa produced from single species (Lefeber *et al*., 2012; Crafack *et al*., 2013). Cocoa with enhanced flavor profiles was obtained using the single species systems (*Pichia kluyveri*, *Lactobacillus fermentum* or *Acetobacter pasteurianus)* compared to the cocoa produced from the spontaneous fermentation (Crafack *et al*., 2013).

Investigating the potential of microbial consortia for sausage production has gained attention recently. The first attempt for the application of a microbial consortium consists of yeast, lactic acid bacteria and acid bacteria (*S. cerevisiae, Lactobacillus lactis, Lactobacillus plantarum, Acetobacter aceti* and *Gluconobacter oxydans subsp. suboxydans*) in cocoa fermentation was done by Schwan in 1998. The cocoa produced by the microbial consortium proved to have the reasonable quality compared to the cocoa obtained from the spontaneous fermentation (Schwan, 1998). Similarly, the study conducted by Lefeber *et al*., (2012), revealed that the cocoa produced by the consortium composed of *Lactobacillus fermentum, Acetobacter pasteurianus, and S. cerevisiae* had a superior quality compared to the cocoa produced from the single culture fermentation of lactic acid and acetic acid bacteria.

In general, up to now, no starter cultures have been used for the commercial production of a large scale (Lefeber *et al*., 2011; Lefeber *et al*., 2012; Crafack *et al*., 2013; Ho *et al*., 2014). The lack of knowledge of the process of cocoa bean fermentation has hampered the production of commercial starter cultures (De Vuyst *et al*., 2010). However, the recent studies have underlined the positive contribution of microbial consortia in organoleptic quality of the fermented cocoa (Crafack *et al*., 2013; Sandhya *et al*., 2016; Visintin *et al*., 2017). Hence, more research needs to be done on exploring the potential of indigenous strains, finding the right combination of strains and the

optimum environmental parameters for the production of cocoa with more complex aromatic profiles (Schwan and Wheals, 2004; Lima *et al*., 2011; Pereira *et al*., 2013; Sandhya *et al*., 2016).

2.5.3.4. Wine fermentation

The art of winemaking by humankind has a history of over 7000 years. Traditionally, wine is produced by the indigenous microbial consortia present in the grape must (Bagheri *et al*., 2015; Ghosh *et al*., 2015; Tofalo *et al*., 2016). Spontaneous fermentations are still being used in boutique wineries in several countries such as France, Italy and South Africa (Pretorius *et al*., 1999; Pretorius, 2000). The fermentation of grape must and production of wine with the premium quality and consistent flavour is a complex process that involves thousands of interactions among the microbial species (Ciani *et al*., 2006; Sadoudi *et al*., 2012; Gobbi *et al*., 2013). Hence, several attempts have been made to isolate and characterize the indigenous strains present during alcoholic fermentation (Di Maro *et al*., 2007; Bagheri *et al*., 2015; Sun *et al*., 2009).

Indeed, wine research before 1998 was mainly focused on single species systems to understand the role of main yeast, *S. cerevisiae,* in wine fermentation, (Visser *et al*. 1990; Walfridsson *et al*. 1995). Application of these single species systems has tremendously increased our understanding of microbial growth, their response to stress and their oenological properties in wine fermentation (summary in table 2.3) (Visser *et al*. 1990; Kolkman *et al*., 2005; Kolkman *et al*. 2006; Pham and Wright, 2008). For instance, oxygen requirement of yeast species was evaluated, using single species systems (Visser *et al*. 1990). The results revealed that with the notable exception of *S. cerevisiae*, all other non-*Saccharomyces* species were unable to grow under strictly anaerobic conditions (Visser *et al*. 1990).

Single species systems have also been used to investigate the mechanisms underlying the superior growth of *S. cerevisiae* under wine fermentation (Kolkman *et al*., 2005; Kolkman *et al*. 2006; Pham and Wright, 2008; Li *et al*., 2010; Aceituno *et al*., 2012). For instance, study the proteomic response of *Candida albicans* and *S. cerevisiae* under amino acid starvation indicated that the proteins closely associated with the glycolysis and gluconeogenesis pathways were downregulated (Yin *et al*. 2004). Similarly, a study by Pham *et al*., (2006), revealed that several proteins involved in the glycolysis/gluconeogenesis pathways such as Pdc1p, Adh1p, and Tal1p were significantly upregulated in *S. cerevisiae* under high-gravity fermentation conditions (high concentration of glucose). Other authors have suggested that upregulation of Pdc1p and Adh1p may provide an advantage for ethanol production whereas; upregulation of Tal1p has proven to enhance the growth of recombinant *S. cerevisiae* strains during ethanol fermentation from xylose (Pham *et al*., 2006; Pham and Wright, 2008; Walfridsson *et al*. 1995).

Furthermore, application of single species model systems has increased our understanding on quorum sensing (Leeder *et al*., 2011; Avbelj *et al*., 2015). For instance, investigating

cell/environment interactions in a single species system has revealed that aromatic alcohols such as 2-phenylethanol and tryptophol are quorum-sensing molecules, stimulating the pseudohyphae formation of *S. cerevisiae* under limited nitrogen conditions (Gimeno *et al*., 1992; Wuster and Babu, 2010; Leeder *et al*., 2011). Further studies on quorum sensing molecules in *S. cerevisiae*, indicated that the production of these molecules (e.g. 2-phenyl ethanol, tryptophol and tyrosol) are positively correlated with cell density and availability of ammonia in culture media, while their production was negatively correlated to ethanol stress (Chen and Fink, 2006; Wuster and Babu, 2010; Avbelj *et al*., 2015).

As a result of the knowledge gained using single-species systems, starter cultures of *S. cerevisiae* and other non-*Saccharomyces* species such as *Torulaspora delbrueckii* (Prelude™, Chr. Hansen, Denmark; Zymaflore® Alpha^{TD}, Laffort, France; Biodiva™ TD291, Lallemand, Canada) and *Lachancea thermotolerans* (Viniflora® Concerto™, Chr. Hansen, Denmark) has been developed. Today, application of commercial starter culture for the production of wine with predictable quality and consistent aroma is a common practice in the wine industry (Fleet and Heard, 1993; Pretorius *et al*., 1999; García-Ríos *et al*., 2014).

Table 2.3. The list of studies that have used single species systems to investigate the role of a single organism in the wine ecosystem.

Objective of the study	Result of the study	Reference
Evaluating the growth of yeast under anaerobic conditions	Lack of growth of non-*Saccharomyces* species	Visser *et al*., 1990
Proteomic response of yeast under amino acid starvation	Down-regulation of some proteins proteins	Yin *et al*., 2004
Proteomic response of yeast under high gravity fermentation conditions	Up-regulation of some proteins	Pham *et al*., 2006
Response of cells under nitrogen starvation	Pseudohyphae formation of *S. cerevisiae* by quorum sensing molecules	Gimeno *et al*., 1992
Factors affecting the quorum sensing	Production of quorum sensing positively correlated with cell density and ammonia availability	Wuster and Babu, 2010; Avbelj *et al*., 2015

Exploring the potential of non-*Saccharomyces* in wine aroma

In face of the defects of single species systems as well as the improved understanding of the potential of non-*Saccharomyces* species, research has shifted toward understanding the microbial interactions in mixed culture systems. Thus, researchers have used microbial consortia, mainly composed of 2 species, as simplified tools to study the oenological properties of non-*Saccharomyces* species in combination with *S. cerevisiae* (Medina *et al*., 2013; Gobbi *et al*., 2013; Sun *et al*., 2014; Ghosh *et al*., 2015).

Modulating the time of inoculation in a yeast-yeast consortium system allows studying the microbial interactions in co-inoculation or sequential approaches (Gobbi *et al*., 2013; Sadoudi *et al*., 2012; Jood *et al*., 2017). Co-culture inoculation in yeast-yeast consortia, involves the simultaneous

inoculation of *Saccharomyces* and non-*Saccharomyces* yeasts, while sequential inoculation strategies involves the inoculation of one species (commonly the non-*Saccharomyces*) first, followed by the other (commonly *S. cerevisiae*) 24 to 48 hours later (Gobbi *et al*., 2013; Sadoudi *et al*., 2012). Similarly, in a yeast-bacterium consortium, co-inoculation is defined as a scenario in which bacteria are inoculated prior to completion of alcoholic fermentation whereas, in sequential inoculation, bacteria are added after completion of primary fermentation (Sun *et al*., 2014; Versari *et al*., 2016; Strickland *et al*., 2016).

For instance, a consortium of *Saccharomyces cerevisiae* and *Oenococcus oeni* was used to investigate the effect of co-inoculation on the chemical and sensory profile of the commercial Cabernet Franc red wine (Versari *et al*., 2016). The authors found that the co-inoculation strategy decreased the length of fermentation, improved the conversion rate of malic into lactic acid and enhanced the sensory property of wine (Versari *et al*., 2016). Similarly, impact of *Pediococcus* spp. on quality of wine and growth of a *Brettanomyces bruxellensis* strain was also investigated in a co-inoculation system. The application of the consortium resulted in the higher population of both species and the production of wine with a lower concentration of 4-ethylphenol, compared to the single culture fermentation (Strickland *et al*., 2016).

Despite numerous studies using microbial consortia in the wine research, the best perspectives on wine ecosystem was usually achieved in studies that compared consortia with single species systems (Sun *et al*., 2014; Suzzi *et al*., 2012; Tofalo *et al*., 2016; Shekhawat *et al*., 2017). For instance, a study conducted by Sun *et al*., (2014), used the combination of a single species system and microbial consortia (*M. pulcherrima/S. cerevisiae* and *T. delbrueckii/S. cerevisiae)* to investigate the effect of two non-*Saccharomyces* species (*M. pulcherrima* and *T. delbrueckii*) on fermentation kinetics and wine aroma in pure cultures and in sequential inoculation with *S. cerevisiae*. While *M. pulcherrima* showed significant differences in cell biomass and fermentation kinetics, *T. delbrueckii* performed similarly in the pure and the mixed culture fermentations (Sun *et al*., 2014). The former result suggested the antagonistic interaction between *M. pulcherrima* and *S. cerevisiae*, whereas the latter suggested a negligible interaction between *T. delbrueckii* and *S. cerevisiae* (Sun *et al*., 2014). Concerning the aroma profiles, the production of esters and acetic acid was significantly higher in both mixed culture fermentations compared to the single culture fermentations. Furthermore, *M. pulcherrima*/*S. cerevisiae* was characterized by high production of higher alcohols, esters, acids, and terpenes, whereas *T. delbrueckii*/*S. cerevisiae* was associated with high production of fruity esters and higher alcohols (Sun *et al*., 2014).

In another attempt, Sadoudi *et al*. (2012), investigated the potential effect of yeast-yeast interaction on aromatic profiles of wine in pure culture (*S. bacillaris, T. delbrueckii,* and *M. pulcherrima*) and in three co-inoculated consortia consisting of individual non-*Saccharomyces* species (*M. pulcherrima, T. delbrueckii* and *S. bacilaris*) in combination with *S. cerevisiae*. In contrast to Sun *et al.* (2014),

Sadoudi *et al.* (2012), reported that due to the synergistic interaction between *M. pulcherrima* and *S. cerevisiae,* higher concentrations of aromatic compounds was produced in the mixed culture compared to the single culture. Interestingly, the author reported the presence of neutral interaction between *T. delbrueckii* and *S. cerevisiae* and therefore confirmed the previous result observed by Sun *et al*., (2014). Furthermore, the antagonistic interaction between *S. bacillaris* and *S. cerevisiae* was identified as a result of the significant decrease in norisoprenoids and terpenol production in the consortium, compared to the pure culture of *S. bacillaris* (Sadoudi *et al*., 2012).

In a different study, fermentation kinetics and microbial interactions in two consortia (*H. uvarum/S. cererviae* and *S. bacillaris/S. cerevisiae*) were compared to single culture fermentations (Suzzi *et al*., 2012). The pure culture of *H. uvarum* was characterized by high production of ethyl acetate and acetoin whereas, low levels of ethyl acetate was produced in the mixed culture of *H. uvarum* and *S. cerevisiae*. Furthermore, the synergistic interaction between *S. bacilaris* and *S. cerevisiae* increased the fermentation kinetics and the production of glycerol while decreasing the production of ethyl acetate and acetic acid (Suzzi *et al*., 2012). In a similar approach, mixed cultures of *S. cerevisiae* with *T. delbrueckii* have shown to produce low amount of ethyl acetate and acetic acid (Renault *et al*., 2015; Comitini *et al*., 2011) whereas, mixed cultures of *M. pulcherrima* and *S. cerevisiae* have shown to enhance the production of fatty acids, esters and terpenols (Comitini *et al*., 2011; Sadoudi *et al*., 2012).

The potential impact of non-*Saccharomyces* species on the production of volatile thiols was evaluated in two yeast consortia composed of *S. cerevisiae, H. uvarum, C. zemplinina, M. pulcherrima, T. delbrueckii* and *I. orientalis* (Zott *et al*., 2011). Non-*Saccharomyces* species were inoculated at 10^{4-5} CFU/mL and *S. cerevisiae* was inoculated at 10^{6} CFU/mL in consortium A, while it was inoculated at 10^{3} CFU/mL in consortium B. It was observed that consortium B, produced a higher amount of volatile thiols (3-sulfanylhexan-1-ol (3SH)) without any negative effect on residual sugar compared to the consortium A (Zott *et al*., 2011). Moreover, these results indicated that lower inoculation of *S. cerevisiae* provided the opportunity for non-*Saccharomyces* species to contribute to volatile thiol formation. In a different approach, the effect of different inoculation strategies (Single culture, two and three-yeast co-inoculations) on the sensory properties of wine was evaluated (King *et al*., 2010). The results indicated that volatile thiols and other flavour compounds were substantially different from the single culture fermentation. Moreover, wine produced from the inoculation of two species and three species were more favoured by the customers than the wine produced from the single culture inoculation, indicating the effect of inoculation strategy on consumer acceptance (King *et al*., 2010).

Based on the existing literature, the comparison between the sequential and co-inoculation strategy in yeast-bacteria, favours the co-inoculation strategy (Bartowsky *et al*., 2015; Zapparoli *et al*., 2009; Izquierdo Cañas *et al*., 2014; Nehme *et al*., 2010) whereas, in case of yeast-yeast

consortia, the results are rather contradictory. Some authors suggested that sequential inoculation strategies increase the persistence of non-*Saccharomyces* species and enhance the aromatic profile of wine (Clemente *et al*., 2004; Benito *et al*., 2015; Domizio *et al*., 2011; Comitini *et al*., 2011; Gobbi *et al*., 2013). In contrast, others have reported a decrease in fermentation rates and an increase in the production of off flavour compounds such as ethyl acetate (Bisson, 1999; Toro and Vazquez, 2002; Fleet, 2003; Ciani *et al*., 2006; Bely *et al*., 2008; Taillandier *et al*., 2014; Suzzi *et al*., 2012).

Overall, regardless of the applied inoculation strategy, there are many studies in which *Saccharomyces* and non-*Saccharomyces* consortia have been shown to bring about improved aroma profiles and complexity, underlining the potential of such consortia for industrial application in wine fermentation (Toro and Vazquez, 2002; Ciani *et al*., 2006; Bely *et al*., 2008; Medina *et al*., 2013; Gobbi *et al*., 2013; Izquierdo Cañas *et al*., 2014).

Exploring the microbial interactions in wine fermentation

Studies on yeast-yeast interactions using microbial consortia have repeatedly indicated that the presence of *S. cerevisiae* can negatively affect the growth of non-*Saccharomyces* species, albeit at varying degrees (Fleet, 2003; Toro and Vazquez, 2002; Suzzi *et al*., 2012). Indeed, *S. cerevisiae* has been shown to suppress the growth of several non-*Saccharomyces* species such as *M. pulcherrima, S. bacillaris,* and *H. uvarum.* However, in most of the scenarios, the mechanisms underlying these interactions are unclear (Hansen *et al*, 2001; Sun *et al*., 2014; Wang *et al*., 2015).

To better understand the dominance behaviour of *S. cerevisiae*, researchers have developed variable consortia to easily study the effect of environmental parameters (e.g. temperature and concentration of dissolved oxygen) on the growth of organisms (Hansen *et al*., 2001; Torija *et al*., 2003; Shekhawat *et al*., 2017). For instance, the effect of oxygen on the survival of two non-*Saccharomyces* species was investigated using a microbial consortium comprised of *L. thermotolerans*/*S. cerevisiae* and *T. delbrueckii*/*S. cerevisiae*. The results revealed that decline in the population of the two non-*Saccharomyces* species and persistence of *S. cerevisiae* in the fermentation was rather due to lack of oxygen and not the production of toxic compounds by *S. cerevisiae* (Hansen *et al*., 2001). A recent study by Shekhawat *et al*., (2017), evaluated the effect of aeration in 3 different consortia (*S. cerevisiae/M. pulcherrima*, *L. thermotolerans*/*S. cerevisiae* and *T. delbrueckii*/*S. cerevisiae*) and demonstrated that effect of oxygen on the growth of non-*Saccharomyces* is species-dependent and oxygen is a limiting factor in the growth of some non-*Saccharomyces* species.

Transcriptomic and proteomic approaches have been widely used to unravel the mechanisms underlying the succession of *S. cerevisiae* throughout fermentation (Egli *et al*., 1998; Andorrà *et*

al., 2010; Mostert, 2013; Kosel *et al*., 2017; Tronchoni *et al*., 2017). An investigation of the transcriptional response to biotic stress in mixed fermentations composed of *D. bruxellensis* and *S. cerevisiae* confirmed that genes involved in thiamine biosynthesis, as well as amino acid and polyamine transport, were induced, suggesting a competitive interaction between the two species (Kosel *et al*., 2017).

Investigating yeast-yeast/yeast-bacterium interactions, using microbial consortia revealed that the growth of some bacteria (*O. oeni)* and some non-*Saccharomyces* species (e.g. *Hanseniaspora guilliermondii, Hanseniapsora uvarum, Lachancea thermotolerans* and *Brettanomyces bruxellensis*) was suppressed by *S. cerevisiae* due to the production of antimicrobial peptides, short to medium-chain fatty acids and killer toxins (Albergaria *et al*., 2010; Gobbi *et al*., 2013; Albergaria *et al*., 2013; Branco *et al*., 2014; Wang *et al*., 2015; Ludovico *et al*., 2001; Fleet, 2003; Schmitt and Breinig, 2002; Comitini *et al*., 2005; Rizk *et al*., 2016).

Currently, studies exploring the use of microbial consortia in wine fermentations have moved beyond the two species interaction level. (Izquierdo Canas *et al*., 2011; Andorrà *et al*., 2012; Suzzi *et al*., 2012; Antalick *et al*., 2013; Zott *et al*., 2012; Padilla *et al*., 2017). Andorrà *et al*., (2012), investigated the microbial interaction in a two-species consortia (*H. uvarum*/*S. cerevisiae* and *S. bacillaris*/*S. cerevisiae)* compared to a multi-species consortium (*H. uvarum, S. bacillaris, and S. cerevisiae*) and could link the production of secondary metabolites with the contribution of individual species (Andorrà *et al*., 2012). The production of higher alcohol and in particular isobutyl alcohol was linked to the presence of *S. bacillaris* whereas; the production of isoamyl acetate and 2- Phenyl ethanol acetate was linked to the presence of *H. uvarum* and *S. cerevisiae,* respectively. Furthermore, the multi-species consortium produced higher levels of fatty acid esters compare to the pure cultures.

Table 2.4. A short summary of the studies that have used microbial consortia to explore microbial interactions in wine ecosystem.

Objective of the study	Result of the study	References
Impact of *Pediococcus* spp. on the growth of *Brettanomyces*	Higher cell biomass of B. *bruxellensis* and *P. parvulus* in the mixed culture	Strickland *et al*., 2016
Effect of yeast-yeast interaction on aromatic profiles of wine in single culture and mixed culture	Modified aromatic profiles of wine in the mixed culture	Sadoudi *et al*., 2012 Suzzi *et al*., 2012
Effect of co-inoculation on the chemical and sensory profile of the commercial wine	Increase in fermentation kinetics and improvement in MLF	Versari *et al*., 2016
Comparative analysis of microbial interactions in different consortia	Correlation between the contribution of individual species and the production of secondary metabolites	Andorrà *et al*., 2012

As a result of the knowledge gained using microbial consortia, several strains such as *M. pulcherrima* (Flavia™ Mp346, Lallemand, Canada), Pichia kluyveri (Frootzen™, Chr. Hansen, Denmark), and *Lachancea thermotolerans* (Viniflora® Concerto™, Chr. Hansen, Denmark) have

been commercialized. Minimal lag phase rehydration, good fermentative capability, moderate tolerance to ethanol, sulfite, and osmotic stress are important criteria (Figure 2.4) for the selection of the starter cultures in wine fermentation (Viana *et al*., 2008; Ciani *et al*., 2009; Comitini *et al*., 2011). Today, commercial application of yeast-yeast consortia such as *T. delbrueckii* and *S. cerevisiae* (Level^{2TD}, Lallemand, France) and *L. thermotolerans* and *S. cerevisiae* (Rhythm/Symphony, Chr. Hansen, Hørsholm, Denmark) has become a common practice in wine fermentations (Takush, 2009). Recently, a multi-species consortium composed of *L. thermotolerans, T. delbrueckii* and *S. cerevisiae* (Melody™, Chr. Hansen, Denmark) was commercialized for wine fermentation (Takush, 2009).

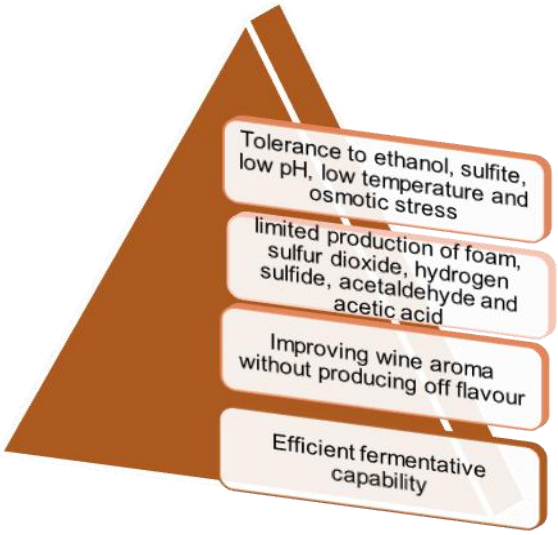

Figure 2.4. The pyramid of criteria required for the selection of yeast species as starter cultures

In conclusion, several attempts have been made to investigate the microbial interactions during alcoholic fermentation. The data suggest that microbial interactions in wine fermentations are affected by biotic and abiotic parameters (Gobbi *et al*., 2013; Sadoudi *et al*., 2012; Shekhawat *et al*., 2017). However, most of the studies have focused on small consortia, comprised of 2 species while wine fermentation is the result of several interactions in wine consortium (Bagheri *et al*., 2015, Shekhawat *et al*., 2017; Jood *et al*., 2017; Del Fresno *et al*., 2017). Thus, we are still unable to decipher the principles that govern the function and dynamics of wine ecosystems. Consequently, more effort is required to develop microbial consortia that can better represent the complex composition of the wine ecosystem. Indeed, studies in other industries have reported that microbial consortia with several and definite strains has good application prospect (Bader *et al*., 2010; Ghosh *et al*., 2016; Jia *et al*., 2016; Jiang *et al*., 2017). Such consortia can be used as a model system to better understand the complex interactions in the wine fermentation.

2.6. Conclusion and future prospect

Overall, our comparison of the single model systems and microbial consortia indicates that the latter can represent the complexity of natural ecosystem more realistically and provides a platform to develop engineered microbial consortia with the potential industrial application (Ver Berkmoes, *et al*., 2009; Klitgord and Segre, 2010; Klitgord and Segre, 2011). Indeed, the application of microbial consortia (natural and engineered) has been widely explored under laboratory conditions in order to overcome the technical barriers associated with single species systems. Nonetheless, successful application of microbial consortia under laboratory conditions does not guarantee their success under industrial conditions. Application of the consortia such as multi-species starter cultures in an industrial setting may result in the lack of efficiency or instability of the system. Thus, developing the successful microbial consortia requires a deep understanding of the existing microbial interactions in the wine ecosystem (Konopka, 2009; Sabra *et al*., 2010; Konopka *et al*., 2015; Lindemann *et al*., 2016). However, gaining a deep understanding of function and composition of the natural ecosystem is difficult mainly due to the complexity of such systems. Thus, more efforts are required to develop microbial consortia with higher diversity and study microbial interactions in a more detailed manner.

2.7. References

Abate, C., Callieri, D., Rodriguez, E., Garro, O., (1996). Ethanol production by a mixed culture of flocculent strains of *Zymomonas mobilis* and *Saccharomyces* sp. *Appl Microbiol Biotechnol*, 45, 580–583.

Abd-Alla, M. H., and Elsadek El-Enany, A. W. (2012). Production of acetone-butanol-ethanol from spoilage date palm (*Phoenix dactylifera* L.) fruits by mixed culture of *Clostridium acetobutylicum* and *Bacillus subtilis*. *Biomass and Bioenergy* 42, 172–178. doi:10.1016/j.biombioe.2012.03.006.

Abdel-Raouf, N., Al-Homaidan, A. A., Ibraheem, I. B. M. (2012). Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences*, *19*(3), 257–275. https://doi.org/10.1016/j.sjbs.2012.04.005.

Aceituno, F. F., Orellana, M., Torres, J., Mendoza, S., Slater, A. W., Melo, F., & Agosin, E. (2012). Oxygen response of the wine yeast *Saccharomyces cerevisiae* EC1118 grown under Carbon-Sufficient, nitrogen-limited enological conditions. *Appl. Environ. Microbiol.*, *78*, 8340–8352. https://doi.org/10.1128/AEM.02305-12.

Alam, M. D. Z., Fakhru'l-razi, A., Abd-aziz, S., H. Molla, A., (2003). Optimization of compatible mixed cultures for liquid state bioconversion of municipal wastewater sludge. *Water Air Soil Pollut* 149: 113–126, 2003.

Albergaria, H., Francisco, D., Gori, K., Arneborg, N., Gírio, F. (2010). *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. *Appl. Microbiol. Biotechnol.*, *86*, 965–972. https://doi.org/10.1007/s00253-009-2409-6.

Albergaria, H., Branco, P., Francisco, D., Coutinho, R., Monteiro, M., Malfeito-Ferreira, M., et al (2013). Dominance of *Saccharomyces cerevisiae* in wine fermentations: secretion of antimicrobial peptides and microbial interactions. Proc. 2nd International Conference on Microbial Diversity: Microbial interactions in complex ecosystems, 23, 98–101.

Andorrà, I., Esteve-Zarzoso, B., Guillamón, J. M., Mas, A. (2010). Determination of viable wine yeast using DNA binding dyes and quantitative PCR. *Int. J. Food Microbiol*, *144*, 257–262.

https://doi.org/10.1016/j.ijfoodmicro.2010.10.003.

Andorrà, I., Berradre, M., Mas, A., Esteve-Zarzoso, B., Guillamón, J. M. (2012). Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT - JFST*, *49*, 8–13.

Angelov, M., Kostov, G., Simova, E., Beshkova, D., & Koprinkova-Hristova, P. (2009). Proto-cooperation factors in yogurt starter cultures. *Revue de Génie Industriel*, *3*, 4–12.

Antalick, G., Perello, M. C., & De Revel, G. (2013). Co-inoculation with yeast and LAB under winery conditions: Modification of the aromatic profile of merlot wines. *S Afr J Enol Vitic* , *34*, 223–232.

Antara, N. S., Sujaya, I. N., Yokota, A., Asano, K., Tomita, F. (2004). Effects of indigenous starter cultures on the microbial and physicochemical characteristics of Urutan, a Balinese fermented *sausage. J. Biosci. Bioeng*. 98, 92–98.

Arnink, K., Henick-Kling, T., (2005). Influence of *Saccharomyces cerevisiae* and *Oenococcus oeni* strains on successful malolactic conversion in wine. *Am J Enol Vitic*, 56, 228-237.

Avbelj, M., Zupan, J., Kranjc, L., Raspor, P. (2015). Quorum-Sensing Kinetics in *Saccharomyces cerevisiae*: A Symphony of ARO Genes and Aromatic Alcohols. *J Agric Food Chem*, *63*, 8544-8550. https://doi.org/10.1021/acs.jafc.5b03400.

Aydemir, E., Demirci, S., Dogan, A., Aytekin, A. O., Sahin, F., (2014). Genetic Modifications of *Saccharomyces cerevisiae* for Ethanol Production from starch fermentation:b A review. *J Bioproces Biotech*, 4 (7)-3-8.

Aziza, M., Amrane, A., (2006). Commensalism during submerged mixed culture of *Geotrichum candidum* and *Penicillium camembertii* on glutamate and lactate. *Process Biochem*, 41, 2452–2457. doi:10. 1016/j.procbio.2006.07.012.

Bader, J., Mast-Gerlach, E., Popović, M. K., Bajpai, R., Stahl, U. (2010). Relevance of microbial coculture fermentations in biotechnology. *J. Appl. Microbiol*, *109*, 371–387. https://doi.org/10.1111/j.1365-2672.2009.04659.x.

Bagheri, B., Bauer, F. F., and Setati, M. E. (2015). The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. *South African J. Enol. Vitic.* 36, 243–251.

Barbosa, A. V., Cerqueira, A. D. M. F., Rusak, F., Falavina dos Reis, C. M., Leal, et al (2015). Characterization of epidemic clones of *Listeria monocytogenes* serotype 4b isolated from humans and meat products in Brazil. *J Infect Dev Ctries*, 9, 962-969.

Bartowsky, E. J., Costello, P. J., & Chambers, P. J. (2015). Emerging trends in the application of malolactic fermentation. *AJGWR*, *21*, 663–669. https://doi.org/10.1111/ajgw.12185.

Baruzzi, F., Quintieri, L., Caputo, L., Cocconcelli, P. S., Borcakli, M., Owczarek, L., et al (2016). Improvement of Ayran quality by the selection of autochthonous microbial cultures. *Food Microbiol*, 60, 92–103. https://doi.org/10.1016/j.fm.2016.07.001.

Bayrock, D. P. and Ingledew, W. M. (2004). Inhibition of yeast by lactic acid bacteria in continuous culture: Nutrient depletion and/or acid toxicity. *J. Ind. Microbiol. Biotechnol*. 31, 362–368.

Beck, M. J., Johnson, R. D., Baker, C. S. (1990). Ethanol production from glucose/xylose mixes by incorporating microbes in selected fermentation schemes. *Appl Biochem Biotechnol*, 25, 415–424.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu, D. (2008). Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. *Int. J. Food Microbiol*, *122*, 312–320. https://doi.org/10.1016/j.ijfoodmicro.2007.12.023.

Bencheqroun, S. K., Bajji, M., Massart, S., Labhilili, M., Jaafari, S. El., Jijakli, M. H. (2007). In vitro and in situ

study of postharvest apple blue mold biocontrol by *Aureobasidium pullulans*: Evidence for the involvement of competition for nutrients. *Postharvest Biol Technol*, *46*, 128-135. https://doi.org/10.1016/j.postharvbio.2007.05.005.

Benito, A., Calderon, F., Palomero, F., Benito, S. (2015). Combine use of selected *Schizosaccharomyces pombe* and *lachancea thermotolerans* yeast strains as an alternative to the traditional malolactic fermentation in red wine production. *Molecules*, *20*, 9510-9523. https://doi.org/10.3390/molecules20069510.

Benkerroum, N., Daoudi, A., Hamraoui, T., Ghalfi, H., Thiry, C., Duroy, M., et al., (2005). Lyophilized preparations of bacteriocinogenic *Lactobacillus curvatus* and *Lactococcus lactis subsp. lactis* as potential protective adjuncts to control *Listeria monocytogenes* in dry-fermented sausages. *J. Appl. Microbiol*, *98*, 56–63. https://doi.org/10.1111/j.1365-2672.2004.02419.x

Bensmira, M., Nsabimana, C., Jiang, B. (2010). Effects of fermentation conditions and homogenization pressure on the rheological properties of Kefir. *LWT - JFST*, *43*, 1180-1184. https://doi.org/10.1016/j.lwt.2010.04.005.

Bernstein, H. C., Carlson, R. P. (2012). Microbial Consortia Engineering for Cellular Factories: in Vitro To in Silico Systems. *Computational and Structural Biotechnology Journal*, *3*(4), e201210017. https://doi.org/10.5936/csbj.201210017.

Bernstein, H. C. (2013). *Systems Analysis of Engineered and Natural Microbial Consortia*. [Doctoral's thesis].Available[http://scholarworks.montana.edu/xmlui/bitstream/handle/1/2723/BernsteinHans2013.pdf;sequence=1]: Montana state university.

Bhatia, S. K., Yi, D.-H., Kim, Y. H., Kim, H. J., Seo, H. M., Lee, J. H., et al. (2015). Development of semi-synthetic microbial consortia of *Streptomyces coelicolor* for increased production of biodiesel (fatty acid methyl esters). *Fuel*, *159*, 189–196. https://doi.org/10.1016/j.fuel.2015.06.084.

Bisson, L. F. (1999). Stuck and sluggish fermentations. *Am J Enol Vitic*, 50, 107–119.

Blattner, F. R. et al (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* 277, 1453–1462.

Boedicker, J. Q., Vincent, M. E., Ismagilov, R. F., (2009). Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behavior of quorum sensing and growth and reveals its variability. *Angew Chem Int Ed Engl*, 48, 5908–5911.

Bogan, R. H., Albertson, O. E., Pluntz, J. C., (1960). Use of algae in removing phosphorus from sewage. Proc. Am. Soc. Civil Engrs. *J. Saint. Eng. Div*., SA5 86, 1–20.

Bolker J. A. (2009). Exemplary and surrogate models: two modes of representation in biology. *Perspect Biol Med*. 52, 485–499.

Boynton, P. J., Greig, D. (2016). Species richness influences wine ecosystem function through a dominant species. *Fungal Ecology*, *22*, 61–72. https://doi.org/10.1016/j.funeco.2016.04.008.

Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M. G., Albergaria, H. (2014). Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol. Biotechnol.*, *98*, 843–853. https://doi.org/10.1007/s00253-013-5411-y.

Branco, P., Viana, T., Albergaria, H., Arneborg, N. (2015). Antimicrobial peptides (AMPs) produced by *Saccharomyces cerevisiae* induce alterations in the intracellular pH, membrane permeability and culturability of *Hanseniaspora guilliermondii* cells. *Int. J. Food Microbiol*, *205*, 112–118.

Branco, P., Francisco, D., Monteiro, M., Almeida, M. G., Caldeira, J., Arneborg, N., et al., (2017). Antimicrobial properties and death-inducing mechanisms of saccharomycin, a biocide secreted by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.*,*101*,159–171.

Brenner, K., You, L., & Arnold, F. H. (2008). Engineering microbial consortia: a new frontier in synthetic biology. *Trends in Biotechnology*, *26*, 483–489.

Brethauer, S., & Studer, M. H. (2014). Consolidated bioprocessing of lignocellulose by a microbial consortium. *Energy & Environmental Science*, *7*, 1446. https://doi.org/10.1039/c3ee41753k

Breton-Toral, A., Trejo Estrada, S. R., and McDonald, A. G. (2017). Lactic Acid Production from Potato Peel Waste, Spent Coffee Grounds and Almond Shells with Undefined Mixed Cultures Isolated from Coffee Mucilage from Coatepec Mexico. *Ferment. Technol.* 6. doi:10.4172/2167-7972.1000139.

Buzzini, P. (2001). Batch and fed-batch carotenoid production by *Rhodotorula glutinis-Debaryomyces castellii* co-cultures in corn syrup. *J Appl Microbiol*, 90, 843–847.

Cappello, M. S., Zapparoli, G., Logrieco, A., and Bartowsky, E. J. (2017). Linking wine lactic acid bacteria diversity with wine aroma and flavour. *Int. J. Food Microbiol.* 243, 16–27.

Chambers, P. J., Pretorius, I. S. (2010). Fermenting knowledge: the history of winemaking, science and yeast research. *EMBO Reports*, *11*, 914–20. https://doi.org/10.1038/embor.2010.179.

Chandrapala, J., Zisu, B., (2016). Novel trends in engineered milk products. *J Dairy Res*, 83, 268-280. https://doi.org/10.1017/S0022029916000479.

Cheirsilp, B., Shoji, H., Shimizu, H., Shioya, S. (2003). Interactions between *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae* in Mixed Culture for Kefiran Production. *Journal of Bioscience and Bioengineering*, *96*, 279–284. https://doi.org/10.1016/S1389-1723(03)80194-9.

Chen, H., Fink, G. R., (2006). Feedback control of morphogenesis in fungi by aromatic alcohols. *Genes Dev,*20, 1150−1161.

Chen, Y., Lin, C. J., Jones, G., Fu, S., Zhan, H. (2009). Enhancing biodegradation of wastewater by microbial consortia with fractional factorial design. *Journal of Hazardous Materials*, *171*, 948-953. https://doi.org/10.1016/j.jhazmat.2009.06.100.

Chen, Y. (2011). Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: A systematic review. *J. Ind. Microbiol. Biotechnol.* 38, 581-597. doi:10.1007/s10295-010-0894-3.

Chr, Hansen. (2015). Viniflora® Yeast specialities. http://www.chr-hansen. com/products/product-areas/wine-ingredients/viniflorar-specialty-yeasts-to-get-closer-to-wild-ferment-complexity-without-the-risk. html (accessed 31 July 2015).

Christensen, B. B., Haagensen, J. A. J., Heydorn, A., Molin, S. (2002). Metabolic commensalism and competition in a two-species microbial consortium. *Appl. Environ. Microbiol.*, *68*, 2495–2502. https://doi.org/10.1128/AEM.68.5.2495-2502.2002.

Chubiz, L. M., Granger, B. R., Segre, D., Harcombe, W. R. (2015). Species interactions differ in their genetic robustness. *Front. Microbiol.* 6, 1–9. doi:10.3389/fmicb.2015.00271.

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

Ciani, M., Comitini, F., Mannazzu, I., Domizio, P. (2010). Controlled mixed culture fermentation: A new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research*, *10*, 123–133. https://doi.org/10.1111/j.1567-1364.2009.00579.x.

Clemente-Jimenez, J. M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F. J., & Rodríguez-Vico, F. (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiology*, *21*, 149–155. https://doi.org/10.1016/S0740-0020(03)00063-7.

Comitini, F., De Ingenis J., Pepe L., Mannazu I. Ciani M. (2004) *Pichia anomala* and *Kluyveromyces wikerhamii* killer toxins as new tool against *Dekkera/Brettanomyces* spoilage yeasts. *FEMS Microbiol Lett* 238, 235–240.

Comitini, F., Ferretti, R., Clementi, F., Mannazzu, I., Ciani, M. (2005). Interactions between *Saccharomyces cerevisiae* and malolactic bacteria: Preliminary characterization of a yeast *proteinaceous* compound(s) active against *Oenococcus oeni*. *J. Appl. Microbiol*, *99*, 105–111. https://doi.org/10.1111/j.1365-2672.2005.02579.x.

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

Cooper, G. M., (2000). The Cell: A Molecular Approach. 2nd edition. Sunderland (M. A.): Sinauer Associates. Cells as experimental models. Available from:

Courtin, P., Courtin, P. (2004). Interactions between microorganisms in a simple ecosystem : yogurt bacteria as a study model To cite this version : Interactions between microorganisms in a simple ecosystem : yogurt bacteria as a study model. https://doi.org/10.1051/lait.

Crafack, M., Mikkelsen, M. B., Saerens, S., Knudsen, M., Blennow, A., Lowor, S., et al. (2013). Influencing cocoa flavour using *Pichia kluyveri and Kluyveromyces marxianus* in a defined mixed starter culture for cocoa fermentation. *Int. J. Food Microbiol.* 167, 103–116.

Dallinger, W. D. (1887) The president's address. *J. R. Microsc. Soc*. 7, 184–199.

De Bari, I., Cuna, D., Nanna, F., Braccio, G., (2004). Ethanol production in immobilized cell bioreactors from mixed sugar syrups and enzymatic hydrolysates of steam-exploded biomass. *Appl Biochem Biotechnol*, 114, 539–557.

De Vuyst, L., and P. Neysens. (2005). The sourdough microflora: biodiversity and metabolic interactions. *Trends Food Sci. Technol*. 16, 43-56.

De Vuyst, L., T. Lefeber, Z. Papalexandratou, and N. Camu. (2010). The functional role of lactic acid bacteria in cocoa bean fermentation, p. 301–326. In F. Mozzi, R. R. Raya, and G. M. Vignolo (ed.), Biotechnology of lactic acid bacteria: novel applications. Wiley-Blackwell, Ames, IA

De Vuyst, L., Wecks, S., (2016). The cocoa bean fermentation process: from ecosystem analysis to starter culture development. *J. Appl. Microbiol* 121, 5-17.

Del Fresno, J. M., Morata, A., Loira, I., Bañuelos, M. A., Escott, C., Benito, S., et al. (2017). Use of non-*Saccharomyces* in single-culture, mixed and sequential fermentation to improve red wine quality. *Eur. Food Res. Technol.* 243, 2175–2185. doi:10.1007/s00217-017-2920-4.

Dethlefsen, L., McFall-Ngai, M., Relman, D. A. (2007). An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature*, *449*, 811-818.

Di Maro, E., Ercolini, D., Coppola, S. (2007). Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. *Int. J. Food Microbiol*, *117*, 201-210.

Dicks, L. M. T., Mellett, F. D. and Hoffman, L. C. (2004). Use of bacteriocin-producing starter cultures of *Lactobacillus plantarum* and *Lactobacillus curvatus* in production of ostrich meat salami. *Meast Sci*, 66, 703–708.

Dizzy, M., Bisson, L. F. (2000). Proteolytic activity of yeast strains during grape juice fermentation. *Am. J. Enol. Vitic*. 51, 155–167. doi: 10.1007/s10482-015- 0578-0.

Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I., Ciani, M. (2011). Outlining a future for non- *Saccharomyces* yeasts : Selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation. *Int. J. Food Microbiol*. https://doi.org/10.1016/j.ijfoodmicro.2011.03.020.

Dortu, C., Huch, M., Holzapfel, W. H., Franz, C. M. A. P., Thonart, P. (2008). Anti-*listerial* activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat. *Letters in Applied Microbiology*, *47*(6), 581–586.

Du, R., Yan, J. B., Li, S. Z., Zhang, L., Zhang, S., Li J., et al (2015). Cellulosic ethanol production by natural bacterial consortia is enhanced by *Pseudoxanthomonas taiwanensis*. *Biotechnol Biofuels*, 8, 1–10.

Ducat, D. C., Avelar-rivas, J. A., Way, J. C., and Silver, P. A. (2012). Rerouting Carbon Flux To Enhance Photosynthetic Productivity. *AEM*, 78, 2660–2668.

Dürre, P., (2007). Biobutanol: an attractive biofuel. *Biotechnol J*. 2, 1525–1534.

Dusseaux, S., Croux, C., Soucaille, P., Meynial-Salles, I., (2013). Metabolic engineering *of Clostridium acetobutylicum* ATCC 824 for the high-yield production of a biofuel composed of an isopropanol/butanol/ethanol mixture. *Metab Eng*, 18, 1–8.

Eddy, S. (1928). Succession of Protozoa in cultures under controlled conditions. *Transact. Am. Micr. Soc*. 47, 283.

Egli, C. M., Edinger, W. D., Mitrakul, C. M., Henick-Kling, T. (1998). Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. *J. Appl. Microbiol*. https://doi.org/10.1046/j.1365-2672.1998.00521.x.

Eiteman M. A., Lee S. A., Altman E., (2008). A co-fermentation strategy to consume sugar mixtures effectively. *J Biol Eng*, 21, 2-9.

Erşan, S., Gültekin-Özgüven, M., Berktaş, I., Erdem, Ö., Tuna, H. E., Gunes, G., & Özçelik, B. (2016). Use of Bacillus indicus HU36 as a probiotic culture in set-type, recombined non fat yoghurt production and its effects on quality. *Int. J. Dairy Technol*, *69*, 81–88. https://doi.org/10.1111/1471-0307.12221.

Escalante-Espinosa, E., Gallegos-Martínez, M. E., Favela-Torres, E., and Gutiérrez-Rojas, M. (2005). Improvement of the hydrocarbon phytoremediation rate by *Cyperus laxus Lam*. inoculated with a microbial consortium in a model system. *Chemosphere* 59, 405–413.

Ezeji, T. C., Qureshi, N., Blaschek, H. P., (2007). Bio production of butanol from biomass: from genes to bioreactors. *Curr. Opin. Biotechnol*, 18, 220–227.

Ezeji, T. C., Qureshi, N., Blaschek, H. P., (2012). Microbial production of a biofuel (acetone-butanol ethanol) in a continuous bioreactor: Impact of bleed and simultaneous product recovery. *Bioproc. Biosyst. Eng.* 36, 109-116.

Ezeji, T. C., Liu, S., and Qureshi, N. (2014). Mixed Sugar Fermentation by Clostridia and Metabolic Engineering for Butanol Production. *Biorefineries Integr. Biochem. Process. Liq. Biofuels*, 191–204. doi:10.1016/B978-0-444-59498-3.00009-9.

Fazzini, R. A. B., Preto, M. J., Quintas, A. C. P., Bielecka, A., Timmis, K. N., dos Santos, V. A. P. M. (2010). Consortia modulation of the stress response: Proteomic analysis of single strain versus mixed culture. *Environmental Microbiology*, *12*, 2436–2449. https://doi.org/10.1111/j.1462-2920.2010.02217.x.

Fisher, S., Palmer, M. (1995). Fermented meat production and consumption in the European Union. In: Campbell-Platt G., Cook P.E. (eds) *Fermented Meats*. Springer, Boston, MA.

Fleet, G. H., Heard, G. M., (1993). Yeasts: growth during fermentation. In: Fleet, G. H. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, Switzerland, pp. 27–54.

Fleet, G. H. (2003). Yeast interactions and wine flavour. *Int. J. Food Microbiol*, *86*, 11–22. https://doi.org/10.1016/S0168-1605(03)00245-9.

Forde, A., Fitzgerald, G. F. (1999). Bacteriophage defence systems in lactic acid bacteria. Antonie van Leeuwenhoek *International Journal of General and Molecular Microbiology*, 76, 89–113.

Foster, K. R., & Bell, T. (2012). Competition, not cooperation, dominates interactions among culturable microbial species. *Current Biology*, *22*(19), 1845–1850. https://doi.org/10.1016/j.cub.2012.08.005.

Frece, J., Kovačevića., Kazazić, S., Mrvcić, J., Vahčić, N., Ježek, D., et al. (2014). Comparison of sensory properties, shelf-life and microbiological safety of industrial sausages produced with autochthonous and commercial starter cultures. *Food Technology and Biotechnology*, *52*, 307–316.

Fu, N., Peiris, P., Markham, J., et al (2009). A novel co-culture process with *Zymomonas mobilis* and *Pichia stipitis* for efficient ethanol production on glucose/xylose mixtures. *Enzyme Microb Technol*, 45, 210–217.

Furukawa, S., Watanabe, T., Toyama, H., & Morinaga, Y. (2013). Significance of microbial symbiotic coexistence in traditional fermentation. *Journal of Bioscience and Bioengineering*, *116*, 533–539. https://doi.org/10.1016/j.jbiosc.2013.05.017.

Gaikwad, L. G. (2014). Development of Microbial Consortia for the Effective Treatment of Complex Wastewater. *J. Bioremediation Biodegrad.* 5, 5–10. doi:10.4172/2155-6199.1000227.

García-Ríos, E., Gutiérrez, A., Salvadó, Z. Z., Arroyo-López, F. N., & Guillamon, J. M. (2014). The Fitness Advantage of Commercial Wine Yeasts in Relation to the Nitrogen Concentration, Temperature, and Ethanol Content under Microvinification Conditions. *Appl. Environ. Microbiol.*, *80*(2), 704–713. https://doi.org/10.1128/AEM.03405-13.

Gardner T. S., Cantor C. R., Collins J. J. (2000). Construction of a genetictoggleswitch in *Escherichia coli. Nature* 403: 339–342.

Gatti, M., Bottari, B., Lazzi, C., Neviani, E., & Mucchetti, G. (2014). Invited review: Microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. *J. Dairy Sci*., 97, 573–591.

Gause, G. F., 1934. The struggle for existence. *Williams &Wilkins, Baltimore* (reprinted 1964 by Hafner, New York).

Ghazali, F. M., Rahman, R. N. Z. A., Salleh, A. B., & Basri, M. (2004). Biodegradation of hydrocarbons in soil by microbial consortium. *International Biodeterioration and Biodegradation*, *54*(1), 61–67. https://doi.org/10.1016/j.ibiod.2004.02.002.

Ghosh, S., Bagheri, B., Morgan, H. H., Divol, B., and Setati, M. E. (2015). Assessment of wine microbial diversity using ARISA and cultivation-based methods. *Ann. Microbiol.* 65, 1833–1840.

Ghosh, S., Chowdhury, R., & Bhattacharya, P. (2016). Mixed consortia in bioprocesses: role of microbial interactions. *Appl. Microbiol. Biotechnol.*, *100*, 4283–4295. https://doi.org/10.1007/s00253-016-7448-1.

Gimeno, C. J., Ljungdahl, P. O., Styles, E. A & Fink, G. R., (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: reg- ulation by starvation and RAS. *Cell*, 68, 1077-1090.

Gobbetti, M., Corsetti, A., Rossi (1994). *J. World Journal of Microbiology & Biotechnology*, 10, 275. https://doi.org/10.1007/BF00414862.

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

Goffeau, et al (1996). Life with 6000 genes. *Science*. 274:563–567

Guest, R. K., and Smith, D. W. (2002). A potential new role for fungi in a wastewater MBR biological nitrogen reduction system. *J. Environ. Eng. Sci*., 1, 433–437.

Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J., Goodman, R. M. (1998). Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chemistry & Biology*, *5*, R245–R249. https://doi.org/10.1016/S1074-5521(98)90108-9.

Hansen E. H., Nissen P., Sommer P., Nielsen J. C., Arneborg N. (2001). The effect of oxygen on the survival of *non-Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. *J Appl Microbiol* 91:541–547

Harish Kumar Reddy, Y., Srijana, M., Madhusudhan Reddy, D., Reddy, G. (2010) Coculture fermentation of banana agro-waste to ethanol by cellulolytic thermophilic *Clostridium thermocellum* CT2. Afr *J Biotechnol,* 9,1926–1934.

Hatoum, R., Labrie, S., Fliss, I. (2012). Antimicrobial and probiotic properties of yeasts: From fundamental to novel applications. *Frontiers in Microbiology*, *3*, 1–12.

Henze, M., (2008). Biological Wastewater Treatment: principles, Modelling and Design. *IWA* Pub, London.

Hernandez, J. P.,de-Bashan, L. E., Rodriguez, D. J., Rodriguez, Y., Bashan, Y. (2009). Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. *Eur J Soil Biol*, 45:88–93.

Hibbing, M. E., Fuqua, C., Parsek, M. R., Peterson, S. B. (2010). Jungle. *Natl. Rev. Microbiol.* 8, 15–25. doi:10.1038/nrmicro2259.Bacterial.

Hirooka, T., Akiyama, Y., Tsuji, N., Nakamura, T., Nagase, H., Hirata, K., et al. (2003). Removal of hazardous phenols by microalgae under photoautotrophic conditions. *J Biosci Bioeng*, 95:200–3.

Ho, V. T. T., Zhao, J., & Fleet, G. (2014). Yeasts are essential for cocoa bean fermentation. *Int. J. Food Microbiol*, *174*, 72–87. https://doi.org/10.1016/j.ijfoodmicro.2013.12.014.

Hong, X., Chen, J., Liu, L., Wu, H., Tan, H., Xie, G., et al. (2016). Metagenomic sequencing reveals the relationship between microbiota composition and quality of Chinese Rice Wine. *Sci. Rep.* 6, 26621. doi:10.1038/srep26621.

Huang, L. P., Jin, B., Lant, P., & Zhou, J. (2003). Biotechnological production of lactic acid integrated with potato wastewater treatment by *Rhizopus arrhizus*. *Journal of Chemical Technology and Biotechnology*, *78*, 899–906. https://doi.org/10.1002/jctb.877.

Hubbell, S. P. (2001) The Unified Neutral Theory of Biodiversity and Biogeography. Princeton, NJ, USA: Princeton University Press. Hugenholtz.

Imran, M., Desmasures, N., Vernoux, J. P. (2010). From undefined red smear cheese consortia to minimal model communities both exhibiting similar anti-listerial activity on a cheese-like matrix. *Food Microbiology*, *27*, 1095–1103. https://doi.org/10.1016/j.fm.2010.07.016.

Inui, M., Suda, M., Kimura, S., Yasuda, K., Suzuki, H, Toda, H., et al (2008). Expression of the *clostridium acetobutylicum* butanol synthetic genes *in Escherichia coli. Appl Microbiol Bioechnol* 77, 1305-1316. doi: 10.1007/s00253-007-1257-5.

Izquierdo Cañas, P. M., García-Romero, E., Heras Manso, J. M., Fernández-González, M. (2014). Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. *European Food Research and Technology*, *239*, 279–286. https://doi.org/10.1007/s00217-014-2220-1.

Jacob, F.ß. Monod, J. (1961). On the regulation of gene activity. Cold Spring Harb. Symp. *Quant. Biol*. 26, 193–211.

Jagmann, N., & Philipp, B. (2014). Reprint of Design of synthetic microbial communities for biotechnological production processes. *Journal of Biotechnology*, *192*, 293–301. https://doi.org/10.1016/j.jbiotec.2014.11.005

Janisiewicz, W. J., Tworkoski, T. J., Sharer, C., (2000). Characterizing the mechanism of biological control of postharvest diseases on fruits with a simple method to study competition for nutrients. *Phytopathology* 90, 1196–1200.

Jeffries, T. W. (2006). Engineering yeasts for xylose metabolism. *Curr Opin Biotechnol* 17:320–326.

Jurgens, G., Survase, S., Berezina, O., Sklavounos, E., Linnekoski, J., Kurkijärvi, A., Granström, T., (2012). Butanol production from lignocellulosics. Biotechnol. *Lett.* 34, 1415-1434.

Jia, X., Liu, C., Song, H., Ding, M., Du, J., Ma, Q., Yuan, Y. (2016). Design, analysis and application of synthetic microbial consortia. *Synthetic and Systems Biotechnology*, *1*, 109–117. https://doi.org/10.1016/j.synbio.2016.02.001.

Jiang, L. L., Zhou, J. J., Quan, C. S., Xiu, Z. L. (2017). Advances in industrial microbiome based on microbial consortium for biorefinery. *Bioresour. Bioprocess.* 4, 11. doi:10.1186/s40643-017-0141-0.

Jin, B., van Leeuwen, J., Patel, B., Yu, Q. (1998). Utilization of starch processing wastewater for production of microbial biomass protein and fungal α-amylase by *Aspergillus oryzae*. *Bioresour. Technol*., 66, 201–206.

Joo, H., Hirai, M., Shoda, M. (2007). Improvement in Ammonium Removal Efficiency in Wastewater Treatment by Mixed Culture of *Alcaligenes faecalis* No . 4 and *L1*. 103, 66–73.

Jood, I., Hoff, J. W., Setati, M. E. (2017). Evaluating fermentation characteristics of *Kazachstania spp*. and their potential influence on wine quality. *World J Microbiol Biotechnol.* https://doi.org/10.1007/s11274-017-2299-1.

Jouzani, G. S., and Taherzadeh, M. J. (2015). Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: a comprehensive review. *Biofuel Res. J.* 5, 152–195. doi:10.18331/BRJ2015.2.1.4.

Kargozari, M., Emam-Djomeh, Z., Gandomi, H., Partovi, R., Ghasemlou, M., & Martin, I. R. (2015). Identification of selected *Lactobacillus* strains isolated from Siahmazgi cheese and study on their behavior after inoculation in fermented-sausage model medium. *LWT - JFST*, *62*, 1177-1183. https://doi.org/10.1016/j.lwt.2015.01.030.

Kato, S., Yoshida, R., Yamaguchi, T., Sato, T., Yumoto, I., Kamagata, Y. (2014). The effects of elevated CO2 concentration on competitive interaction between aceticlastic and syntrophic methanogenesis in a model microbial consortium. *Front in Microbiol*, *5*, 1–8.

Kim, H. J., Boedicker, J. Q., Choi, J. W., Ismagilov, R. F. (2008). Defined spatial structure stabilizes a synthetic multispecies bacterial community. *Proceedings of the National Academy of Sciences*, *105*, 18188–18193. https://doi.org/10.1073/pnas.0807935105.

King, E. S., Kievit, R. L., Curtin, C., Swiegers, J. H., Pretorius, I. S., Bastian, S. E. P., Leigh Francis, I. (2010). The effect of multiple yeasts co-inoculations on Sauvignon Blanc wine aroma composition, sensory properties and consumer preference. *Food Chemistry*.

Kirkup, B. C., Riley, M. A. (2004). Antibiotic-mediated antagonism leads to a bacterial game of rock–paper–scissors in vivo. *Nature*, *428*, 412–414. https://doi.org/10.1038/nature02429.

Klitgord, N., and Segrè, D. (2010). Environments that induce synthetic microbial ecosystems. *PLoS Comp. Biol*. 6:e1001002. doi: 10.1371/journal.pcbi.1001002.

Klitgord, N., and Segrè, D. (2011). Ecosystems biology of microbial metabolism. *Curr. Opin. Biotech*. 22, 541–546. doi: 10.1016/j.copbio.2011.04.018.

Kolkman, A., Slijper, M., & Heck, A. J. R. (2005) “Development and application of proteomics technologies in *Saccharomyces cerevisiae*”, *Trends in Biotechnology*, 23, 598–604.

Kolkman, A., Daran-Lapujade, P., Fullaondo, A., Olsthoorn, M.M.A., Pronk, J.T., Slijper, M. and Heck, A.J.R. (2006). Proteome analysis of yeast response to various nutrient limitations. *Mol Syst Biol* 2, 2006-0026.

Konopka, A., (2009). What is microbial community ecology? *ISME* J, 3, 1223–1230.

Konopka, A., Lindemann, S., & Fredrickson, J. (2015). Dynamics in microbial communities: unraveling mechanisms to identify principles. *The ISME Journal*, *9*(7), 1488–1495.

Kosel, J., Cadez, N., Schuller, D., Carreto, L., Franco-Duarte, R., Raspor, P. (2017). The influence of *Dekkera bruxellensis* on the transcriptome of *Saccharomyces cerevisiae* and on the aromatic profile of synthetic wine must. *FEMS Yeast Research*, 17, 4.

Laffort (2015). Zymaflore® Aplha$^{TDn.Sacch.}$ www.laffort.com/en/ products/470-zymaflore-alpha (accessed 31 July 2015).

Lallemand (2015). Products portfolio. www.lallemandwine.com/ products/catalogue/range=9 (accesssed 31 July 2015).

Latif, F., Rajoka, M. I., (2001). Production of ethanol and xylitol from corn cobs by yeasts. *Bioresour Technol*, 77, 57–63.

Lebeau, T., Jouenne, T., Junter, G. A., (1997). Simultaneous fermentation of glucose and xylose by pure and mixed cultures of *Saccharomyces cerevisiae* and *Candida shehatae* immobilized in a two-chambered bioreactor. *Enzyme Microb Technol* 21, 265–272.

Leclercq, C., Arcella, D., Piccinelli, R., Sette, S., & Le Donne, C. (2009). The Italian National Food Consumption Survey INRAN-SCAI 2005–06: Main results in terms of food consumption. *Public Health Nutrition, 12*(12), 2504-2532. doi:10.1017/S1368980009005035.

Lee, W.J., Lucey, J.A., (2004). Structure and Physical Properties of Yogurt Gels: Effect of Inoculation Rate and Incubation Temperature. *Journal of Dairy Science*, 87, 3153-3164. https://doi.org/10.3168/jds.S0022-0302(04)73450-5.

Lee, J. W., (2012a). Advanced biofuels and bioproducts. *Adv. Biofuels Bioprod.*, 1–1122. doi:10.1007/978-1-4614-3348-4.

Lee, J., Jang, Y. S., Choi, S. J., Im, J. A., Song, H., Cho, J. H., et al., (2012b). Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol–butanol–ethanol fermentation. *Appl. Environ. Microbiol*. 78, 1416–1423.

Lee, S. Y., Kim, H. U. (2015). Systems strategies for developing industrial microbial strains. *Nature Biotechnology*, *33*, 1061–1072. https://doi.org/10.1038/nbt.3365.

Leeder, A. C.; Palma-Guerrero, J.; Glass, N. L. The social network: deciphering fungal language. *Nat. Rev.: Microbiol*. 2011, 9, 440−451.

Lefeber, T., Janssens, M., Moens, F., Gobert, W., De Vuyst, L. (2011). Interesting starter culture strains for controlled cocoa bean fermentation revealed by simulated cocoa pulp fermentations of cocoa-specific lactic acid bacteria. *Appl. Environ. Microbiol.*, *77*, 6694–6698.

Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N. De Vuyst, L. (2012) On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. *Food Microbiol* , 30, 379–392.

Leite, A .M. O., Mayo, B., Rachid, T. C. C., Peixoto, R. S., Silva, J. T., Paschoalin, V. M. F., Delgado, S., (2012). Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. *Food Microbiology*. 31, 215-221.

Levy, A., Currie, A., (2015). Model organisms are not (theoretical) models. *Brit. J. Phil. Sci*. 66, 327–348 doi:10.1093/bjps/axt055.

Li, J., Zhao, J. B., Zhao, M., Yang, Y. L., Jiang, W. H., & Yang, S. (2010). Screening and characterization of butanol-tolerant micro-organisms. *Letters in Applied Microbiology*, *50*, 373–379.

Lima, L. J. R., Almeida, M. H., Nout, M. J. R., Zwietering, M. H., (2011). *Theobroma cacao* L., "The Food of the Gods": quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. Crit. Rev. *Food Sci. Nutr*. 51, 731–761.

Lin, Y., Tanaka, S., (2006). Ethanol fermentation from biomass resources: current state and prospects. *Appl Environ Microbiol* 69:627–642.

Lindemann, S. R., Bernstein, H. C., Song, H. S., Fredrickson, J. K., Fields, M. W., Shou, W. et al. (2016). Engineering microbial consortia for controllable outputs. *The ISME Journal*, *10*, 2077–2084. https://doi.org/10.1038/ismej.2016.26.

Ling, J., Nip, S., Cheok, W. L., de Toledo, R. A., Shim, H. (2014). Lipid production by a mixed culture of oleaginous yeast and microalga from distillery and domestic mixed wastewater. *Bioresource Technology*, *173*, 132–139. https://doi.org/10.1016/j.biortech.2014.09.047.

Little, A. E., Robinson, C. J., Peterson, S. B., Raffa, K. F., and Handelsman, J. (2008). Rules of Engagement: Interspecies Interactions that Regulate Microbial Communities. *Annu Rev Microbiol* 62, 375–401. doi:10.1146/annurev.micro.030608.101423.

Liu Laplace, J M., Delgenes, J P., Moletta, R., Navarro, J M., (1993). Co-fermentation of glucose and xylose to ethanol by a respiratory deficient mutant of *Saccharomyces cerevisiae* co-cultivated with a xylose-fermenting yeast. *J Ferment Bioeng*, 75,207–212.

Liu, Y., Rousseaux, S., Tourdot-Maréchal, R., Sadoudi, M., Gougeon, R., Schmitt-Kopplin, P., et al. (2017). Wine microbiome: A dynamic world of microbial interactions. *Crit. Rev. Food Sci. Nutr.* 57, 856–873. doi:10.1080/10408398.2014.983591.

Lleixà, J., Martín, V., Portillo, M. d. C., Carrau, F., Beltran, G., & Mas, A. (2016). Comparison of fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*. *Front in Microbiol*, 7, 338.

Long, T., Tu, K C., Wang, Y., Mehta, P., Ong, NP., et al. (2009) Quantifying the integration of quorum-sensing signals with single-cell resolution. *PLoS Biol* 7-68.

Lopez, CC., Paparella, A., Tofalo, R., Suzzi, G., (2011). Proteolytic activity of *Saccharomyces cerevisiae* strains associated with Italian dry-fermented sausages in a model system. *Int. J. Food Microbiol*. 150, 50–58. https://doi.org/10.1016/j.ijfoodmicro.2011.07.017.

López, C. M., Sentandreu, M. A., Vignolo, G. M., Fadda, S. G. (2015). Low molecular weight peptides derived from sarcoplasmic proteins produced by an autochthonous starter culture in a beaker sausage model. *EuPA Open Proteomics*, *7*, 54–63. https://doi.org/10.1016/j.euprot.2015.05.001.

Luo, H., Zeng, Q., Han, S. Wang, Z., Dong, Q., Zhao, Y. (2017). High-efficient n-butanol production by co-culturing Clostridium acetobutylicum and Saccharomyces cerevisiae integrated with butyrate fermentative supernatant addition. *World J Microbiol Biotechnol*, 33, 76. https://doi.org/10.1007/s11274-017-2246-1.

Ludovico, P., Sousa, M J., Silva, M T., Leao, C., and Corte-Rea, l M. (2001). *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiology*, 147, 2409-2415.

Lynch, J.M., Poole, N. J., (1979). *Microbial Ecology*. Blackwell, London.

M. Gancedo, J., (2001). Control of *pseudohyphae* formation in *Saccharomyces cerevisiae*, *FEMS Microbiology Reviews*, 25, 107–123, https://doi.org/10.1111/j.1574-6976.2001.tb00573.x.

Magistà, D., Ferrara, M., Del Nobile, M. A., Gammariello, D., Conte, A., Perrone, G. (2016). Penicillium salamii strain ITEM 15302: A new promising fungal starter for salami production. *Int. J. Food Microbiol*, *231*, 33–41. https://doi.org/10.1016/j.ijfoodmicro.2016.04.029.

Maron, P. A., Ranjard, L., Mougel, C., and Lemanceau, P. (2007). Metaproteomics: A new approach for

studying functional microbial ecology. *Microb. Ecol.* 53, 486–493. doi:10.1007/s00248-006-9196-8.

Medina, K., Boido, E., Fariña, L., Gioia, O., Gomez, M. E., Barquet, M. et al. (2013). Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chemistry*, *141*, 2513–2521. https://doi.org/10.1016/j.foodchem.2013.04.056.

Mendes-Ferreira, A., Del Olmo, M., García-Martínez, J., Jiménez-Martí, E., Mendes-Faia, A., Pérez-Ortín, J. E., & Leão, C. (2007). Transcriptional response of *Saccharomyces cerevisiae* to different nitrogen concentrations during alcoholic fermentation. *Appl. Environ. Microbiol*. 73, 3049–3060. https://doi.org/10.1128/AEM.02754-06.

Mehlomakulu, N. N., Setati, M. E., Divol, B. (2014). Characterization of novel killer toxins secreted by wine-related *non-Saccharomyces* yeasts and their action on *Brettanomyces spp*. *Int. J. Food Microbiol*, 188, 83–91. https://doi.org/10.1016/j.ijfoodmicro.2014.07.015.

Mielenz, J. R., (2001). Ethanol production from biomass: technology and commercialization status. *Curr Opin Microbiol,* 4, 324–329.

Mishra, P., Roy, S., Das, D., (2015). Comparative evaluation of the hydrogen production by mixed consortium, synthetic co-culture and pure culture using distillery effluent. *Bioresource Technology* 198, 593–602. http://dx.doi.org/10.1016/j.biortech.2015.09.074.

Minty, J. J., Singer, M. E., Scholz, S. A., Bae, C. H., Ahn, J. H., Foster, C. E., et al. (2013). Design and characterization of synthetic fungal-bacterial consortia for direct production of isobutanol from cellulosic biomass. *Proc. Natl. Acad. Sci.* 110, 14592–14597. doi:10.1073/pnas.1218447110.

Montanari, C., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F., Tabanelli, G. (2016). Correlation between volatile profiles of Italian fermented sausages and their size and starter culture. *Food Chemistry*. 192, 736–744. http://dx.doi.org/10.1016/j.foodchem.2015.07.062.

Mounier, J., Monnet, C., Vallaeys, T., Arditi, R., Sarthou, A. S., Hélias, A., & Irlinger, F. (2008). Microbial interactions within a cheese microbial community. *Appl. Environ. Microbiol.*, *74*, 172–181. https://doi.org/10.1128/AEM.01338-07.

Mostert, T. T. (2013). Investigating the secretome of *non Saccharomyces* yeast in model wine. *Unpublished M. Sc. Thesis. South Africa, Stellenbosch: Institute for Wine Biotechnology, Faculty of AgriSciences. Stellenbosch University Institute*, (March), 98. Retrieved from http://scholar.sun.ac.za/handle/10019.1/79989.

Mujtaba, G., Lee, K. (2016). Advanced Treatment of Wastewater Using Symbiotic Co-culture of Microalgae and Bacteria. *Appl. Chem. Eng*. 27, 1-9, doi:10.14478/ace.2016.1002.

Nanda, S., Dalai, A. K., and Kozinski, J. A. (2014). Butanol and ethanol production from lignocellulosic feedstock: Biomass pretreatment and bioconversion. *Energy Sci. Eng.* 2, 138–148. doi:10.1002/ese3.41.

Nehme, N., Mathieu, F., & Taillandier, P. (2010). Impact of the co-culture of *Saccharomyces cerevisiae-Oenococcus oeni* on malolactic fermentation and partial characterization of a yeast-derived inhibitory peptidic fraction. *Food Microbiology*, *27*, 150–157.

Nielsen, D R., Leonard, E., Yoon, S H., et al (2009). Engineering alternative butanol production platforms in heterologous bacteria. *Metab Eng* 11:262–273.

Nissen, P., Nielsen, D., Arneborg, N., (2003). Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of *non-Saccharomyces* yeasts in mixed cultures by a cell-cell contact-mediated mechanism. *Yeast* 20, 331-341.

O'Malley, M. A., Travisano, M., Velicer, G. J., and Bolker, J. A. (2015). How do microbial populations and communities function as model systems? *Q. Rev. Biol.* 90, 269–293. doi:10.1086/682588.

Okuda, N., Ninomiya, K., Katakura, Y., Shioya, S., (2008). Strategies for reducing supplemental medium cost in bioethanol production from waste house wood hydrolysate by ethanologenic *Escherichia coli*: inoculum size increase and co-culture with *Saccharomyces cerevisiae. J Biosci Bioeng*,105, 90–96.

Öner, Z., Karahan, A. G., Çakmakçı, M. L. (2009). effects of different milk types and starter cultures on kefir, *GIDA*, *7*, 1–6.

Oswald W, Gotaas H, Ludwig H, Lynch V (1953). Algae symbiosis in oxidation ponds: III. Photosynthetic oxygenation. *Sewage Indust Wastes* 25:692–705.

Padilla, B., Zulian, L., Ferreres, À., Pastor, R., Esteve-Zarzoso, B., Beltran, G., Mas, A. (2017). Sequential inoculation of native *non-Saccharomyces* and *Saccharomyces cerevisiae* strains for wine making. *Front in Microbiol*, *8*, 1–12. https://doi.org/10.3389/fmicb.2017.01293.

Palavecino Prpich, N. Z., Garro, O. A., Romero, M., Judis, M. A., Cayré, M. E., Castro, M. P. (2016). Evaluation of an autochthonous starter culture on the production of a traditional dry fermented sausage from Chaco (Argentina) at a small-scale facility. *Meast Sci*, *115*, 41–44.

Parapouli, M., Delbès-Paus, C., Kakouri, A., Koukkou, A. I., Montel, M. C., and Samelis, J. (2013). Characterization of a wild, novel nisin A-Producing *Lactococcus* strain with an *L. lactis subsp*. *cremoris* genotype and an *L. lactis subsp. lactis* phenotype, isolated from Greek raw milk. *Appl. Environ. Microbiol.* 79, 3476–3484. doi:10.1128/AEM.00436-13.

Park, M. J., Lee, S. Y. (2015). Quality characteristics of soy yogurt produced using proteases and mixed microbial consortia. *Journal of the Korean Society for Applied Biological Chemistry*, *58*, 761–769. https://doi.org/10.1007/s13765-015-0105-z.

Pasotti, L., Zucca, S., Casanova, M., Micoli, G., Cusella, M., Magni, P. (2017). Fermentation of lactose to ethanol in cheese whey permeate and concentrated permeate by engineered *Escherichia coli*. *BMC Biotechnology*, *17*, 48. https://doi.org/10.1186/s12896-017-0369-y.

Passoth, V., Blomqvist, J., and Schnürer, J. (2007). *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. *Appl. Environ. Microbiol.* 73, 4354-4356. doi:10.1128/AEM.00437-07.

Pereira, G. V., Magalhaes, K. T., de Almeida, E. G., Coelho, I. and Schwan, R. F. (2013). Spontaneous cocoa bean fermentation carried out in a novel design stainless steel tank: influence on the dynamics of microbial populations and physical-chemical properties. *Int J Food Microbiol* 161, 121–133.

Pereira, G. V., Pedrozo, M. G., Ramos, C .L. and Schwan, R. F. (2012). Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture. *Appl Environ Microbiol*, 78, 5395– 5405.

Pham, T. K., Chong, P. K., Gan, C. S., Wright, P. C., (2006). Proteomic analysis of *Saccharomyces cerevisiae* under high gravity fermentation conditions. *J. Proteome Res*. 5, 3411–3419.

Pham, T. K.,Wright, P. C., (2008). The proteomic response of *Saccharomyces cerevisiae* in very high glucose conditions with amino acid supplementation. J*. Proteome Res*. 7, 4766–4774.

Poleto, L., Souza, P., Magrini, F. E., Beal, L. L., Torres, A. P. R., de Sousa, M. P. et al. (2016). Selection and identification of microorganisms present in the treatment of wastewater and activated sludge to produce biohydrogen from glycerol*. Int. J. Hydrogen Energy* 41, 4374-4381.

Prado, F., Kerr, B., (2008). The evolution of restraint in bacterial biofilms under nontransitive competition. *Evolution*, 62, 538-548.

Prado, M. R., Blandón, L. M., Vandenberghe, L. P. S., Rodrigues, C., Castro, G. R., Thomaz-Soccol, V., Soccol, C. R. (2015). Milk kefir: Composition, microbial cultures, biological activities, and related products. *Front in Microbiol*, 6, 1–10.

Pretorius, I. S., van der Westhuizen, T. J., & Augustyn, O. P. H. (1999). Yeast Biodiversity in Vineyards and

Wineries and Its Importance to the South African Wine Industry-A Review, *S Afr J Enol Vitic* , 20, 61–75.

Pretorius I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16, 675–729.

Preyer-Buitenzorg, A., (1901). Über Kakaofermentation. *Tropenpflanzer*, 5, 157–173.

Qureshi, N., Saha, B., Dien, B., Hector, R., Cotta, M., (2010a). Production of butanol (a biofuel) from agricultural residues: part I - use of barley straw hydrolysate. *Biomass Bioenerg*. 34, 559-565.

Quinto, E. J., Marín, J. M., and Schaffner, D. W. (2016). Effect of the competitive growth of *Lactobacillus sakei* MN on the growth kinetics of *Listeria monocytogenes* Scott A in model meat gravy. *Food Control* 63, 34–45. doi:10.1016/j.foodcont.2015.11.025.

Qureshi, N., Saha, B.C., Hector, R.E., et al., (2010b). Production of butanol (a biofuel) from agricultural residues: part II-use of corn stover and switchgrass hydrolysate. *Biomass Bioenerg*. 34, 566-571.

Qureshi, N., Liu, S., Ezeji T.C., (2013). Cellulosic Butanol Production from Agricultural Biomass and Residues: Recent advances in technology, in Lee J.W. (ed.), Adv. *Biofuels Bioprod*. doi: 10.1007/978-1-4614-3348-4_15, 247.

Radke Mitchell L., Sandine W. E. (1984). Associative growth and differential enumeration of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*: a review, *J. Food Prot*. 47, 245–248.

Reinke, J. (1872a). Ueber die anatomischen Verhältnisse einiger Arten von Gunnera L. Nachrichten von der Königl. Gesellschaft der Wissenschaften und der Georg-Augusts-Universität zu Göttingen, 9, 100-108.

Reinke, J. (1872b). Über gonidienartige Bildungen in einer dikotylischen Pflanze (Gunnera). Botanischer Zeitung, 30, 59–61. cit. in Höxtermann 2007.

Reinke, J. (1873). Zur Kenntniss des Rhizoms von Corallorhiza und Epipogon. *Flora*, 56, 145–152. 161–167, 177–184, 209–224.

Renault, P., Coulon, J., de Revel, G., Barbe, J. C., Bely, M. (2015). Increase of fruity aroma during mixed *T. delbrueckii/S. cerevisiae* wine fermentation is linked to specific esters enhancement. Int. J. Food Microbiol (Vol. 207). *Elsevier* B.V. https://doi.org/10.1016/j.ijfoodmicro.2015.04.037.

Rivett, D. W., Scheuerl, T., Culbert, C. T., Mombrikotb, S. B., Johnstone, E., Barraclough, T. G., et al. (2016). Resource-dependent attenuation of species interactions during bacterial succession. *ISME J.* 10, 2259–2268. doi:10.1038/ismej.2016.11.

Rizk, Z., El Rayess, Y., Ghanem, C., Mathieu, F., Taillandier, P., Nehme, N. (2016). Impact of inhibitory peptides released by *Saccharomyces cerevisiae* BDX on the malolactic fermentation performed by *Oenococcus oeni Vitilactic* F. *Int. J. Food Microbiol*, 233, 90–96.

Rossignol, T., Kobi, D., Jacquet-Gutfreund, L., Blondin, B. (2009). The proteome of a wine yeast strain during fermentation, correlation with the transcriptome. *J. Appl. Microbiol*, 107, 47–55. https://doi.org/10.1111/j.1365-2672.2009.04156.x.

Rouhollah, H., Iraj, N., Giti, E., Sorah, A., (2007). Mixed sugar fermentation by *Pichia stipitis*, *Saccharomyces cerevisiae*, and an isolated xylose-fermenting *Kluyveromyces marxianus* and their co-cultures. *Afr J Biotechnol* 6, 1110–1114.

Routray, W., Mishra, H. N. (2011). Scientific and Technical Aspects of Yogurt Aroma and Taste: A Review. Comprehensive *Reviews in Food Science and Food Safety*, 10, 208–220. https://doi.org/10.1111/j.1541-4337.2011.00151.x.

Ruiz-Marin, A., Mendoza-espinosa, L. G., Stephenson, T. (2010). Bioresource Technology Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. *Bioresource* Technology, 101, 58–64.

Sabra, W., Dietz, D., Tjahjasari, D., & Zeng, A. P. (2010). Biosystems analysis and engineering of microbial consortia for industrial biotechnology. *Engineering in Life Sciences*, 10, 407–421. https://doi.org/10.1002/elsc.201000111.

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J. J., Ballester et al (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of *non-Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol,* 32, 243–253. https://doi.org/10.1016/j.fm.2012.06.006.

Salimi, F., Mahadevan, R., (2013). Characterizing metabolic interactions in a *clostridial* co-culture for consolidated bioprocessing, *BMC Biotechnol*. 13, 95.

Sandhya, M. V. S., Yallappa, B. S., Varadaraj, M. C., Puranaik, J., Rao, L. J., Janardhan, P., & Murthy, P. S. (2016). Inoculum of the starter consortia and interactive metabolic process in enhancing quality of cocoa bean (*Theobroma cacao*) fermentation. *LWT - JFST*, 65, 731–738.

Sankaran, S., Khanal, S. K., Jasti, N., Jin, B., Iii, A. L. P., Leeuwen, J. H. Van. (2010). Use of Filamentous Fungi for Wastewater Treatment and Production of High Value Fungal Byproducts : A Review, C*ritical Reviews in Environmental Science and Technology,* 40, 400-449, 2, Copyright. doi: 10.1080/10643380802278943.

Santa, O. R. D., Macedo, R. E. F. de, Santa, H. S. D., Zanette, C. M., Freitas, R. J. S. de, & Terra, N. N. (2014). Use of starter cultures isolated from native microbiota of artisanal sausage in the production of Italian sausage. *JFST (Campinas),* 34(4), 780–786. https://doi.org/10.1590/1678-457X.6467.

Schwan R. F. (1998). Cocoa fermentations conducted with a defined microbial cocktail inoculum. *Appl. Environ.Microbiol*. 64, 1477–83.

Schwan, R. F., Wheals, A. E. (2004). The microbiology of cocoa fermentation and its role in chocolate quality. *Critical Reviews in Food Science and Nutrition*, 44, 205-221.

Seman, D. L., Borger, A. C., Meyer, J. D., Hall, P. A., and Milkowski, A. L. (2002). Modeling the growth of *Listeria monocytogenes* in cured ready-to-eat processed meat products by manipulation of sodium chloride, sodium diacetate, potassium lactate, and product moisture content. *J. Food Prot*. 65, 651-658. doi:10.4315/0362-028X-65.4.651.

Sen, R. (2008). Biotechnology in petroleum recovery: The microbial EOR. Prog. *Energy Combust. Sci*. 34, 714–724. doi:10.1016/j.pecs.2008.05.001.

Settachaimongkon, S., Nout, M. J. R., Antunes Fernandes, E. C., Hettinga, K. A., Vervoort, J. M., van Hooijdonk, T. C. M., et al. (2014). Influence of different proteolytic strains of *Streptococcus thermophilus* in co-culture with *Lactobacillus delbrueckii* subsp. *bulgaricus* on the metabolite profile of set-yoghurt. Int. *J. Food Microbiol*. 177, 29–36. doi:10.1016/j.ijfoodmicro.2014.02.008.

Shekhawat, K., Bauer, F. F., & Setati, M. E. (2017). Impact of oxygenation on the performance of three *non-Saccharomyces* yeasts in co-fermentation with *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol*, https://doi.org/10.1007/s00253-016-8001-y.

Shong, J., Jimenez Diaz, M. R., and Collins, C. H. (2012). Towards synthetic microbial consortia for bioprocessing. *Curr. Opin. Biotechnol*. 23, 798-802. doi:10.1016/j.copbio.2012.02.001.

Sieuwerts, S., De Bok, F. A. M., Hugenholtz, J., & Van Hylckama Vlieg, J. E. T. (2008). Unravelling microbial interactions in food fermentations: From classical to genomics approaches. *Appl. Environ. Microbiol.*, 74, 4997–5007. https://doi.org/10.1128/AEM.00113-08.

Sieuwerts, S., Molenaar, D., Van Hijum, S.A.F.T., Beerthuyzen, M., Stevens, M. J. A., Janssen, P. W. M., et al., (2010). Mixed-culture transcriptome analysis reveals the molecular basis of mixed-culture growth in *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, *Appl.Environ. Microbiol*. 76, 7775–7784.

Sieuwerts S (2016). Microbial Interactions in the Yoghurt Consortium: Current Status and Product

Implications. *SOJ Microbiol Infect*, Dis 4, 1-5.

Sillers, R., Chow, A., Tracy, B., Papoutsakis, E. T., (2008). Metabolic engineering of the non-sporulating, non-solventogenic *Clostridium acetobutylicum* strain M5 to produce butanol without acetone demonstrate the robustness of the acid-formation pathways and the importance of the electron balance. *Metab Eng*, 10, 321–32.

Simova, E.D., Frengova, G.I. and Beshkova, D.M. (2004a). *Exopolysaccharides* produced by mixed culture of yeast *Rhodotorula rubra* GED10 and yogurt bacteria (*Streptococcus thermophilus* 13a + *Lactobacillus bulgaricus* 2-11). *J Appl Microbiol*, 97, 512–519.

Simova, E.D., Frengova, G.I. and Beshkova, D.M. (2004b) Synthesis of carotenoids by *Rhodotorula rubra* GED8 co-cultured with yogurt starter cultures in whey ultrafiltrate. *J Ind Microbiol Biotechnol*, 31, 115–121.

Smid, E. J., Lacroix, C. (2013). Microbe-microbe interactions in mixed culture food fermentations. *Current Opinion in Biotechnology*, 24, 148–154. https://doi.org/10.1016/j.copbio.2012.11.007.

Schmitt, M J., Breinig, F. (2002). The viral killer system in yeast: from molecular biology to application. *FEMS Microbiol Rev*, 26, 257– 276

Soomro, A. H., Masud, T. (2008). Selection of yoghurt starter culture from indigenous isolates of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* on the basis of technological properties. *Annals of Microbiology*, 58, 67–71.

Sparo, M., Nunez, G. G., Castro, M., Calcagno, M. L., Garcia Allende, M. A., Ceci, M., Et al (2008). Characteristics of an environmental strain, *Enterococcus faecalis* CECT7121, and its effects as additive on craft dry-fermented sausages. *Food microbiol*, 25, 607-615.

Stadie, J., Gulitz, A., Ehrmann, M. A., Vogel, R. F. (2013). Metabolic activity and symbiotic interactions of lactic acid bacteria and yeasts isolated from water kefir. *Food Microbiology*, 35, 92–98. https://doi.org/10.1016/j.fm.2013.03.009.

Steen, E. J., Chan, R., Prasad, N., Myers, S., Petzold, C. J., Redding, A., et al. (2008). Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. *Microb. Cell Fact*. 7, 36. doi:10.1186/1475-2859-7-36.

Steinkraus, KH., (2004). Industrialization of indigenous fermented foods, 2nd ed. CRC Press, Boca Raton, FL.

Stouthamer, A. H., de Boer, A. P. N., van der Oost, J., and van Spanning, R. J. M. (1997). Emerging principles of inorganic nitrogen metabolism in *Paracoccus denitrificans* and related bacteria. *Antonie van Leeuwenhoek*, 71, 33–41.

Strickland, M. T., Schopp, L. M., Edwards, C. G., & Osborne, J. P. (2016). Impact of *Pediococcus spp*. on pinot noir wine quality and growth of *Brettanomyces*. *Am. J. Enol. Vitic*, 67, 188–198. https://doi.org/10.5344/ajev.2015.15011.

Sun, H., Ma, H., Hao, M., Pretorius, I. S., and Chen, S. (2009). Identification of yeast population dynamics of spontaneous fermentation in Beijing wine region, China. *Ann. Microbiol.* 59, 69–76. doi:10.1007/BF03175601.

Sun, S. Y., Gong, H. S., Jiang, X. M., Zhao, Y. P. (2014). Selected *non-Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae* on alcoholic fermentation behaviour and wine aroma of cherry wines. *Food Microbiology*, 44, 15–23. https://doi.org/10.1016/j.fm.2014.05.007.

Suntornsuk, W., Pochanavanich, P., and Suntornsuk, L. (2002). Fungal chitosan production on food processing by-products. *Process Biochem*, 37, 727–729.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., Tofalo, R. (2012). Multi-starter from organic viticulture for red wine Montepulciano d'Abruzzo production. *Front in Microbiol,* 3, 1–10.

https://doi.org/10.3389/fmicb.2012.00135.

Swenson, W., Arendt, J., & Wilson, D. S. (2000a). Artificial selection of microbial ecosystems for 3-chloroaniline biodegradation. *Environmental Microbiology*, 2, 564–571.

Swenson, W., Wilson, D. S., & Elias, R. (2000b). Artificial ecosystem selection. Proceedings of the National Academy of Sciences, 97, 9110–9114. https://doi.org/10.1073/pnas.150237597.

Talon, R., Leroy, S., Lebert, I. (2007). Microbial ecosystems of traditional fermented meat products: The importance of indigenous starters. *Meast Sci*, 77, 55-62.

Taillandier, P., Lai, Q. P., Julien-Ortiz, A., and Brandam, C. (2014). Interactions between *Torulaspora delbrueckii* and Saccharomyces cerevisiae in wine fermentation: Influence of inoculation and nitrogen content. World *Journal of Microbiology and Biotechnology*, 30, 1959–1967.

Takush, D. G. (2009). The Impact of *Saccharomyces* and *non-Saccharomyces* Yeast on the Aroma and Flavor of *Vitis vinifera* L. cv. 'Pinot Noir' Wine. [master's thesis]. Oregon State University.

Tan, C. H., Lee, K. W. K., Burmølle, M., Kjelleberg, S., Rice, S. A. (2017). All together now: experimental multispecies biofilm model systems. *Environmental Microbiology*, 19, 42–53.

Tang, X., Dang, Z., He, L., Lu, G., Yi, X. T. X. (2012). Biodegradation of Crude Oil by an Artificial Microalgal-Bacterial Consortium, 1, 10–13. https://doi.org/10.4172/scientificreports.118.

Taniguchi, M., Itaya, T., Tohma, T., Fujii, M., (1997). Ethanol production from a mixture of glucose and xylose by a novel co-culture system with two fermenters and two microfiltration modules. *J Ferment Bioeng,* 84, 59–64.

Tochikura T, Yamada H, Beppu T, Soda K. (2001). Hakko-handbook. Tokyo: Kyoritsu Shuppan; 445–446. Japanese.

Tofalo, R., Patrignani, F., Lanciotti, R., Perpetuini, G., Schirone, M., Di Gianvito, P., et al (2016). Aroma profile of montepulciano d'abruzzo wine fermented by single and co-culture starters of autochthonous *Saccharomyces* and *non-Saccharomyces* yeasts. *Front in Microbiol*. https://doi.org/10.3389/fmicb.2016.00610.

Tomas, C. A., Welker, N. E. and Papoutsakis, E. T. (2003). Overexpression of groESL in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program, 69, 4951–4965.

Tramer, J., (1973). Yogurt cultures. *Int. J. Dairy Technol.,* 26, 16-21. doi: 10.1111/j.1471-0307.1973.tb02053.x.

Toro M. E., Vazquez F. (2002). Fermentation behaviour of controlled mixed and sequential cultures of *Candida cantarellii* and *Saccharomyces cerevisiae* wine yeasts. *World J Microb Biot* 18: 347–354.

Tronchoni, J., Curiel, J. A., Morales, P., Torres-Perez, R., & Gonzalez, R. (2017). Early transcriptional response to biotic stress in mixed starter fermentations involving *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. Int. *J. Food Microbiol*, 241, 60–68.

Tsai, S. L., Oh, J., Singh, S., Chen, R., & Chen, W. (2009). Functional assembly of mini-cellulosomes on the *Saccharomyces cerevisiae* cell surface for cellulose hydrolysis and ethanol production. *Appl. Environ. Microbiol*, 75, 6087–6093. https://doi.org/10.1128/AEM.01538-09.

Venters, M., Carlson, R. P., Gedeon, T., and Heys, J. J. (2017). Effects of spatial localization on microbial consortia growth. *PLoS One* 12, 1–20. doi:10.1371/journal.pone.0168592.

Verachtert, H., Shanta Kumara, H. M. C. and Dawoud, E. (1990). Yeasts in mixed cultures with emphasis on lambic beer. In: Yeast: *Biotechnology and Biocatalysis*, 42, 429-449. Verachtert, H. and de Mot, R., Eds., Marcel Dekker, New York.

Ver Berkmoes, N. C., Denef, V. J., Hettich, R. L., & Banfield, J. F. (2009). Functional analysis of natural microbial consortia using community proteomics. *Nat Rev Microbiol*, 7, 196–205.

Versari, A., Patrizi, C., Parpinello, G. P., Mattioli, A. U., Pasini, L., Meglioli, M., & Longhini, G. (2016). Effect of co-inoculation with yeast and bacteria on chemical and sensory characteristics of commercial Cabernet Franc red wine from Switzerland. *Journal of Chemical Technology and Biotechnology*, 91, 876–882. https://doi.org/10.1002/jctb.4652.

Viana, F., Gil, J. V., Genovés, S., Vallés, S., & Manzanares, P. (2008). Rational selection of non-Saccharomyces wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiology*. https://doi.org/10.1016/j.fm.2008.04.015.

Visintin, S., Ramos, L., Batista, N., Dolci, P., Schwan, F., & Cocolin, L. (2017). Impact of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* starter cultures on cocoa beans fermentation. *Int. J. Food Microbiol*, 257(January), 31–40.

Visser, W., Scheffers, WA., Batenburg-van Der Vegte, WH., Van Dijken, JP., (1990). Oxygen requirements of yeasts. *Appl EnvironMicrobiol* 56: 3785–3792.

Walfridsson, M., Hallborn, J., Penttilä, M., Keränen, S., Hahn-Hägerdal, B. (1995). Xylose-metabolizing *Saccharomyces cerevisiae* strains overexpressing the TKL1 and TAL1 genes encoding the pentose phosphate pathway enzymes transketolase and transaldolase. *Appl Environ Microbiol* 61, 4184-4190.

Wang, H. H., Mee, M. T., & Church, G. M. (2013). Applications of Engineered Synthetic Ecosystems. *Synthetic Biology*, 317–325. https://doi.org/10.1016/B978-0-12-394430-6.00017-0.

Wang, C., Esteve-Zarzoso, B., & Mas, A. (2014). Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence in situ hybridization. *Int. J. Food Microbiol*, 191, 1–9. https://doi.org/10.1016/j.ijfoodmicro.2014.08.014.

Wang, Z., Cao, G., Zheng, J., Fu, D., Song, J., Zhang, J. et al (2015). Developing a mesophilic co-culture for direct conversion of cellulose to butanol in consolidated bioprocess. *Biotechnology for Biofuels*, 8, 84. https://doi.org/10.1186/s13068-015-0266-3.

Wang, E-X., Ding, M. Z., Ma, Q., Dong, X. T., Yun, Y. J. (2016). Reorganization of a synthetic microbial consortium for one-step vitamin C fermentation. *Microb Cell Fact*, 15:21 DOI 10.1186/s12934-016-0418-6.

Watanabe, K., (2001). Microorganisms relevant to bioremediation. *Curr Opin Biotechnol*.12, 237-241.

Weckx, S., Van Der Meulen, R., Allemeersch, J., Huys, G., Vandamme, P., Van Hummelen, P., et al. (2010). Community dynamics of bacteria in sourdough fermentations as revealed by their metatranscriptome. *Appl. Environ. Microbiol*. 76, 5402–5408. doi:10.1128/AEM.00570-10.

Wen, Z., Wu, M., Lin, Y., Yang, L., Lin, J., Cen, P., (2014a). Artificial symbiosis for acetone-butanol-ethanol (ABE) fermentation from alkali extracted deshelled corn cobs by co-culture of *Clostridium beijerinckii* and *Clostridium cellulovorans*. *Microb. Cell Fact*. 13, 92.

Wen, Z., Wu, M., Lin, Y., Yang, L., Lin, J., Cen, P., (2014b). A novel strategy for sequential co-culture of *Clostridium thermocellum* and *Clostridium beijerinckii* to produce solvents from alkali extracted corn cobs. *Process Biochem*. 49(11), 1941-1949.

Widder, S., Allen, R. J., Pfeiffer, T., Curtis, T. P., Wiuf, C., Sloan, W. T., et al. (2016). Challenges in microbial ecology: building predictive understanding of community function and dynamics. *ISME J.* 10, 2557–2568. doi:10.1038/ismej.2016.45.

Wolfe, B. E., Dutton, R. J. (2015). Fermented foods as experimentally tractable microbial ecosystems. *Cell*, 161, 49–55. https://doi.org/10.1016/j.cell.2015.02.034.

Woodruff, L.L. (1912) Observations on the origin and sequence of the protozoan fauna of hay infusions. *J.*

Exp. Zool. 12, 205–264.

Wu, P., Wang, G., Wang, G., Børresen, B. T., Liu, H., and Zhang, J. (2016). Butanol production under microaerobic conditions with a symbiotic system of *Clostridium acetobutylicum* and *Bacillus cereus*. *Microb. Cell Fact*. 15, 8. doi:10.1186/s12934-016-0412-z.

Wuster, A.; Babu, M. M. (2010). Transcriptional control of the quorum sensing response in yeast. *Mol. Biosyst*. 6, 134−141.

Xiao H, Li Z, Jiang Y, Yang Y, Jiang W, Gu Y, et al. (2012). Metabolic engineering of D-xylose pathway in *Clostridium beijerinckii* to optimize solvent production from xylose mother liquid. *Metab Eng*, 14, 569–78.

Xu, L., Tschirner, U., (2011). Improved ethanol production from various carbohydrates through anaerobic thermophilic co-culture. *Bioresour Technol*, 102, 10065-10071.

Xu, B., Zhang, H., Chen, K. et al. *Curr Microbiol* (2013) 67: 255. https://doi.org/10.1007/s00284-013-0359-9. DOIhttps://doi.org/10.1007/s00284-013-0359-9.

Xue, F., Miao, J., Zhang, X., Tan, T., (2010). A new strategy for lipid production by mix cultivation of *Spirulina platensis* and *Rhodotorula glutinis*. *Appl. Biochem. Biotechnol*. 160, 498–503.

Yen, H., Chen, P., and Chen, L. (2015). The synergistic effects for the co-cultivation of oleaginous yeast-*Rhodotorula glutinis* and microalgae- *Scenedesmus obliquus* on the biomass and total lipids accumulation. *Bioresour. Technol*. 184, 148–152. doi:10.1016/j.biortech.2014.09.113.

Yin, Z., Stead, D., Selway, L., Walker, J., Riba-Garcia, I., McLnerney, T., Gaskell, S., Oliver, S. G., Cash, P., Brown, A. (2004) "Proteomic response to amino acid starvation in *Candida albicans* and *Saccharomyces cerevisiae*", *Proteomics*, 4, pp. 2425-36.

Young, I. M., & Crawford, J. W. (2004). Interactions and Self-Organization in the Soil-Microbe Complex. *Science*, 304(5677), 1634–1637. https://doi.org/10.1126/science.1097394.

Zapparoli, G., Tosi, E., Azzolini, M., Vagnoli, P., & Krieger, S. (2009). Bacterial inoculation strategies for the achievement of Malolactic fermentation in high-alcohol wines. *S Afr J Enol Vitic*, 30, 49-55. https://doi.org/10.21548/30-1-1424.

Zomorrodi, A. R., Islam, M. M., & Maranas, C. D. (2014). D-OptCom: Dynamic Multi-level and Multi-objective Metabolic Modeling of Microbial Communities. *ACS Synthetic Biology*, 3(4), 247–257. https://doi.org/10.1021/sb4001307.

Zott, K., Thibon, C., Bely, M., Lonvaud-Funel, A., Dubourdieu, D., & Masneuf-Pomarede, I. (2011). The grape must *non-Saccharomyces* microbial community: Impact on volatile thiol release. *Int. J. Food Microbiol*, 151(2), 210–215. https://doi.org/10.1016/j.ijfoodmicro.2011.08.026.

Chapter 3

Research results

The Impact of *Saccharomyces cerevisiae* on a wine yeast consortium in natural and inoculated fermentations

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Author Contributions

FFB and MES conceptualized the study.
BB, FFB and MES designed the experimental layout.
BB performed the experiments, analyzed the data and wrote the first draft of the manuscript. BB, FFB and MES edited subsequent drafts, read and approved the final manuscript

3.1. Abstract

Natural, also referred to as spontaneous wine fermentations, are carried out by the native microbiota of the grape juice, without inoculation of selected, industrially produced yeast or bacterial strains. Such fermentations are commonly initiated by non-*Saccharomyces* yeast species that numerically dominate the must. Community composition and numerical dominance of species vary significantly between individual musts, but *Saccharomyces cerevisiae* will in most cases dominate the late stages of the fermentation and complete the process. Nevertheless, non-*Saccharomyces* species contribute significantly, positively or negatively, to the character and quality of the final product. The contribution is species and strain dependent and will depend on each species or strain's absolute and relative contribution to total metabolically active biomass, and will therefore, be a function of its relative fitness within the microbial ecosystem. However, the population dynamics of multi-species fermentations are not well understood. Consequently, the oenological potential of the microbiome in any given grape must, can currently not be evaluated or predicted. To better characterize the rules that govern the complex wine microbial ecosystem, a model yeast consortium comprising eight species commonly encountered in South African grape musts and an ARISA based method to monitor their dynamics were developed and validated. The dynamics of these species were evaluated in synthetic must in the presence or absence of *S. cerevisiae* using direct viable counts and ARISA. The data show that *S. cerevisiae* specifically suppresses certain species while appearing to favor the persistence of other species. Growth dynamics in Chenin blanc grape must fermentation was monitored only through viable counts. The interactions observed in the synthetic must, were upheld in the natural must fermentations, suggesting the broad applicability of the observed ecosystem dynamics. Importantly, the presence of indigenous yeast populations did not appear to affect the broad interaction patterns between the consortium species. The data show that the wine ecosystem is characterized by both mutually supportive and inhibitory species. The current study presents a first step in the development of a model to predict the oenological potential of any given wine mycobiome.

3.2. Introduction

The alcoholic fermentation of grape must, whether inoculated or not with commercial starter cultures, is initiated by a complex yeast community comprising a high proportion of oxidative and weakly fermentative yeasts (Jolly *et al*., 2003a; Ghosh *et al*., 2015; Wang *et al*., 2015). These species are rapidly outgrown by strongly fermentative yeasts that dominate the middle and end of fermentation (Pretorius *et al*., 1999; Jolly *et al*., 2003b; Zott *et al*., 2008; Bagheri *et al*., 2015; Ghosh *et al*., 2015; Setati *et al*., 2015; Wang *et al*., 2015; Morgan, 2016; Portillo *et al*., 2016; Tristezza *et al*., 2016). The growth and metabolic activity of these yeast species are influenced by physicochemical conditions that prevail during the fermentation process including the rapid depletion of nutrients and oxygen and the accumulation of ethanol (Sainz *et al*., 2003; Mendoza *et al*., 2009). However, beyond such environmental or chemical factors, ecological interactions between yeast species will primarily determine the wine fermentation dynamics and the outcome of the fermentation process (Nissen and Arneborg, 2003; Pina *et al*., 2004; Sadoudi *et al*., 2012; Renault *et al*., 2013; Morales *et al*., 2015; Wang *et al*., 2015; Shekhawat *et al*., 2017). For many years, research evaluated interactions between strains of *S. cerevisiae*, the main wine fermenting yeast, with a focus on killer toxin-producing strains (Branco *et al*., 2014; Williams *et al*., 2015; Albergaria and Arneborg, 2016; Pérez-Torrado *et al*., 2017). However, with the growing interest in non-*Saccharomyces* yeast species and the commercialization of a few species for use as coinoculants in controlled mixed starter fermentations, attention has turned toward evaluating yeast–yeast interactions holistically (Ciani and Comitini, 2015; Albergaria and Arneborg, 2016; Ciani *et al*., 2016; Wang *et al*., 2016). Undoubtedly, wine microbial consortia are difficult to scrutinize. Consequently, some studies have employed simplified models in which the interaction between two species mainly *S. cerevisiae* and non-*Saccharomyces* species were investigated (Andorra *et al*., 2011; Wang *et al*., 2014; Englezos *et al*., 2015; Shekhawat *et al*., 2017). Several aspects, including inoculum ratio, the timing of inoculation of *S. cerevisiae*, cell-cell contact and production of inhibitory metabolites, have been investigated in order to decipher the mechanisms underlying yeast–yeast interactions during wine fermentation (Gobbi *et al*., 2013; Branco *et al*., 2014, 2015; Izquierdo Cañas *et al*., 2014; Kemsawad *et al*., 2015; Lencioni *et al*., 2016). Despite these efforts, the overall interactions among wine yeast species in a fermentation modulated by multiple species remain unclear. Synthetic microbial consortia composed of a subset of culturable strains that simulate the natural community and preserve the indigenous interactions shaped by coadaptation/evolution, provide a tractable model system with reduced complexity (De Roy *et al*., 2014; Ponomarova and Patil, 2015), which makes it easier to study interspecific interactions (Jagmann and Philipp, 2014; Jiang *et al*., 2017). Such a model system also opens opportunities to employ methods inapplicable to complex systems, e.g., species quantitation can easily be done with selective plating, quantitative PCR, fluorescent *in situ* hybridization (*FISH*), and flow cytometry

(Xufre *et al*., 2006; Grube and Berg, 2009; Zott *et al*., 2010; Ponomarova and Patil, 2015). These methods have been applied successfully to monitor population dynamics in wine fermentation. However, they are not without limitations. For instance, FISH and qPCR, require species-specific probes and primers whereas, flow cytometry requires prior knowledge of initial microbial population in order to label different species (Deere *et al*., 1998; Malacrinò *et al*., 2001; Prakitchaiwattana *et al*., 2004; Hierro *et al*., 2006a; Xufre *et al*., 2006; Andorrà *et al*., 2010a and b; Zott *et al*., 2010). In contrast, Automated Ribosomal Intergenic Spacer Analysis (ARISA), which mainly relies on the heterogeneity of the ITS1-5.8S rRNA-ITS2 gene, has been used successfully in several ecological studies (Brežná *et al*., 2010; Kraková *et al*., 2012; Ghosh *et al*., 2015). Like other methods, ARISA may also introduce bias since it is unable to differentiate live and dead cells. However, ARISA is an efficient and rapid tool that can provide a snapshot of the population dynamics (Hierro *et al*., 2006a; Ramette, 2009; Brežná *et al*., 2010; Kraková *et al*., 2012; O'Sullivan *et al*., 2013; Cangelosi and Meschke, 2014; Ženišová *et al*., 2014; Ghosh *et al*., 2015). The current study aimed to evaluate the application of a multi-species yeast consortium as a tool to investigate population dynamics and yeast-yeast interactions in wine fermentation. The constructed model consortium resembles natural wine yeast consortia in so far as comprising species with different fermentative capacities (i.e., weakly fermentative, medium fermentation capacity and strongly fermentative). Moreover, the consortium was formulated based on species that have been encountered and found in sometimes dominant numbers in grape musts from different South African wine regions and cultivars (Jolly *et al*., 2003a; Weightman, 2014; Bagheri *et al*., 2015; Ghosh *et al*., 2015; Morgan, 2016). The model consortium was evaluated in synthetic must in the presence and absence of *S. cerevisiae*, as well as in a real grape juice that differed significantly from the synthetic must. To allow for a rapid and accurate monitoring of the population dynamics, ARISA was optimized and assessed for its suitability and reliability as a tool to semi-quantitatively monitor yeast dynamics in the model consortium. The data show that *S. cerevisiae* strongly and specifically suppresses certain non-*Saccharomyces* yeast species, while also favoring the persistence of other species. The findings suggest that the complex modulation of the yeast ecosystem by *S. cerevisiae* will influence the outcome of wine fermentation by selectively changing the contribution of non-*Saccharomyces* species.

3.3. Materials and methods

3.3.1. Yeast Strains and Culture Conditions

Sixteen yeast isolates obtained from the culture collection of the Institute for Wine Biotechnology (IWBT) and two commercial yeast species, *S. cerevisiae* Lalvin EC1118 (Lallemand, Canada) and *Torulaspora delbrueckii* BIODIVA (Lallemand, Canada) were used in this study (Table 3.1). The yeast stock cultures were maintained in 20% (v/v) glycerol at −80°C and were streaked out on

Wallerstein Laboratory Nutrient agar (WLN) (Sigma–Aldrich, Spain) when required. The plates were incubated at 30°C for 3–5 days

Table 3.1. Strains used in this study and their ITS1-5.8S rRNA-ITS2 gene sizes

Species	Strains number	ITS Size (bp)
Hanseniaspora uvarum (Hu)	Y1104	747
Hanseniaspora vineae (Hv)	Y980	740
Hanseniaspora opuntiae (*Ho*)	Y866	748
Pichia terricola (Pt)	Y974	419
Issatchenkia orientalis (Io)	Y1130	490
Starmerella bacillaris (*Sb*)	Y975	458
Candida apicola (*Cap)*	Y957	457
Candida azyma (Ca)	Y979	436
Candida parapsilosis (Cp)	Y842	522
Candida glabrata (Cg)	Y800	884
Torulaspora delbrueckii (Td)	BIODIVA	797
Rhodotorula glutinis (Rg)	Y824	614
Rhodosporidium diobovatum (Rd)	Y840	618
Kazachstania aerobia (Ka)	Y845	751
Lachancea thermotolerans (*Lt*)	Y973	675
Saccharomyces cerevisiae (Sc)	EC1118	842
Wickerhamomyces anomalus (Wa)	Y934	618
Metschnikowia pulcherrima (Mp)	Y981	377

3.3.2. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Single colonies of each yeast species were inoculated into 5 mL YPD broth (10 g/L yeast extract, 20 g/L peptone and, 20 g/L glucose) and incubated for 16 h at 30°C. Two milliliters of cultures were centrifuged at 5630 × *g* for 10 min to collect the cells. Genomic DNA was extracted using the method described by Sambrook and Russell, (2006). DNA concentration was determined spectrophotometrically, using the NanoDrop^{R} ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, United States). The ITS1-5.8S rRNA-ITS2 gene was amplified using the carboxy-fluorescein labeled ITS1 primer (50-6-FAM- TCC GTA GGT GAA CCT TGC GG-30) and ITS4 (50 - TCC GTA GGT GAA CCTTGC GG-30) in a 25 µL reaction, containing 50 ng DNA, 1U Takara Ex Taq, DNA polymerase (TaKaRa Bio Inc., Olsu, Shiga, Japan), 1 × Taq buffer, 0.25 µM of each primer, 400 µM dNTP mix and 1 mM $MgCl_2$. The PCR reaction was performed under the following conditions: an initial denaturation of 3 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 45 s and a final extension step of 72°C for 10 min (Slabbert *et al*., 2010). Three independent PCR reactions were performed. The PCR products were excised from the gel and purified using the Zymoclean™Gel DNA Recovery Kit Short Protocol (Zymo Research Corporation, Irvine, CA, United States). The ARISA fragments were separated by capillary electrophoresis at the Stellenbosch University Central Analytical Facility on an ABI 3010x

Genetic Analyzer (Applied Biosystems) with a ROX 1.1 labeled size standard (75-1121 base pairs). ARISA profiles were analyzed using Genemapper software version 4.1 (Applied Biosystems). Only fragments with peak area larger than 0.5% of the total fluorescence were considered for further analysis. A bin size of 3 bp for species with ITS region below 700 and 5 bp for species with ITS region above 700 bp, was employed to minimize the inaccuracies in the ARISA analysis (Slabbert *et al*., 2010). The relative abundance of each peak was calculated by dividing individual peak area with the total peak areas for the respective sample.

3.3.3. Micro-Fermentations

3.3.3.1. Fermentation in Synthetic Grape Must

Eight yeast species viz. *Metschnikowia pulcherrima*, *Pichia terricola*, *Starmerella bacillaris*, *Candida parapsilosis*, *Wickerhamomyces anomalus*, *Lachancea thermotolerans*, *Hanseniaspora vineae*, and *S. cerevisiae* were selected to establish a consortium based on (i) their frequent occurrence in grape juices from SA and other wine producing regions, (ii) easy and consistent resolution in ARISA, and (iii) easy morphological detection on WL agar (Jolly *et al*., 2003a; Combina *et al*., 2005; Di Maro *et al*., 2007; Lopandic *et al*., 2008; Romancino *et al*., 2008; Salinas *et al*., 2009; Sun *et al*., 2009; Suzzi *et al*., 2012; Weightman, 2014; Maturano *et al*., 2015; Morgan, 2016). Fermentations were carried out, by inoculating the selected yeast species, in synthetic grape juice medium (pH 3.5) adapted from Bely *et al*. (1990) and Henschke and Jiranek (1993). The medium contained 200 g/L sugars (100 g/L glucose and 100 g/L fructose) and 300 mg/L assimilable nitrogen (460 mg/L NH4Cl and 180 mg/L amino acids). Five hundred milliliters of the juice was dispensed into 500 mL Erlenmeyer flasks, fitted with fermentation locks. The juice was inoculated with the NS-Sc (non-*Saccharomyces*-*Saccharomyces*) consortium comprising of 7 non-Saccharomyces yeast species (*M. pulcherrima*, *P. terricola*, *S. bacillaris*, *C. parapsilosis, W. anomalus, L. thermotolerans*, and *H. vineae*), each inoculated at 10^6 cells/mL and *S. cerevisiae* at 10^3 cells/mL, and the NS (non-*Saccharomyces*) consortium which only consisted of the seven non-*Saccharomyces* yeasts. The fermentations were performed at 25°C with no agitation. Fermentations were monitored by weighing the flasks regularly to measure CO_2 loss. Furthermore, samples were collected regularly to determine sugar concentrations using Fourier Transform Infra-Red Spectroscopy on the Foss Wine scan 2000 (Rhine Ruhr, Denmark). Samples were withdrawn at 2-day intervals and yeast population dynamics was monitored by direct plating on WLN agar and ARISA.

3.3.3.2. Real Must Fermentation

Fifty liters of clarified Chenin blanc grape juice was obtained from a commercial cellar. The chemical composition of juice was measured, using spectroscopy technique by Foss wine scan

2000 (Rhine Ruhr, Denmark). The yeast community composition of the juice was determined by serial dilution and direct plating on WL-agar, followed by identification through ITS-5.8S rRNA amplification, RFLP, and sequencing as described in Bagheri *et al*. (2015). Subsequently, 1.5 L Chenin blanc grape juice was dispensed into 2 L fermentation bottles. Three sets of fermentations were performed: (i) spontaneous (ii) *Sc*-inoculated fermentation (at 10^3 cells/mL, *S. cerevisiae* EC1118), and (iii) NS-Sc consortium inoculated (7 non-*Saccharomyces* at 10^6 cells/mL. vs. *S. cerevisiae* at 10^3 cells/mL). The fermentations were performed in triplicate, at 25°C, and without SO_2 addition. The fermentations were weighed daily to monitor CO_2 release and samples were withdrawn at 2-day intervals to monitor population dynamics. The residual sugar at the end of fermentation was measured. The fermentations were considered complete when residual sugars in wine were less than 2 g/L and the yeast population dynamics was monitored by direct plating on WLN agar.

3.3.4. Statistical Analysis

The DNA extraction, ARISA analysis, and fermentations were performed in triplicate. The values were presented as means ± SD. The differences between treatments were determined using analysis of variance (ANOVA) with the statistical software Statistica version 13.0 (StatSoft Inc., Tulsa, OK, United States). The differences were considered significant should the *p*-values were equal or less than 0.05. For multivariate data analysis, the Principal Component Analysis was performed, using XLSTAT in Microsoft R Excel (2016).

3.4. RESULTS

3.4.1. Selection of Yeast Species for the Consortium

Eighteen yeast species commonly isolated from South African grape musts (Jolly *et al*., 2003a; Weightman, 2014; Bagheri *et al*., 2015; Morgan, 2016), were initially evaluated for DNA extractability and resolvability in ARISA analysis. The ARISA profile of the mixed community only revealed 13 peaks (Figure 3.1).

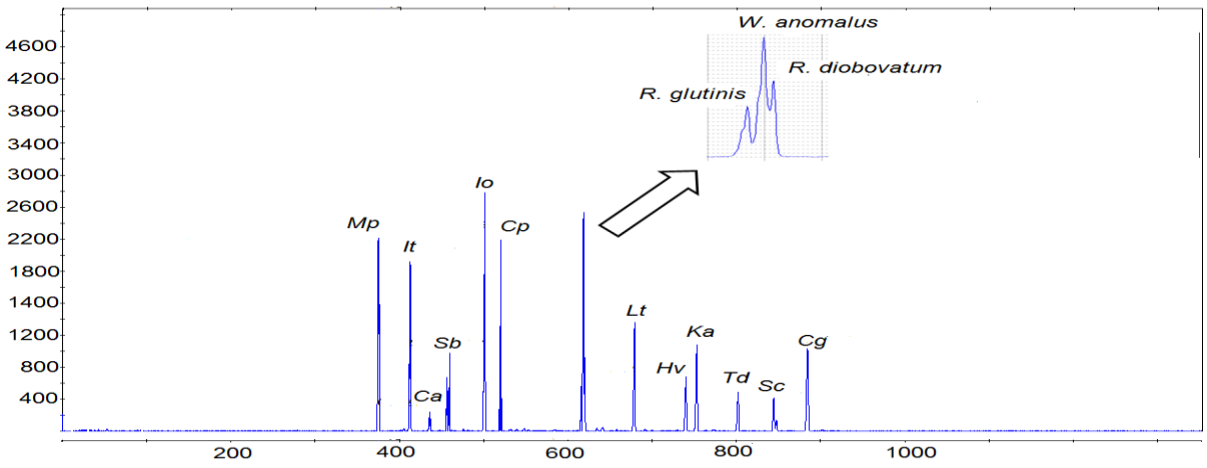


Figure 3.1. Electropherogram of a mixed culture of 18 yeast species, generated via PCR amplification with ITS1F-ITS4 primers. The x-axis represents the fragment size (bp) and the y-axis represents the relative fluorescence intensity. The following abbreviations were used for names of yeast species. *Mp, Metschnikowia pulcherrima; Pt, Pichia terricola; Ca, Candida azyma; Sb, Starmerella bacillaris; Io, Issatchenkia orientalis; Cp, Candida parapsilosis; Lt, Lachancea thermotolerans; Hv, Hanseniaspora vineae; Ka, Kazachstania aerobia; Td, Torulaspora delbrueckii; Sc, Saccharomyces cerevisiae*; *Cg, Candida glabrata*.

An overlap between *Rhodotorula glutinis* (614 bp), *R. diobovatum* (618 bp) and *W. anomalus* (618 bp) was observed. Similarly, *H. uvarum* (747 bp), *H. opuntiae* (748 bp), and *Kazachstania aerobia* (751 bp), as well as *S. bacillaris* (458 bp) and *C. apicola* (458 bp) co-migrated and could not be resolved. Consequently, eight species (*M. pulcherrima*, *P. terricola*, *S. bacillaris*, *C. parapsilosis*, *W. anomalus*, *L. thermotolerans*, *H. vineae*, and *S. cerevisiae*), which could be reliably resolved in ARISA, and could be distinguished based on their colony morphology on WLN agar, were selected to establish a model consortium. The efficiency of DNA extraction method and ARISA on the consortium was evaluated. In addition, standard curves of optical density (OD_{600} nm) vs. colony forming units (CFU/mL) were established for each species (data not shown). A cell suspension containing approximately each at 10^5 CFU/mL was prepared. Total genomic DNA was extracted from the mixed culture and ARISA was performed. Similar peak heights and peak areas were observed for all species, suggesting that the DNA extraction method and ARISA were efficient for all of them (Figure 3.2).

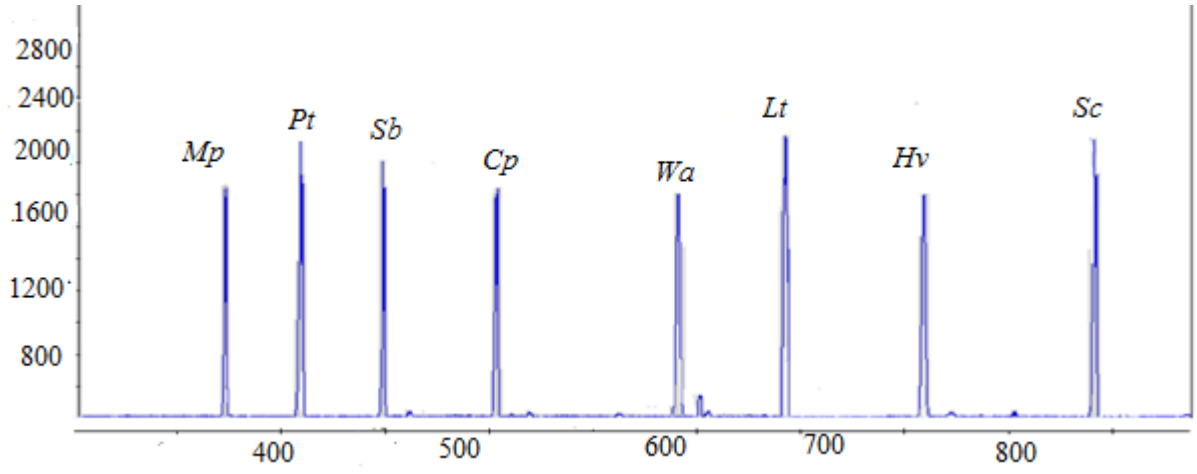


Figure 3.2. Quantitative validation between the ARISA peaks of eight selected yeast species and CFU/mL. All yeast species were inoculated at 10^5 CFU/mL. The x-axis represents the fragment size (bp) and the y-axis represents the relative fluorescence intensity.

3.4.2. Validation of ARISA in the Model Consortium

The detection limit of ARISA was investigated in different inoculation scenarios, representing low and high levels of selected yeast species (Table 3.2).

Table 3.2. Yeast inoculum combinations used to determine ARISA detection limits

Yeasts species	A	B	C
H. vineae	10^3	10^4	10^3
S. bacillaris (C. zemplinina)	10^3	10^4	10^3
C. parapsilosis	10^3	10^4	10^3
P. terricola	10^3	10^4	10^3
L. thermotolerans	10^3	10^6	10^3
W. anomalus	10^3	10^4	10^3
M. pulcherrima	10^3	10^4	10^3
S. cerevisiae	10^3	10^4	10^6

The data indicated that when all species were inoculated at the same level, they could be detected even at 10^3 CFU/mL while, in a situation where one species was significantly higher in concentration (≥10^6 CFU/mL), other species could be detected if present at 10^4 CFU/mL but not at 10^3 CFU/mL (Figure 3S1). Therefore, the detection limit of ARISA was defined as the lowest cell concentration (10^4 CFU/mL) that resulted in a positive signal and fluorescence intensity above 50 relative fluorescence units (RFU). To test the repeatability and reliability of ARISA for monitoring the yeast dynamics throughout the fermentation, three independent DNA extractions were performed from a sample in which the yeasts were mixed in different concentrations. In each case, similar peak profiles were observed for triplicates with minor variations in peak intensities (Figure 3S2). For better quantification of the individual yeast species, standard curves correlating colony forming units and peak areas were established. Strong linear correlation between CFU/mL and ARISA peak area, with an R_2 value of ≈0.9 was observed, for individual yeast species (Figure 3.3). However, at lower biomass, the correlation between peak area and viable counts was nonlinear.

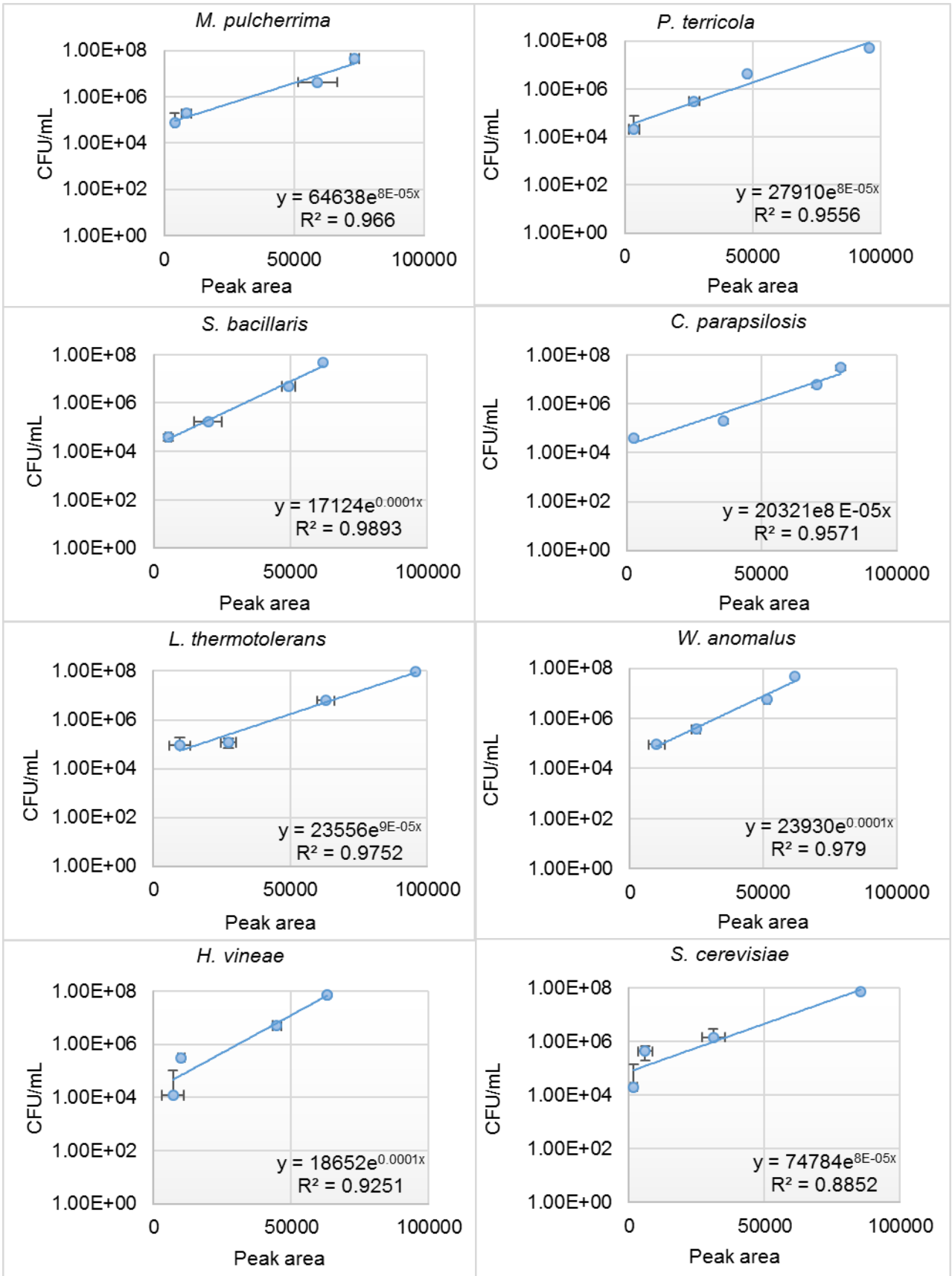


Figure 3.3. Standard curves of individual yeast species in the consortium. The correlation between the colony forming unit and peak area (bp) was investigated at different dilutions (10^3–10^7 CFU/mL) for individual yeast species in the consortium.

3.4.3. Fermentation in Synthetic Grape Juice

3.4.3.1. Fermentation and Growth Kinetics

The applicability of the consortium and ARISA as a model was tested in the synthetic grape juice fermentation, inoculated with NS-Sc and NS only. The two sets of fermentations displayed distinct kinetics, with the NS-Sc fermentation reaching dryness (residual sugar < 2 g/L) within 21 days, while the fermentation with the NS consortium was sluggish and still had a total of 88 g/L residual sugar by day 30 (Figure 3.4). The NS fermentation got stuck at this level since the residual sugar was found to be the same after 40 days.

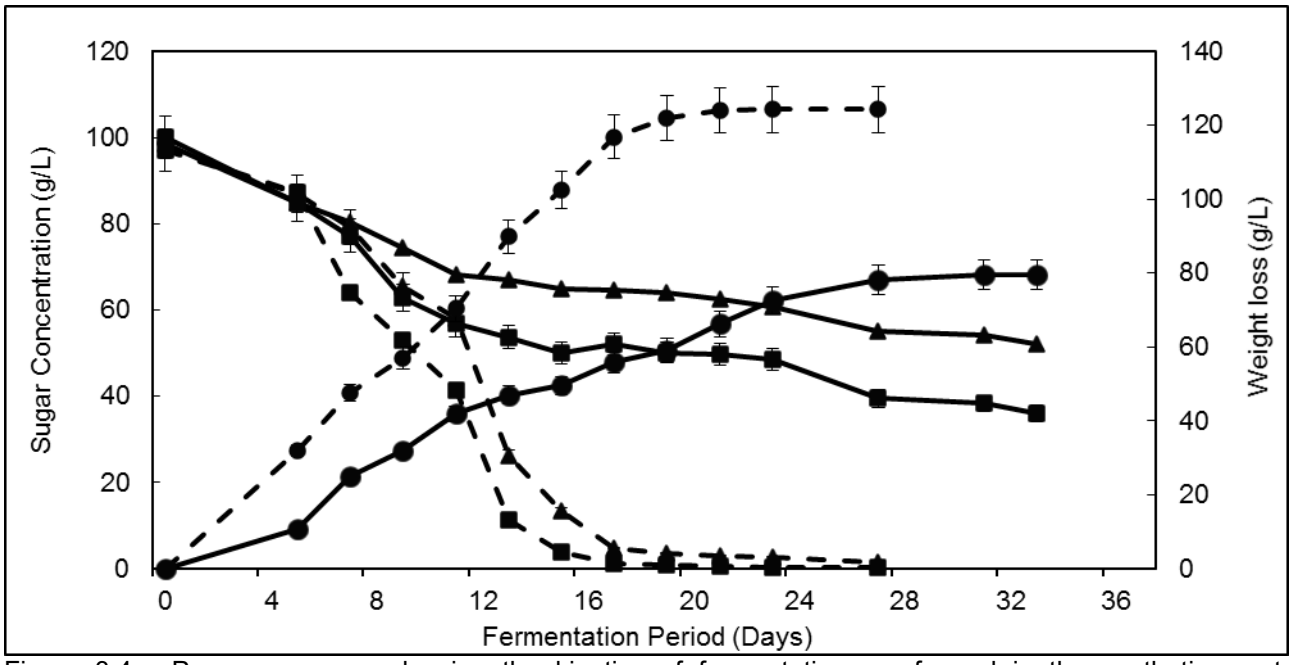


Figure 3.4. Progress curves showing the kinetics of fermentations performed in the synthetic must. Fermentation performed with NS-Sc consortium is indicated with broken lines while fermentation with NS consortium is indicated with solid lines. Glucose (■), fructose (▲) and weight loss (●) were monitored throughout fermentation.

3.4.3.2. Yeast Population Dynamics in Synthetic Grape Juice

Comparison of ARISA and viable counts from the NS-Sc fermentation revealed similar trends in the relative abundance of the individual species in the early stage of fermentation (Figure 3.5). However, in the middle and final fermentation stages, ARISA consistently showed higher levels of *S. cerevisiae* and lower levels of *H. vineae* than direct plating (Table 3S1). In addition, *M. pulcherrima* and *P. terricola* were detectable by ARISA until the end of fermentation while, they could not be observed and enumerated on agar plates.

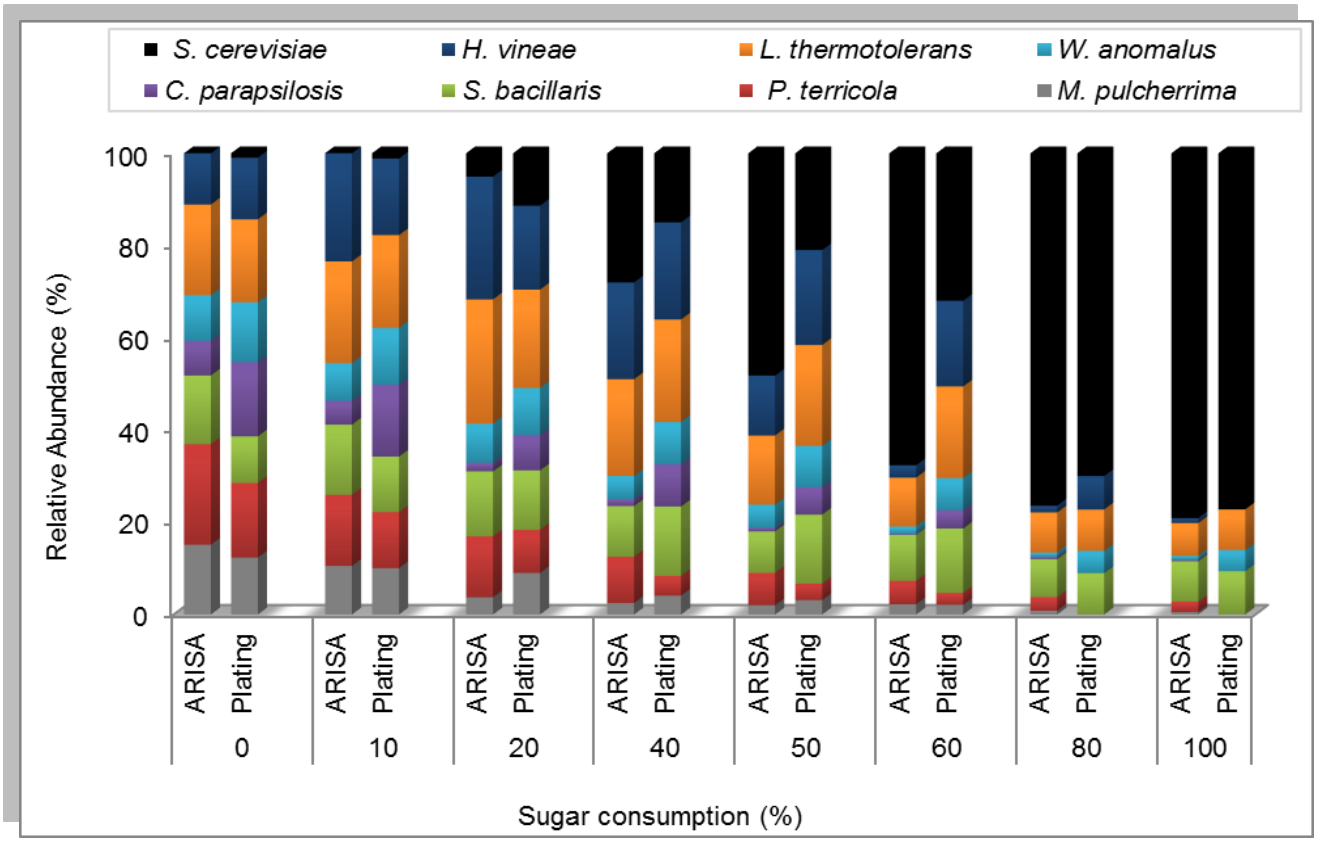


Figure 3.5. Relative abundance of yeast species throughout the NS-Sc fermentation in synthetic grape must. Yeast population dynamics were monitored using ARISA and plating methods.

Analysis of the yeast dynamics in the NS-Sc fermentation by standard plating on WLN agar revealed an initial increase in the population of non-*Saccharomyces* species until 10% of the sugar was consumed. The individual non-*Saccharomyces* species reached up to 10^7–10^8 CFU/mL and maintained viability at these levels for a brief period, before starting to decline. *P. terricola* and *C. parapsilosis*, dropped below detection by 50% sugar consumption, whereas *M. pulcherrima* and *H. vineae* were below detection after 70% and 90% sugar consumption, respectively (Figure 3.6).

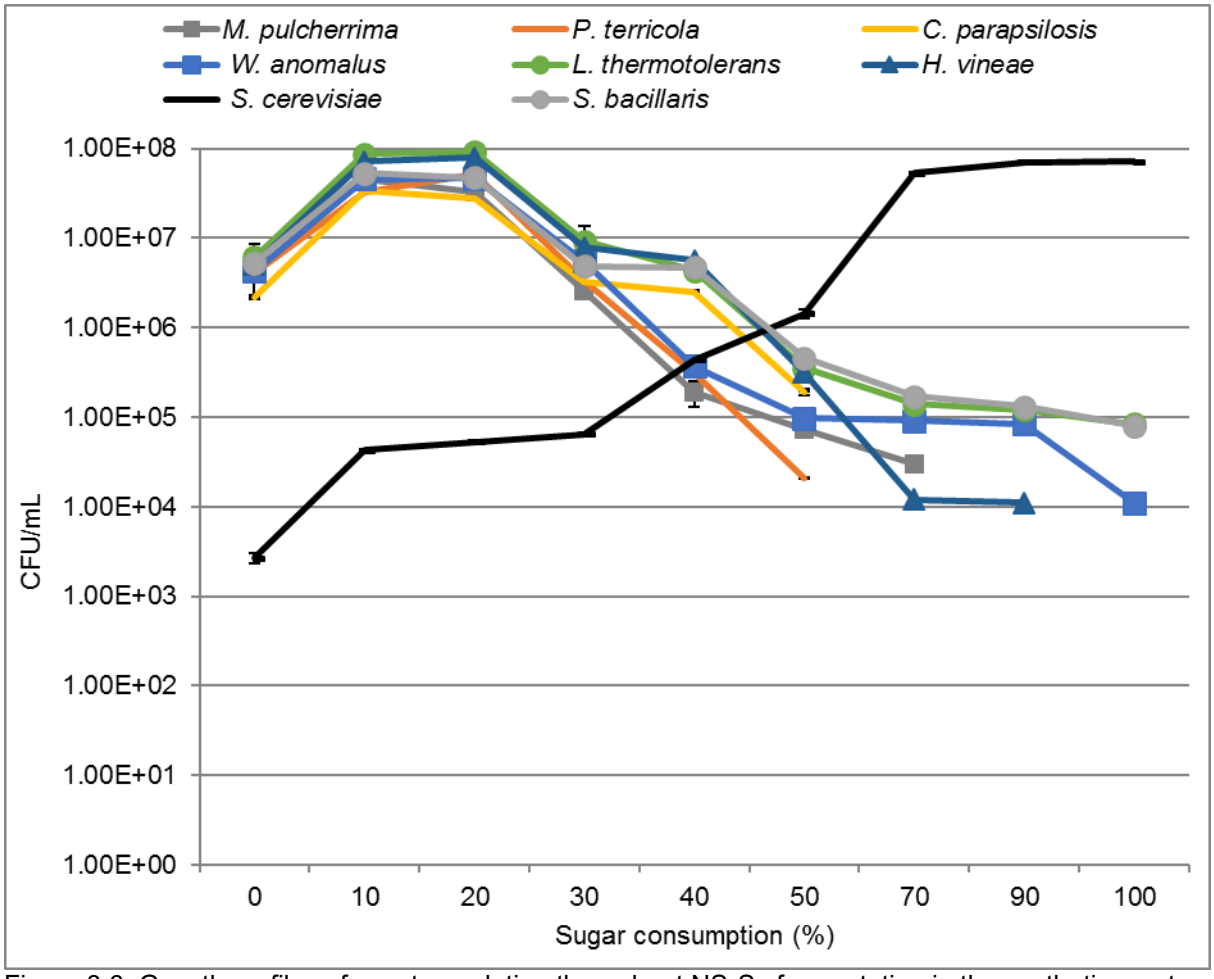


Figure 3.6. Growth profiles of yeast population throughout NS-Sc fermentation in the synthetic must.

In contrast, the population of *S. cerevisiae* increased steadily from 10^3 CFU/mL to 4.37 × 10^4 CFU/mL (20% sugar consumption) where the population of all non-*Saccharomyces* species declined to 10^6 CFU/mL. When *S. cerevisiae* reached to 6.47 × 10^4 , a decline in the population of *W. anomalus* (3.70 × 10^5), *P. terricola* (3.10 × 10^5) and *M. pulccherrima* (1.90 × 10^5) was observed whereas, the population of *C. parapsolosis*, *H. vineae*, *S. bacillaris*, and *L. thermotolerans* remained at 10^6 CFU/mL. Finally, *S. cerevisiae* dominated the fermentation and reached to 7.19 × 10^7 CFU/mL. *L. thermotolerans* (8.40 × 10^4), *S. bacillaris* (8.03 × 10^4), and *W. anomalus* (1.10 × 10^4) remained viable until the end of fermentation. In the NS fermentation, the levels of *S. bacillaris*, *P. terricola*, and *L. thermotolerans* increased moderately and maintained dominance until 40% of the sugar was consumed while, *M. pulcherrima* and *C. parapsilosis* declined steadily from the onset of fermentation. Using the standard curves constructed as described in the previous section, the population of *S. bacillaris*, *P. terricola*, and *L. thermotolerans* was estimated to be 1.48 × 10^5, 5.33 × 10^5, and 2.82 × 10^5 CFU/mL, respectively, whereas the population of *M. pulcherrima* and *C. parapsilosis* was 1.22 × 10^3 and 1.69 × 10^3 CFU/mL. The population of *H. vineae* at 40% sugar consumption was estimated to be 2.07 × 10^3 CFU/mL. After 50% of the sugar was consumed, only four species (*L. thermotolerans, S. bacillaris, P. terricola,* and *W. anomalus*) were detected, with *W. anomalus*, accounting for 65% of the population. The population of *L.*

thermotolerans, *S. bacillaris, P. terricola*, and *W. anomalus* based on the standard curves were 2.74 × 10^5, 5.58 × 10^4, 2.77 × 10^4, and 7.23 × 10^6 CFU/mL, respectively. The fermentation got stuck at 60% of sugar consumption and *W. anomalus* was the only detectable yeast at this stage (Figure 3.7). The level of *W. anomalus* based on the standard curve was estimated to be 9.67 × 10^6 CFU/mL by 60% of sugar consumption in NS fermentation while *S. cerevisiae* reached up to 7.19 × 10^7 CFU/mL by the end of the NS-Sc fermentation.

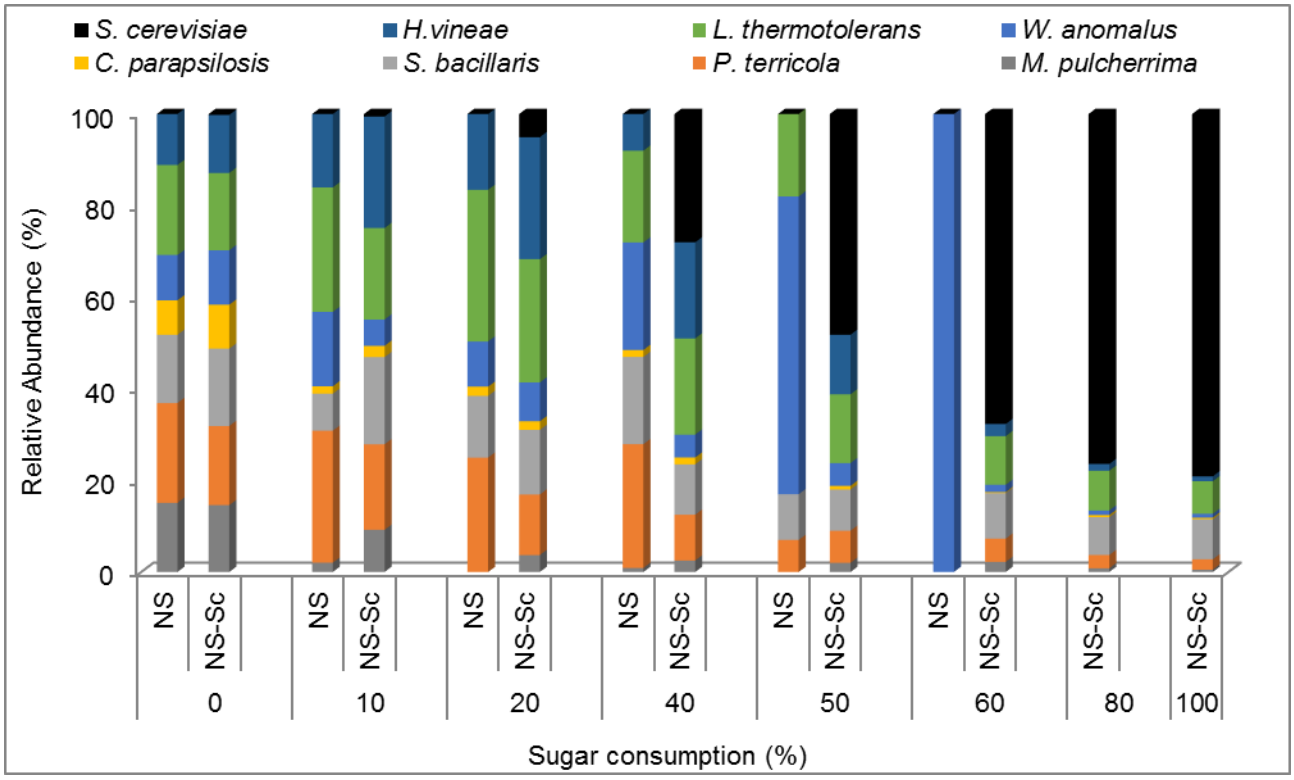


Figure 3.7. Relative abundance of yeast species during fermentations performed with NS-Sc and NS. Yeast population dynamics was monitored using ARISA.

3.4.4. Chemical Parameters and Yeast Diversity in Chenin blanc Juice

The Chenin blanc juice used in the current study was at 21.7 °Brix with a total acidity of 3.23 g/L, pH 3.37 and a yeast assimilable nitrogen (YAN) of 195 mg/L. Sugar content and YAN concentration were higher in Chenin blanc juice compared to the synthetic must (Table 3.3). One hundred and eighty four yeast isolates obtained from the Chenin blanc juice were identified and revealed that the initial indigenous yeast population comprised *M. pulcherrima* (2.39 × 10^3 CFU/mL), *H. uvarum* (4.21 × 10^3 CFU/mL), *L. thermotolerans* (2.70 × 10^3 CFU/mL), *W. anomalus* (3.34 × 10^3 CFU/mL) and *S. cerevisiae* (4.85 × 10^3 CFU/mL).

Table 3.3. Chemical parameters of Chenin blanc must compared to the synthetic grape must.

Chemical parameter	Chenin blanc must	Synthetic must
Sugar (°Brix)	21.7	20
YAN (mg/L)	195	300
pH	3.37	3.5

3.4.5. Chenin blanc Fermentations

A comparison of the spontaneous fermentation, the Scinoculated, and the NS-Sc inoculated fermentations, revealed that the Sc fermentation was the fastest and reached dryness in 24 days, followed by the spontaneous fermentation at 26 days, while, NS-Sc fermentation took 28 days to reach dryness (Figure 3.8).

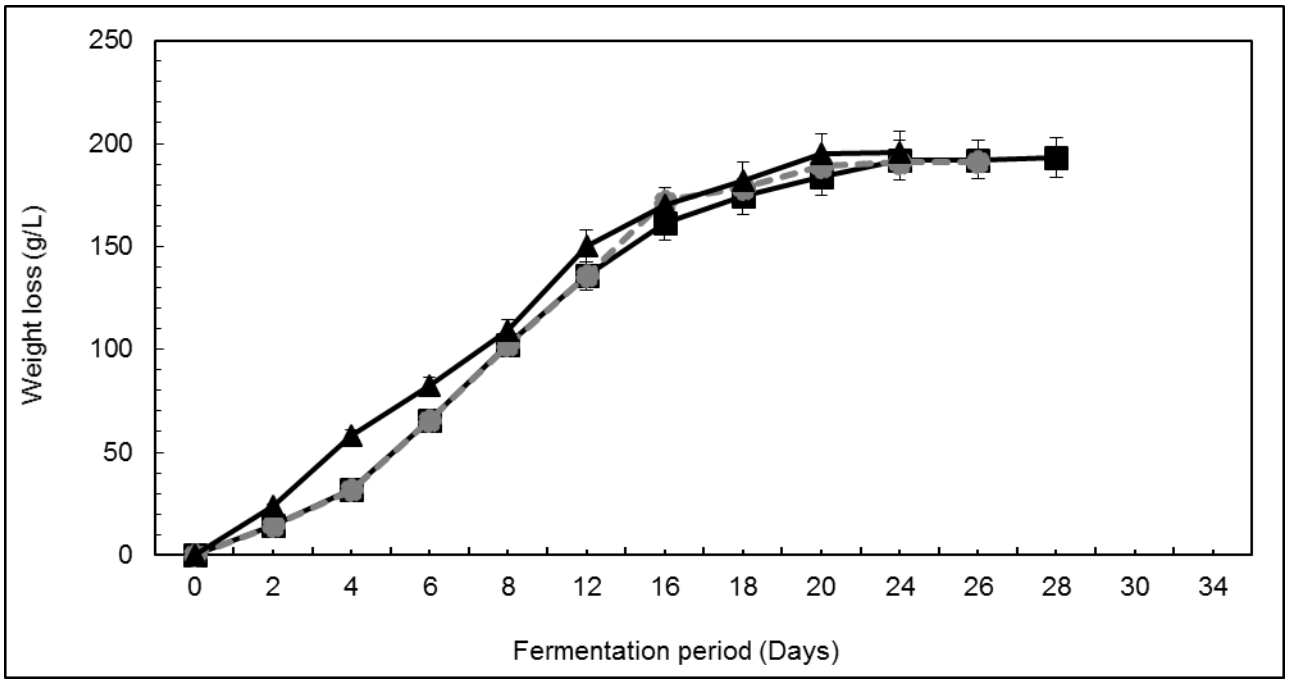
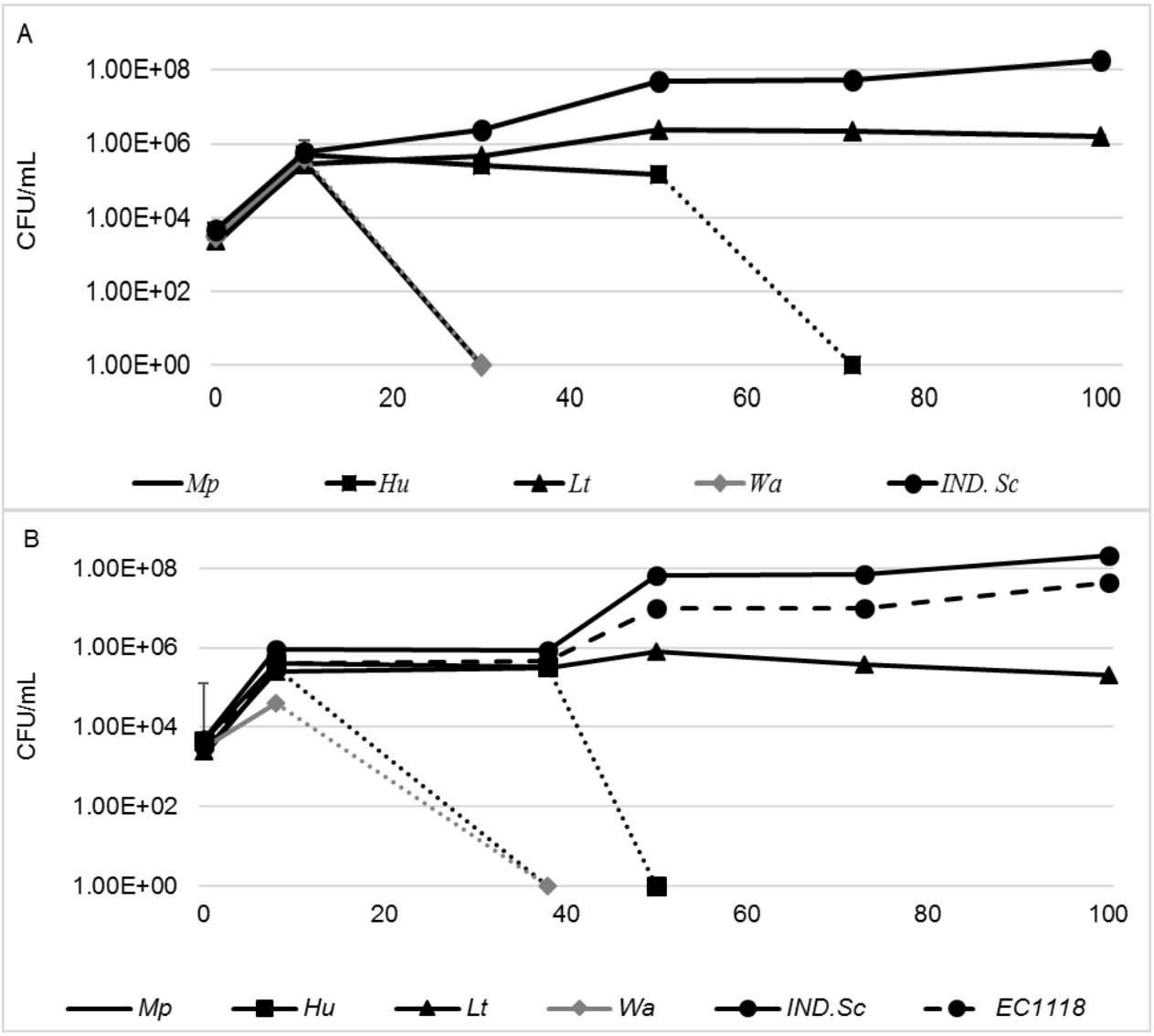


Figure 3.8. Progress curves displaying the kinetics of spontaneous fermentation (●), fermentation inoculated with *Sc* (▲) and fermentation inoculated with NS-Sc consortium (■).

The spontaneous fermentation of the juice was characterized by an initial increase in the yeast population from ≈10^3 CFU/mL to 6.27 × 10^5 CFU/mL, by 10% sugar consumption. Subsequently, a decline in some non-*Saccharomyces* species was observed; amongst them, *W. anomalus* and *M. pulcherrima* declined rapidly and could not be detected by 30% sugar consumption, while *H. uvarum* persisted until 50% of the sugar was consumed. In contrast, *L. thermotolerans* increased in growth up to 2.3 × 10^6 CFU/mL at 50% sugar consumption and persisted until the end of fermentation. The indigenous *S. cerevisiae* (IND-Sc) increased from ≈10^3 CFU/mL to a maximum of 1.82 × 10^8 CFU/mL (Figure 3.9A). Similar trends were observed in the *Sc*-inoculated fermentation. However, *W. anomalus* only grew up to 4 × 10^4 CFU/mL and *H. uvarum* persisted until 40% sugar consumption (Figure 3.9B). In addition, *L. thermotolerans* only reached a maximum of 8 × 10^5 CFU/mL. Within the *S. cerevisiae* population, IND-Sc and EC1118 displayed similar growth patterns. However, IND-Sc persisted at a higher level, reaching a maximum of 2.1 × 10^8 CFU/mL, while EC1118 reached 4.5 × 10^7 CFU/mL (Figure 3.9B). When the NS-Sc consortium was inoculated, *H. uvarum* (the only indigenous non-*Saccharomyces* yeast that was not part of the consortium), grew from 4.4 × 10^3 to 6.20 × 10^4 CFU/mL by 10% sugar consumption followed by a

steady decline until it could not be detected by 50% sugar consumption (Figure 3.9C). Amongst the remainder of the non-*Saccharomyces* yeasts which were inoculated at ≈10^6 CFU/mL, *P. terricola* and *C. parapsilosis* declined below detection after 10% sugar consumption, followed by *M. pulcherrima* and *W. anomalus* by 28% sugar consumption. In contrast, *H. vineae* declined gradually until 78% sugar consumption; *S. bacillaris* persisted at 10^6 CFU/mL until 78% sugar consumption before dropping to 8 × 10^4 CFU/mL at the end of fermentation, while, *L. thermotolerans* persisted at 10^6 CFU/mL until the end of fermentation. The *S. cerevisiae* population behaved in a similar way as observed in the *S. cerevisiae* inoculated fermentation, albeit at 10 times less cell concentrations. For instance, IND-Sc reached a maximum of 3.2 × 10^7 CFU/mL, while EC1118 reached 6.9 × 10^6 CFU/mL.



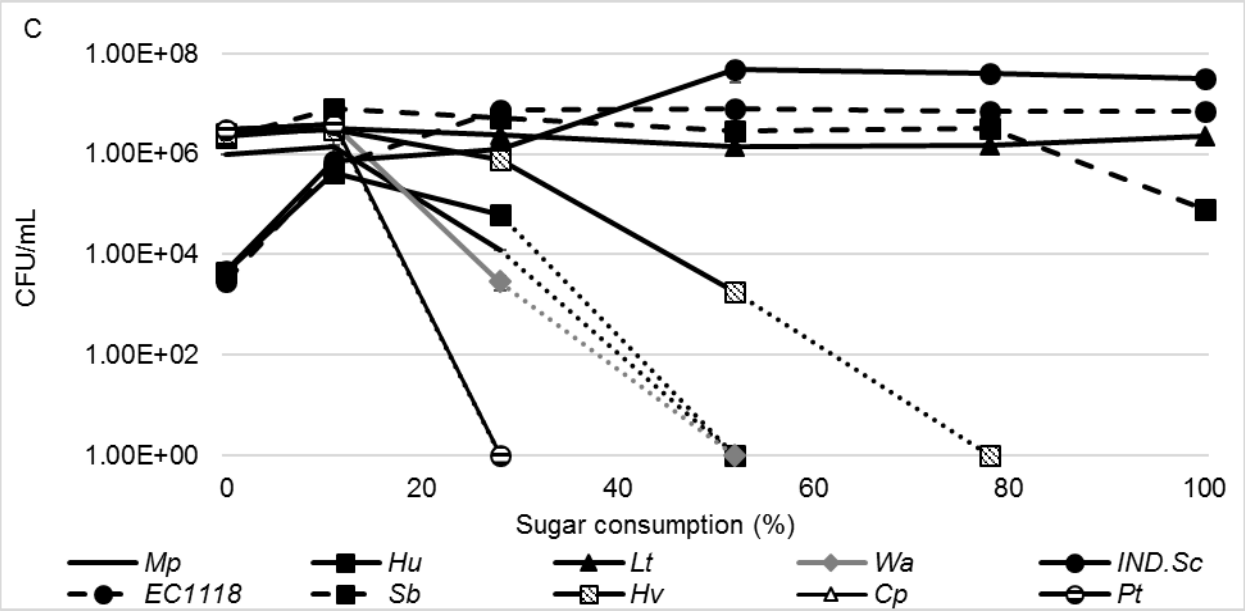


Figure 3.9. Yeast population dynamics in Chenin blanc spontaneous fermentation (A), S. cerevisiae inoculated fermentation (B) and NS-Sc consortium fermentation (C). The following abbreviations were used for names of yeast species. *Mp, M. pulcherrima; Pt, P. terricola; Sb, S. bacillaris; Cp, C. parapsilosis; Lt, L. thermotolerans; Hv, H. vineae; Hu, H. uvarum* and IND.Sc, Indigenous *S. cerevisiae.*

3.5. Discussion

The current study aimed to establish and validate a model system for reliable monitoring and prediction of the temporal trajectories of yeast populations within the wine fermentation ecosystem. To this end, a yeast consortium comprising *S. cerevisiae* and seven non-*Saccharomyces* yeast species of varying fermentative capacities was constructed. These yeast species are all regularly encountered in SA grape juices, and some species have sometimes been detected in significant numbers. Furthermore, all of these non-*Saccharomyces* species have been isolated in countries with several wine producing regions such as Italy, France, Argentina, China, and Brazil (Jolly *et al*., 2003a; Combina *et al*., 2005; Di Maro *et al*., 2007; Lopandic *et al*., 2008; Romancino *et al*., 2008; Salinas *et al*., 2009; Sun *et al*., 2009; Suzzi *et al*., 2012; Tofalo *et al*., 2012; Weightman, 2014; Maturano *et al*., 2015; Morgan, 2016). These yeast species also differed in their ITS1- 5.8S rRNA-ITS2 gene sizes, which made ARISA a suitable method to monitor their dynamics. Our data show that in this semi-complex consortium, the detection limit of ARISA could be as low as 10^3 CFU/mL when all species are present at low levels. However, at lower biomass (10^{3-4} CFU/mL) larger deviations were observed, possibly due to the bias introduced by DNA extraction or preferential amplification in PCR (Giraffa, 2004; Ramette, 2009). Furthermore, in a typical wine fermentation scenario where dominant taxa grow up to 10^{7-8} CFU/mL, minor taxa would not be detected below 10^4 CFU/mL. ARISA is also unable to differentiate between strains of the same species, limiting its ability to monitor strain-specific dynamics. However, species-specific interactions of significantly contributing species can be easily detected and quantified (Ramette, 2009; Ženišová *et al*., 2014; Ghosh *et al*., 2015; Setati *et al*., 2015). The limits are similar to those obtained for *FISH* (Xufre *et*

al., 2006) and PCR-DGGE (Prakitchaiwattana *et al*., 2004) and they are less sensitive than qPCR (10^2 CFU/mL) and flow cytometry (10^3 CFU/mL) methods (Malacrinò *et al*., 2001; Hierro *et al*., 2006a and b; Zott *et al*., 2010). However, ARISA does not require species-specific primers and is less technically demanding than qPCR and flow cytometry. Overall, ARISA generated similar growth patterns for individual yeast species in the consortium as observed with viable counts. However, some discrepancies were observed in the middle and final stage of fermentation. These discrepancies might reflect biases and limitation in both methods. For instance, plating method might show bias against cells in a VBNC state and injured population (Divol and Lonvaud-Funel, 2005; Renouf *et al*., 2007) while ARISA is unable to differentiate between live and dead cells (Xie *et al*., 2007; O'Sullivan *et al*., 2013). Consequently, an overestimation of most of the species (e.g., *M. pulcherrima*, *P. terricola*, *H. vineae*, *L. thermotolerans, S. bacillaris*, and *S. cerevisiae*) by one order of magnitude was evident with ARISA compared to the plating method. The data in the current study suggest that up to 3% of dead cells could possibly be detected by ARISA. Similarly, Salinas *et al*., (2009), indicated that qPCR overestimate the number of live cells in average one order higher compared to microscopy analysis, which according to Hierro *et al*., (2006a), could represent up to 1% of the dead cells. Our study showed that the yeast species constituting the consortium responded differently to the wine fermentation ecosystem, and the behavior of the non-*Saccharomyces* species was differentially influenced by the presence of *S. cerevisiae*. The data showed that in the absence of *S. cerevisiae*, some non-*Saccharomyces* species such as *M. pulcherrima* and *C. parapsilosis* experienced a decline from the onset of fermentation whereas, species such as *S. bacillaris*, *P. terricola*, and *L. thermotolerans* experienced a moderate increase followed by a steady decline in the absolute numbers by the middle of fermentation. On the contrary, *W. anomalus* suppressed the rest of non-*Saccharomyces* species and increased in cell concentration back to the initial inoculum level. This suggests that *W. anomalus* can withstand the chemical milieu created in the early stages of the fermentation better than the other yeast species and may utilize the nitrogen released by dead cells. In contrast, in the presence of *S. cerevisiae*, specifically, this yeast declines early in fermentation, suggesting that *S. cerevisiae* creates an unconducive environment, which suppresses *W. anomalus*. Indeed, an antagonistic interaction between *S. cerevisiae* and *W. anomalus*, has been proposed in other fermentation ecosystems (Ye *et al*., 2014). *S. cerevisiae* may inhibit other organisms through a variety of mechanisms including the production of short chain fatty acids and glycoproteins (killer toxin), and the specific antagonism exerted by *S. cerevisiae* modulates the ecosystem (Vannette and Fukami, 2014; Boynton and Greig, 2016). Conversely, other yeast species such as *M. pulcherrima*, *P. terricola*, and *C. parapsilosis* consistently declined in the early stages of the fermentation, both in the presence and in the absence of *S. cerevisiae*, suggesting that the decline could be due to another factor such as oxygen limitation. Several studies have shown that the growth and survival rate of *M. pulcherrima* and *C. parapsilosis* was markedly enhanced in aerated fermentations (Oh *et al*.,

1998; Rossignol *et al*., 2009; Morales *et al*., 2015; Shekhawat *et al*., (2017). Furthermore, in the presence of *S. cerevisiae*, *L. thermotolerans*, and *S. bacillaris* could survive until late fermentation. The survival of *L. thermotolerans* until end of the fermentation has been shown previously (Gobbi *et al*., 2013). In addition, *S. bacillaris* strains are typically fructophilic and therefore preferentially utilize fructose, which is less preferred by *S. cerevisiae*. Interestingly, our study revealed that *H. vineae* survives better in the presence *S. cerevisiae* suggesting a positive interaction between the two yeasts. Such an interaction is perhaps not coincidental since other studies have shown that in nutrientrich conditions, co-fermentations using strains of these two species often reflect a significant contribution of *H. vineae* to wine aroma and flavor (Viana *et al*., 2011; Medina *et al*., 2013). Based on our current findings, we can infer that the mutualism (*S. cerevisiae* and *H. vineae*) and antagonism (*S. cerevisiae* and *W. anomalus*) observed in the wine ecosystem, could be a species-specific interaction that occurs as a result of the presence of *S. cerevisiae*. However, the strength of the mutualism or antagonism in the wine consortium may vary between different strains of one species requires further investigation. Indeed, species-specific patterns throughout the wine fermentation process are probable and comprehensible. For instance, it is well established that some species decline rapidly by early or midfermentation (*Cryptococcus carnescens*, *Aureobasidium pullulans*, *P. terricola*, and *M. pulcherrima*), others repeatedly persist until late fermentation (*S. bacillaris*, *L. thermotolerans*, *T. delbrueckii*) regardless of the strain variability (Jemec *et al*., 2001; Sun *et al*., 2009; Cordero-Bueso *et al*., 2011; Bezerra-Bussoli *et al*., 2013; Gobbi *et al*., 2013; Milanovic *et al*., 2013; Bagheri *et al*., 2015).

One of the goals of the current study was to establish a consortium that would serve as a representative model to predict yeast dynamics in wine fermentation. In order to validate the suitability of this consortium, it was used as an inoculum in Chenin blanc must and the dynamics was monitored throughout the fermentation. Interestingly, four of the yeast species (*M. pulcherrima*, *L. thermotolerans*, *W. anomalus*, and *S. cerevisiae*) which form part of the consortium were also present in the natural yeast community of the Chenin blanc must, confirming once more the representative nature of our consortium. Our study shows that all the species in the consortium could compete with the native yeast species in a non-sterilized must. While we were unable to differentiate between the indigenous strains and inoculated strains (e.g., *W. anomalus*), the population dynamics observed were similar to those described for the synthetic grape juice, suggesting species, and not strain specific drivers of interactions. This is further supported by the fact that the dynamics were preserved although the environmental conditions, including nitrogen and sugar levels, differed consoderably between the two matrices (Table 3S2). We also observed that the indigenous *S. cerevisiae* population displayed better growth than the EC1118 inoculated strain although they were at similar levels at the beginning of the fermentation, further indicating that the selective drivers were species and not strain-dependent. Our data show that the consortium constructed in the current study serves as a viable and robust model to assess yeast

population dynamics during wine fermentation since the matrix did not have a considerable influence on the dynamics as such. We suggest that the yeast dynamics observed in the current study is mainly due to species-specific interactions and the selective pressure applied by *S. cerevisiae* to other species. Our data suggest that inoculation with *S. cerevisiae* favors the persistence of some non-*Saccharomyces* species in wine fermentation whereas; it clearly suppresses the growth and contribution of other non-*Saccharomyces* species. The dynamics of the wine ecosystem is driven by a multitude of positive and negative yeast-yeast interactions.

The main challenge in microbial ecology is to link microbial composition to function. Here, we demonstrate that a model consortium approach can be used as a tool to predict the microbial behavior in a complex natural environment. Such a model consortium can be easily perturbed under well-controlled conditions in order to gain a deep understanding of the effect of environmental parameters on yeast–yeast interactions. In-depth insight on yeast–yeast interactions may allow us to manipulate the microbial community and enhance the population of the beneficial microbes or suppress the population of undesirable yeast species. The study presents a first step in the development of a model to predict the oenological potential of any given wine mycobiome.

3.6. References

Albergaria, H., and Arneborg, N. (2016). Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: role of physiological fitness and microbial interactions. *Appl. Microbiol. Biotechnol.* 100, 2035–2046. doi:10.1007/s00253-015-7255-0.

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2010a). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *Eur. Food Res. Technol.* 231, 215–224. doi:10.1007/s00217-010-1272-0.

Andorrà, I., Esteve-Zarzoso, B., Guillamón, J. M., and Mas, A. (2010b). Determination of viable wine yeast using DNA binding dyes and quantitative PCR. *Int. J. Food Microbiol.* 144, 257–262. doi:10.1016/j.ijfoodmicro.2010.10.003.

Andorra, I., Monteiro, M., Esteve-Zarzoso, B., Albergaria, H., and Mas, A. (2011). Analysis and direct quantification of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* populations during alcoholic fermentation by fluorescence *in situ* hybridization, flow cytometry and quantitative PCR. *Food Microbiol.* 28, 1483-1491. doi:10.1016/j.fm.2011.08.009.

Bagheri, B., Bauer, F. F., and Setati, M. E. (2015). The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. *S. Afr. J. Enol. Vitic.* 36, 243–251.

Bely, M., Sablayrolles, J. M., and Barre, P. (1990). Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *J. Ferment. Bioeng.* 70, 246–252. doi:10.1016/0922-338X(90)90057-4.

Bezerra-Bussoli, C., Baffi, M. A., Gomes, E., Da-Silva, R., (2013). Yeast Diversity Isolated from Grape Musts During Spontaneous Fermentation from a Brazilian Winery. *Current Microbiol.* 67, 356-361. doi: 10.1007/s00284-013-0375-9.

Boynton, J. P., and Greig, D. (2016). Species richness influences wine ecosystem function through a dominant species. Fungal Ecol. 22, 61–72. doi: 10.1016/j.dib.2016.05.038

Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M. G., et al. (2014). Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol. Biotechnol.* 98, 843-8536. doi:10.1007/s00253-013-5411-y.

Branco, P., Viana, T., Albergaria, H., and Arneborg, N. (2015). Antimicrobial peptides (AMPs) produced by *Saccharomyces cerevisiae* induce alterations in the intracellular pH, membrane permeability and culturability of *Hanseniaspora guilliermondii* cells. *Int. J. Food Microbiol.* 205, 112–118. doi:10.1016/j.ijfoodmicro.2015.04.015.

Brežná, B., Ženišová, K., Chovanová, K., Chebeňová, V., Kraková, L., Kuchta, T., et al. (2010). Evaluation of fungal and yeast diversity in Slovakian wine-related microbial communities. *Antonie van Leeuwenhoek.* 98, 519–529. doi:10.1007/s10482-010-9469-6.

Cangelosi, G. A., and Meschke, J. S. (2014). Dead or alive: Molecular assessment of microbial viability. *Appl. Environ. Microbiol.* 80, 5884–5891. doi:10.1128/AEM.01763-14.

Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., and Romano, P. (2016). Yeast interactions in inoculated wine fermentation. *Front. Microbiol.* 7:555 doi:10.3389/fmicb.2016.00555.

Ciani, M., and Comitini, F. (2015). Yeast interactions in multi-starter wine fermentation. *Curr. Opin. Food Sci.* 1, 1–6. doi:10.1016/j.cofs.2014.07.001.

Combina, M., Elía, A., Mercado, L., Catania, C., Ganga, A., and Martinez, C. (2005). Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. *Int. J. Food Microbiol.* 99, 237–243.

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M.D., Valero, E., (2011). Influence of the farming system and vine variety on yeast communities associated with grape-berries. *Int. J. Food Microbiol.* 145, 132– 139. doi: 10.1016/j.ijfoodmicro.2010.11.040.

De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., and Boon, N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environ. Microbiol.* 16, 1472–1481. doi:10.1111/1462-2920.12343.

Deere, D., Jian, S., Vesey, G., Bell, P., Bissinger, P., and Veal, D. (1998). Flow Cytometry and Cell Sorting for Yeast Viability Assessment and Cell Selection. *Yeast.* 14, 147–160. doi:10.1002/(SICI)1097-0061(19980130)14.

Di Maro, E., Ercolini, D., and Coppola, S. (2007). Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. *Int. J. Food Microbiol.* 117, 201–210. doi:10.1016/j.ijfoodmicro.2007.04.007.

Divol, B., and Lonvaud-Funel, A. (2005). Evidence for viable but nonculturable yeasts in botrytis-affected wine. *J. Appl. Microbiol.* 99, 85–93. doi:10.1111/j.1365-2672.2005.02578.x.

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., and Cocolin, L. (2015). Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: Physiological and molecular characterizations. *Int. J. Food Microbiol.* 199, 33–40. doi:10.1016/j.ijfoodmicro.2015.01.009.

Ghosh, S., Bagheri, B., Morgan, H. H., Divol, B., and Setati, M. E. (2015). Assessment of wine microbial diversity using ARISA and cultivation-based methods. *Ann. Microbiol.* 65, 1833–1840. doi:10.1007/s13213-014-1021-x.

Giraffa, G., (2004). Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiology Reviews*. 28, 251–260. doi:10.1016/j.femsre.2003.10.005.

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

Grube, M., and Berg, G. (2009). Microbial consortia of bacteria and fungi with focus on the lichen symbiosis. *Fungal Biol. Rev.* 23, 72–85. doi:10.1016/j.fbr.2009.10.001.

Henschke, PA., Jiranek, V. (1993). Yeasts metabolism of nitrogen compounds. *In* Wine Microbiology and Biotechnology, Fleet GH *(ed). Harwood Academic:* Chur, Switzerland; 77–164.

Hierro, N., Esteve-Zarzoso, B., González, Á., Mas, A., and Guillamón, J. M. (2006a). Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. *Appl. Environ. Microbiol.* 72, 7148–7155. doi:10.1128/AEM.00388-06.

Hierro, N., González, Á., Mas, A., and Guillamón, J. M. (2006b). Diversity and evolution of non-*Saccharomyces* yeast populations during wine fermentation: Effect of grape ripeness and cold maceration. *FEMS Yeast Res.* 6, 102–111. doi:10.1111/j.1567-1364.2005.00014.x.

Izquierdo Cañas, P.M., Esteban, C., Romero, G., Heras, J.M., Mónica, M., González, F. (2014). Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. *Eur. Food Res. Technol.* 239, 279–286.

Jagmann, N., and Philipp, B. (2014). Design of synthetic microbial communities for biotechnological production processes. *J. Biotechnol.* 184, 209–218. doi:10.1016/j.jbiotec.2014.05.019.

Jemec, K. P., Cadez, N., Zagorc, T., Bubic, V., Zupec, A., Raspor, P., (2001). Yeast population dynamics in five spontaneous fermentations of Malvasia must. *Food Microbiol*. 18, 247–259. https://doi.org/10.1006/fmic.2001.0396.

Jiang, L.-L., Zhou, J.-J., Quan, C.-S., and Xiu, Z.-L. (2017). Advances in industrial microbiome based on

microbial consortium for biorefinery. *Bioresour. Bioprocess.* 4, 11. doi:10.1186/s40643-017-0141-0.

Jolly, N. P., Augustyn, O. P. H., and Pretorius, I. S. (2003a). The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. *S. Afr. J. Enol. Vitic.* 24, 63–69.

Jolly, N. P., Augustyn, O. P. H., and Pretorius, I. S. (2003b). The effect of Non-*Saccharomyces* yeasts on fermentation and wine quality. *S. Afr. J. Enol. Vitic.* 24, 55–62.

Kemsawad, V., Branco, P., Almeida, M. G., Caldeira, J., Albergaria, H., and Arneborg, N. (2015). Cell-to-cell contact and antimicrobial peptides play a combined role in the death of *Lachancea thermotolerans* during mixed-culture alcoholic fermentation with *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* 362, 1–8. doi:10.1093/femsle/fnv103.

Kraková, L., Chovanová, K., Selim, S. A., Šimonovičová, A., Puškarová, A., Maková, A., et al. (2012). A multiphasic approach for investigation of the microbial diversity and its biodegradative abilities in historical paper and parchment documents. *Int. Biodeterior. Biodegrad.* 70, 117–125. doi:10.1016/j.ibiod.2012.01.011.

Lencioni, L., Romani, C., Gobbi, M., Comitini, F., Ciani, M., and Domizio, P. (2016). Controlled mixed fermentation at winery scale using *Zygotorulaspora florentina* and *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 234, 36–44. doi:10.1016/j.ijfoodmicro.2016.06.004.

Lopandic, K., Tiefenbrunner, W., Gangl, H., Mandl, K., Berger, S., Leitner, G., et al. (2008). Molecular profiling of yeasts isolated during spontaneous fermentations of Austrian wines. *FEMS Yeast Res.* 8, 1063–1075. doi:10.1111/j.1567-1364.2008.00385.x.

Malacrinò, P., Zapparoli, G., Torriani, S., and Dellaglio, F. (2001). Rapid detection of viable yeasts and bacteria in wine by flow cytometry. *J. Microbiol. Methods* 45, 127–134. doi:10.1016/S0167-7012(01)00243-3.

Maturano, Y. P., Mestre, M. V., Esteve-Zarzoso, B., Nally, M. C., Lerena, M. C., Toro, M. E., et al. (2015). Yeast population dynamics during pre-fermentative cold soak of Cabernet Sauvignon and Malbec wines. Int. J. Food Microbiol. 199, 23–32. doi: 10.1016/j.ijfoodmicro.2015.01.005

Medina, K., Boido, E., Fariña, L., Gioia, O., Gomez, M.E., Barquet, M., Gaggero, C., Dellacassa, E., and Carrau, F. (2013) Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem*. 141, 2513-2521. doi:10.1016/j.foodchem.2013.04.056

Mendoza, L. M., De Nadra, M. C. M., Bru, E., and Farıas, M. E. (2009). Influence of wine-related physicochemical factors on the growth and metabolism of non-*Saccharomyces* and *Saccharomyces* yeasts in mixed culture. *J. Ind. Microbiol. Biotechnol.* 36, 229–237. doi:10.1007/s10295-008-0489-4.

Milanović, V., Comitini, F., Ciani, M., (2013). Grape berry yeast communities: Influence of fungicide treatments. *Int. J. Food Microbiol.* 161, 240– 246. https://doi.org/10.1016/j.ijfoodmicro.2012.12.019.

Morales, P., Rojas, V., Quirós, M., and Gonzalez, R. (2015). The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. *Appl. Microbiol. Biotechnol.* 99, 3993–4003. doi:10.1007/s00253-014-6321-3.

Morgan, H. H. (2016). Investigating the Effect of Leaf Removal on the Grape-Associated Microbiome Through Culture-Dependent and Independent Approaches. Master's thesis, Stellenbosch University, Stellenbosch.

Maturano, Y. P., Mestre, M. V., Esteve-Zarzoso, B., Nally, M. C., Lerena, M. C., Toro, M. E., et al. (2015). Yeast population dynamics during prefermentative cold soak of Cabernet Sauvignon and Malbec wines. *Int. J. Food Microbiol.* 199, 23–32. doi:10.1016/j.ijfoodmicro.2015.01.005.

Nissen, P., and Arneborg, N. (2003). Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. *Arch. Microbiol.* 180, 257–263. doi:10.1007/s00203-003-0585-9.

Oh, D., Kim, S., and Kim, J. (1998). Increase of Xylitol Production Rate by Controlling Redox Potential in

Candida parapsilosis. Biotechnol. Bioeng, 58-4.

O'Sullivan, D. J., Giblin, L., McSweeney, P. L. H., Sheehan, J. J., and Cotter, P. D. (2013). Nucleic acid-based approaches to investigate microbial-related cheese quality defects. *Front. Microbiol.* 4, 1–15. doi:10.3389/ fmicb.2013.00001.

Pérez-Torrado, R., Rantsiou, K., Perrone, B., Navarro-Tapia, E., Querol, A., and Cocolin, L. (2017). Ecological interactions among *Saccharomyces cerevisiae* strains: insight into the dominance phenomenon. *Sci. Rep.* 7, 43603. doi:10.1038/ srep43603.

Pina, C., Couto, J. A., António, J., and Hogg, T. (2004). Inferring ethanol tolerance of *Saccharomyces* and non-*Saccharomyces* yeasts by progressive inactivation. *Biotechnol. Lett.* 26, 1521–7. doi:10.1023/ B:BILE.0000044456.72347.9f.

Ponomarova, O., and Patil, K. R. (2015). Metabolic interactions in microbial communities: Untangling the Gordian knot. *Curr. Opin. Microbiol.* 27, 37–44. doi:10.1016/j.mib.2015.06.014.

Portillo, M. del C., and Mas, A. (2016). Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing. *LWT - Food Sci. Technol.* 72, 317–321. doi:10.1016/j.lwt.2016.05.009.

Prakitchaiwattana, C. J., Fleet, G. H., and Heard, G. M. (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Res.* 4, 865–877. doi:10.1016/ j.femsyr.2004.05.004.

Pretorius, I. S., Van der Westhuizen, T. J. J., and Augustyn, O. P. H. (1999). Yeast Biodiversity in Vineyards and Wineries and Its Importance to the South African Wine Industry-A Review. *S. Afr. J. Enol. Vitic.* 20, 61–75.

Ramette, A. (2009). Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. *Appl. Environ. Microbiol.* 75, 2495–2505. doi:10.1128/AEM.02409-08.

Renault, P. E., Albertin, W., and Bely, M. (2013). An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Appl. Microbiol. Biotechnol.* 97, 4105–4119. doi:10.1007/s00253-012-4660-5.

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2007). Inventory and monitoring of wine microbial consortia. *Appl. Microbiol. Biotechnol.* 75, 149–164. doi:10.1007/s00253-006-0798-3.

Rossignol, T., Kobi, D., Jacquet-Gutfreund, L., and Blondin, B. (2009). The proteome of a wine yeast strain during fermentation, correlation with the transcriptome. *J. Appl. Microbiol. 107*(1), 47–55. https://doi.org/10.1111/ j.1365-2672.2009.04156.x.

Romancino, D. P., Di Maio, S., Muriella, R., and Oliva, D. (2008). Analysis of non-*Saccharomyces* yeast populations isolated from grape musts from Sicily (Italy). *J. Appl. Microbiol.* 105, 2248–2254. doi:10.1111/j.1365-2672.2008.03894.x.

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J. J., Ballester, J., et al. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol.* 32, 243–253. doi:10.1016/j.fm.2012.06.006.

Sainz, J., Pizarro, F., Perez- Correa, J. R., and Agosin, E. (2003). Modeling of yeast metabolism and process dynamics in batch fermentation. *Biotechnol. Bioeng.* 81, 818–828. doi:10.1002/bit.10535.

Salinas, F., Garrido, D., Ganga, A., Veliz, G., and Martínez, C. (2009). Taqman real-time PCR for the detection and enumeration of *Saccharomyces cerevisiae* in wine. *Food Microbiol.* 26, 328–332. doi:10.1016/j.fm.2008.12.001.

Sambrook, J., and W. Russell, D (2006). Rapid Isolation of Yeast DNA Cold Spring Harb Protoc; 2006; doi:10.1101/pdb.prot4039.

Setati, M. E., Jacobson, D., and Bauer, F. F. (2015). Sequence-based analysis of the *Vitis vinifera* L. cv Cabernet sauvignon grape must mycobiome in three South African vineyards employing distinct agronomic systems. *Front. Microbiol.* 6:1358. doi:10.3389/fmicb.2015.01358.

Shekhawat, K., Bauer, F. F., and Setati, M. E. (2017). Impact of oxygenation on the performance of three non-*Saccharomyces* yeasts in co-fermentation with *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 101, 2479–2491. doi:10.1007/s00253-016-8001-y.

Slabbert, E., Van Heerden, CJ., Jacobs, K. (2010). Optimisation of automated ribosomal intergenic spacer analysis for the estimation of microbial diversity in fynbos soil. *S Afr J Sci* 106: 1–4.

Sun, H., Ma, H., Hao, M., Pretorius, I. S., and Chen, S. (2009). Identification of yeast population dynamics of spontaneous fermentation in Beijing wine region, China. *Ann. Microbiol.* 59, 69–76. doi:10.1007/BF03175601.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., et al. (2012). Multistarter from organic viticulture for red wine Montepulciano d'Abruzzo production. *Front. Microbiol.* 3, 1–10. doi:10.3389/fmicb.2012.00135.

Tristezza, M., Tufariello, M., Capozzi, V., Spano, G., Mita, G., and Grieco, F. (2016). The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Front. Microbiol.* 7, 1–14. doi:10.3389/fmicb.2016.00670.

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., et al. (2012). Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiol*. 29, 18-26. doi: 10.1016/ismej. 2016.45.

Vannette, L. R., and Fukami, T. (2014). Historical contingency in species interactions: towards niche-based predictions. *Ecol. Lett*. 17, 115–124. doi: 10.1111/ele.1220

Viana, F., Belloch, C., Vallés, S., and Manzanares, P. (2011) Monitoring a mixed starter of *Hanseniaspora vineae-Saccharomyces cerevisiae* in natural must: Impact on 2-phenylethyl acetate production. *Int. J. Food Microbiol*. 151, 235-240. doi:10.1016/j.ijfoodmicro.2011.09.005.

Wang, C., Esteve-Zarzoso, B., Cocolin, L., Mas, A., and Rantsiou, K. (2015). Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during Barbera must fermentation. *Food Res. Int.* 78, 195–200. doi:10.1016/j.foodres.2015.10.014.

Wang, C., Esteve-Zarzoso, B., and Mas, A. (2014). Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum,* and *Starmerella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence *in situ* hybridization. *Int. J. Food Microbiol.* 191, 1–9. doi:10.1016/j.ijfoodmicro.2014.08.014.

Wang, C., Mas, A., and Esteve-Zarzoso, B. (2016). The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. *Front. Microbiol.* 7, 1–11. doi:10.3389/fmicb.2016.00502.

Weightman, C. J. (2014). Characterization of Chenin blanc wines produced by natural fermentation and skin contact : focus on application of rapid sensory profiling method. [master's thesis]. [Available at: http://scholar.sun.ac.za/handle/10019.1/96008]: Stellenbosch University.

Williams, K. M., Liu, P., and Fay, J. C. (2015). Evolution of ecological dominance of yeast species in high-sugar environments. *Evolution (N. Y).* 69, 2079–2093. doi:10.1111/evo.12707.

Xie, G., Li, W., Lu, J., Cao, Y., Fang, H., Zou, H., et al. (2007). Isolation and Identification of representative fungi from Shaoxing rice wine wheat Qu using a polyphasic approach of Culture-based and molecular-based methods. *J. Inst. Brew.* 113, 272–279. doi:10.1002/j.2050-0416.2007.tb00287.x.

Xufre, A., Albergaria, H., Inácio, J., Spencer-Martins, I., and Gírio, F. (2006). Application of fluorescence *in situ* hybridisation (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. *Int. J. Food Microbiol.* 108,376-384. doi:10.1016/j.ijfoodmicro.2006.01.025.

Ye, M., Yue, T., and Yuan, Y. (2014). Effects of sequential mixed cultures of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* on apple cider fermentation. *FEMS Yeast Res.* 14, 873–882. doi:10.1111/1567-1364.12175.

Ženišová, K., Chovanová, K., Chebeňová-Turcovská, V., Godálová, Z., Kraková, L., Kuchta, T., et al. (2014). Mapping of wine yeast and fungal diversity in the Small Carpathian wine-producing region (Slovakia): evaluation of phenotypic, genotypic and culture-independent approaches. *Ann. Microbiol.* 64, 1819–1828. doi:10.1007/s13213-014-0827-x.

Zott, K., Claisse, O., Lucas, P., Coulon, J., Lonvaud-Funel, A., and Masneuf-Pomarede, I. (2010). Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiol.* 27, 559–567. doi:10.1016/j.fm.2010.01.006.

Zott, K., Miot-Sertier, C., Claisse, O., Lonvaud-Funel, A., and Masneuf-Pomarede, I. (2008). Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. *Int. J. Food Microbiol.* 125, 197–203. doi:10.1016/j.ijfoodmicro.2008.04.001.

3.7. Supplementary materials

3.7.1. Supplementary Tables

Table 3S1. Comparison between the actual viable counts as determined by plating (PL) and the total counts calculated based on ARISA peak area standard curves (STC).

Yeast Species	CFU/mL PL	CFU/mL STC	CFU/mL PL	CFU/mL STC	CFU/mL PL	CFU/mL STC
	0% Sugar		50% Sugar		100% Sugar	
M. pulcherrima	(42.7 ±1.56) E+05	(15.8 ± 1.01) E+06	(73.3 ±2.03) E+03	(31.3 ± 3.52) E+03	0	(22.4 ±1.12) E+03
P. terricola	(41.0 ±2.85) E+05	(18.7 ±2.41) E+05	(21.1± 2.43) E+03	(47 ± 3.85) E+03	0	(12.5 ± 1.14) E+03
S. bacillaris	(52.7 ±3.06) E+05	(44.9 ±2.47) E+06	(45.7±3.06) E+04	(35.1± 1.52) E+03	(80 ± 2.74) E+03	(15.9 ± 1.85) E+03
C. parapsilosis	(22 ± 1.2) E+05	(22.5 ± 2.74) E+04	(19± 1.18) E+04	(83.8 ± 3.17) E+03	0	(73.5 ± 4.41) E+02
W. anomalus	(42.7 ±1.03) E+05	(25.9 ± 2.03) E+04	(96.7± 2.31) E+04	(12 ±2.85) E+03	(11 ±1.65) E+03	(22.4 ± 2.44) E+02
L. thermotoleranns	(61.7±2.08) E+05	(50.1 ± 2.31) E+06	(35±1.29) E+04	(14.1 ± 2.17) E+04	(84 ± 3.74) E+03	(19.2± 1.17) E+03
H. vineae	(51 ± 1.61) E+05	(21.5 ±2.17) E+06	(31.6 ±1.37) E+04	(16.9± 2.07) E+05	0	(82 ± 2.92) E+02
S. cerevisiae	(26.7 ±1.51) E+02	(25.5 ±1.81) E+03	(14.4 ±2.41) E+06	(83.6± 4.32) E+06	(71.9 ± 2.6) E+06	(11.3 ± 1.74) E+07

Table 3S2. Comparison between the yeast population (CFU/mL) in synthetic must and Chenin blanc must at the time of inoculation (T_{INC}) and in the end of fermentation (T_{EF}).

Yeast Species	Yeast population T_{INC} (CFU/mL) synthetic must (*NS-Sc*)	Yeast population T_{EF} (CFU/mL) synthetic must (*NS-Sc*)	Yeast population T_{INC} (CFU/mL) Chenin blanc must (*NS-Sc*)	Yeast population T_{EF} (CFU/mL) Chenin blanc must (*NS-Sc*)
S. cerevisiae (EC1118)	(26.7 ± 1.51) E+02	(71.9± 2.6) E+06	(29+ 1.07) E+02	(69± 2.87) E+05
S. cerevisiae (*IND-Sc*)	0	0	(48± 2.41) E+02	(32 ± 2.17) E+06
S. bacillaris	(52.7 ± 3.06) E+05	(80 ± 2.74) E+03	(21± 1.65) E+05	(80±3.14) E+03
L. thermotolerans	(61.7 ± 2.08) E+05	(84 ± 3.74) E+03	(23 ± 2.85) E+05	(23±1.19) E+05
W. anomalus	(42.7 ± 1.03) E+05	(11 ±1.65) E+03	(30 ± 1.12) E+05	
H. vineae	(51± 1.61) E+05	0	(26.3± 1.01) E+05	0
H. uvarum	0	0	(44± 1.14) E+05	0

M. pulcherrima	(42.7± 1.56) E+05	0	(10 ± 1.65) E+05	0
P. terricola	(41±2.85) E+05	0	(31.1± 2.54) E+05	0
C. parapsilosis	(22± 1.2) E+05	0	(31.2 ± 1.12) E+05	0

3.7.2. Supplementary Figures

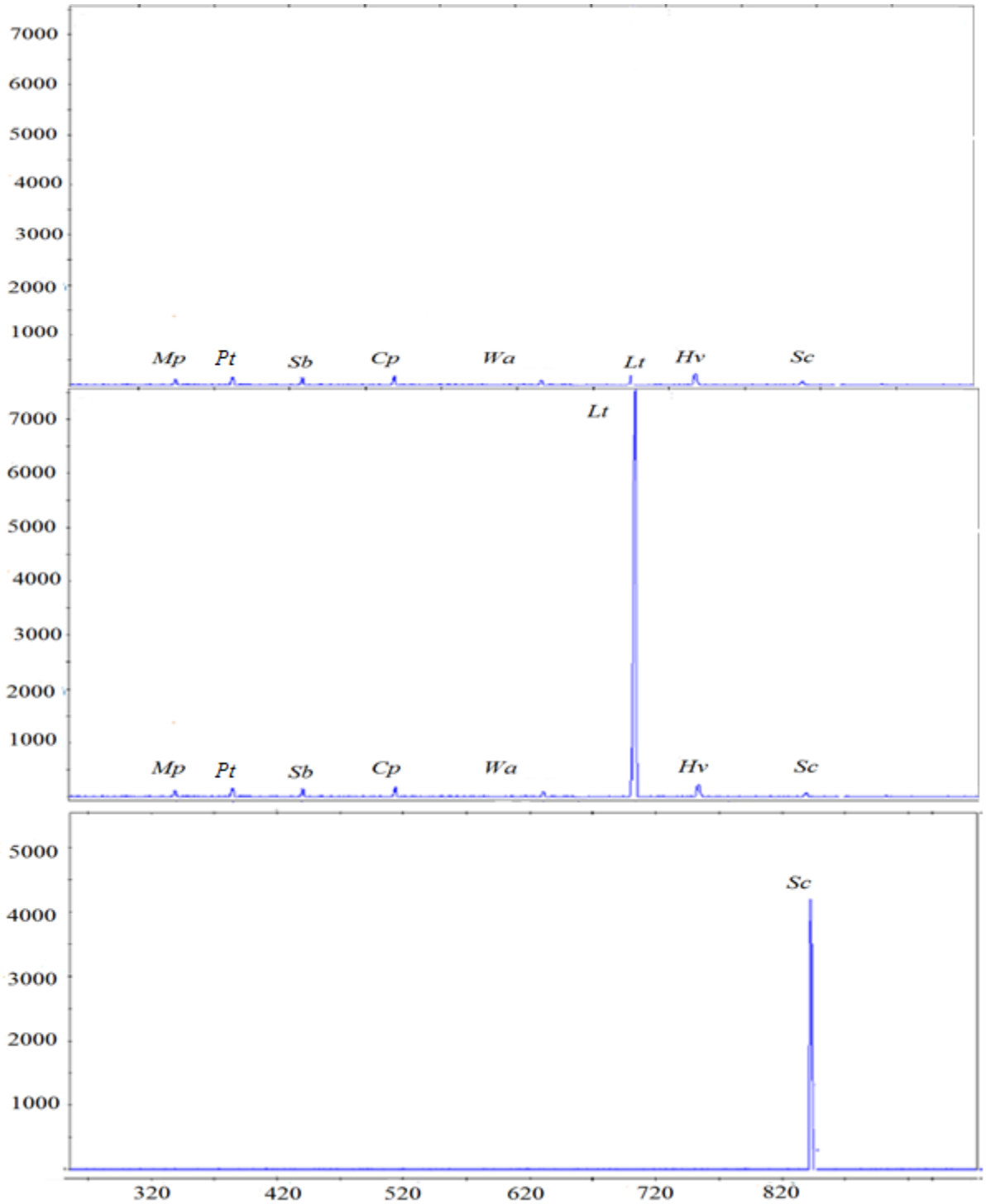


Figure 3S1. Electropherograms of ARISA detection limit. The x-axis represents the fragment size (bp) and the y-axis represents the relative fluorescence intensity.

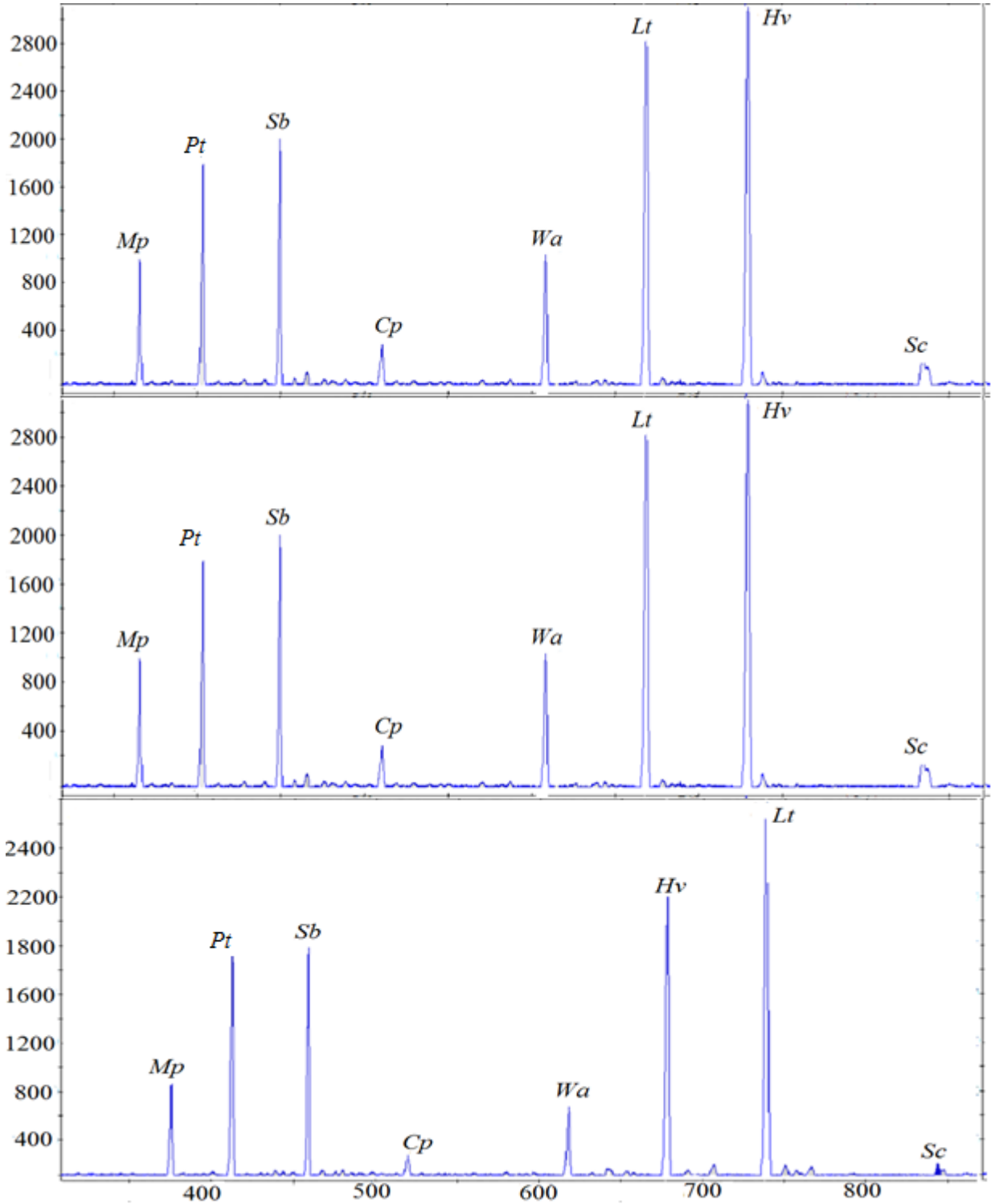


Figure 3S2. ARISA Electropherograms for triplicates of one sample. The x-axis represents the fragment size (bp) and the y-axis represents the relative fluorescence intensity. Three independent DNA extractions were performed for one sample. Minor variations were observed between the triplicates.

Chapter 4

Research results

Effect of inoculum dosage on the dynamics of yeast consortium

This manuscript will be submitted for publication in
Applied Microbiology and Biotechnology

4.1. Abstract

Conversion of grape must to wine is a complex process that is initiated by a diverse population of microorganisms, including many non-*Saccharomyces* yeast species, that are present at different concentrations in grape must. These species will grow, interact and compete with each other, and the final chemical composition, flavour and, aroma of the wine will be affected by the nature, diversity and relative metabolic performance of the different species. Many factors impact on the relative performance of each yeast species, including yeast initial cell density, competition for nutrients, ecological interactions and time of inoculation. However, the fundamental rules underlying the working of the wine ecosystem are not fully understood. Here we report on the effect of initial cell density on population dynamics in a multispecies wine-like yeast consortium and on its impact on wine aroma. Presence of individual species at a high cell density affected the fermentation kinetics. Fermentation kinetics was enhanced in some treatments while it was delayed in others, indicating the presence of positive and negative interactions in the wine ecosystem. The data revealed that the growth of other members of the yeast consortium was affected by the applied inoculation treatment. Inoculation density of individual species supported or inhibited the growth and persistence of other species in the wine ecosystem. The data suggest that *S. cerevisiae* applied selective pressure to suppress the growth of the main competitor in the wine ecosystem. The production of major volatiles was also affected by the treatments. The aromatic signature of some non-*Saccharomyces* species was observed in wine suggesting the presence of strong yeast-yeast interactions between species in the consortium. The data identified the underlying ecological interactions between the yeast species in a complex environment and reveal the contribution of individual species to the wine aroma.

4.2. Introduction

The inoculation of grape must with the commercial *Saccharomyces cerevisiae* strains is a common practice in the wine industry. This practice offers better management of wine fermentation, resulting in a wine with a reliable and consistent quality (Molina *et al*., 2009; Ciani *et al*., 2016). However, since our understanding of the oenological properties and potential contribution of non-*Saccharomyces* species has increased, there is a growing interest in mixed culture fermentations (Gobbi *et al*., 2013; Suzzi *et al*., 2012). Several non-*Saccharomyces* species have been commercialized as single culture or in combination with *S. cerevisiae*, and studies have also indicated that despite inoculation with commercial starter cultures, indigenous non-*Saccharomyces* species can persist throughout fermentation and contribute to the aromatic profile of wine (Ciani and Maccarelli, 1998; Fleet, 2008; Renault *et al*., 2009; Sadoudi *et al*., 2012; Gobbi *et al*., 2013; Vigentini *et al*., 2014). Persistence of these species throughout fermentation and their consequent effect on wine aroma is dependent on their relative fitness within the wine microbial ecosystem which is affected by several parameters including the initial cell density, competition for nutrients, and ecological interactions (Medina *et al*., 2013; Sadoudi *et al*., 2012; Taliander *et al*., 2014).

In our previous study, we have reported that the persistence of many non-*Saccharomyces* species was positively correlated with their initial cell densities (Bagheri *et al*., 2015). For instance, when *L. thermotolerans* accounted for 15% of total population in must, it persisted until the middle of fermentation whereas, it could only be detected in early fermentation when it accounted for 7% of the total population (Bagheri et al., 2015). Similar results have been reported for other combinations of non-*Saccharomyces* and *S. cerevisiae* including for the species *Starmerella bacillaris*, *Lachanceae thermotolerans* and *Torulaspora delbruecki* (Sun *et al*., 2014; Gobbi *et al*., 2013; Lee *et al*., 2013). In contrast, high initial cell densities had only a marginal effect on the competitiveness and persistence of *Metchnikowia pulcherrima* and *Williopsis* (formerly *Hansenula*) *saturnus* (Sun *et al*., 2014; Lee *et al*., 2013).

Many of these studies also support the conclusion that high initial cell density and longer persistence of such strains correlates with a distinctive aromatic profile of wine, which is different from those produced by single cultures of *S. cerevisiae* (Moreira *et al*., 2005; Sadoudi *et al*., 2012). For instance, mixed cultures of *H. vineae* and *S. cerevisiae* were shown to produce a larger amount of 2-Phenylethyl acetate whereas mixed cultures of *L. thermotolerans* and *S. cerevisiae* were shown to increase the production of 2-phenyl ethanol and glycerol in final wines (Viana *et al*., 2011; Medina *et al*., 2013; Gobbi *et al*., 2013).

In this context, very few studies have reported on yeast-yeast interactions in multispecies wine fermentations, primarily because of the complexity of such multispecies systems. Studies of ecological interactions in wine fermentation have mainly focused on mixed culture fermentations composed of 2 species, and have reported on the antagonistic interaction between *S. cerevisiae*

and *L. thermotolerans*, and between *S. cerevisiae* and *S. bacillaris* (Gobbi *et al*., 2013; Sun *et al*., 2014; Taillander *et al*., 2014). Other data show a mutualistic interaction between *S. cerevisiae* and *M. pulcherrima* (Sadoudi *et al*., 2012; Sun *et al*., 2014).

Thus, we have only a limited understanding of the effect of individual species in a multi-species yeast community. To address this issue, we have previously established a multi-species yeast consortium of eight species that are representative of the South African wine microbiota and proved to be a suitable approximation of a grape must ecosystem (Bagheri *et al*., 2017). Using this system, we have previously reported on the effect of presence of *S. cerevisiae* on dynamics of this yeast consortium. In particular, the study revealed specific antagonistic interactions between *S. cerevisiae* and *W. anomalus*, whereas a mutualistic support was observed between *S. cerevisiae* and *H. vineae*.

The current study aims to use variations in initial cell densities of each member of this consortium as a tool to better understand the impact of individual species on others in the multi-species wine ecosystem.

4.3. Material and methods

4.3.1. Yeast consortium and culture conditions

A yeast consortium comprising seven yeast strains obtained from the culture collection of the Institute for Wine Biotechnology (IWBT) and a commercial yeast *S. cerevisiae* Lalvin EC1118 (Lallemand, Canada) were constructed as described in Bagheri *et al*., 2017. The yeast stock cultures were maintained in 20% (v/v) glycerol at -80°C and were streaked out on Wallerstein Laboratory Nutrient agar (WLN) (Sigma-Aldrich, Spain), when required. The plates were incubated at 30°C for 3-5 days.

Table.4.1. Strains used in this study. The strain codes are abbreviation of the name of each strain.

Strains	Strains codes	Strains number
Hanseniaspora vineae	*Hv*	Y980
Pichia terricola	*Pt*	Y974
Starmerella bacillaris	*Sb*	Y975
Candida parapsilosis	*Cp*	Y842
Lachancea thermotolerans	*Lt*	Y973
Saccharomyces cerevisiae	*Sc*	EC1118
Wickerhamomyces anomalus	*Wa*	Y934
Metschnikowia pulcherrima	*Mp*	Y981

4.3.2. Microfermentations

Fermentations were performed in synthetic grape juice medium (pH 3.5) adapted from Henschke and Jiranek (1993), and Bely *et al*., (1990). The medium contained 200 g/L sugars (100 g/L

glucose and 100 g/L fructose) and 300 mg/L assimilable nitrogen (460 mg/L NH_4Cl and 180 mg/L amino acids).

The effect of increased cell density (high dosage) on dynamics of the yeast consortium was evaluated in the presence and in absence of *S. cerevisiae*. Therefore, different fermentations were inoculated with one non-*Saccharomyces* species at 4×10^6 cells/mL, the remaining six non-*Saccharomyces* species and *S. cerevisiae* at 10^4 cells/mL (Figure 4.1A) or alternatively without *S. cerevisiae* (Figure 4.1B). In addition, a control fermentation inoculated with 7 non-*Saccharomyces* species inoculated at 10^6 cells/mL and *S. cerevisiae* at 10^3 cells/mL (NS-SC consortium) was conducted.

The fermentations were performed in 500 mL synthetic grape juice dispensed in 500 mL Erlenmeyer flasks fitted with fermentation locks. Static fermentations were carried out in triplicate, at 25°C. The fermentations were weighed regularly to monitor CO_2 release and the samples were withdrawn at two-day intervals. The fermentations were considered finished when the residual sugar (glucose and fructose) was less than 2 g/L.

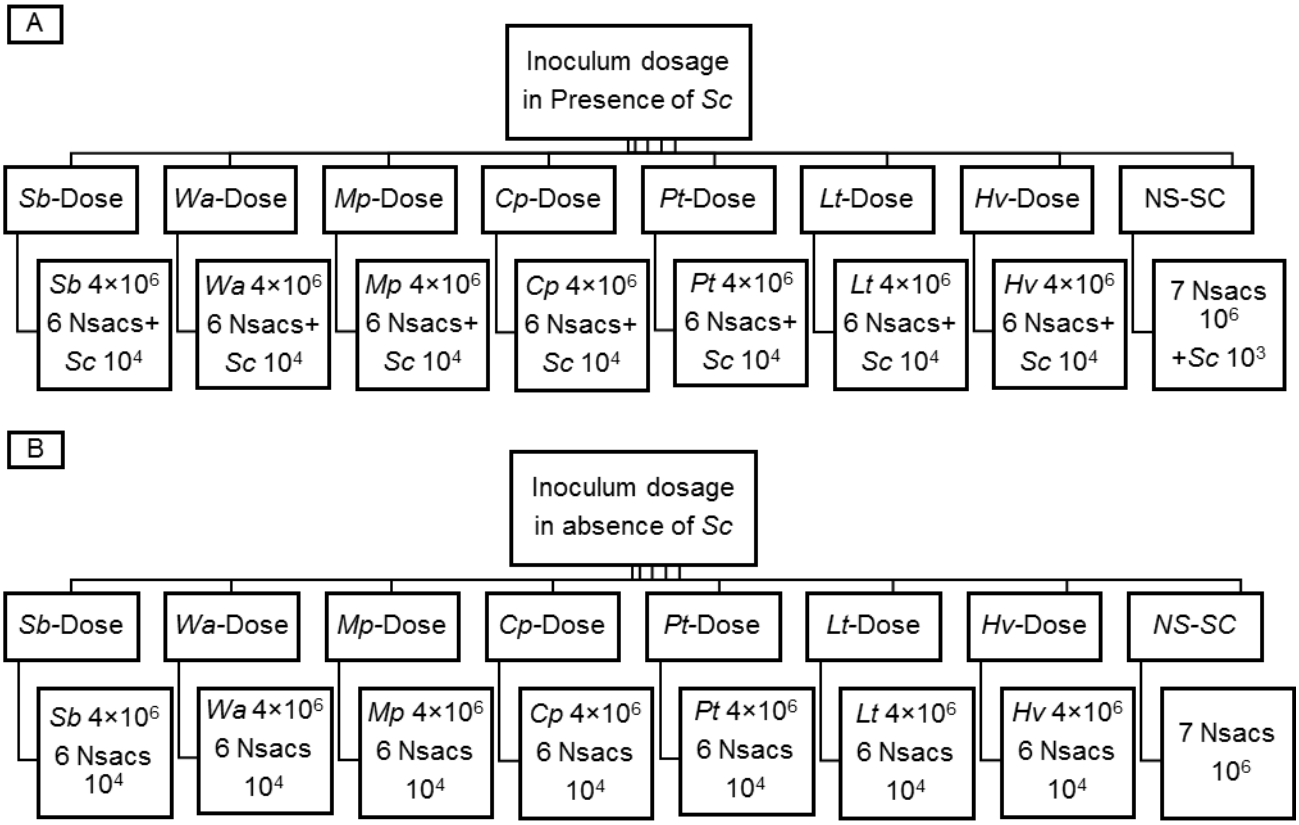


Figure.4.1. Outline of the experimental plan showing the dosage treatments with *S. cerevisiae* included in the inoculum (A) and in the absence of *S. cerevisiae* (B). Treatments are defined based on the strain codes. The following abbreviations were used in this figure. Nsacs: Non-*Saccharomyces species*, Sc: *S. cerevisiae, Mp*: *M. pulcherrima, Pt: P. terricola, Sb: S. bacillaris, Cp: C. parapsilosis, Wa:, W. anomalus, Lt: L. thermotolerans, Hv: H. vineae.*

4.3.3. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Two milliliter samples were withdrawn from fermentation flasks. Samples were centrifuged at 5630 × *g* for 10 min to collect the cells. Genomic DNA was extracted using the method described by Sambrook and Russell, (2006). The concentrations of DNA were determined spectrophotometrically using the NanoDrop®ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA). Concentration of all DNA samples was adjusted to 100 ng/µL. ITS1-5.8S rRNA-ITS2 gene was amplified using the carboxy-fluorescein labeled ITS1 primer (5′-6-FAM- TCC GTA GGT GAA CCT TGC GG-3′) and ITS4 (5′- TCC GTA GGT GAA CCTTGC GG-3′) as described in Slabbert *et al*., (2010).

ARISA fragments were separated by capillary electrophoresis on an ABI 3010x Genetic Analyser (Applied Biosystems) with a ROX 1.1 labeled size standard (75-1121 base pairs). ARISA profiles were analyzed using Genemapper software version 4.1 (Applied Biosystems). Only fragments with peak area larger than 0.5% of the total fluorescence were considered for further analysis. A bin size of 3 bp for species with ITS region below 700 bp and 5 bp for species with ITS region above 700 bp, was employed to minimize the inaccuracies in the ARISA analysis (Slabbert *et al*., 2010). The abundance of each peak was calculated, dividing individual peak area by the total peak areas for the respective sample.

4.3.4. Analytical methods

Samples were centrifuged at 7000 × *g* for 10 min. Cell-free supernatants were used to measure glucose and fructose using enzymatic kits, Enzytec™Fluid D-glucose (E5140), fructose (E5120) (Boehringer Mannheim, R-biopharm, Darmstadt, Germany).

The volatile compounds of wines from different treatments were analyzed by liquid-liquid extraction method, using GC-FID as described by Louw *et al*., 2010. In brief, the extraction was performed by the addition of 4-methyl-2-pentanol as the internal standard (final concentration 5 mg/L) and 1 mL diethyl ether to each sample. The samples were sonicated for 5 min followed by centrifugation at 4000 × *g* for 5 min. The ether layer (supernatant) was removed and dried over Na_2SO_4. A DB-FFAP capillary column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length × 0.32 mm i.d. × 0.5 µm film thickness and a Hewlett Packard 6890 Plus GC instrument (Little Falls, USA) equipped with a split/splitless injector and a flame ionization detector (FID) were used for gas chromatography (GC). The gas chromatography was performed under the following conditions: an initial oven temperature of 33°C for 17 min, followed by an increase in temperature up to 240°C, for 5 min (12°C/min). Finally, three microliters of the diethyl-ether extract were injected at 200°C in split mode, with the split ratio of 15:1 and the split flow rate of 49.5 mL/min. The column flow rate was 3.3 mL/min, using hydrogen as carrier gas. The detector temperature was 250°C.

4.3.5. Data analysis of aromatic compounds

The contribution of volatile compounds in wine aroma was evaluated based on the odour activity values (OAVs). The values were calculated by dividing the concentrations of aroma compounds with their odour thresholds as described previously (Etiévant, 1991; Guth, 1997; Ferreira *et al*., 2001; Grosch *et al*., 2001). The compounds with an OAV greater than 1 were considered to have the main contribution to wine aroma (Etiévant, 1991; Guth, 1997). The odor descriptors were grouped into different aromatic series based on previous literature (Peinado *et al*., 2004; Sánchez-Palomo *et al*., 2010). The descriptors, floral, fruity, balsamic (vinegar), solvent and fatty (rancid) were selected for this analysis based on their extensive use for describing wines (Peinado *et al*., 2004; Sánchez-Palomo *et al*., 2010). The total intensities for every aromatic series were calculated as the sum of the OAVs of each one of the compounds assigned to the series.

4.3.6. Statistical analysis

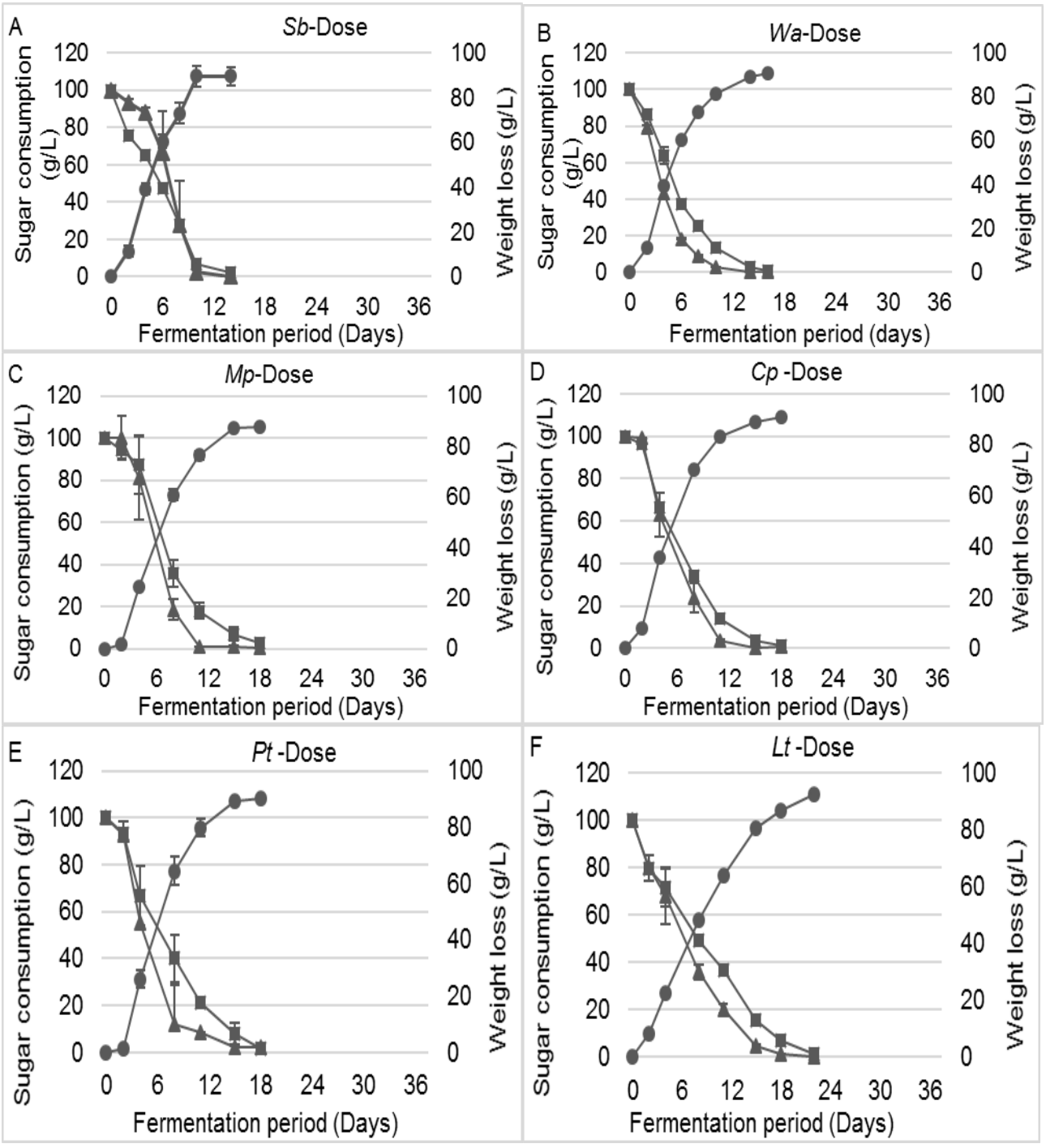
All the fermentations and the chemical analysis were performed in at least three repeats. The values were presented as means ± SD. The differences between treatments were determined using analysis of variance (ANOVA) using the statistical software, Statistica version 13.0 (StatSoft Inc., Tulsa, Oklahoma, USA). The differences were considered significant should the *p*-values be equal to or less than 0.05. For multivariate data analysis, the principal component analysis was performed, using XLSTAT in Microsoft® Excel (2016).

4.4. Results

4.4.1. Fermentation kinetics of dosage treatments

Overall, fermentations conducted in the absence of *S. cerevisiae* were sluggish and did not ferment to dryness. Residual sugar ranging between 88 and 107 g/L was still detected after 28-32 days when the fermentations were terminated. In contrast, all the fermentations in which *S. cerevisiae* was included in the consortium fermented to dryness, albeit at different rates. For instance, the fermentation inoculated with *S. bacillaris* (*Sb*-dose) were faster, reaching dryness within 14 days (Figure 4.2A), followed by the *W. anomalus (Wa*-dose), which took 16 days to reach dryness (Figure 4.2B). Conversely, the fermentations inoculated with either a high dosage of *M. pulcherrima (Mp*-dose*), C. parapsilosis (Cp*-dose*),* or *P. terricola (Pt*-dose*)* displayed similar kinetics and fermented to dryness in 18 days (Figure 4.2C-2E), while the *L. thermotolerans (Lt*-dose*)* and *H. vineae (Hv*-dose*)* fermentations took 22 and 28 days to reach dryness, respectively (Figure 4.2F and 4.2G). The control fermentation (NS-SC), on the other hand, finished in 21 days (data not shown).

Differences in sugar consumption were observed between the treatments. For instance, the *Sb*-dose (Figure 4.2A) displayed preferential consumption of fructose from the onset of fermentation while the rest of the treatments showed a similar consumption rate for both glucose and fructose in the early stages of fermentations and a faster consumption of glucose towards the middle of fermentations. *Wa*-Dose (Figure 4.2B) and *Pt*-Dose (Figure 4.2E) exhibited the fastest glucose consumption whereas *Hv*-Dose (Figure 4.2G) treatment showed the slowest fructose consumption compared to the rest of the treatments.



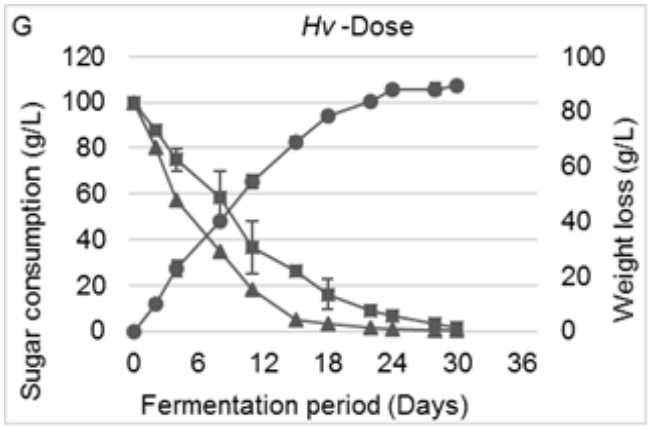
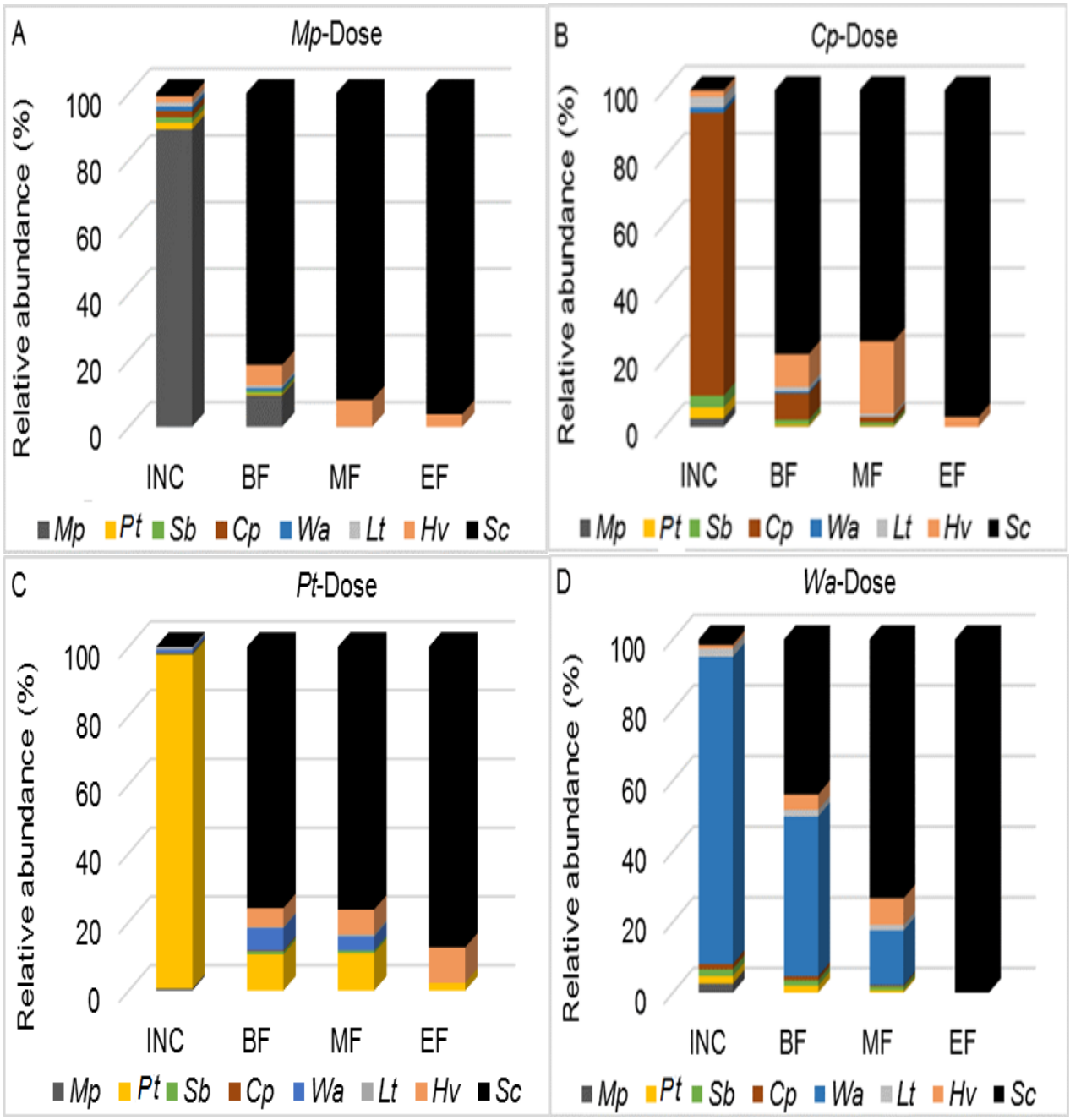


Figure.4.2. Fermentation kinetics of the dose treatments in presence of *S. cerevisiae*. Treatments are presented based on the species that is inoculated at a higher inoculum dosage. For instance, the treatment in which *M. pulcherrima* was inoculated at a higher dosage is represented with *Mp*-dose. Fermentation weight loss (◯) glucose (▲) and fruectose (■) were monitored throughout the fermenntations.

4.4.2. Culture-independent analysis of dosage treatments

In this study, we evaluated the influence of variation in cell density of individual non-*Saccharomyces* yeast species on population dynamics within the model consortium. The dynamics were assessed at beginning of fermentation (10-20% sugar consumption), middle (40-60% consumption) and end of fermentation (over 95% sugar consumption). In each scenario, a selected non-*Saccharomyces* species was inoculated at a 100:1 ratio relative to the other seven species in the consortium and population dynamics were monitored using ARISA.

Overall, a decline in the population of non-*Saccharomyces* species and an increase in *S. cerevisiae* were apparent in all scenarios. However, the rate of decline of non-*Saccharomyces* species varied considerably. For instance, *M. pulcherrima* and *C. parapsilosis* declined rapidly, despite the higher cell densities and only accounted for 0.7% and 1.68% of the population, respectively, by the middle of fermentation (Figure 4.3A and 4.3B). In contrast, *P. terricola*, *W. anomalus* and *H. vineae* accounted for the 10.5%, 15.5% and 15.2% of the population in their respective dose treatments in the middle of fermentation (Figure 4.3C, 4.3D, and 4.3E), while *L. thermotolerans* was at 30% (Figure 4.3F). *S. bacillaris* was the only species that persisted until the end of fermentation at a considerable level (12.7%) when inoculated at a higher dosage relative to the other yeasts (Figure 4.3G). The NS-SC treatment on the other hand, was characterized by a rapid decline in the *M. pulcherrima* and *C. parapsilosis* population at the beginning of fermentation, followed by *W. anomalus*, *P. terricola* and *H. vineae* (Figure 4.3H). In this fermentation, *L. thermotolerans* and *S. bacillaris* were the only two non-*Saccharomyces* species that persisted until the end of fermentation, accounting for 7.1% and 8.8% of population, respectively.



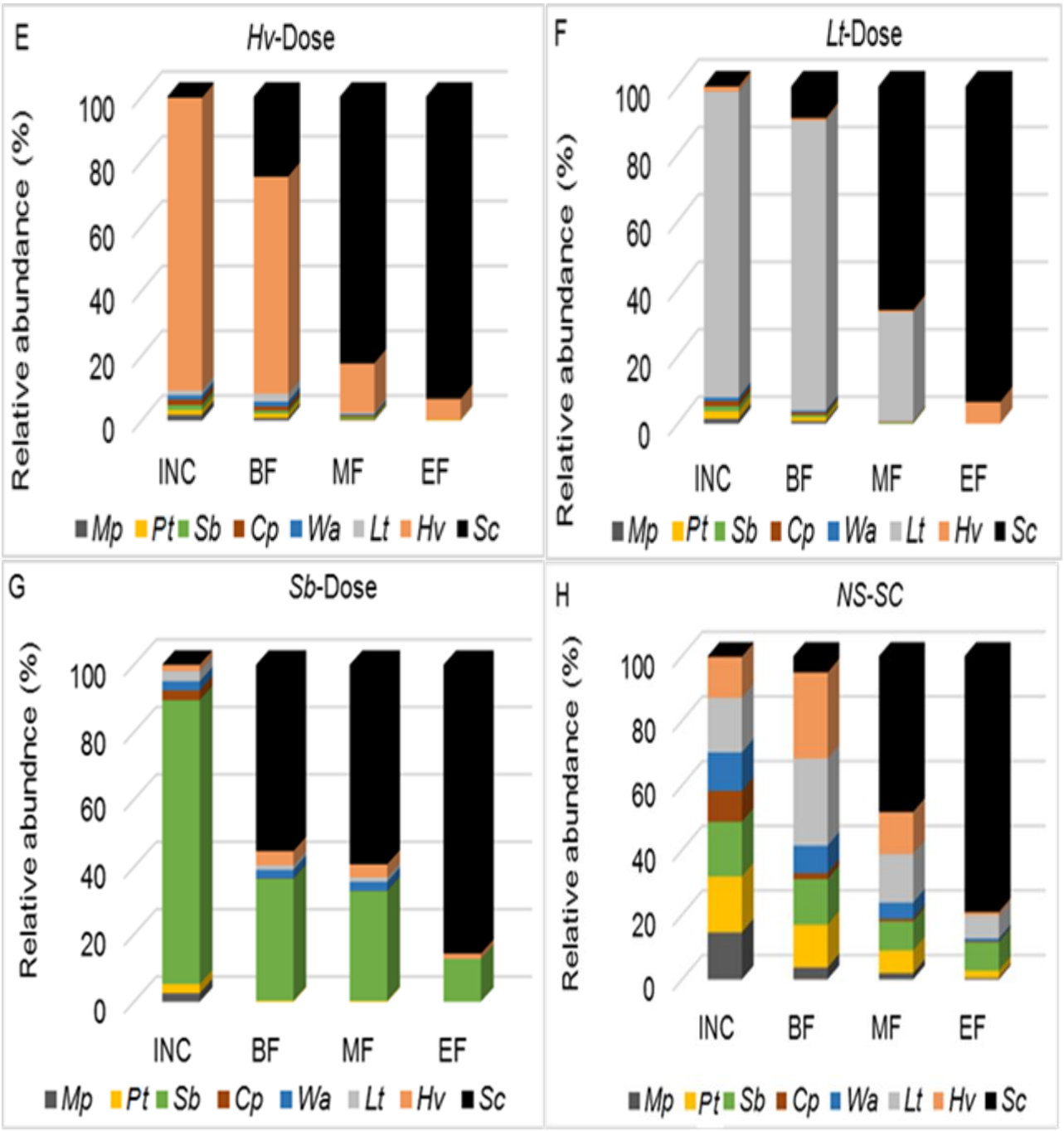


Figure.4.3. Distribution of yeast species (%) during fermentations at the inoculation time (INC), the beginning (BF), middle (MF) and the end of fermentation (EF).

4.4.3. Production of major volatiles

The production of major volatiles was also affected by dosage treatments. Chemical analysis of the wines produced from different treatments revealed that production of acetic acid varied considerably between treatments ranging from 614.36 mg/L in the NS-SC to 1105 mg/L in the *Hv*-dose fermentation (Table 4.2). In addition, the wine from the *Hv*-dose generated the highest level of ethyl acetate (114.73 mg/L) and total volatile fatty acids (mainly attributed to hexanoic acid),

while the *Lt*-dose wine had the highest total higher alcohols (Table 4.2). In contrast, the NS-SC fermentation resulted in the lowest level of total volatile fatty acids, total ethyl esters and the second highest level of ethyl acetate (82.71 mg/L). The *Mp*-dose and *Pt*-dose treatments produced significantly lower levels of higher alcohols, while *Lt*-dose, *Wa*-dose, and NS-SC produced the highest. The *Sb*-dose treatment produced a significant amount of acetoin (15.89 mg/L) compared to the rest of the treatments (Table 4.2).

Odour activity values were calculated for all the compounds to evaluate their potential contribution to wine aroma (Table 4.3). Sixteen out of 23 compounds exhibited odour activity (OAV) values above 1 in all dosage treatments. All the acetate esters were found to be active odorants (OAV>1) while amongst the ethyl esters, only ethyl caprylate and ethyl caprate were active odorants. Furthermore, the total intensities for every aromatic series were calculated in order to relate quantitative data obtained by chemical analysis to sensory perception.

Table.4.2. The concentration of major volatile compounds obtained in wines produced from different treatments. Values are represented in mg/L ± standard deviations.

Compound	NS-SC	*Sb*-dose	*Wa*-dose	*Cp*-dose	*Mp*-dose	*Pt*-dose	*Lt*-dose	*Hv*-dose
Ethyl Esters								
Ethyl Caprylate	0.22± 0.02^{c}	0.09± 0.01^{a}	0.17± 0.02^{bc}	0.40± 0.05^{e}	0.33± 0.01^{d}	0.34± 0.04^{d}	0.14± 0.01^{ab}	0.1± 0^{a}
Ethyl Caprate	0.02± 0^{a}	0.12± 0.01^{ab}	0.28± 0.04^{bc}	0.93± 0.30^{e}	0.59± 0.04^{d}	0.71± 0.21^{d}	0.50± 0.05^{cd}	0.23± 0.02^{ab}
Ethyl Lactate	5.60± 0.06^{a}	10.05± 0.09^{e}	9.50± 0.07^{cd}	9.14± 0.08^{bc}	8.98± 0.23^{b}	9.08± 0.11^{b}	9.73± 0.27^{de}	9.31± 0.22^{bc}
Diethyl Succinate	0.68± 0.13^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
∑ Esters	6.52± 0.21	10.26± 0.11	9.95± 0.13	10.47 ±0.43	9.9± 0.28	10.13± 0.36	10.37 ±0.33	9.64± 0.24
Acetate Esters								
Ethyl Acetate	82.71± 4.57^{c}	38.1± 1.16^{b}	43.85± 1.45^{b}	39.48± 2.3^{b}	21.97± 7.1^{a}	23.80± 0.47^{a}	45.19± 2.8^{b}	114.73± 8.78^{d}
Ethyl phenylacetate	1.02± 0.1^{a}	1± 0.02^{a}	1.37+ 0.08^{b}	1.74± 0.12^{c}	1.46± 0.04^{c}	1.61± 0.6^{c}	1.34± 0.01^{b}	1.33±. 0.02^{b}
2-Phenylethyl Acetate	0.84± 0.03^{a}	0.79± 0.03^{a}	1.3± 0.01^{b}	1.41± 0.01^{bc}	1.67± 0.01^{d}	1.55± 0.01^{cd}	0.86± 0.01^{a}	0.89± 0.03^{a}
Isoamyl Acetate	0.69± 0.04^{e}	0.35± 0.03^{ab}	0.45± 0.01^{cd}	0.38± 0.03^{abc}	0.32± 0.01^{a}	0.32± 0.01^{a}	0.48± 0.01^{d}	0.41± 0.08^{bcd}
∑ Acetates	85.28± 4.74	40.24± 1.24	46.97± 1.55	43.01± 2.46	25.42± 7.16	27.28± 1.09	47.87± 2.83	117.36± 8.91
Alcohol								
Isoamyl alcohol	100.39± 4.81^{de}	96.60± 5.38^{d}	102.26± 1.60^{de}	79.95± 2.36^{bc}	67.64± 1.83^{a}	74.12± 3.81^{ab}	106.07± 4.96^{e}	84.44± 1.57^{c}
2-Phenyl ethanol	22.56± 3.13^{bc}	25.81± 2.41^{e}	24.53± 3.17^{de}	21.33± 2.41^{b}	22.01± 1.23^{bc}	21.33± 1.73^{ab}	23.40± 2.25^{cd}	18.72± 1.9^{a}
Isobutanol	15.96± 0.35^{a}	26.71± 0.79^{d}	28.33± 1.88^{d}	18.95± 2.2^{b}	19.39± 0.8^{b}	20.1± 0.55^{bc}	22.6± 0.67^{c}	18.31± 1.41^{ab}
Butanol	0.88± 0.09^{c}	0.93± 0.08^{c}	0.87± 0.14^{c}	0.58± 0.05^{ab}	0.52± 0.01^{a}	0.55± 0.04^{a}	0.99± 0.10^{c}	0.81± 0.14b^{c}
Propanol	46.52± 6.03^{e}	31.16± 0.23^{bc}	35.60± 3.34^{cd}	30.05± 4.48^{bc}	15.18± 3.69^{a}	21.55± 4.11^{ab}	47.28± 6.53^{de}	43.32± 7.2^{de}
3-ethoxy-1-propanol	2.65±	3.29±	6.33±	5.48±	4.73±	5.97±	2.59±	3.04±

	0.27[a]	0.21[a]	0.31[c]	0.96[bc]	0.37[b]	0.62[c]	0.23[a]	0.26[a]
∑ Higher Alcohols	188.96± 14.68	184.5± 9.1	197.92± 10.44	156.34± 12.46	129.47± 7.93	143.62± 10.86	202.93± 14.74	168.64± 12.48
Volatile acids								
Acetic Acid	614.36± 8.95[a]	742.4± 26.7[c]	719.9± 59.16[bc]	853.24± 25.35[d]	837.3± 1.89[d]	881.8± 3.15[d]	639.9± 2.29[ab]	1105± 1.87[e]
Propionic Acid	0.96± 0.04[a]	4.07± 0.21[f]	3.77± 0.02[ef]	3.68± 0.6[def]	2.15± 0.06[b]	2.61± 0.17[c]	3.47± 0.2[de]	3.28± 0.17[d]
Isobutyric acid	0.89± 0.02[a]	1.44± 0.03[e]	1.35± 0.14[cd]	1.19± 0.02[bc]	1.07± 0.03[ab]	1.18± 0.04[bc]	1.30± 0.05[cd]	1.65± 0.21[f]
Butyric acid	2.27± 0.06[d]	0.74± 0[ab]	0.77± 0.01[bc]	0.82± 0.04[c]	0.85± 0.03[c]	0.85± 0.06[c]	0.65± 0[a]	0.71± 0.02[ab]
Iso-Valeric acid	0.84± 0.19[a]	1.15± 0.01[c]	1.06± 0.03[bc]	0.89± 0.04[ab]	0.85± 0.01[ab]	0.88± 0.04[ab]	0.96± 0.01[abc]	0.91± 0.04[ab]
Valeric acid	1.11± 0.03[d]	0.79± 0.02[abc]	1.09± 0[d]	1.78± 0.06[e]	0.81± 0.04[bc]	0.93± 0.14[c]	0.74± 0.07[ab]	0.65± 0.06[a]
Medium chain fatty acids (MCFAs)								
Hexanoic acid	1.20± 0.17[b]	0.41± 0.02[a]	0.40± 0.02[a]	1.13± 0.03[b]	0.44± 0.01[a]	0.42± 0.02[a]	0.31± 0.08[a]	5.72± 0.19[c]
Octanoic acid	1.70± 0.01[b]	1.20± 0.04[a]	2.32± 0.09[c]	3.28± 0.01[d]	3.36± 0.02[d]	3.45± 0.08[d]	1.37± 1.01[ab]	1.39± 0.8[ab]
Decanoic acid	0.96± 0.01[a]	0.83± 0.01[a]	1.47± 0.01[b]	2.55± 0.05[d]	2.38± 0.04[dc]	2.72± 0.08[d]	1.85± 0.01[bc]	1.75± 0.09[b]
∑ Volatile acids without acetic acid	9.93± 0.53	10.63± 0.34	12.23± 0.32	15.32± 0.85	11.91± 0.24	13.04± 0.63	10.65± 1.43	16.06± 1.58
Aldehydes and ketones								
Acetoin	2.07± 1.07[a]	15.89± 3.57[c]	5.23± 0.09[ab]	5.43± 0.57[ab]	7.88± 1.30[b]	7.98± 0.42[b]	5.28± 0.51[ab]	5.44± 0.45[ab]

Table.4.3. Sensory descriptors, odour threshold (mg/L) and odour activity value (OAV) of aroma compounds in wines with different treatments.

Compound	Sensory Description	Odorant Series	Odour Threshold (mg/L)	Odour Activity Values (OAV)							
				NS-SC	*Sb*	*Wa*	*Cp*	*Mp*	*Pt*	*Lt*	*Hv*
Acetate esters											
Ethyl Acetate	Fruity	1. 5 [5]	7.5 [1]	11.1^{c}	5.08^{b}	5.85^{b}	5.26^{b}	2.93^{a}	3.17^{a}	6.03^{b}	15.3^{d}
Isoamyl Acetate	Banana	1	0.03 [3]	23.76^{e}	11.84^{ab}	15.20^{cd}	12.9^{abc}	10.82^{a}	10.95^{a}	16.14^{d}	13.88^{bcd}
Ethyl Phenyl Acetate	Honey	2	0.25 [3]	4.10^{a}	4.03^{a}	5.50^{ab}	6.46^{b}	5.87^{b}	6.40^{b}	5.39^{ab}	5.33^{ab}
2-Phenylethyl Acetate	Floral	2 [4]	0.25 [1]	3.31^{a}	3.20^{a}	5.24^{b}	5.64^{bc}	6.71^{d}	6.22^{cd}	3.45^{a}	3.57^{a}
Ethyl Esters											
Ethyl Caprylate	Banana Floral	1.2.	0.005 [2]	46.59^{c}	19.19^{a}	35.74^{bc}	80.40^{e}	66.17^{d}	69.29^{de}	28.91^{ab}	20.58^{a}
Ethyl Caprate	Fruity Floral	1.2	0.2 [3]	0.13^{a}	0.63^{a}	1.41^{abc}	4.67^{e}	2.95^{b}	3.60^{de}	2.54^{bcd}	1.16^{ab}
Alcohol											
3-ethoxy-1-Propanol	Solvent	5	0.1 [7]	26.48^{a}	32.91^{a}	63.30^{c}	54.85^{bc}	47.34^{c}	59.77^{b}	25.92^{a}	30.42^{a}
Propanol	Ripe fruit	3, 5[5]	0.83 [2]	58.69^{e}	37.55^{bc}	42.9^{cd}	36.21^{bc}	18.30^{a}	25.97^{ab}	56.96^{e}	52.20^{de}
2-phenyl Ethanol	Rose	2 [4]	10 [1]	2.28^{cd}	2.58^{f}	2.45^{ef}	2.14^{bc}	2.20^{bc}	2.13^{b}	2.34^{de}	1.87^{a}
Isoamyl Alcohol	Solvent	3, 5 [4]	30 [1]	3.43^{de}	3.22^{d}	3.41^{de}	2.67^{bc}	2.25^{ab}	2.47^{a}	3.54^{e}	2.81^{c}
Volatile Acids											
Acetic acid	Rancid	4	200 [1]	3.03^{a}	3.71^{cd}	3.60^{bc}	4.27^{e}	4.19^{de}	4.41^{e}	3.20^{ab}	5.53^{f}
Butyric acid	Rancid	4 [4]	0.173 [2]	13.28^{e}	$4.29a^{b}$	4.49^{abc}	4.78^{cd}	4.97^{d}	4.92^{d}	3.80^{a}	4.15^{ab}
Iso- Valeric acid	Rancid	4	0.33 [3]	28.06^{ab}	35.13^{d}	32.16^{c}	27.07^{ab}	25.99^{a}	26.95^{ab}	29.14^{b}	27.68^{ab}
(Medium chain fatty acids (MCFAs)											
Hexanoic acid	Rancid	4 [4]	0.42 [2]	2.84^{b}	0.99^{a}	0.96^{a}	3.03^{b}	1.05^{a}	1^{a}	0.75^{a}	13.62^{c}
Octanoic acid	Rancid	4 [4]	0.5 [3]	3.37^{b}	2.42^{a}	4.65^{c}	6.54^{d}	6.73^{d}	6.91^{d}	2.76^{ab}	2.79^{ab}
Decanoic acid	Rancid	4 [4]	1 [2]	0.94^{ab}	0.84^{a}	1.47^{bc}	2.47^{e}	2.38^{de}	2.72^{e}	1.86^{cd}	1.76^{c}

The references, from which odour thresholds and odorant series have been taken, are given in the parentheses. [1]: Guth, (1997). The matrix was a 8.10 g/100 g water/ethanol solution, [2]: Etiévant, (1991), thresholds were calculated in wine. [3]: Ferreira *et al*., (2001), thresholds were calculated in the 11% water/ethanol solution comprising 7 g/L glycerol and 5 g/L tartaric acid, pH adjusted to 3.4 with 1 M NaOH. [4]: Sánchez-Palomo *et al*., 2010, [5]: Peinado *et al*., 2004. Odour activity values were calculated by dividing concentration by odour threshold value of the compound.1, fruity; 2, floral; 3, balsamic, fresh; 4, fatty; 5, solvent.

Based on the total intensities of odorant series calculated for all the treatments, the *Cp*-dose fermentation exhibited the highest potential to generate wines with a fruity and floral aroma, followed by the *Pt*-dose and *Mp*-dose while the *Wa*-dose would result in wines with a strong solvent and balsamic/vinegar aroma (Figure 4.4). Similarly, *Hv*-dose, *Lt*-dose, and *Sb*-dose displayed high potentials to generate wine with solvent characteristics. However, they may produce wines with low floral and intermediate fruity characteristics. In contrast, the NS-SC showed an intermediate floral and fatty character and a high fruity and balsamic character.

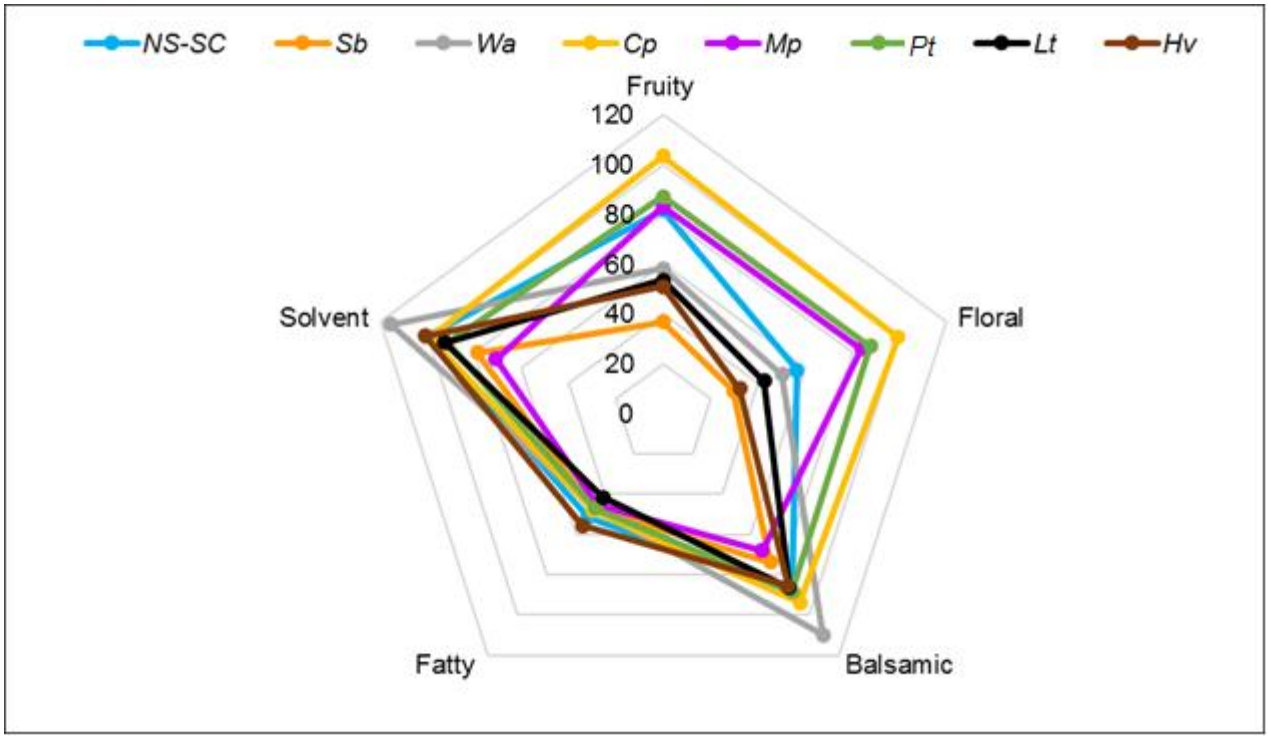


Figure.4.4. Odorant series in different wines. The odorant series were calculated as the sum of the OAVs of each one of the compounds assigned to these series.

The principal component analysis (PCA) was applied to all quantifiable major volatiles to decipher the compounds which would drive the differentiation between the wines. PC1 and PC2 explained 66.21% of the variance. The PCA plot showed that the NS-SC derived wine was mainly associated with butyric acid, ethyl acetate, isoamyl acetate and diethyl succinate, while the *Cp*-dose, *Pt*-dose, and *Mp*-dose formed one grouping mainly associated with the ethyl esters (ethyl caprate and ethyl caprylate), acetate esters (2-phenylethyl acetate and ethyl phenylacetate) as well as the medium chain fatty acids (octanoic acid and decanoic acid). *Sb*-dose, *Hv*-dose, and *Lt*-dose clustered closer together and were mainly characterized by elevated levels of higher alcohols and short

chain fatty acids. However, the wines could be distinguished from each other. For instance, the *Sb*-dose wine was associated with iso-valeric acid, isobutanol and phenyl ethanol, while, the *Hv*-dose, and *Lt*-dose were characterized by higher levels of hexanoic acid, isoamyl alcohol, butanol, and propanol (Figure. 4.5). Conversely, the *Wa*-dose wine forms a distinct cluster mainly associated with isobutyric acid, propionic acid, ethyl lactate and acetoin.

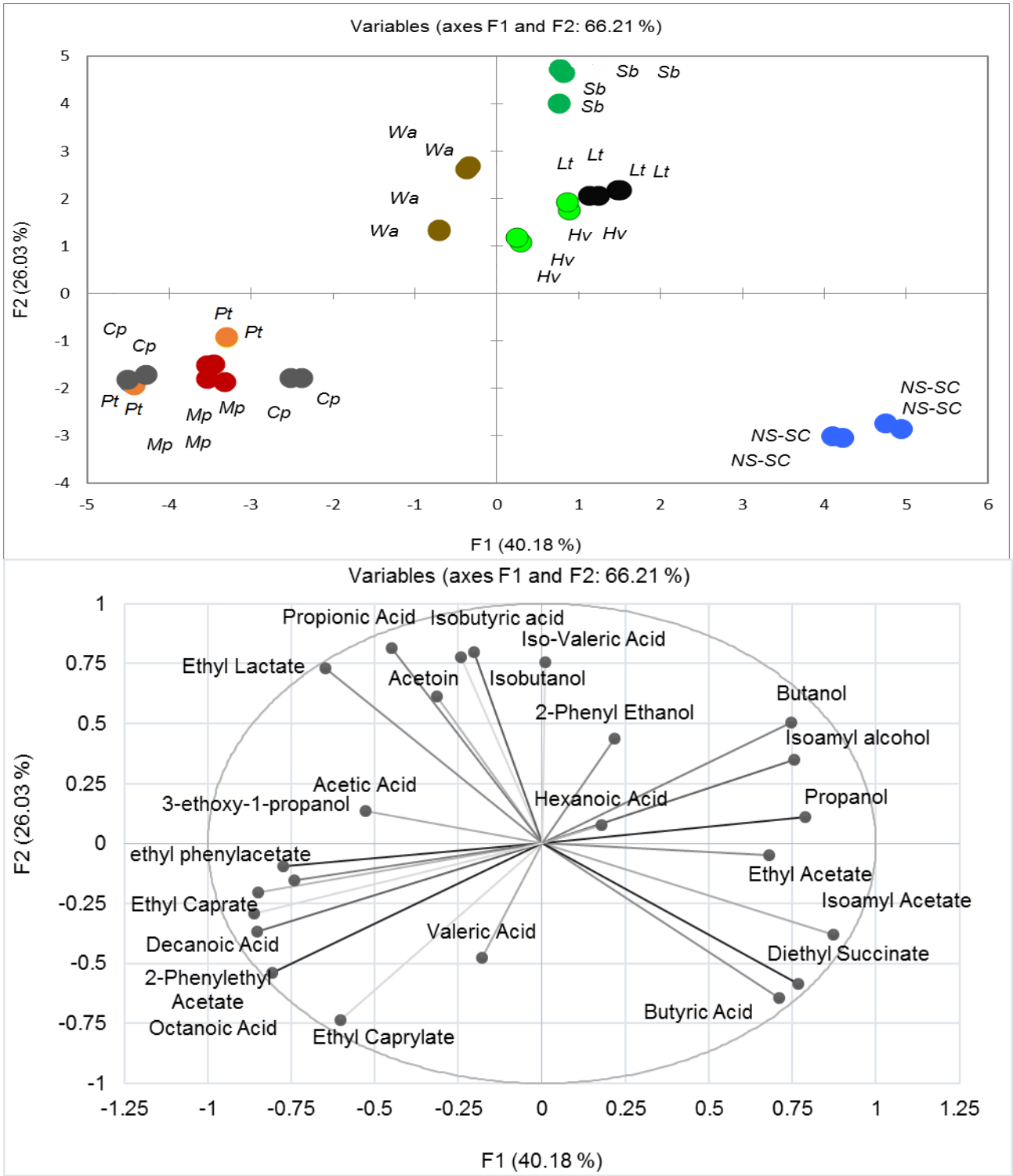


Figure.4.5. A biplot of the first and second principal components (PC) derived from PC analysis of the major volatile compounds produced in synthetic must fermentations. The major volatile compounds are represented by light grays whereas the treatments are as follows: *Sb*-dose ( ), *Wa*-dose( ), *Hv*-

dose ( ), *Lt*-dose ( ), *Mp*-dose ( ), *Cp*-dose ( ), *Pt*-dose (and NS-SC ( ). Labels for dosage trials are denoted by their abbreviation (*Sb, Wa, Lt, Hv, Mp, Cp, pt*, and NS-SC).

4.5. Discussion

The current study evaluated the influence of cell density of yeast species on population dynamics in a complex community and on the final wine aroma. For this purpose, one out of 8 species were inoculated at a dosage 100 times higher than the rest of the species. Evidently, a higher inoculum dosage influenced fermentation kinetics, promoted the dominance, and enhanced persistence in a species-specific manner. For instance, *Sb*-dose fermentation in which *S. bacillaris* was inoculated at a higher dose resulted in a faster fermentation which could be attributed to the rapid simultaneous utilization of both fructose and glucose, and improved persistence of *S. bacillaris*. The data reflect a clear synergistic interaction between the two dominant yeasts viz. *S. bacillaris* and *S. cerevisiae* which have different sugar preferences in this fermentation. Indeed, many strains of *S. bacillaris* have been shown to be fructophilic while *S. cerevisiae* is glucophilic (Magyar and Toth, 2011; Masneuf-Pomarede *et al*., 2015). The cooperative interaction between these yeasts has been observed with other strains (Suzzi *et al*. 2012), suggesting that this could be more species-specific rather than strain specific. This positive interaction on fermentation kinetics is however only maintained in scenarios where both species are dominant. However, this positive interaction seems to rather diminish in the presence of other species and when the inoculum level of *S. bacillaris* is low, as is the case for the rest of the fermentations in the current study. Similarly, Suzzi *et al*. (2012), showed that when *H. uvarum* was co-inoculated together with *S. bacillaris* and *S. cerevisiae* in a 25:25:50 ratio, the fermentation rate was slower than that of the *S. bacillaris*-*S. cerevisiae* co-fermentation. On the other hand, when *H. vineae* was inoculated at a higher dose (*Hv*-dose), the slowest fermentation rate was observed. Our data clearly demonstrated that 60% of fructose was still available in day 8 of *Hv*-dose fermentation whereas 50% of glucose was consumed in the first 4 days of fermentation, suggesting the glucophilic characteristics of both species. Similarly, Viana *et al*., (2009), showed that when *H. vineae* was inoculated sequentially with *S. cerevisiae* in 90:10 and 75:25, the rate of glucose consumption was slower in the first scenario.

The slow fermentation kinetics observed in *Hv*-dose treatment suggests that there is a negative interaction between the *H. vineae* and *S. cerevisiae* which can be attributed to the high competition for nutrients such as glucose. Indeed, Medina *et al*., (2013), showed that sequential inoculation of *H. vineae* and *S. cerevisiae*, limited the nutrient availability for *S. cerevisiae*. Furthermore, the author demonstrated that addition of yeast assimilable nitrogen and vitamin mixture improved *S. cerevisiae* growth, resulting in higher population levels and increased fermentation rate (Medina *et al*., 2013).

The second slowest kinetics was observed in *Lt*-dose fermentation, suggesting that there was a competition between these strongly fermentative species. A similar interaction has been previously reported by Gobbi *et al*., (2013), who demonstrated that the growth of *L. thermotolerans* was negatively affected in co-inoculation with *S. cerevisiae*, while its persistence increased in sequential fermentation. Nissen and Arneborg (2003), showed that lack of oxygen decreased the relative glucose uptake abilities of *L. thermotolerans*, suggesting that oxygen increased the ability *of L. thermotolerans* to compete for space in mixed culture fermentation with *S. cerevisiae*. Similarly, Shekhawat *et al*., (2017), have demonstrated that in presence of 1, 5 and 21% dissolved oxygen, *L. thermotolerans* dominated *S. cerevisiae* in mixed culture fermentations. These data suggest that the slow fermentation in the *Lt*-dose could be due to a slow metabolic activity of *L. thermotolerans* under anoxic conditions, coupled with reduced nutrient availability which retards the establishment of *S. cerevisiae*.

The second fastest rate of glucose consumption was observed in the *Wa*-dose fermentation, suggesting the rapid glucose uptake ability of *W. anomalus* and *S. cerevisiae*. We have previously reported the antagonistic interaction between *W. anomalus* and *S. cerevisiae* in NS-SC fermentation (Bagheri *et al*., 2017). Our data showed that higher inoculation enhanced the persistence of *W. anomalus* possibly due to the rapid utilization of glucose in early fermentation. This observation suggested that the negative interaction between the two is biomass-dependent. On the contrary, the dosage treatment had no effect on the persistence of *M. pulcherrima*, *C. parapsilosis*, and *P. terricola*, suggesting that their growth and survival was curtailed by other factors such as high oxygen demand. Indeed, several strains of *M. pulcherrima* for were reported to have lower growth rates than *S. cerevisiae* (Contreras *et al*., 2014) and required significantly higher oxygen input to grow in high sugar environments (Shekhawat *et al*., 2017). A similar correlation was observed observed for the growth of *C. parapsilosis* and oxygen availability (Oh *et al*., 1998), while the rapid decline in the population of *P. terricola* in *Pt*-dose could be due to many factors, which requires further investigation.

Comparative analysis of population dynamics in all inoculation strategies suggests that high inoculation of one species may support the growth of others in the ecosystem. This behaviour was observed in *Sb*-dose and *Pt*-dose treatments where the growth of *W. anomalus* was either maintained or it was enhanced throughout the fermentation. Thus, the results suggested that high inoculation with *P. terricola* and *S. bacillaris* supported the growth of *W. anomalus*. Another interesting observation was the persistence of *H. vineae* along with *S. cerevisiae* in all dosage treatments except in *Lt*-dose and *Sb*-dose. Higher inoculations with *C. parapsilosis, M. pulcherrima* and to a lesser extend *W. anomalus* and *P. terricola*, appeared to support the persistence of *H. vineae*. We have previously reported the synergistic interaction between *H. vineae* and *S.*

cerevisiae in NS-SC treatment. However, the result clearly suggested that synergistic interaction between the two only occurred in all dosage treatments except *H. vineae* was inoculated at a higher dosage (*Hv*-dose). Thus, we hypothesize that in a multi-species consortium *S. cerevisiae* uses different strategies to first outcompete the species which is present at a higher cell density compared to other members of the consortium.

Concerning the major volatile production, a clear separation was observed between NS-SC and dosage treatments. The rate in which S. *cerevisiae* dominated the fermentation drove the separation of PC1 and PC2. *S. cerevisiae* dominated the fermentation much faster in the treatments which were in the negative dimension of PC1 (*Cp*-dose, *Pt*-dose, *Mp*-dose, and *Wa*-dose) compared to the treatments which were in the positive dimension of PC1 (NS-SC, *Lt*-dose, *Sb*-dose and *Hv*-dose). Furthermore, the aromatic signature of some non-*Saccharomyces* species was observed in wine suggesting the presence of strong yeast-yeast interactions between species in the consortium.

A significant amount of decanoic acid, octanoic acid and their fatty acid esters (ethyl caprate, ethyl caprylate) as well as ethyl phenylacetate were produced in wines obtained from *Cp*-dose, *Pt*-dose and *Mp*-dose fermentations. Sadoudi *et al*., (2012), has previously reported that production of esters was enhanced in the mixed culture of *S. cerevisiae* and *M. pulcherrima* whereas, our result for the first time highlighted the positive contribution of *C. parapsilosis* and *P. terricola* in wine aroma. Furthermore, a significant level of isobutanol and 2-phenyl ethanol was produced in *Sb*-dose and *Wa*-dose whereas high levels of propionic acid and acetoin were produced in *Sb*-dose wine. In contrast, isobutyric acid, hexanoic acid, acetic acid and ethyl acetate were produced at high concentrations in *Hv*-dose wine. *H. vineae* strains have been shown to produce high levels of volatile acidity (Viana *et al*., 2008; Viana *et al*., 2011). Medina *et al*., (2013), has reported the acceptable volatile acidity in wine inoculated with *H. vineae* in grape must; although, strain variability was reported previously (Viana *et al*., 2009; Medina *et al*., 2013; Lleixà *et al*., 2016). *Lt*-dose produced a low amount of acetic acid which was not significantly different from NS-SC wine, suggesting that low concentration of acetic acid produced in NS-SC, could be the positive contribution of *L. thermotolerans*. Similarly, Gobbi *et al*., (2013), as well as Benito *et al*., (2015), have reported that wine obtained from sequential inoculation or mixed inoculation of *S. cerevisiae* with *L. thermotolerans* contain a low concentrations of acetic acid.

Overall, the data confirmed that cell density of individual species affects the growth of other species in the consortium. The observations made in this study required further investigation to unravel the mechanisms underlying these interactions (metabolically, physically, etc). Furthermore, the result revealed that evaluating the effect of cell density on the dynamics of a multi-species

consortium is a useful tool to unravel the real contribution of individual non-*Saccharomyces* species in wine ecosystem.

4.6. References

Bagheri, B., Bauer, F. F., Setati, M. E. (2015). The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. *S. Afr. J. Enol. Vitic*. 36, 243–251.

Bagheri, B., Bauer, F. F., Setati, M. E. (2017). The Impact of *Saccharomyces cerevisiae* on a wine yeast consortium in natural and inoculated fermentations. *Front. Microbiol*. 8:1988. doi: 10.3389/fmicb.2017.01988.

Bely, M., Sablayrolles, J. M., Barre, P. (1990). Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *J. Ferment. Bioeng.* 70, 246–252. doi:10.1016/0922-338X(90)90057-4.

Benito, A, Fernando, C., Palomero, F., and Benito, S., (2015). Quality and Composition of Airén Wines Fermented by Sequential Inoculation of *Lachancea thermotolerans* and *Saccharomyces cerevisiae*. *Food Technol. Biotechnol*. 54, 135–144.

Ciani, M., Maccarelli, F., (1998). Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World J. Microbiol. Biotechnol*. 14, 199–203.

Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., Romano, P. (2016). Yeast interactions in inoculated wine fermentation. *Front. Microbiol.* doi:10.3389/fmicb.2016.00555.

Contreras, A., Hidalgo, C., Henschke, P. A., Chambers, P. J., Curtin, C., and Varela, C. (2014). Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Appl. Environ. Microbiol.* 80, 1670–1678. doi:10.1128/AEM.03780-13.

Etievant, P.X. (1991). "Wine" In volatile compounds in food and beverages. ed H. Maarse (New York, NY: Dekker), 483–546.

Ferreira, V.,AznarM., Lopez, R., Cacho, J. (2001). Quantitative gas chromatography- olfactometry carried out at different dilutions of an extract. Key differences in the odor profiles of four high-quality Spanish aged red wines. *J. Agric. Food Chem*. 49, 4818- 4824.

Fleet, G.H., (2008). Wine yeasts for the future. *FEMS Yeast Res*. 8, 979–995.

Grosch, W., (2001). Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. *Chem Senses* 26:533–545.

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

Guth, H. (1997). Quantitation and Sensory Studies of Character Impact Odorants of Different White Wine Varieties. *J. Agric. Food Chem*, *45*, 3027–3032. https://doi.org/10.1021/jf970280a.

Henschke, PA., Jiranek, V. (1993). Yeasts metabolism of nitrogen compounds. *In* Wine Microbiology and Biotechnology, Fleet GH *(ed). Harwood Academic:* Chur, Switzerland; 77-164.

Lee, P. R., Kho, S. H. C., Yu, B., Curran, P., and Liu, S. Q. (2013). Yeast ratio is a critical factor for sequential fermentation of papaya wine by *Williopsis saturnus* and *Saccharomyces cerevisiae*. *Microb. Biotechnol.* 6, 385–393. doi:10.1111/1751-7915.12008.

Lleixà, J., Martín, V., Portillo, M. del C., Carrau, F., Beltran, G., & Mas, A. (2016). Comparison of fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*. *Frontiers in Microbiology*, 7, 338, https://doi.org/10.3389/fmicb.2016.00338.

Louw, L., Tredoux, AGJ., van Rensburg, P., Kidd, M., Naes, T., Nieuwoudt, HH., (2010). Fermentation-derived aroma compounds in varietal young wines from South Africa. *S Afr J Enol Vitic* 31, 213-225.

Magyar, I., and T. Toth. (2011). Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiol*, 28:94-100.

Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F., et al (2015). The yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) shows high genetic diversity in winemaking environments. *FEMS Yeast Res* 15:fov045. https://doi.org/10.1093/ femsyr/fov045.

Medina, K., Boido, E., Fariña, L., Gioia, O., Gomez, M. E., Barquet, M., Carrau, F. (2013). Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chemistry*, *141*, 2513–2521. https://doi.org/10.1016/j.foodchem.2013.04.056.

Molina, A.M., Guadalupe, V., Varela, C., Swiegers, J.H., Pretorius, I.S., Agosin, E., (2009). Differential synthesis of fermentative aroma compounds of two related commercial wine yeast strains. *Food Chem*. 117, 189-195.

Moreira, N., Mendes, F., Hogg, T., and Vasconcelos, I. (2005). Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. *Int. J. Food Microbiol.* 103, 285–294. doi:10.1016/j.ijfoodmicro.2004.12.029.

Nissen, P., Arneborg, N., (2003). Characterization of early death of non-*Saccharomyces* yeaststst in mixed cultures with *Saccharomyces cerevisiae. Arch Microbiol,* 180:257-263.

Oh, D., Kim, S., and Kim, J. (1998). Increase of xylitol production rate by controlling redox potential in *Candida parapsilosis*. *Biotechnol. Bioeng*. 58, 440–444. doi: 10.1002/(SICI)1097-0290(19980520)58:43.0. CO;2-F.

Peinado, R. A., Moreno, J., Medina, M., Mauricio, J. C. (2004). Changes in volatile compounds and aromatic series in sherry wine with high gluconic acid levels subjected to aging by submerged flour yeast cultures. *J Biotechnol Lett*, 26, 757e762.

Renault, P., Miot-Sertier, C., Marullo, P., Hernandez-Orte, P., Lagarrigue, L., Lonvaud-Funel, A., et al. (2009). Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: potential applications in the wine industry. *Int J Food Microbiol*. 134, 201–210.

Sadoudi, M.,Tourdot-Maréchal, R.,Rousseaux, S., Steyer, D.,GallardoChacón, J. J., Ballester, J., *et al*. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non- *Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol*. 32, 243–253. doi: 10.1016/j.fm.2012.06.006.

Sambrook, J., and W. Russell, D (2006). Rapid Isolation of Yeast DNA Cold Spring Harb Protoc; doi:10.1101/pdb.prot4039.

Sánchez-Palomo, E., Gómez García-Carpintero, E., Alonso-Villegas, R., González-Viñas, M. A. (2010). Characterization of aroma compounds of Verdejo white wines from the La Mancha region by odour activity values. *Flavour Frag. J. 25*, 456–462. https://doi.org/10.1002/ffj.2005.

Shekhawat, K., Bauer, F. F., Setati, M. E. (2017). Impact of oxygenation on the performance of three non-Saccharomyces yeasts in co-fermentation with Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol*. 101, 2479–2491. doi: 10.1007/s00253-016-8001-y.

Slabbert, E., Van Heerden, C. J., Jacobs, K. (2010). Optimisation of automated ribosomal intergenic spacer analysis for the estimation of microbial diversity in fynbos soil. *S. Afr. J. Sci*, *106*, 8–11. https://doi.org/10.4102/sajs.v106i7/8.329.

Sun, S. Y., Gong, H. S., Jiang, X. M., Zhao, Y. P. (2014). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae* on alcoholic fermentation behaviour and wine aroma of cherry wines. *Food Microbiol.* 44, 15–23. doi:10.1016/j.fm.2014.05.007.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., et al. (2012). Multistarter from organic viticulture for red wine Montepulciano d'Abruzzo production. *Front. Microbiol.* 3, 1–10. doi:10.3389/fmicb.2012.00135.

Taillander, P., Lai, Q. P., Julien-Ortiz, A., Brandam, C. (2014). Interaction between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: influence of inoculation and nitrogen content. *World J.Microbiol. Biotechnol.* 30, 1959–1967. doi: 10.1007/s11274-014-1618-z.

Viana, F., Gil, J. V., Vallés, S., Manzanares, P. (2009). Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. *I Int. J. Food Microbiol*, *135* (1), 68–74. https://doi.org/10.1016/j.ijfoodmicro.2009.07.025.

Viana, F., Belloch, C., Vallés, S., Manzanares, P. (2011). Monitoring a mixed starter of Hanseniaspora vineae–Saccharomyces cerevisiae in natural must: impact on 2-phenylethyl acetate production. *Int. J. Food Microbiol*. 151, 235–240. doi: 10.1016/j.ijfoodmicro.2011.09.005.

Vigentini, I., Fabrizio, V., Faccincani, M., Picozzi, C., Comasio, A., and Foschino, R. (2014). Dynamics of *Saccharomyces cerevisiae* populations in controlled and spontaneous fermentations for Franciacorta D.O.C.G. base wine production. *Ann. Microbiol*. 64, 639–651. doi: 10.1007/s13213-013- 0697-7.

Chapter 5

Research results

Effect of environmental parameters on the dynamics of yeast consortium

This manuscript will be submitted for publication in

Applied and Environmental Microbiology

5.1. Abstract

Conversion of grape must to wine is a complex process that is initiated by a diverse population of microorganisms, including many non-*Saccharomyces* yeast species. These species grow and interact with one another physically and metabolically. Ultimately, the species with greater fitness dominate the remaining population. The fitness of each strain in wine fermentation is affected by several biotic and abiotic parameters. Environmental parameters such as temperature and SO_2 have shown to significantly affect the contribution of individual species and consequently affect the wine aroma. However, most studies have focused on single species or mixed culture fermentations composed of two species. Thus, the fundamental rules underlying the effect of these parameters in a multi-species ecosystem are not fully understood. The current study evaluated the effect of temperature and sulphur addition on the dynamics of a multi-species yeast consortium in synthetic must and real grape must fermentations. The pattern of yeast population dynamics was affected by temperature and sulphur addition. The fermentations performed at a lower temperature and without sulphur dioxide were characterized by high population of non-*Saccharomyces* species whereas the fermentations with a higher temperature and sulphur dioxide were mainly dominated by *S. cerevisiae* strains. The differences in the population dynamics between the treatments influenced the production of majority of volatile compounds, However, few compounds remained unaffected. The results suggest that environmental parameters modify the population dynamics. However, ecological interactions seem to drive the wine ecosystem and affect the aromatic profiles of wine. Overall, a similar pattern of population dynamics was observed in the synthetic must and two real grape must fermentations, despite the differences among three matrices. The results demonstrated ecological interactions are largely independent of matrix. These observations confirm that the constructed multi-species consortium is a robust model that can be used as a tool to predict the microbial behavior from a simple ecosystem to the complex natural environment. The current study for the first time underlined the potential of a model yeast consortium in understanding the ecological interactions in wine fermentation.

5.2. Introduction

Alcoholic fermentation is commonly initiated by complex microbial consortia composed of oxidative and weakly non-*Saccharomyces* species (Jolly *et al*., 2003a; Ghosh *et al*., 2015; Bagheri *et al*., 2015; Wang *et al*., 2015). The weakly fermentative non-*Saccharomyces* species are sequentially replaced by stronger fermentative species which may persist along with *Saccharomyces cerevisiae* throughout the fermentation (Eder *et al*., 2017; De Filippis *et al*., 2017; Del Fresno *et al*., 2017). The persistence of each strain is influenced by its relative fitness within the wine consortium (Pérez-Torrado *et al*., 2017). Therefore, the fitness of each strain is affected by several biotic (grape variety and ecological interactions) as well as abiotic parameters (temperature, sulphur dioxide, oxygen and nutrient availability, pH, osmotic pressure and ethanol) (Ciani *et al*., 2016; Liu *et al*., 2017).

Temperature and sulphur dioxide are two of the most important parameters, affecting yeast growth and wine aroma (Torija *et al*., 2003; Reddy and Reddy, 2011; Sun *et al*., 2016). Investigating the effect of temperature on wine microbiota has been the focus of several studies (Torija *et al*., 2003; Reddy and Reddy, 2011; García-Ríos *et al*., 2014; Rollero *et al*., 2015). The existing data suggest that temperature strongly affects fermentation kinetics, yeast population dynamics as well as yeast metabolism (Torija *et al*., 2003; Reddy and Reddy, 2011; García-Ríos *et al*., 2014; Rollero *et al*., 2015; Maturano *et al*., 2016; Alonso-del-Real *et al*., 2017). Fermentations conducted at a lower temperature (e.g. 15°C) have shown to increase the growth of some non-*Saccharomyces* species (e.g. *Candida stellata*, *Lachancea thermotolerans, Metschnikowia pulcherrima* and *Hanseniaspora uvarum*) whereas, fermentations performed at higher temperatures have been shown to increase the relative fitness of *S. cerevisiae* in wine fermentation (Mills *et al*., 2002; Erten, 2002; Salvadò *et al*., 2011; Alonso-del-Real *et al*., 2017). Indeed, some studies have shown that in fermentations at low temperatures, yeasts such as *L. thermotolerans* and *H. uvarum* may sometimes surpass the growth of *S. cerevisiae* (Fleet *et al*., 1984; Mora *et al*., 1990; Gao and Fleet, 1988; Ciani, 1997; Ciani *et al*., 2006; Alonso-del-Real *et al*., 2017). Furthermore, the reports indicated that the chemical composition of wine is also affected by temperature. However, the results suggest that the effect of temperature on the production of aroma compounds depends upon several variables such as strains and conditions that the experiments were performed (Hammond, 1993; Monila *et al*., 2007; Beltran *et al*., 2006; Beltran *et al*., 2008; Reddy and Reddy *et al*., 2011; Suzzi *et al*., 2012; Gobbi *et al*., 2013; Fairbairn *et al*., 2014; Rollero *et al*., 2015).

Addition of sulphur dioxide as an antioxidant and antimicrobial compound to grape must is a common practice in wine industry (Reed and Nagodawithana, 1991). Sulphur is added to must mainly to suppress the growth of undesired microorganisms (Sun *et al*., 2016; Bokulich *et al*., 2015). Addition of sulphur dioxide ranging from 40-50 mg/L has been shown to negatively affect the growth of *H. uvarum*, *L. thermotolerans* and *Torulaspora delbrueckii* while supporting the growth of *S. cerevisiae* (Cocolin and Mills, 2003; Albertin *et al*., 2014; Chandra *et al*., 2015; Sun *et al*., 2016). Concerning the effect of sulphur dioxide on the aromatic profiles of wine, there is no clear direct correlation between sulphur addition and production of volatile compounds and the results are rather contradictory (Coetzee *et al*., 2013; Sun *et al*., 2016).

Previous studies have mainly focused on understanding the effect of these parameters in single or mixed culture fermentations composed of 2-3 species (Fleet, 1992; Romano and Suzzi, 1993; Redon *et al*., 2011; Alonso-del-Real *et al*., 2017). Consequently, despite all efforts made, limited information is available on how temperature and sulphur addition affect yeast-yeast interactions in a multi-species ecosystem. Since multispecies ecosystems are considered more robust in terms of resistance to environmental variation, it is also essential to ascertain whether the primary driver of changes in population growth and dynamics are linked to environmental factors, or are rather a function of yeast ecosystem itself. We have previously evaluated the effect of two biotic parameters (presence of *S. cerevisiae* and variation in cell density of individual species) on the dynamics of yeast consortium. The data confirmed that presence of *S. cerevisiae* dramatically changed the pattern of population dynamics (Bagheri *et al*., 2017). On the other hand, variation in cell density of individual members of consortium underlined that presence of one species at a high density may promote or inhibit the growth of others in the wine ecosystem. The current study aimed to evaluate the effect of two abiotic parameters (temperature and sulphur dioxide) on yeast dynamics and wine chemical composition.

5.3. Materials and methods

5.3.1. Yeast consortium and culture conditions

The yeast consortium comprising seven yeast strains obtained from the culture collection of the Institute for Wine Biotechnology (IWBT) and a commercial yeast *S. cerevisiae* Lalvin EC1118 (Lallemand, Canada) were constructed as described in the first chapter (table 5.1). The yeast stock cultures were maintained in 20% (v/v) glycerol at -80°C and were streaked out as previously described.

Table.5.1. Strains used in this study. The strain codes are the abbreviation of the name of each strain.

Strains	Strains codes	Strains number
Hanseniaspora vineae	*Hv*	Y980

Pichia terricola	*Pt*	Y974
Starmerella bacillaris	*Sb*	Y975
Candida parapsilosis	*Cp*	Y842
Lachancea thermotolerans	*Lt*	Y973
Saccharomyces cerevisiae	*Sc*	EC1118
Wickerhamomyces anomalus	*Wa*	Y934
Metschnikowia pulcherrima	*Mp*	Y981

5.3.2. Micro-fermentations

5.3.2.1. Synthetic must fermentation

Fermentations were performed in synthetic grape juice medium (pH 3.5) adapted from Henschke and Jiranek, (1993) and Bely *et al*., (1990). The medium contained 200 g/L sugars (100 g/L glucose and 100 g/L fructose) and 300 mg/L assimilable nitrogen (460 mg/L NH_4Cl and 180 mg/L amino acids).

The effect of temperature (15, 25 and 30˚C) and sulphur dioxide (0, 30 mg/L) on the dynamics of yeast consortium was evaluated in the presence and absence of *S. cerevisiae* (Figure 5.1). In the presence of *S. cerevisiae*, fermentations were inoculated with the yeast consortium (NS-SC) comprising 7 *non-Saccharomyces* species (each inoculated at 10^6 CFU/mL) and *S. cerevisiae* (inoculated at 10^3 CFU/mL). On the contrary, in absence of *S. cerevisiae,* 7 non-*Saccharomyces* species were inoculated at 10^6 CFU/mL whereas *S. cerevisiae* was excluded from the yeast consortium (NS).

The fermentations were performed in the final volume of 500 mL and the Erlenmeyer flasks were fitted with fermentation locks. The fermentations were conducted in triplicate and without agitation. The fermentations were weighed to monitor CO_2 release and samples were withdrawn regularly. The fermentations were considered dry and terminated when the residual sugar (glucose and fructose) was less than 2 g/L.

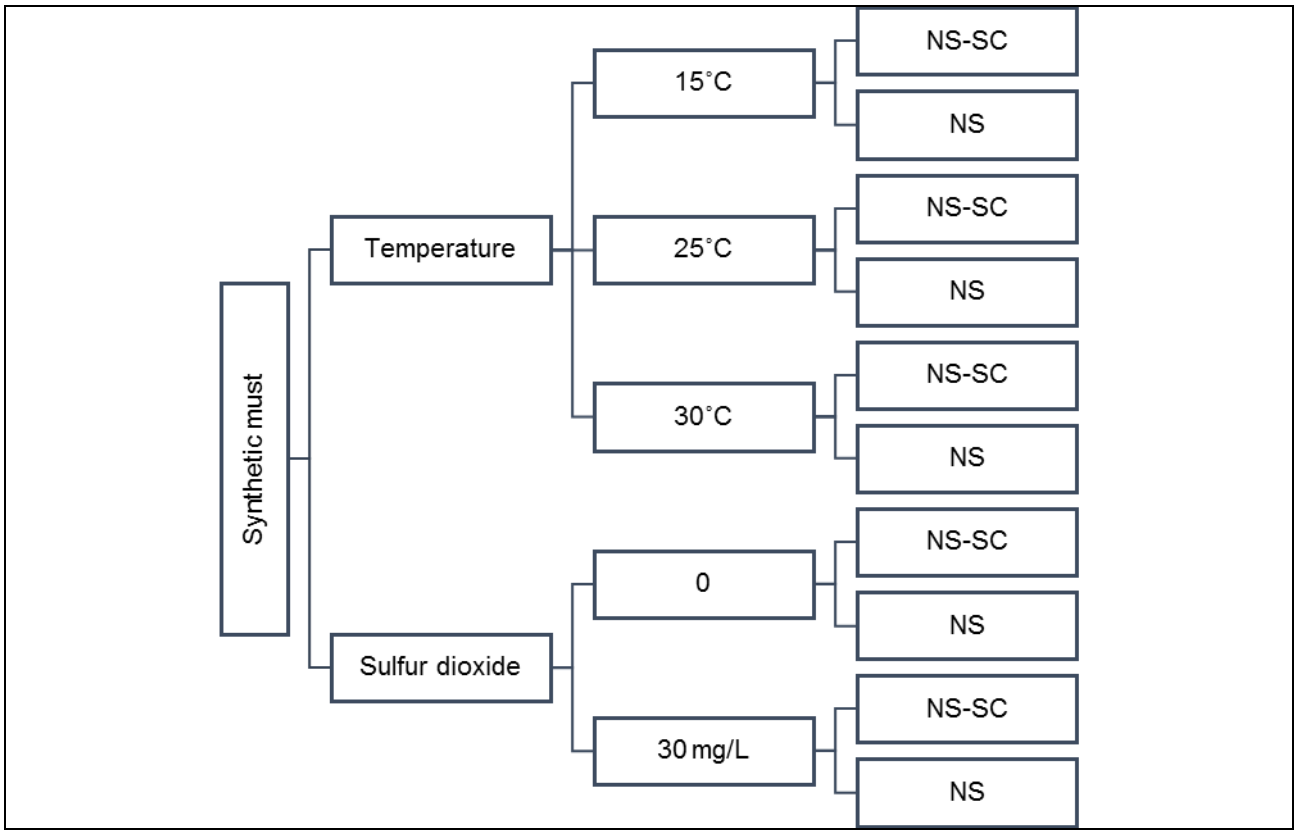


Figure.5.1. Schematic representation of fermentations conducted in the synthetic must. Effect of temperature and sulphur dioxide on the yeast population dynamics was evaluated in the presence (NS-SC) and in absence of *S. cerevisiae* (NS).

5.3.2.2. Real grape juice fermentation

Fermentations were performed during two harvests, one in Stellenbosch, South Africa and another in Perugia, Italy. Chenin blanc cultivar was used to conduct the fermentation in South Africa (CHEN) whereas Grechetto Bianco cultivar was selected to perform the fermentations in Italy (GREC.) Fifty liters of clarified juice was obtained from the commercial cellars. The yeast diversity of grape juice was determined by serial dilution and plating on WL-agar. Hundred and eighty-four (184) isolates from South African must and 212 isolates from Italian must was identified through ITS-5.8S rRNA amplification, RFLP, and sequencing as previously described in Bagheri *et al*., (2015).

Fermentations were conducted at 15˚C and 25˚C, with and without sulphur dioxide (30 mg/L and 0) (Figure 5.2). The fermentations were conducted: (i) spontaneously (SP), (ii) inoculated with commercial *S. cerevisiae*, EC1118 at 10^3 cells/mL (EC) and (iii) inoculated with a consortium of 7 *non-Saccharomyces* yeast at 10^6 cells/mL and *S. cerevisiae* at 10^3 cells/mL (NS-SC).

The Chenin blanc juice was divided into 1.5 L samples and fermentations were conducted in 2 L fermentation bottles in South Africa whereas, in Italy, the fermentations were conducted by dividing the juice in 500 mL Erlenmeyer flasks. Fermentations were performed in triplicate, without agitation and the fermentation vessels were fitted with fermentation locks. The fermentation progress was determined by monitoring the CO_2 release. The fermentations were considered complete when the residual sugar (glucose and fructose) was less than 2 g/L.

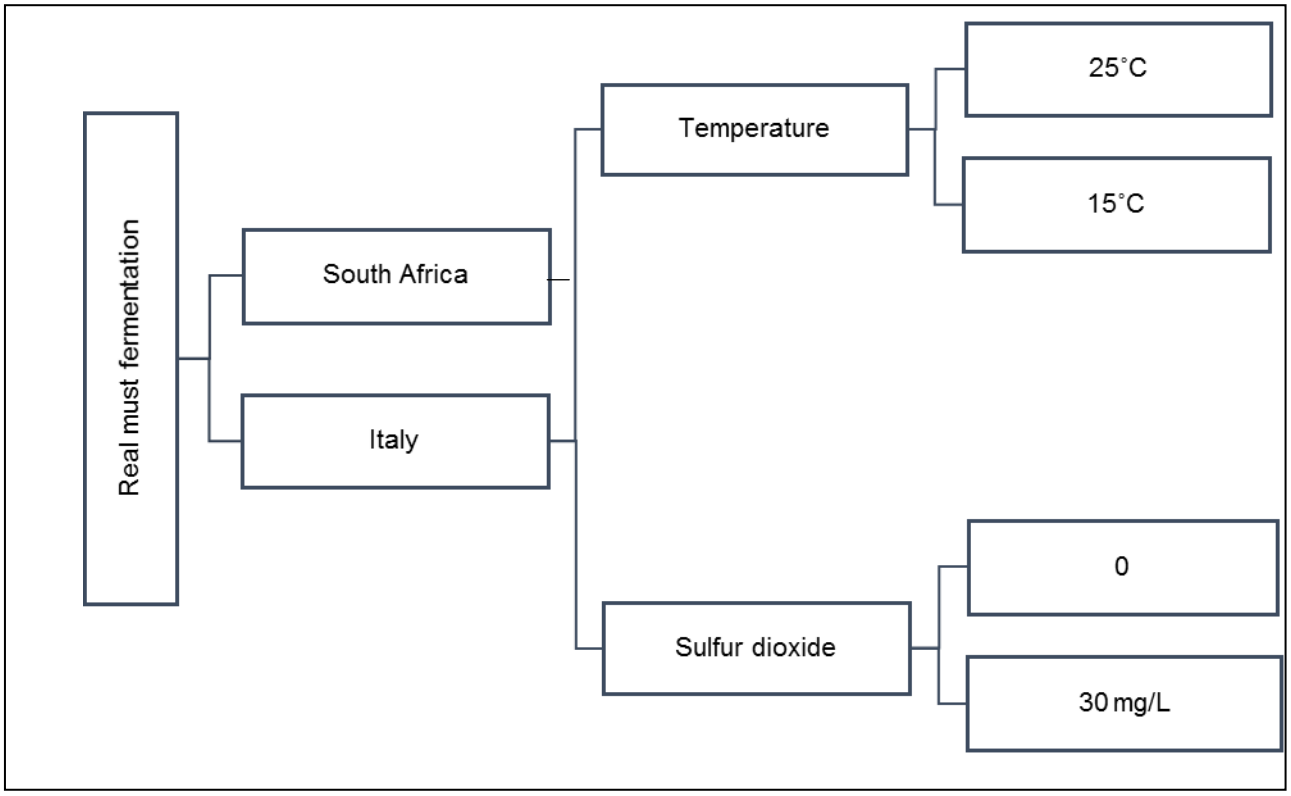


Figure.5.2. Schematic representation of the fermentations conducted in real must in South Africa and Italy. The figure represents the parameters selected for the experimental plan including temperature (15°C and 25°C) and sulphur dioxide (0 and 30 mg/L), respectively.

5.3.3. Analysis of yeast population dynamics

Samples were withdrawn regularly for both synthetic and real must fermentations. The population dynamics in synthetic must fermentations were monitored using ARISA analysis as previously described in Bagheri *et al*., 2017. In contrast, the population dynamics of real must fermentations in both harvests (South Africa and Italy), were monitored by direct plating on WL-agar. Four stages of fermentation including the inoculation (INC), beginning (BF), middle (MF) and end (EF), were selected for the analysis of all treatments (synthetic and real must fermentation). The beginning of fermentation was defined as 10-20% sugar consumption whereas, the middle and the end of fermentation were defined as 40-60% and over 95% sugar consumption, respectively.

5.3.4. Analytical methods

Wines obtained from the fermentation of synthetic must at three different temperatures (NS-SC-T15, NS-SC-T25, and NS-SC-T30) as well as the wines produced from the fermentation of Chenin

blanc must were selected for further analysis on aromatic compounds. The aromatic profiles of wine were analyzed by Liquid-liquid extraction method, using GC-FID as described by Louw *et al*., 2010.

5.3.5. Statistical analysis

All the fermentation and chemical analysis were reported as means ± SD of three repeats. The effect of temperature and sulphur dioxide on yeast population dynamics and wine aroma was evaluated by conducting an analysis of variance (ANOVA) using the statistical software, Statistica version 13.0 (StatSoft Inc., Tulsa, Oklahoma, USA). The treatments were considered significant should the *p*-values be equal to or less than 0.05. For multivariate data analysis, the principal component analysis was performed, using XLSTAT in Microsoft® Excel (2016).

5.4. Results

5.4.1. The impact of temperature on fermentation kinetics and population dynamics in synthetic must

White wine fermentations are typically conducted at a cooler temperature such as 15°C whereas red wine fermentations are performed at a higher temperature 25-30°C (Bauer and Pretorius, 2000). Thus, the temperatures used in the wine industry for white and red wine fermentations was the main criteria for the selection of the following temperatures (15, 25 and 30˚C) in the experimental plan. Therefore, the fermentations were conducted in the synthetic grape must at three temperatures in the presence and in absence of *S. cerevisiae*, using the yeast consortium as an inoculum.

All the fermentations in which *S. cerevisiae* was included in the consortium (NS-SC treatments) reached dryness whereas the fermentations without *S. cerevisiae* became sluggish (NS treatments) albeit at varying time points (data not shown). For instance, the fermentation at 15˚C (NS-T15) became sluggish after 39 days when 62% of sugar had been consumed whereas, 57.5% and 59% of the sugar was consumed after 40 and 42 days in NS-T25 and NS-T30 fermentations, respectively. However, none of the differences were statistically significant. In contrast, in the presence of *S. cerevisiae,* the fermentation at 30˚C (NS-SC-T30) was the fastest and reached dryness within 20 days, followed by fermentation at 25˚C (NS-SC-T25) which was completed within 22 days. The fermentation at 15˚C (NS-SC-T15) was the slowest and only reached dryness after 28 days (Figure 5.3).

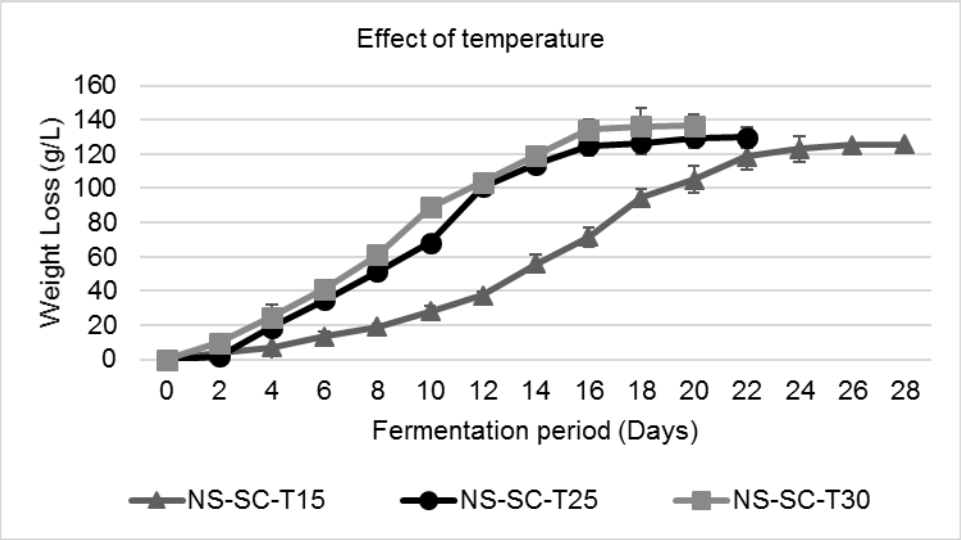


Figure.5.3. Progress curves representing fermentation kinetics in the presence of *S. cerevisiae* at 15˚C (NS-SC-T15), 25˚C (NS-SC-T25) and 30˚C (NS-SC-T30).

Although the fermentation conducted in absence of *S. cerevisiae* became sluggish, they revealed valuable information about the dynamics of non-*Saccharomyces* species. Overall none of the species increased in growth from the initial inoculum level. However, they displayed varying patterns of survival rate and decline depending on the fermentation temperature. For instance, at 15˚C, only *M. pulcherrima* and *C. parapsilosis* declined to levels below detection by mid-fermentation, while *S. bacillaris* displayed considerable dominance over the other species. At the end of NS-SC-T15 fermentation, *S. bacillaris* accounted for 39.64% of the population while *L. thermotolerans*, *W. anomalus*, *H. vineae* and *P. terricola* accounted for 25%, 20.44%, and 6.77%, respectively (Figure 5.4). In contrast, at 25˚C and 30˚C, *H. vineae* declined to levels below detection by the middle of fermentation whereas *W. anomalus* accounted for 65% and 73.77% of the total population at this stage and was the only detectable species at the end of both fermentations. *S. bacillaris* and *P. terricola*, survived until the middle of fermentation at 25˚C while *L. thermotolerans* accounted for 18% and 24.98% of the total population by the middle of 25˚C and 30˚C, respectively.

The inclusion of *S. cerevisiae* in the consortium influenced the population dynamics of non-*Saccharomyces* species at 25˚C and 30˚C considerably. For instance, *H. vineae* could persist until the middle of fermentation while *W. anomalus* declined rapidly and was undetectable in the middle of fermentation at 30˚C. Overall, the dominance of *S. cerevisiae* increased rapidly with an increase in the temperature. For instance, *S. cerevisiae* accounted for 68.4% and 93.8% of the population by middle and end of fermentation at 30 ˚C whereas it was only accounted for 6.19% and 29.41% of the total population by middle and end of fermentation at 15˚C.

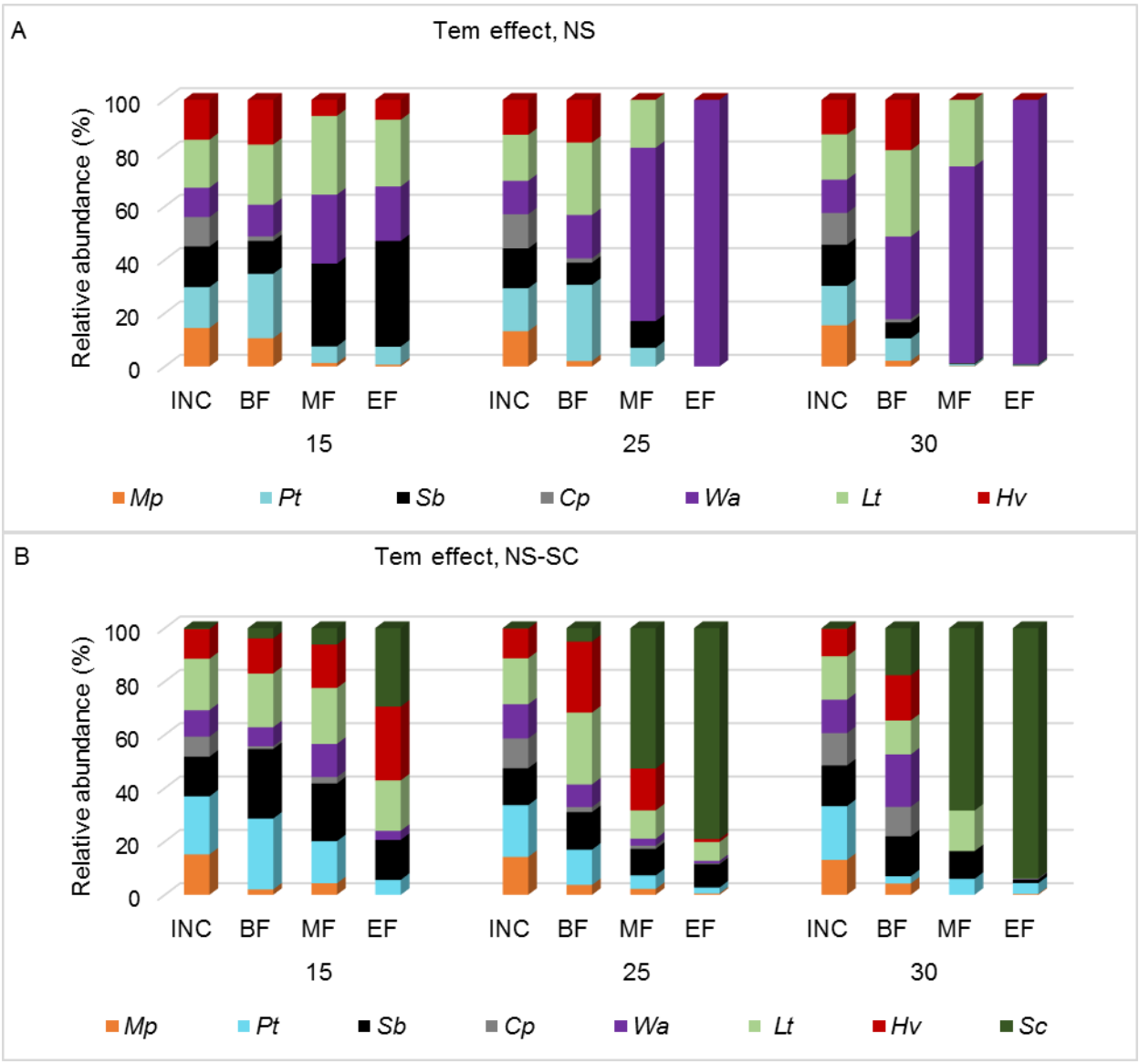


Figure.5.4. Dynamics of the yeast consortium in presence of *S. cerevisiae* at 15˚C, 25˚C, and 30˚C. Figure A and B, represents the relative abundance of yeast species throughout NS-SC and NS fermentations (at inoculation time (INC), beginning (BF), middle (MF), end (EF)). However, since NS fermentations got stock, the last sampling point was considered as the end point of fermentation (EF).

5.4.2. The impact of sulphur dioxide on fermentation kinetics and population dynamics in synthetic must

The yeast consortium was used as an inoculum to perform the fermentations in synthetic must, in the presence and in absence of *S. cerevisiae*, with (30 mg/L) and without sulphur dioxide. The concentration of sulphur dioxide was selected based on the concentrations which are commonly used in the wine industry (Vinlab, South Africa). Taking into consideration the pH of grape juice, 30-50 mg/L of sulphur dioxide (free and bound in total) is frequently added to juice in the wine industry. However, 25-40 mg/L free SO_2 (equivalent to 0.5-0.8 mg/L molecular SO_2), is sufficient to eliminate undesirable organisms in red and white wine fermentations (Vinlab, South Africa). Thus, the concentration of 30 mg/L SO_2 was added to the synthetic grape juice. However, the

concentration of free sulphur dioxide was 22 mg/L after 24 hours (702 SM Titrino, Metrohm, Switzerland), equivalent to 0.6 mg/L molecular SO_2.

In general, SO_2 addition seemed to increase the fermentation kinetics. For instance, the NS-SC fermentation reached dryness within 16 days while without SO_2, the fermentation took 21 days to complete (Figure 5.5).

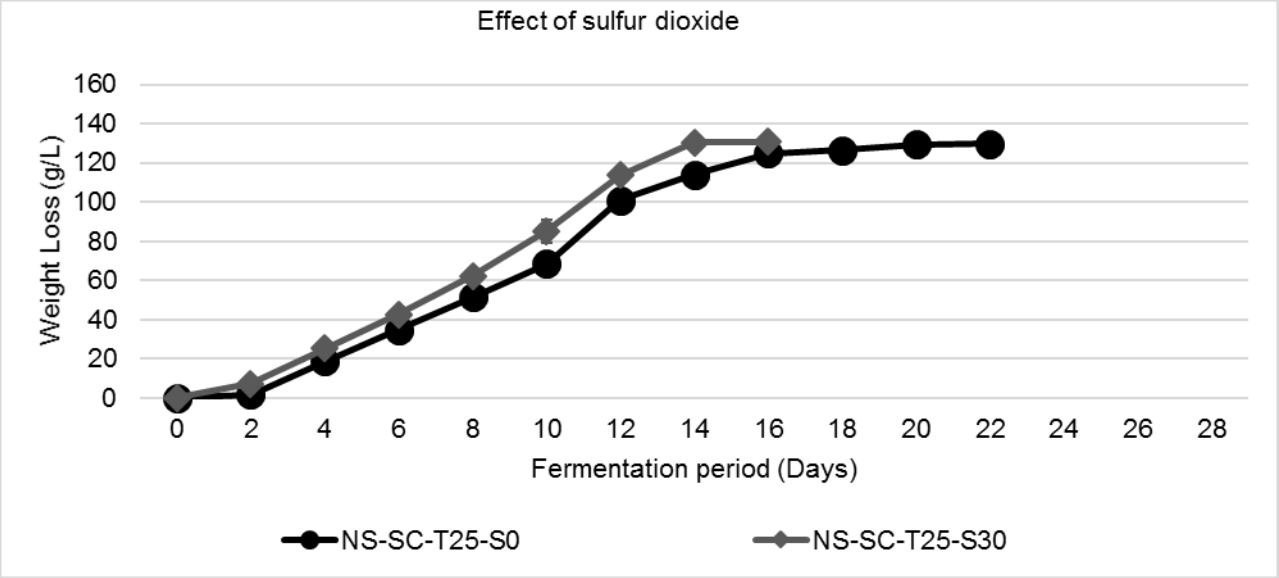
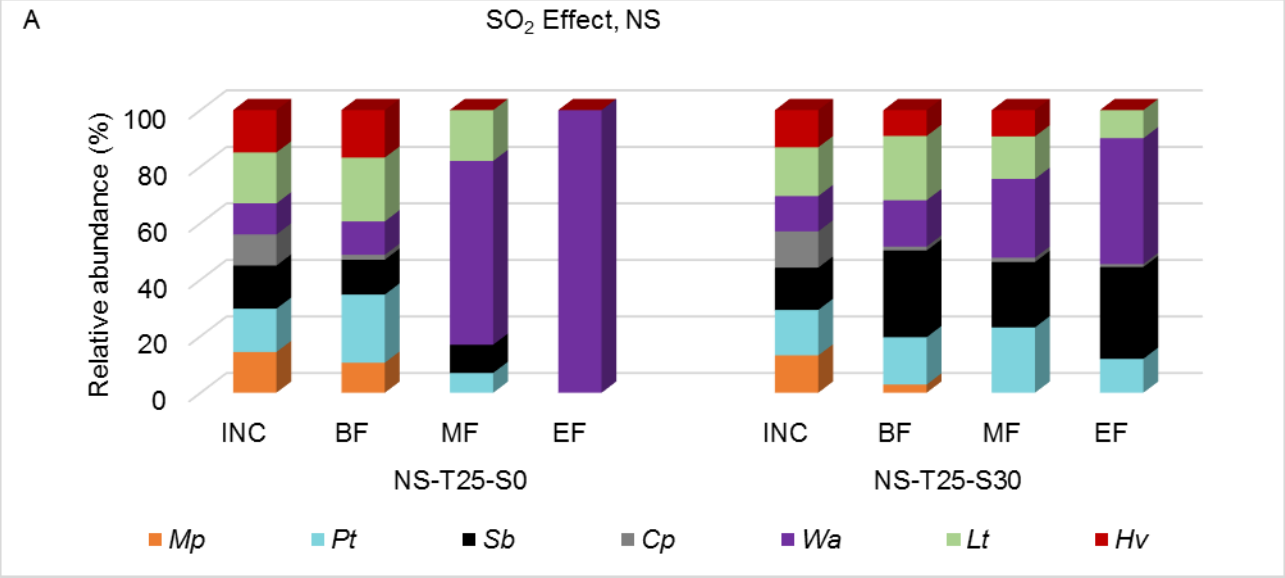


Figure.5.5. Progress curves representing the fermentation kinetics in presence of *S. cerevisiae* with and without sulphur dioxide (NS-SC-T25-S30 and NS-SC-T25-S0).

In the absence of *S. cerevisiae*, SO_2 addition enhanced the growth of *P. terricola*, *S. bacillaris* and *L. thermotolerans* beyond the middle of fermentation while *H. vineae* could only survive until the middle of fermentation (Figure 5.6A). Conversely, in the presence of *S. cerevisiae* at 30 mg/L SO_2, the persistence of *H. vineae* was diminished while *W. anomalus* survived until the end of fermentation where together with *L. thermotolerans* and *S. bacillaris*, they accounted for 10.56%, 9.25% and 8.81% of the total population (Figure 5.6B).



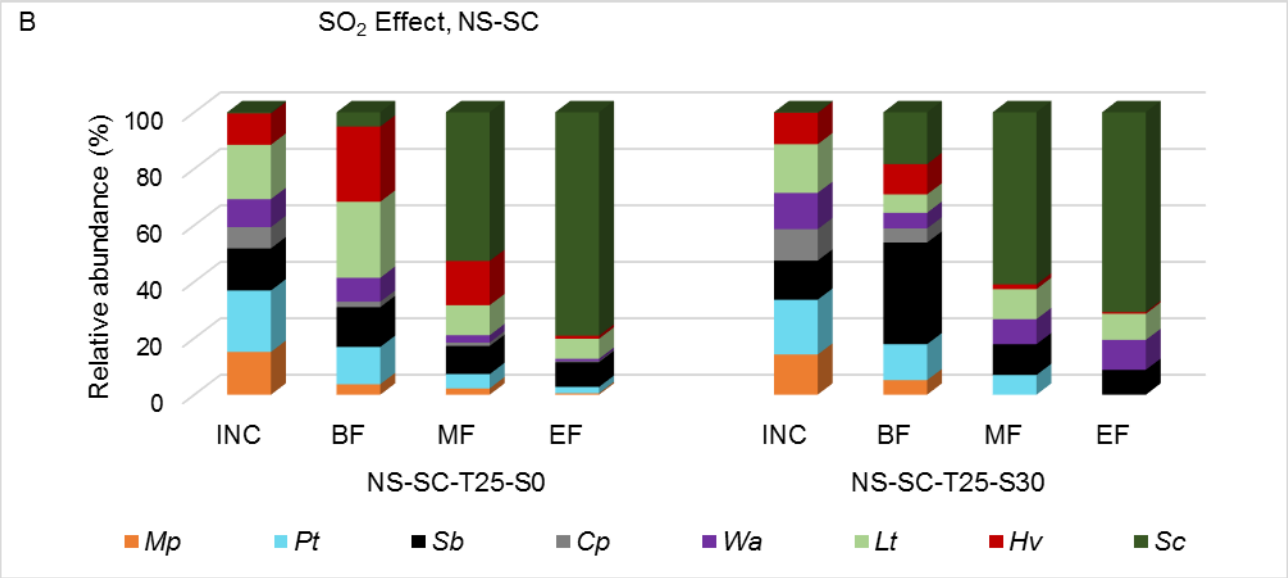


Figure.5.6. The dynamics of yeast consortium in the presence and in absence of *S. cerevisiae* (NS-SC and NS) with and without sulphur dioxide (S30 and S0). Figure A, represents the relative abundance of yeast species throughout the NS fermentation at inoculation time (INC), beginning (BF), middle (MF) and last sampling point (EF) with and without sulphur addition (NS-T25-S30 and NS-T25-S0). Figure B represents the population dynamics in NS-SC fermentation at inoculation time (INC), beginning (BF), middle (MF) and end of fermentation (EF) with and without sulphur addition (NS-SC-T25-S30 and NS-SC-T25-S0).

A one-way analysis of variance (ANOVA) confirmed that *S. bacillaris and L. thermotolerans* were significantly affected by temperature, *W. anomalus* was significantly affected by SO_2 while *H. vineae* and *S. cerevisiae* were affected by both temperature and SO_2 (Table 5.S1).

5.4.3. Grape juice chemical parameters and yeast diversity in real must fermentations

Chenin blanc was used to conduct the fermentation in South Africa and Greccheto Bianco was used in Italy. Must analysis showed that the Chenin blanc contained 21.7 ˚Brix, a total acidity of 3.23 g/L, a pH of 3.37 and a yeast assimilable nitrogen (YAN) of 195 mg/L, while the Greccheto Bianco must was at 25 ˚Brix, a total acidity of 4.41 g/L, pH 3.17 and a yeast assimilable nitrogen (YAN) of 191 mg/L. The indigenous microbiota in these musts was determined by plate counts. The data show that the Chenin blanc juice contained *S. cerevisiae* (4.85×10^3 CFU/mL), *H. uvarum* (4.20×10^3 CFU/mL), *W. anomalus* (3.34×10^3 CFU/mL), *L. thermotolerans* (2.60×10^3 CFU/mL), and *M. pulcherrima* (2.20×10^3 CFU/mL) (Figure 5.7A). In contrast, the Greccheto Bianco must consisted of *H. uvarum* (5.20×10^4 CFU/mL), *R. mucilaginosa* (4.8×10^4 CFU/ mL), *P. terricola* (4.30×10^4 CFU/mL), *M. pulcherrima* (3.70×10^4 CFU/mL) and *S. cerevisiae* (2.10×10^3 CFU/mL) (Figure 5.7B). Both grape matrices contained some of the species present in the yeast consortium. However, they were present at levels considerably below the inoculation density of individual yeast strains in the consortium.

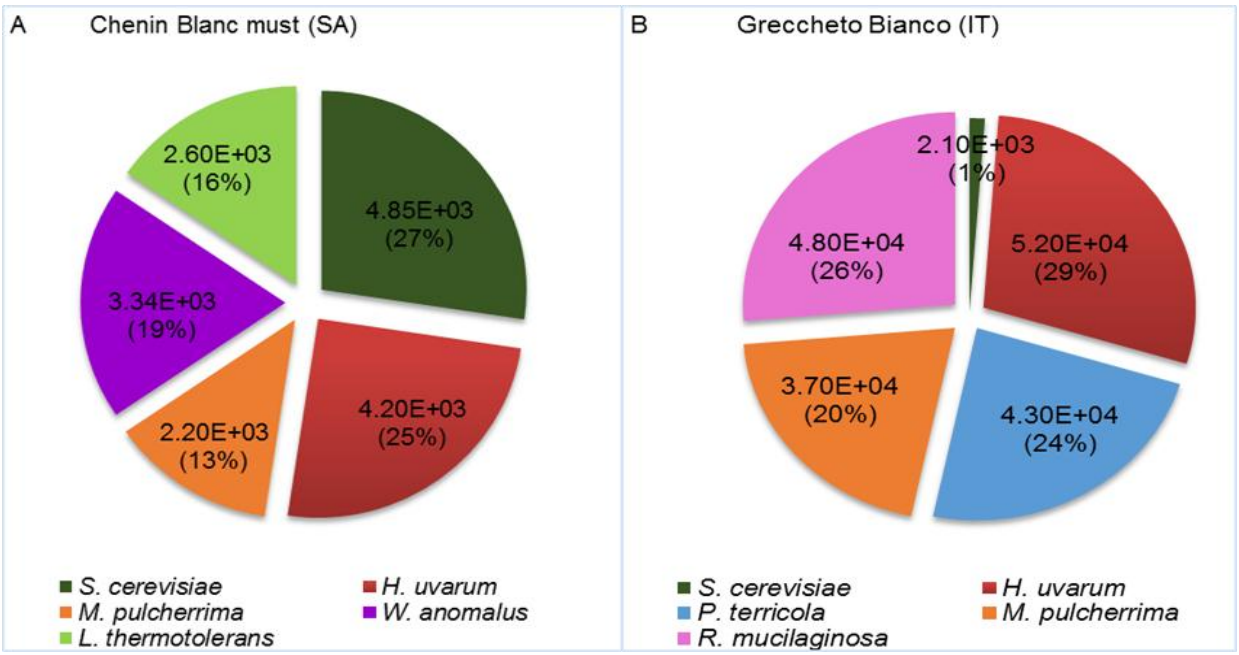


Figure.5.7. Representation of the yeast diversity in Chenin blanc and Greccheto Bianco musts. Yeast species are presented with CFU/mL and with their relative abundances in the grape musts.

5.4.4. Fermentation kinetics and yeast population dynamics in grape must fermentations

In general, the fermentations were performed at 15˚C and 25˚C, with and without sulphur dioxide (30 mg/L and 0). The yeast consortium was used as an inoculum to perform the fermentations in grape musts (NS-SC). Two additional sets of fermentations including (i) inoculation of grape must with the commercial strain of *S. cerevisiae* (EC) and (ii) spontaneous fermentations (SP) were conducted as control fermentations.

All the fermentations reached dryness albeit at varying time points. Overall, in all treatments, the fermentations performed at 25˚C, with 30 mg/L SO_2 displayed the fastest kinetics whereas the fermentations conducted at 15˚C without SO_2 exhibited the slowest kinetics. A comparison among the fermentations (NS-SC, EC, and SP) indicated that EC fermentations reached dryness faster than SP and NS-SC treatments (Figure 5.8). For instance, EC-T25-S30, SP-T25-S30 and NS-SC-T25-S30 took 18, 24 and 26 days to complete the fermentations whereas EC-T15-S0, SP-T15-S0 and NS-SC-T15-S0 reached dryness within 26, 30 and 34 days, respectively. Similar trends of fermentations kinetics were observed among the treatments in both South Africa and Italy. Hence, the fermentations conducted in South Africa was selected for the purpose of presentation in this section.

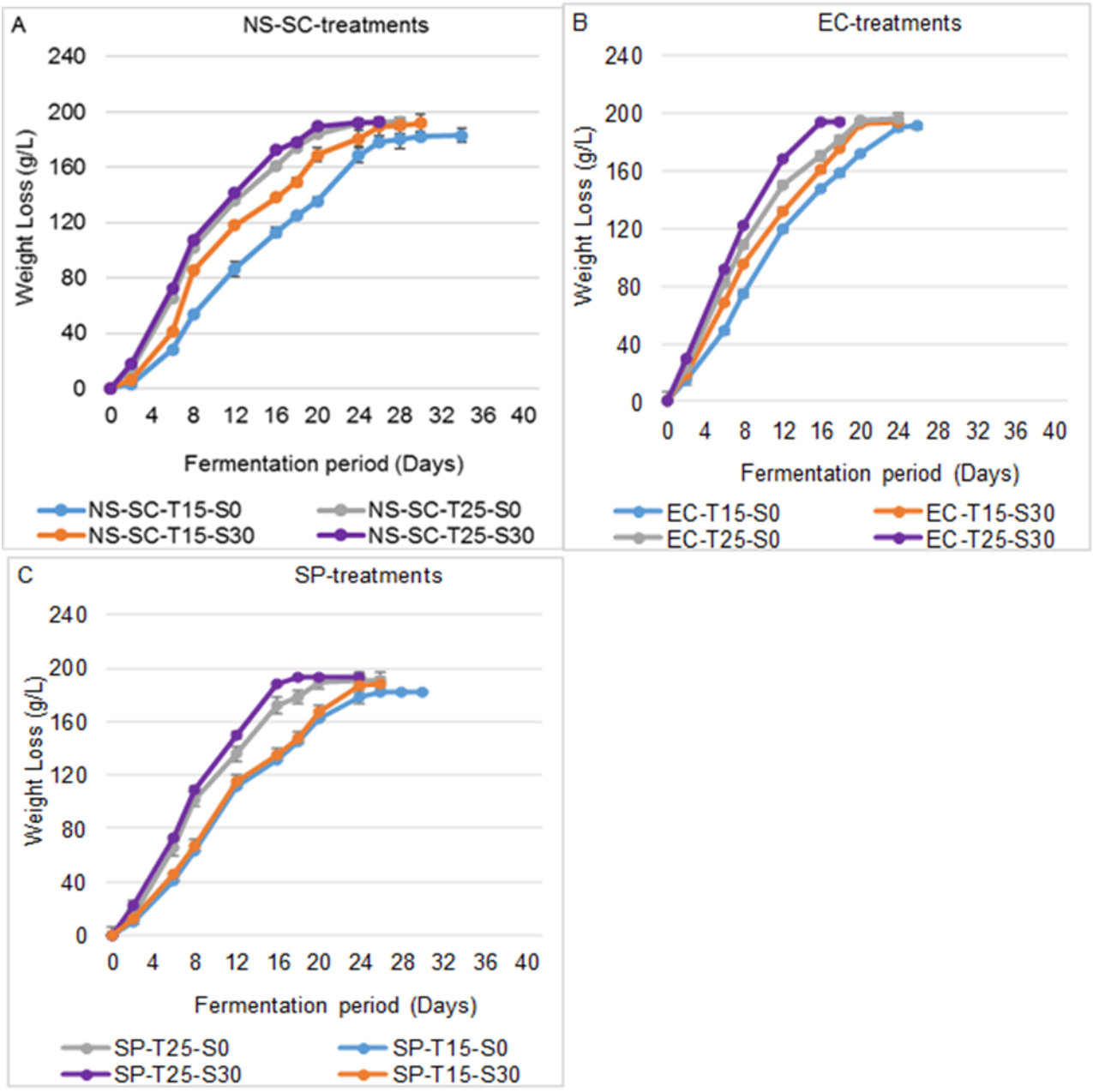


Figure.5.8. Progress curves displaying the kinetics of Chenin blanc fermentations inoculated with the consortium (NS-SC), inoculated with EC1118 (EC) and the spontaneous (SP) fermentation. Fermentations conducted at 15˚C with sulphur dioxide (SO_2: 30 mg/L) and without sulphur dioxide (SO_2: 0 mg/L) are represented in all the treatments with orange and blue whereas, fermentations performed at 25˚C with and without sulphur dioxide are indicated with purple and gray.

In general, the population dynamics within the NS-SC consortium in Chenin blanc and Greccheto Bianco to a large extent closely resembled the trends observed in the synthetic grape juice fermentations. For instance, the behaviour of the indigenous *S. cerevisiae*, *M. pulcherrima*, *L. thermotolerans* and *W. anomalus* in the Chenin blanc SP fermentation was similar to that of the same species in the NS-SC inoculated in synthetic juice and in Chenin blanc. Briefly, *M. pulcherrima* declined rapidly irrespective of fermentation temperature or sulphur addition whereas, in SP and EC fermentations, Indigenous *M. pulcherrima* strains persisted slightly longer in absence

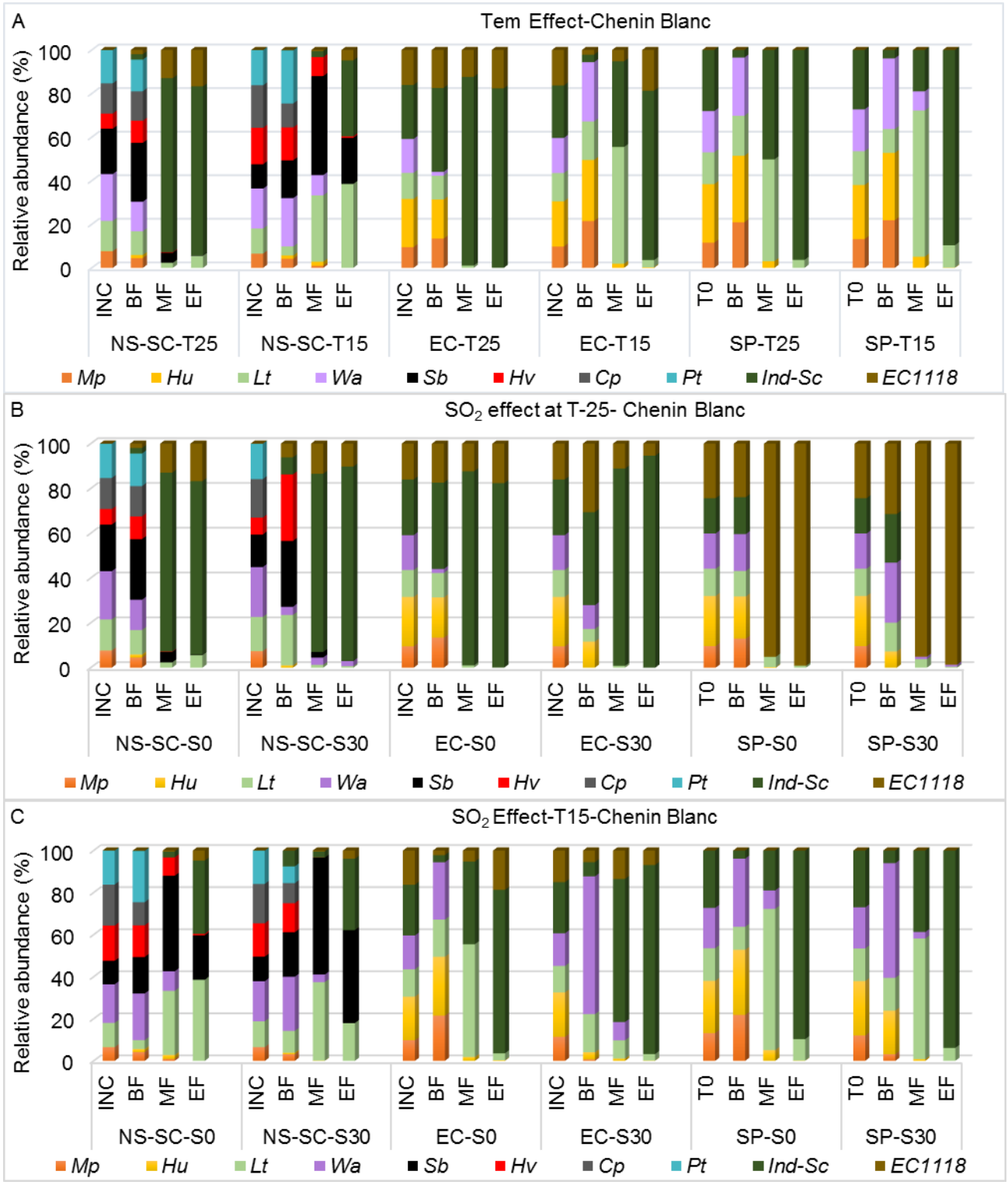
of sulphur dioxide. On the other hand, the growth of *W. anomalus* was enhanced at 15°C and in the presence of sulphur. However, the survival of *W. anomalus* was severely curtailed by high *S. cerevisiae* levels. In contrast, *L. thermotolerans* persisted until the end of fermentation in all fermentation scenarios. However, the growth of this species was enhanced at 15°C, particularly in NS-SC-T15-S0 and SP-15. EC-T15-S30 was the only treatment where the growth of indigenous *L. thermotolerans* was negatively affected by sulphur addition (Table 5.S2). In contrast, the growth of Ind-*Sc* and EC1118 was enhanced at the higher temperature and in the presence of sulphur dioxide. The remaining species in the NS-SC consortium viz. *S. bacillaris*, *P. terricola*, *C. parapsilosis* and *H. vineae* maintained similar growth patterns in Chenin blanc fermentation as in synthetic grape juice.

The growth of indigenous *H. uvarum* in Chenin blanc fermentation was enhanced in the absence of sulphur dioxide whereas temperature appeared to have a marginal effect on the growth of *H. uvarum*. In contrast, the persistence of indigenous *H. uvarum* and *R. mucilaginosa* in Greccheto Bianco fermentations was enhanced at 15°C and in the absence of sulphur dioxide in SP and EC treatments. The population of *C. parapsilosis* and *M. pulcherrima* in Greccheto Bianco fermentations declined in the presence of sulphur dioxide at both temperatures, although their population diminished faster in NS-SC-T25-S30 compared to NS-SC-T15-S30. On the other hand, the indigenous *M. pulcherrima* responded differently to temperature and sulphur addition in control treatments (SP and EC). For instance, the growth of indigenous *M. pulcherrima* was enhanced at 15°C in SP treatments whereas the marginal effect of sulphur dioxide on the growth of this species was observed in EC treatment. The growth of *P. terricola* was favoured at 15°C and in the absence of sulphur dioxide.

The behaviour of *S. cerevisiae* strains (Ind-*Sc* and EC1118) in the Greccheto Bianco fermentations (NS-SC, SP, and EC), was similar to that of the same species in the NS-SC fermentation in synthetic juice. For instance, the growth of both strains (Ind-*Sc* and EC1118) was enhanced in the presence of sulphur dioxide and at 25°C. However, SP fermentation was the only scenario where the growth of Ind-*Sc* was significantly affected by SO_2 (Table 5.S3). On the other hand, the growth of *W. anomalus* and *H. vineae* in NS-SC fermentation was favoured at the lower temperature. However, the presence of sulphur dioxide suppressed the growth of *H. vineae* while supporting the growth of *W. anomalus*.

L. thermotolerans and *S. bacillaris* persisted until the end of NS-SC fermentations in both synthetic must and Greccheto Bianco. However, the population of both species was enhanced at 15°C. Concerning the effect of sulphur dioxide, the growth of *S. bacillaris* and *L. thermotolerans* was significantly affected by sulphur dioxide in Greccheto Bianco fermentations whereas their population was not significantly affected by SO_2 in synthetic must (Table 5.S1& 5.S3). Briefly, the population of *S. bacillaris* was increased in BF in NS-SC-T25-S30, followed by a decline by mid-fermentation and maintaining the population by EF, in both synthetic and Greccheto Bianco

fermentations. On the other hand, *S. bacillaris* declined rapidly from the middle of fermentation in NS-SC-T25-S30 fermentation in Greccheto Bianco. Furthermore, decrease in the population of *L. thermotolerans* in presence of sulphur dioxide was evident in the middle and end of NS-SC-T25-S30 and NS-SC-T15-S30 in Greccheto Bianco fermentation whereas, in the synthetic must, *L. thermotolerans* maintained the population at similar levels by the middle and end of fermentation irrespective of sulphur dioxide levels.



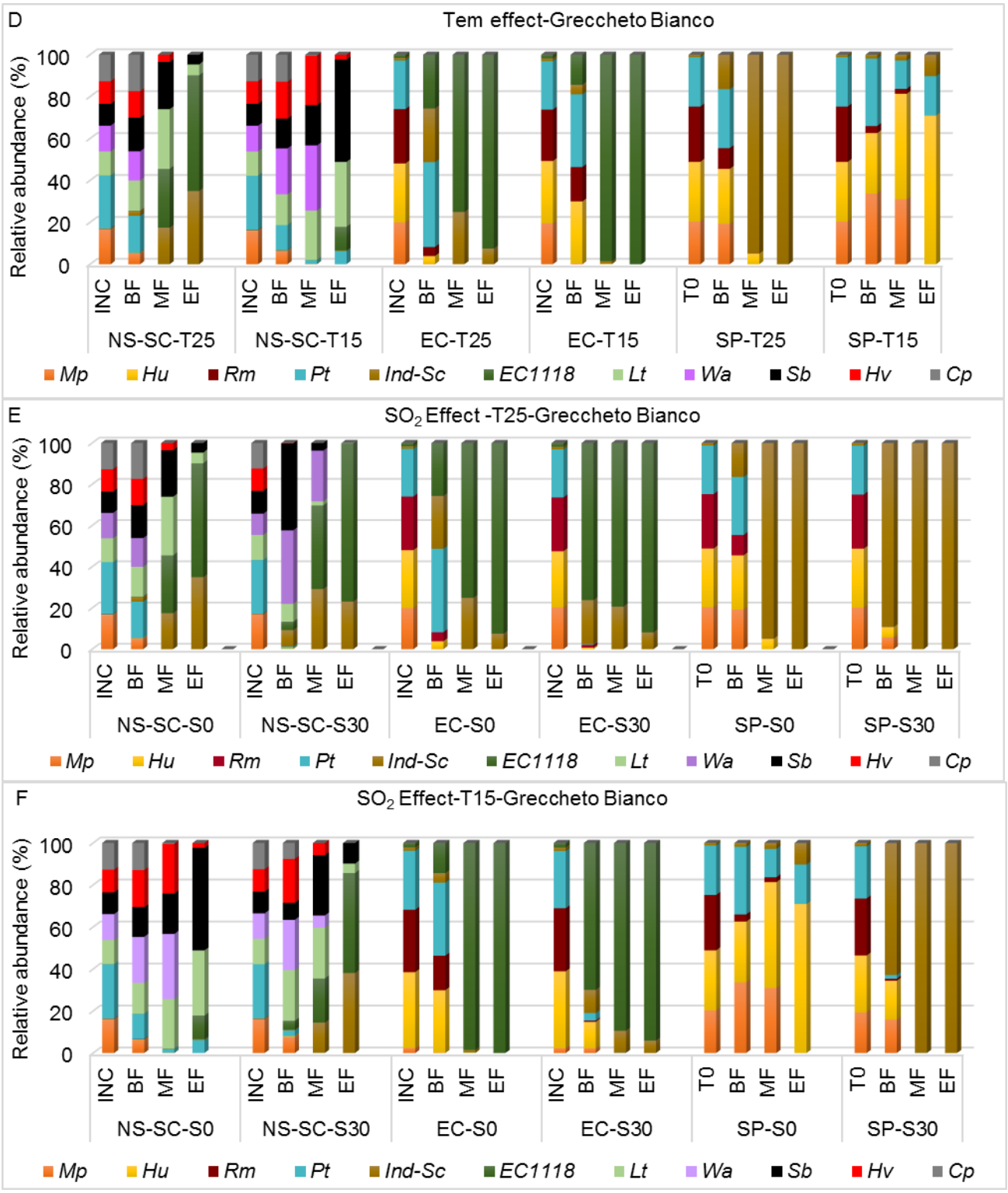


Figure.5.9. Effect of temperature and sulphur addition on the distribution of yeast species (%) at different stages of Chenin blanc and Greccheto Bianco fermentations. The Chenin blanc and Greccheto Bianco were fermented spontaneously (SP) or they were inoculated with the yeast consortium (NS-SC) and with the commercial *S. cerevisiae* strain, EC1118 (EC).

The result of a two-way ANOVA confirmed that in Chenin Banc fermentations, the growth of indigenous *L. thermotolerans* in EC, as well as the growth of *M. pulcherrima* in EC and SP, was significantly affected by SO_2 whereas, growth of none of the species except *H. uvarum* was

significantly affected by SO_2 in NS-SC fermentations. The growth of *P. terricola, H. vineae*, *S. bacillaris*, EC1118 and Ind-Sc was significantly affected by temperature in all treatments in Chenin blanc fermentations. In contrast, the growth of *L. thermotolerans* was only affected in NS-SC and SP whereas, the growth of *W. anomalus* was only affected in NS-SC (Table 5.S2).

On the other hand, in Greccheto Bianco fermentations, the growth of all indigenous species was significantly affected by SO_2 in SP fermentation. However, *P. terricola* was the only species which was affected by sulphur addition in SP and EC treatment. Furthermore, *L. thermotolerans, S. bacillaris*, and *H. vineae* were the only species affected by sulphur addition in NS-SC Greccheto Bianco fermentations. The growth of Ind-*Sc* and EC1118 in all fermentations (NS-SC, SP, and EC), *M. pulcherrima* and *H. uvarum* in SP and *H. vineae* in NS-SC was significantly affected by temperature (Table 5.S3).

5.4.5. Production of major volatiles in synthetic must

Synthetic must fermentations at 15, 25, and 30°C produced wines with different aroma profiles. Overall, wine generated from the fermentation at 30°C produced the highest level of ethyl esters and volatile acids. However, with the exception of ethyl lactate and acetic acid, no trend was observed in the production of the remaining ethyl esters and volatile acids between the treatments. For instance, the production of ethyl caprylate (0.55 mg/L) and ethyl caprate (0.61 mg/L) was significantly higher in NS-SC-T15. Furthermore, wine generated from the fermentation at 15°C produced the highest level of acetate esters with Ethyl acetate being the main contributing compound. In contrast, the fermentation at 25°C (NS-SC-T25) displayed the highest production of higher alcohols (188.96 mg/L), with isoamyl alcohol, propanol, 2-phenyl ethanol and isobutanol being the main contributors. Isobutanol was the only alcohol produced at a higher concentration in NS-SC-30 (20.92 mg/L) compared to NS-SC-T25 (15.96 mg/L) and NS-SC-T15 (13.77 mg/L). NS-SC-T30 followed by NS-SC-T25 and NS-SC-T15 produced 693.27 mg/L, 614.36 mg/L, and 411.52 mg/L acetic acid respectively. Propionic acid and decanoic acid were mainly produced in NS-SC-T15 whereas butyric acid and valeric acid was produced at a significantly higher level in NS-SC-T25 compare to NS-SC-T15 and NS-SC-T30.

Table.5.2. Concentrations of volatile compounds at the fermentations conducted at 15°C (NS-SC-T15), 25°C (NS-SC-T25) and 30°C (NS-SC-T30) in synthetic must. Values are represented in mg/L with standard deviations.

Compound	NS-SC-T15	NS-SC-T25	NS-SC-T30
Ethyl Esters			
Ethyl Caprylate	0.55± 0.07^{b}	0.22± 0.02^{a}	0.21± 0.01^{a}
Ethyl Caprate	0.61± 0.01^{c}	0.02± 0^{a}	0.39± 0.04^{b}
Ethyl Lactate	3.24±	5.60±	9.51±

	0.09[a]	0.06[ab]	0.07[b]
Diethyl Succinate	0.15± 0.01[a]	0.68± 0.13[b]	0.00[a]
∑ Esters	4.55± 1.98	6.52± 0.21	10.11± 0.12
Acetate esters			
Ethyl Acetate	98.80± 2.87[b]	82.71± 4.57[a]	84.39± 8.83[a]
Ethyl phenyl acetate	0.36± 0.01[a]	1.02± 0.1[b]	0.67+ 0.03[ab]
2-Phenylethyl acetate	1.04± 0.1[b]	0.84± 0.03[a]	0.72± 0.05[a]
Isoamyl acetate	0.25± 0.06[a]	0.69± 0.04[b]	0.25± 0.05[a]
∑ Acetates	100.45± 3.04	85.26± 4.74	86.03± 8.96
Alcohols			
Isoamyl alcohol	71.99± 4.38[a]	100.39± 4.81[b]	78.25± 5.12[a]
2-Phenyl ethanol	16.22± 2.16[a]	22.56± 3.13[b]	18.72± 1.24[ab]
Isobutanol	13.77± 6.54[a]	15.96± 0.35[a]	20.92± 0.8[b]
Butanol	0.27± 0[a]	0.88± 0.09[b]	0.76± 0.04[b]
Propanol	20.42± 2.89[a]	46.52± 6.03[b]	23.32± 2.10[a]
3-ethoxy-1-propanol	3.93± 0.32[b]	2.65± 0.27[a]	2.36± 0.28[a]
∑ Higher alcohols (no methanol)	126.6± 16.29	188.96± 14.68	144.33± 9.58
Volatile acids			
Acetic Acid	411.52± 3.88[a]	614.36± 8.95[ab]	693.27± 39.65[b]
Propionic Acid	1.78± 0.1[b]	0.96± 0.04[a]	1.20± 0.02[a]
Isobutyric acid	0.5± 0.03[a]	0.89± 0.02[b]	0.78± 0.04[ab]
Butyric acid	0.99± 0.07[b]	2.27± 0.06[c]	0.73± 0.01[a]
Iso-Valeric acid	1.03± 0.03[b]	0.84± 0.19[a]	1.29± 0.01[b]
Valeric acid	0.42± 0.55[a]	1.11± 0.03[b]	0.50± 0.18[a]
Hexanoic acid	1.36± 0.85[b]	1.20± 0.17[b]	0.79± 0.2[a]
Octanoic acid	1.97± 1.29[b]	1.70± 0.01[b]	1.29± 0.3[a]
Decanoic acid	1.65± 0.18[c]	0.96± 0.01[a]	1.45± 0.02[b]
∑ Volatile acids without acetic acid	9.7± 3.1	9.93± 0.53	8.03± 0.77
Acetoin	7.83± 0.57[b]	2.07± 1.07[a]	13.88± 2.09[c]

The principal component analysis was applied on all quantifiable major volatiles to determine the compounds that drive the differences between the wines. PC1 and PC2 explained 53.85% and

33.88% of the variance (Figure 5.11). The PCA plot indicated that the wine produced at 15°C (NS-SC-T15) was mainly associated with fatty acids (hexanoic acid and octanoic acid), esters (2-phenylethyl acetate, ethyl acetate, and ethyl caprate and ethyl caprylate) as well as 3-ethoxy-1-propanol. In contrast, wine produced at 30°C (NS-SC-T30) was characterized by higher alcohols (butanol and isobutanol), short chain fatty acids (isobutyric acid, isovaleric acid, and acetic acid), acetoin and ethyl lactate. NS-SC-T25, on the other hand, was mainly associated with esters (ethyl phenylacetate, isoamyl acetate, and diethyl succinate) and alcohols (isoamyl alcohol, 2-phenyl ethanol, and propanol) as well as butyric acid.

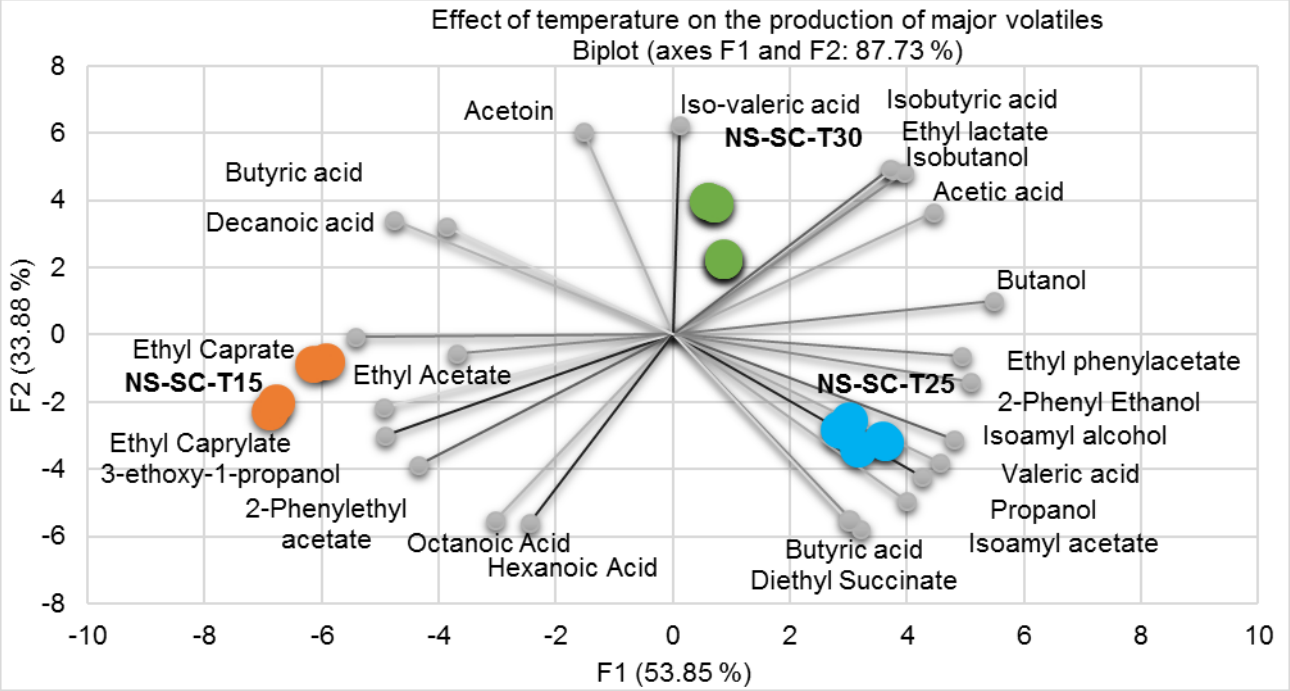


Figure.5.11. A biplot of the first and second principal components (PC) derived from PC analysis (PCA) of the major volatile compounds of wines obtained from the fermentation of synthetic must at 15°C, 25°C and 30°C. NS-SC-T15 is presented by orange ( ) while NS-SC-T25 and NS-SC-T30 are presented by blue ( ) and green ( ) respectively.

A one-way analysis of variance (ANOVA) was performed to test which major volatiles was significantly affected by the temperature in the wines produced from the synthetic must. The results indicated that with the exception of propanol, pentanol, ethyl butyrate and 3-ethoxy-1-propanol, production of all other major volatiles was significantly affected by temperature (Table 5S4).

5.4.6. Production of major volatiles in Chenin blanc fermentation

Overall, the production of some compounds followed a similar pattern in both wines (Chenin blanc wines and synthetic juice wines) whereas, inconsistent results were observed for other compounds. For instance, the production of total ethyl esters was significantly higher at 25°C fermentations compared to 15°C in Chenin blanc juice (NS-SC, SP, and EC) and synthetic juice. In contrast, ethyl acetate, as the most important acetate ester was produced at high levels in NS-SC-T25-S30 (93.03 mg/L) followed NS-SC-T15-S0 (76.62 mg/L) in Chenin blanc wines while the

opposite trend was observed in the synthetic wine. Furthermore, no significant differences were observed in ethyl acetate production within the control treatments (SP and EC).

Addition of SO_2 negatively affected the production of some esters. For instance, the production of ethyl caprylate and ethyl caprate was significantly higher in the absence of sulphur dioxide in NS-SC wines. Effect of SO_2 addition on ethyl esters production in EC-T25 wines was similar to the NS-SC wine, with the exception of ethyl butyrate. However, no significant differences were observed in the production of ethyl esters in SP wines (T15 and T25) as well as EC-T15 wines. Addition of SO_2 negatively affected the production of ethyl acetate, 2-phenylethyl acetate and isoamyl acetate in the NS-SC-T15 fermentations. For instance, a larger amount of ethyl acetate and 2-phenylethyl acetate was produced in NS-SC-T25-S30 (93.03 mg/L and 1.86 mg/L) compared to NS-SC-T25-S0 (66.35 mg/L and 1 mg/L) whereas the opposite trend was observed in NS-SC-T15 fermentations. Hexyl acetate was the only acetate ester produced at a higher concentration in EC-T15-S0 (0.27 mg/L) compared to EC-T15-S30 (0.24 mg/L) whereas, no significant differences were observed in the production of the remaining acetate esters in EC and SP wines.

Similar to synthetic wine, larger concentrations of some higher alcohols were produced in wines generated at 25°C compared to 15°C. For instance, isoamyl alcohols, 2-phenyl ethanol, and total higher alcohols were produced at 187.64 mg/L, 41.84 mg/L, and 274.678 mg/L in SP-T25-S0 while they were produced at 146.61 mg/L, 23.88 mg/L and 24.48 mg/L in SP-T15-S0. The only exception was NS-SC-T15-S0 (195.44 mg/L) which produced a larger amount of higher alcohols than NS-SC-T25-S0 (179.07 mg/L). Overall, SO_2 addition enhanced the production of total higher alcohols in NS-SC-T25-S30 (263.73 mg/L), EC-T25-S30 (287.74 mg/L) and SP-T15-S30 (213.65 mg/L) fermentations, due to the high production of isoamyl alcohol, isobutanol, and 2-phenyl ethanol in these treatments. However, the opposite trend was observed in SP-T25 fermentations.

EC-T25-S0 wine followed by NS-SC-T25-S0 stood out for the high production of acetic acid whereas, there was no significant differences in acetic acid production among other treatments. Furthermore, NS-SC-T25-S30 and SP-T15-S0 produced the highest (1.55 mg/L) and the lowest (0.6 mg/L) levels of propionic acid, respectively. However, no significant differences were observed in the production of propionic acid among EC treatments. In contrast, butyric acid and octanoic acid were produced at higher concentrations in all the wines fermented at the lower temperature (15°C) compared to the wines fermented at the higher temperature (25°C). For instance, hexanoic acid was produced at 5.73 mg/L in both NS-SC-T15-S0 and NS-SC-T15-S30 although it was produced at 4.9 mg/L and 4.23 mg/L in NS-SC-T25-S0 and NS-SC-T25-S30, respectively. No acetoin was produced in the wines fermented spontaneously. However, in EC and NS-SC, acetoin was only produced in the wines fermented at 25°C. NS-SC-T25-S30 (24.61 mg/L) and EC-T25-S0 (14.69 mg/L) produced the highest levels of acetoin between the treatments.

Table.5.3. The concentration of major volatile compounds obtained in wines produced from different treatments. Values are represented in mg/L ± standard deviations.

Compound	Spontaneous fermentation				Inoculated with EC1118				Inoculated with consortium			
	SP-T25-S0	**SP-T25-S30**	**SP-T15-S0**	**SP-T15-S30**	**EC-T25-S0**	**EC-T25-S30**	**EC-T15-S0**	**EC-T15-S30**	**NS-SC-T25-S0**	**NS-SC-T25-S30**	**NS-SC-T15-S0**	**NS-SC-T15-S30**
Ethyl Esters												
Ethyl Caprylate (octanoate)	0.96± 0.09^{ab}	0.88± 0.06^{a}	1.01± 0.18^{abc}	1.02± 0.07^{abc}	1.16± 0.02^{bcd}	0.95± 0.04^{ab}	1.26± 0.15^{de}	1.19± 0.01^{cde}	2.03± 0.03^{g}	1.41± 0.01^{ef}	1.55± 0.04^{f}	1.30± 0.05^{de}
Ethyl Caprate (decanoate)	0.67± 0.14^{ab}	0.61± 0.02^{a}	0.60± 0.06^{a}	0.58± 0.12^{a}	0.94± 0.06^{cde}	0.58± 0.13^{a}	0.77± 0.25^{abc}	0.59± 0.07^{a}	1.30± 0.09^{f}	1.08± 0.08^{def}	1.13± 0.1^{ef}	0.86± 0.06^{bcd}
Ethyl Lactate	8.55± 0.05^{b}	8.50± 0.05^{b}	0^{a}	0^{a}	28.19± 5.06^{c}	8.46± 0.09^{b}	0^{a}	0^{a}	25.91± 1.42^{c}	9.22± 0.5^{b}	0^{a}	0^{a}
Diethyl Succinate	0.49± 0.02^{ab}	0.46± 0.02^{ab}	0.38± 0.01^{ab}	0.41± 0.04^{ab}	0.52± 0.05^{bc}	0.50± 0.06^{abc}	0.54± 0.13^{bc}	0.27± 0.02^{a}	0.49± 0.01^{ab}	0.49± 0.01^{ab}	0.73± 0.25^{c}	0.53± 0.13^{bc}
Ethyl Butyrate	0.27± 0.02^{a}	0.28± 0.02^{a}	0.35± 0.04^{bc}	0.37± 0.01^{bcd}	0.27± 0.01^{a}	0.31± 0.02^{a}	0.38± 0.02^{cd}	0.44± 0.04^{fg}	0.37± 0.01^{bcd}	0.31± 0.011^{a}	0.46± 0.03^{g}	0.41± 0.01^{dfg}
Ethyl Hexanoate	0.82± 0.03^{a}	0.79± 0.03^{a}	0.99± 0.17^{bc}	1.00± 0.01^{bc}	0.88± 0.03^{ab}	0.91± 0.03^{ab}	1.15± 0.01^{cd}	1.30± 0.04^{d}	1.03±0^{b}	0.93± 0.04^{ab}	1.23± 0.06^{d}	1.16± 0.09^{d}
∑ Esters	11.76± 0.33	11.52± 0.18	3.33± 0.46	3.38± 0.25	31.96± 5.23	11.71± 0.37	4.1± 0.56	3.79± 0.18	31.13± 1.56	13.44± 1.44	5.1± 0.48	4.26± 0.34
Acetates												
Ethyl Acetate	39.83± 1.08^{ab}	37.60 ±1.84^{a}	36.38± 1.79^{a}	36.13± 0.78^{a}	34.34± 3.93^{a}	40.33± 1.36^{ab}	35.57± 0.52^{a}	38.28± 3.11^{ab}	66.35± 3.75^{b}	93.03± 15.40^{c}	76.62± 6.08^{c}	47.12± 8.72^{ab}
Ethylphenyl acetate	0.13± 0.06^{ab}	0.11± 0.07^{ab}	0.15± 0.01^{ab}	0.09± 0.03^{ab}	0.05± 0^{ab}	0.14± 0.16^{ab}	0.08± 0.03^{ab}	0.29± 0.22^{b}	0^{a}	0.12± 0.05^{ab}	0^{a}	0.02± 0.03^{a}
2-Phenylethyl Acetate	0.83± 0.04^{a}	0.77± 0.01^{a}	0.66± 0.11^{a}	0.70± 0.01^{a}	0.79± 0.07^{a}	0.98± 0.1^{ab}	0.73± 0.01^{a}	0.74± 0.04^{a}	1.00± 0.06^{ab}	1.86± 0.86^{c}	1.68± 0.67^{bc}	1.19± 0.48^{abc}
Isoamyl Acetate	2.20± 0.17^{abc}	2.00± 0.05^{a}	2.48± 0.54^{abcd}	2.70± 0.02^{bcd}	2.11± 0.13^{ab}	2.78± 0.24^{bcd}	2.88± 0.12^{cd}	3.14± 0.32^{de}	2.06± 0.06^{a}	2.28± 0.08^{abc}	3.67± 0.68^{e}	3.04± 0.3^{d}
Hexyl acetate	0.11± 0.01^{a}	0.11± 0.01^{a}	0.21± 0.01^{b}	0.24± 0.01^{bc}	0.13± 0^{a}	0.13± 0.01^{a}	0.27± 0^{d}	0.24± 0.04^{bc}	0.09± 0.01^{a}	0.07± 0.03^{a}	0.24± 0.05^{bc}	0.27± 0.01^{d}
∑ Acetates	43.1± 1.36	40.58± 1.98	39.88± 2.46	39.86± 0.85	37.42± 4.13	44.36± 1.87	39.53± 0.68	42.69± 3.73	69.5± 3.88	97.36± 16.42	82.21± 7.48	51.64± 9.54
Alcohols												
Isoamyl alcohol	187.64± 4.1^{d}	170.94± 3^{cd}	146.61± 4.72^{abc}	154.49±3. 14^{bcd}	161.50± 5.5^{bcd}	198.64± 5^{d}	161.05± 2.19bcd	181.41± 6.35^{cd}	113.41± 3.70^{a}	177.59± 13.98^{cd}	138.76± 4.45^{abc}	129.89± 4.11^{ab}

2-Phenyl ethanol	41.84± 2.41^{gh}	37.59± 0.84^{fgh}	23.88± 7.71^{cd}	26.18± 0.67^{cd}	35.71± 6.23^{efgh}	46.05± 8.09^{h}	27.02± 2.44^{cdef}	31.13± 4.37^{efd}	16.135± 0.54^{ab}	33.4± 3.08^{efg}	14.28± 1.30^{a}	19.52± 5.62^{abc}
Isobutanol	29.64± 1.56^{bd}	25.78± 1.08^{bcd}	17.94± 4.02^{a}	17.52± 2.50^{a}	22.92± 2.29^{abc}	29.02± 3.44^{bd}	18.45± 2.18^{ac}	19.19± 3.47^{ac}	28.07± 5.26^{bd}	32.96± 2.31^{d}	23.66± 4.86^{abcd}	17±2.91^{a}
Butanol	0.77± 0.05^{ab}	0.75± 0.04^{ab}	0.64± 0.05^{a}	0.64± 0.0^{a}	0.73± 0.0^{ab}	0.75± 0.07^{ab}	0.61± 0.0^{a}	0.72± 0.09^{ab}	0.94± 0.0^{c}	0.92± 0.1^{c}	0.86± 0.04^{c}	0.67± 0.0^{a}
Propanol	10.67± 0.48^{a}	10.02± 0.3^{a}	11.35± 1.30^{ab}	10.56± 0.64^{a}	9.84± 0.33^{a}	9.14± 0.39^{a}	9.87± 0.27^{a}	9.49± 0.37^{a}	16.04± 0.91^{c}	14.56± 1.55^{b}	13.57± 0.48^{b}	10.37± 0.54^{a}
Hexanol	1.23± 0.03^{ab}	1.21± 0.04^{a}	1.22± 0.06^{ab}	1.25± 0.03^{ab}	1.29±0^{ab}	1.22± 0.04^{ab}	1.31± 0.01^{b}	1.32± 0.04^{b}	1.28± 0.02^{ab}	1.32± 0.03^{ab}	1.23± 0.04^{ab}	1.27± 0.05^{ab}
3-ethoxy-1-propanol	2.88± 0.1^{ab}	2.89± 0.08^{ab}	2.84± 0.14^{a}	3.01± 0.07^{bcd}	2.98± 0.06^{abcd}	2.92± 0.02^{abc}	2.93± 0.03^{abc}	3.16± 0.05^{de}	3.2± 0.06^{e}	2.98± 0.04^{abcd}	3.05± 0.04^{bcde}	3.07± 0.02^{cde}
∑ Higher alcohols (no methanol)	274.67± 8.73	249.18± 5.38	204.48± 18.00	213.65± 17.91	234.97± 14.41	287.74± 17.05	221.24± 7.12	246.42± 14.74	179.07± 10.49	263.73± 21.09	195.41± 11.21	181.79± 13.25
Volatile acids												
Acetic Acid	300.67± 4.94^{a}	296.98± 21.47^{a}	285.86± 4.55^{a}	302.46± 16.38^{a}	433.41± 3.09^{b}	261.09± 15.38^{a}	265.31± 8.73^{a}	257.12± 10.12^{a}	428.80± 2.36^{b}	272.51± 17.11^{a}	266.45± 9.89^{a}	292.06± 12.42^{a}
Propionic Acid	1.22± 0.02^{bc}	1.18± 0.03^{abc}	0.6± 0.02^{a}	1.15± 0.03^{abc}	1.2± 0.01^{abc}	1.14± 0.01^{abc}	1.13± 0.03^{abc}	1.16± 0.02^{abc}	1.19± 0.01^{abc}	1.55± 0.41^{c}	1.16± 0.04^{abc}	0.92± 0.02^{ab}
Isobutyric acid	2.09± 0.1^{b}	1.89± 0.04^{b}	1.12± 0.22^{a}	1.19± 0.05^{a}	1.87± 0.28^{b}	2.21± 0.42^{b}	1.20± 0.14^{a}	1.21± 0.16^{a}	1.05± 0.02^{a}	2.23± 0.12^{b}	0.83± 0.1^{a}	1.1± 0.12^{a}
Butyric acid	1.2± 0^{ab}	1.17± 0.02^{a}	1.39± 0.14^{cde}	1.39± 0.06^{cde}	1.17± 0.03^{ab}	1.21± 0.05^{ab}	1.41± 0.04^{bcd}	1.48± 0.09^{ef}	1.31± 0.04^{bcd}	1.26± 0^{abc}	1.62± 0.05^{f}	1.48± 0.01^{e}
Iso-valeric acid	1.63± 0.07^{ef}	1.54± 0.01^{def}	1.2± 0.23^{bc}	1.27± 0.02^{bcd}	1.5± 0.19^{cdef}	1.81± 0.27^{f}	1.34± 0.15^{bcde}	1.47± 0.16^{cde}	0.89± 0.01^{a}	1.4± 0.05^{cde}	0.9± 0.05^{a}	1.1± 0.14^{ab}
Valeric acid	0.31± 0.01^{d}	0.29± 0.01^{d}	0.12± 0.05^{c}	0.2± 0.02^{cd}	0.31± 0.04^{d}	0.32± 0.02^{d}	0.21± 0.01^{bcd}	0.24± 0.04^{cd}	0.03± 0^{a}	0.31± 0.01^{d}	0.07± 0^{a}	0.21± 0.04^{cd}
Hexanoic acid	4.14± 0.25^{a}	4.1± 0.21^{a}	4.75± 0.52^{bc}	4.8± 0.09^{bc}	4.38± 0.02^{abc}	4.49± 0.09^{abc}	5.37± 0.08^{de}	5.42± 0.15^{de}	4.9± 0.14^{cd}	4.23± 0.14^{ab}	5.73± 0.06^{e}	5.73± 0.13^{e}
Octanoic acid	6.57± 0.31^{ab}	6.29± 0.28^{a}	7.42± 0.73^{c}	7.74± 0.07^{c}	7.32± 0.15^{bc}	7.4± 0.22^{bc}	8.54± 0.18^{ef}	8.93± 0.49^{e}	7.06± 0.18^{abc}	6.66± 0.34^{ab}	8.33± 0.17^{de}	8.97± 0.57^{e}
Decanoic acid	2.59± 0.09^{bcd}	2.45± 0.04^{ab}	2.58± 0.28^{bcd}	2.69± 0.12^{bcd}	2.74± 0.06^{bcde}	2.95± 0.12^{de}	2.90± 0.14^{cde}	3.11± 0.05^{e}	2.2± 0.03^{a}	2.47± 0.2^{ab}	2.52± 0.12^{bc}	2.8± 0.26^{cde}
∑ Volatile acids without acetic acid	19.75± 0.85	18.91± 0.64	19.18± 2.19	20.43± 0.46	20.49± 0.78	21.53± 1.2	22.1± 0.77	23.02± 1.16	18.63± 0.43	20.11± 1.27	21.16± 0.59	22.31± 1.25
Acetoin	0^{a}	0^{a}	0^{a}	0^{a}	14.69± 5.87^{ab}	2.08± 0.03^{a}	0^{a}	0^{a}	11.76± 1.95^{ab}	24.61± 8.16^{b}	0^{a}	0^{a}

The principal component analysis (PCA) was applied on the quantifiable major volatiles of all treatments to determine the main compounds, driving the differences among the wines. PC1 and PC2 explained 33.26 and 25.92% of the variance respectively (Figure 5.12). PC1 separated the NS-SC-T25-S0, NS-SC-T15-S0, NS-SC-T25-S30, and EC-T25-S0 from the rest of the treatments. In contrast, PC2 separated all the wines produced at 25°C (e.g. EC-T25-S0, SP-T25-S0 and NS-SC-T25-S0) from wines produced at 15°C (SP-T15-S0, EC-T15-S0, and NS-SC-T15-S30). NS-SC-T25-S0 and NS-SC-T15-S0 made a cluster on the upper right corner of PC2 and were mainly associated with esters (e.g. ethyl acetate, ethyl caprate and ethyl caprylate) and higher alcohols (propanol, hexanol, and 3-ethoxy-1-propanol). In contrast, the remaining treatments in 15°C including NS-SC-T15-S0, EC-T15-S0, EC-T15-S0, EC-T15-S30, SP-T15-S0 and SP-T15-S30 formed one grouping and were characterized by the higher accumulation of medium chain fatty acids (butyric acid, octanoic, decanoic and hexanoic acids) and isoamyl acetate. All the wines produced at 25°C including SP and EC, clustered together in the negative dimension of PC1. Thus, EC-T15-S0, EC-T15-S30, SP-T15-S0 and SP-T25-S30 were associated with the production of short chain fatty acids (valeric acid, isovaleric acid, and isobutyric acid) as well as 2-phenyl ethanol, isoamyl alcohol, and ethyl phenylacetate. On the other hand, NS-SC-T25-S30 was located in the upper left corner of PC2 and was characterized by high accumulation of alcohols (butanol and isobutanol), short chain fatty acids (propionic acid and acetic acid) as well as ethyl lactate and acetoin.

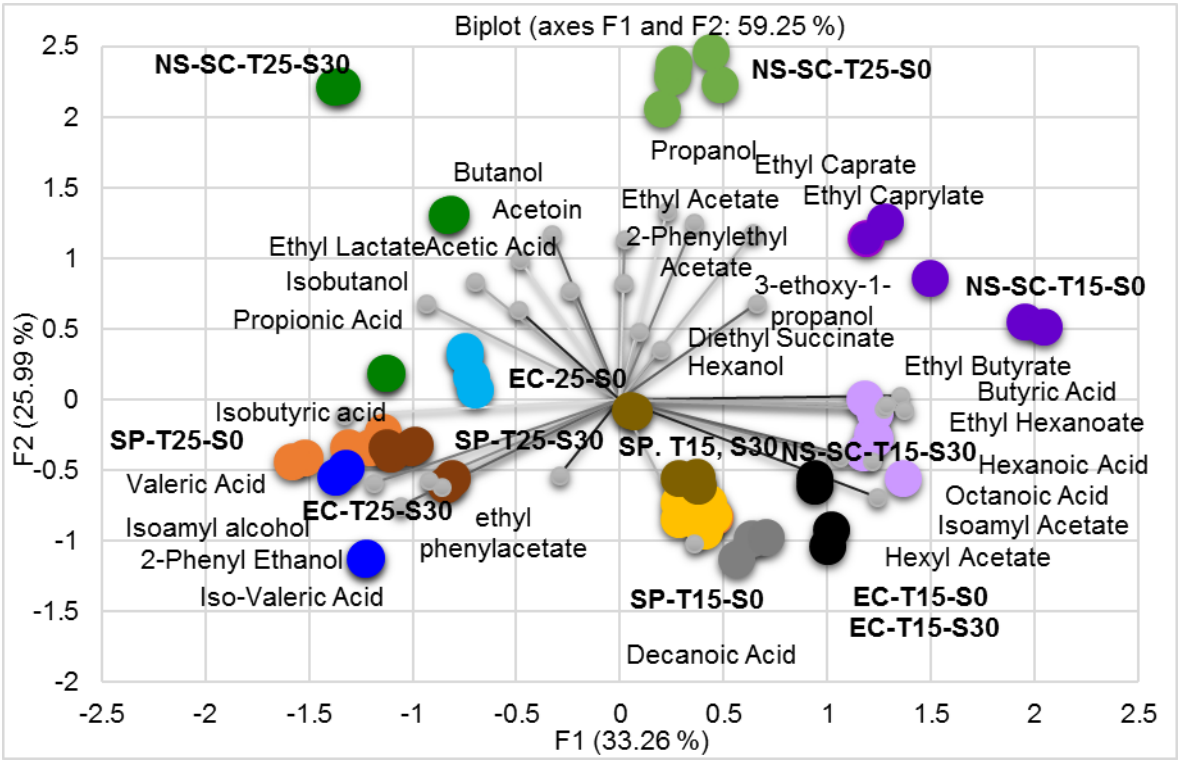


Figure.5.12. A biplot of the first and second principal components (PC) derived from PC analysis of the major volatile compounds produced in Chenin blanc fermentations in South African. The fermentations were conducted spontaneously (SP), inoculated with EC1118 (EC) and inoculated with the consortium (NS-SC) at two temperatures (T15 and T25), with and without sulphur addition (S0 and S30).The major volatile compounds are represented by light greys whereas the treatment are as follows: SP-T25-S0 ( ), S0-T25-S30 ( ), SP-T15-S0 ( ), SP-T15-S30 ( ), NS-SC-T25-S0 ( ), NS-SC-T25-S30 ( ), NS-SC-T15-S0- ( ), NS-SC-T15-S30 ( ), EC-T25-S0 ( ) and EC-T25-S30 ( ), EC-T15-S0 ( ) and EC-T15-S30 ( ).

Principal component analysis (PCA) was applied on all the major volatiles obtained from the wines fermented at 25°C and 15°C to determine the effect of temperature on the production of major volatiles (Figure 5.13). PC1 explained 37.21% of the variation and separated the fermentations which were conducted at 25°C (NS-SC-T25-S0, EC-T25-S0, and SP-T25-S0) in the positive dimension of PC, from the fermentation performed at 15°C (NS-SC-T15-S0, EC-T15-S0, and SP-T15-S0) in the negative dimension of PC. In contrast, PC2 separated the NS-SC treatments (NS-SC-T25-S0 and NS-SC-T15-S0) from EC and SP treatments (EC-T15-S0, EC-T25-S0, SP-T15-S0, and SP-T25-S0). EC-T25-S0 and SP-T25-S0 made a small cluster and were mainly associated with the short-chain fatty acids (valeric, isovaleric, isobutyric acid and acetic acid), alcohols (isobutanol and 2-phenyl ethanol) and ethyl lactate. In contrast, EC-T15-S0 and SP-T15-S0 formed a group together and were characterized by the high accumulation of acetate esters (hexyl acetate and ethyl phenylacetate), medium chain fatty acids (octanoic acid and decanoic acid) as well as isoamyl alcohol. On the other hand, NS-SC-T15-S0 was associated with esters including 2-phenyl ethyl acetate, diethyl succinate, ethyl hexanoate, ethyl butyrate and isoamyl acetate, as well as hexanoic acid. NS-SC-T25-S0 was characterized by high production of ethyl esters (ethyl acetate,

ethyl caprate, and ethyl caprylate), alcohols including aliphatic alcohols (butanol and hexanol), propanol and 3-ethoxy-1-propanol as well as propionic acid and acetoin.

A two-way ANOVA confirmed that the production of 21 major volatiles was significantly affected by temperature. In contrast, only 10 volatile compounds, mainly within the group of volatile acids (e.g. acetic acid, valeric acid, isovaleric acid, isobutyric acid and decanoic acid) and higher alcohols (propanol, isoamyl alcohol and 2-phenyl ethanol) were significantly affected by SO_2 whereas the production of few esters (ethyl lactate, ethyl caprate, and ethyl caprylate) was affected by SO_2. Furthermore, the production of few compounds including octanoic acid, acetic acid, 3 ethoxy-1-propanol, isobutanol, ethyl lactate and ethyl acetate were significantly affected by the combined effect of temperature and sulphur dioxide (Table 5S5).

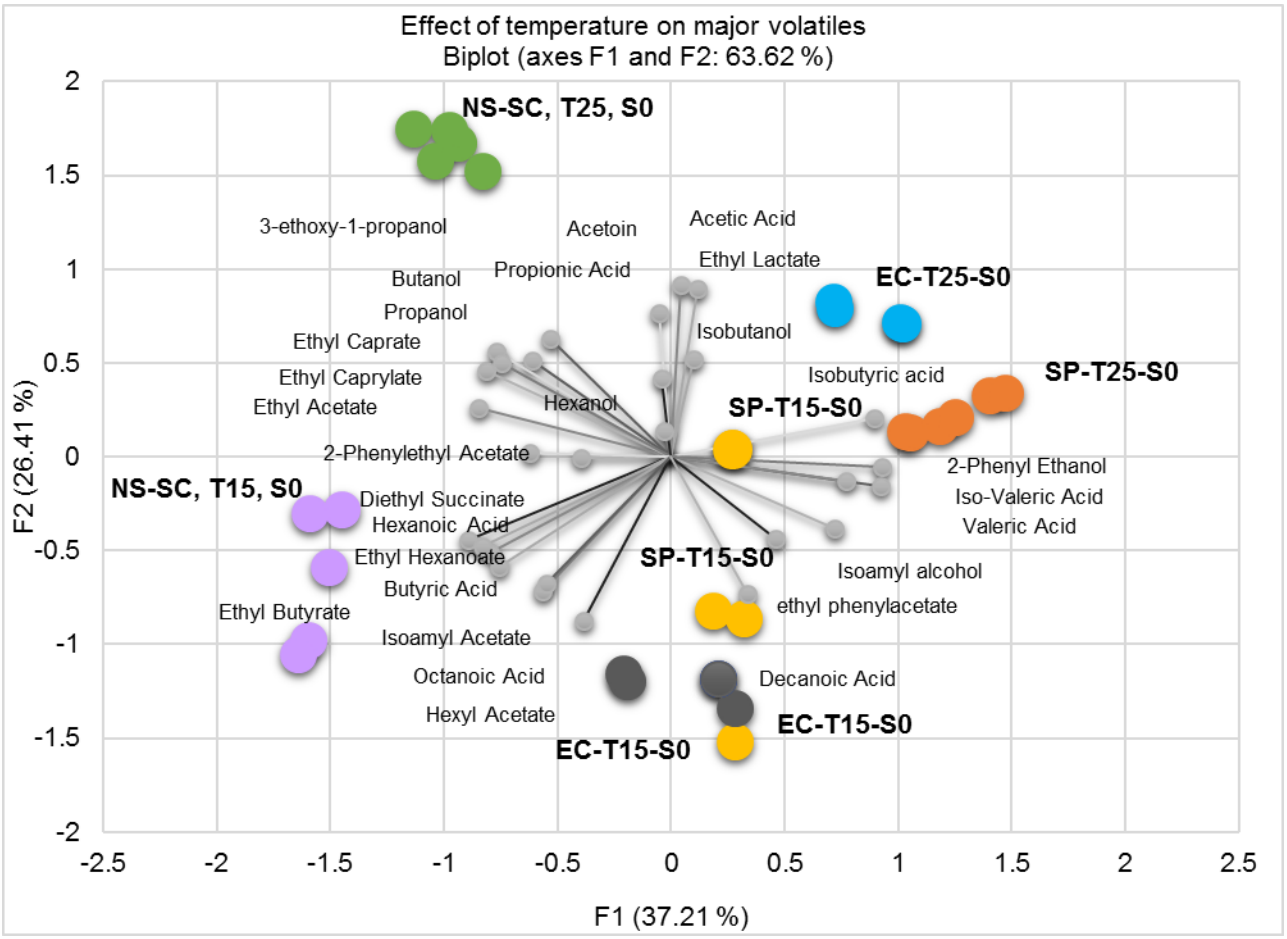


Figure.5.13. A biplot of the first and second principal components (PC) derived from PC analysis of the major volatile compounds produced in Chenin blanc fermentations in the South African harvest. The fermentations were conducted spontaneously (SP), inoculated with EC1118 (EC) or inoculated with the consortium (NS-SC) at two temperatures (T15 and T25), with and without sulphur addition (S0 and S30). The major volatile compounds are represented by light greys whereas the treatments are as follows: SP-T25-S0 (), SP-T15-S0 (), NS-SC-T25-S0 (), NS-SC-T15-S0 (), EC-T25-S0 () and EC-T15-S0 ().

5.5. Discussion

The current study evaluated the influence of temperature and sulphur dioxide on the yeast population dynamics as well as wine aroma in a complex community. For this purpose, three grape matrices including synthetic juice, Chenin blanc, and Greccheto Bianco were selected. Overall, the yeast diversity in both Chenin blanc and Grechceto Bianco juice was highly similar to the model consortium, confirming that the yeast consortium was a reasonable approximation of yeast diversity in the wine ecosystem. Since some non-*Saccharomyces* species (*P. terricola, M. pulcherrima*, and *W. anomalus*) were present in the natural grape juice as well as the model consortium, we could compare the effect of SO_2 and temperature on the growth of these species in NS-SC and control fermentations (SP and EC). Similar trends in population dynamics were observed between NS-SC and control fermentations even though the strains present in the consortium and grape must were most probably different.

The data show that fermentation kinetics (rate and length) and yeast metabolism were affected by temperature and sulphur addition. Fermentations performed at a higher temperature and with SO_2 provided an ecological advantage for *S. cerevisiae* in the yeast consortium. In contrast, fermentations conducted at a lower temperature and without SO_2 enhanced the growth of some non-*Saccharomyces* species, while decreasing the growth of *S. cerevisiae*. Similar results have been reported previously in the mixed culture fermentation (Mills *et al*., 2002; Erten, 2002; Salvadò *et al*., 2011; Alonso-del-Real *et al*., 2017). Furthermore, our data confirmed that the effect of temperature on the growth of non-*Saccharomyces* species in a multi-species ecosystem is species dependent. For instance, the growth of *H. vineae*, *L. thermotolerans* and *S. bacillaris* was enhanced at 15°C whereas the growth of *M. pulcherrima* and *C. parapsilosis* was not significantly affected by temperature in the synthetic must fermentation (Table 5S1). Some studies have suggested that yeast species modify the cell wall lipid compositions to adapt with the temperature stress (Alexandre *et al*., 1994; Pina *et al*., 2004; Arroyo-Lopez *et al*., 2010; Redón *et al*., 2011; Salvadó *et al*., 2011; García-Ríos *et al*., 2017). In this context, our data underlined the importance of ecological interaction in the persistence of non-*Saccharomyces* yeast throughout the fermentation. For instance, increased persistence or maximal growth of some indigenous species such as *M. pulcherrima, P. terricola* and *H. uvarum* in SP fermentations (Chenin blanc/Greccheto Bianco) compared to NS-SC suggested that increased competition between the species in the yeast consortium may have negatively affected the growth of these species. In contrast, the growth of these indigenous species was enhanced in the ecosystem with a limited number of species and limited competition (SP). Similarly, *C. parapsilosis* and *P. terricola* persisted until the end of NS-SC-GREC fermentation whereas, their population diminished earlier in NS-SC-CHEN and NS-SC-synthetic must fermentations. This result can be explained by the differences in the initial diversity of grape must, highlighting the importance of ecological interactions in the wine ecosystem.

Concerning sulphur dioxide, some interesting results were observed. Firstly, our result was in agreement with the previous reports by Jolly *et al*., (2003b) and Albertin *et al*., (2014), confirming that the degree to which sulphur dioxide affects the growth of yeast species in a multi-species ecosystem was species/strain dependent. For instance, sulphur addition displayed an immediate inhibitory effect on the growth of *M. pulcherrima* and *L. thermotolerans* in synthetic must fermentation while the growth of *H. vineae* and *P. terricola* was inhibited gradually throughout the fermentation. The growth of some species was unaffected by SO_2 (e.g. *L. thermotolerans* in NS-SC-synthetic must fermentation) while the supportive effect of SO_2 on the growth of *S. cerevisiae* and *W. anomalus* was observed in NS-SC-CHEN. Better implantation of *S. cerevisiae* in the yeast consortium in the presence of SO_2 was in agreement with the previous report by Albertin *et al*., (2014). *S. cerevisiae* has been shown to apply different strategies to adapt to sulphur stress (Casalone *et al*. 1992; Park and Bakalinsky, 2000; Divol *et al*., 2012).

Studies have previously reported that the growth of some species remained unaffected with 30-50 mg/L sulphur addition while others have reported that the growth of some non-*Saccharomyces* species was inhibited with higher SO_2 dosage such as 60 mg/L-100 mg/L (Albertin *et al*., 2014; Jolly *et al*., 2003b). However, the supportive effect of sulphur dioxide at a low dosage (30 mg/L), on the growth of non-*Saccharomyces* species was not reported previously. Our result suggests that *W. anomalus* has an adaptation mechanism to survive under sulphur stress which opens up new avenues for further investigations. Recently, Bokulich *et al*., (2015), and Morgan *et al*., (2017), confirmed that SO_2 affect the growth of organisms in a dose-dependent manner. Thus, further investigation is required to evaluate the dose-dependent effect of SO_2 on the growth of yeast species in a multi-species consortium. Furthermore, our data confirmed that the inhibitory effect of SO_2 on the growth of non-*Saccharomyces* yeasts was amplified at a higher temperature. For instance, *S. bacillaris* was present at 2-fold higher population in NS-SC-T15-S30 compared to NS-SC-T25-S30 in GREC and CHEN fermentations, suggesting that *S. bacillaris* is more resistant to SO_2 at a lower temperature (Figure 5.S2C&D, 5.S3C& D).

The data suggest that temperature and SO_2 modify the pattern of population dynamics in the yeast consortium. However, the growth and persistence of yeast species throughout fermentation highly depend on their ability to compete with the rest of species in the yeast consortium. Therefore, the dynamics observed in the yeast consortium under different treatments seemed to heavily rely on the ecological interactions. Furthermore, our data suggest that ecological interactions are largely independent of the matrix. These observations confirm that the constructed model consortium is a suitable tool to study the microbial interactions in wine fermentations.

Concerning the production of aroma compounds, a clear separation between the fermentations performed at 25°C and 15°C in the positive and negative dimensions of PC1, suggesting that temperature affected the production of wine aroma. The persistence of non-*Saccharomyces*

species seemed to be the main contributor to the differences among the treatments. Furthermore, the number of volatile compounds which were significantly affected by temperature and SO_2 were greater in the NS-SC wines compared to SP and EC wines. Nonetheless, temperature displayed a stronger effect compared to SO_2 on the production of the volatile compound. This result was in agreement with the greater effect of temperature compared to sulphur addition on the growth of individual species in the yeast consortium (Table 5S2& 3). However, other authors have suggested that higher production of some esters at a lower temperature might be due to reduced evaporation loss of these compounds at 15°C compared to 25 and 30°C (Walker 1998; Torija et al. 2003).

Overall, population dynamics followed a similar trend in synthetic must as well as the real must fermentations, despite the differences among three matrices. Our results confirmed that the constructed consortium is a robust model that can be used as a tool to predict the microbial behavior from a simple ecosystem to the complex natural environment, irrespective of the grape matrix. The current study provided deep insight into yeast-yeast interactions and the significance of ecological interactions in wine ecosystem. Our results suggest that microbial diversity of grape must and the ecological interactions occurring throughout wine fermentation drive the dynamics of wine consortium and affect the quality of the wine. Thus, the effect of ecological interactions seems to be more important than temperature and sulphur dioxide in the wine ecosystem. Furthermore, our study suggests that inoculating the fermentation with a commercial strain of *S. cerevisiae* at a lower dosage (10^3-10^4) may solve the problem of stuck fermentation at a lower temperature in the wine industry. Inoculating the fermentation with a commercial strain of *S. cerevisiae* (i) ensure the completion of wine fermentations at a low temperature (ii) ensure the persistence of some non-*Saccharomyces* yeasts throughout fermentation due to the slow metabolism of *S. cerevisiae* (iii) can enhance the organoleptic properties of wine.

5.6. References

Albertin, W., Miot-Sertier, C., Bely, M., Marullo, P., Coulon, J., Moine, V., et al. (2014). Oenological pre-fermentation practices strongly impact yeast population dynamics and alcoholic fermentation kinetics in Chardonnay grape must. *Int. J. Food Microbiol.* 178, 87–97. doi:10.1016/j.ijfoodmicro.2014.03.009.

Alexandre, H., Long, T.N., Feuillat, M., Charpentier, C. (1994). Contribution à l'étude des bourbes : influence sur a fermentiscibilité des moûts. *Rev. Fr. Enol*. 146, 11–19.

Alonso-del-Real, J., Lairón-Peris, M., Barrio, E., Querol, A. (2017). Effect of Temperature on the Prevalence of *Saccharomyces* Non *cerevisiae* Species against a *S. cerevisiae* Wine Strain in Wine Fermentation: Competition, Physiological Fitness, and Influence in Final Wine Composition. *Front in Microbiol*. 8, 1-15. https://doi.org/10.3389/fmicb.2017.00150.

Arroyo-Lopez, F. N., Salvadó, Z., Tronchoni, J., Guillamón, J. M., Barrio, E., Querol, A. (2010). Susceptibility and resistance to ethanol on *Saccharomyces* strains isolated from wild and fermentative environments. *Yeast*, 27, 1005–1015.

Bagheri, B., Bauer, F. F., Setati, M. E. (2015). The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. S*. Afr. J. Enol. Vitic.* 36, 243–251.

Bagheri, B., Bauer, F. F., Setati, M. E. (2017). The Impact of *Saccharomyces cerevisiae* on a Wine Yeast Consortium in Natural and Inoculated Fermentations. *Front in Microbiol*, *8*, 1–13. https://doi.org/10.3389/fmicb.2017.01988.

Bauer, E. F., and Pretorius, L. S. (2000). Yeast Stress Response and Fermentation Efficiency : How to Survive the Making of Wine - A Review. *South African J. Enol. Vitic.* 21, 27–51.

Beltran, G., Novo, M., Leberre, V., Sokol, S., Labourdette, D., Guillamon, J. M., et al (2006). Integration of transcriptomic and metabolic analyses for understanding the global responses of low-temperature winemaking fermentations. *FEMS Yeast Research*. *6*, 1167–1183. https://doi.org/10.1111/j.1567-1364.2006.00106.x.

Beltran, G., Novo, M., Guillamón, J. M., Mas, A., Rozès, N. (2008). Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *Int. J. Food Microbiol*, *121*, 169–177. https://doi.org/10.1016/j.ijfoodmicro.2007.11.030.

Bely, M., Sablayrolles, J. M., Barre, P. (1990). Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *J. Ferment. Bioeng.* 70, 246–252. doi:10.1016/0922-338X(90)90057-4.

Bokulich, N. A., Swadener, M., Sakamoto, K., Mills, D. A., Bisson, L. F. (2015). Sulfur dioxide treatment alters wine microbial diversity and fermentation progression in a dose-dependent fashion. *Am. J. Enol. Vitic*. *66*, 73–79. https://doi.org/10.5344/ajev.2014.14096.

Casalone, E., Colella, CM., Daly, S., Gallori, E., Moriani, L., Polsinelli, M. (1992). Mechanism of 614 resistance to sulfite in *Saccharomyces cerevisiae. Curr Genet.* 22:435-440.

Chandra, M., Oro, I., Ferreira-Dias, S., Malfeito-Ferreira, M. (2015). Effect of ethanol, sulfur dioxide and glucose on the growth of wine spoilage yeasts using response surface methodology. *PLoS One* 10, 1–15. doi:10.1371/journal.pone.0128702.

Ciani, M., (1997). Role, enological properties and potential use of non-*Saccharomyces* wine yeasts*. Recent Res. Devel in Microbiology*. 1, 317–331.

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

Ciani, M., Comitini, F., Mannazzu, I., Domizio, P. (2010). Controlled mixed culture fermentation: A new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research*, *10*, 123–133. https://doi.org/10.1111/j.1567-1364.2009.00579.x.

Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., Romano, P. (2016). Yeast interactions in inoculated wine fermentation. *Front. Microbiol.* doi:10.3389/fmicb.2016.00555.

Cocolin, L., Mills, D. A. (2003). Wine yeast inhibition by sulfur dioxide: A comparison of culture-dependent and independent methods. *Am. J. Enol. Vitic*. *54*, 125–130.

Coetzee, C., Lisjak, K., Nicola, L., Kilmartin, P., Du toit, W J., (2013). Oxygen and sulfur dioxide additions to Sauvignon blanc must: effect on must and wine composition. *Flavour Fragr. J*. 28, 155–167.

De Filippis, F., Parente, E., and Ercolini, D. (2017). Metagenomics insights into food fermentations. *Microb. Biotechnol.* 10, 91–102. doi:10.1111/1751-7915.12421.

Del Fresno, J. M., Morata, A., Loira, I., Bañuelos, M. A., Escott, C., Benito, S., et al. (2017). Use of non-*Saccharomyces* in single-culture, mixed and sequential fermentation to improve red wine quality. *Eur. Food Res. Technol.* 243, 2175–2185. doi:10.1007/s00217-017-2920-4.

Divol, B., du Toit, M., Duckitt, E., (2012). Surviving in the presence of sulfur dioxide: strategies developed by wine yeasts. *Appl Microbiol Biotechnol*. 95:601-613.

Eder, M. L. R., Reynoso, C., Lauret, S. C., Rosa, A. L., and Rosa, A. L. (2017). Isolation and Identification of the Indigenous Yeast Population during Spontaneous Fermentation of Isabella (*Vitis labrusca L* .) Grape Must. 8, 1–8. doi:10.3389/fmicb.2017.00532.

Erten, H., (2002). Relations between elevated temperatures and fermentation behaviour of *Kloeckera apiculata* and *Saccharomyces cerevisiae* associated with winemaking in mixed cultures. *JMB*, 18, 373–378.

Fairbairn, S. C., Smit, A. Y., Jacobson, D., Prior, B. A., Bauer, F. F. (2014). Environmental stress and aroma production during wine fermentation. S*. Afr. J. Enol. Vitic*. *35*(2), 168–177.

Fleet, G.H., Lafon-Lafourcade, S., Ribe´reau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Appl. Environ. Microbiol*, 48, 1034-1038.

Fleet, G.H., (1992). Spoilage yeasts. *Crit. Reviews Biotech*. 12, 1-44.

García-Ríos, E., Gutiérrez, A., Salvadó, Z. Z., Arroyo-López, F. N., Guillamon, J. M. (2014). The Fitness Advantage of Commercial Wine Yeasts in Relation to the Nitrogen Concentration, Temperature, and Ethanol Content under Microvinification Conditions. *Appl. Environ. Microbiol*, *80* (2), 704–713. https://doi.org/10.1128/AEM.03405-13.

García-Ríos, E., Morard, M., Parts, L., Liti, G., Guillamón, J. M. (2017). The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae*. *BMC Genomics*, *18* (1), 159. https://doi.org/10.1186/s12864-017-3572-2.

Gao, C., Fleet, G.H., (1988). The effects of temperature and pH on the ethanol tolerance of the wine yeasts, *Saccharomyces cerevisiae, Candida stellata,* and *Kloeckera apiculata*. *J. Appl. Bacteriol*. 65, 405-410.

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

Hammond, J. R .M., (1993). Brewer's yeasts. In: Rose, A.H., Harrison, J.S. (Eds.), *Yeast Technology*, vol. 5. Academic Press Limited, London, pp. 7–67.

Henschke, PA., Jiranek, V. *(*1993). Yeasts metabolism of nitrogen compounds*. In Wine Microbiology and Biotechnology,* Fleet GH *(ed). Harwood Academic:* Chur, Switzerland*;* 77–164*.*

García-Ríos, E., Gutiérrez, A., Salvadó, Z. Z., Arroyo-López, F. N., Guillamon, J. M. (2014). The Fitness Advantage of Commercial Wine Yeasts in Relation to the Nitrogen Concentration, Temperature, and Ethanol Content under Microvinification Conditions. *Appl. Environ. Microbiol*. *80*, 704–713. https://doi.org/10.1128/AEM.03405-13.

García-Ríos, E., Morard, M., Parts, L., Liti, G., Guillamón, J. M. (2017). The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae*. *BMC Genomics*. *18*, 159. https://doi.org/10.1186/s12864-017-3572-2.

Ghosh, S., Bagheri, B., Morgan, H. H., Divol, B., Setati, M. E. (2015). Assessment of wine microbial diversity using ARISA and cultivation-based methods. *Ann Microbiol*, *65*, 1833–1840. https://doi.org/10.1007/s13213-014-1021-x.

Jolly, N. P., Augustyn, O. H. P. H., Pretorius, I. S. (2003a). The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. S*. Afr. J. Enol. Vitic*, *24*, 63–69.

Jolly, N. P., Augustyn, O. H. P. H., Pretorius, I. S. (2003b). The Use of *Candida pulcherrima* in Combination with *Saccharomyces cerevisiae* for the Production of Chenin blanc Wine. *S. Afr. J. Enol. Vitic*, 24, 2.

Liu, Y., Rousseaux, S., Tourdot-Maréchal, R., Sadoudi, M., Gougeon, R., Schmitt-Kopplin, P., et al. (2017). Wine microbiome: A dynamic world of microbial interactions. *Crit. Rev. Food Sci. Nutr.* 57, 856–873. doi:10.1080/10408398.2014.983591.

Louw, L., Tredoux, A. G. J., Rensburg, P. Van, Kidd, M., Naes, T., Nieuwoudt, H. H. (2010). Fermentation-derived Aroma Compounds in Varietal Young Wines from South Africa, *Sasev, 31*, 213–225.

Maturano, Y. P., Mestre, M. V., Combina, M., Toro, M. E., Vazquez, F., Esteve-Zarzoso, B. (2016). Culture-dependent and independent techniques to monitor yeast species during cold soak carried out at different temperatures in winemaking. *Int. J. Food Microbiol*, *237*, 142–149. https://doi.org/10.1016/j.ijfoodmicro.2016.08.013

Mills, D. A., Johannsen, E. A., Cocolin, L. (2002). Yeast diversity and persistence in botrytis-affected wine fermentations. *Appl. Environ. Microbiol*, *68*, 4884–4893. https://doi.org/10.1128/AEM.68.10.4884-4893.2002.

Molina, A. M., Swiegers, J. H., Varela, C., Pretorius, I. S., Agosin, E (2007). Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. *Appl. Microbiol. Biotechnol*, 77, 675−687.

Morgan, S. C., Scholl, C. M., Benson, N. L., Stone, M. L., Durall, D. M. (2017). Sulfur dioxide addition at crush alters *Saccharomyces cerevisiae* strain composition in spontaneous fermentations at two Canadian wineries. *Int. J. Food Microbiol.* 244, 96–102. doi:10.1016/j.ijfoodmicro.2016.12.025.

Padilla, B., Gil, J. V., Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Front in Microbiol*, *7*, 1–20. https://doi.org/10.3389/fmicb.2016.00411.

Park, H., and Bakalinsky, A. T. (2000). SSUI mediates sulphite efflux in *Saccharomyces cerevisiae*. *Yeast* 16, 881–888.

Pérez-Torrado, R., Rantsiou, K., Perrone, B., Navarro-Tapia, E., Querol, A., Cocolin, L. (2017). Ecological interactions among *Saccharomyces cerevisiae* strains: insight into the dominance phenomenon. *Sci. Rep.* 7, 43603. doi:10.1038/srep43603.

Pina, C., Couto, J. A., António, J., Hogg, T. (2004). Inferring ethanol tolerance of *Saccharomyces* and non-*Saccharomyces* yeasts by progressive inactivation. *Biotechnol Lett*, *26*(19), 1521–7. https://doi.org/10.1023/B:BILE.0000044456.72347.9f.

Reddy, L. V. A., Reddy, O. V. S. (2011). Effect of fermentation conditions on yeast growth and volatile composition of wine produced from mango (*Mangifera indica* L.) fruit juice. *FBP*, *89*, 487–491. https://doi.org/10.1016/j.fbp.2010.11.007.

Redón, M., Guillamón, J. M., Mas, A., Rozès, N. (2011). Effect of growth temperature on yeast lipid composition and alcoholic fermentation at low temperature. *Eur. Food Res. Technol.***,** *232*, 517–527. https://doi.org/10.1007/s00217-010-1415-3.

Reed, G., Nagodawithana, T.W., (1991). Wine yeast. In: Yeast Technology. Van Nostrand Reinhold, NewYork, pp. 151–224.

Rollero, S., Bloem, A., Camarasa, C., Sanchez, I., Ortiz-Julien, A., Sablayrolles, et al (2015). Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. *Appl. Microbiol. Biotechnol, 99*, 2291–2304. https://doi.org/10.1007/s00253-014-6210-9.

Romano, P., Suzzi, G., (1993). Sulfur dioxide and wine microorganisms. In: Fleet, G.H. (Ed.), *Wine Microbiology and Biotechnology*. Harwood Academic, Switzerland, pp. 373–393.

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J. J., Ballester, J., et al. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol.* 32, 243–253. doi:10.1016/j.fm.2012.06.006.

Salvadó, Z., Arroyo-López, F. N., Barrio, E., Querol, A., Guillamón, J. M. (2011). Quantifying the individual effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*. *Food Microbiol*. *28*, 1155–1161. https://doi.org/10.1016/j.fm.2011.03.008.

Sun, Y., Zhang, T., Lu, H., Yu, Z., Li, X. (2016). Effect of added sulfur dioxide levels on the fermentation characteristics of strawberry wine. *J. Inst. Brew, 122* (3), 446–451. https://doi.org/10.1002/jib.342.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., et al. (2012). Multistarter from organic viticulture for red wine Montepulciano d'Abruzzo production. *Front. Microbiol.* 3, 1–10. doi:10.3389/fmicb.2012.00135.

Torija, M. J., Rozès, N., Poblet, M., Guillamón, J. M., Mas, A. (2003). Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol*. *80*, 47–53. https://doi.org/10.1016/S0168-1605(02)00144-7.

Vinlab, SA, Sulphur dioxide additions and calculations, available at: [http://www. Airqualityontario.com/science/ pollutants/sulphur.php].

Wang, C., Esteve-Zarzoso, B., Cocolin, L., Mas, A., Rantsiou, K. (2015). Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during Barbera must fermentation. *Food Res Int*, *78*, 195–200. https://doi.org/10.1016/j.foodres.2015.10.0.

Walker, G. (1998). Yeast physiology and biotechnology. Wiley, New York.

5.7. Supplementary materials

5.7.1. Supplementary tables

Table.5.S1. Results of one way ANOVA indicating the effect of temperature (tem) on the growth of individual species in NS-SC fermentation conducted in synthetic must at three different temperatures (15˚C, 25˚C, and 30˚C) as well as the effect of and sulphur addition 0 and 30 mg/L). The growth of species with the *p*-values lower than 0.05 was significantly affected by the treatment. Red. represents the statistically significant values**.** The following abbreviations were used for names of yeast species. *Mp*: *M. pulcherrima, Pt: P. terricola, Sb: S. bacillaris, Cp: C. parapsilosis, Wa, W. anomalus, Lt: L. thermotolerans, Hv: H. vineae and Ind-Sc:* Indigenous *S. cerevisiae*.

Species	Values	Tem	SO_2
Mp	F	0.13	0.15
	p	0.87	0.64
Pt	F	2.89	2.95
	p	0.06	0.05
Sb	F	5.31	6.32
	p	0.00	0.05
Cp	F	0.05	0.08
	p	0.94	0.14
Wa	F	2.74	3.22
	p	0.07	0.00
Lt	F	3.40	3.14
	p	0.03	0.25
Hv	F	3.54	2.25
	p	0.03	0.04
EC1118	F	7.21	7.52
	p	0.00	0.00

Table.5.S2. Results of a two-way ANOVA, indicating the effect of temperature (Tem), sulphur dioxide (SO_2), and the combined effect of two factors (Tem*SO_2) on the growth of individual species in fermentations conducted in Chenin blanc juice in South Africa. The growth of species with the *p*-values lower than 0.05 was significantly affected by the treatment/combination of treatments. Red. represents the statistically significant values.

		NS-SC			EC1118			Spontaneous		
Species	Values	Temp	"SO_2"	Temp *"SO_2"	Temp	"SO_2"	Temp *"SO2"	Temp	"SO_2"	Temp *SO_2"
Mp	F	3.35	3.00	0.44	0.00	9.82	0.00	0.00	8.86	0.09
	p	0.07	0.08	0.50	0.92	0.00	0.97	0.95	0.00	0.75
Hu	F	0.10	5.18	0.06	0.19	2.76	2.46	3.68	1.55	0.66
	p	0.74	0.02	0.79	0.65	0.10	0.12	3.68	0.54	0.66
Lt	F	17.74	0.00	0.20	3.08	4.60	4.10	10.55	1.45	0.38
	p	0.00	0.93	0.65	0.08	0.03	0.04	0.00	0.23	0.53
	F	15.36	0.00	0.03	3.66	1.63	0.64	0.34	2.72	8.16

Wa	p	0.00	0.93	0.86	0.06	0.20	0.42	0.55	0.10	0.00
	F	14.43	3.38	3.32	14.19	1.15	1.11	10.64	0.24	0.31
Ind-Sc	p	0.00	0.07	0.07	0.00	0.28	0.29	0.00	0.62	0.57
	F	20.53	2.66	2.64	15.73	0.34	0.37			
EC1118	p	0.00	0.10	0.10	0.00	0.55	0.54			
	F	10.47	0.08	1.35						
Sb	p	0.00	0.76	0.24						
	F	8.52	1.37	0.52						
Hv	p	0.00	0.24	0.47						
	F	1.09	2.90	1.61						
Cp	p	0.30	0.09	0.20						
	F	5.44	0.74	0.99						
Pt	p	0.02	0.39	0.32						

Table.5.S3. The result of two-way ANOVA analysis, indicating the effect of temperature (Tem), sulphur dioxide (SO_2), and the combined effect of two factors (Tem*SO_2) on the growth of individual species in the fermentations conducted in Grecchedto Bianco juice in Italy. The growth of species with the *p*-values lower than 0.05 was significantly affected by the treatment/combination of treatments. Red. represents the statistically significant values

Species	**Values**	**NS-SC**			**EC1118**			**Spontaneous**		
		Temp	"SO_2"	Temp *"SO_2"	Temp	"SO_2"	Temp *"SO_2"	Temp	"SO_2"	Temp *"SO_2"
Mp	F	0.07	0.10	0.04	5.71	0.00	0.00	10.14	9.76	9.77
	p	0.78	0.74	0.83	0.02	0.96	0.98	0.00	0.00	0.00
Hu	F	0.33	0.31	0.00	1.03	0.97	1.30	19.42	27.05	18.43
	p	0.56	0.57	0.98	0.31	0.32	0.25	0.00	0.00	0.00
Rm	F	0.46	1.19	0.65	0.00	0.13	0.96	2.91	5.46	2.42
	p	0.49	0.28	0.42	0.97	0.71	0.33	0.09	0.02	0.12
Pt	F	0.00	1.05	0.01	0.00	4.80	0.06	8.86	16.92	8.04
	p	0.99	0.31	0.90	0.95	0.03	0.79	0.00	0.00	0.00
Ind-Sc	F	7.72	3.96	2.01	21.39	0.49	0.39	10.38	4.16	0.06
	p	0.00	0.05	0.16	0.00	0.48	0.53	0.00	0.04	0.80
EC1118	F	5.14	3.63	2.85	11.29	0.08	1.51			
	p	0.02	0.06	0.09	0.00	0.76	0.22			
Lt	F	2.16	11.74	0.53						
	p	0.14	0.00	0.46						
Wa-F	F	0.09	1.71	0.01						
	p	0.76	0.19	0.89						
Sb	F	0.00	15.31	0.66						

	p	0.94	0.00	0.42
Hv	F	6.55	7.32	0.38
	p	0.01	0.00	0.53
Cp	F	0.01	2.61	0.35
	p	0.91	0.11	0.55

Table.5.S4. The results of one way ANOVA, indicating the effect of temperature on the aromatic compounds of wine obtained from synthetic must at three different temperatures (15˚C, 25˚C, and 30˚C). The compounds with the *p*-values lower than 0.05 were significantly affected by the treatment. The statistically significant values are presented by red.

Variable	F	p
Ethyl Acetate	12.64	0.00
Isoamyl Acetate	114.07	0
ethyl phenylacetate	4.72	0.02
2-Phenylethyl Acetate	10.73	0.00
Diethyl Succinate	64.71	0
Ethyl Lactate	7.16	0.00
Ethyl Caprylate	13.74	0.00
Ethyl Caprate	333.49	0
Diethyl Succinate	64.71	0
Propanol	51.04	0
Isobutanol	5.53	0.01
Butanol	10.1	0.00
Isoamyl alcohol	20.2	0.00
2-Phenyl Ethanol	7.9	0.00
Hexanol	62.34	0
3-ethoxy-1-propanol	10.37	0.00
Acetic Acid	4.36	0.03
Propionic Acid	15.21	0.00
Isobutyric acid	6.05	0.01
Butyric Acid	136.35	0
Iso-Valeric Acid	13.02	0.00
Valeric Acid	32.3	0.00
Hexanoic Acid	12.46	0.00
Octanoic Acid	12.31	0.00
Decanoic Acid	38.86	0.00
Acetoin	21.85	0.00

Table.5.S5. The result of a two-way ANOVA analysis, indicating the effect of temperature (Tem), sulphur dioxide (SO_2), and the combination of treatments on the major volatiles of wine obtained from fermentations conducted in South Africa. The compounds with the p-values lower than 0.05 were significantly affected by the treatment/ combination of treatments. The statistically significant values are represented by red.

Effect		"SO_2"	Tem	"SO2"*Tem
Ethyl Acetate	F	0.16	2.38	3.83
	p	0.68	0.12	0.05
Ethyl Butyrate	F	0.08	69.90	0.13
	p	0.77	0.00	0.71
Ethyl Lactate	F	19.15	130.73	19.15
	p	0.00	0.00	0.00
Ethyl Caprate	F	5.49	3.23	0.03
	p	0.02	0.07	0.85
Ethyl Hexanoate	F	0.03	55.90	0.69
	p	0.86	0.00	0.40
Isoamyl Acetate	F	0.44	46.55	1.25
	p	0.50	0.00	0.26
Diethyl Succinate	F	4.27	0.16	2.29
	p	0.04	0.68	0.13
Ethyl Caprylate	F	4.63	0.11	1.11
	p	0.03	0.73	0.29
Isoamyl Acetate	F	0.44	46.55	1.25
	p	0.50	0.00	0.26
Ethyl phenylacetate	F	1.70	0.03	0.24
	p	0.19	0.84	0.62
2-Phenylethyl Acetate	F	0.94	0.75	3.94
	p	0.33	0.38	0.05
Hexyl Acetate	F	0.30	296.54	2.97
	p	0.58	0.00	0.08
Butanol	F	0.43	21.77	0.38
	p	0.51	0.0	0.53
Propanol	F	6.53	1.61	0.049
	p	0.01	0.20	0.82
Isobutanol	F	0.00	86.47	4.65
	p	0.97	0.00	0.03
2-Phenyl Ethanol	F	5.16	29.20	0.60
	p	0.02	0.00	0.44
Isoamyl alcohol	F	4.45	7.20	2.32
	p	0.03	0.00	0.13
Hexanol	F	0.00	0.18	2.00
	p	0.98	0.67	0.16
3-ethoxy-1-propanol	F	0.47	0.99	13.35
	p	0.49	0.32	0.00
Acetic Acid	F	16.66	21.68	29.35
	p	0.00	0.00	0.00
	F	2.25	9.75	0.02

Propionic Acid	p	0.13	0.00	0.86
Isobutyric acid	F	9.18	93.82	2.61
	p	0.00	0.00	0.11
Butyric Acid	F	0.58	102.42	0.03
	p	0.45	0.00	0.85
Iso-Valeric Acid	F	6.15	15.45	0.41
	p	0.01	0.00	0.52
Valeric Acid	F	14.46	13.50	0.00
	p	0.00	0.00	1.00
Hexanoic Acid	F	0.21	72.21	1.27
	p	0.64	0.00	0.26
Octanoic Acid	F	0.82	86.41	4.95
	p	0.36	0.00	0.03
Decanoic Acid	F	4.52	10.81	0.44
	p	0.03	0.00	0.50
Acetoin	F	0.30	15.63	0.30
	p	0.58	0.00	0.58

5.7.2. Supplementary Figures

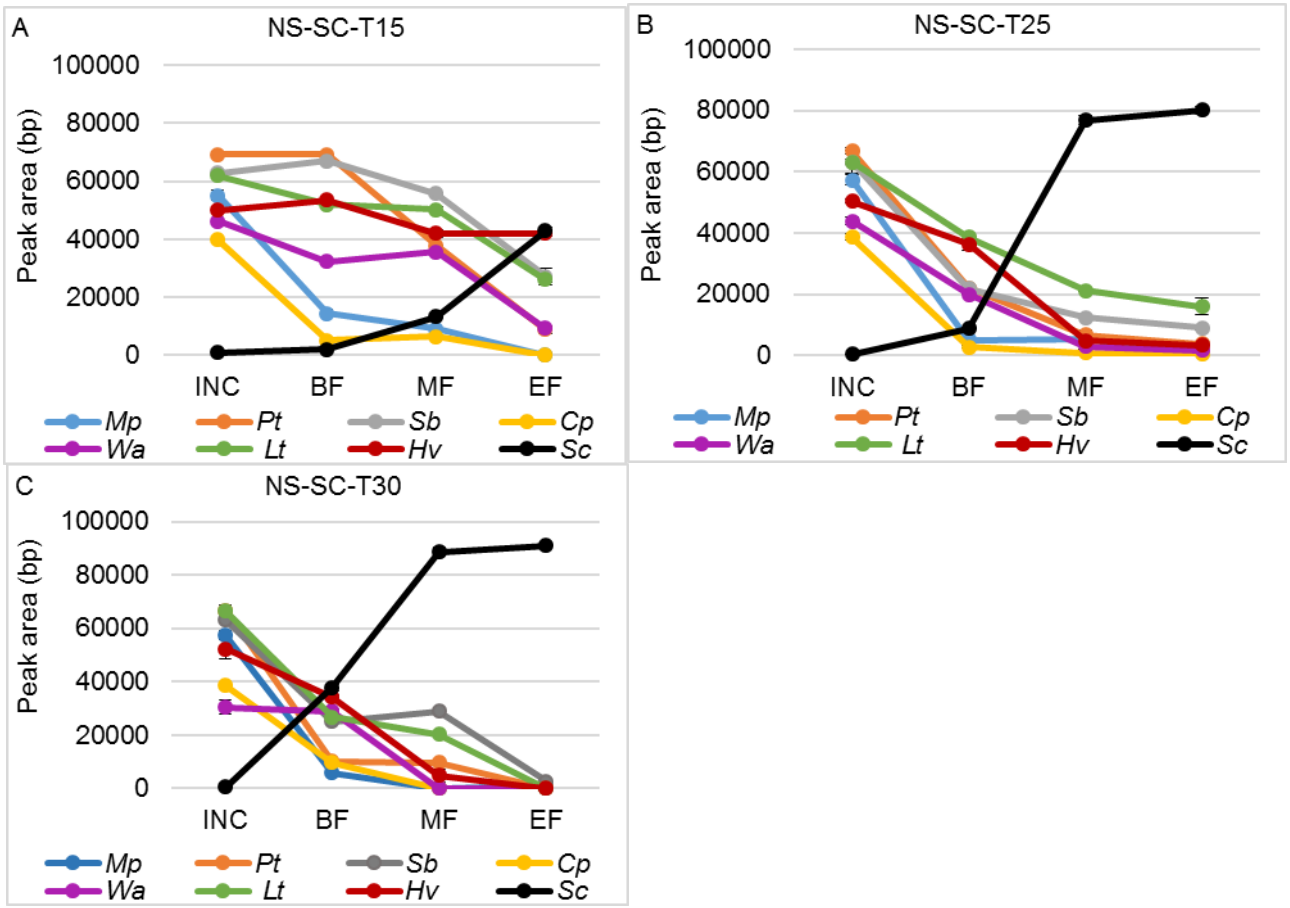
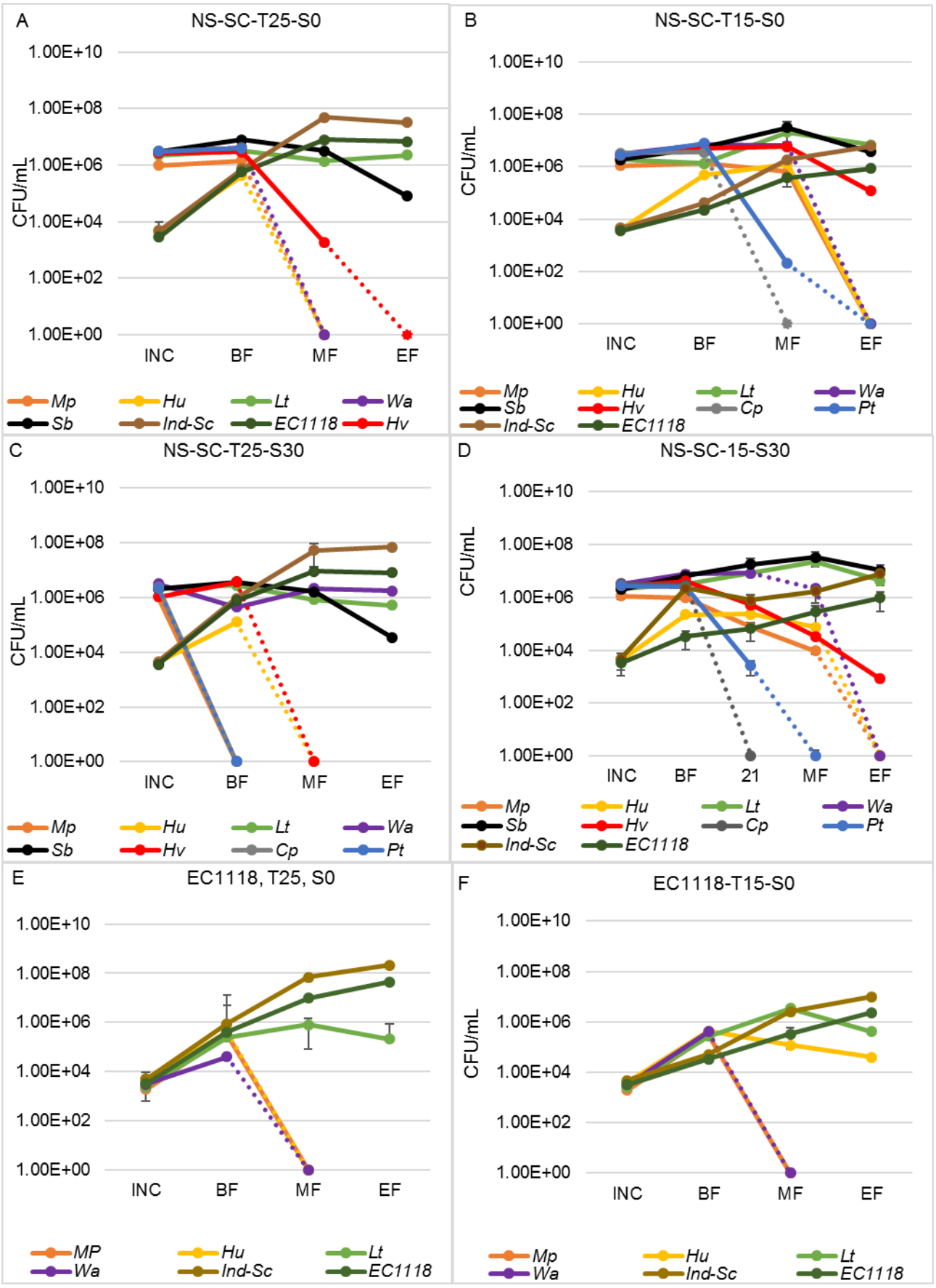
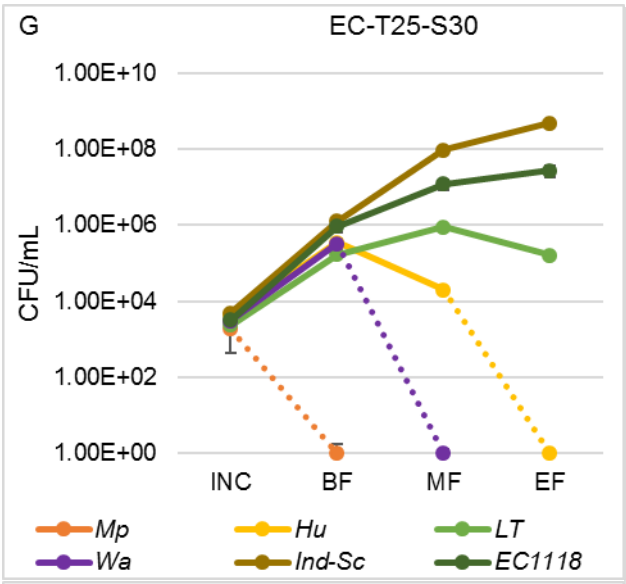
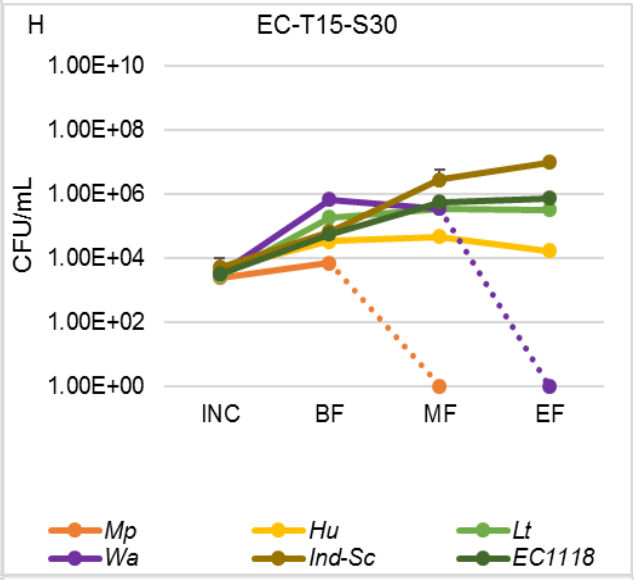
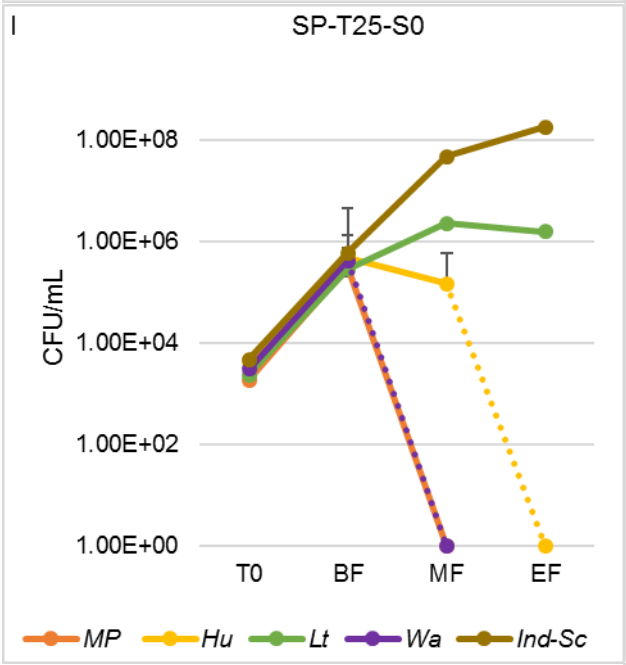


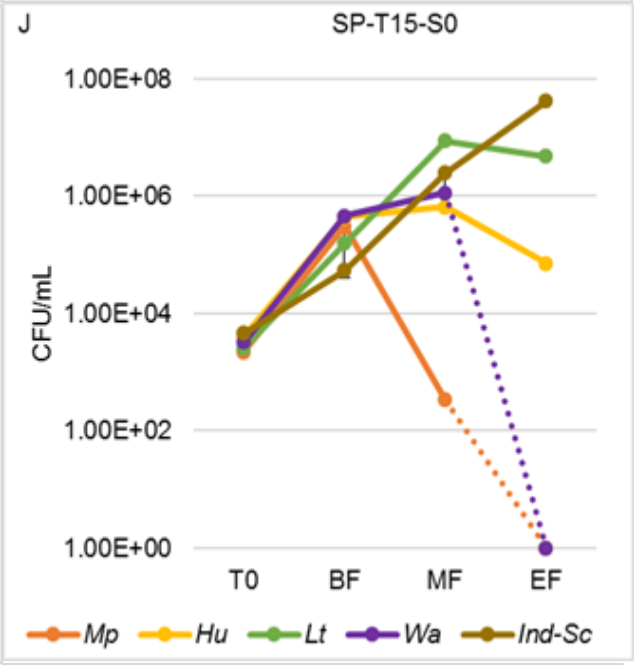
Figure.5.S1. The growth pattern of yeast species throughout the fermentations performed by inoculating the yeast consortium in the synthetic must.

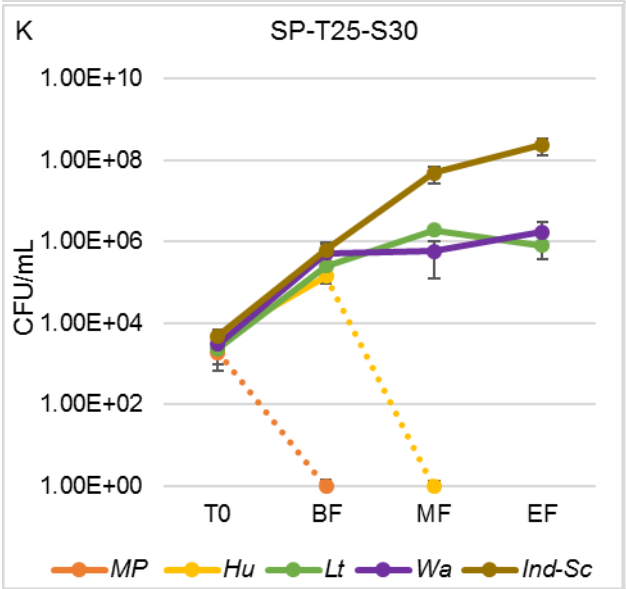












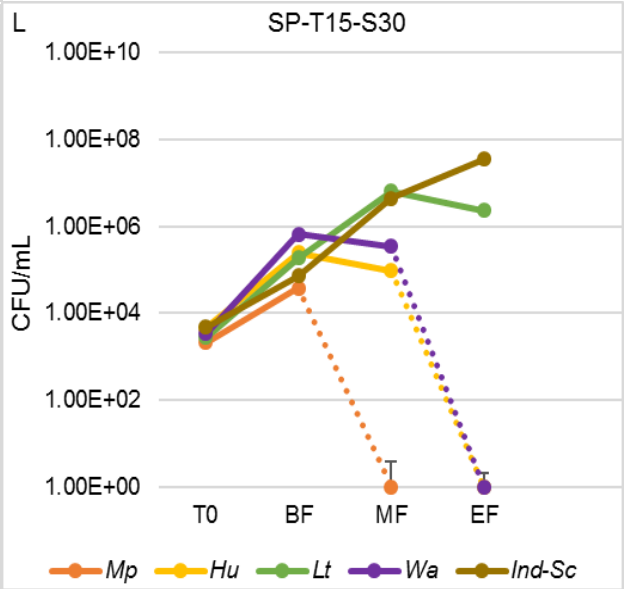
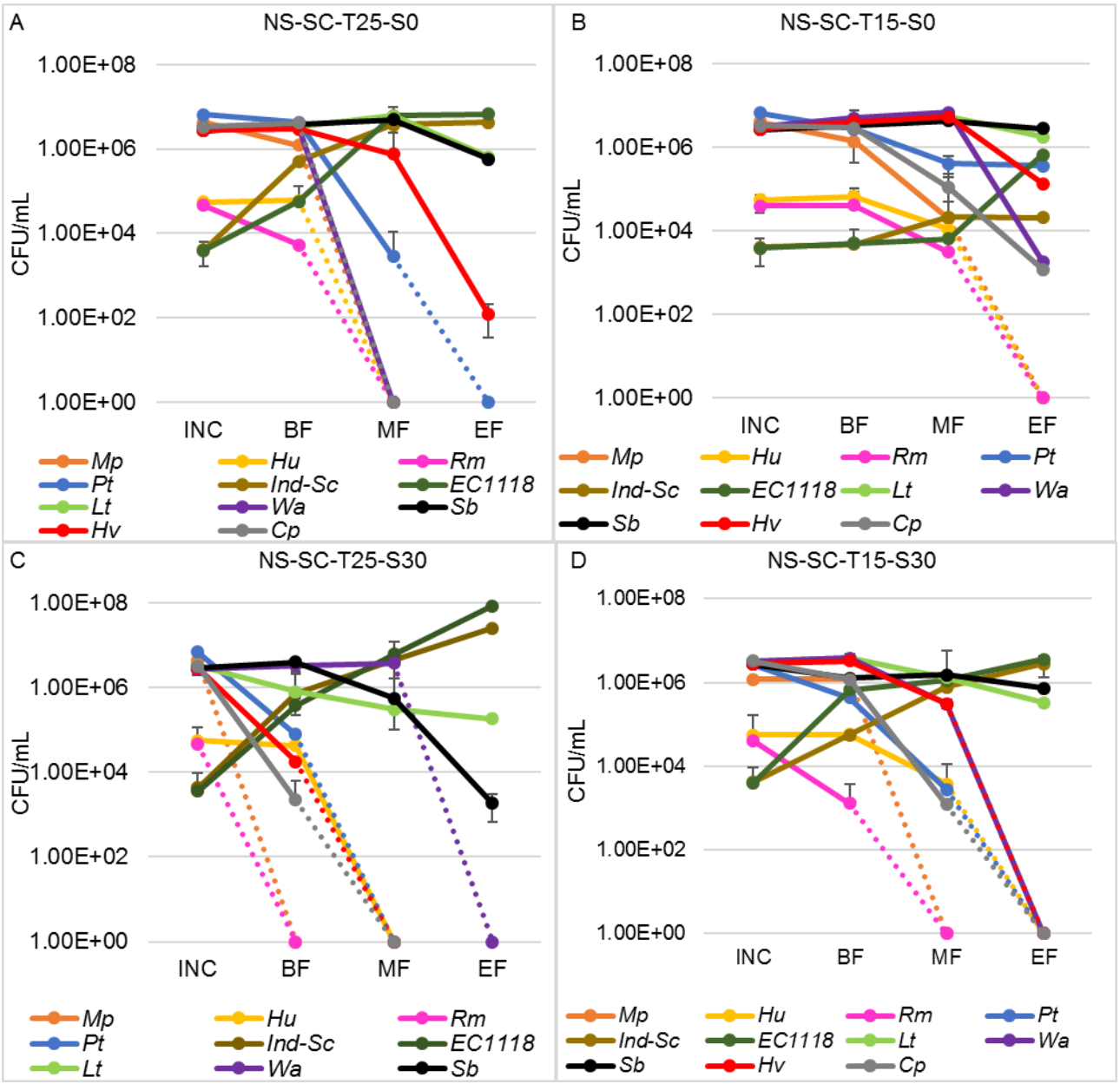
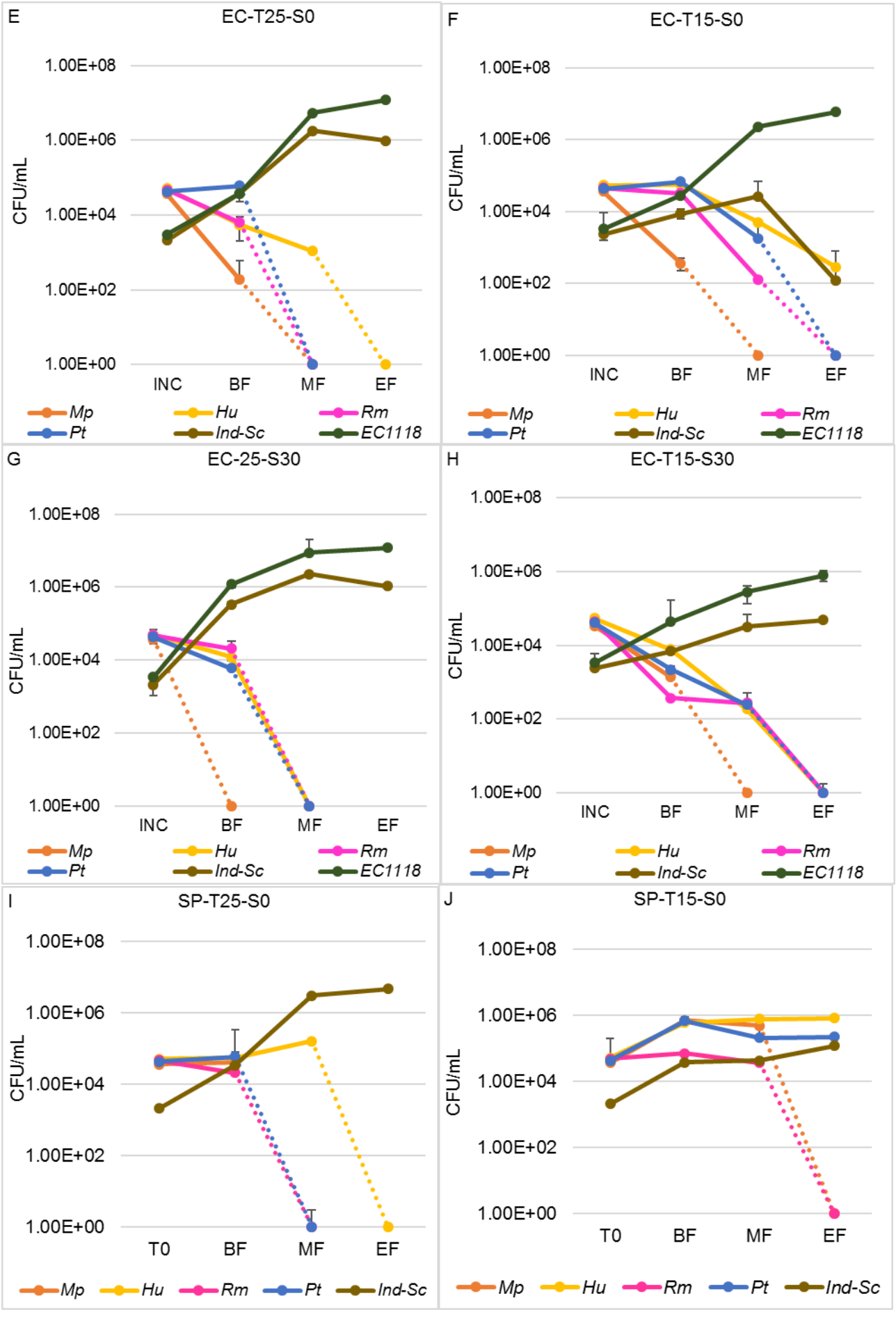


Figure.5.S2. Yeast population dynamics in the Chenin blanc fermentations inoculated with the consortium (NS-SC), with EC1118 (EC) as well as the spontaneous fermentations in South Africa. The fermentations were conducted at 25°C and 15°C degrees (T25 and T15) with and without sulphur dioxide (S30 and S0).





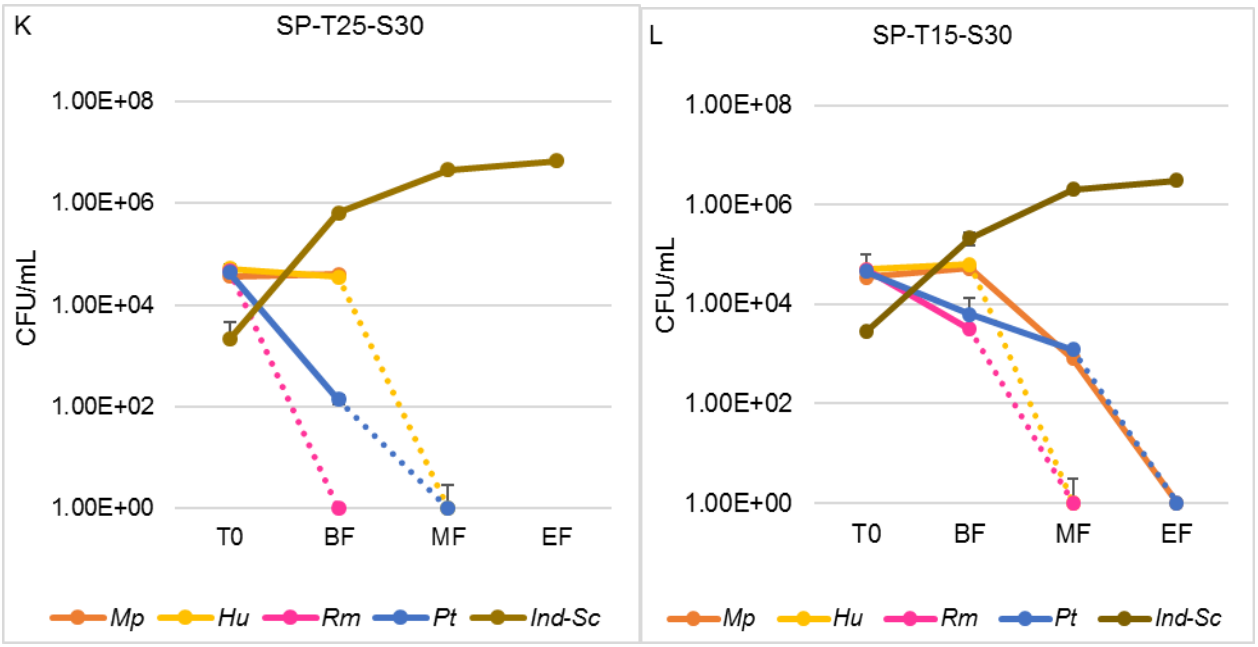


Figure.5.S3. The dynamics of yeast species in Greccheto Bianco fermentations inoculated with the consortium (NS-SC), with EC1118 (EC) and the spontaneous fermentation (SP) in Italy. The fermentations were performed at two temperatures (T15 and T25) with and without sulphur dioxide (S30 and S0).

Chapter 6

General discussion and conclusion

6.1. General discussion and conclusion

Wine is traditionally produced by spontaneous fermentation of grape must where the complex interactions among indigenous microbial population play the fundamental role in the process (Sun *et al*., 2009; Bagheri *et al*., 2015; Eder *et al*., 2017; De Filippis *et al*., 2017). Among other organisms present in grape must, yeasts are the most relevant organism in wine fermentation (Ciani *et al*., 2006; Ciani *et al*., 2010). Consequently, safety, quality, and acceptability of the final product mainly rely on yeast-yeast interactions (Fleet, 2003; Ciani *et al*., 2006; Belda *et al*., 2017). Thus, *Saccharomyces* species and single non-*Saccharomyces* species have been studied extensively. However, due to the complexity of the wine ecosystem, most studies have limited their investigations on the mixed culture fermentations, mostly of two species (Sadoudi *et al*., 2012; Gobbi *et al*., 2013; Shekhawat *et al*., 2017). Consequently, the yeast-yeast interactions in the wine ecosystem have not been studied systematically (Padila *et al*., 2016; Mateo and Maicas, 2016).

To address this issue we have developed a multi-species yeast consortium of 8 species that are representative of the South African grape must microbiota. The study aimed to use this model consortium as a tool to understand the yeast-yeast interactions in the wine fermentation. A reliable culture-independent method, (ARISA) was also developed to monitor the population dynamics of the yeast consortium in the synthetic must. The model consortium was used to evaluate the effect of the presence of *Saccharomyces cerevisiae* on the dynamics of yeast consortium. In order to evaluate the suitability of the yeast consortium to predict the population dynamics in wine fermentation, the consortium was used as an inoculum and the fermentations were performed in Chenin blanc must. Taking into consideration the limitation of ARISA, the standard plating method was used to monitor the yeast population dynamics in the Chenin blanc fermentation.

Firstly, the data indicated that the selective pressure applied by *S. cerevisiae* suppressed the growth of some species (*Wickerhamomyces anomalus*) while promoting the growth of others (*Hanseniaspora vineae*) in the synthetic must fermentation. Our data confirmed that *S. cerevisiae* is the keystone species in the wine ecosystem. Identifying the interactions between the keystone species, *S. cerevisiae* and other species in wine fermentation can play significant roles in the final quality of the wine. Previous studies have confirmed that the aromatic property of wine is affected positively or negatively by the interactions between *S. cerevisiae* and non-*Saccharomyces* species (Suzzi *et al*., 2012; Gobbi *et al*., 2013). To the best of our knowledge, this was the first time that the supportive and inhibitory interactions between *S. cerevisiae* and other non-*Saccharomyces* species have been reported in a multi-species wine consortium. However, further investigations are required to untangle the mechanism underlying the antagonism and mutualism observed in the wine ecosystem. Based on the existing data, antagonistic activities of yeast species have made positive contributions to several fields including food fermentation, agriculture, and medicine

(Hatoum *et al*., 2012). For instance, the growth of *Brettanomyces* wine spoilage yeast) was inhibited by the killer toxin produced by *W. anomalus* (Comitini *et al*., 2004a&b).

Secondly, the data showed that there was a high similarity between the yeast diversity in the Chenin blanc must and the selected consortium. Despite the considerable differences between the two matrices, Chenin blanc fermentation confirmed the interactions observed in the synthetic must. These observations suggested that the model consortium has a broad applicability to predict the population dynamics from a simple consortium to the wine fermentation. Such model systems can have a great potential to study microbial interactions in various industries. Food and beverage industry today is dealing with huge economic losses as a consequence of a limited understanding of ecological interactions in food fermentation. Phage attack in the dairy industry, the growth of foodborne pathogen *L. monocytogenes* in salami fermentation and production of off-flavour compounds by spoilage organisms in wine, have led to huge economic losses in the food and beverage industries (Seman *et al*., 2001; Malherbe *et al*., 2007; Sieuwerts.*et al*., 2008; Sablayrolles, 2009).

In order to investigate how far we can use the model consortium to learn about yeast-yeast interactions, variation in cell density was used as a tool to better understand how an increase in the cell density of one species can affect the growth of others in the consortium. Furthermore, we aimed to evaluate the contribution of these consortia to the wine aroma and attempted to link such changes to the modified population dynamics. Our data revealed that presence of one species at a high density may promote or inhibit the growth of others in the wine ecosystem. Furthermore, variation in the initial cell densities of the yeast species changed the nature of the ecological interactions in the wine ecosystem. The synergistic interaction between *H. vineae* and *S. cerevisiae* was modified when *H. vineae* was the only member of the consortium inoculated at significantly high cell density. In this scenario, the fermentation kinetics was negatively affected possibly due to the presence of strong competition between the yeast species for the nutrient. In contrast, the fermentation kinetics was enhanced when *S. bacillaris* was inoculated at a high cell density, suggesting the synergistic interaction between *S. cerevisiae* and *S. bacillaris*. The mechanisms underlying these interactions require further investigation.

It is worth mentioning that we might have missed some information by not measuring the other fermentation metabolites such as glycerol and ethanol. However, several studies have demonstrated that some non-*Saccharomyces* species *(e.g. S. bacillaris* and *M. pulcherrima* and *Hanseniaspora uvarum*) produce wine with higher levels of glycerol and lower levels of ethanol (Moreira *et al*., 2008; Sadineni *et al*., 2012; Englezos *et al*., 2015; Morales *et al*., 2015; Shekhawat *et al*., 2017).

Concerning the effect of the treatments on the aroma profiles of wine, our results are in line with previous studies confirming that the signature of non-*Saccharomyces* species can be detected on the aromatic profile of wine when they persist longer in the wine fermentation (Sadoudi *et al*., 2012; Gobbi *et al*., 2013; Jood *et al*., 2017). Thus, using the variation of cell densities, we could confirm that the production of specific major volatiles was enhanced by the higher inoculation of specific species, suggesting that some species may contribute more to the production of specific compounds in the wine aroma.

The results obtained by modifying the cell densities can have huge implications for the wine industry as well as other food industries. Boutique wineries are still using spontaneous fermentations that rely on the indigenous population present in the grape microflora (Steensels and Verstrepen, 2014). Previous studies have shown that microbial ecosystems diverge significantly according to the region (microbial *terroir*), suggesting the regional signature of wine microflora to wine aroma (Bokulich *et al*., 2013; Gilbert *et al*., 2014). Our results confirmed that investigating the microbial interactions in a simplified model system which represent the regional grape microflora, may allow us to unravel the potential of indigenous species in the aromatic profile of the wine. Such studies can also provide valuable information for winemakers that use inoculation strategy to produce wine. Nowadays several non-*Saccharomyces* species have been commercialized as a single culture (e.g. *Lachancea thermotolerans* (Viniflora® Concerto™, Chr. Hansen, Denmark) or in combination with *S. cerevisiae* (e.g. *L. thermotolerans* and *S. cerevisiae* (Rhythm/Symphony, Chr. Hansen, Hørsholm, Denmark). In case of inoculated fermentation, the result underlined that the growth of some indigenous non-*Saccharomyces* species present in grape must can be enhanced by the inoculated starter culture. Consequently, in order to ascertain the successful application of the starter cultures, it is important to evaluate their interactions with other members of the wine consortia, particularly the species with potential to produce off-flavour compounds.

In the last objective of the project, we have evaluated the effect of temperature and sulphur dioxide on the population dynamics in synthetic must and two grape must matrices. Ultimately, this objective aimed to evaluate the robustness of the yeast consortium to predict the yeast-yeast interaction in wine fermentation. First, our results confirmed the previous reports, indicating that the growth of *S. cerevisiae* was negatively affected by low temperature whereas the growth of some non-*Saccharomyces* species was enhanced by the low temperature (Torija *et al*., 2003; Salvadò *et al*., 2011; Alonso-del-Real *et al*., 2017). Our data underlined the high possibility of stuck fermentation in spontaneously fermented juice at a lower temperature, particularly in the scenario where a grape must has a high diversity of non-*Saccharomyces* species. We propose that inoculation of grape juice with a commercial *S. cerevisiae* at a low inoculum dosage (10^3-10^4 CFU/mL) may provide a solution. Such a strategy can ensure the completion of fermentation and

can simultaneously ensure the contribution of non-*Saccharomyces* species in the organoleptic properties of wine.

Concerning the effect of sulphur dioxide, the result indicated that some non-*Saccharomyces* species were less sensitive while others were inhibited by sulphur addition. This result was in agreement with the previous report by Jolly *et al*., (2003), and Albertin *et al*., (2014). However, our result for the first time underlined that the growth of some non-*Saccharomyces* (e.g. *W. anomalus*) was promoted by sulphur addition, suggesting that this species may have developed adaptation mechanism to survive under sulphur stress. It has been previously reported that sulphur dioxide affects the growth of non-*Saccharomyces* species in a dose-dependent manner. However, the dosage of sulphur dioxide (30 mg/L) in the current experiment was selected with the reference to the applied levels in the wine industry in South Africa (Morgan et al., 2017; Bokulich et al., 2015). Furthermore, sulphur dioxide displayed a stronger inhibitory effect on the growth of some non-*Saccharomyces* species at a lower temperature, suggesting that effect of sulphur dioxide on the growth of yeast species is temperature-dependent.

Taken together, our results opened up new avenues to investigate the mechanism underlying the persistence of *W. anomalus* under sulphur addition. Currently, we are unable to confirm if the persistence of some individuals in the presence of ionsulphur dioxide is due to the high resistance of this strain to SO_2 or it is due to the fact that ecosystems are most robust to the perturbations. Thus, evaluating the resistance of the individual strains against sulphur dioxide would add more insight in the yeast-yeast interactions observed in this consortium. Furthermore, there is no information available on why some non-*Saccharomyces* species are more resistant to sulphur dioxide at low- temperature fermentation. Thus, future studies need to include the spoilage organisms in the multi-species consortium to investigate how these species react to the presence of sulphur dioxide at a low temperature and how they interact with the other members of the consortium.

Our result revealed the significance of the ecological interaction in the wine ecosystem. The ecological interactions and wine aroma were different when the consortium was used as an inoculum compared to the control fermentations. The data underlined that temperature and sulphur dioxide triggered the shift in the population dynamics. However, species richness and the ecological interactions seem to be the main drivers of the ecosystem.

Lastly and most importantly, the population dynamics of yeast consortium in the real grape matrices to a large extent resembled the trends observed in the synthetic must fermentation. This observation confirmed that the yeast consortium is a robust model that can be used as a tool to predict the population dynamics in wine fermentation. Deep understanding of yeast-yeast interactions would be critical for the proper control of the fermentation processes. This approach can eventually provide guidelines to winemakers on how to boost positive or eliminate negative

contributions of specific species in wine fermentations. However, in order to fully utilize the knowledge of yeast-yeast interactions in wine fermentation, a fast, easy-to-use and cost-effective method is required to assess the composition of the grape microflora. Such a method is currently not available. However, taking into account the advances in metagenomics, metatranscriptomics and, metaproteomics over the last two decades, developing such a technology might not be far-reaching.

In conclusion, the current study for the first time evaluated the yeast-yeast interactions in a multi-species yeast consortium. The result underlined the importance of ecological interactions for the proper control of wine fermentation. Furthermore, the study confirmed the suitability and applicability of the model consortium as a reliable tool to predict ecological interactions in wine ecosystem. This result has an important implication in the future of microbial ecology and food industry. The amount of knowledge that can be extracted by developing model consortia has not been recognized before in wine research. Wine research needs to shift from mixed culture fermentation toward developing model consortia with more complex compositions to study the ecological interactions that may ultimately lead to better management of wine fermentation. However, the synergy between top-down and bottom-up approach can have great applications in the microbial ecology.

6.2. References

Alonso-del-Real, J., Contreras-Ruiz, A., Castiglioni, G. L., Barrio, E., and Querol, A. (2017). The use of mixed populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to reduce ethanol content in wine: Limited Aeration, inoculum proportions, and sequential inoculation. *Front. Microbiol.* 8. doi:10.3389/fmicb.2017.02087.

Bagheri, B., Bauer, F. F., Setati, M. E. (2015). The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. S*. Afr. J. Enol. Vitic.* 36, 243–251.

Belda, I., Ruiz, J., Fernandez, A-E., Navascues, E., Marquina, D., Santos, A., et al., (2017). Microbial contribution to wine aroma and its intended use for wine quality improvement. *Molecules*. 22, 189.

Bokulich, N. A., Ohta, M., Richardson, P. M., and Mills, D. A. (2013). Monitoring Seasonal Changes in Winery-Resident Microbiota. *PLoS One* 8. doi:10.1371/journal.pone.0066437.

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

Ciani, M., Comitini, F., Mannazzu, I., Domizio, P. (2010). Controlled mixed culture fermentation: A new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research*, *10*, 123–133. https://doi.org/10.1111/j.1567-1364.2009.00579.x.

Comitini, F., De Ingeniis, J., Pepe, L., Mannazzu, I., Ciani, M. (2004a). *Pichia anomala* and *Kluyveromyces wickerhamii* killer toxins as new tools against *Dekkera/Brettanomyces* spoilage yeasts. *FEMS Microbiol*. *Lett*. 238, 235–240.

Comitini, F., Di Pietro, N., Zacchi, L., Mannazzu,I., Ciani,M.(2004b). *Kluyveromyces phaffii* killer toxin active against wine spoilage yeasts: purification and characterization. Microbiology 150, 2535–2541.

De Filippis, F., Parente, E., and Ercolini, D. (2017). Metagenomics insights into food fermentations. *Microb. Biotechnol.* 10, 91–102. doi:10.1111/1751-7915.12421.

Eder, M. L. R., Reynoso, C., Lauret, S. C., Rosa, A. L., and Rosa, A. L. (2017). Isolation and Identification of the Indigenous Yeast Population during Spontaneous Fermentation of Isabella (*Vitis labrusca L .*) Grape Must. 8, 1–8. doi:10.3389/fmicb.2017.00532.

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., and Cocolin, L. (2015). Exploitation of the non-Saccharomyces yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: Physiological and molecular characterizations. *Int. J. Food Microbiol.* 199, 33–40.

Fleet, G. H., (2003). . Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11-22.

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

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

Hatoum, R., Labrie, S., and Fliss, I. (2012). Antimicrobial and probiotic properties of yeasts: From fundamental to novel applications. *Front. Microbiol.* 3, 1–12. doi:10.3389/fmicb.2012.00421.

Jood, I., Hoff, J. W., and Setati, M. E. (2017). Evaluating fermentation characteristics of *Kazachstania* spp. and their potential influence on wine quality. *World J. Microbiol. Biotechnol.* doi:10.1007/s11274-017-2299-1.

Mateo, J. H., Maicas, S (2016). Application of Non-*Saccharomyces* Yeasts to Wine-Making Process.

Fermentation, 2, 14.

Marais, J., (1998). Effect of grape temperature, oxidation and skin contact on Sauvignon blac juice and wine composition and wine quality. *S. Afr. J. Enol. Vitic*,19, 1.

Malherbe, S., Bauer, F.F., Du toit, M., (2007). Understanding Problem Fermentations – A Review. *S. Afr. J. Enol. Vitic*., 28,2.

Morales, P., Rojas, V., Quirós, M., and Gonzalez, R. (2015). The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. *Appl. Microbiol. Biotechnol.* 99, 3993–4003. doi:10.1007/s00253-014-6321-3.

Moreira, N., Mendes, F., Guedes de Pinho, P., Hogg, T., and Vasconcelos, I. (2008). Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *Int. J. Food Microbiol.* 124, 231–238. doi:10.1016/j.ijfoodmicro.2008.03.025.

Padilla, B., Gil, J. V., Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Front in Microbiol*, *7*, 1–20.

Sablayrolles, J. M., (2009). Control of alcoholic fermentation in winemaking: Current situation and prospect. *Food research international*. 42, 418-424.

Sadineni, V., Kondapalli, N., and Reddy Obulam, V. S. (2012). Effect of co-fermentation with Saccharomyces cerevisiae and Torulaspora delbrueckii or Metschnikowia pulcherrima on the aroma and sensory properties of mango wine. *Ann. Microbiol.* 62, 1353–1360. doi:10.1007/s13213-011-0383-6.

Sadoudi, M., Tourdot-Marechal, R., Rousseaux, S., Steyer, D., Gallardo-Chacon, J. J., Ballester, J., *et al* (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol* 32: 243–253.

Salvadó, Z., Arroyo-López, F. N., Barrio, E., Querol, A., and Guillamón, J. M. (2011). Quantifying the individual effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 1155–1161. doi:10.1016/j.fm.2011.03.008.

Seman, D. L., Borger, A. C., Meyer, J. D., Hall, P. A., Milkowski, A. L., (2002). Modeling the Growth of *Listeria monocytogenes* in Cured Ready-to-Eat Processed Meat Products by Manipulation of Sodium Chloride, Sodium Diacetate, Potassium Lactate, and Product Moisture Content. *Journal of Food Protection*, 65, 651–658.

Shekhawat, K., Bauer, F. F., and Setati, M. E. (2017). Impact of oxygenation on the performance of three non-Saccharomyces yeasts in co-fermentation with Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-016-8001-y.

Sieuwerts, S., de Bok, F.A.M., Hugenholtz, J., van Hylckama Vlieg, J.E.T., (2008). Unraveling microbial interactions in food fermentations: from classical to genomics approaches. *Appl. Environ. Microbiol*. 74, 4997–5007.

Steensels, J., Verstreoen, K. J., (2014). Taming Wild Yeast: Potential of Conventional and Nonconventional Yeasts in Industrial Fermentations. *Annu. Rev. Microbiol.* 68:61–80.

Sun, H., Ma, H., Hao, M., Pretorius, I. S., and Chen, S. (2009). Identification of yeast population dynamics of spontaneous fermentation in Beijing wine region, China. *Ann. Microbiol.* 59, 69–76.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., et al. (2012). Multistarter from organic viticulture for red wine Montepulciano d'Abruzzo production. *Front. Microbiol.* 3, 1–10.

Torija, M. J., Beltran, G., Novo, M., Poblet, M., Guillamón, J. M., Mas, A., et al. (2003). Effects of fermentation temperature and *Saccharomyces* species on the cell fatty acid composition and presence of volatile compounds in wine. *Int. J. Food Microbiol.* 85, 127–136. doi:10.1016/S0168-1605(02)00506-

Viana, F., Gil, JV., Valles, S., Manzanares, P., (2009). Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae. Int J Food Microbiol,* 135:68–74.

Vigentini, I., Fabrizio, V., Faccincani, M., Picozzi, C., Comasio, A., and Foschino, R. (2014). Dynamics of *Saccharomyces cerevisiae* populations in controlled and spontaneous fermentations for Franciacorta D.O.C.G. base wine production. *Ann. Microbiol*. 64, 639–651.

APPENDIX I

The Impact of *Saccharomyces cerevisiae* on a wine yeast consortium in natural and inoculated fermentations

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Author Contributions

FFB and MES conceptualized the study.
BB, FFB and MES designed the experimental layout.
BB performed the experiments, analyzed the data and wrote the first draft of the manuscript. BB, FFB and MES edited subsequent drafts, read and approved the final manuscript

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The Impact of *Saccharomyces cerevisiae* on a Wine Yeast Consortium in Natural and Inoculated Fermentations

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Natural, also referred to as spontaneous wine fermentations, are carried out by the native microbiota of the grape juice, without inoculation of selected, industrially produced yeast or bacterial strains. Such fermentations are commonly initiated by non-*Saccharomyces* yeast species that numerically dominate the must. Community composition and numerical dominance of species vary significantly between individual musts, but *Saccharomyces cerevisiae* will in most cases dominate the late stages of the fermentation and complete the process. Nevertheless, non-*Saccharomyces* species contribute significantly, positively or negatively, to the character and quality of the final product. The contribution is species and strain dependent and will depend on each species or strain's absolute and relative contribution to total metabolically active biomass, and will therefore, be a function of its relative fitness within the microbial ecosystem. However, the population dynamics of multispecies fermentations are not well understood. Consequently, the oenological potential of the microbiome in any given grape must, can currently not be evaluated or predicted. To better characterize the rules that govern the complex wine microbial ecosystem, a model yeast consortium comprising eight species commonly encountered in South African grape musts and an ARISA based method to monitor their dynamics were developed and validated. The dynamics of these species were evaluated in synthetic must in the presence or absence of *S. cerevisiae* using direct viable counts and ARISA. The data show that *S. cerevisiae* specifically suppresses certain species while appearing to favor the persistence of other species. Growth dynamics in Chenin blanc grape must fermentation was monitored only through viable counts. The interactions observed in the synthetic must, were upheld in the natural must fermentations, suggesting the broad applicability of the observed ecosystem dynamics. Importantly, the presence of indigenous yeast populations did not appear to affect the broad interaction patterns between the consortium species. The data show that the wine ecosystem is characterized by both mutually supportive and inhibitory species. The current study presents a first step in the development of a model to predict the oenological potential of any given wine mycobiome.

Keywords: yeast consortium, population dynamics, yeast interactions, wine fermentation, ARISA

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INTRODUCTION

The alcoholic fermentation of grape must, whether inoculated or not with commercial starter cultures, is initiated by a complex yeast community comprising a high proportion of oxidative and weakly fermentative yeasts (Jolly et al., 2003a; Ghosh et al., 2015; Wang et al., 2015). These species are rapidly outgrown by strongly fermentative yeasts that dominate the middle and end of fermentation (Pretorius et al., 1999; Jolly et al., 2003b; Zott et al., 2008; Bagheri et al., 2015; Ghosh et al., 2015; Setati et al., 2015; Wang et al., 2015; Morgan, 2016; Portillo et al., 2016; Tristezza et al., 2016). The growth and metabolic activity of these yeast species are influenced by physicochemical conditions that prevail during the fermentation process including the rapid depletion of nutrients and oxygen and the accumulation of ethanol (Sainz et al., 2003; Mendoza et al., 2009). However, beyond such environmental or chemical factors, ecological interactions between yeast species will primarily determine the wine fermentation dynamics and the outcome of the fermentation process (Nissen and Arneborg, 2003; Pina et al., 2004; Sadoudi et al., 2012; Renault et al., 2013; Morales et al., 2015; Wang et al., 2015; Shekhawat et al., 2017). For many years, research evaluated interactions between strains of *S. cerevisiae*, the main wine fermenting yeast, with a focus on killer toxin-producing strains (Branco et al., 2014; Williams et al., 2015; Albergaria and Arneborg, 2016; Pérez-Torrado et al., 2017). However, with the growing interest in non-*Saccharomyces* yeast species and the commercialization of a few species for use as co-inoculants in controlled mixed starter fermentations, attention has turned toward evaluating yeast–yeast interactions holistically (Ciani and Comitini, 2015; Albergaria and Arneborg, 2016; Ciani et al., 2016; Wang et al., 2016). Undoubtedly, wine microbial consortia are difficult to scrutinize. Consequently, some studies have employed simplified models in which the interaction between two species mainly *S. cerevisiae* and non-*Saccharomyces* species were investigated (Andorra et al., 2011; Wang et al., 2014; Englezos et al., 2015; Shekhawat et al., 2017). Several aspects, including inoculum ratio, the timing of inoculation of *S. cerevisiae*, cell-cell contact and production of inhibitory metabolites, have been investigated in order to decipher the mechanisms underlying yeast–yeast interactions during wine fermentation (Gobbi et al., 2013; Branco et al., 2014, 2015; Izquierdo Cañas et al., 2014; Kemsawad et al., 2015; Lencioni et al., 2016). Despite these efforts, the overall interactions among wine yeast species in a fermentation modulated by multiple species remain unclear.

Synthetic microbial consortia composed of a subset of culturable strains that simulate the natural community and preserve the indigenous interactions shaped by co-adaptation/evolution, provide a tractable model system with reduced complexity (De Roy et al., 2014; Ponomarova and Patil, 2015), which makes it easier to study interspecific interactions (Jagmann and Philipp, 2014; Jiang et al., 2017). Such a model system also opens opportunities to employ methods inapplicable to complex systems, e.g., species quantitation can easily be done with selective plating, quantitative PCR, fluorescent *in situ* hybridization (FISH), and flow cytometry (Xufre et al., 2006; Grube and Berg, 2009; Zott et al., 2010; Ponomarova and Patil, 2015). These methods have been applied successfully to monitor population dynamics in wine fermentation. However, they are not without limitations. For instance, FISH and qPCR, require species-specific probes and primers whereas, flow cytometry requires prior knowledge of initial microbial population in order to label different species (Deere et al., 1998; Malacrinò et al., 2001; Prakitchaiwattana et al., 2004; Hierro et al., 2006a; Xufre et al., 2006; Andorrà et al., 2010a,b; Zott et al., 2010). In contrast, Automated Ribosomal Intergenic Spacer Analysis (ARISA), which mainly relies on the heterogeneity of the ITS1-5.8S rRNA-ITS2 gene, has been used successfully in several ecological studies (Brežná et al., 2010; Kraková et al., 2012; Ghosh et al., 2015). Like other methods, ARISA may also introduce bias since it is unable to differentiate live and dead cells. However, ARISA is an efficient and rapid tool that can provide a snapshot of the population dynamics (Hierro et al., 2006a; Ramette, 2009; Brežná et al., 2010; Kraková et al., 2012; O'Sullivan et al., 2013; Cangelosi and Meschke, 2014; Ženišová et al., 2014; Ghosh et al., 2015).

The current study aimed to evaluate the application of a multi-species yeast consortium as a tool to investigate population dynamics and yeast–yeast interactions in wine fermentation. The constructed model consortium resembles natural wine yeast consortia in so far as comprising species with different fermentative capacities (i.e., weakly fermentative, medium fermentation capacity and strongly fermentative). Moreover, the consortium was formulated based on species that have been encountered and found in sometimes dominant numbers in grape musts from different South African wine regions and cultivars (Jolly et al., 2003a; Weightman, 2014; Bagheri et al., 2015; Ghosh et al., 2015; Morgan, 2016). The model consortium was evaluated in synthetic must in the presence and absence of *S. cerevisiae*, as well as in a real grape juice that differed significantly from the synthetic must. To allow for a rapid and accurate monitoring of the population dynamics, ARISA was optimized and assessed for its suitability and reliability as a tool to semi-quantitatively monitor yeast dynamics in the model consortium.

The data show that *S. cerevisiae* strongly and specifically suppresses certain non-*Saccharomyces* yeast species, while also favoring the persistence of other species. The findings suggest that the complex modulation of the yeast ecosystem by *S. cerevisiae* will influence the outcome of wine fermentation by selectively changing the contribution of non-*Saccharomyces* species.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

Sixteen yeast isolates obtained from the culture collection of the Institute for Wine Biotechnology (IWBT) and two commercial yeast species, *S. cerevisiae* Lalvin EC1118 (Lallemand, Canada) and *Torulaspora delbrueckii* BIODIVA (Lallemand, Canada) were used in this study (**Table 1**). The yeast stock cultures were maintained in 20% (v/v) glycerol at −80°C and were streaked out on Wallerstein Laboratory Nutrient agar (WLN) (Sigma–Aldrich,

TABLE 1 | Strains used in this study and their ITS1-5.8S rRNA-ITS2 gene sizes.

Species	Strains number	ITS Size (bp)
Hanseniaspora uvarum (Hu)	Y1104	747
Hanseniaspora vineae (Hv)	Y980	740
Hanseniaspora opuntiae (Ho)	Y866	748
Pichia terricola (Pt)	Y974	419
Issatchenkia orientalis (Io)	Y1130	490
Starmerella bacillaris (Sb)	Y975	458
Candida apicola (Cap)	Y957	457
Candida azyma (Ca)	Y979	436
Candida parapsilosis (Cp)	Y842	522
Candida glabrata (Cg)	Y800	884
Torulaspora delbrueckii (Td)	BIODIVA	797
Rhodotorula glutinis (Rg)	Y824	614
Rhodosporidium diobovatum (Rd)	Y840	618
Kazachstania aerobia (Ka)	Y845	751
Lachancea thermotolerans (Lt)	Y973	675
Saccharomyces cerevisiae (Sc)	EC1118	842
Wickerhamomyces anomalus (Wa)	Y934	618
Metschnikowia pulcherrima (Mp)	Y981	377

Spain) when required. The plates were incubated at 30°C for 3–5 days.

Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Single colonies of each yeast species were inoculated into 5 mL YPD broth (10 g/L yeast extract, 20 g/L peptone and, 20 g/L glucose) and incubated for 16 h at 30°C. Two milliliters of cultures were centrifuged at 5630 × *g* for 10 min to collect the cells. Genomic DNA was extracted using the method described by Sambrook and Russell (2006). DNA concentration was determined spectrophotometrically, using the NanoDrop®ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, United States). The ITS1-5.8S rRNA-ITS2 gene was amplified using the carboxy-fluorescein labeled ITS1 primer (5′-6-FAM- TCC GTA GGT GAA CCT TGC GG-3′) and ITS4 (5′- TCC GTA GGT GAA CCTTGC GG-3′) in a 25 μL reaction, containing 50 ng DNA, 1U Takara Ex Taq, DNA polymerase (TaKaRa Bio Inc., Olsu, Shiga, Japan), 1 × Taq buffer, 0.25 μM of each primer, 400 μM dNTP mix and 1 mM $MgCl_2$. The PCR reaction was performed under the following conditions: an initial denaturation of 3 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 45 s and a final extension step of 72°C for 10 min (Slabbert et al., 2010). Three independent PCR reactions were performed. The PCR products were excised from the gel and purified using the Zymoclean™ Gel DNA Recovery Kit Short Protocol (Zymo Research Corporation, Irvine, CA, United States). The ARISA fragments were separated by capillary electrophoresis at the Stellenbosch University Central Analytical Facility on an ABI 3010x Genetic Analyzer (Applied Biosystems) with a ROX 1.1 labeled size standard (75-1121 base pairs). ARISA profiles were analyzed using Genemapper software version 4.1 (Applied Biosystems). Only fragments with peak area larger than 0.5% of the total fluorescence were considered for further analysis. A bin size of 3 bp for species with ITS region below 700 and 5 bp for species with ITS region above 700 bp, was employed to minimize the inaccuracies in the ARISA analysis (Slabbert et al., 2010). The relative abundance of each peak was calculated by dividing individual peak area with the total peak areas for the respective sample.

Micro-Fermentations

Fermentation in Synthetic Grape Must

Eight yeast species *viz. Metschnikowia pulcherrima, Pichia terricola, Starmerella bacillaris, Candida parapsilosis, Wickerhamomyces anomalus, Lachancea thermotolerans, Hanseniaspora vineae,* and *S. cerevisiae* were selected to establish a consortium based on (i) their frequent occurrence in grape juices from SA and other wine producing regions, (ii) easy and consistent resolution in ARISA, and (iii) easy morphological detection on WL agar (Jolly et al., 2003a; Combina et al., 2005; Di Maro et al., 2007; Lopandic et al., 2008; Romancino et al., 2008; Salinas et al., 2009; Sun et al., 2009; Suzzi et al., 2012; Weightman, 2014; Maturano et al., 2015; Morgan, 2016). Fermentations were carried out, by inoculating the selected yeast species, in synthetic grape juice medium (pH 3.5) adapted from Bely et al. (1990) and Henschke and Jiranek (1993). The medium contained 200 g/L sugars (100 g/L glucose and 100 g/L fructose) and 300 mg/L assimilable nitrogen (460 mg/L NH_4Cl and 180 mg/L amino acids). Five hundred milliliters of the juice was dispensed into 500 mL Erlenmeyer flasks, fitted with CO_2 traps. The juice was inoculated with the *NS-Sc* (non-*Saccharomyces-Saccharomyces*) consortium comprising of 7 non-*Saccharomyces* yeast species (*M. pulcherrima*, *P. terricola*, *S. bacillaris*, *C. parapsilosis*, *W. anomalus*, *L. thermotolerans,* and *H. vineae*), each inoculated at 10^6 cells/mL and *S. cerevisiae* at 10^3 cells/mL, and the *NS* (non-*Saccharomyces*) consortium which only consisted of the seven non-*Saccharomyces* yeasts. The fermentations were performed at 25°C with no agitation. Fermentations were monitored by weighing the flasks regularly to measure CO_2 loss. Furthermore, samples were collected regularly to determine sugar concentrations using Fourier Transform Infra-Red Spectroscopy on the Foss Wine scan 2000 (Rhine Ruhr, Denmark). Samples were withdrawn at 2-day intervals and yeast population dynamics was monitored by direct plating on WLN agar and ARISA.

Real Must Fermentation

Fifty liters of clarified Chenin blanc grape juice was obtained from a commercial cellar. The chemical composition of juice was measured, using spectroscopy technique by Foss wine scan 2000 (Rhine Ruhr, Denmark). The yeast community composition of the juice was determined by serial dilution and direct plating on WL-agar, followed by identification through ITS-5.8S rRNA amplification, RFLP, and sequencing as described in Bagheri et al. (2015). Subsequently, 1.5 L Chenin blanc grape juice was dispensed into 2 L fermentation bottles. Three sets of fermentations were performed: (i) spontaneous (ii) *Sc*-inoculated fermentation (at 10^3 cells/mL, *S. cerevisiae* EC1118), and (iii) *NS-Sc* consortium inoculated (7 non-*Saccharomyces* at 10^6 cells/mL

vs. *S. cerevisiae* at 10^3 cells/mL). The fermentations were performed in triplicate, at 25°C, and without SO_2 addition. The fermentations were weighed daily to monitor CO_2 release and samples were withdrawn at 2-day intervals to monitor population dynamics. The residual sugar at the end of fermentation was measured. The fermentations were considered complete when residual sugars in wine were less than 2 g/L and the yeast population dynamics was monitored by direct plating on WLN agar.

Statistical Analysis

The DNA extraction, ARISA analysis, and fermentations were performed in triplicate. The values were presented as means ± SD. The differences between treatments were determined using analysis of variance (ANOVA) with the statistical software Statistica version 13.0 (StatSoft Inc., Tulsa, OK, United States). The differences were considered significant should the *p*-values were equal or less than 0.05. For multivariate data analysis, the Principal Component Analysis was performed, using XLSTAT in Microsoft®Excel (2016).

RESULTS

Selection of Yeast Species for the Consortium

Eighteen yeast species commonly isolated from South African grape musts (Jolly et al., 2003a; Weightman, 2014; Bagheri et al., 2015; Morgan, 2016), were initially evaluated for DNA extractability and resolvability in ARISA analysis. The ARISA profile of the mixed community only revealed 13 peaks (**Figure 1**). An overlap between *Rhodotorula glutinis* (614 bp), *R. diobovatum* (618 bp) and *W. anomalus* (618 bp) was observed. Similarly, *H. uvarum* (747 bp), *H. opuntiae* (748 bp), and *Kazachstania aerobia* (751 bp), as well as *S. bacillaris* (458 bp) and *C. apicola* (458 bp) co-migrated and could not be resolved. Consequently, eight species (*M. pulcherrima, P. terricola, S. bacillaris, C. parapsilosis, W. anomalus, L. thermotolerans, H. vineae,* and *S. cerevisiae*), which could be reliably resolved in ARISA, and could be distinguished based on their colony morphology on WLN agar, were selected to establish a model consortium. The efficiency of DNA extraction method and ARISA on the consortium was evaluated. In addition, standard curves of optical density ($OD_{600\ nm}$) vs. colony forming units (CFU/mL) were established for each species (data not shown). A cell suspension containing approximately each at 10^5 CFU/mL was prepared. Total genomic DNA was extracted from the mixed culture and ARISA was performed. Similar peak heights and peak areas were observed for all species, suggesting that the DNA extraction method and ARISA were efficient for all of them (**Figure 2**).

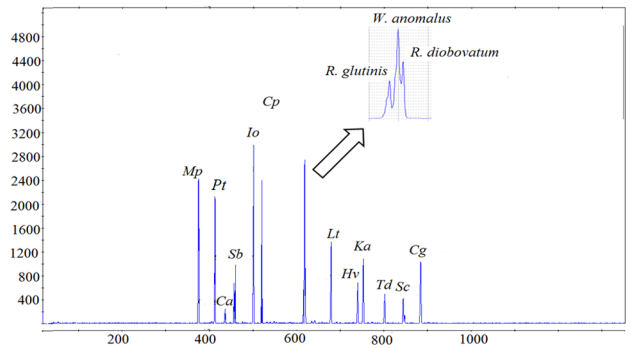

FIGURE 1 | Electropherogram of a mixed culture of 18 yeast species, generated via PCR amplification with ITS1F-ITS4 primers. The *x*-axis represents the fragment size (bp) and the *y*-axis represents the relative fluorescence intensity. The following abbreviations were used for names of yeast species. *Mp*, *Metschnikowia pulcherrima; Pt, Pichia terricola; Ca, Candida azyma; Sb, Starmerella bacillaris; Io, Issatchenkia orientalis; Cp, Candida parapsilosis; Lt, Lachancea thermotolerans; Hv, Hanseniaspora vineae; Ka, Kazachstania aerobia; Td, Torulaspora delbrueckii; Sc, Saccharomyces cerevisiae; Cg, Candida glabrata.*

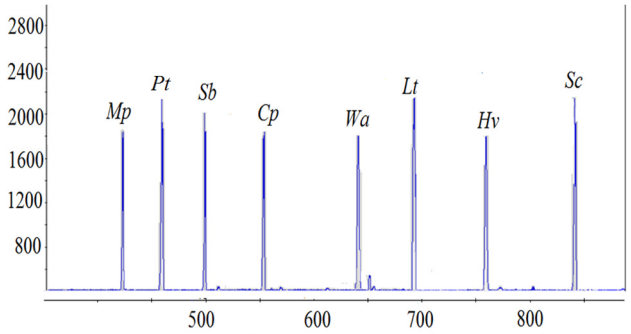

FIGURE 2 | Quantitative validation between the ARISA peaks of eight selected yeast species and CFU/mL. All yeast species were inoculated at 10^5 CFU/mL. The *x*-axis represents the fragment size (bp) and the *y*-axis represents the relative fluorescence intensity.

Validation of ARISA in the Model Consortium

The detection limit of ARISA was investigated in different inoculation scenarios, representing low and high levels of selected yeast species (**Table 2**). The data indicated that when all species were inoculated at the same level, they could be detected even at 10^3 CFU/mL while, in a situation where one species

TABLE 2 | Yeast inoculum combinations used to determine ARISA detection limits.

Yeast species	A	B	C
H. vineae	10^3	10^4	10^3
S. bacillaris	10^3	10^4	10^3
C. parapsilosis	10^3	10^4	10^3
P. terricola	10^3	10^4	10^3
L. thermotolerans	10^3	10^6	10^3
W. anomalus	10^3	10^4	10^3
M. pulcherrima	10^3	10^4	10^3
S. cerevisiae	10^3	10^4	10^6

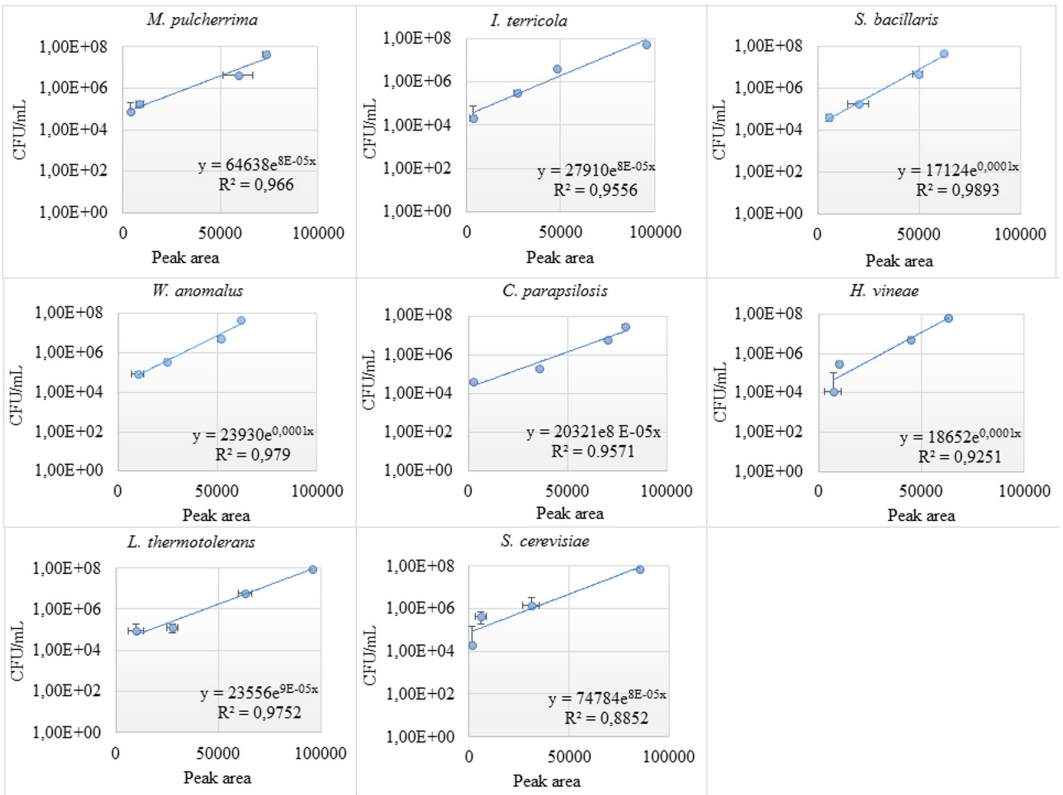

FIGURE 3 | Standard curves of individual yeast species in the consortium. The correlation between the colony forming unit and peak area (bp) was investigated at different dilutions (10^3–10^7 CFU/mL) for individual yeast species in the consortium.

was significantly higher in concentration ($\geq 10^6$ CFU/mL), other species could be detected if present at 10^4 CFU/mL but not at 10^3 CFU/mL (Supplementary Figure S1). Therefore, the detection limit of ARISA was defined as the lowest cell concentration (10^4 CFU/mL) that resulted in a positive signal and fluorescence intensity above 50 relative fluorescence units (RFU).

To test the repeatability and reliability of ARISA for monitoring the yeast dynamics throughout the fermentation, three independent DNA extractions were performed from a sample in which the yeasts were mixed in different concentrations. In each case, similar peak profiles were observed for triplicates with minor variations in peak intensities (Supplementary Figure S2).

For better quantification of the individual yeast species, standard curves correlating colony forming units and peak areas were established. Strong linear correlation between CFU/mL and ARISA peak area, with an R_2 value of ≈0.9 was observed, for individual yeast species (**Figure 3**). However, at lower biomass, the correlation between peak area and viable counts was non-linear.

Fermentation in Synthetic Grape Juice

Fermentation and Growth Kinetics

The applicability of the consortium and ARISA as a model was tested in the synthetic grape juice fermentation, inoculated with *NS-Sc* and *NS* only. The two sets of fermentations displayed distinct kinetics, with the *NS-Sc* fermentation reaching dryness (residual sugar < 2 g/L) within 21 days, while the fermentation with the *NS* consortium was sluggish and still had a total of 88 g/L residual sugar by day 30 (**Figure 4**). The *NS* fermentation got stuck at this level since the residual sugar was found to be the same after 40 days.

Yeast Population Dynamics in Synthetic Grape Juice

Comparison of ARISA and viable counts from the *NS-Sc* fermentation revealed similar trends in the relative abundance of the individual species in the early stage of fermentation (**Figure 5**). However, in the middle and final fermentation stages, ARISA consistently showed higher levels of *S. cerevisiae* and lower levels of *H. vineae* than direct plating (Supplementary Table S1). In addition, *M. pulcherrima* and *P. terricola* were detectable by ARISA until the end of fermentation while, they could not be observed and enumerated on agar plates.

Analysis of the yeast dynamics in the *NS-Sc* fermentation by standard plating on WLN agar revealed an initial increase in the population of non-*Saccharomyces* species until 10% of the sugar was consumed. The individual non-*Saccharomyces* species reached up to 10^7–10^8 CFU/mL and maintained viability at these levels for a brief period, before starting

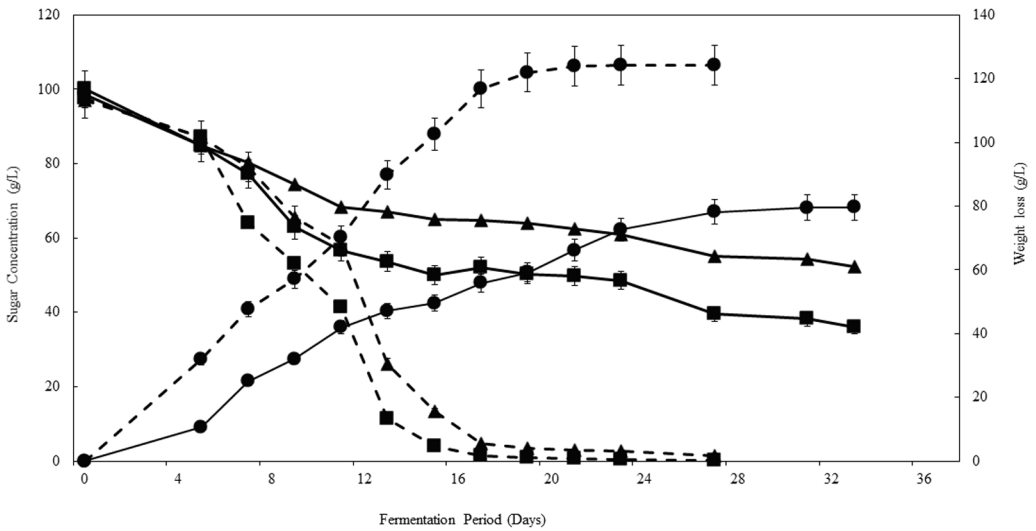

FIGURE 4 | Progress curves showing the kinetics of fermentations performed in the synthetic must. Fermentation performed with *NS-Sc* consortium is indicated with broken lines while fermentation with *NS* consortium is indicated with solid lines. Glucose (■), fructose (▲) and CO_2 release (●) were monitored throughout fermentation.

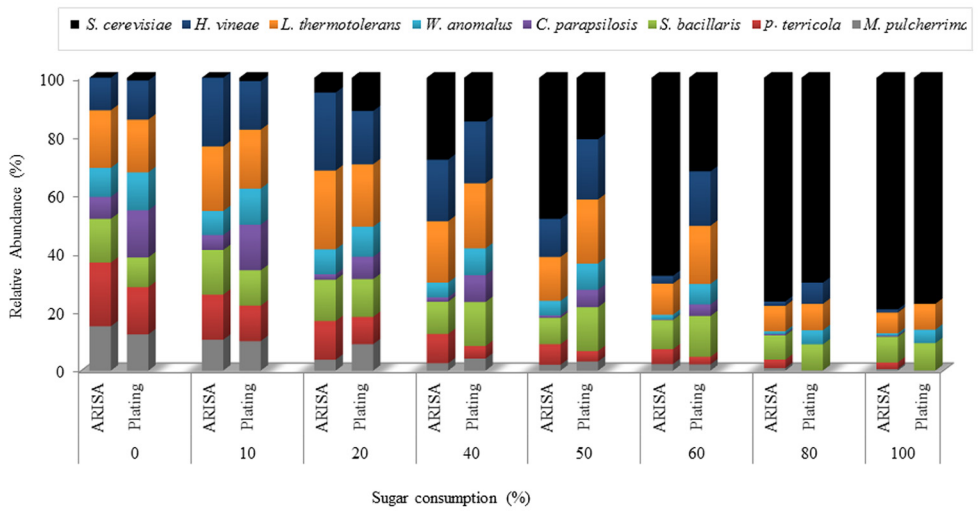

FIGURE 5 | Relative abundance of yeast species throughout the *NS-Sc* fermentation in synthetic grape must. Yeast population dynamics were monitored using ARISA and plating methods.

to decline. *P. terricola* and *C. parapsilosis,* dropped below detection by 50% sugar consumption, whereas *M. pulcherrima* and *H. vineae* were below detection after 70 and 90% sugar consumption, respectively (**Figure 6**). In contrast, the population of *S. cerevisiae* increased steadily from 10^3 CFU/mL to 4.37×10^4 CFU/mL (20% sugar consumption) where the population of all non-*Saccharomyces* species declined to 10^6 CFU/mL. When *S. cerevisiae* reached to 6.47×10^4, a decline in the population of *W. anomalus* (3.70×10^5), *P. terricola* (3.10×10^5) and *M. pulccherrima* (1.90×10^5) was observed whereas, the population of *C. parapsolosis, H. vineae, S. bacillaris,* and *L. thermotolerans* remained at 10^6 CFU/mL. Finally, *S. cerevisiae* dominated the fermentation and reached to 7.19×10^7 CFU/mL. *L. thermotolerans* (8.40×10^4), *S. bacillaris* (8.03×10^4), and *W. anomalus* (1.10×10^4) remained viable until the end of fermentation.

In the *NS* fermentation, the levels of *S. bacillaris, P. terricola,* and *L. thermotolerans* increased moderately and maintained dominance until 40% of the sugar was consumed while, *M. pulcherrima* and *C. parapsilosis* declined steadily from the onset of fermentation. Using the standard curves constructed as described in the previous section, the population of *S. bacillaris,*

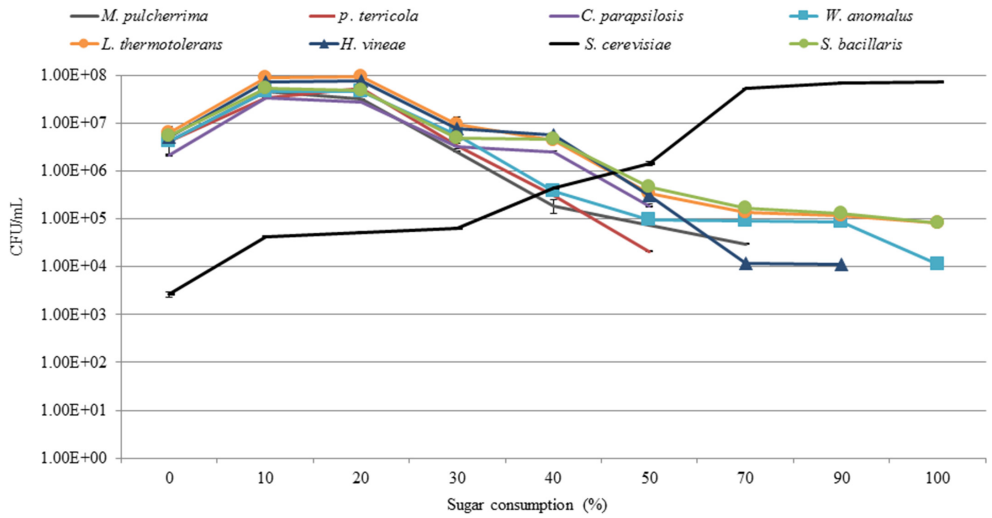

FIGURE 6 | Growth profiles of yeast population throughout *NS-Sc* fermentation in the synthetic must.

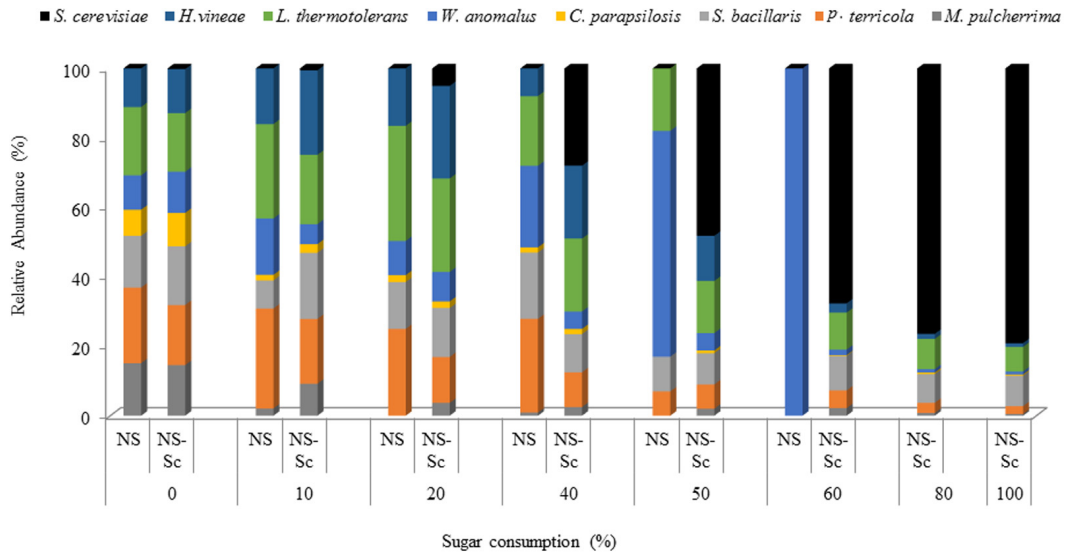

FIGURE 7 | Relative abundance of yeast species during fermentations performed with *NS-Sc* and *NS*. Yeast population dynamics was monitored using ARISA.

P. terricola, and *L. thermotolerans* was estimated to be 1.48×10^5, 5.33×10^5, and 2.82×10^5 CFU/mL, respectively, whereas the population of *M. pulcherrima* and *C. parapsilosis* was 1.22×10^3 and 1.69×10^3 CFU/mL. The population of *H. vineae* at 40% sugar consumption was estimated to be 2.07×10^3 CFU/mL.

After 50% of the sugar was consumed, only four species (*L. thermotolerans*, *S. bacillaris, P. terricola,* and *W. anomalus*) were detected, with *W. anomalus*, accounting for 65% of the population. The population of *L. thermotolerans*, *S. bacillaris, P. terricola,* and *W. anomalus* based on the standard curves were 2.74×10^5, 5.58×10^4, 2.77×10^4, and 7.23×10^6 CFU/mL, respectively. The fermentation got stuck at 60% of sugar consumption and *W. anomalus* was the only detectable yeast at this stage (**Figure 7**). The level of *W. anomalus* based on the standard curve was estimated to be 9.67×10^6 CFU/mL by 60% of sugar consumption in *NS* fermentation while *S. cerevisiae* reached up to 7.19×10^7 CFU/mL by the end of the *NS-Sc* fermentation.

Chemical Parameters and Yeast Diversity in Chenin Blanc Juice

The Chenin blanc juice used in the current study was at 21.7 °Brix with a total acidity of 3.23 g/L, pH 3.37 and a yeast assimilable nitrogen (YAN) of 195 mg/L. Sugar content and YAN concentration were higher in Chenin blanc juice compared to the synthetic must (**Table 3**). One hundred and eighty four yeast isolates obtained from the Chenin blanc juice were identified

TABLE 3 | Chemical parameters of Chenin blanc compared to the synthetic grape juice.

Chemical parameter	Chenin blanc juice	Synthetic grape juice
Sugar (°Brix)	21.7	20
YAN (mg/L)	195	300
pH	3.37	3.5

and revealed that the initial indigenous yeast population comprised *M. pulcherrima* (2.39×10^3 CFU/mL), *H. uvarum* (4.21×10^3 CFU/mL), *L. thermotolerans* (2.70×10^3 CFU/mL), *W. anomalus* (3.34×10^3 CFU/mL) and *S. cerevisiae* (4.85×10^3 CFU/mL).

Chenin Blanc Fermentations

A comparison of the spontaneous fermentation, the *Sc*-inoculated, and the *NS-Sc* inoculated fermentations, revealed that the *Sc* fermentation was the fastest and reached dryness in 24 days, followed by the spontaneous fermentation at 26 days, while, *NS-Sc* fermentation took 28 days to reach dryness (**Figure 8**).

The spontaneous fermentation of the juice was characterized by an initial increase in the yeast population from $\approx 10^3$ CFU/mL to 6.27×10^5 CFU/mL, by 10% sugar consumption. Subsequently, a decline in some non-*Saccharomyces* species was observed; amongst them, *W. anomalus* and *M. pulcherrima* declined rapidly and could not be detected by 30% sugar consumption, while *H. uvarum* persisted until 50% of the sugar was consumed. In contrast, *L. thermotolerans* increased in growth up to 2.3×10^6 CFU/mL at 50% sugar consumption and persisted until the end of fermentation. The indigenous *S. cerevisiae* (*IND-Sc*) increased from $\approx 10^3$ CFU/mL to a maximum of 1.82×10^8 CFU/mL (**Figure 9A**). Similar trends were observed in the *Sc*-inoculated fermentation. However, *W. anomalus* only grew up to 4×10^4 CFU/mL and *H. uvarum* persisted until 40% sugar consumption (**Figure 9B**). In addition, *L. thermotolerans* only reached a maximum of 8×10^5 CFU/mL. Within the *S. cerevisiae* population, *IND-Sc* and EC1118 displayed similar growth patterns. However, *IND-Sc* persisted at a higher level, reaching a maximum of 2.1×10^8 CFU/mL, while EC1118 reached 4.5×10^7 CFU/mL (**Figure 9B**). When the *NS-Sc* consortium was inoculated, *H. uvarum* (the only indigenous non-*Saccharomyces* yeast that was not part of the consortium), grew from 4.4×10^3 to 6.20×10^4 CFU/mL by 10% sugar consumption followed by a steady decline until it could not be detected by 50% sugar consumption (**Figure 9C**). Amongst the remainder of the non-*Saccharomyces* yeasts which were inoculated at $\approx 10^6$ CFU/mL, *P. terricola* and *C. parapsilosis* declined below detection after 10% sugar consumption, followed by *M. pulcherrima* and *W. anomalus* by 28% sugar consumption. In contrast, *H. vineae* declined gradually until 78% sugar consumption; *S. bacillaris* persisted at 10^6 CFU/mL until 78% sugar consumption before dropping to 8×10^4 CFU/mL at the end of fermentation, while, *L. thermotolerans* persisted at 10^6 CFU/mL until the end of fermentation. The *S. cerevisiae* population behaved in a similar way as observed in the *S. cerevisiae* inoculated fermentation, albeit at 10 times less cell concentrations. For instance, *IND-Sc* reached a maximum of 3.2×10^7 CFU/mL, while EC1118 reached 6.9×10^6 CFU/mL.

DISCUSSION

The current study aimed to establish and validate a model system for reliable monitoring and prediction of the temporal trajectories of yeast populations within the wine fermentation ecosystem. To this end, a yeast consortium comprising *S. cerevisiae* and seven non-*Saccharomyces* yeast species of varying fermentative capacities was constructed. These yeast species are all regularly encountered in SA grape juices, and some species have sometimes been detected in significant numbers. Furthermore, all of these non-*Saccharomyces* species have been isolated in countries with several wine producing regions such as Italy, France, Argentina, China, and Brazil (Jolly et al., 2003a; Combina et al., 2005; Di Maro et al., 2007; Lopandic et al., 2008; Romancino et al., 2008; Salinas et al., 2009; Sun et al., 2009; Suzzi et al., 2012; Tofalo et al., 2012; Weightman, 2014; Maturano et al., 2015; Morgan, 2016). These yeast species also differed in their ITS1-5.8S rRNA-ITS2 gene sizes, which made ARISA a suitable method to monitor their dynamics. Our data show that in this semi-complex consortium, the detection limit of ARISA could be as low as 10^3 CFU/mL when all species are present at low levels. However, at lower biomass (10^{3-4} CFU/mL) larger deviations were observed, possibly due to the bias introduced by DNA extraction or preferential amplification in PCR (Giraffa, 2004; Ramette, 2009). Furthermore, in a typical wine fermentation scenario where dominant taxa grow up to 10^{7-8} CFU/mL, minor taxa would not be detected below 10^4 CFU/mL. ARISA is also unable to differentiate between strains of the same species, limiting its ability to monitor strain-specific dynamics. However, species-specific interactions of significantly contributing species can be easily detected and quantified (Ramette, 2009; Ženišová et al., 2014; Ghosh et al., 2015; Setati et al., 2015). The limits are similar to those obtained for FISH (Xufre et al., 2006) and PCR-DGGE (Prakitchaiwattana et al., 2004) and they are less sensitive than qPCR (10^2 CFU/mL) and flow cytometry (10^3 CFU/mL) methods (Malacrinò et al., 2001; Hierro et al., 2006a,b; Zott et al., 2010). However, ARISA does not require species-specific primers and is less technically demanding than qPCR and flow cytometry. Overall, ARISA generated similar growth patterns for individual yeast species in the consortium as observed with viable counts. However, some discrepancies were observed in the middle and final stage of fermentation. These discrepancies might reflect biases and limitation in both methods. For instance, plating method might show bias against cells in a VBNC state and injured population (Divol and Lonvaud-Funel, 2005; Renouf et al., 2007) while ARISA is unable to differentiate between live and dead cells (Xie et al., 2007; O'Sullivan et al., 2013). Consequently, an overestimation of most of the species (e.g., *M. pulcherrima, P. terricola, H. vineae, L. thermotolerans, S. bacillaris, and S. cerevisiae*) by one order of magnitude was evident with ARISA compared to the plating method. The data in the current study suggest that up to 3% of dead cells could possibly be detected

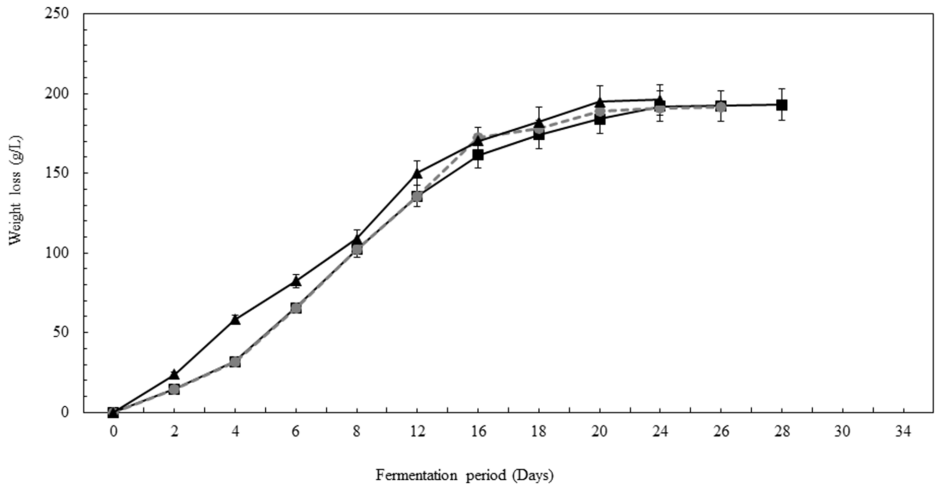

FIGURE 8 | Progress curves displaying the kinetics of spontaneous fermentation (●), fermentation inoculated with *Sc* (▲), and fermentation inoculated with *NS-Sc* consortium (■).

by ARISA. Similarly, Salinas et al., (2009) indicated that qPCR overestimate the number of live cells in average one order higher compared to microscopy analysis, which according to Hierro et al. (2006a) could represent up to 1% of the dead cells.

Our study showed that the yeast species constituting the consortium responded differently to the wine fermentation ecosystem, and the behavior of the non-*Saccharomyces* species was differentially influenced by the presence of *S. cerevisiae*. The data showed that in the absence of *S. cerevisiae*, some non-*Saccharomyces* species such as *M. pulcherrima* and *C. parapsilosis* experienced a decline from the onset of fermentation whereas, species such as *S. bacillaris, P. terricola,* and *L. thermotolerans* experienced a moderate increase followed by a steady decline in the absolute numbers by the middle of fermentation. On the contrary, *W. anomalus* suppressed the rest of non-*Saccharomyces* species and increased in cell concentration back to the initial inoculum level. This suggests that *W. anomalus* can withstand the chemical milieu created in the early stages of the fermentation better than the other yeast species and may utilize the nitrogen released by dead cells. In contrast, in the presence of *S. cerevisiae*, specifically, this yeast declines early in fermentation, suggesting that *S. cerevisiae* creates an unconducive environment, which suppresses *W. anomalus*. Indeed, an antagonistic interaction between *S. cerevisiae* and *W. anomalus*, has been proposed in other fermentation ecosystems (Ye et al., 2014). *S. cerevisiae* may inhibit other organisms through a variety of mechanisms including the production of short chain fatty acids and glycoproteins (killer toxin), and the specific antagonism exerted by *S. cerevisiae* modulates the ecosystem (Vannette and Fukami, 2014; Boynton and Greig, 2016). Conversely, other yeast species such as *M. pulcherrima*, *P. terricola,* and *C. parapsilosis* consistently declined in the early stages of the fermentation, both in the presence and in the absence of *S. cerevisiae*, suggesting that the decline could be due to another factor such as oxygen limitation. Several studies have shown that the growth and survival rate of *M. pulcherrima* and *C. parapsilosis* was markedly enhanced in aerated fermentations (Oh et al., 1998; Rossignol et al., 2009; Morales et al., 2015; Shekhawat et al., 2017). Furthermore, in the presence of *S. cerevisiae*, *L. thermotolerans,* and *S. bacillaris* could survive until late fermentation. The survival of *L. thermotolerans* until end of the fermentation has been shown previously (Gobbi et al., 2013). In addition, *S. bacillaris* strains are typically fructophilic and therefore preferentially utilize fructose, which is less preferred by *S. cerevisiae*. Interestingly, our study revealed that *H. vineae* survives better in the presence *S. cerevisiae* suggesting a positive interaction between the two yeasts. Such an interaction is perhaps not coincidental since other studies have shown that in nutrient-rich conditions, co-fermentations using strains of these two species often reflect a significant contribution of *H. vineae* to wine aroma and flavor (Viana et al., 2011; Medina et al., 2013).

Based on our current findings, we can infer that the mutualism (*S. cerevisiae* and *H. vineae*) and antagonism (*S. cerevisiae* and *W. anomalus*) observed in the wine ecosystem, could be a species-specific interaction that occurs as a result of the presence of *S. cerevisiae*. However, the strength of the mutualism or antagonism in the wine consortium may vary between different strains of one species requires further investigation. Indeed, species-specific patterns throughout the wine fermentation process are probable and comprehensible. For instance, it is well established that some species decline rapidly by early or mid-fermentation (*Cryptococcus carnescens, Aureobasidium pullulans, P. terricola,* and *M. pulcherrima)*, others repeatedly persist until late fermentation (*S. bacillaris, L. thermotolerans, T. delbrueckii*) regardless of the strain variability (Jemec et al., 2001; Sun et al., 2009; Cordero-Bueso et al., 2011; Bezerra-Bussoli et al., 2013; Gobbi et al., 2013; Milanović et al., 2013; Bagheri et al., 2015).

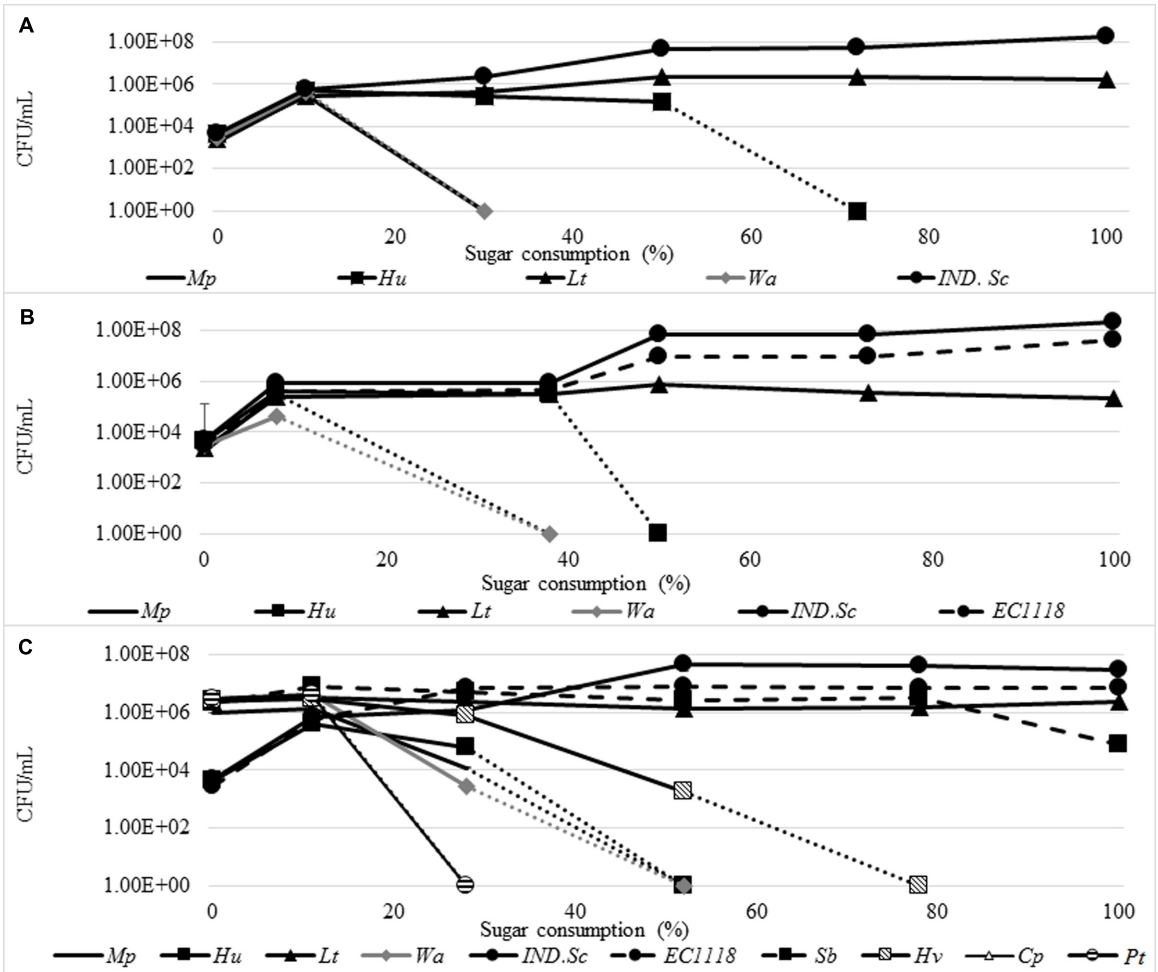

FIGURE 9 | Yeast population dynamics in Chenin blanc spontaneous fermentation **(A)**, *S. cerevisiae* inoculated fermentation **(B)** and *NS-Sc* consortium fermentation **(C)**. The following abbreviations were used for names of yeast species. *Mp*, *M. pulcherrima; It, P. terricola; Sb, S. bacillaris; Cp, C. parapsilosis; Lt, L. thermotolerans; Hv, H. vineae; Hu, H. uvarum; IND.Sc,* Indigenous *S. cerevisiae.*

One of the goals of the current study was to establish a consortium that would serve as a representative model to predict yeast dynamics in wine fermentation. In order to validate the suitability of this consortium, it was used as an inoculum in Chenin blanc must and the dynamics was monitored throughout the fermentation. Interestingly, four of the yeast species (*M. pulcherrima*, *L. thermotolerans, W. anomalus*, and *S. cerevisiae*) which form part of the consortium were also present in the natural yeast community of the Chenin blanc must, confirming once more the representative nature of our consortium. Our study shows that all the species in the consortium could compete with the native yeast species in a non-sterilized must. While we were unable to differentiate between the indigenous strains and inoculated strains (e.g., *W. anomalus*), the population dynamics observed were similar to those described for the synthetic grape juice, suggesting species, and not strain specific drivers of interactions. This is further supported by the fact that the dynamics were preserved although the environmental conditions, including nitrogen and sugar levels, differed consoderably between the two matrices (Supplementary Table S2). We also observed that the indigenous *S. cerevisiae* population displayed better growth than the EC1118 inoculated strain although they were at similar levels at the beginning of the fermentation, further indicating that the selective drivers were species and not strain-dependent. Our data show that the consortium constructed in the current study serves as a viable and robust model to assess yeast population dynamics during wine fermentation since the matrix did not have a considerable influence on the dynamics as such. We suggest that the yeast dynamics observed in the current study is mainly due to species-specific interactions and the selective pressure applied by *S. cerevisiae* to other species. Our data suggest that inoculation with *S. cerevisiae* favors the persistence of some non-*Saccharomyces* species in wine fermentation whereas; it clearly suppresses the growth and contribution of other non-*Saccharomyces* species.

The dynamics of the wine ecosystem is driven by a multitude of positive and negative yeast–yeast interactions. The main

challenge in microbial ecology is to link microbial composition to function. Here, we demonstrate that a model consortium approach can be used as a tool to predict the microbial behavior in a complex natural environment. Such a model consortium can be easily perturbed under well-controlled conditions in order to gain a deep understanding of the effect of environmental parameters on yeast–yeast interactions. In-depth insight on yeast–yeast interactions may allow us to manipulate the microbial community and enhance the population of the beneficial microbes or suppress the population of undesirable yeast species. The study presents a first step in the development of a model to predict the oenological potential of any given wine mycobiome.

AUTHOR CONTRIBUTIONS

FB and MS conceptualized the study. BB, FB, and MS designed the experimental layout. BB performed the experiments, analyzed the data and wrote the first draft of the manuscript. BB, FB, and MS edited subsequent drafts, read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01988/full#supplementary-material

REFERENCES

Albergaria, H., and Arneborg, N. (2016). Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: role of physiological fitness and microbial interactions. *Appl. Microbiol. Biotechnol.* 100, 2035–2046. doi: 10.1007/s00253-015-7255-0

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2010a). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *Eur. Food Res. Technol.* 231, 215–224. doi: 10.1007/s00217-010-1272-0

Andorrà, I., Esteve-Zarzoso, B., Guillamón, J. M., and Mas, A. (2010b). Determination of viable wine yeast using DNA binding dyes and quantitative PCR. *Int. J. Food Microbiol.* 144, 257–262. doi: 10.1016/j.ijfoodmicro.2010.10.003

Andorra, I., Monteiro, M., Esteve-Zarzoso, B., Albergaria, H., and Mas, A. (2011). Analysis and direct quantification of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* populations during alcoholic fermentation by fluorescence *in situ* hybridization, flow cytometry and quantitative PCR. *Food Microbiol.* 28, 1483–1491. doi: 10.1016/j.fm.2011.08.009

Bagheri, B., Bauer, F. F., and Setati, M. E. (2015). The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. *S. Afr. J. Enol. Vitic.* 36, 243–251.

Bely, M., Sablayrolles, J. M., and Barre, P. (1990). Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *J. Ferment. Bioeng.* 70, 246–252. doi: 10.1016/0922-338X(90)90057-4

Bezerra-Bussoli, C., Baffi, M. A., Gomes, E., and Da-Silva, R. (2013). Yeast diversity isolated from grape musts during spontaneous fermentation from a Brazilian Winery. *Curr. Microbiol.* 67, 356–361. doi: 10.1007/s00284-013-0375-9

Boynton, J. P., and Greig, D. (2016). Species richness influences wine ecosystem function through a dominant species. *Fungal Ecol.* 22, 61–72. doi: 10.1016/j.dib.2016.05.038

Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M. G., et al. (2014). Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol. Biotechnol.* 98, 843–853. doi: 10.1007/s00253-013-5411-y

Branco, P., Viana, T., Albergaria, H., and Arneborg, N. (2015). Antimicrobial peptides (AMPs) produced by *Saccharomyces cerevisiae* induce alterations in the intracellular pH, membrane permeability and culturability of *Hanseniaspora guilliermondii* cells. *Int. J. Food Microbiol.* 205, 112–118. doi: 10.1016/j.ijfoodmicro.2015.04.015

Brežná, B., Ženišová, K., Chovanová, K., Chebeòová, V., Kraková, L., Kuchta, T., et al. (2010). Evaluation of fungal and yeast diversity in Slovakian wine-related microbial communities. *Antonie Van Leeuwenhoek* 98, 519–529. doi: 10.1007/s10482-010-9469-6

Cangelosi, G. A., and Meschke, J. S. (2014). Dead or alive: molecular assessment of microbial viability. *Appl. Environ. Microbiol.* 80, 5884–5891. doi: 10.1128/AEM.01763-14

Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., and Romano, P. (2016). Yeast interactions in inoculated wine fermentation. *Front. Microbiol.* 7:555. doi: 10.3389/fmicb.2016.00555

Ciani, M., and Comitini, F. (2015). Yeast interactions in multi-starter wine fermentation. *Curr. Opin. Food Sci.* 1, 1–6. doi: 10.1016/j.cofs.2014.07.001

Combina, M., Elía, A., Mercado, L., Catania, C., Ganga, A., and Martinez, C. (2005). Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. *Int. J. Food Microbiol.* 99, 237–243. doi: 10.1016/j.ijfoodmicro.2004.08.017

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M. D., et al. (2011). Influence of the farming system and vine variety on yeast communities associated with grape-berries. *Int. J. Food Microbiol.* 145, 132–139. doi: 10.1016/j.ijfoodmicro.2010.11.040

De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., and Boon, N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environ. Microbiol.* 16, 1472–1481. doi: 10.1111/1462-2920.12343

Deere, D., Jian, S., Vesey, G., Bell, P., Bissinger, P., and Veal, D. (1998). Flow cytometry and cell sorting for yeast viability assessment and cell selection. *Yeast* 14, 147–160. doi: 10.1002/(SICI)1097-0061(19980130)14

Di Maro, E., Ercolini, D., and Coppola, S. (2007). Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. *Int. J. Food Microbiol.* 117, 201–210. doi: 10.1016/j.ijfoodmicro.2007.04.007

Divol, B., and Lonvaud-Funel, A. (2005). Evidence for viable but non-culturable yeasts in botrytis-affected wine. *J. Appl. Microbiol.* 99, 85–93. doi: 10.1111/j.1365-2672.2005.02578.x

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., and Cocolin, L. (2015). Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterizations. *Int. J. Food Microbiol.* 199, 33–40. doi: 10.1016/j.ijfoodmicro.2015.01.009

Ghosh, S., Bagheri, B., Morgan, H. H., Divol, B., and Setati, M. E. (2015). Assessment of wine microbial diversity using ARISA and cultivation-based methods. *Ann. Microbiol.* 65, 1833–1840. doi: 10.1007/s13213-014-1021-x

Giraffa, G. (2004). Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiol. Rev.* 28, 251–260. doi: 10.1016/j.femsre.2003.10.005

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

Grube, M., and Berg, G. (2009). Microbial consortia of bacteria and fungi with focus on the lichen symbiosis. *Fungal Biol. Rev.* 23, 72–85. doi: 10.1016/j.fbr.2009.10.001

Henschke, P. A., and Jiranek, V. (1993). "Yeasts metabolism of nitrogen compounds," in *Wine Microbiology and Biotechnology*, ed. G. H. Fleet (Chur: Harwood Academic), 77–164.

Hierro, N., Esteve-Zarzoso, B., González, Á., Mas, A., and Guillamón, J. M. (2006a). Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. *Appl. Environ. Microbiol.* 72, 7148–7155. doi: 10.1128/AEM.00388-06

Hierro, N., González, Á, Mas, A., and Guillamón, J. M. (2006b). Diversity and evolution of non-*Saccharomyces* yeast populations during wine fermentation: effect of grape ripeness and cold maceration. *FEMS Yeast Res.* 6, 102–111. doi: 10.1111/j.1567-1364.2005.00014.x

Izquierdo Cañas, P. M., Esteban, C., Romero, G., Heras, J. M., Mónica, M., and González, F. (2014). Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. *Eur. Food Res. Technol.* 239, 279–286. doi: 10.1007/s00217-014-2220-1

Jagmann, N., and Philipp, B. (2014). Design of synthetic microbial communities for biotechnological production processes. *J. Biotechnol.* 184, 209–218. doi: 10.1016/j.jbiotec.2014.05.019

Jemec, K. P., Cadez, N., Zagorc, T., Bubic, V., Zupec, A., and Raspor, P. (2001). Yeast population dynamics in five spontaneous fermentations of Malvasia must. *Food Microbiol.* 18, 247–259. doi: 10.1006/fmic.2001.0396

Jiang, L.-L., Zhou, J.-J., Quan, C.-S., and Xiu, Z.-L. (2017). Advances in industrial microbiome based on microbial consortium for biorefinery. *Bioresour. Bioprocess.* 4, 11. doi: 10.1186/s40643-017-0141-0

Jolly, N. P., Augustyn, O. P. H., and Pretorius, I. S. (2003a). The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. *S. Afr. J. Enol. Vitic.* 24, 63–69.

Jolly, N. P., Augustyn, O. P. H., and Pretorius, I. S. (2003b). The effect of Non-*Saccharomyces* yeasts on fermentation and wine quality. *S. Afr. J. Enol. Vitic.* 24, 55–62.

Kemsawad, V., Branco, P., Almeida, M. G., Caldeira, J., Albergaria, H., and Arneborg, N. (2015). Cell-to-cell contact and antimicrobial peptides play a combined role in the death of *Lachancea thermotolerans* during mixed-culture alcoholic fermentation with *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* 362, 1–8. doi: 10.1093/femsle/fnv103

Kraková, L., Chovanová, K., Selim, S. A., Šimonovičová, A., Puškarová, A., Maková, A., et al. (2012). A multiphasic approach for investigation of the microbial diversity and its biodegradative abilities in historical paper and parchment documents. *Int. Biodeterior. Biodegrad.* 70, 117–125. doi: 10.1016/j.ibiod.2012.01.011

Lencioni, L., Romani, C., Gobbi, M., Comitini, F., Ciani, M., and Domizio, P. (2016). Controlled mixed fermentation at winery scale using *Zygotorulaspora florentina* and *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 234, 36–44. doi: 10.1016/j.ijfoodmicro.2016.06.004

Lopandic, K., Tiefenbrunner, W., Gangl, H., Mandl, K., Berger, S., Leitner, G., et al. (2008). Molecular profiling of yeasts isolated during spontaneous fermentations of Austrian wines. *FEMS Yeast Res.* 8, 1063–1075. doi: 10.1111/j.1567-1364.2008.00385.x

Malacrinò, P., Zapparoli, G., Torriani, S., and Dellaglio, F. (2001). Rapid detection of viable yeasts and bacteria in wine by flow cytometry. *J. Microbiol. Methods* 45, 127–134. doi: 10.1016/S0167-7012(01)00243-3

Maturano, Y. P., Mestre, M. V., Esteve-Zarzoso, B., Nally, M. C., Lerena, M. C., Toro, M. E., et al. (2015). Yeast population dynamics during pre-fermentative cold soak of Cabernet Sauvignon and Malbec wines. *Int. J. Food Microbiol.* 199, 23–32. doi: 10.1016/j.ijfoodmicro.2015.01.005

Medina, K., Boido, E., Fariña, L., Gioia, O., Gomez, M. E., Barquet, M., et al. (2013). Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem.* 141, 2513–2521. doi: 10.1016/j.foodchem.2013.04.056

Mendoza, L. M., De Nadra, M. C. M., Bru, E., and Farıas, M. E. (2009). Influence of wine-related physicochemical factors on the growth and metabolism of non-*Saccharomyces* and *Saccharomyces* yeasts in mixed culture. *J. Ind. Microbiol. Biotechnol.* 36, 229–237. doi: 10.1007/s10295-008-0489-4

Milanović, V., Comitini, F., and Ciani, M. (2013). Grape berry yeast communities: Influence of fungicide treatments. *Int. J. Food Microbiol.* 161, 240–246. doi: 10.1016/j.ijfoodmicro.2012.12.019

Morales, P., Rojas, V., Quirós, M., and Gonzalez, R. (2015). The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. *Appl. Microbiol. Biotechnol.* 99, 3993–4003. doi: 10.1007/s00253-014-6321-3

Morgan, H. H. (2016). *Investigating the Effect of Leaf Removal on the Grape-Associated Microbiome Through Culture-Dependent and Independent Approaches*. Master's thesis, Stellenbosch University, Stellenbosch.

Nissen, P., and Arneborg, N. (2003). Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. *Arch. Microbiol.* 180, 257–263. doi: 10.1007/s00203-003-0585-9

Oh, D., Kim, S., and Kim, J. (1998). Increase of xylitol production rate by controlling redox potential in *Candida parapsilosis*. *Biotechnol. Bioeng.* 58, 440–444. doi: 10.1002/(SICI)1097-0290(19980520)58:4<440::AID-BIT11>3.0.CO;2-F

O'Sullivan, D. J., Giblin, L., McSweeney, P. L. H., Sheehan, J. J., and Cotter, P. D. (2013). Nucleic acid-based approaches to investigate microbial-related cheese quality defects. *Front. Microbiol.* 4:1. doi: 10.3389/fmicb.2013.00001

Pérez-Torrado, R., Rantsiou, K., Perrone, B., Navarro-Tapia, E., Querol, A., and Cocolin, L. (2017). Ecological interactions among *Saccharomyces cerevisiae* strains: insight into the dominance phenomenon. *Sci. Rep.* 7:43603. doi: 10.1038/srep43603

Pina, C., Couto, J. A., António, J., and Hogg, T. (2004). Inferring ethanol tolerance of *Saccharomyces* and non-*Saccharomyces* yeasts by progressive inactivation. *Biotechnol. Lett.* 26, 1521–1527. doi: 10.1023/B:BILE.0000044456.72347.9f

Ponomarova, O., and Patil, K. R. (2015). Metabolic interactions in microbial communities: untangling the Gordian knot. *Curr. Opin. Microbiol.* 27, 37–44. doi: 10.1016/j.mib.2015.06.014

Portillo, M., del, C., and Mas, A. (2016). Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing. *LWT Food Sci. Technol.* 72, 317–321. doi: 10.1016/j.lwt.2016.05.009

Prakitchaiwattana, C. J., Fleet, G. H., and Heard, G. M. (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Res.* 4, 865–877. doi: 10.1016/j.femsyr.2004.05.004

Pretorius, I. S., Van der Westhuizen, T. J. J., and Augustyn, O. P. H. (1999). Yeast Biodiversity in Vineyards and Wineries and its importance to the South African wine industry-a review. *S. Afr. J. Enol. Vitic.* 20, 61–75.

Ramette, A. (2009). Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. *Appl. Environ. Microbiol.* 75, 2495–2505. doi: 10.1128/AEM.02409-08

Renault, P. E., Albertin, W., and Bely, M. (2013). An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Appl. Microbiol. Biotechnol.* 97, 4105–4119. doi: 10.1007/s00253-012-4660-5

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2007). Inventory and monitoring of wine microbial consortia. *Appl. Microbiol. Biotechnol.* 75, 149–164. doi: 10.1007/s00253-006-0798-3

Romancino, D. P., Di Maio, S., Muriella, R., and Oliva, D. (2008). Analysis of non-*Saccharomyces* yeast populations isolated from grape musts from Sicily (Italy). *J. Appl. Microbiol.* 105, 2248–2254. doi: 10.1111/j.1365-2672.2008.03894.x

Rossignol, T., Kobi, D., Jacquet-Gutfreund, L., and Blondin, B. (2009). The proteome of a wine yeast strain during fermentation, correlation with the transcriptome. *J. Appl. Microbiol.* 107, 47–55. doi: 10.1111/j.1365-2672.2009.04156.x

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J. J., Ballester, J., et al. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol.* 32, 243–253. doi: 10.1016/j.fm.2012.06.006

Sainz, J., Pizarro, F., Perez- Correa, J. R., and Agosin, E. (2003). Modeling of yeast metabolism and process dynamics in batch fermentation. *Biotechnol. Bioeng.* 81, 818–828. doi: 10.1002/bit.10535

Salinas, F., Garrido, D., Ganga, A., Veliz, G., and Martínez, C. (2009). TaqMan real-time PCR for the detection and enumeration of *Saccharomyces cerevisiae* in wine. *Food Microbiol.* 26, 328–332. doi: 10.1016/j.fm.2008.12.001

Sambrook, J., and Russell, D. W. (2006). *Rapid Isolation of Yeast DNA*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Setati, M. E., Jacobson, D., and Bauer, F. F. (2015). Sequence-based analysis of the *Vitis vinifera* L. cv Cabernet sauvignon grape must mycobiome in three South African vineyards employing distinct agronomic systems. *Front. Microbiol.* 6:1358. doi: 10.3389/fmicb.2015.01358

Shekhawat, K., Bauer, F. F., and Setati, M. E. (2017). Impact of oxygenation on the performance of three non-*Saccharomyces* yeasts in co-fermentation with *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 101, 2479–2491. doi: 10.1007/s00253-016-8001-y

Slabbert, E., Van Heerden, C. J., and Jacobs, K. (2010). Optimisation of automated ribosomal intergenic spacer analysis for the estimation of microbial diversity in fynbos soil. *S. Afr. J. Sci.* 106, 1–4. doi: 10.4102/sajs.v106i7/8.329

Sun, H., Ma, H., Hao, M., Pretorius, I. S., and Chen, S. (2009). Identification of yeast population dynamics of spontaneous fermentation in Beijing wine region, China. *Ann. Microbiol.* 59, 69–76. doi: 10.1007/BF03175601

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., et al. (2012). Multistarter from organic viticulture for red wine Montepulciano d'Abruzzo production. *Front. Microbiol.* 3:135. doi: 10.3389/fmicb.2012.00135

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., et al. (2012). Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiol.* 29, 18–26. doi: 10.1016/j.fm.2011.08.014

Tristezza, M., Tufariello, M., Capozzi, V., Spano, G., Mita, G., and Grieco, F. (2016). The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Front. Microbiol.* 7:670. doi: 10.3389/fmicb.2016.00670

Vannette, L. R., and Fukami, T. (2014). Historical contingency in species interactions: towards niche-based predictions. *Ecol. Lett.* 17, 115–124. doi: 10.1111/ele.1220

Viana, F., Belloch, C., Vallés, S., and Manzanares, P. (2011). Monitoring a mixed starter of *Hanseniaspora vineae-Saccharomyces cerevisiae* in natural must: impact on 2-phenylethyl acetate production. *Int. J. Food Microbiol.* 151, 235–240. doi: 10.1016/j.ijfoodmicro.2011.09.005

Wang, C., Esteve-Zarzoso, B., Cocolin, L., Mas, A., and Rantsiou, K. (2015). Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during Barbera must fermentation. *Food Res. Int.* 78, 195–200. doi: 10.1016/j.foodres.2015.10.014

Wang, C., Esteve-Zarzoso, B., and Mas, A. (2014). Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence *in situ* hybridization. *Int. J. Food Microbiol.* 191, 1–9. doi: 10.1016/j.ijfoodmicro.2014.08.014

Wang, C., Mas, A., and Esteve-Zarzoso, B. (2016). The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. *Front. Microbiol.* 7:502. doi: 10.3389/fmicb.2016.00502

Weightman, C. J. (2014). *Characterization of Chenin Blanc Wines Produced by Natural Fermentation and Skin Contact?: Focus on the Application of Rapid Sensory Profiling Method*. Master's thesis, Stellenbosch University, Stellenbosch.

Williams, K. M., Liu, P., and Fay, J. C. (2015). Evolution of ecological dominance of yeast species in high-sugar environments. *Evolution (N. Y).* 69, 2079–2093. doi: 10.1111/evo.12707

Xie, G., Li, W., Lu, J., Cao, Y., Fang, H., Zou, H., et al. (2007). Isolation and Identification of representative fungi from Shaoxing rice wine wheat Qu using a polyphasic approach of Culture-based and molecular-based methods. *J. Inst. Brew.* 113, 272–279. doi: 10.1002/j.2050-0416.2007.tb00287.x

Xufre, A., Albergaria, H., Inácio, J., Spencer-Martins, I., and Gírio, F. (2006). Application of fluorescence *in situ* hybridisation (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. *Int. J. Food Microbiol.* 108, 376–384. doi: 10.1016/j.ijfoodmicro.2006.01.025

Ye, M., Yue, T., and Yuan, Y. (2014). Effects of sequential mixed cultures of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* on apple cider fermentation. *FEMS Yeast Res.* 14, 873–882. doi: 10.1111/1567-1364.12175

Ženišová, K., Chovanová, K., Chebeňová-Turcovská, V., Godálová, Z., Kraková, L., Kuchta, T., et al. (2014). Mapping of wine yeast and fungal diversity in the Small Carpathian wine-producing region (Slovakia): evaluation of phenotypic, genotypic and culture-independent approaches. *Ann. Microbiol.* 64, 1819–1828. doi: 10.1007/s13213-014-0827-x

Zott, K., Claisse, O., Lucas, P., Coulon, J., Lonvaud-Funel, A., and Masneuf-Pomarede, I. (2010). Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiol.* 27, 559–567. doi: 10.1016/j.fm.2010.01.006

Zott, K., Miot-Sertier, C., Claisse, O., Lonvaud-Funel, A., and Masneuf-Pomarede, I. (2008). Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. *Int. J. Food Microbiol.* 125, 197–203. doi: 10.1016/j.ijfoodmicro.2008.04.001

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