Exploring multispecies interactions between wine-associated yeasts

by

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Declaration

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Summary

The fermentation of grape must to wine is catalysed by a diverse microbial community. Yeast are primary drivers of the associated alcoholic fermentation process and have therefore garnered considerable research interest. The diversity of yeast species present during wine fermentation influences the chemical composition and related sensory properties of wine as a result of the metabolic functioning of particular yeast species in response to abiotic and biotic factors. The latter is a relatively new research field, given that microbiological science has a significant monoculture bias, and as such, there is much still to be understood about the role and mechanisms of biotic stress in wine yeast ecosystems. Moreover, while the wine yeast ecosystem was the model used in this study, there are several other yeast ecosystems of biotechnological importance, including in biofuels production, bioremediation and other food and beverage industries, that would benefit from insight into these biotic stress mechanisms. The current basis of our understanding of the molecular mechanisms of yeast interactions in the wine ecosystem is based on two-species pairings, which keeps the system interaction network uncomplicated. However, there are many more role-players in natural ecosystems, and they do not interact in a linear fashion. At the micro- and macroscopic level, the importance of these often overlooked higher-order interactions has been highlighted in other ecosystems. There is very little information on higher-order interactions in the yeast ecology field, and this must be remedied for predictive understanding of these systems.

Here, we sought to address the current status quo in multispecies yeast research, by aiming to develop new tools to investigate the mechanistic basis of interaction in systems comprised of more than two species. Furthermore, the study aimed to generate a greater depth of understanding of these systems, by investigating transcriptional responses of *Saccharomyces cerevisiae* to co-culture in mixed-species cultures of increasing complexity.

Firstly, these aims were achieved by developing a fluorescence-based multi-colour flow cytometric method for tracking of a consortium consisting of wine-associated yeast species. This involved optimizing the genetic modification of the selected environmentally isolated yeast species, followed by extensive validation to confirm the representativeness of the system as well as development of the flow cytometric protocol. This was followed by addressing the pertinent issue of reproducibility in multispecies cultures, and showing the role of the physiological state of pre-cultures in determining their growth performance in three-species and four-species consortia. Finally, to contribute to our understanding of the molecular mechanisms of interaction in non-linear yeast systems, we showed that *Saccharomyces cerevisiae* expresses a combination of known pair-wise as well as unique genes when grown in a three-species system. By using interactive network visualizations of the generated transcriptomic data, we were able to functionally characterize the cellular responses in more detail than has been done before in similar studies.

This study contributes to the knowledgebase on multispecies interactions in microbial ecosystems by improving methodologies to study these systems more efficiently and suggesting potential mechanisms of interaction that govern yeast consortia.

Opsomming

Die fermentasie van druiwemos tot wyn word deur 'n diverse mikrobiese gemeenskap gekataliseer. Gis is die primêre drywers van die gepaardgaande alkoholiese fermentasie proses en het dus 'n aansienlike hoeveelheid navorsing belangstelling ontlok. Die diversiteit van gis spesies teenwoordig tydens wyn fermentasie beïnvloed die chemiese samestelling en verwante sensoriese eienskappe van wyn. Hierdie gebeur as gevolg van die metaboliese funksionering van bepaalde gis spesies in reaksie op abiotiese en biotiese faktore. Die invloed van biotiese faktore is 'n relatiewe nuwe navorsing veld, gegewe dat mikrobiologiese wetenskap 'n beduidende monokultuur vooroordeel het, en dus is daar nog baie om te verstaan oor die rol en meganismes van biotiese stres in wyngis-ekosisteme. Boonop, terwyl die wyngis-ekosisteem die model was wat in hierdie studie gebruik is, is daar 'n aantal ander gis-ekosisteme van biotegnologiese belang, insluitend in biobrandstof produksie, bioremediëring en ander voedsel- en drank bedrywe, wat baat sal vind by insig in hierdie biotiese stres meganismes. Die huidige basis van ons begrip van die molekulêre meganismes van gis-interaksies in die wyn-ekosisteem is gebaseer op twee-spesie parings, wat die stelsel interaksie netwerk ongekompliseerd hou. In natuurlike ekosisteme is daar egter baie meer rolspelers, en hulle werk nie op 'n lineêre wyse met mekaar nie. In ander ekosisteme, op die mikro- en makroskopiese vlak, is die belangrikheid van hierdie hoër-orde interaksies al reeds uitgelig. Daar is egter vergelykend baie min inligting oor hoër-orde interaksies in die gis ekologie veld, en moet dus reggestel word om ‘n voorspellende begrip te hê van hierdie stelsels.

Hier het ons gepoog om die huidige *status quo* in multi-spesie gis navorsing te opdateer, deur nuwe metodes te ontwikkel vir die ondersoek van die meganistiese basis van gis-gis interaksies in sintetiese-ekosisteme wat uit meer as twee spesies bestaan. Verder was die studie daarop gemik om 'n beter begrip van hierdie sisteme te genereer, deur om na die transkripsie reaksie van *Saccharomyces cerevisiae* op mede-kultuur in gemengde-spesie kulture van toenemende kompleksiteit te ondersoek.

Hierdie doelwitte is bereik deur eerstens 'n fluoressensie-gebaseerde multi-kleur vloeisitometriese metode te ontwikkel vir die monitering van 'n konsortium wat uit wyn-geassosieerde gis spesies bestaan. Hierdie het die optimalisering van die genetiese modifikasie van die geselekteerde omgewings-geïsoleerde gis spesies behels, gevolg deur uitgebreide validering om die verteenwoordigendheid van die gis in die sisteem te bevestig asook die ontwikkeling van die vloeisitometriese protokol. Dit was gevolg deur die tersaaklike kwessie van reproduseerbaarheid in multi-spesie kulture aan te spreek, en die rol van die fisiologiese toestand van voor-kulture in die bepaling van hul groei prestasie in drie-spesie en vier-spesie konsortia. Ten slotte, om by te dra tot die begrip van die molekulêre meganismes van interaksie in nie-lineêre gis stelsels, het ons getoon dat *Saccharomyces cerevisiae* 'n kombinasie van bekende paar gewyse sowel as heeltemal unieke gene uitdruk wanneer dit in 'n drie-spesie sisteem gekweek word. Deur interaktiewe netwerk visualisering van die gegenereerde transkriptomiese data,

was ons in staat om die sellulêre reaksie in meer detail funksioneel te karakteriseer as wat voorheen in soortgelyke studies gedoen is.

Hierdie studie dra by tot die kennisbasis oor multi-spesie interaksies in mikrobiese ekosisteme deur metodologieë te verbeter om hierdie stelsels meer doeltreffend te bestudeer en potensiële meganismes van interaksie voor te stel wat gis konsortia beheer.

This dissertation is dedicated to:
my parents, for all their sacrifices to further my education,
and my Omi: the original Gertrud

Biographical sketch

Cleo Conacher completed her undergraduate BSc-degree in Molecular Biology and Biotechnology (*Cum Laude*) at Stellenbosch University in 2014, and pursued an Honours degree in Microbiology the year after, graduating *Cum Laude* in 2015. She then completed her Master's in Process Engineering at the Department of Chemical Engineering, Stellenbosch University, in 2017. She spent the better part of 2018 in industry, working for a food safety laboratory. She then enrolled for a PhD in Wine Biotechnology in 2019 at the South African Grape and Wine Research Institute, Stellenbosch University. Her research interests are largely based in yeast biotechnology and ecology, spanning from enzyme bioprocessing to fundamental yeast ecology.

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Preface

This dissertation is presented as a compilation of six chapters and four appendices. Each chapter is written according to the style of the journal to which the manuscript was/will be submitted for publication. The general research topic is introduced in Chapter 1, followed by a two-part review of the literature. These parts have been published as stand-alone reviews and echo the construction of the study itself, which focused on both the fundamental ecological mechanisms of yeast-yeast interactions (Part I) as well as the tools required to observe them (Part II). The first research chapter, Chapter 3, deals with the development of a novel multicolour flow cytometric tool for observation of population dynamics and physical interactions in a synthetic yeast consortium, which is applied throughout all research chapters. The second research chapter (Chapter 4) dealt with the optimization of the multispecies system itself. Chapter 5 reports the fundamental component of the study, which explores the transcriptomic response of *Saccharomyces cerevisiae* to growth within a consortium context. Finally, the results are contextualized in the general discussion and conclusions chapter (Chapter 6), where the appendices, which are all related to providing tools for future experimental work, are discussed as well.

Chapter 1	**General introduction and project aims**
Chapter 2	**Literature review** **Part I:** Peer pressure: evolutionary responses to biotic pressures in wine yeasts Published: DOI: 10.1093/femsyr/foz072 **Part II:** The ecology of wine fermentation: a model for the study of complex microbial ecosystems Published: DOI: 10.1007/s00253-021-11270-6
Chapter 3	**Research results I** Real-time monitoring of population dynamics and physical interactions in a synthetic yeast ecosystem by use of multicolour flow cytometry Published: DOI: 10.1007/s00253-020-10607-x
Chapter 4	**Research results II** The importance of the physiological state of inocula in determining population dynamics in synthetic yeast consortia studies
Chapter 5	**Research results III** Evidence for higher-order interactions in yeast ecosystems
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Appendix III	If two is company, but three is a crowd, then what is four? Answers from a yeast ecosystem

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List of Abbreviations

AA	Amino acids
ARISA	Automated ribosomal intergenic spacer analysis
BFP	Blue fluorescent protein
CFU	Colony forming units
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FISH	Fluorescence in situ hybridization
FT	Fluorescent tag
GFP	Green fluorescent protein
LOG2FC	Logarithm of fold change
Lt	*Lachancea thermotolerans*
OD	Optical density
OYNB	Optimized yeast nitrogen base
PBS	Phosphate buffered saline
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
RFP	Red fluorescent protein
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
RPM	Revolutions per minute
Sc	*Saccharomyces cerevisiae*
SGM	Synthetic grape must
Td	*Torulaspora delbrueckii*
Wa	*Wickerhamomyces anomalus*
WT	Wild-type
YNB	Yeast nitrogen base
YPD	Yeast peptone dextrose
YPDS	Yeast peptone dextrose sorbitol

Chapter 1

General Introduction and Project Aims

General Introduction and Project Aims

General Introduction

Managing risk and reward in wine fermentation is a fine balance. Natural, otherwise known as spontaneous, fermentations are performed by the naturally present microbiota, but are known to be unpredictable. If they go right, the product has a distinct sensorial profile with biogeographical exclusivity that is a marketing dream. Unfortunately, they often go wrong too, wasting valuable resources, time, and money. As such, industrial-scale winemaking practices moved towards inoculating strains of the most robust of fermentation yeast, *Saccharomyces cerevisiae*, to make the fermentation process more predictable and economically favourable. In doing so, anecdotal evidence suggests that these wines have lowered flavour complexity and lowered regional uniqueness. This led to the realization that the diversity of microbes catalysing the fermentation, whether indigenous or inoculated, were important contributors to the final properties of wine. Research has indeed shown the positive role of indigenous wine yeast other than *S. cerevisiae*, often termed non-*Saccharomyces* yeasts, in contributing to improved wine chemical complexity (Comitini et al. 2011; Ciani and Comitini 2015; Vilela 2020; Zhu et al. 2021). Such insights have led to the application of mixtures of yeast species, so-called 'multi-starters', in wine fermentations. However, this solution is arguably a superficial band-aid for industry; while introducing other yeast species does add some *je ne sais pas quoi* to wine, there is very little knowledge on the fundamental ecological mechanisms driving these assemblages. The ideal way to combine the good qualities of both spontaneous and inoculated fermentations relies on understanding the establishment and functioning of the natural ecosystem to the point of manipulating it for better fermentation outcomes.

This challenge is not unique to wine fermentation; in the general realm of microbial ecology, understanding the inner workings of natural multispecies systems for ultimate human exploitation is a major research challenge. A significant component of this lack of predictive understanding stems from the fact that as systems become more complex, the depth of analyses decreases, as a result of the highly exponential increase in system intricacy. As such, in terms of the molecular mechanisms that govern yeast-yeast interactions, the current state of knowledge is largely based on binary, i.e. two-species systems (Rivero et al. 2015; Wang et al. 2016; Curiel et al. 2017; Tronchoni et al. 2017; Englezos et al. 2019; Peng et al. 2019; Shekhawat et al. 2019; Tondini et al. 2020; Roullier-Gall et al. 2020; Mencher et al. 2021). Research focussed on the transcriptome, proteome, and metabolome of interacting yeast has demonstrated specific metabolic and physical interactions during intra-species and inter-species yeast co-cultures. The major findings in these studies agree that physical interactions and structural rearrangements at the cell envelope, competition for limiting trace

elements, and activation of environmental stress response are major components of biotic stress. However, these binary systems are not representative of the complex biotic heterogeneity of the wine ecosystem, and avoid a very pressing issue: the contribution of higher-order interactions. A major contributor to the issue of exploding complexity in multispecies systems is unpredictable, non-linear impacts driven by higher-order interactions which occur in systems comprised of more than two components. Higher-order interactions are not well understood and remain a significant barrier to the efficient use of microbial ecosystems in any bioprocess, including wine. Some progress has been made in understanding higher-order interactions in bacterial systems but there is little to no mechanistic information on this issue in yeast ecosystems (Billick and Case 1994; Guo and Boedicker 2016; Morin et al. 2018; Sanchez-Gorostiaga et al. 2019).

The wine yeast ecosystem presents an excellent model from which to research the gaps in our understanding of the mechanistic bases of complex microbial ecosystems. The argument for this stems from the unique evolutionary history of this ecosystem. Fermenting grape must is a highly selective, harsh environment characterized by temporal successions of different yeast species. This specific environment only exists in the confines of the man-made process for wine-making. There is genetic and phenotypic evidence that the evolutionary trajectory of wine-associated yeast species has been strongly influenced by an adaptation to this anthropogenic environment (Borneman et al. 2014; Albertin et al. 2014; Marsit et al. 2017; Guillamón and Barrio 2017; Dujon and Louis 2017; Legras et al. 2018). Other data strongly suggest that, besides adapting to the specific chemical and physical requirements of this environment, these species have also been subjected to consistent biotic selection pressures. Indeed, the same keystone yeast species are consistently isolated from wine fermentations around the world (Liu et al. 2017; Liu et al. 2020). The combination of these consistent abiotic and biotic selection pressures within an evolutionarily isolated, and anthropogenically linked niche has created a rather neat model ecosystem from which to study eco-evolutionary feedback in yeasts (Conacher et al. 2021).

Aims and Objectives

The following study aimed to address the lack of mechanistic understanding of multispecies yeast ecosystems by using a model consortium of wine-associated yeast species. Over-all, the generated data was envisioned to contribute to the fundamental understanding of yeast-yeast interactions in natural yeast communities, which is inextricably linked to an improved capacity to control these systems for biotechnological applications.

This aim was achieved by completing the following objectives:

- Address technical shortcomings of the field with the development of a higher throughput, fluorescence-based flow cytometry technique for monitoring and observing interactions in multispecies yeast systems
- Improve reproducibility of the synthetic multispecies system by evaluating the impact of pre-culture strategies
- Improve mechanistic understanding of the role of higher-order interactions in yeast ecosystems by investigating emergent higher-order responses in *S. cerevisiae* at the transcriptional level

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Chapter 2

Literature Review Part I

Peer pressure: evolutionary responses to biotic pressures in wine yeasts

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Minireview

MINIREVIEW

Peer pressure: evolutionary responses to biotic pressures in wine yeasts

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One sentence summary: The review highlights the current understanding of yeast ecosystem dynamics and evolution, with a particular focus on wine fermentation as an appropriate model.

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ABSTRACT

In the macroscopic world, ecological interactions between multiple species of fauna and flora are recognised as major role-players in the evolution of any particular species. By comparison, research on ecological interactions as a driver of evolutionary adaptation in microbial ecosystems has been neglected. The evolutionary history of the budding yeast *Saccharomyces cerevisiae* has been extensively researched, providing an unmatched foundation for exploring adaptive evolution of microorganisms. However, in most studies, the habitat is only defined by physical and chemical parameters, and little attention is paid to the impact of cohabiting species. Such ecological interactions arguably provide a more relevant evolutionary framework. Within the genomic phylogenetic tree of *S. cerevisiae* strains, wine associated isolates form a distinct clade, also matched by phenotypic evidence. This domestication signature in genomes and phenomes suggests that the wine fermentation environment is of significant evolutionary relevance. Data also show that the microbiological composition of wine fermentation ecosystems is dominated by the same species globally, suggesting that these species have co-evolved within this ecosystem. This system therefore presents an excellent model for investigating the origins and mechanisms of interspecific yeast interactions. This review explores the role of biotic stress in the adaptive evolution of wine yeast.

Keywords: wine yeast ecosystem; biotic stress; yeast–yeast interaction; evolution; physical contact; yeast ecology

INTRODUCTION

In their natural habitat, free living yeasts adapt to changing environmental conditions to survive and proliferate. Rapid sensing of environmental changes, anthropocentrically referred to as 'stress', leads to the execution of intracellular responses (Mager and De Kruijff 1995; Galhardo, Hastings and Rosenberg 2007). Environmental stresses can be defined as abiotic or biotic. Abiotic stressors may include physical and chemical parameters such as temperature, pressure, pH, radiation, solutes and water concentration, nutrient availability, presence/absence of certain ions, and toxic chemical agents. Biotic stressors encompass the effect of other organisms (micro- and macro-) in the environment (Ciani *et al.* 2016). Cellular stress responses aim to protect the cell from the particular stress (immediately and in the future), as well as repair damage that may have occurred as a result of the stressor (Bauer and Pretorius 2000; Siderius and Mager 2003; Wadskog and Adler 2003). Yeast have proven to be excellent models for the study of environmentally-induced transcriptional changes, due to their ability to rapidly sense and adapt to extracellular stressors, and the availability of advanced

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functional genomics techniques, which allow researchers to recognise and interpret these transcriptional changes (López-Maury, Marguerat and Bähler 2008). With the use of microarray technology, and more recently, RNA-seq techniques, it has been possible to observe global transcriptional changes in yeast exposed to a multitude of conditions.

In eukaryotes, the study of stress response has been studied in most detail in *Saccharomyces cerevisiae*, generating a wealth of fundamental mechanistic knowledge that can be modelled and applied to more complex life forms due to a significant degree of evolutionary conservation (Mager and De Kruijff 1995; Bauer and Pretorius 2000; Estruch 2000; Gasch *et al.* 2002; Siderius and Mager 2003; Matallana and Aranda 2017). While abiotic stress responses have been well characterised, biotic stress remains largely unexplored. From a biological and evolutionary perspective, this might appear rather surprising, since the evolutionary framework of any given species is primarily defined by interactions with other species, and abiotic conditions and stresses only provide a framework to which individual species in any given ecosystem will have to be adapted to (Little et al. 2008). Arguably, in this context, the primary evolutionary pressure (stress) experienced by any given species is therefore exerted by the presence of other interacting species (Ley, Peterson and Gordon 2006; Bailey *et al.* 2013; Andrade-Domínguez *et al.* 2014). The reasons for this gap in our understanding on the other hand is quite simple: most microbiology has focused on studying individual species as pure cultures, and almost all data sets on molecular mechanisms or high-throughput phenotyping in yeast have been generated in single species cultures (Chambers *et al.* 2015; Marsit and Dequin 2015). This is largely due to factors such as biotechnological bias, where single species cultures were the option of choice, and the fact that multispecies cultures are rather difficult to control experimentally (Goers, Freemont and Polizzi 2014). Indeed, an approach to better comprehend biotic stress would have to integrate evolutionary biology, molecular biology and community ecology data (Turcotte, Corrin and Johnson 2012).

Nevertheless, several studies, most of which are primarily descriptive in nature, have characterised interactions amongst yeast, mainly for the purpose of manipulating these ecological relationships for use in industry (Fleet 2003; Di Maro, Ercolini and Coppola 2007; Curiel *et al.* 2017; Liu *et al.* 2017; Tronchoni *et al.* 2017; Rollero *et al.* 2018; Shekhawat *et al.* 2019). These studies are particularly prevalent in the wine industry, given the fact that during wine fermentation a number of microbial role-players, including yeast, filamentous fungi and bacteria are present, and that the interactions between these microbes have a significant impact on the characteristics of the final product, wine (Barbosa *et al.* 2015; Rollero *et al.* 2018). Specifically, interspecies interactions between wine yeasts has become a highly investigated topic because of the positive or negative properties that so-called 'non-*Saccharomyces*' yeasts can contribute to wine (Fleet 2003; Ciani *et al.* 2010). While wine-associated bacteria and filamentous fungi are also significant role-players in the complex evolutionary history of the wine ecosystem, this review will focus specifically on yeast–yeast interactions. From an evolutionary perspective, wine yeasts occupy a relatively isolated niche with high selective pressure, and this anthropogenic environment provides an opportunity to study adaptive evolution on a molecular level, integrating various approaches such as multispecies omics, laboratory-based evolution and synthetic ecology (Little *et al.* 2008; Borneman *et al.* 2014; Marsit and Dequin 2015; Dujon and Louis 2017; Marsit *et al.* 2017; Legras *et al.* 2018).

The following review examines this concept by contextualising the evolutionary relevance of the wine ecosystem, which leads to a brief discussion of the major mechanisms of adaptive evolution in wine yeast, with a specific focus on potential biotic stress adaptation. The final section discusses the current state of knowledge of evolved interaction mechanisms in yeast, the challenges of complex interaction studies, and the way forward in elucidating how yeast–yeast interactions shape associated adaptive evolution trajectories.

EVOLUTIONARY CONTEXT OF WINE-ASSOCIATED YEAST SPECIES

For millennia humans have inadvertently altered the evolutionary trajectory of yeast by exploiting them for their desirable fermentative properties (Marsit and Dequin 2015). Domesticated yeasts that have evolved in anthropogenic environments provide an interesting case study for adaptive evolution. In particular, the evolutionary history of wine yeasts is an especially relevant topic, given the highly selective nature of the wine-making process (Chambers *et al.* 2015; Marsit and Dequin 2015). In contrast to the fermentation matrices of beer and bread, which are biotechnologically well controlled and species-poor environments, grape must is an especially challenging, dynamic habitat for yeasts, where a high selection pressure exists not only as a result of physical (osmolarity, low pH) and chemical (limited nitrogen availability, high ethanol concentrations) parameters (Bauer and Pretorius 2000), but also because of the many different competing microbial species. The genomic evolutionary footprints of this isolated niche is illustrated by the phylogenetic separation of wine yeasts into a distinct lineage from their wild counter-parts, as well as from brewing and baking yeasts (Dujon *et al.* 2004; Borneman *et al.* 2014; Marsit *et al.* 2017; Legras *et al.* 2018). There is also phenotypic evidence of improved fermentative performance and increased fermentation-related stress resistance in wine yeasts (Guillamón and Barrio 2017). Notably, the genomic and phenotypic data shows the link between heritable ecological specialisation of these yeast strains to their anthropogenic or biotechnological uses. Besides the hallmark wine-associated *S. cerevisiae* strains, there is also evidence of domestication in other common wine yeast species, including *Lachancea thermotolerans* and *Torulaspora delbrueckii* (Albertin *et al.* 2014; Hranilovic et al. 2017, 2018).

Interestingly, similar diversity of abundant yeast species is seen globally across spontaneous wine fermentation (Fleet 2008; Liu *et al.* 2017; Binati *et al.* 2019). The wine ecosystem therefore provides a relatively consistent ecological framework in which biotic pressures, in the form of ecological interactions, would have significantly altered the evolutionary trajectory of the participant yeasts, and presents a model to investigate the evolutionary origins of yeast ecological interactions.

ADAPTIVE EVOLUTION OF WINE YEAST

The gene–environment interaction influences adaptive phenotypes in yeast (Harrison, Wright and Mank 2012; Yadav and Sinha 2018). A central, emerging theme within this context is phenotypic heterogeneity within a genetically homogenous population. This phenomenon is advantageous in challenging, unpredictable environments, since it increases the likelihood that a particular representative will be primed for any given stress, and therefore survive, continuing the population (Holland *et al.* 2014). Given the physiological challenges associated

with fermenting grape juice, the gene–environment interaction, which also encompasses phenotypic heterogeneity within wine-associated yeast populations, is notable as a potential origin for adaptive phenotypes. Expectedly, wine yeasts show a number of evolutionary adaptions to the stressful wine environment, illustrating the mechanisms of the gene-environment link in adaptive evolution.

Genome studies have highlighted mutations that have influenced the selection of many traits that are relevant for fitness within the physical and chemical framework of wine fermentation, including resistance to inhibitory compounds and improved nutrient acquisition and metabolism (Marsit and Dequin 2015). Interestingly, there appears to be a link between yeast stress response systems and mutation rates (Galhardo, Hastings and Rosenberg 2007; Chen *et al.* 2012; Shor, Fox and Broach 2013). A major role-player in this proposed link is Hsp90, which has been suggested to act as an intermediate between environmental fluctuations and an altered genotype–phenotype map, since variations in Hsp90 functionality can be linked to mutations, epigenetic variations, protein conformational changes, as well as general genetic variation (Brudvig *et al.* 2011; Schell, Mullis and Ehrenreich 2016; Zabinsky *et al.* 2019). Genes with high trait variation caused by mutations are typically not associated with essential biological functions such as cell growth and maintenance, cell cycle regulation, metabolic processes and transcription (Basehoar, Zanton and Pugh 2004; Landry *et al.* 2007; Park and Lehner 2013). Rather, a positive correlation to evolvability, which was defined as the potential for regulatory change due to spontaneous mutation, was seen in genes that contain a TATA box in their promoter region or in genes with a large trans-mutational size, i.e. whose expression is influenced by a high number of other genes (Basehoar, Zanton and Pugh 2004; Landry *et al.* 2007). The TATA box is essential in recruiting transcriptional machinery to promoter regions during transcription initiation (Basehoar, Zanton and Pugh 2004). The presence of a TATA box in a promoter is linked to an increased likelihood of the associated gene being subtelomeric, rapidly induced during stress response, and more prone to evolutionary selective pressure (Basehoar, Zanton and Pugh 2004). In addition, the TATA box is linked to stochasticity in gene expression, also known as transcriptional 'noise', which increases expression variability (Blake *et al.* 2006). Cell–cell variability is beneficial in stressed populations as it provides a type of bet-hedging strategy, which increases the likelihood that there will be a population representative that is better adapted to a particular transient stress (Beaumont *et al.* 2009). This bet-hedging has indeed been described as a selected trait in yeast populations (Holland *et al.* 2014). From this, it can be deduced that genes involved in rapid adaptation to cohabitants and therefore ecological interactions are likely to contain TATA boxes since this presents as a transient environmental stress.

A more direct source of adaptive evolution that is influenced by population ecology is horizontal gene transfer (HGT) and interspecies hybridisation (Hall, Brachat and Dietrich 2005; Sipiczki 2008; Morales and Dujon 2012; Marsit *et al.* 2015; Milner *et al.* 2019). These processes are reliant on proximity in nature, since some level of cellular contact or uptake of genetic material from the environment is required (Dujon and Louis 2017). Interestingly, but not surprisingly, some genomic regions that are remnants of hybridisation or horizontal gene transfer encode for genes that increase fitness in the challenging fermentation environment (Novo *et al.* 2009; Guillamón and Barrio 2017).

The apparent link between the general stress response and adaptive mutation presents an interesting angle for the argument of biotic selection pressure in yeast evolution (Fig. 1). In this way, community-based stressors may have affected the phenotypic landscape upon which evolutionary forces acted. Zhou *et al.* (2018) provided evidence for the strength of biotic selection pressures in a long term cross-kingdom competitive experiment, where it was shown that heritable genomic changes occurred in a yeast response to bacteria-driven selection pressure. Similarly, Morrison-Whittle *et al.* (2018) evolved *Candida glabrata* and *Pichia kudriavzevii* in co-culture for 65 generations and observed significant differences in the metabolic profiles and growth rates of the evolved versus parental yeasts. These metabolic changes were not investigated at the genome level, however, it was shown that the altered metabolic phenotypes were heritable, demonstrating the power of using biotic selection pressures to evolve novel or desirable phenotypes. The combination of synthetic ecology and evolutionary analysis offers a promising new approach to evaluate the specific effects of biotic stressors and ecosystem-derived selection pressures on the evolutionary trajectory of participating microorganisms.

BIOTIC STRESS IN YEAST–YEAST INTERACTIONS

Many types of interactions occur simultaneously within microbial communities, ultimately affecting population dynamics, phenotypic diversity and genetic diversity within the community (Andrade-Domínguez *et al.* 2014). Hence, community composition acts as a driver of natural selection. To survive, microorganisms must adapt to their living counterparts. This biotic selection results in ecological interaction types such as mutualism, competition, antagonism, predation or commensalism, in combination with the specific environment (Little *et al.* 2008). Simulating biotic stress and assessing interaction mechanisms between microbial community members can shed light on the effect of biotic stressors on the evolutionary fate of strains within consortia as well as the functionality of the community (Song *et al.* 2014). Interactions within microbial communities include communication and coordination of collective activities including signalling, biofilm construction, reproduction and even chemical warfare (West *et al.* 2006). Elucidating the molecular mechanisms of these interactions will provide insights on the adaptive influence and properties of community behaviours. The aforementioned approach is defined as a top-down approach to microbial ecology, where the properties of a synthetic ecosystem are evaluated, and the effects of interaction dynamics can be inferred from the collected data (Dolinšek, Goldschmidt and Johnson 2016).

Yeast–yeast interaction mechanisms

Interactions have been defined based on a simplified binary model, consisting of an actor and a recipient. Interactions may be positive, negative or neutral to the fitness of the actor or recipient. Interactions are grouped according to a range, from parasitic, to benign or neutral, to mutualistic (Little *et al.* 2008). For pairwise interactions there are six main categories: mutualism (+/+), commensalism (+/0), neutralism (0/0), amensalism (0/-), parasitism (+/-) or predation (+/-) and competition (-/-) (Little *et al.* 2008; Song *et al.* 2014). Microbial interaction mechanisms can be either contact-dependent (direct), or

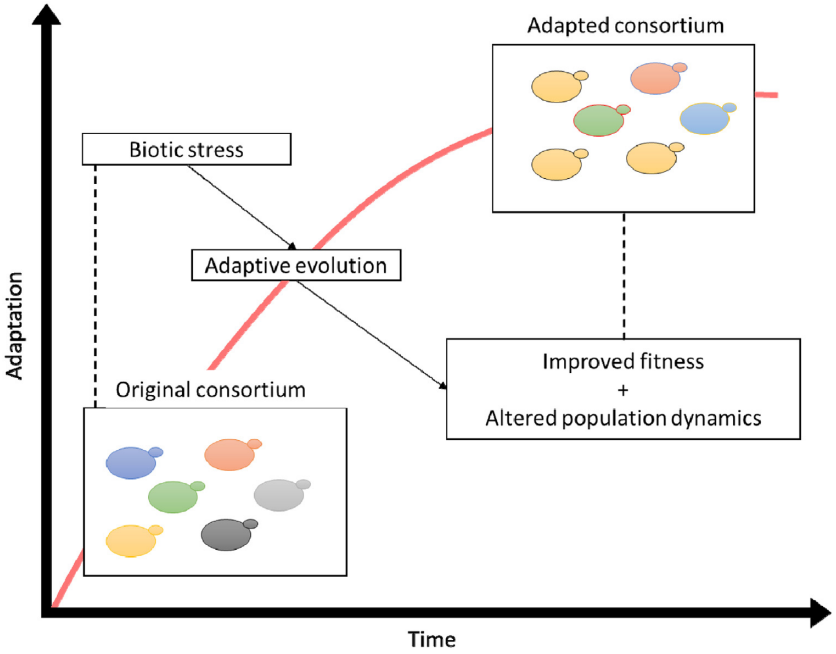

Figure 1. Illustration of the accelerating effect that biotic stress has on adaptive evolution of different yeast species in defined ecological niches.

contact-independent (indirect). Contact dependent mechanisms involve exchange of biomolecules and electrons through direct cell–cell contact (Fig. 2). Contact independent interaction mechanisms indirectly occur by extracellular exchange of diffusible metabolites and signalling molecules between cells (Fig. 3) (Song *et al.* 2014).

Direct interaction mechanisms

Microorganisms within a particular environmental niche are often in direct contact with each other. For direct contact to occur, there must be a physical interface. This interface is the cell wall surface. Nissen, Nielsen and Arneborg (2003) were among the first to suggest a cell–cell contact mechanism for induced cell death of *L. thermotolerans* and *T. delbrueckii*. Years later, this was confirmed in a study that evaluated direct versus indirect contact of *S. cerevisiae* and *T. delbreuckii* in a double-compartment bioreactor, where it was found that cell–cell contact induced cell death in *T. delbreuckii* (Renault, Albertin and Bely 2013). The involvement of toxic compounds secreted by *S. cerevisiae* strains in causing early death of *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* was subsequently demonstrated (Pérez-Nevado *et al.* 2006). Further analyses identified the toxic compounds as small antimicrobial peptides, composed of glyceraldehyde-3-phosphate dehydrogenase fragments (Branco *et al.* 2014). Interestingly, these antimicrobial peptides accumulate on the cell surface, which has been hypothesised as one of the factors that contributes to cell–cell mediated weapons of *S. cerevisiae* (Branco et al. 2017, 2019). Cell–cell contact between *L. thermotolerans* and *S. cerevisiae* reportedly resulted in significant differences in volatile compound production as well as exometabolite levels (Petitgonnet *et al.* 2019). While informative, these studies were mainly descriptive, and lacked any investigation into the molecular mechanisms of the interactions at play. With the advent of omics-based technologies, more mechanistic studies have been done. Recently, a transcriptomic study that sought to characterise the gene expression profiles of *S. cerevisiae* and *L. thermotolerans* in co-culture showed significant effects in genes involved in cell wall structure and integrity, further confirming the importance of cell–cell contact based interaction mechanisms (Shekhawat *et al.* 2019).

Cell–cell adhesion is a central mechanism in the structural organisation of yeast communities (Honigberg 2011). In *S. cerevisiae*, *FLO* genes play an integral role in mediating flocculation, a type of cell–cell adhesion interaction, which forms aggregates of cells (Teunissen and Steensma 1995). Flocculation is seen as an adaptive trait, since it can serve as a protective mechanism during stress, as the inner cells are protected by the outer layer of cells. This mechanism has been described as a cooperative mechanism for genetically similar yeast cells (Smukalla *et al.* 2008). Interestingly, the key *FLO1* gene shows high rates of genetic and phenotypic variability in nature, implying a route for accelerated adaptive evolution. In addition to this, the *FLO* genes are localised to the subtelomeric regions of chromosomes, which are prone to adaptive mutations, as previously discussed (Fidalgo *et al.* 2006). The adaptive evolution of the flocculation phenotype appears to be influenced by transposable element insertion into the regulatory regions of *FLO1* (Hope *et al.* 2017). Rossouw *et al.* (2015) demonstrated that co-flocculation, governed by *FLO* genes, acts as a contact-mediated mechanism that influences population dynamics in wine yeasts. The same research group explored this concept further by over-expressing *FLO* gene family members in *S. cerevisiae* and observing population dynamics in co-culture with *L. thermotolerans*, *Wickerhamomyces anomalus* and *Hanseniaspora opuntiae*. Interspecies adhesions were observed, which significantly affected population dynamics (Rossouw, Meiring and Bauer 2018). Therefore, flocculation appears to be an evolved trait involved in cell–cell interactions in yeast communities.

In-direct interaction mechanisms

Extracellular molecules are also central to many yeast–yeast interactions (Pérez-Nevado *et al.* 2006; Kemsawasd *et al.* 2015; Wang, Mas and Esteve-Zarzoso 2016). Metabolomics, the study of the total intracellular and extracellular metabolites, has significantly contributed to our understanding of microbial interactions. In the case of indirect interactions, the focus is on

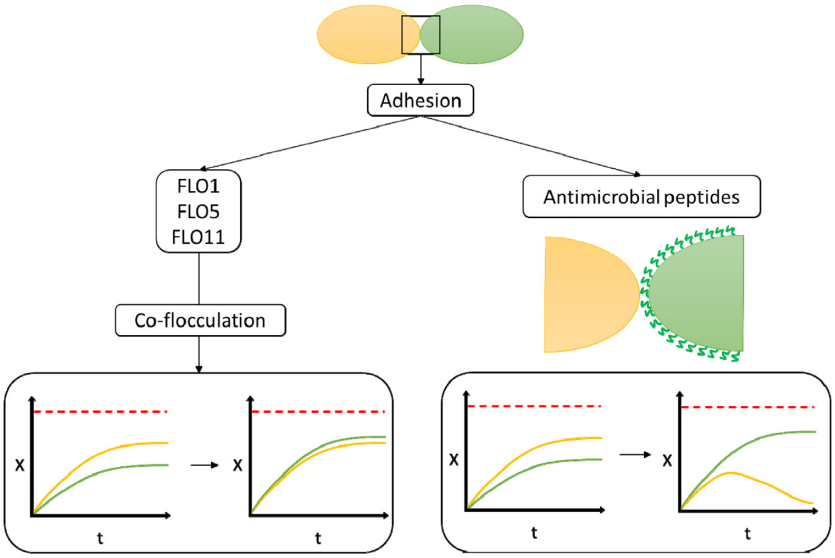

Figure 2. Direct, cell contact-based yeast interaction mechanisms and their effect on interspecies population dynamics. Adhesion is central to flocculation, and the action of cell-surface located antimicrobial peptides.

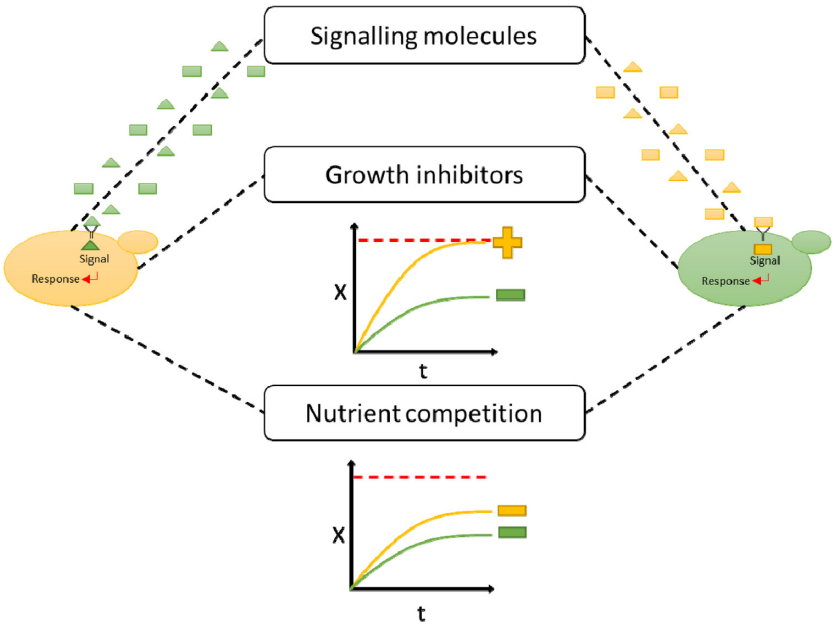

Figure 3. Indirect, non-contact based yeast interaction mechanisms and their effect on interspecies population dynamics.

exometabolomics, which quantifies and identifies extracellular metabolites (Mashego *et al.* 2007). Many studies have demonstrated the significant effects of yeast–yeast interactions on the exometabolome, known as metabolic interactions (Sadoudi *et al.* 2012; Bagheri, Bauer and Setati 2017; Pinu and Villas-Boas 2017; Hassa *et al.* 2018; Peng *et al.* 2018). Exometabolites of significance in indirect interactions include growth inhibitors, signalling molecules and available nutrients (Fig. 3).

Growth inhibitors An extensively studied example of growth inhibiting metabolites is killer toxins. Killer toxins are proteins and glycoproteins secreted by killer yeast strains, which induce death in sensitive yeast strains (Liu *et al.* 2017). The killer phenotype is associated with inherited viral double stranded RNA, linear plasmid DNA or chromosomal DNA (Schmitt and Breinig 2006). These toxins may be associated with the cell surface or are secreted. Historically, only three groups of toxins, K1, K2 and K28, were recognised, but recent studies have shown the involvement of different toxin-like compounds in the killer phenotype, including antimicrobial peptides (Branco *et al.* 2014; Brou *et al.* 2018). As discussed in the previous section, Branco *et al.* identified a killer toxin-like antimicrobial peptide secreted in *S. cerevisiae* as GAPDH fragments, which are active against several non-*Saccharomyces* yeasts (Branco et al. 2014, 2017). This antagonistic property is widespread in yeasts, having been identified in several genera, including *Aureobasidium*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Mrakia*, *Pichia*, *Torulopsis*, *Ustilago*, *Williopsis*, *Zygosaccharomyces* and *Zygowilliopsis* (Liu *et al.* 2015). The release of killer toxins is a competitive advantage,

increasing likelihood of niche dominance in killer yeasts, and causes changes in yeast population dynamics (Pérez-Nevado *et al.* 2006). Interestingly, the killer phenotype is not constitutive and is affected by environmental parameters such as temperature, nutrient availability and presence of neutral or sensitive strains (Perez, Ramirez and Regodon 2001; Gobbi *et al.* 2013).

Besides antimicrobial peptides, other growth inhibiting factors also play a role in antagonistic interactions. These include short- to medium-chained fatty acids, ethanol, acetic acid and acetaldehyde (Liu *et al.* 2017). Gonzalez *et al.* (2018) demonstrated that aromatic amino-acid derived compounds (serotonin and tryptamine) and aromatic alcohols (tryptophol, phenylethanol, and tyrosol) commonly produced during wine fermentations have species-specific impacts on the growth of wine-associated yeasts. Serotonin, tryptamine and tryptophol inhibited growth to varying extents of all tested yeasts.

Signalling molecules Cell–cell signalling, where diffusible exometabolites are emitted, recognised and generate a cellular response, plays a role in several coordinated activities, such as mating, biofilm formation and community organisation (Honigberg 2011). Mating in haploid *S. cerevisiae* cells is guided by pheromone signals between cells of opposite mating types, which triggers the intracellular mating pathway (Bardwell 2004). Ammonia causes metabolic reprogramming and cell differentiation within yeast colonies, and synchronises growth phases between yeast colonies (Váchová and Palková 2018). During growth as a colony, yeast alter the pH of their growth substrate, in an oscillatory fashion, between acidic and alkaline (Palková and Váchová 2003). During the alkaline growth phase, volatile ammonia is released, and acts as a signalling molecule within and between yeast colonies (Palkova *et al.* 1997). This was demonstrated in several genera, including *Candida*, *Cryptococcus, Endomyces, Hansenula, Kluyveromyces, Rhodosporidium, Rhodotorula, Saccharomyces* and *Schwanniomyces*. Similiarly, acetaldehyde was shown to act as a signalling molecule for synchronizing glycolytic oscillation, which affects redox potential, between yeast cells (Richard *et al.* 1996).

Cell density-based signalling also occurs in yeast cells, generically termed quorum sensing, however, this definition is under debate. An example of cell-density based signalling in yeast is a dimorphic switch that occurs at high cell densities. In *Candida albicans,* at low cell densities, the switch is repressed by the presence of farnesol, while at high cell densities, the aromatic alcohol, tyrosol, is secreted, which triggers the dimorphic switch (Honigberg 2011). This switch also occurs in *S. cerevisiae,* however, the signalling molecules differ (Wadskog and Adler 2003). Interestingly, the dimorphic switch of *S. cerevisiae* is also linked to aromatic alcohols, namely tryptophol and phenylethanol (Chen and Fink 2006; González *et al.* 2018). The production of these aromatic alcohols in *S. cerevisiae* is regulated by nutrient starvation stress as well as cell density (Winderickx *et al.* 2003; Chen and Fink 2006). The genes responsible for the first two steps of aromatic alcohol biosynthesis, *ARO9* and *ARO10,* are regulated by nitrogen availability and cell density, via the common transcription factor, Aro80p. At low nitrogen concentrations and high cell density, *FLO11* mediated morphogenic changes occur in *S. cerevisiae* (Chen and Fink 2006). These quorum-sensing-like signalling molecules appear to elicit species specific responses—different aromatic alcohols cause varied effects on morphogenesis and growth rate in different yeast species (González *et al.* 2018). This highlights the potential role that these signalling molecules play in regulating yeast interaction dynamics during ecosystem growth.

Nutrient availability *Saccharomyces cerevisiae* has several finely-tuned genetic and physiological responses to fluctuations of available nutrients, and this has been reviewed extensively (Winderickx *et al.* 2003). In the context of adaptation involving metabolic interactions, competition for nutrients is a notable concept. Indeed, the dominance of *S. cerevisiae* in yeast communities has partially been attributed to its ability to speedily outcompete other yeasts for nutrients (Nissen, Neilsen and Arneborg 2004; Tronchoni *et al.* 2017). This is widely studied in wine fermentation, since grape musts are naturally low in available nitrogen and vitamins (Liu *et al.* 2017). The depletion of nutrients, especially vitamins and assimilable nitrogen, by certain yeast affect the metabolism and, therefore, growth performances and fermentative ability of successive yeast (Ciani, Beco and Comitini 2006; Curiel *et al.* 2017; Bagheri *et al.* 2018; Rollero *et al.* 2018). Competition for nutrients simulates a low nutrient environment, and activates starvation stress response pathways, which are also associated with adaptive evolution (Gasch *et al.* 2002)

Stress response in yeast–yeast interaction studies

Ecological interactions will largely define the evolutionary trajectory of all species (Little *et al.* 2008; Andrade-Domínguez *et al.* 2014; Khan *et al.* 2018). Simply put, the rate of evolution, and the forces of evolution, select for different traits in individuals, depending on the co-inhabiting species in any given ecosystem. Within a mixture of species, there are three broad mechanisms that may influence evolutionary dynamics: (i) competition for resources, (ii) ecological sorting, a post-evolutionary mechanism that describes the fact that species that co-occur in a particular environment are there as a result of habitat suitability or biotic interactions (Warren *et al.* 2014) and (iii) combinatory adaptation to both abiotic and biotic factors, which results in trade-offs between the two, dependent on fitness gains (Lawrence *et al.* 2012). These concepts can be applied to any ecological niche, but when focussing on yeast evolution, the diversity and competitiveness of the wine fermentation environment presents an accessible model, since it represents a relatively recent ecological niche, which has already been demonstrated to be of evolutionary significance since it has been generally accepted that wine yeast strains *S. cerevisiae* are an example of microbial domestication (Borneman *et al.* 2014; Chambers *et al.* 2015; Marsit and Dequin 2015; Almeida *et al.* 2017). In fact, the same yeast species are found globally in naturally fermenting grape juices, suggesting that the entire wine fermentation ecosystem can be considered as domesticated, and that it is likely that the interactions between species in this system have been shaped by evolution (Fleet 2008; Liu *et al.* 2017; Binati *et al.* 2019).

Several studies have been published that evaluate the impact of co-cultures by using transcriptomic and proteomic techniques (Barbosa *et al.* 2015; Rivero *et al.* 2015; Curiel *et al.* 2017; Tronchoni *et al.* 2017; Alonso-del-Real *et al.* 2019; Peng *et al.* 2019; Shekhawat *et al.* 2019). Most of these studies, however, have analysed data only from the perspective of *S. cerevisiae* (Barbosa *et al.* 2015; Rivero *et al.* 2015; Curiel *et al.* 2017; Peng *et al.* 2019).

Barbosa *et al.* compared the transcriptional response of *S. cerevisiae* during mono- and co-culture with *Hanseniaspora guilliermondii*. In mixed culture, *S. cerevisiae* showed a reduced fermentative ability, and upregulation of genes involved in biotin, vitamin and co-factor biosynthesis, cell wall regeneration/degradation, as well as stress response. The transcriptomic responses were then aligned to their hypothesised effects on flavour-active compounds.

In a more ecologically focussed study, Rivero *et al.* (2015) demonstrated the involvement of the stress response protein, Hsp12, as well as *PAU* genes in interactions between natural *S. cerevisiae* strains. A metabolic interaction as well as cell contact-based interaction was seen between relatively fit and unfit strains. Mass spectrometry analyses were conducted on the exometabolome, and no significant differences in non-proteinaceous metabolites were found. However, significant differences in the protein content was found: increased cell-wall proteins, ribosomally regulated proteins, and the heat shock proteins Eno1p, Hsp12p, Tdh3p and Ssb1p were observed. The largest quantitative difference was found with Hsp12p, but it stands to reason that the other proteins may also have an effect, showing potential for further studies. The over-all effect of extracellular compounds on the investigated strains was assessed by comparative transcriptome analyses, where the majority of differentially expressed genes were involved in stress response pathways, including heat shock proteins and the general stress response transcription factor, Msn4p. Interestingly, 22 out of 24 genes of the subtelomeric *PAU* family were downregulated. The role of *PAU* genes in the adaptive response of *S. cerevisiae* to the fermentation environment was previously identified in a functional analysis of the *PAU* gene family (Luo and van Vuuren 2009). From the data generated by Rivero, it appears that the Hsp12p protein is released by dying cells, which may act as a high cell density signalling molecule to trigger stress responses in other genetically similar cells, thereby conferring a fitness advantage. In addition, copy number variation in *PAU* genes seems to affect fitness of *S. cerevisiae* strains in wine fermentation conditions.

Recently, Peng *et al.* (2019) investigated the proteomic response of *S. cerevisiae* to the presence of *L. thermotolerans* during alcoholic fermentation. A tandem mass tag proteomic approach was used, where cellular and extracellular proteins were extracted, pooled and evaluated at two time points during fermentation. At the earlier time point, *S. cerevisiae* showed increased levels of metabolic and stress response proteins, and decreased levels of proline synthesis and apoptosis proteins. The most upregulated metabolic proteins included two proteins involved in the glycine decarboxylase complex, and an intermediate in methionine synthesis. Methionine synthesis has been shown to act as a protective mechanism in yeast stress (Vinci and Clarke 2007). The stress response proteins included heat shock proteins, ergosterol biosynthesis proteins and endocytosis intermediates. At the later time point, protein synthesis was highly upregulated, while the stress response was repressed. This study confirms the significance of the stress response in wine yeast ecological interactions at the translational level.

As annotated genomes of more yeast genera became available, studies could analyse transcriptomic responses of non-*Saccharomyces* yeasts (Curiel *et al.* 2017; Tronchoni *et al.* 2017; Shekhawat *et al.* 2019). Tronchoni *et al.* (2017) evaluated the gene expression programs of *S. cerevisiae* and *T. delbrueckii* in anaerobic co-culture over time. For analysis of co-culture RNA-Seq data, they developed a chimeric genome by concatenating the genome builds representative of the two species. For *S. cerevisiae*, after about 3 hours (T1) in co-culture, there was a pronounced upregulation in genes involved in glycolysis, glucose uptake and mitochondrial activity. This was seen in *T. delbrueckii* as well, but only after 12 hours (T2) of co-culture, suggesting that *S. cerevisiae*'s speedy metabolic response is a competitive advantage during co-culture. The early sampling point is taken to increase the likelihood that differential gene expression is because of interaction, and not metabolic stresses such as nutrient depletion. Similar to Rivero *et al.* (2015), Tronchoni also observed overexpression of Hsp12p and *PAU* genes across both time points in *S. cerevisiae*. Interestingly, Hsp12p was also overexpressed in *T. delbrueckii* across both time points, indicating that this protein may play a role in interspecies interaction as well as intraspecies interaction as suggested by Rivero. In fact, three other stress response genes involved in osmotic and oxidative stress were also upregulated in *T. delbrueckii* at T1. This is notable since oxidative stress is directly linked to adaptive mutation (Heidenreich 2007).

Building on this work, the same research group then applied similar methodologies to study interspecies interaction between *S. cerevisiae* and other yeast species, namely *Hanseniaspora uvarum* and *Candida sake*, under aerobic conditions (Curiel *et al.* 2017). The results were reported and discussed only from the perspective of *S. cerevisiae*. The major contribution of this study was showing that different species elicited different gene expression programs in *S. cerevisiae*, while also highlighting the commonalities observed in all the evaluated co-cultures. This difference in metabolic reaction to different species was also observed in studies focussed in nutrient consumption comparisons between *S. cerevisiae* and non-*Saccharomyces* yeasts in co-culture (Wang, Mas and Esteve-Zarzoso 2016). In Curiel's study, the presence of *T. delbrueckii* appeared to induce *S. cerevisiae* genes under nitrogen catabolite repression, while *C. sake* upregulated *S. cerevisiae* genes involved in replication as well as genes related to cell wall and membrane composition. Lastly, *H. uvarum* highly upregulated genes involved in stimulus response, stress response and cell wall mannoproteins. The stress protein Hsp12p, mentioned by Rivero and Tronchoni, was only upregulated in response to *T. delbrueckii*, seemingly disproving its role as a general signalling molecule, however, this result poses an interesting question. Since Hsp12p appears to only be upregulated during co-culture with more closely related species, could it perhaps be a species-specific signalling molecule? Indeed, there was apparently a stronger transcriptomic response in *S. cerevisiae* to *T. delbrueckii* than for *C. sake* and *H. uvarum*, implying that more genetically similar species may result in fiercer competition or physiological reaction in *S. cerevisiae*.

Alternatively, the degree of competitive response in yeast may be influenced by the evolutionary proximity of two species within a niche, rather than their genetic similarity. Alonso-del-Real *et al.* (2019) recently demonstrated this by assessing the transcriptomic response of *S. cerevisiae* strains and *S. kudriavzevii* to each other in co-culture. As expected, the *S. cerevisiae* strain of wine origin outcompeted the *S. kudrivzevvi* strain during fermentations in synthetic grape must, and this was as a result of the speedy, extensive transcriptomic response of *S. cerevisiae* to direct contact with a competing yeast, which accelerated nutrient uptake and metabolism. Notably, substituting the competitive co-culture with an *S. cerevisiae* strain originating from oak showed an entirely different transcriptomic response compared to the wine *S. cerevisiae* strain. Transcriptomic remodelling in the oak *S. cerevisiae* strain was significantly delayed and less extensive than that of the wine *S. cerevisiae* strain, showing that a unique competitive phenotype may have evolved within the highly selective and competitive wine fermentation niche.

Lastly, a recent study evaluated gene expression responses of both *S. cerevisiae* and *L. thermotolerans* in aerobic and anoxic co-culture (Shekhawat *et al.* 2019). During anaerobic co-culture, *S. cerevisiae* increased expression of iron and copper acquisition systems as well as 5 *PAU* genes, and decreased expression of

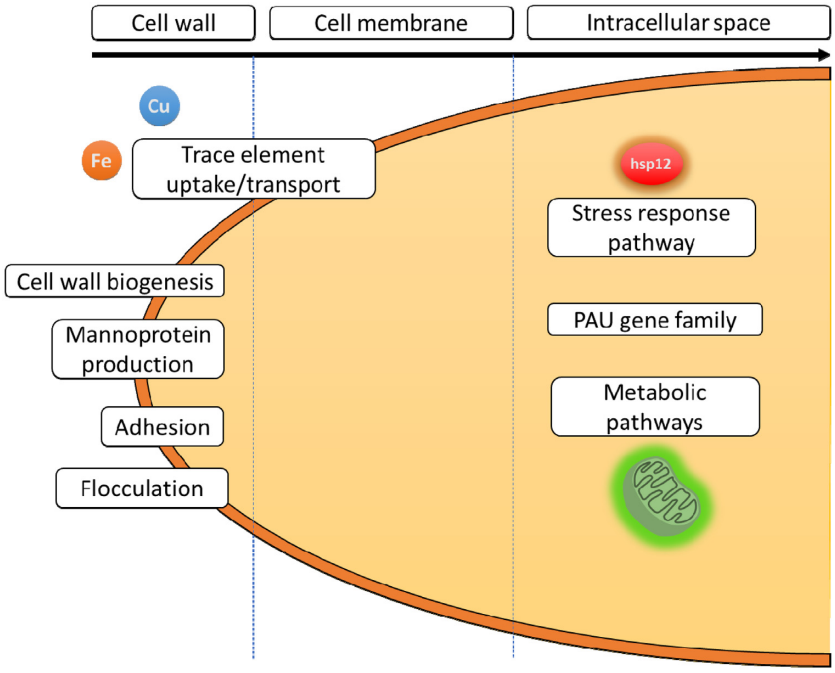

Figure 4. Common gene expression programs affected during interspecies yeast co-culture, as determined by transcriptomic and proteomic analyses (Barbosa *et al.* 2015; Rivero *et al.* 2015; Curiel *et al.* 2017; Tronchoni *et al.* 2017; Peng *et al.* 2019; Shekhawat *et al.* 2019)

genes associated with cell–cell adhesion, aggregation and flocculation. In contrast, *L. thermotolerans* decreased expression of genes involved in iron and copper uptake and transport, and upregulated genes involved in amino acid metabolism. Both species showed significant differential expression of cell wall biogenesis genes. Increased expression of two mannoprotein-encoding genes was also observed in *S. cerevisiae*, similar to Curiel *et al.* (2017), who made use of aerobic cultures. This overlap may imply that this overexpression of mannoproteins is independent of oxygenation and might be significant for cell wall mediated interaction mechanisms.

From the above research, it is clear that both direct and indirect interactions occur amongst different yeast species and strains. That is, biotic stressors elicit a physiological response, to which cells must adapt to maintain fitness. Transcriptomic and proteomic studies have aided in elucidating some of the molecular mechanisms as play. The cell wall interface, competition for nutrients and trace elements, and activation of stress response appear to be the most significant factors in all of these studies (Fig. 4). However, all of the discussed studies only attempted to characterise interactions between two species. In addition, there is a definite underrepresentation of yeast species other than *S. cerevisiae*. In terms of the methodology used, there is certainly room for more detailed confirmatory experiments of transcriptomic data. For a more complete picture of characterizing biotic stress adaptation in yeast, these factors should be considered in future work.

Three is a crowd?

This apparent lack of more complex interaction studies in yeast is understandable, given the challenges already associated with analysing only two species. The addition of another member to a synthetic ecosystem will cause non-linear effects, and disentangling the different layers of binary interactions in such a system is difficult. This non-linearity in synthetic ecosystems is caused by several factors. First, the differences in growth rates and unbalanced uptake and production of metabolites between consortium members. Second, the fluctuations in abiotic factors caused by the consortium itself benefits or harms certain strains, thereby affecting the population dynamics, which in turn causes fluctuations in abiotic conditions. Lastly, the ecological interactions, including chemical warfare at play during production of secondary metabolites, also affects the microbial system (Weibel 2008). Adding to this, spatial organisation of the consortium also plays an integral role in governing any interactions at work (Kim *et al.* 2008). A small number of researchers have tackled interaction studies with more than two populations in bacterial or multi-kingdom systems, but this type of study is extremely limited in yeast–yeast interactions (Kato *et al.* 2005; Kim *et al.* 2008; Virtanen, Hattula and Arstila 2010). In addition, there is a definite absence in attempting to create predictive models of how the addition of more cohabitants affects the metabolic and physiological functioning of a community (Weibel 2008).

Besides controlling the factors involved in experimental set-up, which is a major task in synthetic ecology systems, a significant challenge lies in meaningfully interpreting the massive data sets generated in these studies (Goers, Freemont and Polizzi 2014). In the case of transcriptomic and proteomic studies, the mapping of RNA or peptide data to particular members of the consortium from a mixed population sample becomes an inexact task in more closely related (genetically homologous) communities. This is challenging in two-member systems, and it becomes exponentially more complex by adding more members. To illustrate this difficulty in data manipulation, in the manuscripts discussed previously, authors chose only to discuss certain pathways that show the highest differential expression, since discussion of each significant finding would most likely produce an overwhelmingly long and broad discussion (Curiel *et al.* 2017; Tronchoni *et al.* 2017; Shekhawat *et al.* 2019). There is a clear lack of rigorous and statistically significant data

evaluation from a global cellular perspective, which can largely be attributed to a lack of data science support that is needed to give a basis for such a comprehensive discussion. This type of selective reporting in data sets allows authors to retrospectively construct any number of convincing arguments for the molecular workings within the cell, generating largely descriptive instead of mechanistic explanations. Despite these limitations, pair-wise studies have contributed significant preliminary data that has illuminated the way forward for more complex, targeted studies.

Considering the above, it is imperative to design studies with the power of available data analyses and computational models in mind. For instance, techniques to separate mixed populations of closely related community members will enhance the accuracy of aligning transcriptomic and proteomic data, which allows for more specific evaluation of metabolic functioning of individual members. The number of biological repeats in experiments must be such that established statistical methods can extrapolate meaningful results. Inclusion of confirmatory experiments after transcriptomic or proteomic analyses, by use of targeted molecular manipulations, should be the norm given the complexity of the genotype-phenotype map in these studies. The skill of managing and interpreting large sets of computational data should be emphasised among molecular biologists. Lastly, researchers are becoming increasingly aware of the importance of functionality versus diversity within microbial communities. This is especially important in the design of synthetic ecosystems for particular applications. This involves a shift from linking functionality to species, and rather linking functionality to associated genetic elements. If these factors are considered, the evaluation of more complex ecological interactions may become a more realistic, meaningful undertaking.

CONCLUSIONS AND PERSPECTIVES

Owing to the short generation time and advanced molecular techniques available, microorganisms make for ideal models to study adaptive evolution. Domestication of wine yeasts has altered their evolution and recounting the molecular happenings that occurred during adaptation to the highly stressful wine fermentation environment has provided a compelling case study in adaptive evolution. With the use of well-annotated genome sequences, comparative evolutionary genomics shed light on the genetic contributors to the evolved phenotypes we observe today. Phenotypic heterogeneity remains an actively researched phenomenon, due to variable gene expression that is observed because of gene-environment interactions, epigenetics, as well as epistasis within genomes. Researchers have managed to create a detailed, albeit incomplete account of how abiotic stressors contributed to the evolutionary force of natural selection in the creation of adapted phenotypes. However, a more elusive question remains the impact of how these yeasts have adapted to their cohabitant microorganisms.

With the use of synthetic ecology, it has become possible to simulate ecological interactions, and attempt to determine the physiological characteristics and mechanisms at play. Genome wide expression data combined with proteomic and metabolomic studies, has demonstrated that there are definite metabolic and physical interactions during intraspecies and interspecies co-culture in wine yeast. The major findings in these studies agree that the cell wall interface, competition for trace elements and activation of stress response are major effects of biotic stress. These preliminary studies have laid the foundation for future studies, which must address the shortcomings brought to light. The effects and mechanisms of these yeast–yeast interactions still remain unclear. The majority of data that has been reported is based on differential gene expression and lacks confirmatory experimental work in the form of targeted expression analyses or targeted proteomics to confirm the downstream significance of the RNA transcripts. Furthermore, post-transcriptional mechanisms also contribute to controlling gene expression programs but have been neglected. There is also a clear need for better global cellular data analysis and modelling, which will contribute to generating a more holistic hypothesis in describing the cellular and genetic effects of co-culture. Lastly, there is limited data on yeast–yeast interaction studies that involve more than two species. This is understandable, given the complexity of characterising pair-wise interactions, and the unpredictable changes that the addition of another cohabitant causes. However, given the diversity of natural yeast ecosystems, accurate predictions of community dynamics require consideration of the joint influence of multiple species (and functional gene diversity), as opposed to pair-wise co-evolution only. The impact of addressing these challenges will be two-fold: it has the potential to contribute to the fundamental knowledge of yeast–yeast ecological interactions, and to the industrial manipulation of these yeast for better quality fermentations.

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Literature Review Part II

The ecology of wine fermentation: a model for the study of complex microbial ecosystems

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MINI-REVIEW

The ecology of wine fermentation: a model for the study of complex microbial ecosystems

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Abstract

The general interest in microbial ecology has skyrocketed over the past decade, driven by technical advances and by the rapidly increasing appreciation of the fundamental services that these ecosystems provide. In biotechnology, ecosystems have many more functionalities than single species, and, if properly understood and harnessed, will be able to deliver better outcomes for almost all imaginable applications. However, the complexity of microbial ecosystems and of the interactions between species has limited their applicability. In research, next generation sequencing allows accurate mapping of the microbiomes that characterise ecosystems of biotechnological and/or medical relevance. But the gap between mapping and understanding, to be filled by "functional microbiomics", requires the collection and integration of many different layers of complex data sets, from molecular multi-omics to spatial imaging technologies to online ecosystem monitoring tools. Holistically, studying the complexity of most microbial ecosystems, consisting of hundreds of species in specific spatial arrangements, is beyond our current technical capabilities, and simpler model systems with fewer species and reduced spatial complexity are required to establish the fundamental rules of ecosystem functioning. One such ecosystem, the ecosystem responsible for natural alcoholic fermentation, can provide an excellent tool to study evolutionarily relevant interactions between multiple species within a relatively easily controlled environment. This review will critically evaluate the approaches that are currently implemented to dissect the cellular and molecular networks that govern this ecosystem.

Key points

- *Evolutionarily isolated fermentation ecosystem can be used as an ecological model.*
- *Experimental toolbox is gearing towards mechanistic understanding of this ecosystem.*
- *Integration of multidisciplinary datasets is key to predictive understanding.*

Keywords Wine microbiome · Ecology · Synthetic ecology · Higher order interactions · Yeast-yeast interactions

Introduction

The foundations of microbiological science are based on data inferred from studies of monocultures. While informative and necessary for understanding the physiology and metabolism of a given species, this pure culture bias has diminished focus on what role ecological interactions have played in the natural functioning and evolution of these microorganisms. For

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example, the model yeast *Saccharomyces cerevisiae*, arguably the best understood eukaryote to date, is still largely a mystery in the context of its natural, biotically heterogeneous evolutionary niche. This assessment is supported by the obvious lack of ecologically relevant annotations of the *S. cerevisiae* genome sequence. Phenotyping, including in particular high throughput, is almost exclusively carried out on single strain liquid cultures or colonies. This gap is even more remarkable when considering that most evolutionary selection pressures in the life cycle of an organism is likely due to the presence of other, competing or generally interacting, species, and less to physical or chemical challenges.

Understanding the functioning of microbial ecosystems is also an increasingly relevant research challenge. It has long been well established that microbial ecosystems are at the base of all food chains, and provide a generic and essential life-support system for all macroscopic organisms and ecosystems. However, it is only since recently that we appreciate how the fine tuning of these ecosystems directly impacts all aspects of multicellular life, from plant and mammalian health to agricultural food production and human well-being, from carbon sequestration to bioremediation. In biotechnological research, this appreciation has led to a shift away from the improvement of single species through ever more onerous, but in final analyses intrinsically limited approaches such as synthetic biology, to the harnessing of multispecies consortia or of entire ecosystems. Genetically and functionally diverse microbial communities can be more productive and effective in complex biotechnological and therapeutic applications (Little et al. 2008; Bernstein and Carlson 2012; Hays et al. 2015; Said and Or 2017). Furthermore, the functioning of a particular microorganism in monoculture does not predict its functioning within a mixed culture, and there are varying levels of interplay between pairwise and higher order interactions that also govern ecosystem functioning (Sanchez 2019; Sanchez-Gorostiaga et al. 2019). Therefore, characterising microbial communities, to the extent that we know enough mechanistically to manipulate them, requires investigating them within an ecologically relevant framework, including co-habitant microorganisms.

While the advantages of studying microbial communities are clear, the execution of these studies is fraught with conceptual and technical challenges (Peng et al. 2016; Widder et al. 2016; Prosser and Martiny 2020). The effects of these challenges are clear when assessing the scientific aim for most ecosystem-centred research studies, which is currently dominated by descriptive, diversity-based surveys. There are proportionately far fewer reports of mechanistic studies which address our fundamental understanding of the establishment, functioning, and evolution of microbial communities (Prosser 2020). This scarcity of key information needs to be addressed to make the intelligent design and control of microbial communities a realistic undertaking.

We propose that the wine yeast ecosystem, besides being biotechnologically relevant, is an ideal model to address some of the gaps in our understanding of the mechanistic bases of complex microbial ecosystems. The importance of the evolutionary context of ecological interaction has been highlighted (Conacher et al. 2019; Gorter et al. 2020), and the wine microbial ecosystem provides an interesting framework to investigate the role of biotic influences on ecosystem establishment and functioning. Support for this is provided by genetic and phenotypic evidence for adaptation of wine-associated yeasts to the abiotic and biotic parameters within the wine niche—an anthropogenic, biotechnologically relevant microbial niche (Borneman et al. 2014; Albertin et al. 2014; Marsit et al. 2017; Guillamón and Barrio 2017; Dujon and Louis 2017; Legras et al. 2018). In addition, there appears to be several keystone yeast species within spontaneous wine fermentations that are consistently isolated in abundance across temporal and spatial scales (Liu et al. 2017). This implies the presence of consistent biotic pressures that these yeasts would be evolutionarily adapted to. It is therefore possible to investigate ecological interactions of the wine ecosystem within the evolutionary context of a well-defined, evolutionarily isolated niche. Couple this to bottom-up (or top-down) synthetic ecology approaches, and the opportunity to address causality within a complex ecosystem presents itself.

The aim of the following review is to provide a basis for researchers to apply the lessons learnt in wine fermentation ecology to the broader field of microbial community ecology. This review introduces and critically discusses the current state of knowledge in wine fermentation ecology, specifically examining the grape must microbiome, and the application of synthetic ecology approaches to create more manageable microbial assemblies to investigate, including elucidating the molecular mechanisms and evolutionary forces at play within these systems. The focus is then shifted to the specific research tools and techniques used within this field, as well as exciting technological advancements that we predict will be crucial in the quest to predictively understand and model complex microbial ecosystems.

The ecology of grape must fermentation

Fermentation of grape must to wine is a complex process catalysed by the metabolic activity of the indigenous microbial community or inoculated starter cultures. In the case of natural fermentations, the indigenous community originates from the grape berry surface as well as the fermentation environment, including winery equipment. As fermentation proceeds, the diversity of the microbial community decreases, and its composition changes, in response to the chemical alterations that the fermenting must undergoes, as well as biotic factors such as ecological interactions (Fig. 1). The initial

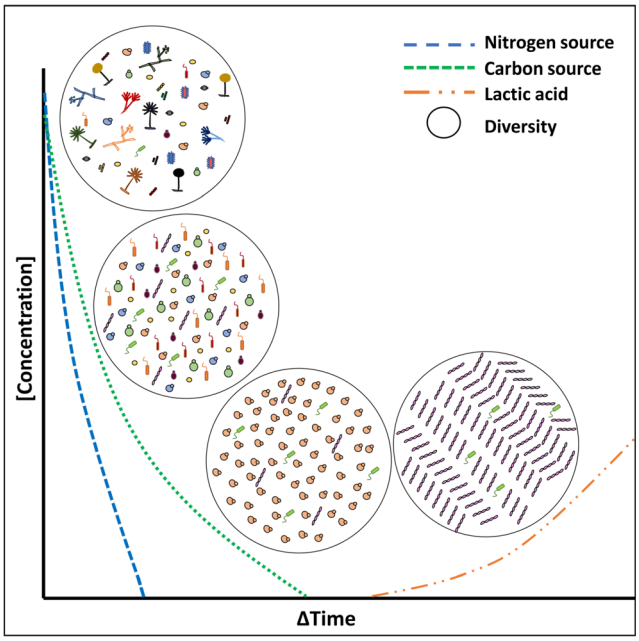

Fig. 1 General microbial diversity succession during spontaneous alcoholic and malolactic fermentation

microbial diversity present within the fermentation, whether it is indigenous or inoculated, has proven to be an important contributor to the final properties of wine, and this has prompted research into understanding the origins, diversity, ecology, and metabolic partitioning of the wine fermentation microbiome.

Within the wine fermentation microbiome, fungi play central roles at all stages of the process. The phylum *Ascomycota* is generally the most predominant in grape must often accounting for 40–80% of the population, followed by *Basidiomycota* (5–15%) while the other phyla are minor. Filamentous fungi frequently detected in grape must irrespective of cultivar or agronomic practices, include members of the genera *Aspergillus*, *Penicillium*, *Alternaria*, *Botryotinia* (anamorph *Botrytis*) as well as *Cladosporium* (Bokulich et al. 2014; David et al. 2014; Pinto et al. 2015; Setati et al. 2015; Kecskeméti et al. 2016; Grangeteau et al. 2017; Morrison-Whittle and Goddard 2018; Zhang et al. 2019). The yeast-like fungus, *Aureobasidium pullulans*, is arguably the most dominant species in grape must followed by ascomycetous non-*Saccharomyces* yeasts, in particular *Hanseniaspora*, *Candida*, *Pichia*, *Metschnikowia*, *Starmerella* and the basidiomycetous species of *Rhodotorula* and *Cryptococcus* (Bokulich et al. 2014; David et al. 2014; Pinto et al. 2015; Setati et al. 2015; Kecskeméti et al. 2016; Grangeteau et al. 2017; Morrison-Whittle and Goddard 2018; Kioroglou et al. 2019). Other genera such as *Torulaspora*, *Lachancea*, *Saccharomycodes*, *Zygosaccharomyces*, *Issatchenkia*, *Meyerozyma*, *Wickerhamomyces*, *Filobasidium*, *Sporobolomyces*, *Rhodosporidium* and *Vishniacozyma* are frequently detected in grape must but are not always abundant (David et al. 2014; Setati et al. 2015; Bokulich et al. 2016; De Filippis et al. 2017; Grangeteau et al. 2017; Kioroglou et al. 2019; Xu et al. 2020). In terms of bacteria, the phylum *Proteobacteria* is typically dominant in grape must from various cultivars and regions followed by *Firmicutes* and other phyla such as *Actinobacteria*, *Acidobacteria* and *Bacteroidetes* with varying abundance levels (Bokulich et al. 2012; Bokulich et al. 2014; Pinto et al. 2015; Zarraonaindia et al. 2015; Bokulich et al. 2016; Portillo et al. 2016; Lleixà et al. 2018). Members of the orders *Bacillales*, *Pseudomonadales*, *Rhodospirillales*, *Enterobacteriales*, *Actinomycetales* and *Sphingomonadales* are frequently encountered in grape must (Bokulich et al. 2012; Bokulich et al. 2014; Portillo et al. 2016; Zhang et al. 2019).

Typically, the population of filamentous fungi decreases sharply in the early stages of fermentation owing to anaerobiosis. Regarding the yeast, an early dominance of weakly fermentative non-*Saccharomyces* yeasts (e.g. *Hanseniaspora* spp.) over non-fermentative species is often apparent. These are gradually replaced by moderate fermenters such as *Torulaspora delbrueckii*, *Lachancea thermotolerans* and *Starmerella bacillaris*, although some species with low fermentation activity might occur sporadically during fermentation (Pinto et al. 2015; Bokulich et al. 2016; De Filippis et al. 2017; Xu et al. 2020). Ultimately, *S. cerevisiae* dominates most fermentation processes at the end of alcoholic fermentation. Regarding the bacteria, *Proteobacteria* typically declines over time during fermentation while the *Firmicutes* increase, particularly the *Lactobacillales*, the predominant catalysers of malolactic fermentation (Fig. 1).

Historically, explanations for the temporal decreases in diversity during grape must fermentation have largely centred on the ability of the observed dominant microorganism, i.e. *S. cerevisiae*, to thrive in the harsh conditions of the fermented must, e.g. high alcohol concentrations, low nitrogen availability and low pH. However, as we delve more into the ecology of the wine microbiome, it has become clear that biotic selection pressures, such as ecological interactions, are also important drivers of succession within the wine microbial community, and generally in any sustained microbial community (Bagheri et al. 2020). Furthermore, using wine as a model of anthropogenic selection means that once the molecular mechanisms of ecological interactions are elucidated, it could also provide information on the evolution of species and communities in this relatively well-defined environment.

The study of fermentation ecosystem ecology and evolution

The approaches that have been employed to understand the ecology of wine fermentation can generally be divided into two major spheres, namely description of the natural microbiome or in the novel application of synthetic ecology to design and characterise simplified synthetic consortia or

develop co-evolution strategies. Furthermore, pairwise interaction studies are a common approach in attempting to understand the molecular basis of microbial community functioning. The following will examine the current state of each of the aforementioned subjects and introduce the ways in which these approaches are aiding in the understanding of complex microbial communities.

Wine microbiome

The most complex level of wine fermentation ecology is the study of the natural grape must microbiome. The grape berry microbiota together with those present on winery equipment during grape processing constitute the grape must microbiome in natural or spontaneous fermentations. Over the past 5 years, the composition of the grape must microbiome has gained much interest owing to early studies which alluded to its contribution to wine quality. Wine microbiome research has largely focussed on surveying and describing the microbial diversity present, and quantifying the effect of various environmental parameters or processing practises on this diversity, and how this influences wine characteristics.

A major theme is the influence of terroir, or local environmental conditions and biogeographical factors, on the grape must microbiome. Using high-throughput amplicon sequencing technologies, studies have shown that an estimated 40% of the fungal diversity in grape must/juice could be attributable to environmental vineyard derived taxa often detected in soil, on grapevine leaves and grape surfaces (Pinto et al. 2015; Morrison-Whittle and Goddard 2018; Alonso et al. 2019). This makes intuitive sense since grapes are ephemeral and therefore not a long-term host; thus, most berry surface microbiota originate from the vineyard soil, fauna and flora, as well as the surrounding environments. Recently, studies investigating the spatial ecology of grape must and wine fermentation processes have demonstrated that grape must samples originating from contiguous vineyards (Setati et al. 2015; Kecskeméti et al. 2016), as well as vineyards within short (1–15 km) distances (Portillo et al. 2016; Knight et al. 2020) and > 100-km regional scales (Bokulich et al. 2014; Knight and Goddard 2014; Taylor et al. 2014; Morrison-Whittle and Goddard 2018; Kioroglou et al. 2019) exhibit distinct microbial communities. Invariably, similar genera and even species are encountered across different locations; however, the microbial community structures (e.g. relative abundances of species, ratios of fermentative and non-fermentative species) result in significant differences in species richness and evenness between locations. These regional distinctions have been shown to correlate with farming practices (Setati et al. 2015; Kecskeméti et al. 2016; Grangeteau et al. 2017; Xu et al. 2020), grape varietal (Bokulich et al. 2014; Portillo et al. 2016; Xu et al. 2020) as well as local weather conditions and viticultural practices that alter the microclimate (Portillo et al. 2016; Morrison-Whittle et al. 2017; Morrison-Whittle and Goddard 2018; Zhang et al. 2019; Knight et al. 2020). Furthermore, grape health status can contribute significantly to inter- and intraspecific variability (Bokulich et al. 2012; Lleixà et al. 2018). For instance, while *Hanseniaspora* is generally the dominant genus in grape must, healthy grapes mainly harbour *Hanseniaspora uvarum*, while botrytized and rotten grapes may display high incidence of *Hanseniaspora osmophila* (Lleixà et al. 2018). Overall, the geographic delineation of the grape must microbiome weakens and collapses as the fermentation progresses, as the community structure shifts to one that is dominated by *S. cerevisiae*.

The research focus at this level is clearly more general, but has generated a wealth of descriptive knowledge that is useful in informing specific viticultural practises and in sustaining regionally distinct wine products. However, the fundamental ecological mechanisms at play are impossible to define, given the vast number of fluctuating, location-specific variables at play. Once the aim of a study shifts to a more mechanistic investigation, it is necessary to also modify the approach to a more controlled experimental design. This is where the value of synthetic ecology is introduced, where a simplified system can be studied under controlled conditions, in the hopes that the results may be quantitatively extrapolated to more complex systems.

Synthetic ecology

Synthetic microbial ecology can be broadly defined as 'the rational design and theory driven manipulation of synthetic ecosystems' to better understand microbial interactions between co-existing microorganisms and their environment (Dunham 2007; Kazamia et al. 2012a; De Roy et al. 2014; Stenuit and Agathos 2015; Dolinšek et al. 2016; Zomorrodi and Segrè 2016; Said and Or 2017). These synthetic ecosystems are usually thoughtfully designed, considering appropriate strain selection, beneficial microbial interactions and optimal culture conditions for the specific biotechnological or industrial application. This allows for more controlled study of interspecies interactions and an improved understanding of the fundamental elements of cooperative and stable ecosystem functioning.

A hallmark feature of these synthetic communities is that they can be intelligently designed to perform novel tasks by exploiting properties such as diversity, resource partitioning, spatial organisation and obligate interdependence which are inherent to the natural environment (Brenner et al. 2008; Hays et al. 2015; Johns et al. 2016; Lindemann et al. 2016; Zomorrodi and Segrè 2016; Roell et al. 2019; Tsoi et al. 2019; Qian et al. 2020). Indeed, microbial communities can perform more functions through the co-ordinated division of labour than their monoculture counterparts with each participating member performing a certain task (niche

differentiation), thus creating more productive, stable, and resilient ecosystems (Hays et al. 2015; Gorter et al. 2020). With the advent of the field of synthetic microbial ecology, synthetic ecosystems, which mimic biologically complex environments, but with reduced complexity, are now routinely used to better understand interspecific interactions and their effects on community dynamics (Kazamia et al. 2012b; Hom and Murray 2014; Hays et al. 2015; Germerodt et al. 2016; La Sarre et al. 2017; Li et al. 2017; Ponomarova et al. 2017; Hillesland 2018; Du et al. 2019; Zuñiga et al. 2019; Liao et al. 2020; Zuñiga et al. 2020).

Consortia

Population dynamics and metabolite profiles of synthetic wine consortia, both in synthetic and real grape must, have shown that ecological interactions between wine yeasts and their environment play an important role in determining fermentation outcomes (Ciani et al. 2006; Ciani et al. 2010; Comitini et al. 2011; Suzzi et al. 2012; Ciani and Comitini 2015; Bagheri et al. 2017; Bagheri et al. 2018; Bagheri et al. 2020; Zhu et al. 2021). However, how these microbial communities have evolved within fermentation ecosystems and the role that ecological processes play in determining the selective conditions which drive evolutionary change, has been underexplored. Some progress has been made in the realm of pairwise interactions (see Pairwise interactions), given that these are comparatively easier to investigate. Still, a major obstacle in the field is understanding and predicting the role of higher order interactions in ecosystem functioning. Higher order interactions, or higher-than-pairwise interactions, occur in systems with more than two discrete populations, where there are deviations from what occurs in the pairwise (i.e. null) system (Sanchez 2019). Especially important is the accurate definition of the null model, which inherently determines the statistical significance of any higher order interactions observed—this is particularly where the use of synthetic consortia assists in creating a manageable subset of a natural community from which to infer ecological dynamics.

In wine, it has become evident that a more diverse fermentative community generates a product with higher perceived sensory quality, albeit unpredictably (Fleet 2003; Andorrà et al. 2012; Varela et al. 2018). This has spurred new research into characterising beneficial microbial contributors within the wine microbial community, which has largely been focussed on population dynamics and metabolite analyses (Ciani et al. 2010; Comitini et al. 2011; Bagheri et al. 2017; Englezos et al. 2018; Englezos et al. 2019b; Bagheri et al. 2020). The major rationale employed is the use of bottom-up approaches, where a synthetic consortium comprised of wine-associated yeasts are applied in a controlled manner to defined growth media or real grape must (Ciani et al. 2006; Wang et al. 2016).

Early studies in the application of controlled, multispecies fermentation starters were the first to highlight and describe the use of simple synthetic consortia in wine (Ciani et al. 2006; Ciani et al. 2010; Comitini et al. 2011; Suzzi et al. 2012; Ciani and Comitini 2015). Bagheri et al. (2017, 2018, 2020) constructed an eight strain synthetic yeast consortium, consisting of one *S. cerevisiae* strain and 7 non-*Saccharomyces* strains and characterised population dynamics and major volatiles in response to varying inoculation ratios and abiotic parameters. A real-time system for monitoring population dynamics in a 4 species synthetic wine yeast consortium has also recently been developed, where species-specific influences on population dynamics of a consortium were observed (Conacher et al. 2020). An inter-kingdom consortium investigation between two wine yeast species (*S. cerevisiae* and *Starmerella bacllarius*) and two lactic acid bacteria (*Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) and *Oenococcus oeni*) showed the importance of these interactions in malolactic fermentation completion and wine properties (Englezos et al. 2019a). The approaches employed by these studies allow for characterising any number of environmental or species-specific effects on consortium population dynamics, as well as their impacts on the composition of resultant wine. However, investigating the mechanistic principles underlying the ecological interactions, more specifically higher order interactions, that take place within these model consortia, has been largely neglected. This is likely as a result of the technical difficulty involved, in both the execution and data analyses stages, as well as limitations of current 'omics' techniques that have been optimised for monoculture investigations. A promising avenue in recognising patterns in large multivariate datasets created by model consortia is in the application of machine learning approaches (Rubbens 2019; DiMucci 2020) or theoretical simulations (Marsland et al. 2020). While this has yet to be applied to any yeast ecosystems, these data-driven mathematical techniques will be necessary to build predictive models from data generated by synthetic consortia.

Adaptive evolution strategies

Synthetic ecology approaches have also accelerated research investigating the evolutionary mechanisms of microbial communities using co-evolution strategies. Recently, it has been demonstrated that by using a combination of ecological engineering, experimental evolution and biotic selection, yeast and microalgae strains with improved growth phenotypes were generated, demonstrating that co-evolution can have significant phenotypic effects on yeast and microalgae populations over a relatively short period (50 generations) in evolutionary timescales (Oosthuizen et al. 2020). Similarly, du Toit et al. (2020) showed that after 100 generations, co-evolved wine yeast and bacterial strains showed improved mutualistic

growth. More importantly, these improvements were also shown to be maintained under non-selective conditions. This indicates the emergence of mutually beneficial adaptations independent of the synthetic selection pressure (du Toit et al. 2020). The benefits of co-evolution are further highlighted in a long-term cross-kingdom study where it was shown that yeast-bacteria competition resulted in the reprogramming of the yeast genome with the generation of phenotypically diverse traits which could be useful industrially (Zhou et al. 2017b; Zhou et al. 2017a).

One of the few studies exploring evolved communities with more than two species was conducted with 5 species of bacteria which had been isolated from beech trees and co-cultured for 70 generations (Lawrence et al. 2012). Here, it was observed that each species evolved more in community than when cultured in isolation. Moreover, resource usage and syntrophy was more diversified in bacteria that were evolved in community resulting in more productive communities than those communities comprised of bacteria which had been evolved in isolation. These findings showed that species interactions can have a major impact on evolutionary dynamics, which can also have positive effects on ecosystem productivity.

In a follow-up study using up to 12 species from the same environment, evolutionary experiments (~ 60 generations) in 3 different conditions were performed. In one of the tested environments, it was seen that community productivity was improved in terms of biomass yields much more due to community evolution than was observed from predictions made for the constituent species. Furthermore, statistical comparisons of community and species yields showed that in diverse communities, species interactions evolved to be less negative, again leading to more productive environments (Fiegna et al. 2015).

Pairwise interactions

The most targeted of fermentation ecology studies are those that seek to elucidate the mechanisms that govern cell-cell interactions at the molecular level. Arguably the most common approach in elucidating ecological mechanisms within more complex communities is to focus on separate pairwise interactions involving only two parties. Most studies have focussed on these interactions in isolation, with two species mutualisms (Hillesland and Stahl 2010; Hom and Murray 2014; Du et al. 2019; Naidoo et al. 2019; Oosthuizen et al. 2020; du Toit et al. 2020), host-microbe symbiosis (McFall-Ngai 2014; Frankowiak et al. 2016; Knight et al. 2017; Cani 2018; Bernasconi et al. 2019) and predator-prey (Herron et al. 2019; Friman et al. 2008; Kaitala et al. 2020) studies being the most common interaction types presented in the literature.

These simplified binary systems have allowed researchers to elucidate some of the mechanistic bases of ecological interaction in wine-associated yeast. These interactions have been reviewed recently (Conacher et al. 2019; Bordet et al. 2020), with one of the major themes being the role of contact (physical) versus non-contact (metabolic) based mechanisms of interaction. Exploring this further, researchers have employed a range of designs to compartmentalise and physically separate different species under investigation while allowing for metabolic exchange of common growth media (Renault et al. 2013; Taillandier et al. 2014; Kemsawasd et al. 2015; Wang et al. 2015b; Branco et al. 2017; Englezos et al. 2019c; Petitgonnet et al. 2019). These studies have highlighted the importance of the cell wall interface in ecological interactions, as well as identifying important metabolites involved in positive, negative or neutral interaction pairs.

Indeed, the impact of binary interspecies interactions on yeast population dynamics is a fast growing collective of research, though much still remains to be known: Importantly, the molecular mechanisms behind these interactions and their effect on yeast gene expression still remains poorly defined. For this reason, recent studies have taken various “omics” approaches in order to better define these interactions, especially the effects that they may have on yeast transcriptomes and proteomes of interacting species (Tronchoni et al. 2017; Peng et al. 2019; Petitgonnet et al. 2019; Shekhawat et al. 2019). However, this is where most of these studies stop, unfortunately not further confirming and characterising the role of the gene expression targets identified. To truly understand the molecular mechanisms at play, it will be necessary to evaluate the function of the genetic targets that have been identified by next generation sequencing technologies.

Tools for investigating fermentation ecosystem dynamics

Advances in experimental methods and equipment are analogous to testing hypotheses and advancing our theoretical understanding of fermentation ecology. Accurate quantification of different populations in ecosystems, whether synthetically constructed or naturally occurring, is central to fermentation ecology studies. It is an intrinsic requirement of knowing ‘who’ performs ‘what’ function, ‘who’ ecologically interacts with ‘who’, and how fluctuations in the population dynamics of an ecosystem affect its functioning. The most widely used methods in monitoring population dynamics, including culture-based or sequencing-based techniques, while generally reliable and simple to execute, are not capable of giving a real-time snapshot of the diversity within a given culture. Recently, trends have moved towards more real-time monitoring techniques such as flow cytometry, microscopic imaging techniques, and online bioreactor technologies. These hold exciting opportunities in the realm of observing physical interactions within microbial communities and in evaluating

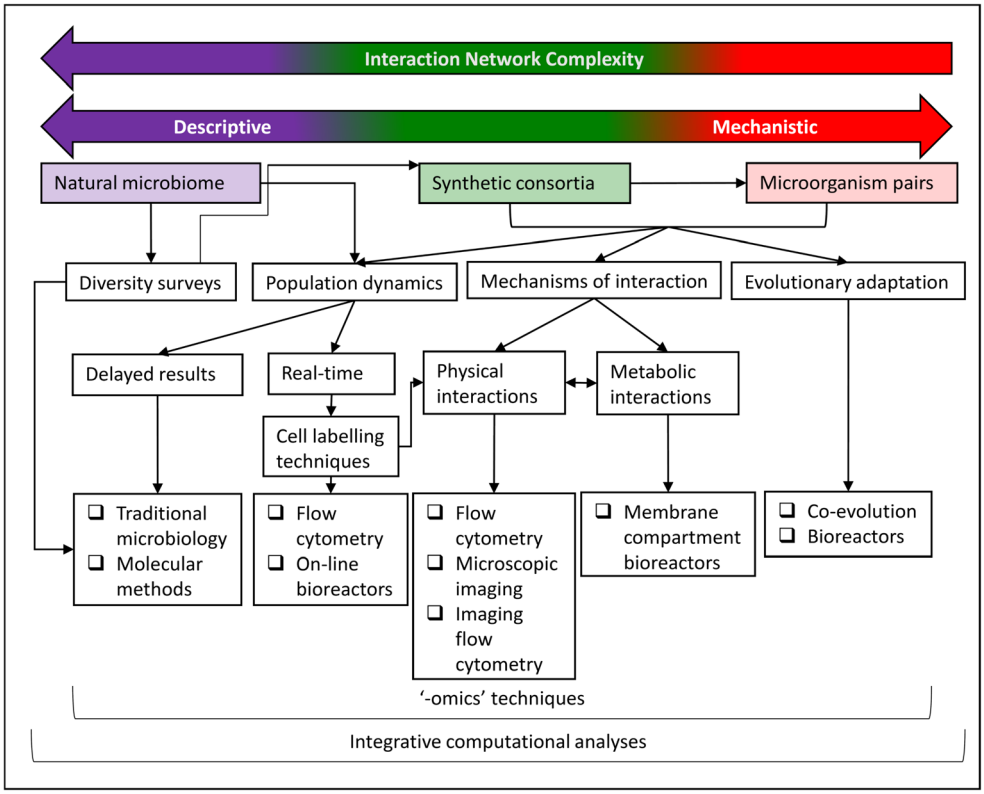

Fig. 2 Concept map for relevant experimental tools in fermentation ecology

community resilience. The following will describe the state of the art in how researchers monitor cells within ecological experiments in wine fermentation, new possibilities in investigating metabolically and/or cell contact–driven interaction mechanisms, and predicted trends moving forward (Fig. 2).

Traditional microbiological methods

The beginnings of monitoring diversity in yeast ecological experiments were based on traditional, culture-based techniques. The major traditional method for differentiative cell enumeration in mixed cultures is culturing samples on solid differential or selective growth medium. This technique relies on differentiating cells based on their colony morphology (i.e. differential) or presence versus absence (i.e. selective). Wallerstein Laboratory (WL) nutrient agar is widely used to differentiate between yeast genera commonly isolated in fermentative environments (Pallmann et al. 2001). The cell count data generated is based on the number of colony forming units within the sample, and is representative of the viable and culturable cells within the sample.

For total biomass determination, with no differentiation between different co-habitants in mixed culture, optical density at 600 nm, wet cell weight or dry cell weight can be used. These techniques all do not give an indication of cell viability. Optical density is a reliable and accurate way to measure biomass within a culture indirectly, but this technique is not ideal in aggregating or flocculating cultures, which often occurs in mixed species populations. Wet and dry cell weight measurements require large amounts of biomass to weigh accurately, which decreases their usefulness in smaller volume, high-throughput experiments.

The advantages of these traditional microbiological techniques are that they are well-established, simple to execute, and require minimal analytical equipment. The disadvantages are their time-consuming nature, which limits high throughput, parallel experiments; the limited opportunity for any real-time analyses given the labour required for each sample; the fact that the data generated by these techniques are statistically weaker, given how few cells per sample are analysed; and the process is more prone to sampling bias and user error.

Molecular methods

DNA-based technologies such as quantitative PCR (qPCR) and Fluorescence *In Situ* Hybridization (FISH) using universal primers or probes targeting rRNA genes can be used to complement culture-based methods. However, such approaches would only allow researchers to quantify total microbial counts. In order to analyse community composition, these methods require prior knowledge of the species to be quantified. Undoubtedly, such methods do not offer a holistic overview of the microbiome as they only allow for quantitation of known species, fast-growing species well-adapted to the prevailing cultivation conditions as well as abundant

species. In contrast, profiling methods, such as PCR-DGGE and ARISA, rely on universal primers and the heterogeneity of the target amplicons. Species are identified either on the basis of amplicon denaturing ability as determined by their base pair composition (for DGGE), or on the basis of their fragment length (for ARISA). These methods reveal more diversity than culture-based methods, though they also mainly allow for detection of abundant species. Species/populations with densities lower than 10^3 cfu/mL in the sample or those which are two-orders of magnitude lower than the most abundant species are often not detected. Moreover, identification to species level from short sequences of PCR-DGGE fragments (≤ 200 bp) is not always reliable, while with ARISA species identification is often not possible since fragment length is not unique between many species.

Recently, most studies have relied on direct phylogenetic surveys commonly based on amplicon sequencing of the variable regions in the bacterial 16S rRNA gene and the internal transcribed spacers (ITS1 or ITS2) of the fungal ITS-5.8S rRNA gene (Bokulich et al. 2014; Bokulich et al. 2015; Bokulich et al. 2016; Kecskeméti et al. 2016; Kioroglou et al. 2019; Zhang et al. 2019; Xu et al. 2020), or in some cases D1–D2 region of the 26S rRNA gene (Taylor et al. 2014; Knight et al. 2015; Wang et al. 2015a; Morrison-Whittle and Goddard 2018; Knight et al. 2020) and the 18S rRNA gene (David et al. 2014; Portillo and Mas 2016; De Filippis et al. 2017). Unsurprisingly, amplicon sequencing retrieves 40–60% more diversity than PCR-DGGE or culture-based methods. This is because all microbial groups (i.e. including those that are deliberately excluded in culture-based approaches) and minor taxa are captured. However, it is important to note that this approach can also lead to overestimation of certain taxonomic groups as detection of dead or non-viable cells is possible. In addition, taxonomic classification of microbial species is not always up to date on reference databases and requires additional curation by researchers to ensure correct designation of taxa. For instance, *Starmerella bacillaris* is still designated *Candida zemplinina*. Nonetheless, amplicon sequencing detects similar genera as those retrieved by culture-based methods and PCR-DGGE although divergent results are sometimes obtained owing to the biases associated with each method (David et al. 2014; Setati et al. 2015; Wang et al. 2015a; Lleixà et al. 2018).

Microscopic imaging techniques

Microscopy with a haemocytometer is a simple, manual technique for generating quantitative cell count data. If differential enumeration is required, the target organisms must be morphologically distinguishable. The cell count data can be generated immediately after serial dilution of the sample, but requires some form of viability staining to give an indication of the viability of the cells being counted. Besides quantitation of cell numbers, the real power of microscopy in microbial ecology is the 'live action' observation of physical interactions and morphological responses in microbial communities.

Physical interactions are known to play important roles in microbial community dynamics and functioning. As such, observing and quantitatively describing physical interaction phenotypes such as aggregation and co-flocculation during fermentation is an important undertaking, and microscopic imaging techniques are ideal for this. In yeast ecosystems, a limitation of microscopy is the lack of clearly distinguishable morphological diversity between wine-associated yeast species and strains, which makes differentiating between them a subjective process. Furthermore, manual microscopy is limited in terms of the number of cells that can be evaluated, and more high-throughput systems, such as flow cytometry, as discussed later in this review, rely on fluorescence-based differentiation. Therefore, in visualising synthetic ecosystems, methods for labelling different yeast ecosystem members are important to evaluate.

Techniques for labelling populations of interest within ecosystems

The major options that have been applied in yeast ecosystems include FISH or genetic manipulation with fluorescent markers. FISH involves design of a specific nucleic acid probe, bound to a fluorescent marker or indirect reporter molecule, which hybridises to and labels complementary nucleic acid sequences within a given sample, allowing identification and quantification of a target organism within a microbial community via microscopy or flow cytometry (Volpi and Bridger 2008). FISH-based techniques have been used to study a number of complex microbial communities, including gut and oral microbiomes, soil communities and activated sludge communities (Geva-Zatorsky et al. 2015; Lukumbuzya et al. 2019; Costa et al. 2020). Samples used for standard FISH are destructively analysed, requiring fixation prior to hybridization—eliminating the possibility of real-time analyses or downstream applications requiring live cells.

The advantages of FISH-based monitoring are that it gives an indication of spatial localization, and can be applied to unculturable microorganisms (Lukumbuzya et al. 2019). New variations on standard FISH analyses have been designed specifically for use in microbial communities, including multicolour FISH, where different fluorophores are used singularly or in combination to distinguish up to eight phylogenetically distinct populations, as well as Raman-FISH, which combines the use of FISH with Raman microspectroscopy (Volpi and Bridger 2008; Lukumbuzya et al. 2019). In wine ecosystems, several FISH labels for different wine-associated yeast species exist, and they have been successfully applied to monitor population dynamics of mixed fermentations. However, their application in wine-like

conditions remains challenging due to difficulty of successful designing FISH probes, species-specific rRNA instability and sample autofluorescence (Wang et al. 2014).

Heterologous expression of fluorescent markers in distinct populations within microbial communities is more suited to live imaging techniques and do not require destructive sample analysis. The expression strategy may involve transformation of a plasmid encoding a fluorescent protein with a selective marker, or integrating the fluorescent protein cassette into the genome of the target organism. While easier to accomplish experimentally, plasmid-based expression requires the presence of selective pressure to maintain expression, and copy number variation may occur within a labelled population. Integrative expression requires more knowledge of the target organism genome, and often results in low transformation efficiencies, especially in non-model microorganisms. The benefits of such a strategy are that it eliminates the need for selective pressure and ensures a similar metabolic burden across a labelled population (Lee et al. 2013).

Integrative expression also allows for tagging of specific genetic elements, enabling quantitative analyses of their expression and visualisation of their localization within the cell. Besides minimising spectral overlap between the selected fluorescent proteins, an important consideration in using fluorescent protein markers are the conditions required for their maturation. For instance, most fluorescent proteins require oxygen to mature, and have different levels of sensitivity in terms of pH and temperature (Sheff and Thorn 2004; Chudakov et al. 2010; Lee et al. 2013; Higuchi-sanabria et al. 2016). Suitable markers should be screened in the growth conditions that they will eventually be evaluated in, and expression of the marker should not influence the behaviour of the organism within the target community. This is an especially important undertaking in an environment as harsh as synthetic or real grape must, which is often used in experimental fermentations. Fluorescent labelling of yeast to track their numbers and whereabouts have been applied in multi-strain *S. cerevisiae* systems, as well as two-species yeast co-cultures (Momeni et al. 2013; Hart et al. 2019; Petitgonnet et al. 2019). In terms of investigating higher order interactions, an integrative fluorescent marker–based system for tracking population dynamics in a constructed wine yeast consortium consisting of four different species grown in synthetic grape must has been developed (Conacher et al. 2020). The findings have shown the usefulness of the platform for real-time tracking and visualisation of such labelled populations.

Once the target organisms in a multispecies system can be visually differentiated, the possibilities for observing their physical interactions or morphological changes within a defined space or condition/s are exponential. With the use of confocal microscopy, a 3D image of cells within a labelled consortium can be generated. Time-lapse recordings are especially useful in observing temporal changes in cell-cell interactions during community establishment or recovery. The limitation of microscopy-based techniques is the relatively few number of cells within a culture that can be analysed at any given time, and manual counting and/or processing of cells can be time consuming. Therefore, for higher throughput quantitative analyses, flow cytometry has proven a powerful tool.

Flow cytometry

Flow cytometers rapidly detect and quantify optical properties, such as size, granularity and fluorescence of particles within a liquid medium. Owing to the speed, accuracy and statistical power of the technique in comparison to culture-based enumeration and detection, flow cytometry is ideal to incorporate into microbial ecology research (Porter et al. 1997; Kron et al. 2007; Wang et al. 2014; Props et al. 2016; Rubbens and Props 2021). Flow cytometers, previously large, cumbersome pieces of equipment that required space and expert knowledge to operate, are now able to fit on a small benchtop. Furthermore, user-friendly designs and software systems have plateaued a previously steep learning curve in operation, making the technique more accessible to any scientist.

Flow cytometry can distinguish between populations within a microbial community based on any specific fluorescent properties (including autofluorescence, fluorescent labels and specific dyes), cell size, cell morphology and nucleic acid content (Kron et al. 2007). It is also possible to evaluate the metabolic state and viability when used in conjunction with specific staining protocols (Longin et al. 2017; Bordet et al. 2020). These properties are detected in parallel, can be in real-time, and at a pace of thousands of cells per second—a vast improvement on the 20–200 cells evaluated on an agar plate or haemocytometer. In a more general microbial ecology context, flow cytometry has previously been applied to determine bacterial diversity in natural aquatic and activated sludge ecosystems where populations were differentiated based on cell morphology and/or nucleic acid content (Davey and Winson 2003; Props et al. 2016; Brown et al. 2019; Heyse et al. 2019). Flow cytometry has also been used to monitor bacterial community stability properties, characterising community shifts and resilience in response to abiotic and biotic stressors (Liu et al. 2018; Liu and Müller 2020). This concept can easily be translated to investigating such ecological questions within the wine-associated microbial community. The use of flow cytometry for population differentiation and enumeration in wine and the effectivity of different staining techniques has previously been reviewed by Longin et al. (2017).

The high statistical power of flow cytometry also allows for robust mathematical modelling approaches. An exciting development in this field is the use of supervised machine learning, where flow cytometry data collected from synthetically constructed consortia informs the development of a model that

can recognise the same populations in natural microbial communities (Rubbens 2019; Rubbens and Props 2021).

In addition to detection and enumeration of populations, flow cytometers may also be coupled to a cell sorting module, enabling physical separation and collection of populations of interest (Mattanovich and Borth 2006). This modality is beneficial when downstream analyses of only a particular population is needed, including transcriptomic and proteomic investigations (key in mechanistic investigations of ecological interactions). For example, the transcriptomic responses of two differentially labelled strains of *S. cerevisiae* in co-culture have been investigated in this way (Pérez-Torrado et al. 2017), as well as the intracellular proteomics of *S. cerevisiae* and *L. thermotolerans* in co-culture (Peng et al. 2019). The application of this sort of approach has been limited, but the insights gained from these studies will be pivotal in addressing the current lack of mechanistic understanding of yeast ecosystems.

While flow cytometry is an established technique for ecology research, one limitation is that each data point collected is just that—a simple dot on a graph. Apart from sorting individual cells of interest for visualisation, conventional flow cytometry does not allow for visual observation of the cells being analysed. This need for combining microscopy with flow cytometry gave rise to imaging flow cytometers.

Imaging flow cytometry

At the intersection of microscopy and flow cytometry are imaging flow cytometers. Imaging flow cytometry is a relatively new technology, but it is already proving to be a trailblazing innovation in several research spheres. It combines the statistical power of high-throughput quantitative flow cytometric data, with the detailed visual images created by fluorescence microscopy. The technology has been used to improve the taxonomic resolution of phytoplankton community quantification (Dashkova et al. 2017; Dunker 2019), observe cell-cell interactions between predator and prey microorganisms (Dey et al. 2019), and evaluate extracellular vesicles—which have been hypothesised to play important roles in yeast-yeast interactions (Clark 2015; Mencher et al. 2020). While still in its infancy in terms of being applied to yeast ecology, imaging flow cytometry may prove useful in characterising population dynamics in synthetic and natural wine ecosystems, allowing for precision in evaluating an otherwise extremely complex community. In terms of applications, any fluorescence microscopy or light microscopy experiment relevant to investigating ecological interaction mechanisms can be scaled up and data acquired in a massively parallel fashion.

Bioreactor technologies

Careful control of culture conditions are intrinsic in investigating causality in microbial ecology experiments. Currently, bioreactors are the best choice for precise control of abiotic factors within pure and mixed cultures, since one can computationally control temperature, pH, oxygenation, agitation and growth kinetics through different growth medium feeding strategies. In addition, bioreactors are central to any research that requires long-term cultivation of microorganisms, especially important in studying adaptation in response to abiotic or biotic selection pressures (Zhou et al. 2018; Liu et al. 2018).

Membrane compartment bioreactors

The physical separation of yeast co-culture populations is important to identify contact-based or metabolically driven mechanisms of ecological interaction. The most logical and suitable means to achieve this goal relies on the use of bioreactor systems (Table 1). Amongst these systems, there are two main approaches: simple formats of dialysis tubing submerged into media contained within a flask or bottle (Nissen et al. 2003; Nissen and Arneborg 2003; Kemsawasd et al. 2015; Wang et al. 2015a, 2015b; Branco et al. 2017; Petitgonnet et al. 2019) or systems consisting of two vessels separated by a membrane (Renault et al. 2013; Lopez et al. 2014; Taillandier et al. 2014; Englezos et al. 2019c).

These fermentations rely on the physical separation of two microbial populations within a medium which is shared and where metabolites are exchanged (depending on the system efficacy and size of the metabolite or compound in question). The non-physical contact fermentation may then be compared to its counterpart: traditional co-culture fermentations where cell-cell contact is present. Any observed differences would allude to a cell-cell contact role in the observed interaction phenotype/s.

The use of dialysis tubes in these studies has proven useful in elucidating physical interaction mechanisms (Table 1) and is a system that should be able to be replicated in most laboratories since it requires a simple setup. With the use of such a system, authors have illustrated that viable *S. cerevisiae* cells cause early cell death of non-*Saccharomyces* yeasts (Nissen et al. 2003; Nissen and Arneborg 2003) and recently provided evidence for the role of antimicrobial peptides produced by *S. cerevisiae* in achieving the latter (Kemsawasd et al. 2015; Branco et al. 2017). In comparison to two-compartment bioreactors (Renault et al. 2013; Lopez et al. 2014; Taillandier et al. 2014; Englezos et al. 2019c), the dialysis system usually has a difference in fermentation volume between its compartments and relies on passive diffusion for the movement of metabolites. The latter means that homogeneity between compartments is achieved at a slower rate compared to two-compartment bioreactors (which utilise either a peristaltic pulp

Table 1 Summary of non-physical contact strategies and bioreactor systems aimed at studying the effects of cell-cell contact

Type of fermentation system	Mode of metabolic exchange	Non-*Saccharomyces* yeast in co-culure fermentations with *S. cerevisiae*	Non-physical contact strategy	References
Dialysis tube	Passive diffusion	*L. thermotolerans* *T. delbrueckii*	Co-inoculation; dialysis tube: 10 ml WYPD *S. cerevisiae* submerged into 70 ml WYPD non-*Saccharomyces*; cut-off 12–14 kDa	Nissen et al. 2003; Nissen and Arneborg 2003
Two-compartment membrane bioreactor	Active movement and passive diffusion via peristaltic pump and membrane	*T. delbrueckii*	Co-inoculation; 2.4 l total synthetic grape juice non-*Saccharomyces* and *S. cerevisiae* in respective vessels; membrane cut-off 1.2 μm	Renault et al. 2013
Two-compartment membrane bioreactor	Active movement via pressurised air	*T. delbrueckii*	Sequential and co-inoculation; 2 l total synthetic grape juice non-*Saccharomyces* and *S. cerevisiae* in respective vessels; membrane cut-off 1 μm	Taillandier et al. 2014
Two-compartment membrane bioreactor	Active movement via pressurised air	*K. marxianus*	Co-inoculation; 2 l total synthetic agave juice non-*Saccharomyces* and *S. cerevisiae* in respective vessels; membrane cut-off 1 μm	Lopez et al. 2014
Dialysis tube	Passive diffusion	*L. thermotolerans*	Co-inoculation; dialysis tube: 5 ml WYPG non-*Saccharomyces* submerged into 245 ml WYPG *S. cerevisiae*; cut-off 3.5–5 kDa and 1000 kDa	Kemsawasd et al. 2015
Dialysis tube	Passive diffusion	*H. uvarum*	Co-inoculation; dialysis tube: A) 20 ml real must non-*Saccharomyces* submerged into 180 ml real must *S. cerevisiae* B) 10 ml real must non-*Saccharomyces* submerged into 10 ml real must *S. cerevisiae*; cut-off 12.4 kDa	Wang et al. 2015b
Dialysis tube	Passive diffusion	*L. thermotolerans* *H. guilliermondii*	Sequential inoculation; dialysis tube: 5 ml modified synthetic grape juice non-*Saccharomyces* submerged into 245 ml modified synthetic grape juice *S. cerevisiae*; cut-off 1000 kDa	Branco et al. 2017
Two-compartment membrane bioreactor	Passive diffusion	*S. bacillaris*	Sequential inoculation; 200 ml total red must non-*Saccharomyces* and *S. cerevisiae* in respective vessels; membrane cut-off 0.45 μm	Englezos et al. 2019a, 2019b, 2019c
Dialysis tube	Passive diffusion	*L. thermotolerans*	Sequential inoculation; dialysis tube: 600 ml white must non-*Saccharomyces* submerged into 1.2 l white must *S. cerevisiae*; cut-off 12-14 kDa	Petitgonnet et al. 2019

or pressurised air to actively move media between the two compartments).

While two-compartment systems have reported problems with fouling (Lopez et al. 2014), Renault et al. 2013 overcomes this by ensuring the system performs an automatic back wash on a regular basis. By no means do these cons outweigh the pros of either systems and both have provided valuable insights into the role of cell-cell contact in interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts. In fact, the setup by Renault et al. 2013 combines both systems in the sense that it separates the two compartments (with equal volumes) by a membrane through which metabolites can diffuse freely, but also actively pumps media from one to the other with the use of a peristaltic pump.

While there is no doubt that dual compartment bioreactors are essential to the future of interaction research, there is clearly no 'perfect', or close-to-perfect system as yet. Each of the systems mentioned show clear limitations and double compartment bioreactor design is an area of research and development in itself. Ongoing and future improvements/optimisation of double compartment bioreactor designs will open up new opportunities in community ecology studies, not just limited to wine research. Double compartment bioreactor systems provide a key platform where complementary, integrated research concepts can be explored that combine elements of both interspecies interaction research and synthetic ecology together.

Online bioreactors

Online monitoring systems coupled to bioreactor technology has made automated real-time monitoring of cultures possible. These in situ monitoring systems can either be based on invasive probes or non-invasive optical sensors and can destructively or non-destructively analyse culture samples (Lourenço et al. 2012). Non-invasive in situ sensors are largely based on

spectroscopy, including vibrational spectroscopy or fluorescence spectroscopy, coupled to chemometric analyses (Lourenço et al. 2012; Faassen and Hitzmann 2015; Wang et al. 2020). These sensors allow for real-time monitoring of general abiotic parameters as well as fluorescence at several wavelengths, making it possible to track fluorescently labelled or stained populations within a mixed microbial culture (Heins and Weuster-Botz 2018; König et al. 2018).

Previously, running parallel bioreactor experiments required a few bulky bioreactors and control modules, and most researchers could realistically only conduct a few parallel experiments at a time, dependent on available equipment, which makes for slow progress. New trends in bioreactors are scaled-down, multi-parallel systems, where up to 48 micro-fermentations can be conducted and monitored at once (Schäpper et al. 2009). These technologies are clearly useful in screening for particular phenotypes, and when coupled to the labelling techniques described previously, have the potential to screen for changes in population dynamics in response to any number of abiotic or biotic challenges. To our knowledge, this has yet to be applied to a synthetic yeast consortium, and up to now, has generally been applied to optimising heterologous expression of bioproducts or to screening monocultures for desired phenotypes. Still, the potential for rapid generation of ecologically relevant data in future is clear. A limitation that should be considered for these systems is the lack of biomass and culture supernatant generated; therefore, any downstream analyses must be sensitive to this, or promising candidates should be scaled up for such analyses.

Towards predictive understanding of wine fermentation ecology and evolution

Wine fermentation ecology is a burgeoning field focussed on elucidating the innerworkings of a microbial community that has evolved in a highly selective niche created by humans. While we know that ecological interactions do play an important role in the structure and functioning of this microbial community, not much is known about how community level properties emerge and even less is known about how ecological processes shape the evolution and maintenance of co-existence. To generate a holistic mechanistic understanding of this complex ecosystem, it is necessary to combine information collected from genomics, transcriptomics, proteomics and metabolomics studies. Evidently, the importance of quantitative modelling and computational biology approaches is paramount to the future of ecosystem ecology. With the wealth of information which now exists on the wine microbiome (Morgan et al. 2017; Liu et al. 2020), transcriptomic (Curiel et al. 2017; Petitgonnet et al. 2019; Shekhawat et al. 2019) and metabolomics data sets (Roullier-Gall et al. 2020), we can combine mathematical modelling with omics profiling to generate metabolic models. There are a number of freely available toolkits and open source platforms now available for use with both natural and synthetic microbial communities. These can be used to model ecosystem metabolism, interspecific interactions, substrate cross-feeding, evolutionary trajectories, microbial community engineering and eco-evolutionary dynamics (La Sarre et al. 2017; Baldini et al. 2019; Liao et al. 2020; Marsland et al. 2020; Zuñiga et al. 2020; Dukovski et al. 2020). This opens the sphere for a new and exciting area of research, whereby we can simulate microbial population dynamics in experimental wine ecosystems in reproducible, transparent and scalable ways to probe community function, structure and evolution—all of which can be applied to other ecosystem niches. The outcomes of such studies will indeed generate highly relevant fundamental advances in microbial ecology for a range of communities/applications. By combining aspects of microbiology, synthetic ecology, experimental evolution and computational biology with the latest advances in imaging and online monitoring systems we can gradually start to build a better understanding of interspecific interactions, the impact thereof on community evolution, ecosystem productivity and population dynamics, which will help to unravel the complexity of the wine ecosystem allowing for more controllable outputs.

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Declarations

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Chapter 3

Research Results I

Real-time monitoring of population dynamics and physical interactions in a synthetic yeast ecosystem by use of multicolour flow cytometry

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ENVIRONMENTAL BIOTECHNOLOGY

Real-time monitoring of population dynamics and physical interactions in a synthetic yeast ecosystem by use of multicolour flow cytometry

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Abstract

Ecological interactions between different species of yeasts have been observed and described extensively, but the mechanisms of interaction remain poorly understood. A hindrance to the characterization of multispecies yeast ecosystems is the lack of accurate methods for rapid real-time analysis of population dynamics in synthetic multispecies consortia. Here, we sought to accelerate and improve the sensitivity of ecological modelling and characterization of a synthetic yeast ecosystem by developing a flow cytometry–based method that tracks and sorts fluorescently tagged individual yeast species in real time during growth in model multispecies consortia. A protocol for integrative genetic modification of non-conventional yeasts was developed. The application of the method was demonstrated in a model four-species synthetic wine-yeast ecosystem that consisted of species commonly isolated from natural wine fermentations. The data show that this method allows for rapid generation of meaningful ecological data that contributes to our understanding of multispecies synthetic yeast ecosystems. Furthermore, interspecies interactions have been shown to impact the evolution of yeasts in natural ecosystems, and this platform will provide an ideal tool to better evaluate the impact of biotic selection pressures.

Key Points
- *Fluorescent labelling of yeast species in a consortium for multicolour flow cytometry*
- *Method developed to track population dynamics of multispecies yeast consortia*
- *Enables real-time visualization, manipulation and response analyses of population dynamics*
- *Produces accurate, reproducible data with powerful visual analyses potential at a rapid rate*

Keywords Wine yeast ecosystem · Biotic stress · Yeast–yeast interactions · Genetic modification of yeast · Multicolour flow cytometry · Cell sorting

Introduction

Yeast ecosystems are of significant biotechnological importance as catalysts for fermentations in the food and beverage industry as well as in varied biorefinery applications (Nandy and Srivastava 2018). However, most of these biotechnological applications, such as in brewing, baking and small metabolite production, are based on single species fermentation, mostly *Saccharomyces cerevisiae*. Similarly, laboratory-based microbial research has almost exclusively characterized single species, whether it is with regards to phenotypes, physiology or molecular and genetic regulation and evolutionary engineering. Yet, in natural ecosystems, microbial organisms would rarely, if ever, find themselves in a single-species system (Song et al. 2014; Said and Or 2017). Instead, microorganisms are continuously exposed to ecological interactions and have evolved competitive or mutualistic traits (Little et al.

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2008; Sanchez and Gore 2013; Andrade-Domínguez et al. 2014). Arguably, such ecosystem interactions provide the majority of evolutionary selection pressures on individual species since evolutionary fitness is a relative concept dependent on the presence of competing and/or cooperating species (Andrade-Domínguez et al. 2014; Friedman and Gore 2017; Venturelli et al. 2018). Yet even for species with extensive databases and genome annotations, very little if any information can be found regarding the molecular or genetic elements involved in these interactions (Jiranek et al. 2019).

Furthermore, to date, little is known about the origins and mechanisms of ecological interactions within complex multispecies yeast communities (Conacher et al. 2019). This gap in our understanding is also handicapping biotechnological applications since it is generally accepted that multispecies systems could have broader applications and a wider range of capabilities, proportional to the genetic and functional diversity present (Song et al. 2014).

The modelling of multispecies systems (i.e. more than two) remains challenging since the parallel interactions between multiple species create non-linear responses. A common approach to understanding ecological interactions in yeast has been to assess binary (two species) interactions in isolation. This has allowed researchers to generate meaningful, reproducible data, which has significantly improved our understanding of yeast ecology (Song et al. 2014). However, given the recent emphasis on the importance of functional diversity within microbial communities, the binary approach to studying microbial interactions is becoming out-dated (Goers et al. 2014). Mechanistic study of complex multispecies ecological dynamics has been attempted in a small number of bacterial and inter-kingdom systems since these are of high interest in human microbiota functioning, but yeast ecosystems have been largely excluded (Kato et al. 2008; Kim et al. 2008; Virtanen et al. 2010). Understanding the ecology of complex yeast communities will allow scientists to better manipulate these systems for desirable outcomes and has the potential to improve the efficiency and sustainability of relevant yeast community–based industrial processes. Furthermore, this will contribute to the growing body of evidence, which suggests that interspecies yeast–yeast interactions have likely been major drivers of the evolutionary history of yeast species in so called 'domesticated' environments (Morrison-Whittle et al. 2018; Zhou et al. 2018)

Unfortunately, the available methods for evaluating natural yeast ecosystems are limited and mainly based on traditional spread-plating or automated ribosomal intergenic spacer analysis (ARISA). The main disadvantages of these methods are (i) the time and labour required, which limits the number of cultures that can simultaneously be evaluated; (ii) the delay between sampling and data availability, which translates to a lack of any real-time measure of population dynamics, thereby excluding the possibility of real-time culture manipulations; and (iii) the many steps at which biases can be introduced during the dilution, spread-plating and enumeration process. In the case of transcriptomic and proteomic studies, the mapping of RNA or peptide data to particular members of a consortium from a mixed population sample becomes an inexact task in more closely related (genetically homologous) communities (Melin 2004; Diz et al. 2012). This is challenging in two-member systems, and it becomes exponentially more complex for multispecies ecosystems. To accelerate ecological research of multispecies yeast cultures, a more rapid, accurate, higher throughput method with real-time applications for evaluating population dynamics is needed. In addition, techniques to separate mixed populations of closely related community members before extraction procedures will enhance the accuracy of aligning transcriptomic and proteomic data and allow for more specific evaluation of the ecological contributions of individual members.

Flow cytometry, an analytical technique that detects and quantifies several optical properties of a given particle, has shown significant potential in advancing the speed and accuracy of characterizing cell populations in the context of microbial ecology (Kron et al. 2007). For a detailed explanation of the fundamentals of flow cytometry, the reader is referred to a number of exhaustive reviews (Kron et al. 2007; Adan et al. 2017; Vembadi et al. 2019). Flow cytometry can delineate sub-populations within a sample based on differences in cell size, morphology, and differences in detected fluorescence. This technique has been used to characterize bacterial diversity metrics within natural aquatic and activated sludge ecosystems by evaluating differences in cell morphology and/or nucleic acid content (Davey and Winson 2003; Props et al. 2016; Brown et al. 2019; Heyse et al. 2019). In yeast, fluorescence tagging has been applied to monitor multistrain *Saccharomyces cerevisiae* systems (Momeni et al. 2013; Hart et al. 2019), as well as two-species *S. cerevisiae* and *Lachancea thermotolerans* co-cultures (Petitgonnet et al. 2019). Additionally, flow cytometers can be equipped with a cell-sorting module, which adds the capability to physically separate distinct sub-populations. This feature allows a myriad of applications, including enrichment or isolation of populations of interest for varied downstream experiments and simplification of 'omics' analyses (Mattanovich and Borth 2006). Cell-sorting based on fluorescence expression has been applied in a two-strain *S. cerevisiae* co-culture (Pérez-Torrado et al. 2017), and cell-sorting based on staining of a small subset of a population has been used in a two-species *S. cerevisiae* and *L. thermotolerans* co-culture (Peng et al. 2019). The potential of flow cytometry–based analyses in yeast community ecology has yet to be exploited fully. There is a definite gap in applying the mentioned methods to more complex ($n > 2$) synthetic yeast systems. Major bottlenecks in method development may include the challenges associated with flow cytometry–based differentiation of

morphologically similar environmental yeast isolates and the general lack of a comprehensive methodology for applying flow cytometry to mixed yeast populations.

To address these bottlenecks, we developed and validated a method where fluorescent tags are expressed as markers in different yeast species (*S. cerevisiae*, *L. thermotolerans*, and *Torulaspora delbrueckii*), which can be analysed and quantified by flow cytometry and fluorescence microscopy. A method for fluorescent tagging of non-*Saccharomyces* yeasts was developed, which can be applied to different yeast species. In this way, population dynamics and physical interactions between species can be rapidly determined in real time. There is no reliance on colony morphology for differentiating species, and given the simplicity of sample preparation, the likelihood of experimental error is significantly reduced in comparison with traditional plating methods. A major contribution of the method developed here is the speed at which meaningful data is generated—samples can be evaluated, and data interpreted, immediately—as well as the real-time nature of the method. This allows for an interventionalist approach to the experimental design, including in situ manipulation and evaluation of responses, affording a vast number of experimental opportunities that are not possible with current methods. This method is anticipated to vastly improve the rate at which population dynamics and ecological interactions can be observed in multispecies yeast cultures.

Methods

Selection of fluorescent tags

This method has been designed for use with a BD FACSMelody (BD Biosciences, La Jolla, CA, USA) cell sorter but can easily be translated to different flow cytometers or cell sorters. For a comprehensive list of fluorescent tags (FTs) available for expression in yeast, the reader is referred to the following chapter (Bialecka-fornal et al. 2016). For recommendations in selecting appropriate combinations of FTs, the reader is referred to the following publications (Sheff and Thorn 2004; Lee et al. 2013; Higuchi-sanabria et al. 2016). Using these criteria, a library of the following FTs was generated: eGFP (GFP), mCherry, mCitrine, mTagBFP2 (BFP) and TagRFP657 (RFP).

Construction of fluorescently tagged yeast species

Fluorescently tagged yeast species (*S. cerevisiae* VIN13 [Anchor Yeast, Cape Town, South Africa], *L. thermotolerans* IWBT Y1240 [CBS: 16374] and *T. delbrueckii* LO544 [CRBO: LO544]) were generated by creating in-frame gene fusions of the FT-cassette with an antibiotic (G418/Geneticin) selection marker. Plasmid-based expression of FTs was a possibility for this method, but it is associated with high cell–cell variation of FT expression and requires more complex controls to ensure even copy number; therefore, we opted for integration of FT cassettes in the genome (Higuchi-sanabria et al. 2016). To obtain reproducible results, constitutive, high-abundance expression of the FTs throughout the intracellular space is required; therefore, a highly conserved glycolytic gene (*TDH3*: glyceraldehyde-3-phosphate dehydrogenase) was selected as the target fusion gene. Previous studies have shown success with using TDH3 for creating FT-fusions in *S. cerevisiae* with minimal impacts on growth properties (Lee et al. 2013). It is possible to create C- or N-terminal FT fusions and dependent on the target protein functioning, either type of fusion can be beneficial or detrimental to the functioning of the fusion protein. It has been found that C-terminal fusions are generally less disruptive to protein functioning, and since the aim here is to minimally alter cellular functioning, we chose to create C-terminal fusions (Higuchi-sanabria et al. 2016; Weill et al. 2019). Integration was accomplished by creating a C-terminal gene fusion, where the FT cassette is inserted before the stop codon of the target gene and expression is coupled to that of the target gene (Janke et al. 2004).

Generating fluorescent tag integration cassettes

Plasmids containing the fluorescent protein and selective marker of interest were ordered from Addgene (Cambridge, MA, USA) (36,226, 44,899, 44,900, 44,903, 44,955). The integration cassette was generated by a PCR reaction, using primers designed to contain 18 bp homology to the donor plasmid and 40 ± 3 bp of homology to the regions upstream and downstream of the integration point, respectively (Supplemental Table S1). The integration point is directly upstream of the target gene stop codon and directly downstream of the stop codon, including the stop codon.

The selection of a high-fidelity *Taq* polymerase was essential to the success of the PCR reaction. Specifically, Q5 (NEB, Ipswich, MA, USA) was found to be superior to Ex *Taq* (TaKaRa, Shiga, Japan) in terms of binding specificity and amplification. The annealing temperature of the PCR reaction was calculated using only the 18 bp primer sequence that bound to the donor plasmid template, not the entire 60 bp primer sequence. The PCR conditions were selected according to manufacturer instructions. The amplification product was a single band approximately 3 kb in size for all FTs. The product was visualized with an agarose gel (1% $w.v^{-1}$). The verified PCR product was purified (FavorPrep PCR clean-up kit, Favorgen, Ping-Tung, Taiwan), and where necessary, the purified PCR reactions were pooled and ethanol precipitated (3 volumes of 100% ethanol and 0.03 M sodium acetate [pH 5.3] for > 2 h at − 20 °C, centrifugation at > 18,000 RPM at 4 °C for 15 min, wash in 70% ethanol,

centrifugation at > 18,000 RPM at 4 °C for 15 min) and concentrated by resuspension in a reduced volume of TE buffer (pH 8).

Transformation of yeast

The transformation process was completed according to the method of Lin-Cereghino et al. (2005), with some modifications. Namely, competent cells were resuspended in 0.01 volumes of BEDS (10 mM bicine-NaOH, pH 8.3, 3% ($v.v^{-1}$) ethylene glycol, 5% ($v.v^{-1}$) dimethyl sulfoxide (DMSO) and 1 M sorbitol) solution and the process following electroporation was adjusted according to the species, as described below. The Gene Pulser® II electroporator (Bio-Rad Laboratories, Hercules, California, USA) was used: cuvette gap, 2.0 mm; charging voltage, 1500 V; resistance, 200 Ω; capacitance, 25 μF. Frozen competent cells were tested by electroporation and showed complete loss of viability; therefore, competent cells must be prepared fresh on the day of transformation. For antibiotic selection, 200 μg l^{-1} Geneticin (G418) was a suitable concentration for all three species evaluated.

Transformation of *S. cerevisiae* VIN13

Fluorescent tagging of *S. cerevisiae* VIN13 was relatively straightforward and did not require significant optimization. An amount of 200 ng purified FT-cassette PCR product was sufficient to yield positive transformants. After electroporation, 4–6 h of recovery in yeast peptone dextrose sorbitol (YPDS) medium was sufficient. The recovered cells were spread-plated onto yeast peptone dextrose (YPD) agar plates supplemented with G418 (200 μg l^{-1}). First, 100 μl of the recovered cells was spread-plated, the rest of the recovered cells were then collected by centrifugation at 1000 RPM for 4 min, resuspended in 100 μl of saline (0.9% NaCl) and spread-plated. The plates were incubated for 3 days at 30 °C, after which screening for positive transformants was performed.

Transformation of non-*Saccharomyces* yeasts

Successful transformation of *L. thermotolerans* and *T. delbrueckii* required significant optimization. Drastically increasing the FT-cassette concentration and introducing transformant enrichment steps were necessary to identify transformants with permanent FT-cassette integration. In terms of DNA concentration, 2 μg per transformation was sufficient. At least eight 50 μl PCR reactions, purified, pooled and concentrated via ethanol precipitation, was required to produce this concentration. The impact of voltage (1.5 1.75, 2 and 3 kV) as well as number of pulses (at 1.5 kV: 1, 2, 3; at 2 kV: 2) during electroporation of *L. thermotolerans* was tested and any condition besides one pulse at 1.5 kV resulted in a complete loss of viability. After electroporation at this setting, ice-cold YPDS was added to the electroporation cuvette and gently mixed by pipetting up and down within 4 s of the pulse. On the last upward pipetting motion, the mixture was gently transferred to a sterile 2 ml Eppendorf tube. The Eppendorf tube was secured with a sealing cap, placed in a test tube and incubated on a test tube rotator for 16 h (recovery times of 4, 6 and 8 h resulted in no successful transformants). If the recovered cells were spread-plated immediately after recovery, it resulted in a matt of colonies, with random and/or temporary expression of antibiotic resistance. Serial dilutions as well as replica plates were not found to solve this issue since the likelihood of selecting the target transformants at this stage is very low. To this end, selection and enrichment of permanent antibiotic-resistance in cells proved to significantly reduce false positives. For the first enrichment step, the recovered cells were added to 5 ml YPD containing G418 at 240 μg l^{-1} (equating to a final G418 concentration of 200 μg l^{-1} after addition of 1 ml volume of recovered cells) and incubated for 8 h at 30 °C with aeration. To further reduce the likelihood of false positives, the enrichment was repeated by adding 1 ml of the first enrichment culture to 5 ml of YPD containing G418 at 240 μg l^{-1}. Test tubes containing the second enrichment were then incubated at 30 °C on a test tube rotator for 16 h and spread-plated onto YPD agar (G418 200 μg l^{-1}). The plates were incubated for 3 days at 30 °C, after which screening for positive transformants was completed.

Screening of transformants

For GFP, BFP and mCherry, positive transformants could be identified by exposing colonies on agar plates to UV (385/30 nm) and selecting fluorescent colonies. For RFP and mCitrine, UV exposure did not identify positive colonies. For all FTs, colonies were further screened by colony PCR, using a forward primer that binds upstream of the integration point and a common reverse primer that binds within the FT cassette of all FTs used (Supplemental Table S1). For positive colonies identified by colony PCR, fluorescence microscopy (Zeiss [Oberkochen, Germany] Axio Scope A1 equipped with Colibri 7 LED system with filter sets FS HE 91, 92, 109) was used to image the transformants and definitively confirm FT expression.

Optimizing flow cytometry analysis and fluorescence-activated cell sorting

The key milestones in method validation are summarized in Fig. 1.

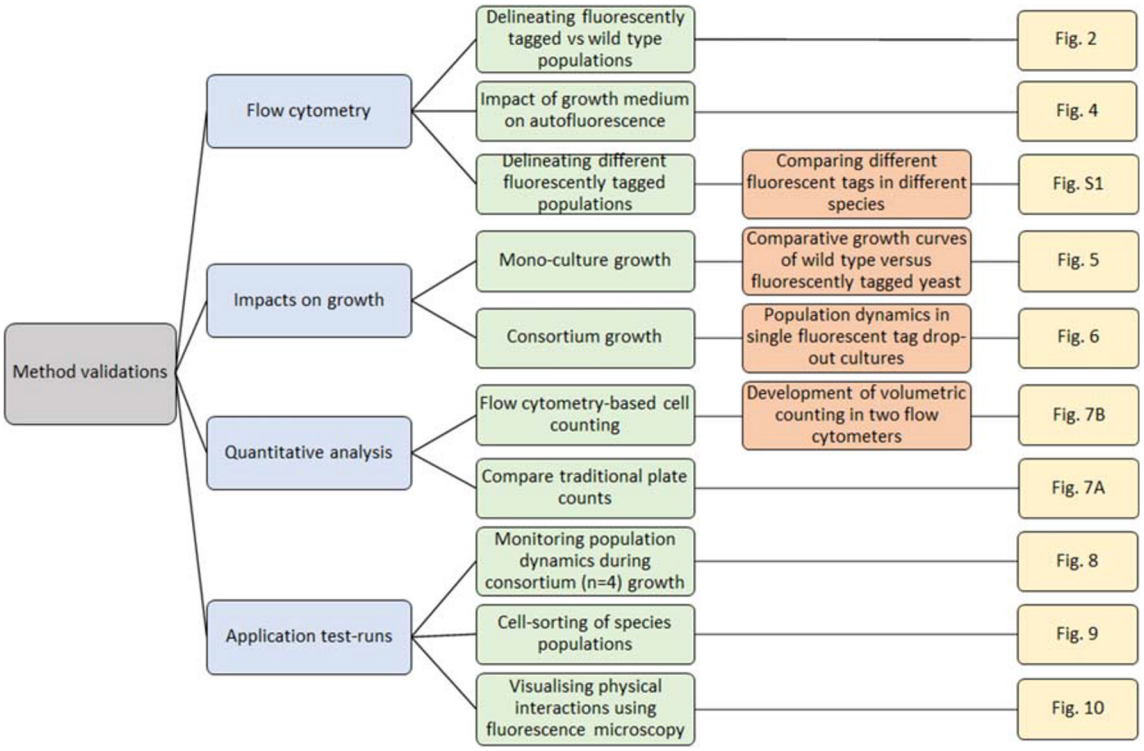

Fig. 1 Flow diagram summary of method validations completed, with references to figures in which validation results are depicted

Flow cytometer set-up

The BD FACSMelody (BD Biosciences, La Jolla, CA, USA), equipped with blue (488 nm), red (640 nm) and violet (405 nm) lasers, measured GFP fluorescence on the FITC channel (527/32; 507 LP), BFP fluorescence on the BV421 channel (448/45) and RFP fluorescence on the APC channel (660/10). Propidium iodide (PI) (Invitrogen, Thermo-fisher, Waltham, MA, USA) was used as a viability stain and was measured on the PerCP channel (700/54; 665 LP). The specifications of the BD FACSMelody used could not analyse mCherry nor mCitrine expression; however, these are still useful constructs that can be used on a different flow cytometer with more laser variety, as well as for fluorescence microscopy applications. All data were analysed using BD FACSChorus (BD Biosciences, La Jolla, CA, USA, 2019) software.

Development of flow cytometry analyses of fluorescently tagged yeast species

To confirm that the fluorescent yeast species could be distinguished from each other using flow cytometry, the tagged yeast species along with each wild type yeast species were inoculated and grown in YPD to late stationary phase, harvested by centrifugation, washed once with phosphate buffered saline (PBS) pH 7.2, diluted to an OD < 0.1 and stained with PI (1 μM). Samples were analysed at a fixed flow rate that ensured less than 1000 events per second, and the data was plotted as side scattered light (SSC) versus fluorescence intensity of the relevant channel. At this stage, an important requirement was that the fluorescent yeast population should be clearly distinguishable from the wild type yeast, and for accuracy of 95%, a maximum of 5% of the fluorescent population should fall in the non-fluorescent population gate.

Gates were drawn to delineate the wild type and fluorescent yeast populations, and the gating hierarchy was established (Fig. 2B). The entire population was firstly gated on viability, which was measured as the extent to which the nucleic acid dye, PI, penetrated the cell membrane. Therefore, the unstained population was gated as the viable population, named 'Live' (Fig. 2A1). The live population was separated based on positive RFP fluorescence, where the positive population is fluorescently tagged *S. cerevisiae* and the RFP negative population includes both fluorescently tagged *L. thermotolerans* and *T. delbrueckii* (Fig. 2A2). The RFP negative population was separated based on positive BFP fluorescence, where the positive population is fluorescently tagged *L. thermotolerans* and the BFP negative population is comprised of the rest of the RFP negative population plus the BFP negative population (Fig. 2A3). The BFP negative population was separated based on positive GFP expression, where the positive population is fluorescently tagged *T. delbrueckii* and the negative population is comprised of cells that are negative for RFP, BFP and GFP expression, named 'Non fluorescent' (Fig. 2A4).

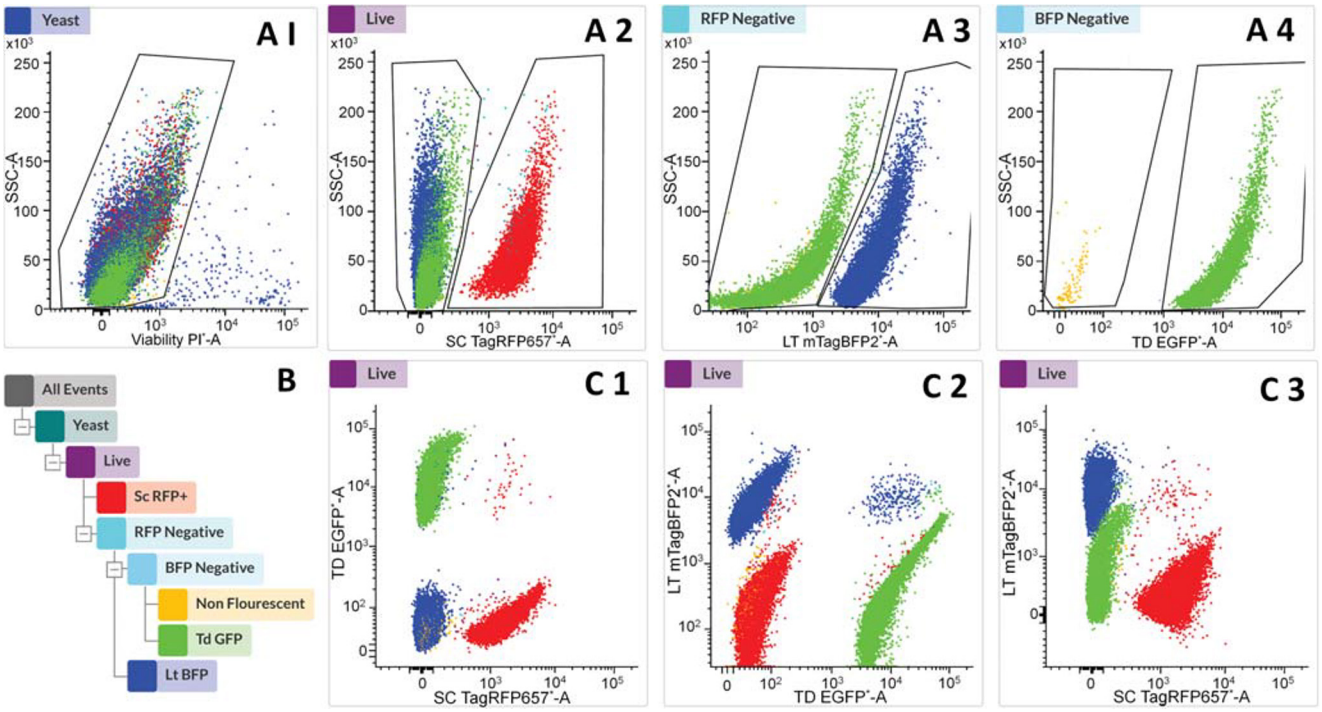

Fig. 2 Determination of gate hierarchy and illustration of expected dot plots in the fluorescent yeast consortium. A1: Viability gate—PI: Propidium Iodide. A2: Positive and negative RFP expression gate. A3: Gates within RFP negative population, for positive and negative BFP expression. A4: Gates within RFP and BFP negative population, for positive and negative GFP expression. Population negative for RFP, BFP and GFP are classified as the non-fluorescent population. B: Population gate hierarchy. The colours of each gate correspond to the population classification of the points in the dot plots. *S. cerevisiae*-RFP (Sc RFP+) are red dots, *L. thermotolerans*-BFP (Lt BFP) are dark blue dots, *T. delbrueckii*-eGFP (Td GFP) are green dots, non-fluorescent cells are yellow dots. C1–C3: Fluorescence intensity dot plots of fluorescent consortium

In terms of the yeast culturing protocol, the effect of growth medium on yeast cell autofluorescence was compared by analysing mixed fluorescent yeast species grown to stationary phase in YPD versus defined synthetic grape must (SGM). The SGM contained: 100 g l^{-1} glucose, 100 g l^{-1} fructose, 200 mg l^{-1} assimilable nitrogen as described by Bely et al. (1990), trace elements and vitamins as described by Henschke and Jiranek (1993) and 10 mg l^{-1} ergosterol.

In addition, to evaluate whether a particular FT was better suited to a particular species, different FTs were expressed in each species, and the ease of population differentiation was compared. Using identical growth and analyses conditions, the expression of GFP and BFP in *L. thermotolerans* and *T. delbrueckii* was compared.

Once the final fluorescent consortium was selected, compensation coefficients were determined. Compensation is a quantitative means of correcting for spectral overlap (interference) between emission spectra of different fluorescent proteins, which ensures accurate determination of fluorescent populations in multicolour experiments (Adan et al. 2017). This is an essential step in multicolour flow cytometric analyses as it removes the signal of a target fluorochrome from all detectors except the specific detector measuring that fluorochrome (Roederer 2002). The BD FACSMelody Chorus software (BD Biosciences, La Jolla, CA, USA, 2019) has an automated compensation set-up, which was used here. The user is prompted to run the following required samples: samples of the different fluorescent yeasts and all wild-type yeast species, as well as known live and dead control samples for PI viability stains. A compensation matrix is then calculated and automatically applied during acquisition.

Evaluating the effects of fluorescent protein expression on growth in mono- and multispecies culture

Monoculture growth

To determine the metabolic load of FT expression in the three target species, comparative growth curves in two different growth media (YPD and SGM) were conducted in triplicate using a CytoFLEX benchtop flow cytometer (Beckman Coulter, Pasadena, CA, USA). Wild-type and FT-expressing strains were inoculated in equal numbers (1×10^6 cells ml^{-1}, determined by volumetric counts using the CytoFLEX) in 5 ml of SGM and 5 ml of YPD. The cultures were grown in

6-well cell culture plates (Lasec, Cape Town, South Africa) and were incubated at 30 °C with agitation (120 RPM). Total viable cell numbers were monitored by volumetric counts of intermittent samples using a CytoFLEX benchtop flow cytometer (Beckman Coulter, California, USA). Samples were diluted to ensure less than 1000 cells per second were analysed (OD < 0.1), and PI was added at a final concentration of 1 μM as a viability stain.

Three-species consortium growth

To determine if there were any differences in growth behaviour of the FT-expressing strains during competitive growth in the synthetic three-species consortium, single FT-expressing strains were substituted for their respective wild type counterparts (while keeping the other two fluorescent strains) (Fig. 3). This is possible given the fact that non-fluorescent populations can be distinguished from the FT-expressing strains. The population dynamics of the single substitution consortium cultures were compared with a fully fluorescent consortium culture that was grown in parallel. Cells of each species were inoculated in 6 ml SGM in a 6-well culture plate at the same density as that of the fluorescent control. Samples were prepared identically to the monoculture experiments. The cultures were monitored by determining volumetric total cell counts on a CytoFLEX flow cytometer (equipped with a blue laser only), and respective ratios of different species were determined using the BDFACS Melody (equipped with a blue, violet, and red laser) (refer to the next paragraph for alternative ways of monitoring absolute cell counts during culture).

Validation of methods for determining absolute counts

Cultures tested in this validation were fluorescent three-species cultures, grown in SGM as described previously for three-species consortium growth. Firstly, to validate the volumetric counting data obtained from the CytoFLEX flow cytometer, total cell counts, as well as species-specific cell counts, were compared with traditional colony forming units (CFU)/ml spread plate counts using Walerstein Laboratory (WL) (Sigma-Aldrich, Johannesburg, South Africa) nutrient agar. In addition, where volumetric count-enabled flow cytometers are unavailable, an alternative method for quantitative counting using the BD FACSMelody, based on sample mass, was also evaluated. For this, sample tubes were weighed on a fine scale before and after sampling, and the samples were each run on the BDFACS Melody for 50,000 events, taking care to keep the timing before data acquisition on each sample equal (4 s). After which, the difference in weight was used to determine an absolute cell count per microgram of cell culture. The use of a counting bead standard was evaluated; however, it was found that the results were highly variable and extremely sensitive to minute dilution errors; therefore, this method was not preferred.

Application of the fluorescent consortium in monitoring population dynamics during multispecies culture and cell sorting

Multispecies culture

To demonstrate the applicability of the fluorescent consortium in monitoring population dynamics, four species cultures were

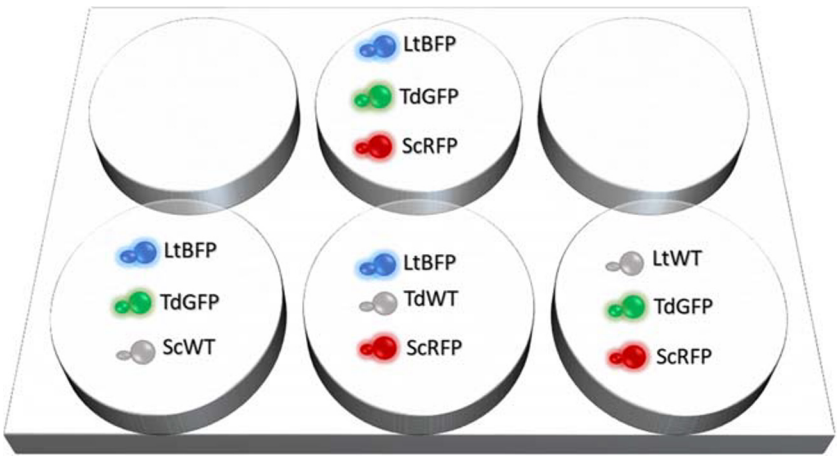

Fig. 3 Illustration of experimental design used when evaluating the impact of fluorescent tag expression during consortium growth. Single fluorescently tagged species were substituted with respective wild type species (B–D). Population dynamics of the single dropout consortia were compared with a fully fluorescent consortium grown in parallel (A). LtBFP: *Lachancea thermotolerans* tagged with BFP; LtWT: Wild type *L. thermotolerans*; TdGFP: *Torulaspora delbrueckii* tagged with GFP; TdWT: Wild type *T. delbrueckii*; ScRFP: *Saccharomyces cerevisiae* tagged with RFP; ScWT: Wild type *S. cerevisiae*

monitored to determine the effect of an additional non-fluorescent species on the population dynamics of the three-species fluorescent consortium. Cultures were grown as described previously for three-species consortium growth. The different species that were evaluated as fourth ecosystem members were *Kluveromyces marxianus*, *Wickerhamomyces anomalus* and *Hansenispora opuntiae*. The effect of initial inoculation ratios was compared by adjusting the inoculation ratios of the different species between cultures. The *K. marxianus* and *W. anomalus* cultures were inoculated as follows: 13% *K.* marxianus/*W. anomalus*, 55% *L. thermotolerans*, 26% *S. cerevisiae* and 6% *T. delbrueckii*. The *H. opuntiae* culture was inoculated at an increased dosage: 33% *H. opuntiae*, 24% *S. cerevisiae*, 23% *L. thermotolerans* and 20% *T. delbrueckii*.

Cell sorting

To demonstrate the efficiency of sorting fluorescently labelled populations during fermentation, species were sorted into 6-well culture plates containing 1 ml of double strength YPD. The cell sorting was based on the gating of the fluorescently labelled species, and 1000 cells were sorted at the 'single cell' purity level setting on BD FACSMelody software. The sorted cells were spread-plated onto WL media, and the precision of the sorting process was evaluated as the ratio of target species colonies versus total colonies.

Results

Optimization of creating fluorescently labelled yeast species

Using the developed method, a multispecies fluorescent library was created, including: *S. cerevisiae*-GFP, *S. cerevisiae*-BFP, *S. cerevisiae*-mCherry, *S. cerevisiae*-RFP, *L. thermotolerans*-GFP, *L. thermotolerans*-BFP, *T. delbrueckii*-GFP, *T. delbrueckii*-BFP and *T. delbrueckii*-mCitrine. Generating transformants with stable integration of the FT-cassette proved challenging in *L. thermotolerans* and *T. delbrueckii*. This can be explained by the fact that many non-*Saccharomyces* yeasts do not favour homologous recombination during double stranded DNA repair, causing random integration of DNA cassettes during transformation and making successful integration of the cassette in these yeast species a rare event (Ito-Harashima and Yagi 2017). CRISPR/Cas9 can be used to improve specificity of integration (Vigentini et al. 2017); however, we found that with a few simple changes to the established transformation protocol, namely increased DNA concentrations during transformation and additional selection steps, successful integration of the FT-cassette in *L. thermotolerans* and *T. delbrueckii* was possible. It is important to note that positive colonies from the same transformation likely originated from the same few cells that have replicated; therefore, if screening of different genetic variants is required, separate transformations should be completed.

Optimization of flow cytometry experimental set-up

The fluorescently tagged yeast species populations were found to be easily distinguishable from each other. The gating strategy developed for the method is summarized in Fig. 2B. As depicted in Fig. 2A2–A4, distinct positively fluorescent populations could be distinguished for RFP (Fig. 2A2), BFP (Fig. 2A3) and GFP (Fig. 2A4), as well as for non-fluorescent populations (Fig. 2A4). The fluorescently labelled species populations could be separated on fluorescence intensity dot plots, with opposing channel fluorescence intensity axes, which is useful for evaluating the efficacy of the compensation, as well as shifts in populations in relation to each other (Fig. 2C1–C3).

There was a significant effect in terms of autofluorescence when using different growth media (Fig. 4). The population labels used here were the same as Fig. 2, except the RFP negative population was labelled 'Not SC' since this population was separated from the RFP tagged *S. cerevisiae* (SC) and consisted of *L. thermotolerans* and *T. delbrueckii*. For cells grown in defined media such as SGM, the autofluorescence was significantly lower, and positive populations were easily distinguishable in unwashed samples (Fig. 4a, b). For cells grown in YPD, it was difficult to separate unwashed BFP positive and negative populations, since the negative population shifted closer to the positive population, resulting in an overlap between the populations (Fig. 4c, d). After washing, the BFP positive and negative populations were easily distinguishable, as the negative population shifted away from the positive population (Fig. 4e, f). It is therefore essential to wash harvested cells grown in YPD at least once in PBS to ensure accurate population delineations.

To determine if a particular FT was better suited to a particular yeast species in the synthetic consortium, different combinations of FTs expressed in the same yeast species were tested (Supplemental Fig. S1). The brightest expression for all tested fluorescent proteins was seen in *S. cerevisiae* (data not shown); therefore, to minimize spectral overlap between FTs, the best-suited FT for *S. cerevisiae* was RFP. This is because of the inherent lowered brightness of this fluorescent protein, and the fact that the emission is in the far-red range, which will not be detected on any blue or green emission channels. When comparing GFP and BFP expression in *L. thermotolerans* and *T. delbrueckii*, no major differences were observed in the ease of differentiating the fluorescently labelled populations from each other, and it was randomly decided to assign BFP expression to *L. thermotolerans* and GFP expression to *T. delbrueckii.*

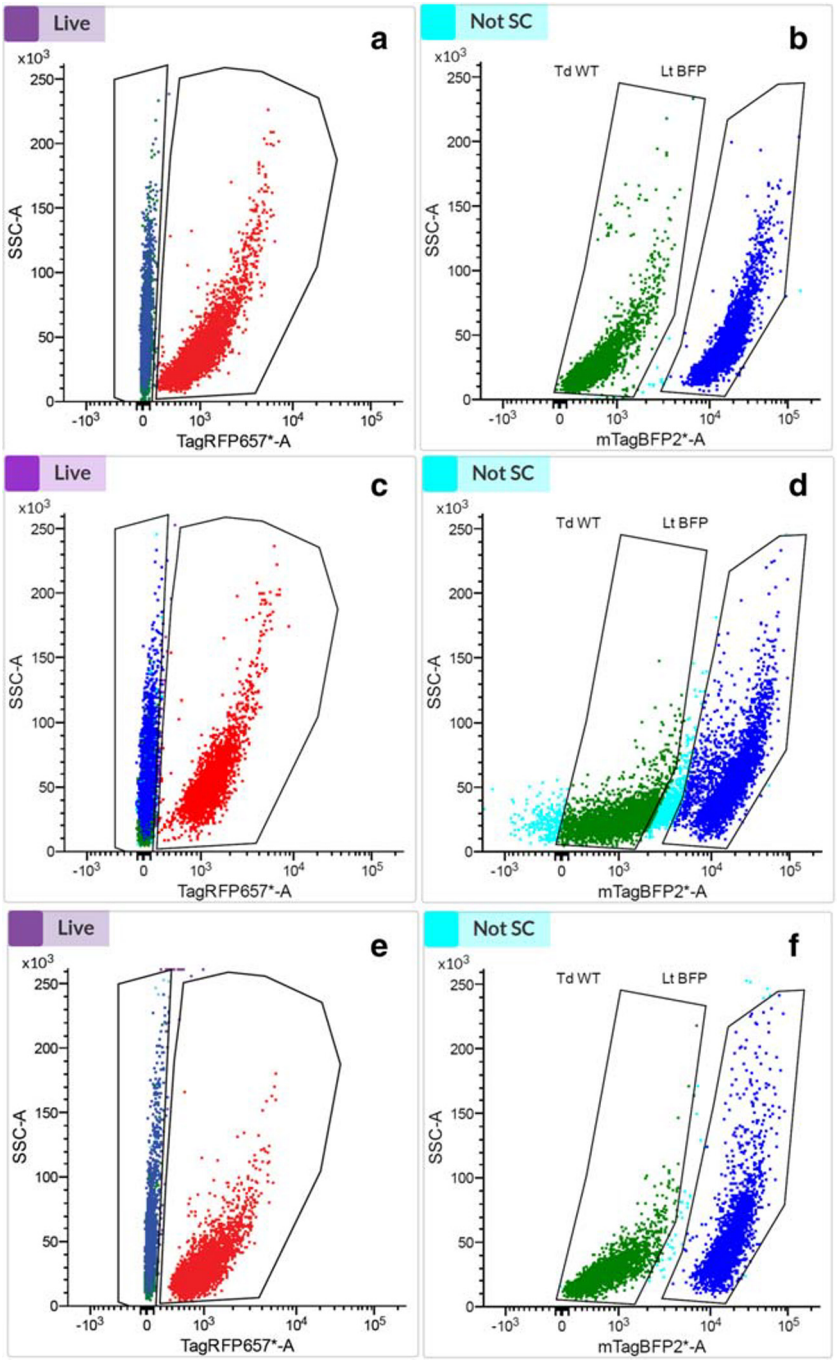

Fig. 4 Differences in population differentiation in unwashed versus washed samples of three-species cultures grown in synthetic grape must (SGM) (**a**, **b**) or yeast peptone broth (YPD) (**d**–**f**). **a**, **b** Unwashed SGM; **c**, **d** Unwashed YPD; **e**, **f** YPD with one PBS wash. The live population was determined using a propidium iodide stain. *S. cerevisiae*-RFP are red dots, *L. thermotolerans*-BFP are dark blue dots, *T. delbrueckii* are green dots, non-*Saccharomyces* cells (Not SC) that do not fall into a clear positive or negative mTagBFP2 population are light blue dots

Effects of fluorescent protein expression on growth in mono- and multispecies culture

The expression of the FTs created here is coupled to an essential glycolytic protein, TDH3, which may impact growth performance of the yeast species. To test the effects of FT-expression on the yeast, both monoculture growth curves (Fig. 5) as well as multispecies competitive growth curves (Fig. 6) were done.

The method used to monitor monoculture yeast growth was highly sensitive and produced viable cell counts, as opposed to usual OD-dependent methods, which cannot differentiate viable versus unviable cells. There were minor differences in the growth curves of FT-expressing strains versus wild type strains (Fig. 5). The differences correlated to slight variations in the initial inoculum concentrations, indicating that this effect is as a result of differences in inoculum concentrations as opposed to a metabolic burden. During the lag and exponential growth phases, the variation between strains in the rich, complex YPD medium was slightly more pronounced in comparison with the defined SGM medium. Overall, there appeared to be no major metabolic burden on the FT-

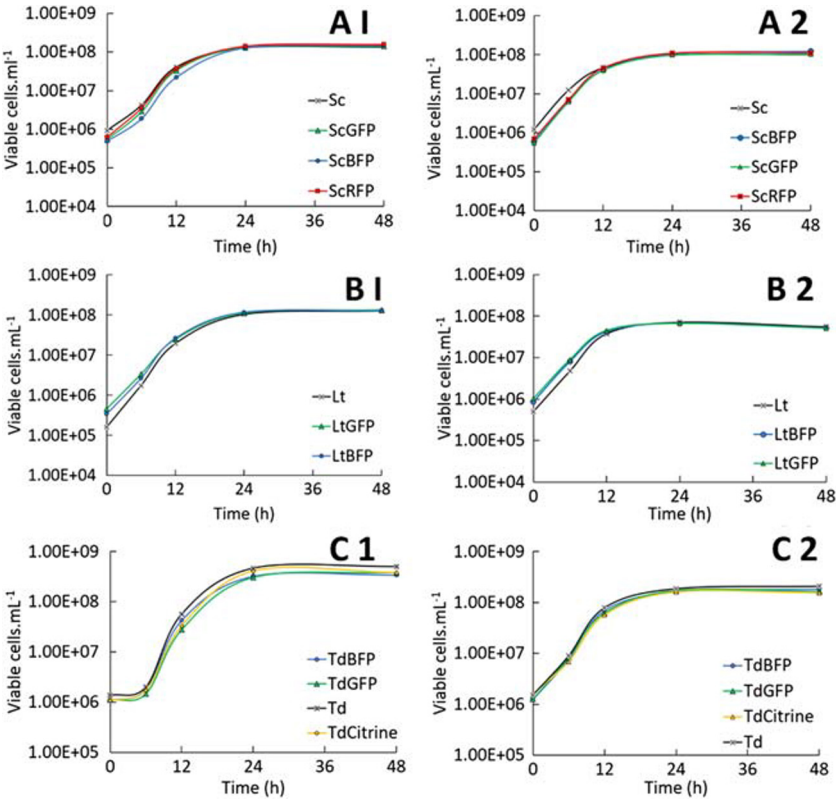

Fig. 5 Comparative growth curves of fluorescently tagged yeast species versus wild type yeast when grown in YPD and SGM growth media. Growth curves were completed in triplicate and error bars depict standard error from the mean. A1: *S. cerevisiae* strains in YPD. A2: *S. cerevisiae* strains in SGM. B1: *L. thermotolerans* strains in YPD. B2: *L. thermotolerans* strains in SGM. C1: *T. delbrueckii* strains in YPD. C2: *T. delbrueckii* in SGM

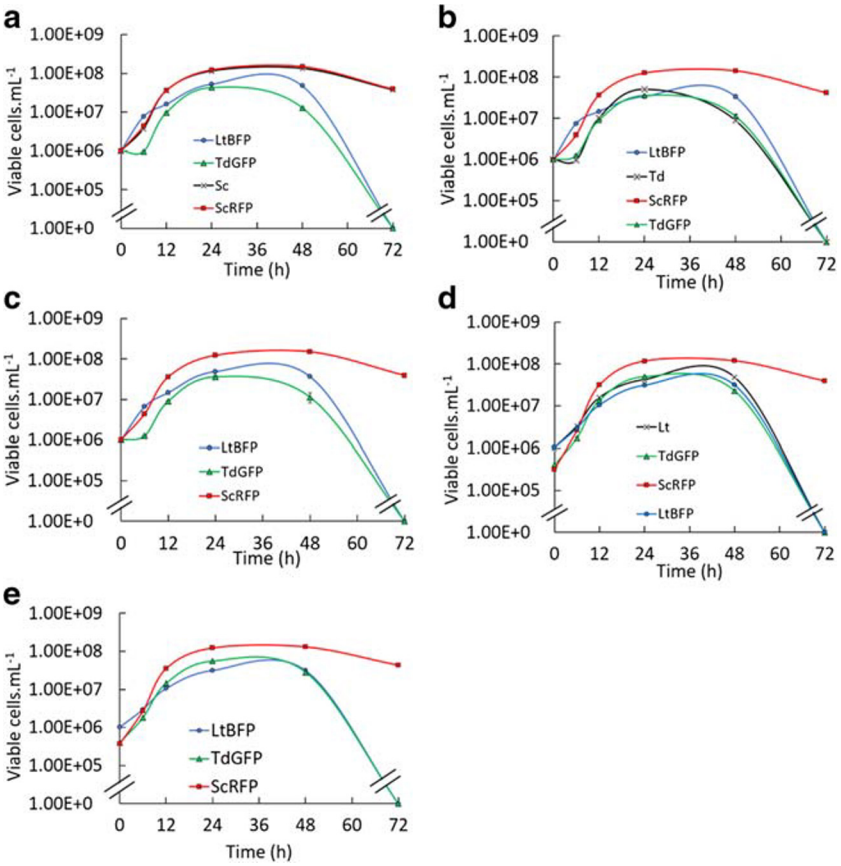

Fig. 6 Comparison of growth behaviour of the FT-expressing strains versus wild type strains during consortium growth in SGM. **a** *S. cerevisiae* strain comparison. **b** *L. thermotolerans* strain comparison. **c** Fully fluorescent consortium control for **a** and **b**. **d** *T. delbrueckii* strain comparison. **e** Fully fluorescent consortium control for **d**. Growth curves were completed in triplicate and error bars depict standard error from the mean

expressing strains, and therefore the growth behaviour of the FT-expressing strains should be representative of the wild type strains.

Since this method is designed to be used during synthetic consortium growth, it was important to compare the growth behaviour of the wild type versus FT-expressing strains during competitive growth (Fig. 3). Both the growth behaviour of the tested strain itself and the growth behaviour of the other yeast in the consortium in response to the tested strain were evaluated (Fig. 6). For *S. cerevisiae*, the FT-expressing strain behaved near identically to the wild type strain (Fig. 6a, c). *T. delbrueckii* strains showed slight variations in absolute cell counts, but the growth trends remained highly correlated and the population dynamics within the consortium were near identical to the control (Fig. 6b, c). The greatest variation in absolute cell numbers was observed between *L. thermotolerans* strains; however, the population dynamics within the consortium remained comparable with the control (Fig. 6d, e). These results validate that the population dynamics of the fluorescent consortium are representative of the wild type consortium.

Validation of methods for determining absolute counts

To track viable biomass accumulation of the yeast consortium during growth, it is necessary to have some form of quantitative measurement. The current method makes use of a CytoFLEX flow cytometer for determining absolute total viable cell numbers and determines the ratio of different species within the consortium by using a BD FACSMelody cell sorter. This combination of flow cytometers is due to the specifications of the machines available to us; however, a CytoFlex flow cytometer equipped with blue, violet and red lasers will be capable of generating the same data sets, if no sorting is required. The standard in tracking synthetic yeast communities is based on traditional spread plating onto selective media and/or ARISA. However, these techniques are prone to experimental error as a result of the many steps involved, rely on subjective colony identification and require a significant amount of time (for preparation, execution, incubation, counting and data analysis) (Comitini et al. 2011; Bagheri et al. 2017; Alonso-del-Real et al. 2019; Shekhawat et al. 2019). When comparing the method developed here with the traditional plating method, plate counts showed similar total cell counts to flow cytometer–based volumetric counting during the first 24 h, after which the plate counts showed significantly higher absolute counts compared to flow cytometry (Fig. 7a). In terms of species ratios, the results are largely comparable, except at the 48 h time point, where a large discrepancy (3.92-fold) between the methods in the amount of *L. thermotolerans* was found. The differences between the enumeration methods during the late exponential and death phases can be attributed to the respective viability measures of the enumeration method. Cells that are membrane-compromised will be excluded from the flow cytometry enumeration, while these cells may still be culturable and therefore be included in the plate count enumeration (Davey and Hexley 2011). The viability measure of each method is an innate feature and does not compromise the validity of either approach.

Secondly, where the use of a single flow cytometer without volumetric counting functionality is required, an alternative method based on the change in sample mass before and after analysis was evaluated. The sample mass–based method showed consistently less total viable cells in comparison with the volumetric-based counting method (Fig. 7b). However, the growth trends were consistent between the two methods, and since a reduced total cell count would not affect the population dynamic trends obtained during analysis, sample mass–based tracking of biomass would be an appropriate alternative method to track total biomass.

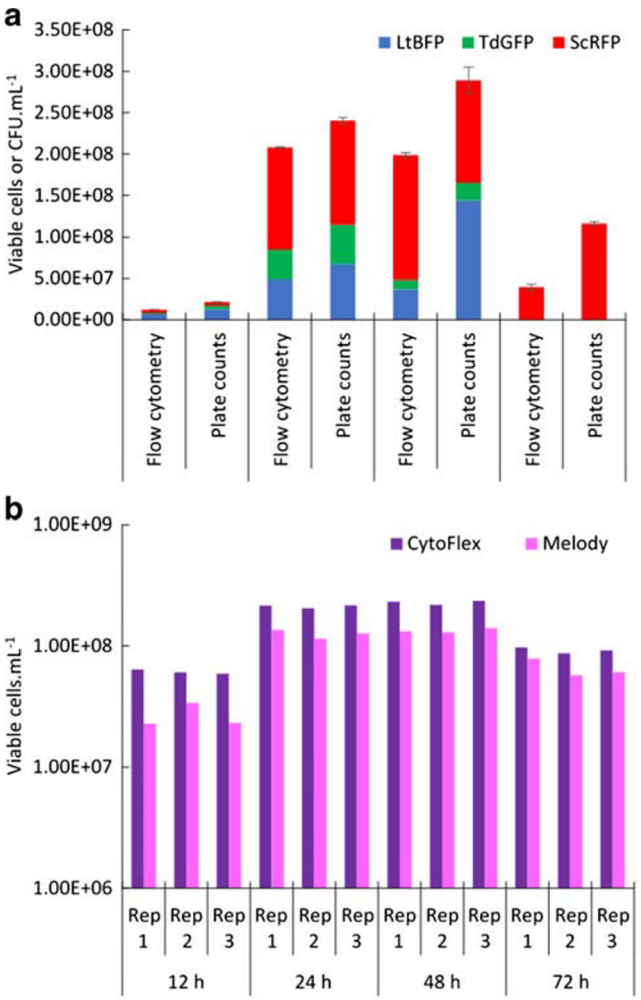

Fig. 7 Validation of absolute counting methods tested. **a** Traditional plate counts versus volumetric flow cytometry counts. **b** Comparison of volumetric counting methods between different flow cytometers, namely the CytoFLEX (Beckman Coulter, Pasadena, CA, USA) and BDFACS Melody (BD Biosciences, La Jolla, CA, USA). Counts were completed in triplicate and error bars depict standard error from the mean

Monitoring population dynamics during multispecies culture and cell sorting

To demonstrate the potential applications of the system developed here, the effect of three additional yeast strains (*K. marxianus*, *W. anomalus* and *H. opuntiae*) on the population dynamics of the fluorescent consortium was evaluated (Fig. 8). In addition, the effect of two different species ratios on the population dynamics was tested (Fig. 8a–c). *K. marxianus* and *W. anomalus* were inoculated into separate fluorescent consortium cultures at a similar percentage (~ 13%) of the total culture (consisting of: 55% *L. thermotolerans*, 26% *S. cerevisiae* and 6% *T. delbrueckii*) and showed similar trends in population dynamics (Fig. 8a, b). That is, the maximum viable cell count was reached at 24 h, after which the species population decreased at a rapid rate to zero viable cells. The cell numbers as well as population dynamics of the fluorescent consortium were near identical between cultures with *W. anomalus* or *K. marxianus*, indicating no species-specific effects at the tested inoculation ratios (Fig. 8a, b). *W. anomalus* appeared to have a faster growth rate than *K. marxianus* in the first 12 h and reached slightly higher maximum cell counts than *K. marxianus* after 24 h, suggesting that *W. anomalus* may be a marginally better competitor in the tested synthetic consortium (Fig. 8a, b). To test the effect of inoculation density, *H. opuntiae* was inoculated at a higher percentage (33%) of the total culture (consisting of: 24% *S. cerevisiae*, 23% *L. thermotolerans* and 20% *T. delbrueckii*) (Fig. 8c). *H. opuntiae* showed a similar trend to the previous cultures, reaching a maximum viable cell count at 24 h, followed by a steep decline. Here, the population dynamics of the fluorescent consortium appeared more affected by the differences in inoculation ratios, with *L. thermotolerans* and *T. delbrueckii* in particular reaching higher cell numbers and *S. cerevisiae* reaching lower cell numbers (Fig. 8c). This significant impact of inoculation density on yeast population dynamics is a known but not well-understood phenomenon (Bagheri et al. 2018). Interestingly, the three species of the fluorescent consortium consistently outcompeted the fourth species by the 48 h time point, suggesting some competitive edge or synergism within the consortium (Fig. 8). One limitation that came to the fore during these experiments is that there is always a small percentage (0.03–1.13%) of the population that appears non-fluorescent (shown in Fig. 2A4). Still, the minimum accuracy was 98.87%, which is well within a confidence interval of 95%. Nevertheless, it remains important to normalize the number of non-fluorescent yeast cells according to these percentages to prevent over-estimating the non-fluorescent population.

Besides monitoring of population dynamics, the system developed here can be used for cell-sorting of different species populations during growth. The sorting efficiency for each species was calculated: *S. cerevisiae*-RFP had 99.92% efficiency, *T. delbrueckii*-GFP had 100% efficiency and *L. thermotolerans*-BFP had 99.86% efficiency (Fig. 9). There was slight contamination between the *L. thermotolerans* and *S. cerevisiae* populations, specifically 0.08% *L. thermotolerans* occurred in *S. cerevisiae* targeted sorts and 0.14% *S. cerevisiae* occurred in *L. thermotolerans* targeted sorts. The sorting process is therefore highly feasible and accurate, allowing for downstream extractions and analyses of the transcriptome or proteome, as well as removal

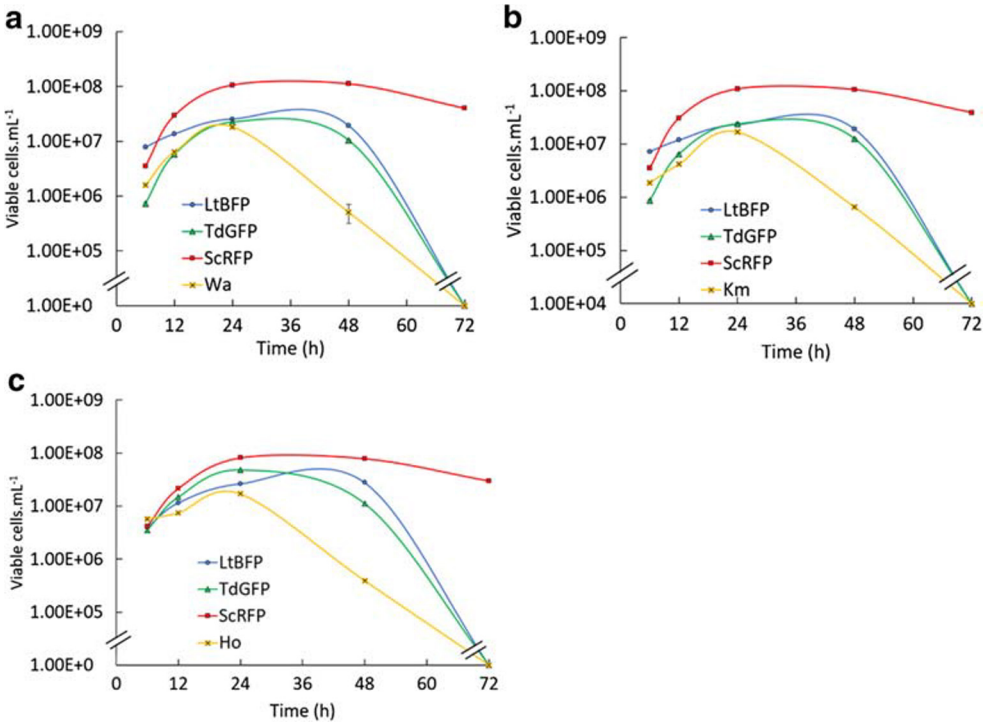

Fig. 8 Screening population dynamics of 4-species synthetic yeast consortium. The fluorescent consortium was challenged with the presence of **a** *Wickerhamomyces anomalus* (Wa), **b** *Kluveromyces marxianus* (Km) and **c** *Hanseniaspora opuntiae* (Ho). LtBFP: *Lachancea thermotolerans* tagged with BFP; TdGFP: *Torulaspora delbrueckii* tagged with GFP; ScRFP: *Saccharomyces cerevisiae* tagged with RFP; ScWT. Growth curves were completed in triplicate and error bars depict standard error from the mean

and/or treatment of a particular population during growth. The latter is a key advantage of the developed method, which is impossible to do with traditional culturing techniques since specific population separation is not feasible.

Lastly, the fluorescent consortium provides an excellent means to visualize physical interactions during growth. Figure 10, consisting of *S. cerevisiae*-mCherry, *L. thermotolerans*-BFP and *T. delbrueckii*-GFP, demonstrates the illustrative power of the fluorescent consortium. This system therefore has potential for any number of fluorescence-based imaging, including time-lapse imaging and imaging flow cytometry.

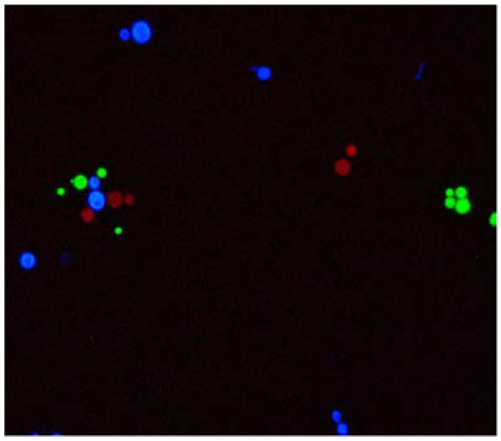

Fig. 10 Fluorescence microscopy image of the three species fluorescent consortium. Red cells are *S. cerevisiae* tagged with mCherry, blue cells are *L. thermotolerans* tagged with BFP, and green cells are *T. delbrueckii* tagged with GFP

Discussion

This work presents a fluorescence-based method for real-time tracking of population dynamics, physical interactions and cell sorting within a synthetic wine yeast ecosystem. While previous studies have used similar approaches to monitor two strain/species yeast systems (Pérez-Torrado et al. 2017; Petitgonnet et al. 2019), this is the first comprehensive method that has been developed for application to a complex multispecies yeast consortium. This method can be used to answer a wide variety of questions within the scope of yeast ecology, including characterizing temporal changes in population dynamics and responses of a synthetic yeast ecosystem to mimicked environmental perturbations (including the addition of more or less species complexity), as well as the more intricate transcriptomic and proteomic responses of individual populations to the presence of another. For the latter application, the cell-sorting functionality of the method is especially useful as a means to target particular species populations for downstream analyses. Further, the visual nature of the fluorescence tags allows for powerful imaging analyses, which can be applied to study the specificity and mechanisms of interspecies aggregation or physical interactions during multispecies growth.

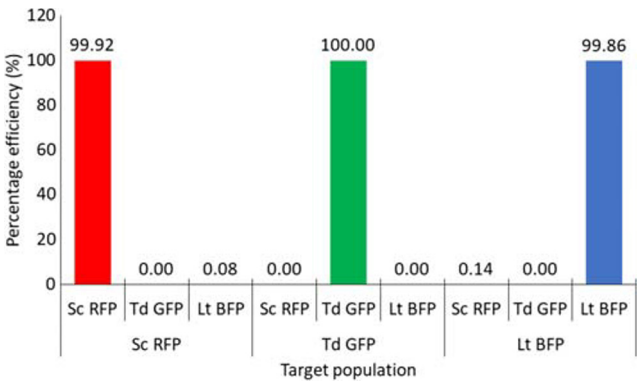

Fig. 9 Sorting efficiency of three species consortium samples. Samples were taken after 48 h of consortium growth in SGM. LtBFP: *Lachancea thermotolerans* tagged with BFP; TdGFP: *Torulaspora delbrueckii* tagged with GFP; ScRFP: *Saccharomyces cerevisiae* tagged with RFP; ScWT

The advantages of the method are that it is much less labour-intensive in terms of sample preparation and analysis than traditional agar-plating-based methods, requiring a simple sample dilution and flow cytometry analysis. This allows a single researcher to analyse many more cultures than usually possible and generate high-quality data of good depth at a significantly faster rate. The method is accurate and precise and generates meaningful ecological data in real time. It removes ambiguity in multispecies analyses since it allows for accurate targeting and physical separation of a particular population (notably, the majority of the population, not a biased subset of the population). A novel and widely applicable feature of the method developed here is the ability to intervene (with any number of abiotic or biotic environmental challenges) and monitor the population response in real time, which generates novel data at a speed that was not possible before. In comparison with fluorescence in situ hybridization methods (Wang et al. 2014), no time-consuming destructive fixation is required, which allows for downstream analysis of live sorted populations. Similarly, the sampling and analyses is simpler to sequencing-based methods such as ARISA (Bagheri et al. 2017), and importantly, samples can be characterized in real time. Lastly, a major advantage is the fluorescence-based imaging applications that can be applied to the fluorescent yeast consortium. This broadens the potential of the method for visualizing and quantifying physical cell-cell interactions, including adhesion, flocculation and preferential interactions—all of which are of great biotechnological relevance, especially in the winemaking process (Rossouw et al. 2018).

The limitations of the system are firstly that there is a finite number of compatible FTs available, limiting the ultimate complexity of the synthetic yeast ecosystems that can be

evaluated. In addition, some information of the target species genome sequence is required to design the integration cassettes used in the tagging process. Further, FTs require molecular oxygen during maturation; therefore, this method will not be effective under true anaerobic conditions. This limitation may be circumvented by incubating samples with aeration following anaerobic growth, which has proven effective in *Clostridium difficile* and *Escherichia coli* (Ransom et al. 2014; Pinilla-Redondo et al. 2018). The method is largely based on the availability of a flow cytometer, but populations can still be quantified with the use of a fluorescence microscope, as well as plating out colonies and exposing colonies on agar plates to a UV light source for enumeration, although these reduce the speed of the method significantly. Finally, while such a system is an improvement in terms of complexity when compared with binary synthetic co-cultures, it is still not fully representative of the complexity within a natural ecosystem. Also, the ecological influence of non-yeast biotic contributors is not evaluated in this system. Still, the simplified model allows for better control of experimental variables and will contribute meaningful insights into the mechanisms and complex functioning within synthetic yeast communities.

This work sought to accelerate the mechanistic understanding of natural yeast ecosystems by developing an optimized, higher throughput method for real-time tracking of population dynamics and physical interactions in synthetic yeast ecosystems. The method, based on multicolour fluorescence flow cytometry, was developed and validated, and potential applications were demonstrated. The key improvements of the method include the accuracy, reproducibility and speediness, as well as the novel applications made possible by the real-time nature of the method. This method has significant potential to assist in rapidly investigating both fundamental and applied aspects of complex multispecies yeast communities, in ways that would not be possible using current standard methodologies.

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Author contributions DR and FFB initiated the research and conceived and conceptualised the project; CC designed and conducted the experimental work; CC, RNB, DR and FFB analysed and interpreted the data; CC wrote the draft version; and CC, DR, RNB and FFB edited the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animal or human participants performed by any of the authors.

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Chapter 4

Research Results II

The importance of the physiological state of inocula in determining population dynamics in synthetic yeast consortia studies

This chapter will be submitted to FEMS Yeast Research and is written according to the journal specifications

The importance of the physiological state of inocula in determining population dynamics in synthetic yeast consortia studies

Abstract

Due to their functional capacity and efficiency, microbial consortia are increasingly being investigated for use in various fields including biomedical research, biofuels, and food and beverage production. However, microbial consortia are notoriously difficult to control. The importance of optimizing the physiological state of inocula has been highlighted in many important biotechnological processes which rely on monoculture growth. In contrast, this issue has been comparatively understudied in the context of consortia-based applications. The impact of a microorganism's initial physiological state on its ecological behaviour and the population dynamics within a consortium thus remains largely unaccounted for. Here, we assess the importance of pre-culture conditions in a synthetic yeast consortium consisting of wine-associated yeasts. It was found that the number of sub-culturing events, the inoculum growth phase, and the pre-culture medium all influenced the population dynamics of the yeast consortia. Furthermore, monoculture growth curves and flocculation phenotypes were found to be non-predictive of the complex impacts observed in the tested yeast consortia. This study emphasises the importance of defining, optimizing and standardising pre-culture conditions in the use of synthetic microbial ecosystems.

Introduction

In traditional bioprocessing and biotechnology based on mono-culture, the impacts of inoculum physiology on measured growth kinetics and product yields are well studied (Orlowski and Barford 1987; Hall et al. 2014; Thomas 2015; Hung et al. 2018). Major factors that have previously been highlighted to impact the physiology of cells in this context are the growth phase of pre-cultures at the time of inoculation, the number of sub-culturing events, as well as the growth medium in which the pre-culture is grown (Bely et al. 2005; Hall et al. 2014; Thomas 2015; Kragh et al. 2018; Keil et al. 2019). Expanding on these three factors, during each growth phase of a typical batch-culture, cells will be in varying physiological states, which are also impacted by the growth medium and growth conditions used (Hall et al. 2014; Li et al. 2018; Keil et al. 2019). Whether complex or defined, rich or minimal, growth media elicit changes in metabolic pathways within microorganisms (Kolkman et al. 2006; Lackner et al. 2012). Similarly, culture conditions, namely the temperature, oxygenation, culture vessel, culture volume, agitation speed, etc; all contribute to determining cellular metabolism and physiological state (de Groot et al. 2007; Knijnenburg et al. 2009; Guan et al. 2017).

In contrast, the extent to which the physiological state of a cell will influence its growth kinetics and phenotype in an ecosystem context has been underexplored. The study of microbial cultures containing more than one species presents numerous technical challenges. In synthetic ecology, thoughtfully designed subsets of natural ecosystems are studied to reduce the technical complexity to a level where accurate deductions can be made regarding ecosystem establishment and functioning (Ben Said and Or 2017). Designing and executing meaningful ecological experiments in mixed cultures is a minefield of optimization, having to consider the needs of each member, as well as what effect the metabolic interplay within the system may have on these needs. As such, within synthetic ecology, there is an emphasis on precise definition and optimization of the culture conditions of any given system. However, the same emphasis has not been placed on pre-culture conditions, i.e. the procedure followed when preparing a culture prior to inoculation into a synthetic ecosystem.

In yeast ecosystem studies, particularly those involved in the alcoholic fermentation of wine, there is a lack of a standard methodology for culturing synthetic ecosystems or yeast species pairs, as recently reviewed by Bordet et al. (2020). Furthermore, pre-culture protocols also vary widely in terms of the number of subculturing events, the inoculum growth phase, growth medium, and growth conditions. To our knowledge, there has been no data reported on the effects of pre-culture conditions on population dynamics in synthetic yeast ecosystems. This raises concern for firstly determining the true ecological mechanisms at play within these ecosystems, and secondly in the reproducibility of these studies.

Here, we sought to investigate how different pre-culture conditions may impact the population dynamics of a synthetic yeast consortium, grown under identical conditions, in batch culture. The number of sub-culturing events, the inoculum growth phase, and inoculum growth media were tested. Flocculation phenotypes were also evaluated since cell adhesion has been shown to be a significant driver of ecosystem dynamics (Rossouw et al. 2015; Rossouw et al. 2018). Monoculture controls were conducted to determine whether the impacts observed during consortium growth could be predicted by monoculture alone. Using quantitative cell count data, there were clear impacts on consortia dynamics based on each of the non-exhaustive list of pre-culture conditions. This study shows that pre-culture methods influence population dynamics and highlights the importance of studying the role of initial physiological state of microbial ecosystem members in synthetic ecosystem functioning. Given the increasing importance of consortia in biotechnological processes, further investigation of this question should aid in better predictive understanding and manipulation of these consortia for desirable outcomes.

Methods

A summary of the methodology is illustrated in Figure 1.

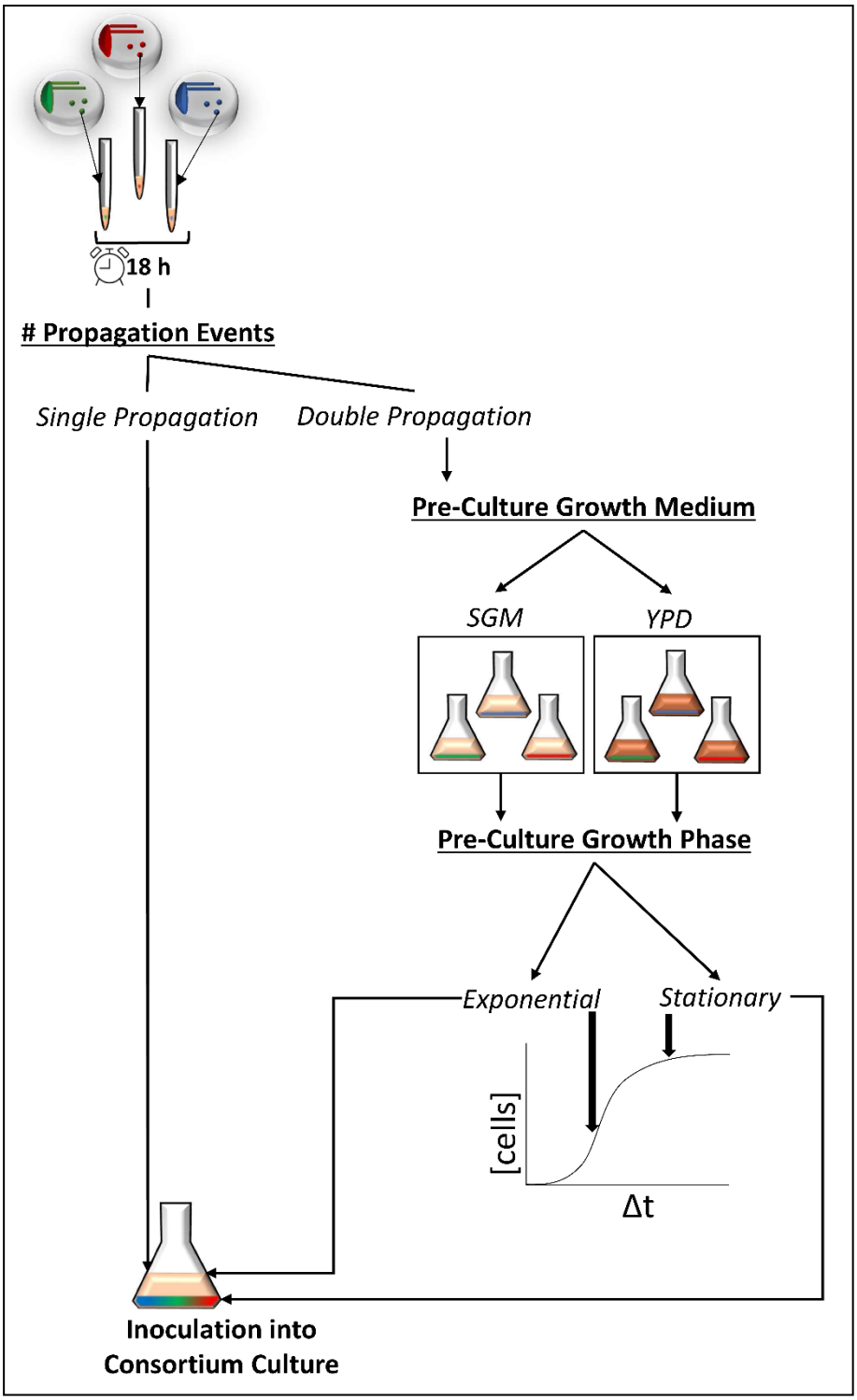


Fig. 1. Summary of the tested pre-culture conditions

Yeast strains

Four yeast species of wine-related origin were used to construct two synthetic yeast consortia. To compare differences in consortia complexity, a three species consortium consisting of *Saccharomyces cerevisiae*, *Lachancea thermotolerans* and *Torulaspora delbrueckii*, as well as a four species consortium, which also contained *Wickerhamomyces anomalus*, were used. Three of these species were fluorescently labelled, each with a different fluorescent label, while the fourth species was not labelled. The strains representing the four species were: *S. cerevisiae* VIN13 (Anchor Yeast, Cape Town, South Africa) labelled with TagRFP657, *L. thermotolerans* IWBT Y1240 [CBS: 16374] labelled with mTagBFP2, *T. delbrueckii* LO544 [CRBO: LO544] labelled with eGFP, and unlabelled *W. anomalus* IWBT Y934 (CBS: 16372) (Conacher et al. 2020). All yeast strains were stored as glycerol stocks (25 % w/v glycerol) at -80 °C.

Growth media

Prior to inoculation, glycerol stocks were streaked out onto Wallerstein Laboratory (WL) nutrient agar (Sigma-Aldrich, Johannesburg, South Africa) and incubated at 30 °C for three days. The Synthetic Grape Must (SGM) used here contained: 100 g l^{-1} glucose, 100 g l^{-1} fructose, 200 mg l^{-1} assimilable nitrogen as described by Bely et al. (1990), trace elements and vitamins as described by Henschke and Jiranek (1993) and 10 mg l^{-1} ergosterol.

Evaluation of effect of pre-culture conditions on consortium growth behaviour

Number of pre-culture propagation steps

For the single pre-culture propagation step, single colonies of each yeast strain were inoculated into 5 ml of Yeast Peptone Dextrose (YPD) broth (Sigma-Aldrich, Johannesburg, South Africa) in a test tube and incubated on a test tube rotator at 30 °C for 18 hours, after which the culture was harvested and inoculated into consortium culture.

For two-step pre-culture propagation, the culture from the single pre-culture propagation step was transferred to 50 ml YPD, at a concentration of 1×10^6 cells.ml^{-1}, in a 250 ml Erlenmeyer flask with a cotton plug and foil covering. The flask was incubated at 30 °C, with agitation (150 RPM), for 24 hours until stationary phase had been reached, after which the culture was harvested and inoculated into consortium culture.

Harvesting pre-cultures at exponential versus stationary phase

Using the two-step pre-inoculation procedure, during the second propagation step, cultures were incubated either for 10 hours until mid/late-exponential phase, or for 24 hours until stationary phase, before being harvested and inoculated into consortium culture. The time at which the pre-

cultures were deemed to be in exponential or stationary phase was based on previous monoculture growth curves (Supplementary Materials).

The impact of pre-culture growth medium

Using the two-step pre-inoculation procedure, during the second propagation step, cultures were incubated either in YPD, or in the final consortium culture growth medium, SGM, for 10 hours until exponential phase, before being inoculated into consortium culture.

Consortium culture inoculation and growth procedure

Pre-cultures were centrifuged at 5000 *x g* for 5 minutes at room temperature, and re-suspended in Phosphate Buffered Saline (PBS), pH 7.2, at a volume of 10X less than the initial culture volume, before being inoculated into a consortium culture. All consortium cultures were conducted as previously described (Conacher et al. 2020) in SGM, at a final volume of 6 ml in a sterile 6-well tissue culture plate, which was sealed with parafilm and incubated at 30 °C, with agitation (150 RPM). Each representative species was inoculated in equal cell numbers, for a final total concentration of 3×10^6 cells.ml^{-1}, measured by volumetric cell counts using the CytoFLEX (Beckman Coulter) flow cytometer. Monocultures of each species were grown in parallel and were inoculated at an initial concentration of 3×10^6 cells.ml^{-1}. Samples of 50 µl were taken at time points 0 h, 6 h, 12 h, 24 h, 48h, and 72 h to quantitate cell numbers of each species within the consortium and in monocultures. Samples were diluted in PBS prior to flow cytometry analysis. Images of the growing cultures were taken at 6 hours of growth to evaluate any early effects on flocculation phenotype.

Monitoring consortium population dynamics

Consortium population dynamics were determined by quantitative flow cytometry as previously described (Conacher et al. 2020), with the exception that all analyses were conducted on a single CytoFLEX (Beckman Coulter) flow cytometer, equipped with blue, violet, and red lasers.

Statistical Analyses

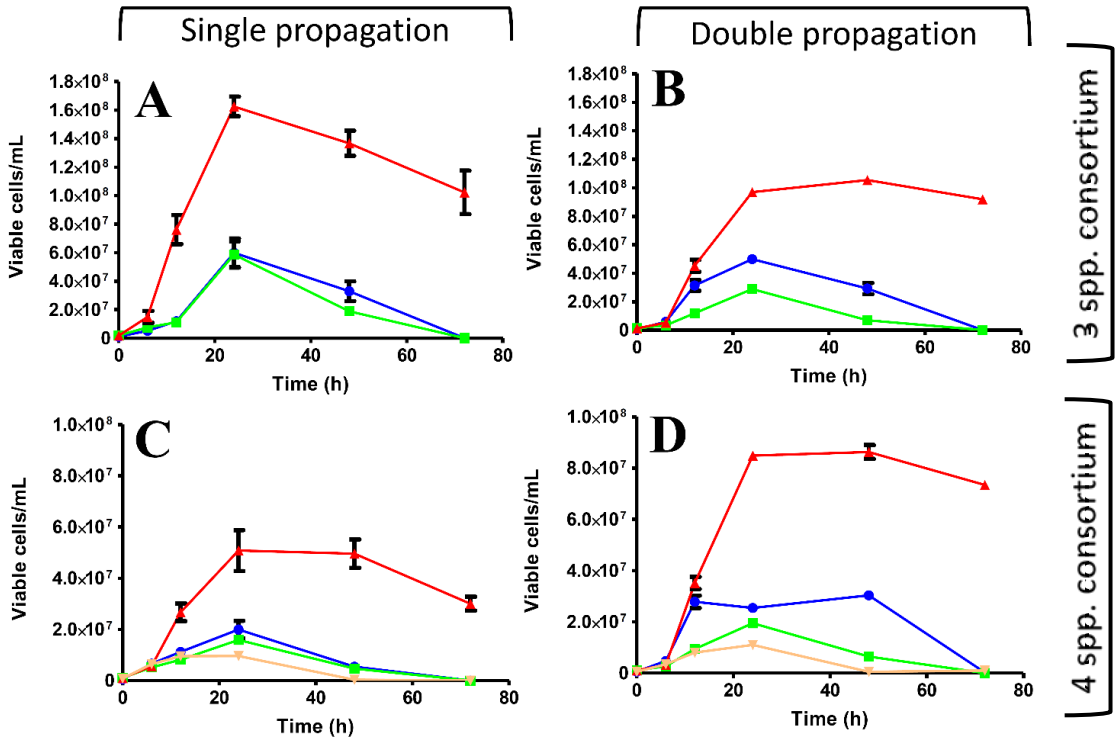
All statistical calculations were performed in GraphPad Prism 5. Two-tailed paired t-tests were performed to compare mean cell numbers of a species between the two propagation conditions. One way Analysis of Variance (ANOVA) was performed to determine significance of cell count differences i) between species in each condition tested for each time point, ii) between conditions for the same species at each time point. Bonferroni's Multiple Comparison Test was performed posthoc at a confidence level of 95 %. All significance values for relevant statistical calculations are given in Supplementary File 1.

Results

More than one pre-culture propagation step impacts consortia population dynamics and improves control of pre-culture growth phase

As a first step, the impact of one versus two propagation steps during pre-culture was evaluated. Differences in population dynamics between cultures were observed (Fig. 2). In both consortia sizes, notable differences in the ratio between *S. cerevisiae* and *L. thermotolerans* were observed (Fig. 2). Namely, *S. cerevisiae* appears to dominate the consortium sooner (from 6 hours onwards) when having undergone a single pre-culture propagation compared to double pre-culture propagation (from 12 hours onwards). The ratio of *L. thermotolerans* to other non-*Saccharomyces* species was increased in the double propagation cultures, particularly at the 12 hour time point for both consortia sizes tested, and at 48 hours in the 4 spp. consortium. The cell numbers of *T. delbrueckii* and *W. anomalus* remained relatively consistent between the two pre-culture conditions.

In monoculture studies, subculturing events have been shown to impact kinetic growth and fermentation parameters, volatile acidity and cell aggregation phenotypes (Orlowski and Barford 1987; Bely et al. 2005; Kragh et al. 2018). However, a limitation of the current comparison is that while the pre-culture medium was kept constant, in the absence of a subculturing event (i.e. inoculation of a colony to growth media), controlling the growth phase of a particular culture becomes an inexact task. In this context, the initial inoculation density and physiological state of a particular colony may differ, resulting in varying growth patterns (Keil et al. 2019). Therefore, while differences were observed, attributing it to subculturing events alone would be dubious and required closer examination. To further investigate what factors were pivotal in causing the observed population dynamics changes, the number of pre-culture propagation steps was kept constant to two, in order to allow more precise definition of the inoculum growth phase, and the impact of growth phase and growth medium was evaluated, since these have been highlighted in the context of defining physiological state in yeast.



E

Consortium Size	Pre-Culture Steps	6h				12h				24h				48h			
		Sc	Lt	Td	Wa	Sc	Lt	Td	Wa	Sc	Lt	Td	Wa	Sc	Lt	Td	Wa
3 spp.	Single Propagation																
3 spp.	Double Propagation																
4spp.	Single Propagation																
4spp.	Double Propagation																

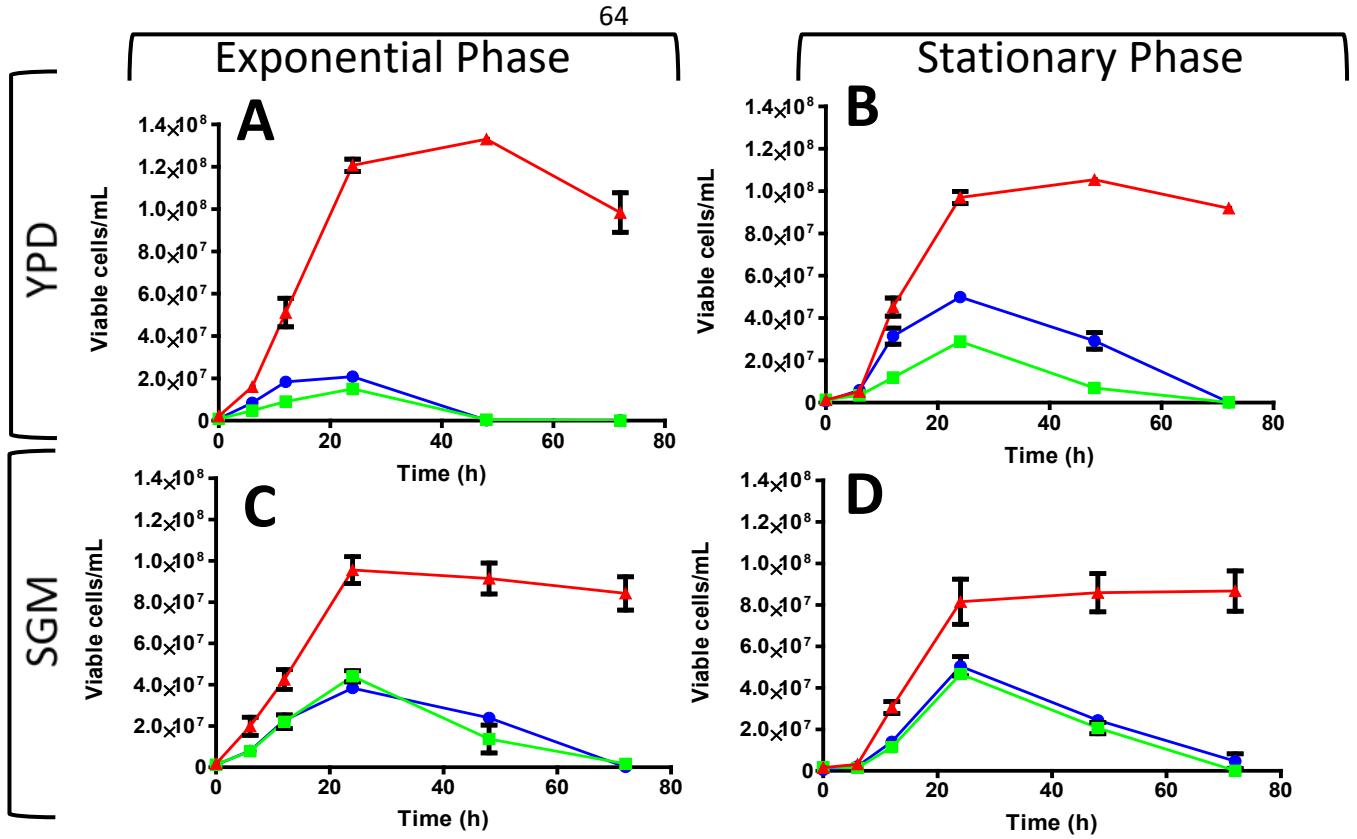
Fig. 2. Synthetic consortium population dynamics after single or double pre-culture propagation steps. Single pre-culture propagation step for 3 species consortia growth (A) and 4 species consortia growth (C). Double pre-culture propagation steps for 3 species consortia growth (B) and 4 species consortia growth (D). Red triangles: *S. cerevisiae*, blue circles: *L. thermotolerans*, green squares: *T. delbrueckii*, cream upside-down triangles: *W. anomalus*. Heat map of cell count numbers of each species (E). The heatmap scale goes from red (highest value) to green (lowest value). Colours are comparable between single and double propagation values at each time point. Colours are not comparable between different consortium sizes or different time points. Error bars represent standard error from the mean, with a minimum of three biological replicates.

Pre-culture growth phase impacts consortia population dynamics

The impact of pre-culture growth phase was determined by comparing pre-cultures in exponential phase versus stationary phase prior to inoculation into the combined consortium cultures. This process was completed in two separate pre-culture growth media, namely YPD and SGM. All consortium cultures inoculated with stationary phase pre-cultures showed an extended lag phase at the six hour point (Fig. 3B, D and Fig. 4B, D). Each species exhibited this same lag phase, confirming the need for metabolic adaption to excess nutrients in the growth medium at the time of inoculation (Yates and Smotzer 2007). In contrast, consortium cultures inoculated with pre-cultures in exponential phase appear to have shorter, more varied lag phases, reaching higher cell numbers at the six hour point (Fig. 3A, C and Fig. 4A, C).

In terms of population dynamics, the three-species consortium showed similar trends between the two pre-culture growth phase conditions (Fig. 3). Namely, for YPD-grown pre-cultures, *L. thermotolerans* is dominant over *T. delbrueckii*, and *S. cerevisiae* is dominant over both non-*Saccharomyces* species (Fig. 3A, B). While this trend was consistent, the cell numbers differed between growth phase conditions for YPD-grown pre-cultures. In terms of cell numbers of non-*Saccharomyces* species, YPD-grown stationary phase pre-cultures resulted in higher cell numbers, particularly for *L. thermotolerans*, while *S. cerevisiae* cell numbers were decreased (Fig. 3A, B). For SGM-grown pre-cultures, the trends and cell numbers were similar, with the non-*Saccharomyces* species being relatively evenly matched, and, in line with expectations and all other treatments, *S. cerevisiae* dominated growth throughout (Fig. 3C, D).

The four species consortium showed more pronounced differences in population dynamics in response to pre-culture growth phase (Fig. 4). In YPD-grown exponential phase pre-cultures, the ratio of the three non-*Saccharomyces* species within the consortium was more evenly matched, with *T. delbrueckii* showing the lowest cell numbers, and *L. thermotolerans* and *W. anomalus* showing very similar cell numbers to each other (Fig. 4A). The YPD-grown stationary phase pre-cultures showed much larger variation in cell numbers between the three non-*Saccharomyces* species (Fig. 4B). Specifically, *L. thermotolerans* showed proportionately higher cell numbers between 12 and 48 hours of growth; *T. delbrueckii* showed the second highest cell number during this time; and *W. anomalus* appeared to be the least competitive non-*Saccharomyces* species under these pre-culture conditions. *W. anomalus* and *T. delbrueckii* reached maximum cell counts later when inoculated from YPD-grown stationary phase versus exponential phase (Fig. 4A, B). In YPD-grown pre-cultures, *L. thermotolerans* inoculated in stationary phase appeared to adapt quicker than the other non-*Saccharomyces* species, resulting in more competitive growth, thereby stifling the growth of *T. delbrueckii* and *W. anomalus* by presumably faster uptake of limiting nutrients



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		6 h			12 h			24 h			48 h		
Pre-Culture Growth Phase	**Pre-Culture Growth Medium**	**Sc**	**Lt**	**Td**	**Sc**	**Lt**	**Td**	**Sc**	**Lt**	**Td**	**Sc**	**Lt**	**Td**
Exponential	YPD												
Exponential	SGM												
Stationary	YPD												
Stationary	SGM												

Fig. 3. Synthetic consortium (3-species) population dynamics after using pre-cultures conducted in YPD or SGM to exponential or stationary growth phase. YPD grown pre-cultures harvested in exponential growth phase (A) or stationary growth phase (B). SGM grown pre-cultures harvested in exponential growth phase (C) or stationary growth phase (D). Red triangles: *S. cerevisiae*, blue circles: *L. thermotolerans*, green squares: *T. delbrueckii*. Heat map of cell count numbers of each species (E). The heatmap scale goes from red (highest value) to green (lowest value). Colours are comparable between all conditions at each time point. Colours are not comparable between different time points. Error bars represent standard error from the mean, with a minimum of three biological replicates.

(Li et al. 2018; Rollero et al. 2018). This effect was not apparent in YPD-grown exponential phase pre-cultures, where it appears all the non-*Saccharomyces* species were similarly physiologically adapted, illustrated by more similar cell numbers throughout growth.

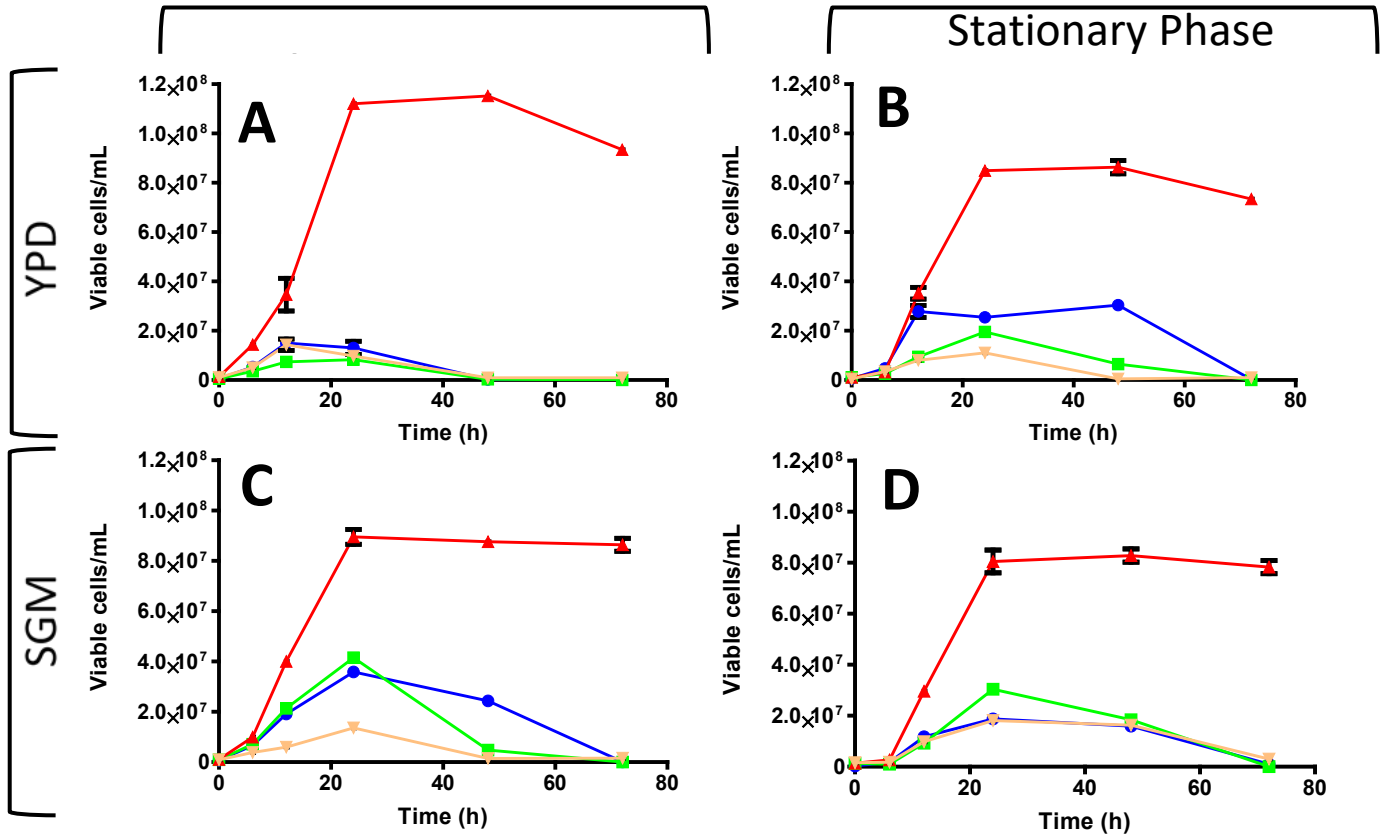
In SGM-grown pre-cultures, the impacts of growth phase on non-*Saccharomyces* dynamics during four-species consortium growth were also apparent (Fig. 4C, D). Namely, *L. thermotolerans* and *T. delbrueckii* pre-cultures grown to exponential phase in SGM showed proportionately higher cell numbers than *W. anomalus* at 12 hours of growth. Further, when pre-cultured in SGM to exponential phase, *L. thermotolerans* and, to a lesser extent, *T. delbrueckii*, reach higher maximum cell numbers after 24 hours of growth. In addition, *T. delbrueckii* undergoes death phase 24 hours sooner when inoculated from exponentially growing cells (Fig. 4C, D).

Pre-culture growth medium impacts consortia population dynamics

Pre-culture medium also significantly impacted on the population dynamics of the synthetic yeast consortium (Fig. 3-4). Here, cultures inoculated in the same growth phase, but grown in different pre-culture media were compared.

In the three-species consortium, when comparing exponential phase pre-cultures, there were slight differences in the ratio of *L. thermotolerans* to *T. delbrueckii*, with *L. thermotolerans* appearing to marginally outcompete *T. delbrueckii* when pre-cultured in YPD (Fig. 3A, C). When stationary phase pre-cultures were used, the ratio of *L. thermotolerans* to *T. delbrueckii* was similarly impacted, with *L. thermotolerans* appearing to have a competitive edge over *T. delbrueckii* when pre-cultured in YPD (Fig. 3B, D). When pre-cultured in SGM to stationary phase, *L. thermotolerans* and *T. delbrueckii* are evenly matched throughout growth in the three-species consortium (Fig. 3B, D).

The four-species consortium showed clear differences in relative ratios of the three non-*Saccharomyces* species between pre-culture growth media (Fig. 4). For exponential phase YPD-grown pre-cultures, there was obvious growth attenuation for all non-*Saccharomyces* species, while *S. cerevisiae* dominated (Fig. 4A, C). Non-*Saccharomyces* species reached lower maximum cell numbers 12 hours sooner than when pre-cultured in SGM (Fig. 4A, C). While the death phases of *T. delbrueckii* and *W. anomalus* were consistent between the two growth media, at 48 hours of growth *L. thermotolerans* had increased cell numbers when pre-cultured in SGM (Fig. 4A, C). Stationary phase pre-cultures also showed interesting impacts on the four species consortium (Fig. 4B, D). The competitive edge of *L. thermotolerans* pre-cultured in YPD to stationary phase, observed in the three species consortium, is also present in the four-species consortium (Fig. 4B, D). The ratio of *L. thermotolerans* to *W. anomalus* was affected in the four-species consortium, where SGM-grown pre-cultures showed even cell numbers of the two species, and YPD-grown pre-cultures showed higher



E

		6h				12h				24h				48h			
Pre-Culture Growth Phase	**Pre-Culture Growth Medium**	**Sc**	**Lt**	**Td**	**Wa**	**Sc**	**Lt**	**Td**	**Wa**	**Sc**	**Lt**	**Td**	**Wa**	**Sc**	**Lt**	**Td**	**Wa**
Exponential	YPD																
Exponential	SGM																
Stationary	YPD																
Stationary	SGM																

Fig. 4. Synthetic consortium (4-species) population dynamics after using pre-cultures conducted in YPD or SGM to exponential or stationary growth phase. YPD grown pre-cultures harvested in exponential growth phase (A) or stationary growth phase (B). SGM grown pre-cultures harvested in exponential growth phase (C) or stationary growth phase (D). Red triangles: *S. cerevisiae*, blue circles: *L. thermotolerans*, green squares: *T. delbrueckii*, cream upside-down triangles: *W. anomalus*. Heat map of cell count numbers of each species (E). The heatmap scale goes from red (highest value) to green (lowest value). Colours are comparable between all conditions at each time point. Colours are not comparable between different time points. Error bars represent standard error from the mean, with a minimum of three biological replicates.

cell numbers of *L. thermotolerans* and lower numbers of *W. anomalus* (Fig. 4B, D). Further, *W. anomalus* and *T. delbrueckii* were outcompeted 24 hours sooner when pre-cultured in YPD. Pre-culturing *T. delbrueckii* in SGM to stationary phase appeared to provide a small competitive edge in the four-species consortium, resulting in *T. delbrueckii* being the dominant non-*Saccharomyces* species at the 24 hour point (Fig. 4D).

Both three and four species consortia inoculated with SGM-grown exponential phase pre-cultures, showed increased cell numbers of *L. thermotolerans* and *T. delbrueckii*, and decreased cell numbers of *S. cerevisiae* (Fig. 3A, C and Fig. 4A, C). For exponential phase pre-cultures, *S. cerevisiae* appeared to adapt and reach dominance much sooner when pre-cultured in YPD and not SGM. This dominance and presumably proportional specific growth rate resulted in non-*Saccharomyces* species reaching attenuated growth sooner. The fast response to competitive growth in *S. cerevisiae* observed here is a well-known phenomenon (Perrone et al. 2013; Pérez-Torrado et al. 2017; Tronchoni et al. 2017). However, for stationary phase pre-cultures, this early dominance of *S. cerevisiae* was not present (Fig. 3B, D and Fig 4B, D). In terms of the non-*Saccharomyces* yeasts, population dynamics were significantly altered between the two growth media when pre-cultures were inoculated at stationary phase, illustrating the compounded impact of rate of adaptation to a new growth medium/condition(s) as well as nutrients in excess. Over-all, the non-*Saccharomyces* species appeared to be advantaged by pre-culture in SGM, which may have physiologically primed the cells for consortia growth in SGM. Species specific adaptation to high sugar growth medium after pre-culture in YPD has been reported, and the data here corroborates this evidence in a consortium context (Tondini et al. 2020).

Effect of pre-culture method on monoculture growth fails to predict impacts on multispecies culture growth

A major trend in microbial ecology research is generating predictive ecosystem models using kinetics data from simplified experiments (Stenuit and Agathos 2015; Guo and Boedicker 2016; Mendes-Soares et al. 2016; D'hoe et al. 2018). In the same vein, we sought to investigate whether a particular species being advantaged or disadvantaged during consortia growth by a particular pre-culture condition could be predicted by monoculture growth data alone. Pre-culture growth phase did impact monoculture growth curves, specifically the lag phase, with some impacts appearing species specific (Fig. 5). A similar trend across all species was that stationary phase pre-culture resulted in a longer lag-phase, with lower cell numbers at the 6 hour point compared to exponential phase pre-cultured monocultures (Fig. 5). In contrast, pre-culture growth medium minimally impacted monoculture growth curves, with a few differences occurring during the death phase (Fig. 5). In addition, all the monocultures reached similar maximum cell numbers, illustrating that there is no clear growth advantage of the chosen growth conditions in the absence of competing species (Fig. 5).

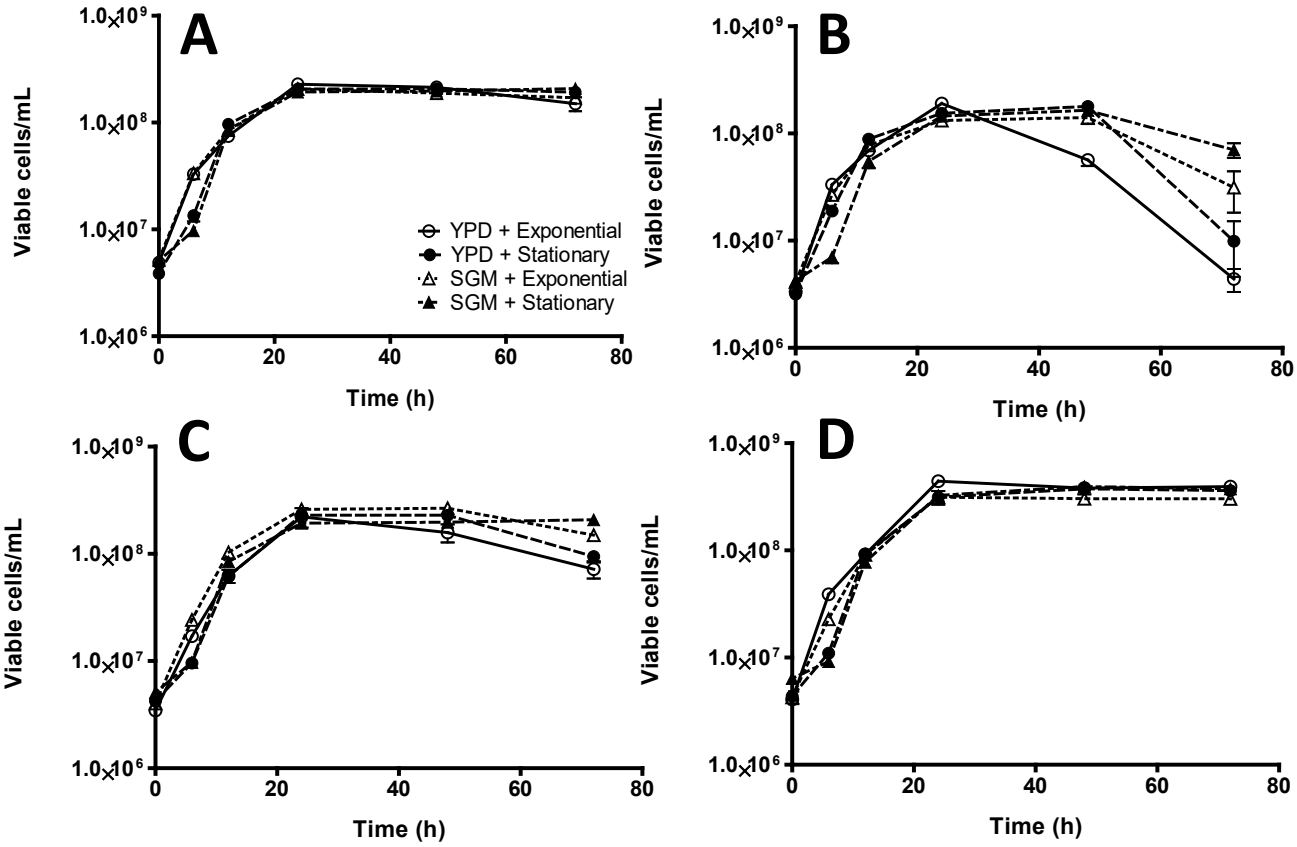


Fig. 5. Monoculture growth curves after using pre-cultures conducted in YPD or SGM to exponential or stationary growth phase. A: *S. cerevisiae*, B: *L. thermotolerans*, C: *T. delbrueckii*, D: *W. anomalus*. Open circles, solid line: YPD + Exponential phase. Filled circles, even long dashed line: YPD + Stationary phase. Open triangles, even short, dashed line: SGM + Exponential phase. Filled triangles, mixed dashed line: SGM + Stationary phase. Error bars represent standard error from the mean, with a minimum of two biological replicates.

The lag phase observed during mono-culture was largely consistent with the lag phase observed during consortia growth. However, predicting the impact on population dynamics from this extended (or shortened) lag phase was unclear. During consortia culture, *S. cerevisiae* consistently reigned as the dominant species, and appeared to adapt faster when inoculated into the consortium, however, this is not reflected in the monoculture growth curves (Fig. 5A). An interesting effect seen in *L. thermotolerans* during consortia growth was that pre-culturing in YPD to stationary phase appeared to give this species an advantage. The lag phase of the *L. thermotolerans* monoculture inoculated with YPD-grown stationary phase cells appeared to be shorter than any other stationary phase pre-culture tested, and while this is not apparent in the consortium culture lag phase, it may indeed have given *L. thermotolerans* a competitive advantage in the initial stages of consortium growth (Fig. 5B). Further, during consortia growth, *T. delbrueckii* was advantaged by pre-culture in SGM, however, this advantage is not present during monoculture growth (Fig. 5C). Lastly, the differences in lag phase observed during monoculture of *W. anomalus* (Fig. 5D) apparently had minimal impact on performance in the consortium, where *W. anomalus* showed consistent trends in all tested conditions.

The majority of monoculture growth curves were relatively consistent, in stark contrast to the major differences in population dynamics observed during consortia growth. This is indeed in line with similar studies in bacterial consortia, where authors tried to use monoculture growth kinetics data to model consortia growth kinetics, but saw that this data was insufficient to accurately describe the system (Guo and Boedicker 2016). While monocultures inoculated with cells in different physiological states recovered and behaved relatively similarly in constant culture conditions, this was not the case in mixed cultures, and ecosystem dynamics were affected. This suggests complex impacts on ecological behaviour within consortia as a result of the physiological state of cells. Therefore, it is recommended to test pre-culture conditions in the context of consortia growth as opposed to monoculture growth alone.

Pre-culture method impacts co-flocculation phenotypes during early stages of consortium growth

Flocculation is known to be an important mechanism of ecological interaction in yeast ecosystems (Rossouw et al. 2015; Rossouw et al. 2018). Co-flocculation phenotypes, particularly during the early stages of growth, were also impacted by pre-culture conditions, and representative images were taken at the 6 hour point to illustrate these (Fig. 6). The clearest effect can be seen in monocultures of *T. delbrueckii*, where flocculation occurs in monocultures inoculated with pre-cultures grown to stationary phase, but not in cultures where exponential phase pre-cultures were used. However, this change in flocculation phenotype is not predictive of whether the same flocculation occurs during consortia growth. While flocculation is observed in consortia where SGM-grown stationary phase pre-cultures were used, it is not present where YPD-grown stationary phase pre-cultures were used, despite this being present in *T. delbrueckii* monoculture. This indicates some impact of pre-culture conditions on the underlying molecular mechanisms that govern co-flocculation in these consortia. Further, since this was observed during early stages of consortium growth, it may also impact ecosystem establishment, which would be an interesting avenue for further research.

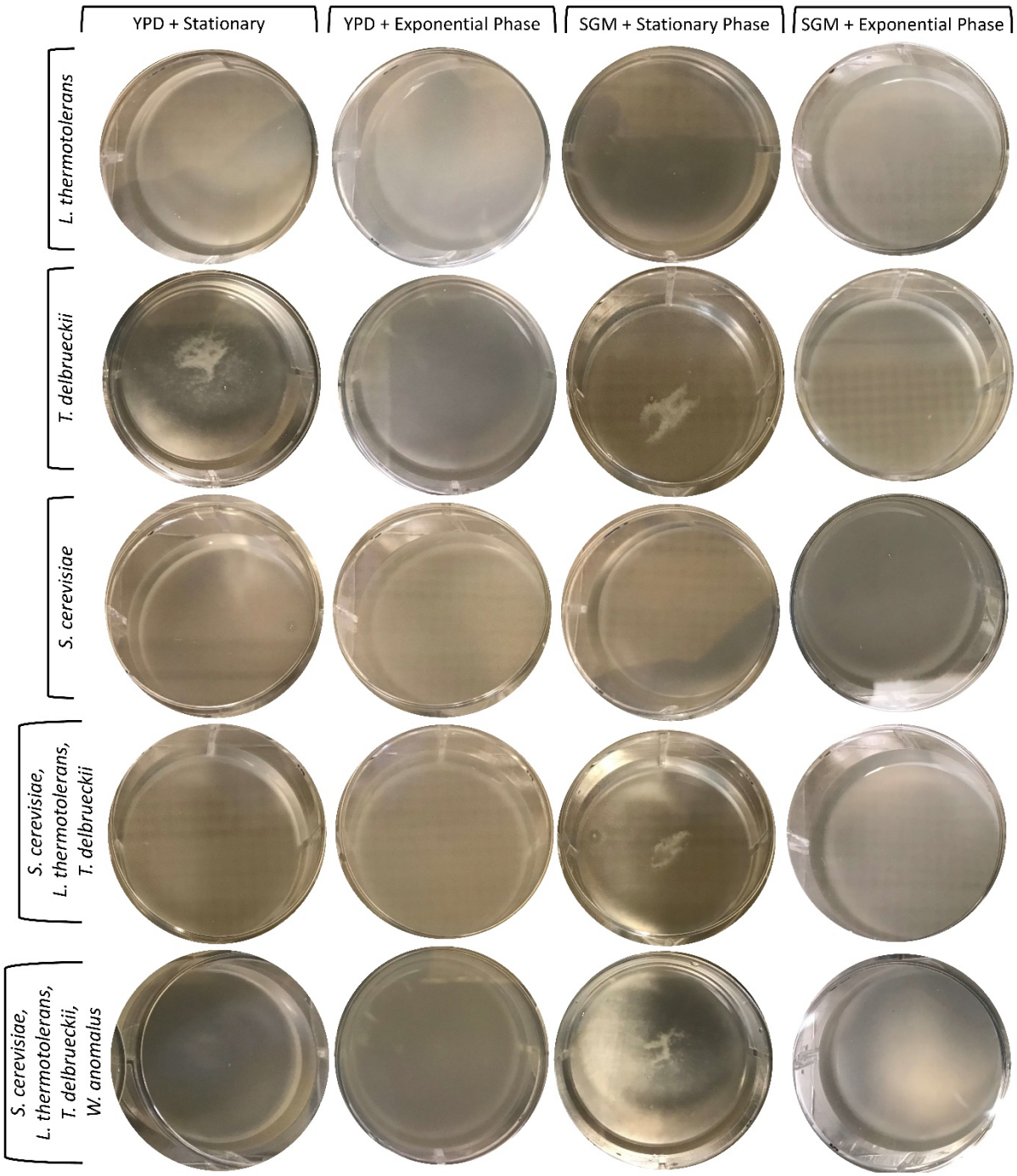


Fig. 6. Flocculation phenotypes in mono- and consortium cultures, using pre-cultures conducted in YPD or SGM to exponential or stationary growth phase, after 6 hours of growth.

Discussion

In an attempt to address current challenges in wrangling multispecies consortia to perform reproducibly, this study sought to clarify whether and how the physiological state of a particular yeast pre-culture would impact its growth behaviour within a synthetic yeast consortium. We saw that each factor tested in this study, namely the number of sub-culturing events, the inoculum growth phase, as well as growth medium, all impacted consortium population dynamics data. A major theme that became apparent was the importance of the adaptation phase in determining a particular species' growth behaviour in a consortium. Specifically, pre-cultures in exponential phase, and grown in the same growth medium as what they will eventually be cultured in, appeared to adapt faster. This emphasises an important balance between adaptation to abiotic versus biotic stress in the initial stages of growth within a consortium; it makes intuitive sense to lessen the stress of adaptation to abiotic stressors, which will allow the data to better show the influence of biotic stressors on growth kinetics. In line with this concept, we showed that monoculture growth kinetics are bad predictors for performance within a competitive growth environment, i.e. evaluating a species' performance in response to abiotic stress will not predict its response to biotic stress. Furthermore, as the consortium became more complex, and presumably the biotic stress as well, the impacts on population dynamics appeared more severe, eluding to the presence of a potential higher order interaction effect at play as well, which would indeed be a good point of further investigation (Sanchez-Gorostiaga et al. 2019).

While we have focussed on reporting what was different between the pre-culture conditions tested here, there were also trends that remained the same. These are also of interest since these trends will be consistent and comparable between studies that have made use of entirely different pre-culture strategies. The dominance and competitiveness of *S. cerevisiae* remained consistent, and the general trends of temporal changes in population dynamics were largely comparable.

In summary, pre-culture conditions tested in this study impacted on consortia dynamics. The comparative data reported here shows that precise definition of pre-culture conditions to suit your study aim is essential. Specifically, the physiological state of the inoculum, in terms of growth phase and adaptation to pre-culture growth medium, influenced the observed population dynamics within a three- and four-species yeast consortium. The dominance of *S. cerevisiae* was consistent regardless of pre-culture, while the dynamics of the non-*Saccharomyces* species were more influenced by pre-culture conditions. The data reported here has major implications for the reproducibility of synthetic consortia studies, and shows the importance of precise definition of pre-culture conditions used in published research. Further, in the quest to resolve the mechanistic basis of higher order ecological interactions, defining the physiological state of your inoculum is paramount, as it clearly impacts the establishment and functioning of synthetic consortia. This study should inform researchers on one of the many

reasons why data derived from consortia appear to be fickle, and emphasise the importance of considering the notion of cell ecophysiology within their ecological studies.

Author Contributions

FFB initiated the research, CC designed and conducted the experimental work, CC, RNB, DR and FFB analysed and interpreted the data, CC wrote the draft version and CC, DR, RNB and FFB edited the final manuscript

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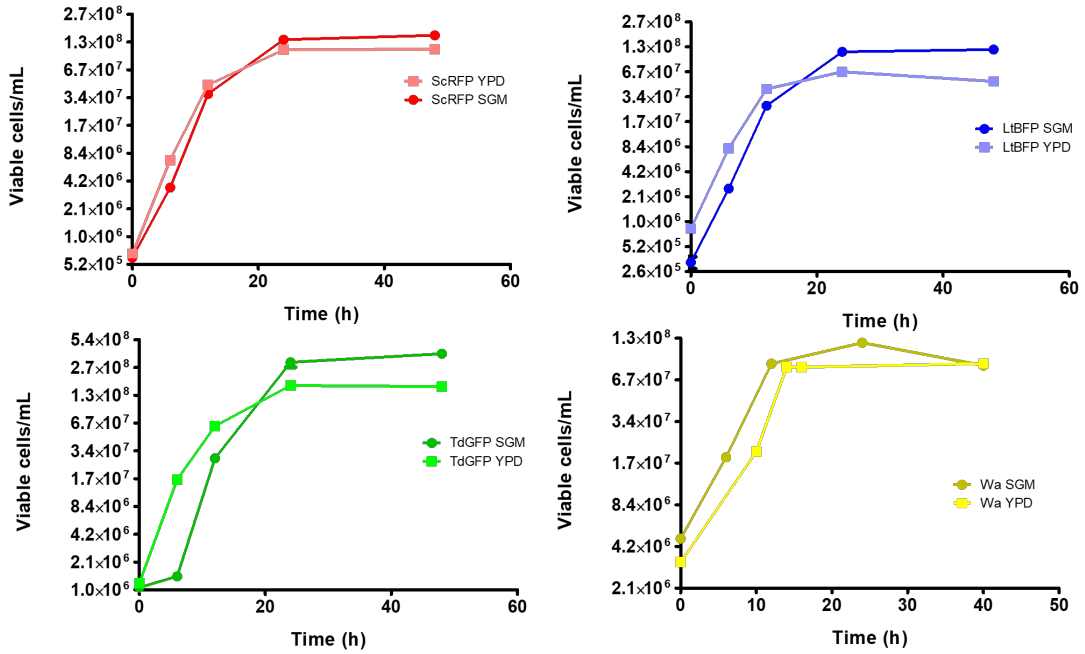
Supplementary Materials for Chapter 4

S1. Growth curves used to confirm pre-culture growth phase



Supplementary File 1: https://figshare.com/s/23e1366fbd85b8d56f88

Chapter 5

Research Results III

Evidence for higher-order interactions in yeast ecosystems

This chapter is in preparation for possible submission to mBio journal

Evidence for higher-order interactions in yeast ecosystems

Abstract

The use of microbial ecosystem-based biotechnology faces significant challenges because of our lack of functional and mechanistic understanding of these biological systems. Non-linear ecological interactions within microbial ecosystems are arguably the main contributors to the infamous unpredictability of ecosystem-based bioprocesses. Higher-order interactions, or interactions in systems comprised of more than two members that cannot be explained by pairwise interaction contribution, are particularly significant and understudied in this context. Wine fermentation presents an excellent model to study yeast ecosystem establishment and functioning. While some progress has been made in characterizing pairwise ecological interactions between wine yeast, very little is known about how more complex, multi-species systems function – an important endeavour, given that this is more relevant to their natural ecological state. Here, we sought to evaluate emergent non-linear ecosystem properties by determining the transcriptomic response of *Saccharomyces cerevisiae* to pairwise versus tri-species consortium culture. mRNA sequencing revealed that genes expressed during pairwise co-culture were enriched in the consortium dataset, and that just under half of the dataset represented expression of genes unique to consortia growth. Through interactive protein-association network visualizations, we were able to provide a holistic cell-wide view of the gene expression data, which highlighted known stress response mechanisms to be specifically activated during growth within the consortium. This provides exciting new evidence showing higher-order interactions within the simplest of synthetic wine yeast ecosystems. The findings are significant both in terms of providing further evidence for the importance of biotic stress in the eco-evolutionary development of wine yeasts, but also in bringing us a step closer to designing more predictable, efficient yeast ecosystem-based bioprocessing.

Introduction

Microbial communities are essential service providers to humans, performing functions ranging from digestion to bioremediation. These ecosystems are genetically and functionally diverse, allowing them to perform a myriad of bioprocesses, and affording them resilience to dynamically respond to fluctuations in their environment (Hays et al. 2015; Tsoi et al. 2019). Historically, humans have exploited the functionality of single microorganisms, for example *Saccharomyces cerevisiae*, for biotechnological applications, such as heterologous enzyme production, bioethanol production, as well as food and beverage fermentations. However, the use of monoculture in biotechnological processes has reached somewhat of an innovation ceiling in more complex bioremediation and fermentative bioprocesses, where emphasis is evermore shifting from attempting to modify a single cell to perform many functions, to rather division of labour between different species within a custom designed microbial ecosystem.

The process of wine fermentation is a prime example of this shift in focus. Spontaneous fermentation of grape must, performed by the natural microbiota present, is a high risk, high reward bioprocess, which gained some natural wine yeasts the reputation of being responsible for spoilage (Jolly et al. 2014). This spurred winemakers to inoculate their must with the strongest of fermenters, *S. cerevisiae*, in an effort to better control fermentation outcomes. While this increased productivity and lowered risk substantially, it came at a cost of sensory complexity and biogeographical exclusivity of the wine (Lambrechts and Pretorius 2000; Fleet 2003; Pretorius 2020; Liu et al. 2020). More recently, the role of the natural yeast other than *S. cerevisiae*, often termed non-*Saccharomyces* yeasts, in contributing to improved sensory quality as well as properties unique to a particular *terroir* of the wine has been realised, and the use of mixtures of yeast species has grown in prevalence (Comitini et al. 2011; Ciani and Comitini 2015; Vilela 2020; Zhu et al. 2021).

As with all bioprocesses that make use of multispecies communities, there are significant challenges that limit their application. One of these is the fact that there is a lack of predictive understanding of the mechanisms that govern the establishment and functioning of these ecosystems (Widder et al. 2016). Within the field of wine yeast ecology, as recently reviewed by Conacher *et al.* 2021, the more complex the system becomes, the less mechanistic the data is, largely relying on either diversity surveys, or cell count data, i.e. population dynamics to describe temporal changes in ecosystem dynamics, which have inherently low resolution in terms of inter-strain evaluations. In terms of the molecular mechanisms that govern yeast-yeast interactions, the current state of knowledge is largely based on binary, i.e. two-species systems (Curiel et al. 2017; Tronchoni et al. 2017; Englezos et al. 2019; Peng et al. 2019; Shekhawat et al. 2019; Tondini et al. 2020; Roullier-Gall et al. 2020; Mencher et al. 2021). These are comparatively easier to investigate than multispecies systems, given their better predictability, and they were prioritised since such simpler systems can provide foundationally important data sets before tackling more complex systems. These studies have investigated the responses of yeast species to each other at the transcriptomic and proteomic level, and have focussed on the response of *S. cerevisiae* to other yeast species. A handful of studies have also reported on non-*Saccharomyces* partner responses, including *Torulaspora delbrueckii*, and *Lachancea thermotolerans*, which are both popular choices for industrial fermentations given their strong fermentative capacity and contribution to positive sensory qualities (Tronchoni et al. 2017; Shekhawat et al. 2019; Tondini et al. 2020). The focus on *S. cerevisiae* in most of these studies is linked to this species playing a dominant role in the wine ecosystem, while also being a model organism, with an excellent molecular toolbox and research archive from which to draw on. The conclusions of these studies have shown that there are definite impacts on *S. cerevisiae* at the transcriptional and translational level in response to mixed species culture, and more interestingly, there are indeed species-specific impacts on *S. cerevisiae* as well.

In contrast, very little is known about the influence of non-binary interactions within yeast ecosystems, and this is indeed a major research challenge within the broader field of microbial ecology as well (Widder et al. 2016). Higher-order interactions are non-linear effects on the existent interactions (and functioning) of a microbial community, which happen when either pairwise interactions are perturbed by the presence of other interactors, or the emergence of completely new properties as a result of a specific combination of microbial role-players (Billick and Case 1994). Currently, the available quantitative data of higher-order interactions in microbial ecology are dominated by bacterial communities only (Guo and Boedicker 2016; D'hoe et al. 2018; Morin et al. 2018; Venturelli et al. 2018; Sanchez-Gorostiaga et al. 2019). These studies have shown that, in general, all possible combinations of pairwise interactions are good predictors of what occurs in the multispecies system, but also that higher-order interactions contribute to unpredictable community dynamics and functioning and are important factors to consider in modelling these ecosystems as well as in the design of synthetic microbial ecosystems. In yeast, far less is known about higher-order interactions, with the best available data being population dynamics that have been collected during fermentations with inoculated yeast consortia, but these are limited in terms of comparing population dynamics in cultures of increasing complexity, so the emergence of any higher-order effects is masked (Ciani et al. 2006; Comitini et al. 2011; Suzzi et al. 2012; Ciani and Comitini 2015; Bagheri et al. 2017; Bagheri et al. 2018; Bagheri et al. 2020). This lack of mechanistic data must be addressed if any yeast ecosystem-based bioprocesses are to be adopted in industry.

Here, we have sought to study the emergence of higher-order interactions at the transcriptomic level in *S. cerevisiae* within the simplest possible consortium of three wine-associated yeast species. The consortium was completed by the addition of *Lachancea thermotolerans* and *Torulaspora delbrueckii*. Population dynamics and the mRNA transcriptome of *S. cerevisiae* were compared between mono-, bi-, and tri-species culture. We report on the species-specific impacts of the pairwise co-cultures on *S. cerevisiae*, contributing to our understanding of the ecological interactions at play and allowing comparison to previous pairwise studies that investigated similar species. By eliminating the signature of pairwise interactions from the consortium dataset, we were able to reveal the presence of a reaction unique to consortia growth, which alludes to a possible higher-order effect in *S. cerevisiae*. This data contributes to our broader understanding of yeast-yeast interactions within the wine fermentation ecosystem, and importantly, gives a first look at the potential mechanisms that allow *S. cerevisiae* to consistently dominate this ecosystem. These findings have implications for the future design of synthetic yeast ecosystems, as well as our fundamental understanding of the role of biotic stress on the establishment and functioning of such ecosystems.

Methods

Yeast strains

Three yeast species representatives of wine-related origin were used to construct a synthetic yeast consortium. The three species were fluorescently labelled, each with a different fluorescent label, namely *S. cerevisiae* VIN13 (Anchor Yeast, Cape Town, South Africa) labelled with TagRFP657, *L. thermotolerans* IWBT Y1240 [CBS: 16374] labelled with mTagBFP2, and *T. delbrueckii* LO544 [CRBO: LO544] labelled with eGFP (Conacher et al. 2020). All yeast strains were stored as glycerol stocks (25 % w/v glycerol) at -80 °C. Prior to inoculation, glycerol stocks were streaked out onto Wallerstein Laboratory (WL) nutrient agar (Sigma-Aldrich, Johannesburg, South Africa) and incubated at 30 °C for three days.

Growth medium design

The synthetic growth medium, Yeast Nitrogen Base (YNB) with amino acids and ammonium sulphate, was adjusted to create a high sugar cultivation medium that supported growth of all three yeast species within the consortium, referred to here as Optimized YNB (OYNB). A summary of the growth medium design process is reported in the Supplementary Materials. The optimized growth medium selected for culturing consisted of 6.7 g/L YNB with amino acids and ammonium sulfate, 100 g/L glucose, 100 g/L fructose, and 1X amino acid stock solution (Table S1.2).

Pre-culture conditions

Single colonies of each yeast strain were inoculated into 5 ml of Yeast Peptone Dextrose (YPD) broth (Sigma-Aldrich, Johannesburg, South Africa) in a test tube and incubated on a test tube rotor at 30 °C for 18 hours. Four biological repeats were conducted, with a biological repeat defined as a culture originating from a separate colony. Cells were harvested by centrifugation, resuspended in OYNB, and transferred to 50 ml OYNB, at a concentration of 1×10^6 cells.ml^{-1}, in a 250 ml Erlenmeyer flask with a cotton plug and foil covering. The flask was incubated at 30 °C, with agitation (150 RPM), for 8 hours, until mid-exponential phase, after which the pre-culture was harvested by centrifugation at 5000 *x g* for 5 minutes at room temperature, and resuspended in OYNB at a volume of 10X less than the initial culture volume, before being inoculated.

Culture conditions

Pre-culture biomass density was measured by OD_{600}, and all cultures were inoculated to a final total density of 0.3 OD_{600}. Four biological repeats of each species were inoculated into either single, double, or triple species cultures (Table 1). Each species representative was inoculated at equal cell biomass, as determined by OD_{600} values. Cultures were conducted in 40 ml OYNB in 100 ml Erlenmeyer flasks with a cotton plug and foil covering. Growth media was pre-warmed to 30 °C with agitation. Cultures were

incubated at 30 °C, with agitation (150 RPM) until samples were removed for RNA extraction. All cultures were conducted on the same day to minimize batch variation.

Table 1. Summary of species composition and inoculation density of cultures tested

Single species culture (OD_{600}/species = 0.3)	**Double species culture (OD_{600}/species = 0.15)**	**Triple species culture (OD_{600}/species = 0.1)**
S. cerevisiae	*S. cerevisiae* + *L. thermotolerans*	*S. cerevisiae* + *L. thermotolerans* + *T. delbrueckii*
	S. cerevisiae + *T. delbrueckii*	

Monitoring consortium population dynamics

Consortium population dynamics were determined by quantitative flow cytometry as previously described (Conacher et al. 2020), with the exception that all analyses were conducted on a single CytoFLEX flow cytometer (Beckman-Coulter), equipped with blue, violet, and red lasers.

Sampling, RNA extraction, sequencing, and data analysis

Samples were taken after approximately 7 hours, when all cultures were in similar phases of early exponential growth, roughly a third of the way through the exponential phase. The sample point was carefully considered to ensure that the monoculture would be in a comparable growth phase to both pairwise cultures as well as the consortium culture. For sampling, 2 ml of culture was removed, centrifuged at 5000 X g for 3 minutes, resuspended in 500 µl cold RNALater (Thermofisher Scientific, South Africa), and stored at 4 °C for 18 hours until extraction. To further confirm the growth phase of the various samples, the sample supernatants were analysed for glucose and fructose concentrations using enzymatic kits (Enzytec™ Fluid D-fructose Id-No: E5120, Roche, R-Biopharm, Enzytec™ Fluid D-fructose Id-No: E5120, Roche, R-Biopharm) and an automated analyser (Konelab Arena 20XT, Thermo Electron Corporation, Finland). Immediately before extraction, 1:1 volume of cold DEPC-treated PBS was added to the sample to reduce sample viscosity and aid in centrifugation of the samples. RNA extraction was performed using the Qiagen AllPrep DNA, RNA, Protein kit. The resultant RNA was checked for gDNA contamination by PCR of the ITS1/ITS4 region, with a positive gDNA control. RNA was stored at -80 °C until sequencing.

The total RNA samples were assessed for RNA integrity (RIN) and quantity on the BioAnalyzer 2100 (Agilent Technologies, Waldbronn, Germany) using the RNA 6000 Nano Chip and reagents. mRNA was captured from 800ng total RNA using the Dynabeads® mRNA DIRECT™ Micro Kit (ThermoFisher Scientific). The diluted mRNA was bound to the Dynabeads® Oligo (dT)25, washed and eluted in 15 µl nuclease-free water. The Ion Total RNA-Seq Kit v2 (ThermoFisher Scientific) was used to convert expressed mRNA transcripts into a representative cDNA library for strand specific RNA sequencing on the Ion Torrent™ Ion S5™system. This library was purified and assessed for yield and fragment size

distribution on the Agilent BioAnalyser 2100 using the High sensitivity DNA chip and kit (Agilent Technologies). The libraries were diluted to a target concentration of 80pM and pooled in equimolar amounts for template preparation using the Ion 540™ Chef Kit (ThermoFisher Scientific). Enriched ion sphere particles were loaded onto an Ion 540™ Chip (ThermoFisher Scientific). Massively parallel sequencing was performed on the Ion Torrent™ GeneStudio™ S5 Prime System using Sequencing Solutions, Reagents according to the protocol. Flow space calibration and basecaller analysis were performed using standard analysis parameters in the Torrent Suite Version 5.12.2 Software.

All sequencing data was processed and analysed using Partek Flow software (Thermofisher) at the Central Analytical Facility for Next Generation Sequencing at Stellenbosch University. During pre-processing of the generated reads, two read-length cut-offs, namely 8 bp and 12 bp, were compared. Processed reads were mapped to a concatenated three-species genome, consisting of *S. cerevisiae* R64, *L. thermotolerans* CBS 6340, and *T. delbrueckii* CBS 1146, as it is planned to include analysis of all species in future manuscripts, thereby keeping the analysis pipeline consistent. Read alignment was performed in two steps, first using STAR (2.6.1), followed by input of unaligned STAR reads into Bowtie2 (2.2.5), and finally combining the two alignment outputs. Non-uniquely mapped reads were randomly assigned to a particular portion of the reference. Aligned reads were then filtered to include only reads aligning to the *S. cerevisiae* genome. Reads mapping to annotated portions of the reference genome were then quantified by the expectation/maximization (E/M) algorithm applied in Partek. PCA plots of the mapped reads per gene were used to identify outlying samples between biological repeats, and these were removed, retaining a minimum of 3 biological repeats for further analysis. Quantified counts were then normalized by counts per million (CPM). Gene Set Analysis (GSA) was then performed to quantify differentially expressed genes, and the list was filtered to include genes with FDR values less than 0.05 and Log2 fold change values less than -1 or greater than 1. Monoculture samples were respectively compared to both pairwise samples, as well as the tri-species (i.e. consortium) samples (Table 1). When comparing the DEGs unique to each of the tested read-length cut-off conditions during pre-processing, it was found that the majority 'unique' DEGs were of borderline statistical significance in the cut-off condition that they did not appear in (Supplementary File 1). Therefore, instead of lowering the FDR significance value, which would generate a highly similar output, it was decided to use the union of the ORFs produced by both of these cut-off conditions. This was deemed appropriate given the main aim of the gene expression analysis was hypothesis generation, and inclusion of all genes would serve this aim better.

Functional enrichment analysis and visualization of gene expression data

Functional enrichment analysis of the generated DEG lists was conducted through the STRING (v11) database functional enrichment tool. To generate a holistic view of the gene expression data, potential protein interaction networks were generated in STRING and interactive visualizations of the networks

were created using Cytoscape (3.8.2) (Cline et al. 2007). Separate analysis of up and down regulated genes was included as a point of reference (data not shown), however, the DEG list input discussed here included both up and downregulated genes to ensure that potential connections between these DEGs were captured. The generated networks were visualized in Cytoscape (3.8.2), and clustered based on the distance matrix calculated from STRING global interaction scores, using the Markov CLustering Algorithm (MCL) within the clusterMaker application (granularity = 2.5, unless otherwise stated). Functional enrichment analysis was repeated on each cluster (with n>4), which assisted in generating a simplified view of the global changes observed within the large dataset (See Supplementary File 2 for per-cluster functional enrichment analyses).

These networks were created for i) DEGs that were commonly differentially expressed between both pairwise and consortia culture conditions, ii) DEGs that were differentially expressed during pairwise co-culture with *L. thermotolerans*, excluding DEGs in (i), iii) DEGs that were uniquely differentially expressed during pairwise co-culture with *T. delbrueckii*, excluding DEGs in (i), iv) All DEGs that were differentially expressed during consortia growth, v) DEGs unique to consortia growth, filtered to remove DEGs that were present in pairwise DEG lists, as well as DEGs on the statistical borderline (FDR > 0.05; <= 0.055) in the pairwise DEG lists (Supplementary File 2). All generated network files are available at the following link: https://figshare.com/s/4ba873d75b96583514d5. Readers are encouraged to make use of these files to visualize the networks within Cytoscape as intended, as the static images presented here are only for illustrative purposes.

Results

Population dynamics of the synthetic yeast consortium

As an initial assessment of ecological trends within the synthetic consortium, pairwise and three-way population dynamics were assessed (Fig. 1). As expected, *S. cerevisiae* was the dominant species in each mixed culture scenario (Albergaria and Arneborg 2016; Boynton and Greig 2016; Ciani et al. 2016; Bagheri et al. 2017; Pérez-Torrado et al. 2017; Bagheri et al. 2020). In the pairwise co-cultures with *S. cerevisiae*, *L. thermotolerans* growth appeared to be more severely attenuated in comparison to *T. delbrueckii* (Fig. 1B-C). Cell numbers of *S. cerevisiae* throughout growth in both of these pairings were near identical. *S. cerevisiae* achieved dominance after 8 hours of co-culture in both pairwise experiments. These pairwise population dynamics were well represented in the three-way population dynamics, with *S. cerevisiae* being the dominant species and *T. delbrueckii* having slightly higher cell numbers than *L. thermotolerans* from 8 hours onwards (Fig. 1E). The growth patterns of *S. cerevisiae* in pairwise and consortia cultures were highly similar, the only notable difference being lower maximum cell numbers, which can be explained by the initial inoculation dosage (Fig. 1). Over-all, these cell number-based trends indicate that the pairwise population dynamics were a good predictor for the dynamics in the more complex tri-species system.

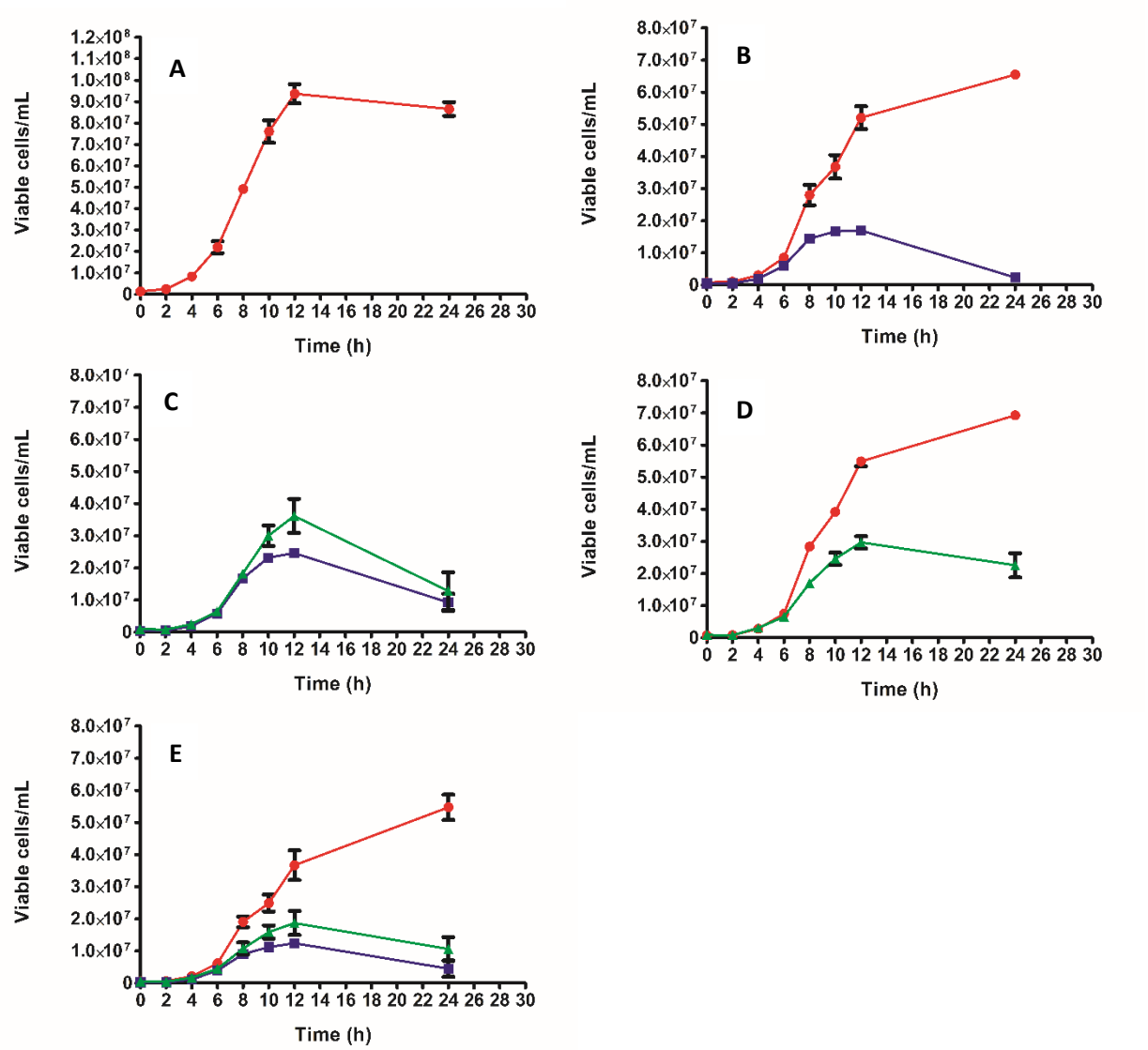


Figure 1. Population dynamics of mono-, bi-, and tri-species cultures, grown at 30 °C in optimized YNB growth medium with aeration and agitation. Red circles: *Saccharomyces cerevisiae*; Purple squares: *Lachancea thermotolerans*; Green triangles: *Torulaspora delbrueckii*

Differential expression analysis of S. cerevisiae *in mixed species cultures*

To characterize gene expression programs of *S. cerevisiae* associated with emergent higher order interactions within a three species yeast consortium, differential expression was compared between the consortium and every possible pairwise combination within the consortium. To contextualize these results, it is important to consider the state of the ecosystem at the sampling point. After 7 hours of growth, in all mixed culture settings, *S. cerevisiae* has just begun to dominate competing species in terms of cell numbers, and is in the early exponential growth phase. This time point represents an important 'turning-point' in the growth of *S. cerevisiae,* where its dominant phenotype is represented in the population dynamics, while still having relatively equal cell numbers to the other species. Thus ensuring that the total RNA extraction would contain enough material from all co-existing species.

Further, the sugar concentrations of the cultures at this time point were all relatively similar (Supplementary Table S1.4), which supports the fact that all the cultures were in similar points of growth, and reduces the likelihood that the differences observed here were due to varying growth phases.

Generalized response of S. cerevisiae *to mixed species culture*

In all conditions tested, there were 24 commonly differentially expressed genes (Fig. 2). These genes were similarly up or down regulated in all conditions, and the differential expression levels were highly correlated between all conditions. Notably, these commonly affected genes were also some of the most highly upregulated in each tested condition.

Two major functional pathways were upregulated within this group, namely thiamine biosynthesis (THI11, THI13, THI22, SNZ3, SNO2) and NAD+ biosynthesis (*de novo* pathway: BNA2, BNA4, BNA5, BNA6; salvage pathway: TNA1). Thiamine (Vitamin B_1) and its phosphorylated derivatives are important co-factors for enzymes involved in carbon metabolism, and is a growth factor of particular interest within fermentative processes because of its influence on glycolytic flux and fermentation efficiency (Brion et al. 2014; Perli et al. 2020; Labuschagne and Divol 2021). Competition for thiamine has also previously been highlighted in interactions of *S. cerevisiae* with *L. thermotolerans* and *Hanseniaspora guilliermondii* (Barbosa et al. 2015; Shekhawat et al. 2019). The pyruvate decarboxylase gene, PDC5 was upregulated within this list, and is repressed by thiamine, signalling low intracellular thiamine levels (Muller et al. 1999; Brion et al. 2014). Interestingly, the upregulation of *de novo* NAD+ biosynthesis and nicotinic acid uptake has direct links to thiamine biosynthesis, given that NAD+ is a necessary co-factor of thiamine biosynthesis enzymes, and that these processes are regulated via the same protein, namely Hst1p, a NAD+ dependent histone deacetylation protein (Li et al. 2010). Thiamine accumulation has also been linked to providing protection against oxidative and osmotic stress, which is of relevance in the high sugar growth medium used here (Wolak et al. 2014). Further, NAD+ homeostasis plays a critical role in maintaining redox balance within the cell (Shi et al. 2016). Here, the osmotic stress marker, HOR7 (GPD1), oxidative stress-associated genes (TSA1, MXR1), and a DNA replication stress gene (SOL4) were all upregulated, providing evidence for a potential link to the stress-protective need for thiamine.

The fact that these genes were impacted regardless of the species or number of co-habitant species, alludes to these responses being indirectly linked to the presence of other species. It is likely that these responses were more as a result of nutritional competition or other impacts of co-habitant species on the growth environment, as opposed to a direct ecological interaction mechanism. The functional analysis of this group of genes indeed corroborates this hypothesis, with many being associated with competition and stress related to the OYNB growth medium formulation.

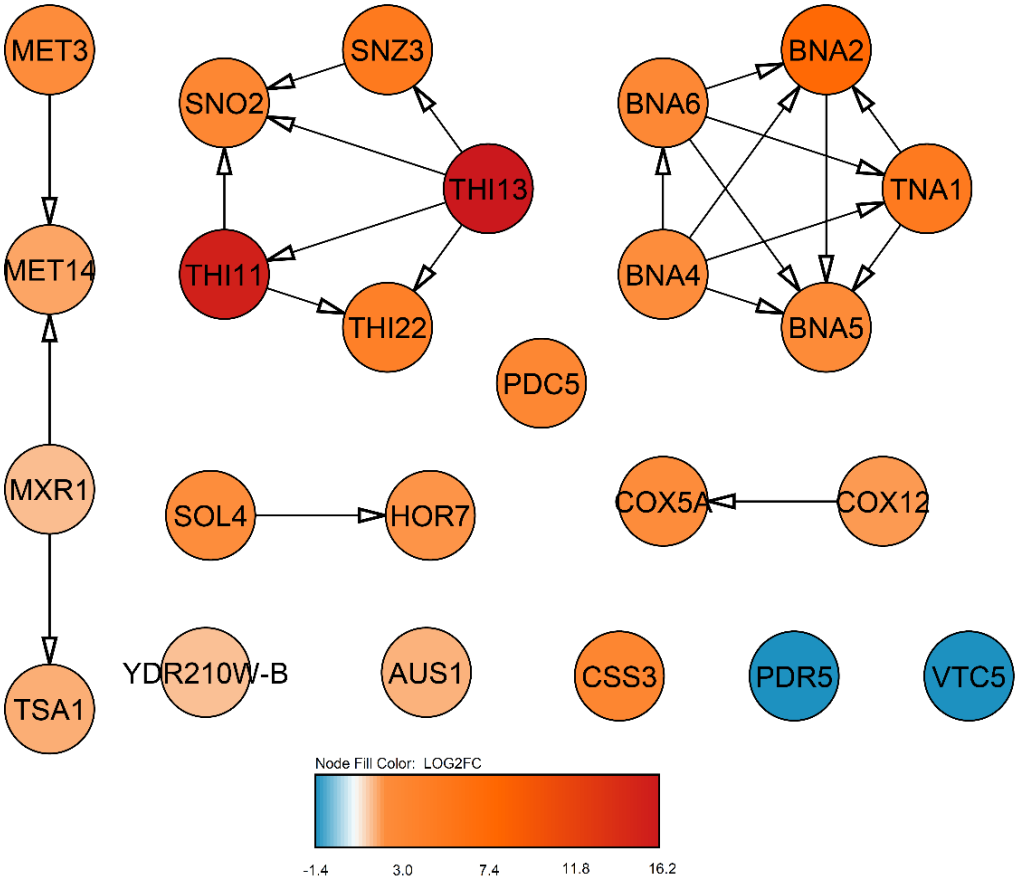


Figure 2. Differentially expressed genes in *S. cerevisiae* that were present in both pairwise co-cultures as well as consortia culture. The nodes are coloured according to Log2 fold-change values, expressed as an average value between all tested conditions. Image and network created in Cytoscape.

S. cerevisiae *shows species-specific transcriptome remodelling during pairwise culture*

Differentially expressed genes unique to each pairing with either *L. thermotolerans* or *T. delbrueckii* were then comparatively assessed. While the primary aim of this analysis was to account for pairwise interactions within the consortium setting, there were notable differences in the transcriptional responses of *S. cerevisiae* to both species that are worth reporting. For instance, there was a remarkable difference in the number of affected genes between the two pairings, with *T. delbrueckii* (811 DEGs) eliciting over 20-fold more of a response than *L. thermotolerans* (35 DEGs). The strong response of *S. cerevisiae* to *T. delbrueckii* has been noted before (Curiel et al. 2017; Tronchoni et al. 2017; Tondini et al. 2020), and is hypothesized to be as a result of very similar metabolism between the two species, given their close evolutionary history (Dujon and Louis 2017). *L. thermotolerans* has a similarly close phylogenetic relationship with *S. cerevisiae*, and has been shown to have similar carbon and nitrogen preferences to *S. cerevisiae* and *T. delbrueckii* under the nutrient conditions applied here (Roca-mesa et al. 2020) – although inter-strain variability in nutritional requirements makes comparisons of this nature difficult (Porter et al. 2018). Previous studies have taken a more targeted approach to discussing potential pairwise interaction mechanisms between these species, focussing on a

small subset of genes within their datasets. Here, with the use of protein interaction networks, we provide a broader cell-wide view of the potential functional reasons behind *S. cerevisiae*'s species-specific responses.

Pairwise interaction with *L. thermotolerans* induced *S. cerevisiae* respiratory metabolism by upregulation of a few genes involved in the respiratory electron transport chain, specifically mitochondrial ATP synthesis (COX4, CYT1, QCR2) and ubiquinone biosynthesis (COQ6) genes (Fig. 3). Further, the lesser-studied putative hexose transporter (HXT8) was downregulated. Taken with the upregulation of pyruvate decarboxylase gene PDC5 (expressed in all conditions), this suggests a shift to simultaneous fermentative and respiratory metabolism, common to Crabtree positive yeasts in high glucose, aerobic conditions. Other upregulated genes included a ubiquitin biosynthesis gene (UBX6), a weak acid stress response gene (YRO1), a transcriptional modulator of meiosis, gene silencing, and stress-induced RNR genes (WTM1), as well as amino acid biosynthesis genes (MET5, MST1). An uncharacterized gene (YJR115W) was downregulated, similar to a previous study that evaluated *S. cerevisiae* and *L. thermotolerans* pairings (Shekhawat et al. 2019), highlighting this gene as a potential target for future functional annotation studies.

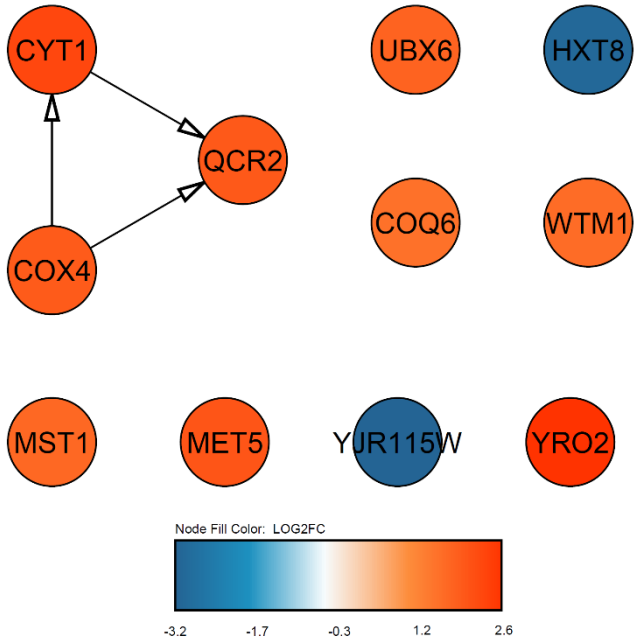


Figure 3. Differentially expressed genes in *S. cerevisiae* in response to co-culture with *L. thermotolerans*, excluding the genes reported in Figure 2. The nodes are coloured according to Log2 fold-change values. Image and network created in Cytoscape.

T. delbrueckii stimulated a more extensive metabolic shift in *S. cerevisiae*. Using the clustered interaction network visualisation, distinguishing the main affected metabolic processes was simplified (Fig. 4).

There was major remodelling of central carbon metabolism, with activation of glucose metabolism, indicating an increase in glucose uptake in response to competition for this preferred carbon source (Fig. 4: Clusters 1, 3, 6). There appeared to also be simultaneous fermentative (Fig. 4: Cluster 10) and respiratory (Fig. 4: Cluster 3) metabolism, albeit with more pronounced impacts on respiration. Specifically, there was upregulation of respiratory genes involved in the mitochondrial electron transport chain (Fig. 4: Cluster 3), re-organization of mitochondrial structure (Cluster 18, 24, 25), increased flux through the TCA cycle (Fig. 4: Cluster 3) and activation of both the oxidative and non-oxidative branch of the pentose-phosphate pathway (Fig. 4: Cluster 1). Glucose sensing and carbon catabolite repression (CCR) regulators were differentially expressed, including target hexose transporters involved in CCR (Fig. 4: Cluster 6). Further, the SIP2 gene, central to glucose starvation response was also upregulated. Interestingly, all genes required for trehalose biosynthesis and regulation (Fig. 4: Cluster 1) were upregulated, as well as the two genes involved in trehalose to glucose catabolism (NTH1, ATH1: Cluster 1), indicating that *S. cerevisiae* may be storing excess glucose and recycling it as a means of competition. Trehalose is also known to be involved in a number of cellular stress responses.

Consistent with a response to starvation, several autophagy and autophagy-associated genes were differentially expressed. Autophagy is induced during nutrient starvation and is the process of the cell cannibalizing organelles and using the resultant by-products to maintain metabolic homeostasis (Gresham et al. 2011; Kaur and Debnath 2015). There were clear signs of activation of signalling cascades mediated by Ser/Thr protein phosphatases (Fig. 4: Cluster 5, 39), which are essential in nutrient sensing, and upregulation of Ras-like protein 2 (RAS2), which is involved in responding to nitrogen starvation (Fig. 4: Cluster 44). Macroautophagy genes were upregulated (Fig. 4: Cluster 8, 30, 40), as were associated intracellular vesicular trafficking and secretion genes, including endocytic genes (Fig. 4: Cluster 4), soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complex-associated genes (Fig. 4: Cluster 20), and ER-associated secretory genes (Fig. 4: Cluster 29). Impacts on transcription were also present, with differential expression of genes involved in transcription by RNA-polymerase II (Fig. 4: Cluster 37), upregulation of RNA helicases (Fig. 4: Cluster 14), and upregulation of transcription activators (Fig. 4: Cluster 42). The second largest network cluster, Cluster 2, illustrated a shift in protein turn-over and stress-related changes in translational programs. Genes involved in cytoplasmic translation were downregulated, indicating a cessation of cytoplasmic translation, while genes involved in proteolysis and protein ubiquitination were upregulated, which is consistent with autophagy-related protein catabolism and recycling. There was upregulation of genes

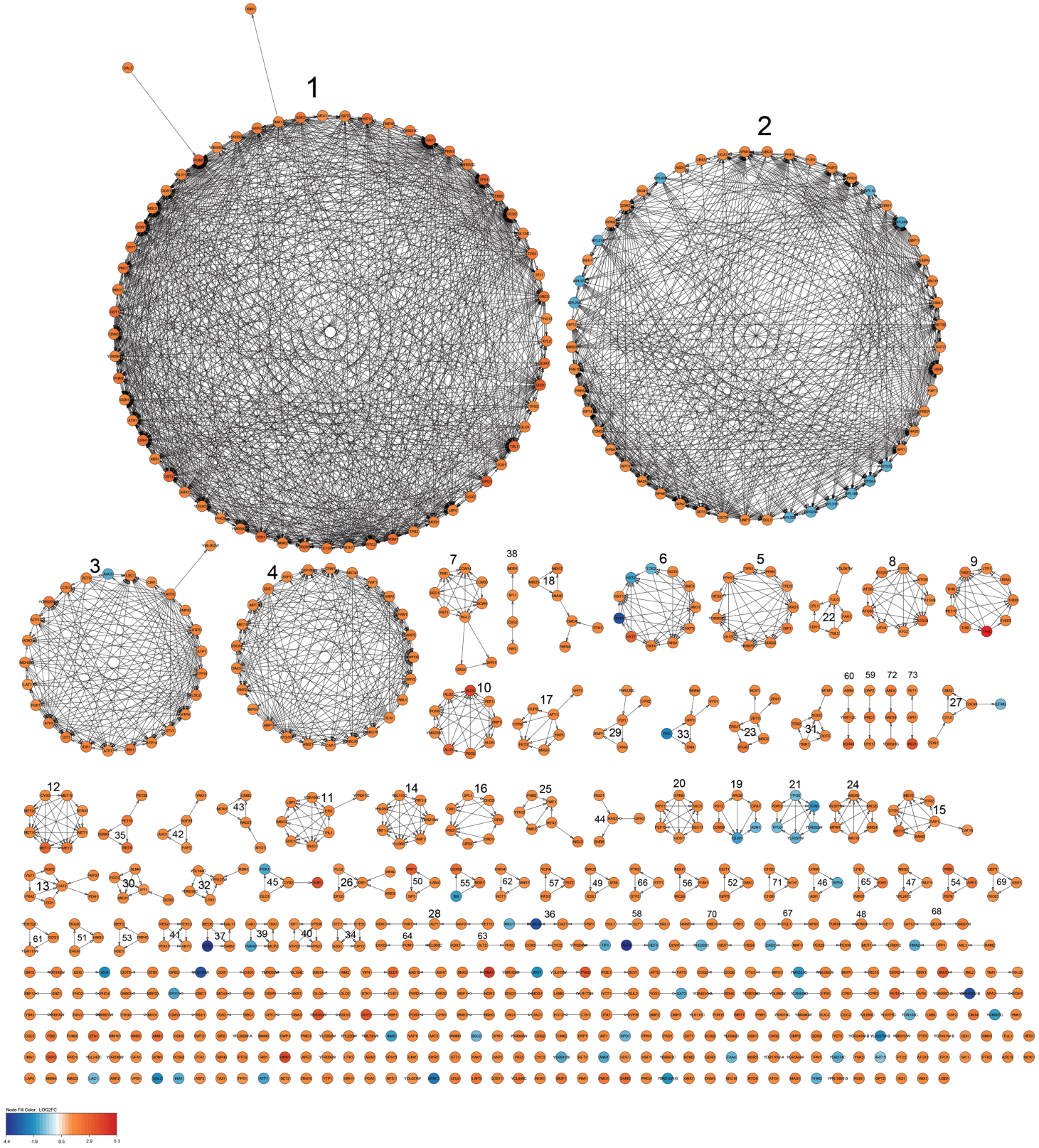


Figure 4. Differentially expressed genes in *S. cerevisiae* in response to co-culture with *T. delbrueckii*, excluding the genes reported in Figure 2. Nodes are coloured according to Log2 fold change values. Image and network created in Cytoscape. Figure is for illustrative purposes only, please see supplementary materials for high quality images and interactive network files.

involved in protein misfolding, including those involved in endoplasmic reticulum (ER)-associated protein degradation (Fig. 4: Cluster 27). Further, lipid droplet catabolism was upregulated, which is also a central autophagic mechanism (Fig. 4: Cluster 16, 22, 35, 38) (Kaur and Debnath 2015). By evaluating the interaction network for clusters enriched in biosynthesis and metabolism of essential nutrients, potential targets for what was limiting, i.e. what caused the starvation response and was therefore stimulating competition during co-culture, can be surmised. In terms of vitamins and trace elements, these include thiamine (Fig. 4: Cluster 9), zinc (Fig. 4: Cluster 23), as well as copper and iron (Fig. 4: Cluster 17). In terms of major carbon and nitrogen metabolism, there appeared to be competition for glucose, as reported above, and there appears to be remodelling in response to available nitrogen sources and a need for sulphur-containing amino acids. Specifically, a general amino acid permease was upregulated (AGP2: Cluster 13), signalling a lack of preferred nitrogen sources, and the uptake of sulphate and biosynthesis of sulphur-containing amino acids was upregulated, in particular, methionine (Fig. 4: Cluster 12, 15).

General oxidative (Fig. 4: Cluster 31) and osmotic (Fig. 4: Cluster 11) stress response genes were upregulated, as well as peroxisome biogenesis genes (Fig. 4: Cluster 41), which are involved in oxidative stress management (Zhao et al. 2015; Sibirny 2016). In addition, genes involved in DNA repair were also upregulated, indicating some DNA replication stress (Fig. 4: Cluster 43).

Finally, in agreement with the majority of previous co-culture analyses, there were significant alterations in the expression of cell envelope-associated genes (Curiel et al. 2017; Tronchoni et al. 2017; Tondini et al. 2020). Cell wall organization and biogenesis genes were upregulated (Cluster 7, 26, 33), and cell wall mannoproteins were also impacted (Fig. 4: Cluster 33). Components of eisosomes, which are distinct, dynamic plasma membrane subdomains which have been shown to play a role in responding to membrane stressors, were also upregulated (Fig. 4: Cluster 11) (Zahumensky and Malinsky 2019).

The over-all response showed similarities to the Environmental Stress Response (ESR) program, a generalized response to varied cellular stresses, which has previously been observed in other *S. cerevisiae* and *T. delbrueckii* co-culture studies (Gasch et al. 2002; Curiel et al. 2017; Tronchoni et al. 2017; Tondini et al. 2020), with remarkable parallels to starvation responses; autophagy in particular. This aligns well with the hypothesis that *S. cerevisiae* and *T. delbrueckii* have very similar nutritional requirements, which stimulates competition.

Growth in a consortium induces a combination of known pairwise responses as well as novel higher order responses in S. cerevisiae

To determine gene expression programs associated with higher-order interactions, we evaluated the DEGs of *S. cerevisiae* during growth within the three-species consortium. First, the extent to which the DEGs present during consortium growth could be predicted by the pairwise co-culture DEGs were

assessed by matching the genes in common between the pairwise and consortium conditions (Fig. 5). Overall, 43 % of the consortium DEGs were unique, i.e. only expressed during consortium growth and not in either pairwise condition, and 57 % were present during pairwise co-culture. Delving into this 57 % of pairwise origin, 100 % of the DEGs expressed during co-culture with *L. thermotolerans* were expressed during consortia growth, and 73 % of DEGs expressed during co-culture with *T. delbrueckii* were expressed during consortia growth. This shows that pairwise ecological interactions are largely retained during consortium growth. The over-all trend is that some prediction of interactions can be made from pairwise data, however there indeed appears to be evidence for higher-order, or unpredictable, expression responses in *S. cerevisiae*.

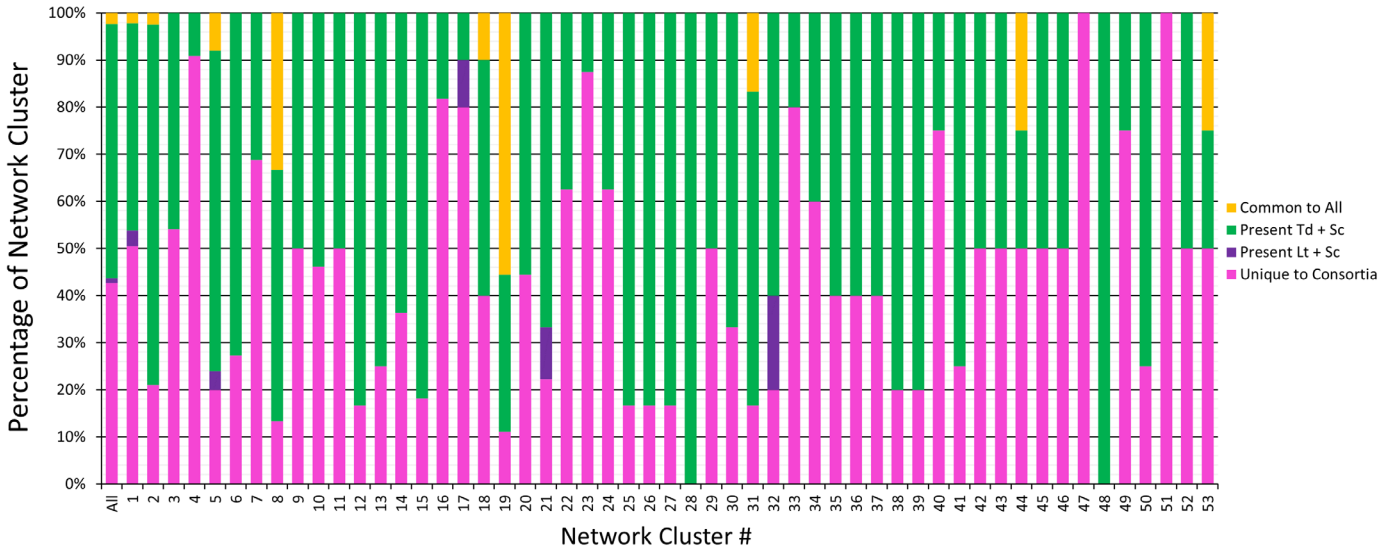


Figure 5. Bar graph showing the gene categories within the differentially expressed gene list of *S. cerevisiae* (Sc) in response to consortia growth with both *T. delbrueckii* (Td) and *L. thermotolerans* (Lt). Percentages were calculated for the entire gene list (All), as well as for clusters 1 – 53. Yellow represents the percentage of genes common between all co-culture conditions tested. Green represents the percentage of genes in common with the pairwise *T. delbrueckii* co-culture. Purple represents the percentage of genes in common with the pairwise *L. thermotolerans* co-culture. Pink represents the percentage of genes unique to consortia growth..

Next, it was necessary to assign the higher-order associated DEGs with their broader cellular functions. The total list of DEGs were used to create a functional network, and were labelled according to their commonality in the pairwise datasets (Fig. 6). This approach was followed to contextualize the higher-order DEGs within the broader functional network, and more easily identify functional clusters that are associated with the higher-order response. To this end, each functional cluster was also represented by the percentage of DEGs that were in common with pairwise co-culture or unique to the consortia setting (Fig. 5). It was found that many higher-order genes are functionally relevant to pairwise genes, illustrated by the distribution of higher-order and pairwise genes within their functional clusters (Fig. 5, 6). For instance, Cluster 9 consists of ergosterol biosynthesis genes, with half being present during the pairwise condition, and the other half being stimulated by consortia growth. Other examples include

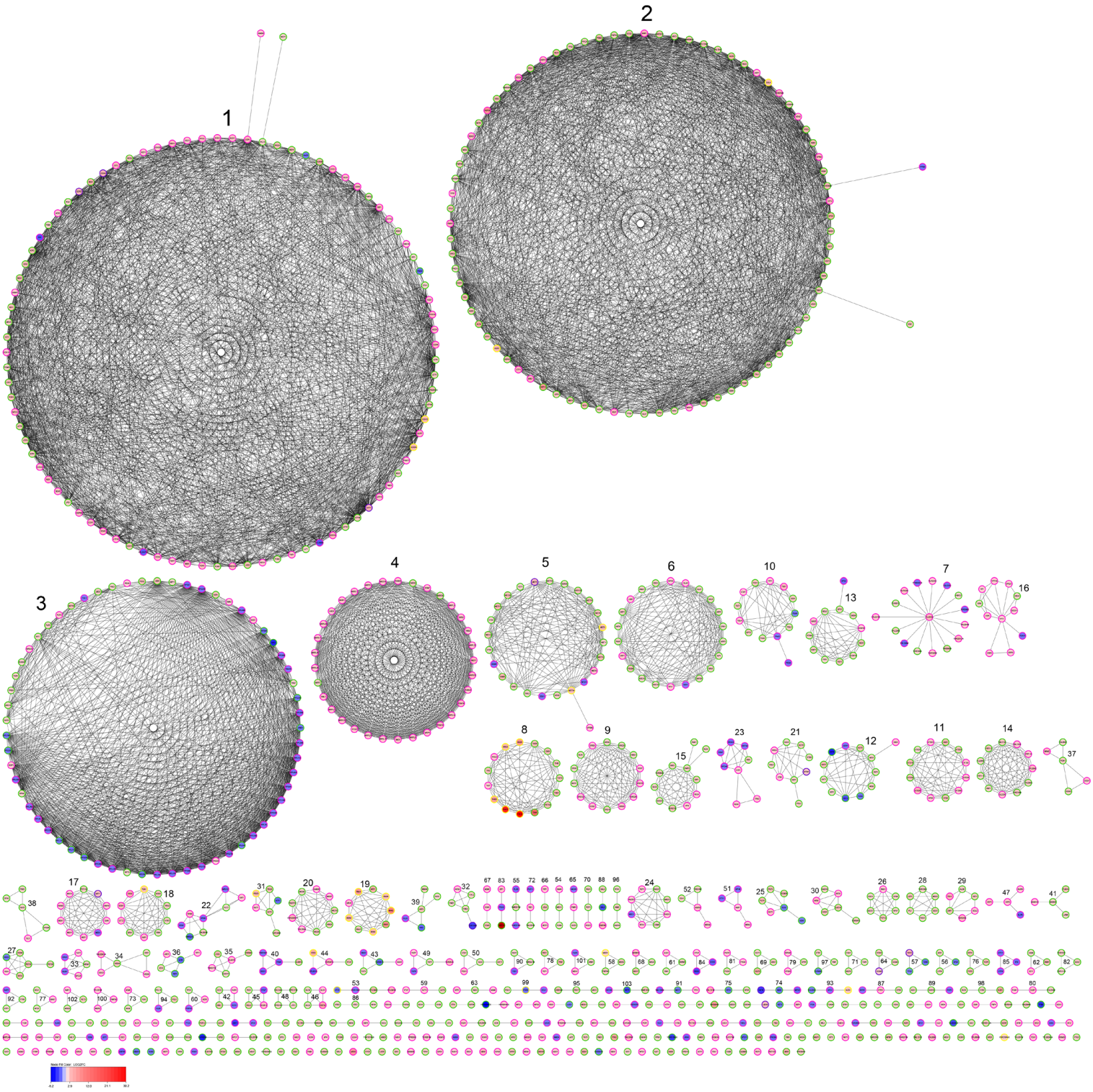


Figure 6. Differentially expressed genes in *S. cerevisiae* in response to consortia culture with *L. thermotolerans* and *T. delbrueckii*. Nodes are coloured according to Log2 fold change values. The borders of the nodes are coloured according to which gene category they belong to, as described in Figure 5. Borders are coloured as follows: Yellow: Common to all conditions, Purple: Common to co-culture with *L. thermotolerans*, Green: common to co-culture with *T. delbrueckii*, Pink: Unique to consortia. Image and network created in Cytoscape. Figure is for illustrative purposes only, please see supplementary materials for high quality images and interactive network files.

clusters that are involved with autophagy (Fig. 6: Cluster 11) and oxidative stress response (Fig. 6: Cluster 18). This suggests that the cellular responses elicited during pairwise growth may be intensified in consortium context.

Within this functional network there were also several clusters that consisted of majority higher-order interaction DEGs, which are notable as they may point to cellular responses that are indeed unique to higher order interactions. This led us to evaluate the DEGs unique to the consortium growth condition in isolation. The statistical stringency of the DEGs was increased to ensure that the considered DEGs were more likely to be involved in higher order interaction (Supplementary File 2) and a new functional network was created (Fig. 7). Within this network, we have focussed on those clusters with novel functional associations, that were not present within the pairwise functional associations.

The data suggests a metabolic shift to respiratory metabolism, with a major increase in energy generation strategies. The largest and most interconnected functional cluster, Cluster 1, showed downregulation in cytoplasmic ribosomal genes and upregulation in mitochondrial ribosomal and translation genes. In accordance with this increase in mitochondrial translation machinery, there was also an increase in amino-acyl tRNA ligases associated with mitochondrial translation (Fig. 7: Cluster 10). In addition, Cluster 2 showed upregulation of respiratory and ATP synthesis genes within the mitochondrion, and Cluster 13 included upregulation of two major glucose-repressed transcriptional activators (HAP4 and HAP5) involved in regulation of respiratory metabolism. Cluster 16 displayed upregulation in mitochondrial organization-related genes as well as stress response genes. The opposing responses in mitochondrial and cytoplasmic translation machinery seen here is an interesting finding, as it is known that these processes are generally regulated in concert (Couvillion et al. 2016), and shows a cellular priority for mitochondrial processes that generate energy. There are also signs of DNA replication stress and alterations to cell cycle checkpoints (Fig. 7: Cluster 3), indicating impacts on proliferation rate as well. Further, protein trafficking within the cell was also upregulated, indicating impacts on protein turn-over within the cell as well (Fig. 7: Cluster 4).

Interestingly, genes of the flocculin family, involved in cell adhesion and flocculation, were largely downregulated (Fig. 7: Cluster 12, 15). These genes have been highlighted in previous studies as a potential regulator of ecosystem dynamics, and are an intuitive target to consider seeing as direct contact between cells influence the nature of their interactions (Kemsawasd et al. 2015; Rossouw et al. 2015; Brou et al. 2018). This seemingly indicates that *S. cerevisiae* may be avoiding direct cell contact with the other species within the culture.

Evaluating highly differentially expressed genes not necessarily associated with large clusters, the aromatic aminotransaminase, ARO9, was the most highly upregulated gene, and is known to be induced by the presence of aromatic amino acids in growth media (Iraqui et al. 1998). This suggests an altered

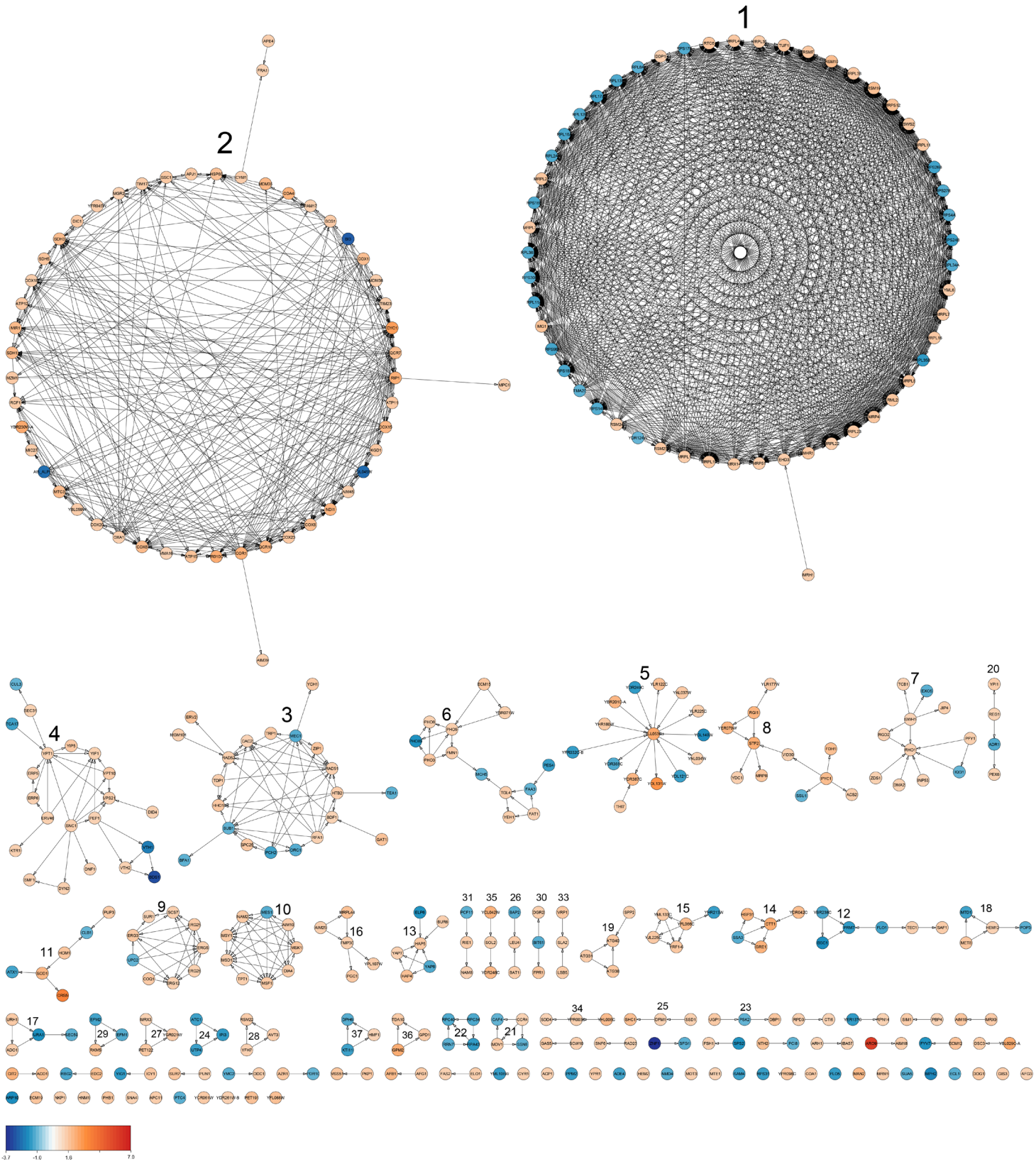


Figure 7. Differentially expressed genes in *S. cerevisiae* that are unique to consortia culture with *L. thermotolerans* and *T. delbrueckii*, filtered for statistically borderline DEGs (See Supplementary File 2). Nodes are coloured according to Log2 fold change values. Figure is for illustrative purposes only, please see supplementary materials for high quality images and interactive network files.

amino acid uptake preference during consortium growth. This phenomenon has been observed in a number of mixed yeast species cultures in the wine fermentation context (Rollero et al. 2018; Rollero et al. 2019; Roca-mesa et al. 2020), and has implications in terms of the generation of higher alcohols, which influence wine flavour and aroma (Cordente et al. 2012). The most downregulated genes included ZNF1, a glucose-repressed transcription factor, with regulatory roles in alternative carbon source utilization and respiration, as well as BDS1, a bacterially derived sulfatase responsible for utilization of sulphate esters, which suggests some impacts on sulphate import and metabolism.

Lastly, a cluster of interest related to the over-arching aim of hypothesis generation, is Cluster 5, which consists of primarily uncharacterized open reading frames. These would be good targets for annotation in a mixed culture context, as opposed to the historic gold standard of high throughput gene function characterization in monoculture.

Discussion

Higher-order interaction mechanisms within microbial ecosystems are of the most poorly understood and quantified phenomena in microbial ecology. This study sought to investigate these mechanisms within a simplified wine yeast consortium, given the biotechnological and fundamental importance of this ecological niche (Conacher et al. 2021). Here, we characterized the emergence of higher order interaction mechanisms of *S. cerevisiae* at the transcriptional level, at the simplest possible degree, in a three-way yeast species interaction model.

At the pairwise level, interesting differences in transcriptional responses of *S. cerevisiae* to either *L. thermotolerans* or *T. delbrueckii* were evident. There was a significantly more extensive response to *T. delbrueckii* than there was to *L. thermotolerans*, with the majority of this response to *T. delbrueckii* being well aligned to known starvation responses, which is in agreement with previous studies (Curiel et al. 2017; Tronchoni et al. 2017; Tondini et al. 2020). This appears to suggest that *T. delbrueckii* was sequestering nutrients with a similar preference to *S. cerevisiae.* When considering the phenotypic evidence, the cell numbers of *S. cerevisiae* are largely identical between the two pairings, which shows that the autophagic strategy to maintain cell growth was successful. This transcriptional response could be as a result of ecological interaction mechanisms, which stimulates *S. cerevisiae* to hoard nutrients, however, the precise mode of how this occurs would require further investigation.

These pairwise responses were well conserved within the consortium context, but there was also a good proportion of genes expressed only within the consortium, which we hypothesize to be involved in higher-order ecosystem mechanisms. In the limited available literature, similar studies in bacteria have also found that pairwise population dynamics and metabolic cross-feeding data are correlated to what occurs within more complex systems, but that there are indeed unpredictable non-linear interactions that distort these interactions as well (Guo and Boedicker 2016; D'hoe et al. 2018; Morin et al. 2018;

Venturelli et al. 2018; Ansari et al. 2019; Sanchez-Gorostiaga et al. 2019). However, this has not been investigated at the transcriptomic level as yet. Here, we highlight that there are unpredictable gene expression responses in *S. cerevisiae* within a yeast consortium. These responses were primarily associated with increasing mitochondrial translation and components of the electron transport chain needed for respiratory metabolism, as well as changes in cellular protein turn-over, similar to the diauxic shift. Alteration of *S. cerevisiae*'s metabolism to favour respiration is well studied, and occurs when extracellular glucose levels drop below a particular level, although the influence of other nutrients on this diauxic shift is less well understood (Galdieri et al. 2010; Olivares-Marin et al. 2018; Di Bartolomeo et al. 2020). However, the sugar concentrations show that glucose in the medium is no-where near exhausted and concentrations are highly similar between the conditions compared here. A more probable scenario, given the low nitrogen availability of the growth medium used here, is that nitrogen may have started to become limiting, which has previously been shown to stimulate respiratory metabolism in *S. cerevisiae* (Larsson et al. 1993). This again emphasises the importance of nitrogen source preference and competition in yeast ecosystem dynamics, and is in line with the highest upregulated gene being an aromatic aminotransaminase (Medina et al. 2012; Curiel et al. 2017; Rollero et al. 2018; Minebois et al. 2020). This switch to respiratory metabolism and adjusted protein turn-over (particularly of the mitochondria) is associated with longevity and ageing mechanisms in *S. cerevisiae* and may perhaps be a mechanism to outlast its competitors (Bonawitz et al. 2007). All of the above also adds to a growing body of evidence that biotic stress is an extremely relevant selection pressure in the context of adaptive evolution.

While the data presented here is the first look at potential gene expression programs that govern higher-order interactions and competitiveness of *S. cerevisiae*, these results must be contextualized within the following boundaries. Firstly, the cultures used here were batch cultures, which limits the exclusion of some growth phase differences between our monoculture and mixed culture settings, although these were very carefully considered for the time point used in this study. Secondly, it is a single snapshot in time, and repeating the study at a different timepoint would allow for conclusions on temporal changes in gene expression programs. Further, the dataset presented here is based only on mRNA, and ultimately requires confirmation of the role of these expressed transcripts through downstream study of these gene targets. Since the data suggests quite an extensive competitive response in terms of nutrients, the study would also benefit from metabolite and amino acid analyses to confirm these observations. Lastly, we have only evaluated the response of *S. cerevisiae*, and the inclusion of the responses of the other two involved species would certainly generate a more robust understanding of the ecological relationships at play here.

Understanding yeast-yeast ecological interactions is a major research challenge, and the importance of characterizing and quantifying higher-order interactions in multispecies systems is clear. We know that

S. cerevisiae strains of wine origin are incredibly competitive, dominate natural fermentations, and act as keystone drivers for the ecological dynamics of these systems. For the first time, we have shown the potential mechanisms behind how *S. cerevisiae* interacts within a multispecies yeast ecosystem at the transcriptional level. The functional networks generated by this study are the first of its kind within the yeast-yeast interaction research field, and provide the most comprehensive functional overview of the complex mRNA transcriptome involved within these interactions. The dataset provided here is also a prime opportunity for quantitative modelling of the emergence of ecosystem properties within yeast ecosystems and contributes to a growing -omics database on yeast ecological interactions. While the data certainly requires further confirmatory studies, it has provided an invaluable resource for hypothesis generation from which to understand wine yeast ecosystem dynamics.

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Author Contributions

FB conceptualized the study. CC designed and executed all experimental work and tertiary bioinformatic analyses. CC, DR, RK, and FB interpreted the data. CC wrote the manuscript, RK and FB edited the manuscript. All authors approved the final manuscript.

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Supplementary Materials for Chapter 5

S1. Growth medium design

A range of conditions were first tested in *S. cerevisiae*, after which selected conditions were tested in *L. thermotolerans* and *T. delbrueckii* (Table 1). Similar to Synthetic Grape Must (SGM), the carbon source was comprised of equal concentrations of glucose and fructose. The impact of carbon source concentration, YNB concentration, the addition of amino acids, and buffering at pH 6 were evaluated in *S. cerevisiae*. A low [2 % (w/v)], medium [10 % (w/v)], and high [20 % (w/v)] carbon source concentration was tested. The concentration of YNB was increased 2-fold in the 2 % carbon medium to adjust carbon:nitrogen ratio. Next, in the high carbon source medium, buffering at pH 6, with 100 mM Potassium Phosphate buffer was evaluated. The addition of complete amino acid stock, based on SGM, was tested. Finally, the effect of combining buffering and additional amino acids was determined.

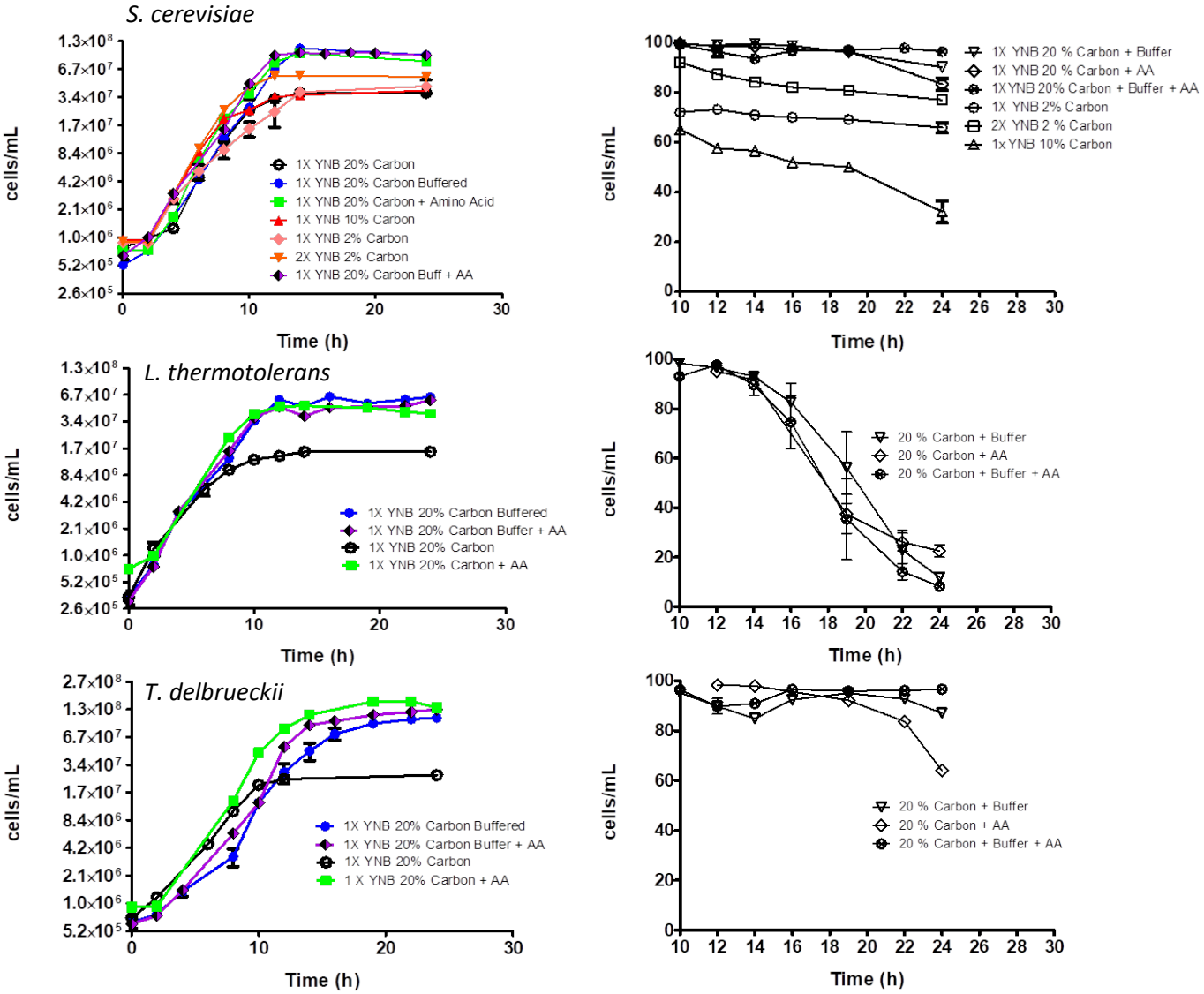
Table S1.1 Summary of conditions tested for Yeast Nitrogen Base growth medium design

Condition	Carbon Source	YNB Concentration	Additional Components	Species Tested
Carbon Source Concentration	Low: 2 % (w/v)	1X: 6.7 g/L	None	*S. cerevisiae*
	Medium: 10 % (w/v)	1X: 6.7 g/L	None	*S. cerevisiae*
	High: 20 % (w/v)	1X: 6.7 g/L	None	*S. cerevisiae*, *L. thermotolerans*, *T. delbrueckii*
YNB Concentration	Low: 2 % (w/v)	1X: 6.7g/L	None	*S. cerevisiae*
		2X: 13.4 g/L	None	*S. cerevisiae*
Buffering	High: 20 % (w/v)	1X: 6.7g/L	100mM Potassium phosphate buffer	*S. cerevisiae*, *L. thermotolerans*, *T. delbrueckii*
Additional amino acids**	High: 20 % (w/v)	1X: 6.7g/L	Amino acid stock solution	*S. cerevisiae*, *L. thermotolerans*, *T. delbrueckii*
Buffering + Additional amino acids	High: 20 % (w/v)	1X: 6.7g/L	100mM Potassium phosphate buffer + Amino acid stock solution	*S. cerevisiae*, *L. thermotolerans*, *T. delbrueckii*

**: Optimized YNB used in cultures

S1.2 Amino acid stock solution composition

Stock of amino acids (150X Stock)	
Prepare in 1l of NaHCO3 2% (20g/l) and add amino acids slowly in following order	
	[g/l]
tyrosine	1.40
tryptophane	13.70
isoleucine	2.50
aspartic acid	3.40
glutamic acid	9.20
arginine	28.60
leucine	3.70
threonine	5.80
glycine	1.40
glutamine	38.60
alanine	11.10
valine	3.40
methionine	2.40
phenylalanine	2.90
serine	6.00
histidine	2.50
lysine	1.30
cysteine	1.00
proline	46.80



S1.3 Growth curves and viability decline comparisons

Initial growth experiments in high sugar YNB (with amino acids, 0.5 % ammonium sulphate, 20 % carbon source) resulted in low biomass yields across all three tested species, and mixed species cultures showed similar attenuated growth. To attempt to improve the biomass yields and assess viability, a number of settings were first screened in *S. cerevisiae*. Initially, lowering the sugar concentration to 2 % YNB resulted in similar low biomass yields and loss in viability from the 10 hour point in *S. cerevisiae*. Increasing the carbon concentration to 10 % did not improve biomass and caused rapid decline in viability in *S. cerevisiae*. It was hypothesized that the carbon:nitrogen ratio was not balanced, and rapid decline in pH may be causing the suboptimal growth. In the low sugar YNB, the concentration of YNB was doubled, and this resulted in better biomass yields and improved viability. This indicated that a limiting nutrient in the YNB components may be attenuating growth. Applying this to improve the high sugar medium, additional amino acids in the form of a complete amino acid stock, usually used in SGM growth medium, were added. This improved biomass yield and allowed *S. cerevisiae* cells to remain

viable until at least 20 hours of growth. Secondly, the role of pH was investigated by buffering the high sugar YNB medium to pH 6. This showed similar improvements in biomass yields and viability. The addition of both buffer and amino acids to high sugar YNB did not improve the biomass yields and viability over addition of only buffer or amino acids. The three best performing high sugar YNB variants, namely, addition of amino acids, or buffer, or both amino acids and buffer were further evaluated in *L. thermotolerans* and *T. delbrueckii*. There were similar improvements in biomass yields and all tested variations showed similar viability. It was however observed that *T. delbrueckii* flocculated severely when grown in the presence of the buffer. This has negative implications for the use of flow cytometry quantitation. Therefore, since the addition of amino acids alone appeared to create an appropriate growth medium for all three species, it was decided to move forward with this growth medium.

S1.4 Glucose and fructose concentrations of supernatant for samples used for RNASeq

Culture	Average Fructose (g/L)	Standard Error Fructose	Average Glucose (g/L)	Standard Error Glucose	Average Total Sugars (g/L)
S. cerevisiae	95.69	0.65	96.28	1.85	191.98
S. cerevisiae* + *L. thermotolerans	94.55	1.11	97.38	0.71	191.93
S. cerevisiae* + *T. delbrueckii	94.54	1.52	97.73	0.49	192.27
S. cerevisiae* + *L. thermotolerans*+ *T. delbrueckii	95.00	0.71	98.86	1.08	193.86

S1.5 Links to supplementary files

Supplementary File 1 and 2: https://figshare.com/s/1ba4fa975bf9af47137a

High quality figures: https://figshare.com/s/5e198e278b67768566f8

Cytoscape network files: https://figshare.com/s/4ba873d75b96583514d5

Chapter 6

General Discussion and Conclusions

General Discussion and Conclusion

Two is company, three is a crowd – a social idiom that is built on rather solid mathematical logic. Condensed into its simplest idea, this phrase was the basis of this project. The wine yeast ecology field has made good inroads at two very different sides of the conceived realm of aims: on the far left, the molecular mechanisms of the simplest (pair-wise) yeast interactions have started to reveal themselves with the aid of transcriptomic, proteomic, and metabolomic datasets; on the far right, great strides have been made in surveying the diversity of the wine microbiome and the roles of several biogeographical factors in maintaining or altering this diversity (Reviewed in: Conacher et al. 2020; Conacher et al. 2021). But, what of the middle? Herein lies a largely unexplored research wilderness where the challenge is to integrate the mechanistic detail of the aforementioned 'far left' with the more naturally representative system complexity of the 'far right'. While a treacherous path to walk, it indeed holds the key to predictive understanding of yeast ecosystem establishment and functioning (Widder et al. 2016). This project aimed to explore this wilderness of wine yeast ecosystems by creating the right tools to do so and using them to understand the inner workings of small crowds in the world of yeast.

The first step in this expedition was a critical evaluation of the tools available to describe temporal changes in yeast ecosystem dynamics. Upon doing so, it was clear that this was a major factor behind why progress had been limited. Techniques for monitoring population dynamics of synthetic or natural yeast ecosystems largely relied on either spread-plating or DNA-based fingerprinting methods such as automated ribosomal intergenic spacer analysis (Reviewed in: Conacher et al. 2021). All of which are time consuming, have low resolution for inter-strain (sometimes species) identification, are limited in terms of downscaling cultures, and the data is only known long after the experiment is complete. The good news was that concepts of synthetic ecology, in particular the design and use of simplified consortia, representative of more complex natural ecosystems, had been applied with success (Ciani et al. 2006; Ciani et al. 2010; Ciani and Comitini 2015; Bagheri et al. 2017; Bagheri et al. 2018; Bagheri et al. 2020). The goal was therefore to design a synthetic consortium but develop a more high-throughput system with better accuracy and real-time data feedback for population dynamics monitoring. Since we were keen on identifying emergent ecosystem properties within consortia, the size of the consortium was initially set to three, and three strain representatives of highly prominent yeast species within the wine industry were selected: *Saccharomyces cerevisiae*, *Lachancea thermotolerans*, and *Torulaspora delbrueckii* (Jolly et al. 2014; Hranilovic et al. 2018; Ramírez and Velázquez 2018). Next, drawing from other real-time reporter systems, we developed and validated a method where fluorescent tags were expressed as markers for each yeast species, which could then be analysed and quantified at a rapid rate by flow cytometry and fluorescence microscopy (Conacher et al. 2020). We could also successfully physically sort these cells based on their fluorescence, which opened a whole new way to test ecological hypotheses. With this base fluorescent consortium, we were able to add any extra non-tagged population, which allowed us to accurately and rapidly track temporal dynamics of a four-species wine

yeast consortium. With the correct tool in hand, the rate at which we could observe and quantify the dynamics of this synthetic consortium was exponentially increased, and the adoption of this within the broader field of yeast ecology should significantly accelerate our understanding of these systems. Additionally, the simplicity of the protocol and very small sample requirements allows for much higher-throughput parallelization of experiments, and the quality and 'density' of the data produced is such that it is ideal for quantitative modelling and computational evaluations (Rubbens and Props 2021). But, it is not perfect. The initial concept could do with some refinement – it is based on fluorescent labels: a finite number of these exist, and even less that can accurately be distinguished from one other. Worse still, fluorescent proteins need molecular oxygen to mature – not ideal for fermentative environments. Finally, it is also only suitable for following known species, which limits its application. Despite this, we have found this tool to be a significant helper in the quest to understand yeast ecosystems. The next challenge would be to evaluate the pros and cons of this system and develop a more elegant solution for fermentative environments with the advantage of hindsight.

The next stage of the study focussed on refining the technical challenges of working with multispecies systems. Working with microbial consortia has a reputation akin to herding cats – difficult, unpredictable, and hard to reproduce consistently (Jawed et al. 2019). We initially found this to indeed be true, especially armed with a monitoring system that very accurately showed (down to the last yeast cell) just how highly varied our quantitative species dynamics were week-to-week. Fortunately, this double-edged sword also allowed us to rapidly pin-point the source of these errors – the physiological state of our pre-cultured yeast cells before they were inoculated into our consortia cultures – and resolve the issue. We saw that how cells are 'primed' before they are inserted into a mixed species culture affects how they assimilate to their new, more competitive environment. Specifically, having pre-cultures that are growing exponentially and have been exposed to the growth medium in which the consortium is set to be cultured in, decreases their adaptation time and levels the playing field between the reigning champion *S. cerevisiae* and the non-*Saccharomyces* contenders. Further, it was clear that making predictions on what the best pre-culture mode would be based on monoculture was not enough and that this should be optimized in a mixed culture context. We also saw an interesting dynamic come to the fore: the more complex the system, the greater the impacts between different pre-culture conditions. The premise of the work conducted here was simple, but it has surprisingly been neglected within the broader microbial ecology field, perhaps since the focus is more commonly based on refining the culture conditions of the consortium, and not what occurs prior to culture. These findings, while largely correlative and superficial in that they only evaluated cell count data at a few time points, are still very important for the field of yeast ecology since the current gold standard of data collection in multispecies systems are indeed population dynamics. This chapter both served as a call to improving reproducibility in multispecies culture research, and also provided a technical foundation from which to continue our work in understanding our yeast consortium.

Having mastered the technical aspects of our study, the focus then shifted from population dynamics to digging deeper into the molecular mechanisms that governed the dynamics in our consortium. In particular, we were interested to know what occurs within yeast when the system moves from a binary to a non-binary conformation. In other words, we set out to characterize the emergence of higher-order interactions in *S. cerevisiae* at the transcriptional level. Higher-order interactions are indeed one of the major blind spots in the quest to predictively understand multispecies systems (Sanchez 2019; Battiston et al. 2020). They are, by definition, the unpredictable, non-linear dynamics observed in systems comprised of more than two entities. This firstly makes them very difficult to quantitatively characterize and secondly is a thorn in the side of any microbial biotechnologist who attempts to design a multispecies system for a particular function (Ben Said and Or 2017; Haruta and Yamamoto 2018). By evaluating the transcriptomic response of *S. cerevisiae* to a stepwise increase in co-culture complexity, i.e. from a two species system to a three species system, we were able to confirm the presence of a unique response in the more complex setting that could not be explained by what occurred at the pairwise level. In addition, the pairwise data was also well represented in the more complex system, making up just over half of the total response. We were also simultaneously able to evaluate differences in the transcriptomic response of *S. cerevisiae* to either *T. delbrueckii* or *L. thermotolerans* at the pairwise level, and the data showed good alignment with previous similar studies, adding credibility to our overall dataset. The putative higher-order response included ramping up cellular constituents required for energy generation from respiration and simultaneous recalibration of cellular protein turn-over – responses that will sound very familiar to any yeast stress biologist (Gasch and Werner-Washburne 2002). Within the field of wine yeast ecology, this is the first dataset of its kind that has broken ground on demystifying unpredictable ecosystem dynamics at the molecular level. This data emphasises the important contribution of non-linear interactions to yeast ecosystem dynamics, and shows that we must have a better grasp of these effects if yeast-ecosystem based bioprocesses are to be a reality. What the data does not show, however, is the biological instigator of these non-linear responses – are they an artifact of purely nutritional limitations in the growth environment or are they driven by genuine yeast-yeast ecological interactions? Indeed, this question is highly relevant to the concept of biotic selection pressures in microbial ecosystem evolution. Not surprisingly, we have very little research that has focussed on teasing apart this question, and the answer will lie in integrating information from the transcriptome, proteome, and metabolome, and conducting causative, targeted experiments, as opposed to the largely correlative approach we are currently taking (Altermann and Hickey 2020; Prosser 2020).

Over-all, this study has made some progress in further developing our understanding of multispecies yeast systems, but there are a number of shortcomings that should also be highlighted. The data presented here is very focused at the cellular level, and suffers from little investigation into the extracellular environment of these systems. We know that extracellular metabolites, signalling

molecules, and extracellular vesicles are important factors in yeast ecological interactions, and it is strongly recommended to complement the findings presented here with these data in future studies (Renault et al. 2013; Mencher et al. 2020; Roullier-Gall et al. 2020). Furthermore, our transcriptomic data was not confirmed at the translational level with proteomic analyses, nor were there any confirmatory studies done to confirm the role of highlighted gene targets. Proteomic studies are, however, extremely challenging in mixed closely-related yeast cultures, due to very high homology between protein sequences (Peng et al. 2019). The good news is that I have made significant progress in developing a tool to address this challenge, which will expedite this recommendation (See: Appendix I). In addition, I conducted whole genome sequencing on the strain representatives of non-model yeast within the consortium, *T. delbrueckii* (CRBO: LO544) and *L. thermolerans* (IWBT: Y1240) and have good quality assembled genomes at the ready to conduct targeted molecular investigations into the role of particular genes in the dynamics of the consortium (See: Appendix II). Finally, the consortium evaluated in this study was the smallest possible size, and our own findings show unpredictable effects at even this level, which indeed emphasises how important it is to increase the consortium size to something more realistic and representative of what occurs in nature. I indeed attempted to increase the consortium size to four, which was convenient with the system developed here, and saw fascinating population dynamics trends that corroborate the need to 'go bigger' to gain better depth of understanding (See: Appendix III). However, this must be balanced with an improved strategy for computation of the generated data to avoid losing depth of insight as the consortium size grows.

Expanding on more specific recommendations, a clear first target for confirmatory studies would be evaluating and comparing the intracellular proteomes of the yeast within this consortium, by following the protocol developed in Appendix I. Secondly, a number of specific gene targets in *S. cerevisiae* were highlighted here, an example being ARO9, which could be genetically knocked-out and/or over-expressed in *S. cerevisiae* to evaluate its role in *S. cerevisiae*'s performance during consortia growth. Finally, the systems and datasets generated during this study are highly suitable for more complex computational analyses and quantitative modelling approaches, such as machine learning, the application of which is sorely lacking and very much needed in the wine yeast ecology field.

In summation, the current project set out to bridge the gap between mechanistic understanding and system complexity in the realm of wine yeast ecology. We developed a novel tool to improve and expedite the way in which yeast ecosystem dynamics and resilience can be evaluated. We have provided new insights into the possible role of pre-culture physiology into the infamous inconsistency in multispecies cultures. Finally, we have shed light on the possible mechanisms by which yeast tackle growth in biotically complex environments, and have provided a comprehensive dataset as well as the relevant tools to conduct causative investigations into the ecological interactions mechanisms at play. The findings of this study are envisioned to significantly impact the way in which yeast ecosystems are

studied and have provided fundamental insights into the role of biotic stress in the natural life cycle of wine yeasts.

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Appendix I

Getting sorted: Method development for RNAseq sample preparation of yeast cells sorted from mixed species cultures

Getting sorted: Method development for RNAseq sample preparation of yeast cells sorted from mixed species cultures

Background

Currently, the best available tools to investigate the molecular mechanisms that govern yeast ecosystem establishment and functioning are so-called '-omics' techniques. These techniques evaluate the transcriptome, proteome, and metabolome of a particular yeast strain or species representative in response to other yeast strains or species representatives (Conacher et al. 2019). However, -omics techniques have been designed and optimized in the context of monoculture investigations, and applying these techniques to mixed species cultures is therefore a considerably more complex challenge. For instance, when extracting total nucleic acids or proteins from mixed species cultures, even if the cell counts are similar within the sample, there may be uneven extraction, due to differing cell wall properties, amounts of nucleic acids/proteins, or levels of respective degradation enzymes, which ultimately results in very low coverage of a particular species within the sample (example: Shekhawat et al. 2019). Furthermore, there are a vast number of experimental situations where there is an uneven population distribution, and we are forced to largely avoid these conditions currently because they would result in a biased sample.

Besides the sample preparation, there are also a number of challenges associated with the data analysis downstream of sequencing. Bioinformatic tools (including commercial software or freeware) are not optimized for evaluating mixed species samples. Pipelines for mixed species transcriptomic or proteomic investigation vary widely and there are several issues that have not yet been addressed. These include methods of normalization to account for differences in the quantity of nucleic acid/RNA per species within the sample, the extent of overlap between homologous genes/proteins across closely related reference genomes, which makes inter-strain evaluations near impossible, and of course the lack of well annotated reference genomes for non-model microorganisms, which limits our interpretation to comparisons to a known model organism. It will certainly become necessary to address these matters as microbial ecology progresses, but we thought there may be a shortcut we can take in the meantime. The premise of the methodology developed here was to create a monoculture sample from a mixed species culture, by separating out the cells of a target species within a synthetic consortium and extracting high quality RNA from these cells, which would theoretically circumvent all the issues we have described above.

Yeast cell sorting and subsequent transcriptomic (Pérez-Torrado et al. 2017) or proteomic (Peng et al. 2019) investigation has previously been done in a two-strain *S. cerevisiae* co-culture, and a *S. cerevisiae*

and *L. thermotolerans* co-culture. However, these studies only evaluated *S. cerevisiae*, and neither validated the impact of the sorting procedure on the final transcriptome/proteome. We have reported on the sorting efficiencies of a mixed culture, however this sample had been taken after 24 hours, corresponding to the stationary phase, and the resultant cells were allowed to recover before we assessed sorting purity (Conacher et al. 2020). For use in an -omics context, we needed to adjust this protocol to firstly be applicable at an earlier growth phase, secondly, the cells would need to be preserved at the sample point before sorting, and finally, cells would have to be ready for extraction immediately after sorting, i.e. there would be no recovery period. To conduct mRNA sequencing, the Next-Gen Sequencing Unit at the Central Analytical Facility of Stellenbosch University requires 300 ng of total RNA to prepare a sequencing library using mRNA enrichment (as opposed to ribodepletion), therefore we set out to meet this RNA yield target. Recovering intact cells (needed for acceptable total RNA extraction yields), especially for the non-*Saccharomyces* species, was a significant challenge. Here, we have summarized the optimized protocol, and report the negative results that lead us to this protocol, which will be an extremely useful resource to expedite future research.

The optimized protocol

The following protocol was developed through many rounds of optimization of the growth conditions, sample preparation, and fluorescence activated sorting (FACS) process (See table: Negative results). Ultimately, the cell recovery values were drastically improved (minimum of 60 % for all species), which allowed for enough total RNA to be isolated (minimum 300 ng total RNA), and this RNA was found to be of good quality for RNA sequencing (RIN > 6.5).

Yeast strains, growth medium, pre-culture conditions, culture conditions, monitoring of population dynamics

A synthetic three-species yeast consortium consisting of *Saccharomyces cerevisiae* VIN13 (Anchor Yeast, Cape Town, South Africa) labelled with TagRFP657, *Lachancea thermotolerans* IWBT Y1240 [CBS: 16374] labelled with mTagBFP2, and *Torulaspora delbrueckii* LO544 [CRBO: LO544] labelled with eGFP was cultured in adjusted YNB growth medium and monitored identical to the process described in Chapter 3, Sections 2.1 – 2.5.

Consortium culture sample preparation

After 8 hours of co-culture, 2 ml samples were removed, centrifuged at 5000 X g for 3 minutes, resuspended in 500 µl cold RNALater (Thermofisher Scientific, South Africa), and stored at 4 °C for 1 hour. This was followed by adding a 1:1 volume of ice-cold DEPC-treated Phosphate Buffered Saline (PBS, pH 7.2) to the sample to reduce sample viscosity and samples were then centrifuged at 5000 X g for 3 minutes and the supernatant discarded. The cells were then resuspended in 2 ml ice-cold DEPC-treated PBS and kept on ice before the FACS procedure.

Fluorescence activated sorting (FACS)

Similar to chapter 2, the BD FACSMelody (BD Biosciences, La Jolla, CA, USA), equipped with blue (488 nm), red (640 nm) and violet (405 nm) lasers, measured GFP fluorescence on the FITC channel (527/32; 507 LP), BFP fluorescence on the BV421 channel (448/45) and RFP fluorescence on the APC channel (660/10). The machine was decontaminated prior to sorting by following manufacturer's instructions for preparing for an aseptic sort, where the fluidics are cleaned with 10 % bleach solution and 70 % ethanol solution. The sheath fluid container was autoclaved prior to use. All the surfaces around the machine were cleaned with RNAse away (Thermofisher Scientific, South Africa).

During the sort itself, the stringency setting selected was 'Purity', and the slowest possible flow rate was selected (1). The cells were sorted into 6-well tissue culture plates that were first rinsed with DEPC treated PBS to wet the entire surface of the well, and were then filled with 1 ml of DEPC treated 2X PBS as recovery fluid. The sorter was instructed to deposit the maximum possible number of cells into each well. The number of wells required for each sample was calculated by multiplying the total number of cells needed by a factor of 1.67 to account for the 40 % loss of intact cells. The total number of cells needed was estimated from the requirements of the extraction kit used here, which was at least 5 million cells.

After the sorting procedure was completed, the liquid containing the cells was aseptically transferred to 50 ml falcon tubes and kept on ice. This procedure was repeated until the total number of required 6-well plates had been completed. The filled falcon tubes were then centrifuged at 5000 RPM for 10 minutes, the supernatant very gently discarded, and the remaining cells were either resuspended in PBS for counting during optimization of cell recovery, or resuspended in cell lysis buffer before RNA extraction.

RNA extraction, quantitation, and quality control

RNA extraction was performed using the Qiagen AllPrep DNA, RNA, Protein kit. The total RNA samples were assessed for RNA integrity (RIN) and quantity on the BioAnalyzer 2100 (Agilent Technologies, Waldbronn, Germany) using the RNA 6000 Nano Chip and reagents.

Cell recovery calculations

Harvested sorted cells were counted using a haemocytometer and the recovery was calculated as a percentage of the actual cell concentration divided by the theoretical cell concentration – which is the expected number of cells that should be sorted according to the BD FACSMelody software.

Negative results

Stage of Protocol	***Troubleshooting Lessons***
Growth medium	Cell recovery in cultures grown in SGM was very low during the exponential phase. Using YNB solved this issue.
Pre-culture conditions	If pre-cultures are left to grow until stationary phase, the recovery of cells within your consortium culture will be better (regardless of the growth phase that your consortium culture is in when the sample is extracted). However, choosing to use pre-cultures in stationary phase will impact the dynamics of your consortium (See Research Chapter 2).
Culture conditions	Monocultures have much better recovery than mixed cultures. The growth phase of the species within the mixed culture impacts the recovery – exponential phase is lower and stationary phase is higher. Recovery rates are species specific, whether in monoculture or mixed species culture. Here, *S. cerevisiae* consistently showed good recovery levels, with *T. delbrueckii* having the second highest recovery yields and *L. thermotolerans* consistently being the worst.
Sample preparation	Live samples (i.e. cells that were not stored in RNALater) were tested and had good cell recovery rates but negligible RNA yields. The length of time that samples are left in RNALater impacts their recovery rate – less is better. RNALater is viscous and directly using the cells stored in RNALater (instead of resuspending them in PBS) vastly increased the sorting time and decreased yield and efficiency.
Fluorescence activated cell sorting	Flow rate was extremely important in keeping the sorted cells intact, and the slower the flow rate during the sort, the better. The volume of recovery fluid in the 6-well plate also significantly impacts recovery yields and the more recovery fluid, the better. However, this must be balanced with the total number of cells that can then be sorted into the well – the more recovery fluid, the less cells can be sorted into a particular well. Do not go over the recommended volume, this results in liquid splashing within the sorting chamber and cross contamination between wells.
Harvesting nucleic acids	Instead of using centrifugation to harvest cells, it was attempted to use standard ethanol and sodium acetate precipitation of the entire cell sorting fluid and use this precipitate in the RNA extraction kit for 'clean-up'. This strategy was unsuccessful and is not recommended.

Recommendations and future outlook

The next step would be to validate this protocol for application in transcriptomic or proteomic studies. This would involve comparing the transcriptome/proteome of a particular species before and after sorting to determine if the sorting process caused any significant changes in the sequencing data. If the protocol passes this validation, the generated datasets would be useful for firstly comparing to known

datasets of mixed species cultures and determining what differences arise when complex normalization is eliminated and simplified bioinformatic pipelines can be used. In particular, we would finally be able to determine the role of highly homologous gene expression in yeast-yeast interactions. Secondly, it would retrospectively lend credibility to the methodology applied by Perez-Torrado et al. and Peng et al. Finally, it would allow new insights into inter-strain interaction evaluations other than *S. cerevisiae* and in particular at the proteomic level.

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Appendix II

Draft genome sequences of *L. thermotolerans* IWBT Y1240 and *T. delbrueckii* LO544

Draft genome sequences of *L. thermotolerans* IWBT Y1240 and *T. delbrueckii* LO544

Background

Whole genome sequencing was conducted on two strain representatives used in all of the chapters mentioned here, namely *Lachancea thermotolerans* IWBT Y1240 [CBS: 16374] and *Torulaspora delbrueckii* LO544 [CRBO: LO544]. These strains are of wine origin, and were isolated from fermenting grape must.

Methods

Genomic DNA Extraction and Sequencing

Single colonies of the two strain representatives were grown in YPD for 16 hours on a test tube rotator. Genomic DNA (gDNA) was extracted using a manual phenyl:chloroform:isoamyl alcohol extraction method, which included RNAse treatment. As a final check, the ITS region was amplified from each gDNA sample and sent for Sanger sequencing at the Central Analytical Facility of Stellenbosch University, where the species identification of the yeast was double-checked. The gDNA was sequenced using the Illumina platform by the Centre for Proteomic and Genomic Research (CPGR) at the University of Cape Town. The quantity and purity of the gDNA was evaluated with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific) as well as a Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Q32853). The gDNA was used for Illumina library preparation using the Nextera DNA Flex Library Prep kit (Illumina, 20018704) according to the manufacturer's instructions. The pooled sequencing library was denatured and diluted as recommended by the Nextera DNA Flex Library Prep Reference Guide, and combined with the denatured PhiX positive control at a spike-in concentration of 1 % v/v. The denatured library was loaded on the Illumina MiSeq instrument and sequenced using the MiSeq Reagent Nano Kit v3 (600 cycles) (Illumina, MS-102-3003).

Genome Assembly

The raw reads were first run through FASTQC (0.11.5) to evaluate their quality, and then run through trimGalore! (0.4.2) (options: paired -q 20 --nextera --fastqc --length 250 --clip_R1 15 --clip_R2 15 --three_prime_clip_R1 5 --three_prime_clip_R2 5) to trim the reads and remove low quality reads. The genomes were then assembled *de novo* by inputting the trimmed reads into SPAdes (3.14.0) (options: --careful). The genome assemblies were then inputted into QUAST (5.0) (options: --fungus) to evaluate their quality.

Results

The *L. thermotolerans* IWBT Y1240 genome was assembled into a 10.41 Mb haploid size genome, comprising of 150 contigs with N50 value of 265055. The *T. delbrueckii* LO544 genome was assembled into a 9.20 Mb haploid size genome, comprising of 97 contigs with N50 value of 510474. Both assemblies show good coverage and according to these statistics, are good quality assemblies with which to move forward.

Conclusion and recommendations

Here, we have provided the first whole genome sequences for respective strains of wine-associated *L. thermotolerans* and *T. delbrueckii*. This data will form the basis for developing a more comprehensive reference genome for both of these strains. The next steps should be to focus on determining the gaps within the genome assemblies with the aim of generating more sequencing data to fill these gaps. The genome assemblies will then be ready to undergo annotation – however, the requirement for annotation is context dependent, and many informative bioinformatic analyses and comparisons can be made without undergoing the mammoth task of genome annotation. It is envisioned that this data will provide a more accurate reference to use in -omics data analysis, for novel genomic comparisons, as well as being a useful tool for molecular manipulations and study of these strains.

Data availability

QUAST Genome assembly reports: https://figshare.com/s/9d9437ed495582e9b484

Genome assembly files are available upon request.

Appendix III

If two is company, but three is a crowd, then what is four?

Answers from a yeast ecosystem

If two is company, but three is a crowd, then what is four? Answers from a yeast ecosystem

Background

In Research Chapter 3, we showed evidence for higher-order interactions in a three-species synthetic yeast consortium. Here, we challenged the same three-species consortium with an additional yeast species, inoculated at an even initial cell density or with the challenger species at seven-fold more than the core consortium. Challenger species were wine-associated yeast species that are known for positive contributions (*Wickerhamomyces anomalus* and *Hanseniaspora opuntiae*) or spoilage (*Brettanomyces bruxellensis*), as well as a predatory yeast species that does not have any relation to the wine ecosystem (*Saccharomycopsis fermentans*). Population dynamics data was compared between the core consortium and the varying four-species consortia with differing initial inoculation ratios. The data presented here shows interesting species-specific impacts on the core fluorescent consortium, and provides targets to further explore complex higher-order interaction mechanisms in this synthetic yeast ecosystem.

Methods

Yeast strains

The three-species fluorescent consortium used in Research Chapters 2, 3, and 4 was also used here, in addition to the following four 'challenger' species representatives: *Saccharomycopsis fermentans* (uncharacterized isolate), *Brettanomyces bruxellensis* IWBT Y169, *Wickerhamomyces anomalus* IWBT Y934 (CBS: 16372), and *Hanseniaspora opuntiae* IWBT Y1055 (CBS: 16375).

Growth medium, pre-culture conditions, culture conditions, monitoring of population dynamics

The three-species fluorescent consortium as well as the varying four-species consortia were cultured in Synthetic Grape Must (SGM) growth medium and monitored according to the methods described in Chapter 2, Sections 2.2, 2.3.1, 2.4, and 2.5. The only deviation from this protocol was changes to the initial inoculation density (Table 1). All species in the consortium were either inoculated in equal cell densities, or the challenger species was inoculated at a cell density of seven-fold more than the fluorescent consortium species. The total initial cell density remained the same throughout all experiments.

Table AIII.1. Inoculation densities used for consortia experiments.

	Even Initial Density (cells.ml^{-1}) Three-species consortium	**Even Initial Density (cells.ml^{-1}) Four-species consortium**	**Higher Challenger Density (cells.ml^{-1}) Four-species consortium**
***S. cerevisiae*-RFP**	1×10^6	7.5×10^5	3×10^5
***L. thermotolerans*-BFP**	1×10^6	7.5×10^5	3×10^5
***T. delbrueckii*-GFP**	1×10^6	7.5×10^5	3×10^5
Challenger species	NA	7.5×10^5	2.1×10^6
Total	3×10^6	3×10^6	3×10^6

Results and Conclusions

Each challenger species evaluated here had differing impacts on the dynamics of the core fluorescent consortium (Fig. AIII.1). These impacts were heightened when the challenger species was inoculated at a higher cell density. *S. fermentans*, a predatory yeast species, stifled the growth of *L. thermotolerans* and *T. delbrueckii*, particularly at high inoculation density (Fig AIII.1 A1-A2). *W. anomalus* presented an impressive competitive relationship with *S. cerevisiae* when inoculated at higher cell densities, and similarly restrained the growth of *L. thermotolerans* and *T. delbrueckii* (Fig AIII.1 B1-B2). *H. opuntiae* appears to compete less with *L. thermotolerans* and *T. delbrueckii* than *W. anomalus* and *S. fermentans*, and seems to favour the growth of *T. delbrueckii* (Fig AIII.1 C1-C2). Finally, *B. bruxellensis*, an infamous wine spoilage yeast, showed notable longevity and persistence in both conditions, and did not appear to compete for nutrients with the fluorescent consortium, evidenced by near identical population dynamics of the fluorescent consortium to the three-species control (Fig AIII1 D1-D2).

The data presented here provides more phenotypic evidence for the presence of complex interactions in yeast ecosystems, and will be a useful resource for further, more detailed studies on the mechanisms that govern these interactions.

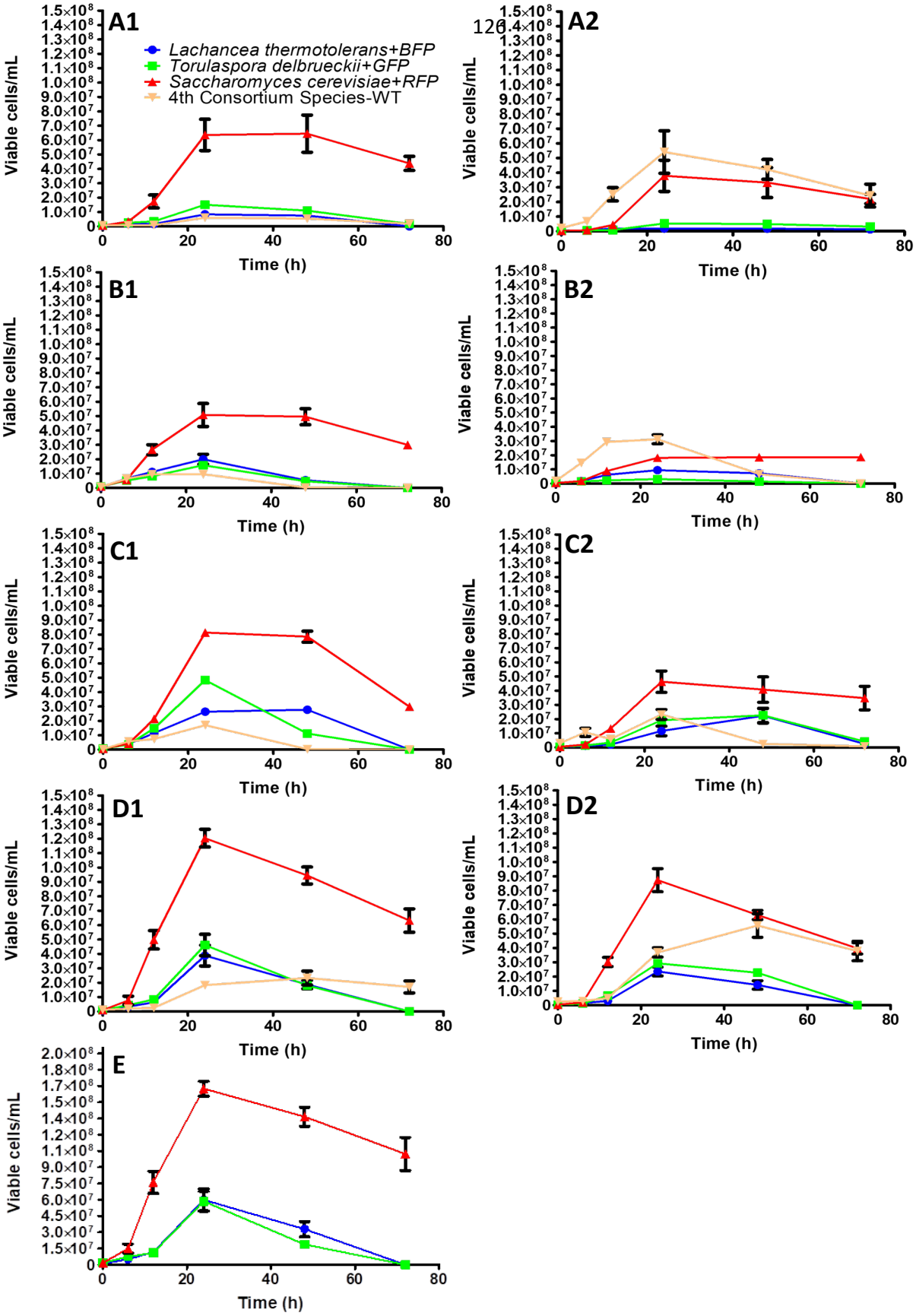

Figure AIII.1. Population dynamics data for three- and four-species yeast consortia. A1, B1, C1, D1, E: All species inoculated at even cell density. A2, B2, C2, D2: 4th Challenger species inoculated at 10-fold more than the fluorescent consortium species (10:1:1:1). A1-2: *Saccharomycopsis fermentans*. B1-2: *Wickerhamomyces anomalus*. C1-2: *Brettanomyces bruxellensis*. D1-2: *Hanseniaspora opuntiae*. E: Fluorescent consortium. See A1 for graphical key.