

The natural ecology of *Saccharomyces* yeasts

Dissertation

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Abstract	1
Zusammenfassung	3
General Introduction	5
1. General information about <i>Saccharomyces</i> yeasts	5
1.1. What are yeasts?	5
1.2. <i>Saccharomyces</i> taxonomy	6
1.3. <i>Saccharomyces</i> life cycle.....	7
2. <i>S. cerevisiae</i>: humanity's pet yeast	8
3. <i>S. cerevisiae</i>: the laboratory model organism	9
3.1. The early days of yeast research.....	9
3.2. <i>S. cerevisiae</i> 's establishment as model organism	10
4. The natural ecology of <i>Saccharomyces</i> yeast	11
4.1. The problematic lack of ecology knowledge – Why should we care?	11
4.1.1. <i>One caveat: the domestication problem of S. cerevisiae</i>	12
4.2. The natural environment of <i>Saccharomyces</i> yeast.....	13
4.2.1. <i>Saccharomyces: adapted to a fruit habitat?</i>	14
4.2.2. <i>Saccharomyces: adapted to oak habitat?</i>	18
4.2.3. <i>Saccharomyces adapted to both; fruit and oak habitat?</i>	19
4.2.4. <i>Saccharomyces: not adapted to any specific habitat</i>	20
Scope of this thesis	22
Chapter I The interaction with <i>Saccharomyces paradoxus</i> with its natural competitors on oak bark – published manuscript	22
Supporting Information Chapter I	39

Chapter II Analysis of the interactions between <i>Saccharomyces paradoxus</i> and two oak bark bacteria (follow up study to Chapter I)	44
1. Introduction	44
1.1. Background	44
1.2. Microbial communities remain poorly understood.....	45
1.3. The paradox of high biodiversity within microbial communities.....	45
1.4. The complexity of microbial communities and the interactions between members	47
1.5. Determining the mechanisms of the interactions between <i>S. paradoxus</i> and two oak bark bacteria	48
2. Methods	50
2.1. Estimating the effects of the microbial species on each other in competition	50
2.2. Characterizing the interaction between <i>S. paradoxus</i> and <i>Pseudomonas</i>	51
2.3. Competition of <i>S. paradoxus</i> with <i>Mucilaginibacter</i> using different initial frequencies.....	52
2.4. Characterizing the interaction between <i>S. paradoxus</i> and <i>Mucilaginibacter</i> on a temperature gradient.....	52
2.5. Conditioned media experiments	53
3. Results	53
3.1. <i>Pseudomonas</i> dominates the interaction with <i>S. paradoxus</i>	53
3.2. <i>S. paradoxus</i> and <i>Mucilaginibacter</i> both benefit from the interaction	54
3.3. Conditioned media has little effect on the growth of <i>S. paradoxus</i>	57
4. Discussion	58
4.1. <i>S. paradoxus</i> and <i>Pseudomonas</i> interaction is uni-directional with a resulting killing of the yeast.....	58
4.2. The interaction between <i>S. paradoxus</i> and <i>Pseudomonas</i> is not frequency-dependent nor strongly regulated by temperature.....	58
4.3. The killing effect from <i>Pseudomonas</i> depends on the direct presence of the bacterium.....	59

4.4.	The interaction between <i>S. paradoxus</i> and <i>Mucilaginibacter</i> benefits the yeast's growth.....	60
4.5.	The interaction of <i>S. paradoxus</i> and <i>Mucilaginibacter</i> is independent of the organism's frequency but strongly influenced by temperature.....	61
4.6.	No big effects on <i>S. paradoxus</i> growth using conditioned medium	61
4.7.	A more natural medium allowed us the detection of more complex interactions	62
5.	Conclusion	63
6.	Supplemental Material	64
Chapter III Is the Crabtree effect an adaptation to high sugar environments?		65
1.	Introduction	65
1.1.	The aerobic and anaerobic metabolism of <i>Saccharomyces</i> spp.	65
1.2.	The Crabtree effect: an adaptation to high sugar environments?	68
1.3.	Competition studies in the scientific literature and their interpretation problem	70
1.4.	Use of a Crabtree positive and negative strain in our study.....	71
1.5.	Project aim	73
2.	Methods	73
2.1.	Transformation of drug resistance genes into yeast strains.....	73
2.2.	Experimental design	74
2.3.	Testing the effects of different media on the benefit of the Crabtree effect.....	76
2.4.	Testing the effects of microbial competitors on the benefit of the Crabtree effect	77
3.	Results	78
3.1.	Being Crabtree positive is advantageous under direct competition in laboratory medium but surprisingly not in grape juice	78
3.2.	Adding natural competitors to grape juice increases the benefit of being Crabtree positive.....	80
4.	Discussion	82

4.1.	Under respiration in non-fermentation medium, fermenter shows a better respiring efficiency than the respirer mutant strain	82
4.2.	In glucose medium the fermenter had an advantage in direct competition due to a better resource competition.....	83
4.3.	In grape juice, fermenter is less fit relative to the respirer both alone and in direct competition.....	83
4.4.	Adding natural competitors to grape juice increases the fitness of the fermenter relative to the respirer.....	84
5.	Conclusion	86
6.	Supplemental material	87
Chapter IV A systematic forest survey showing an association of <i>Saccharomyces</i> with oak leaf litter		88
1.	Introduction	88
2.	Methods	91
2.1.	Leaf litter transects	91
2.2.	Comparison of leaf litter under four tree genera	92
2.3.	Enumeration of the <i>Saccharomyces</i> cell number in oak leaf litter and on oak bark	92
2.4.	Comparison between direct-isolated and enrichment-isolated <i>S. paradoxus</i> strains.....	93
2.5.	Over-the-year sampling study	94
3.	Results	95
3.1.	<i>Saccharomyces</i> isolation success decreases with increasing distance to an oak	95
3.2.	Oaks harbor most <i>Saccharomyces</i> isolates compared to other tree genera	96
3.3.	<i>Saccharomyces</i> is much more abundant in leaf litter than on bark	97
3.4.	No fitness difference between strains isolated with direct plating and enrichment isolation....	99
3.5.	<i>Saccharomyces</i> abundance and distribution varies between trees and over season	99
4.	Discussion	102
4.1.	Proximity to an oak tree predicts <i>Saccharomyces</i> abundance in leaf litter	102

Table of Contents

4.2.	<i>Saccharomyces</i> occurrence is decreasing with increasing distance to oak	103
4.3.	More <i>Saccharomyces</i> in leaf litter than on bark.....	104
4.4.	Seasonal effects on yeast abundance and distribution	104
4.5.	Variation in yeast abundance within tree samples suggest patchy distribution	105
4.6.	Seasonal effects on <i>Saccharomyces</i> abundance and dispersion	106
4.7.	Oak provides a better habitat for <i>Saccharomyces</i> than larch, beech or spruce.	106
4.8.	Strong tree-to-tree variation in harboring <i>Saccharomyces</i>	107
4.9.	<i>S. paradoxus</i> is the dominant <i>Saccharomyces</i> species in Nehnten	108
5.	Conclusion	109
6.	Supplemental Material	110
7.	Acknowledgements	110
	General Discussion	111
	References	118
	Declaration of Author’s Contribution	136
	Acknowledgements	137
	Affidavit	138

Abstract

There are some organisms on Earth that have an especially close relationship with humankind, and one of them is *Saccharomyces cerevisiae*. This species, which is also known as the baker's or brewer's yeast, has been used for thousands of years, and almost all around the world, for the production of alcoholic beverages and bread. Today, we owe even more to this organism than wine, as it became one of the best studied model organisms in biology and is widely used in different scientific disciplines like Cell Biology, Biochemistry, Genetics and Molecular Biology. But surprisingly little is known about its natural ecology. The habitat of wild, undomesticated *Saccharomyces* yeasts cannot be reliably defined and contrary hypotheses exist. There is some evidence that the yeast is adapted to live in sugar rich environments like fruits, but on the other hand there is also indication that wild *Saccharomyces* yeasts are associated with oak trees, especially their bark. In both environments, yeast is only found in a small proportion of samples and most studies additionally suffer from different biases making it impossible to conclude which, if either, environment is truly the natural habitat of the yeast.

In this thesis the natural ecology of *Saccharomyces* spp. was studied, with an emphasis on the natural environment. The oak bark environment was analyzed by characterizing the associated microbial community using culturing and pyrosequencing methods. *S. paradoxus*, the wild and undomesticated relative of *S. cerevisiae*, could indeed grow on nutrients present in oak bark but is only a rare member of the microbial community. Studying the influence of co-isolated oak bark microorganisms on *S. paradoxus*' growth and survival in natural oak bark medium revealed a wide range of effects strongly dependent on temperature. Further experimental analysis of the interactions between the yeast and two of the bacteria gave insights into the diversity and complexity of natural microbial interactions. While one *Pseudomonas* spp. killed the yeast, another bacterium; *Mucilaginibacter* spp. promoted its growth. *Saccharomyces*' metabolic specialty of fermenting under aerobic conditions (Crabtree effect) is taken as evidence that the yeast is adapted to sugary fruit environments. To test this, a Crabtree positive ("fermenter") and its isogenic Crabtree negative mutant strain ("respirer") were competed in laboratory media and grape juice, confirming that the Crabtree effect provides a benefit under resource competition in lab medium. Unexpectedly, this benefit was absent in the more natural grape juice, although adding natural microbial competitors restored the benefit of fermentation over respiration, perhaps by interference competition. Finally, the yeast / oak association could be confirmed by an intense sampling study, but *Saccharomyces* was found to be much more abundant in oak leaf litter than on bark. Oak leaf litter provides a stable habitat over the year from which yeast can

be easily isolated and studied. This is a useful discovery for the ecology and evolutionary history of *Saccharomyces* yeasts, with great promise for future studies.

Zusammenfassung

Es gibt ein paar Organismen auf der Erde, die in einer besonders engen Beziehung zur Menschheit stehen und eine davon ist die Hefe *Saccharomyces cerevisiae*. Diese Spezies, die auch als Bäcker- oder Brauereihefe bekannt ist, wird seit tausenden von Jahren und fast überall auf der Welt für die Produktion von alkoholischen Getränken und Brot verwendet. Heutzutage verdanken wir der Hefe allerdings um einiges mehr als bloß Wein, da sie zu einem der best studierten Modellorganismen in der Biologie geworden ist und weitreichend in verschiedenen wissenschaftlichen Disziplinen wie Zellbiologie, Biochemie, Genetik oder Molekularbiologie Verwendung findet. Allerdings ist erstaunlich wenig über die natürliche Ökologie der Hefe bekannt. Das Habitat von wilden, undomestizierten *Saccharomyces* Hefen kann nicht sicher bestimmt werden und verschiedene, gegensätzliche Hypothesen existieren hierzu. Es gibt einerseits Hinweise, dass die Hefe adaptiert ist, um in zuckerreichen Umwelten wie Früchten zu leben, aber andererseits gibt es auch Hinweise, dass wilde *Saccharomyces* Hefen mit Eichenbäumen, speziell deren Borke assoziiert sind. In beiden Umwelten wird die Hefe allerdings nur in einer geringen Anzahl der Proben gefunden und die meisten Studien leiden obendrein unter unterschiedlichen, systematischen Fehlern, was eine Entscheidung welche, wenn überhaupt eine, der Umwelten das reale natürliche Habitat der Hefe bildet, unmöglich macht.

In dieser Arbeit wurde die natürliche Ökologie von *Saccharomyces* Hefen mit einem Hauptaugenmerk auf die natürliche Umwelt untersucht. Die Eichenborken-Umwelt wurde mittels einer Charakterisierung der assoziierten mikrobiellen Gemeinschaft durch die Verwendung von Kultivierungs- und Hochdurchsatzsequenziermethoden analysiert. *S. paradoxus*, die wilde und undomestizierte verwandte Art von *S. cerevisiae*, konnte tatsächlich auf in Eichenborke präsenten Nährstoffen wachsen, aber ist nur ein seltenes Mitglied der mikrobiellen Gemeinschaft. Studien über den Einfluss von co-isolierten Eichenborken-Mikroorganismen auf *S. paradoxus* Wachstum und Überleben in natürlichem Eichenborkenmedium ergaben eine Bandbreite an Effekten, die stark von Temperatur beeinflusst wurden. Weitere experimentelle Analysen der Interaktionen zwischen der Hefe und zwei der Bakterien gaben Einblicke in die Diversität und Komplexität natürlicher mikrobieller Interaktionen. Während eine *Pseudomonas* Spezies die Hefe tötete, hat ein anderes Bakterium; *Mucilaginibacter*, ihr Wachstum erhöht. *Saccharomyces'* metabolische Besonderheit der Fermentation unter aeroben Bedingungen (Crabtree Effekt) wird als Beweis angesehen, dass die Hefe auf das Leben in zuckerreichen Frucht Umwelten adaptiert ist. Um dies zu überprüfen, wurde eine Crabtree positive („Fermentierer“) Hefe mit ihrer isogenen Crabtree negativen („Respirierer“) Mutante in Labormedium und Traubensaft konkurriert, was bestätigte, dass der

Crabtree Effekt einen Vorteil unter Konkurrenz um Ressourcen liefert. Dieser Vorteil jedoch war im natürlicheren Traubensaft unerwarteterweise nicht vorhanden, obwohl die Zugabe von weiteren, natürlichen mikrobiellen Konkurrenten den Vorteil von Fermentation über Respiration ausbaute, vermutlich durch Interferen-Konkurrenz. Letztlich konnte die Hefe/Eichen Assoziierung durch eine intensive Sammlungsstudie bestätigt werden, allerdings war *Saccharomyces* in viel größerer Anzahl in Eichenlaub, als an Borke präsent. Eichenlaub lieferte ein stabiles Habitat über das Jahr, von dem Hefe einfach isoliert und somit studiert werden kann. Dies ist eine nützliche Entdeckung für die Ökologie und evolutionäre Geschichte von *Saccharomyces* Hefen mit großartiger Aussicht für zukünftige Studien.

General Introduction

Microbes rarely capture public attention and those that do achieve fame are usually cast as villains responsible for human disease and death. One of the most beneficial microbes for humanity is the baker's yeast *Saccharomyces cerevisiae*. This yeast is involved in the production of many different foods (ranging from bread to chocolate), alcohol (wine, beer, and spirits), and life-saving medicines (including insulin and artemisinin). It is also well suited to laboratory studies, providing a basis for huge advances in many fields of biology. However, despite the involvement of *S. cerevisiae* in so many aspects of our daily lives, we know almost nothing about its life outside of industry and the laboratory. This makes it questionable to put any obtained result in a context that exceeds the laboratory or brewery. For a real and deeper understanding of an organism we need to know its ecology and evolutionary history. With a stronger focus on studying these fields, *S. cerevisiae* could become an all-encompassing model organism useful in all biological disciplines in the future.

1. General information about *Saccharomyces* yeasts

1.1. What are yeasts?

Yeasts are eukaryotic and predominantly single-celled microorganisms that span the taxa Ascomycota and Basidiomycota in the kingdom Fungi. Some yeast species can transition between a yeast-phase (unicellular) and a multicellular-phase at different stages in their life cycles. Yeasts as well as other fungi are found in every environment on Earth and play a crucial ecological role: they decompose organic matter by secreting enzymes that break down their food and then absorbing the products. This mechanism allows them to adopt certain highly specialized ecological roles including binding inorganic nitrate or detoxification of substrates (Deak, 2006). However, only 1 % of all the living yeast species are described (Kurtzman and Fell, 2006). Yeasts can physiologically be classified in terms of their sugar metabolism, namely non-fermentative, facultative-fermentative, or obligate-fermentative. Non-fermentative yeasts exclusively respire, whereas obligate fermentative yeasts exclusively ferment nutrients. Most yeasts identified to date are facultative-fermentative ones and it depends on growth conditions, the type and concentration of sugar and/or oxygen availability if either a fully respiratory, a fermentative or a mixed respire-fermentative metabolism is displayed (Rodrigues et al., 2006). The most common yeasts in this sense are the ones belonging to the genus *Saccharomyces*.

1.2. *Saccharomyces* taxonomy

S. cerevisiae is the most famous species belonging to the *Saccharomyces sensu stricto* complex (Figure 1) which are taxa currently assigned to the genus *Saccharomyces* (reviewed in: Boynton and Greig, 2014; Kurtzman, 2003). The first described *S. cerevisiae* came from human-associated fermentations. Its closest related sister species *S. paradoxus* was the first to be acknowledged as a non-domesticated species in 1914 (Bachinskaya, 1914). Afterwards the naturally occurring species *S. kudriavzevii*, *S. mikatae*, *S. arboricola* and *S. bayanus* were described in quick succession (reviewed in: Boynton and Greig, 2014). *S. mikatae* and *S. arboricola* have never been found outside of eastern Asia (Naumov et al., 2012), whereas *S. kudriavzevii*, that has also been isolated in Japan for the first time, was also detected in Europe (Sampaio and Gonçalves, 2008). *S. cerevisiae* is the most common species used in human fermentations and is considered to be the agent of wine, ale beer, sake and palm fermentation as well as the agent of leavened bread (Albertin et al., 2009; Fay and Benavides, 2005; Legras et al., 2007; Spor et al., 2008) but also other *Saccharomyces* species are used in these processes. Hybridizations between species of the *sensu stricto* complex as well as variation of ploidy (Albertin et al., 2009) have occurred several times and strongly participated to the evolution of domesticated yeast (Sicard and Legras, 2011). In low-temperature fermentations (like Lager brewing) *S. cerevisiae* which has a high temperature growth profile generally tends to be replaced by hybrids that combine the genomes of *S. cerevisiae* with those of other cryotolerant *Saccharomyces* species: *S. eubayanus*, *S. kudriavzevii* and *S. uvarum*. The latter forms hybrids with *S. cerevisiae* as well as *S. eubayanus* that are involved in lager beer, wine and cider fermentations conducted at low temperatures (Demuyter et al., 2004; Naumov et al., 2000; Nakao et al., 2009; Naumov et al., 2001; Naumova et al., 2005; Rementeria et al., 2003; Sipiczki et al., 2001; Torriani et al., 1999; Valles et al., 2007). The recently discovered *S. eubayanus* also showed to be the second parent besides *S. cerevisiae* of the lager-brewing hybrid species *S. pastorianus* (Libkind et al., 2011). Other hybrids harboring genomic contributions from *S. cerevisiae* and *S. kudriavzevii*, are commonly found among strains used to produce Belgian-style beers and wines fermented at low temperatures (González et al., 2006, 2008; Lopandic et al., 2007).

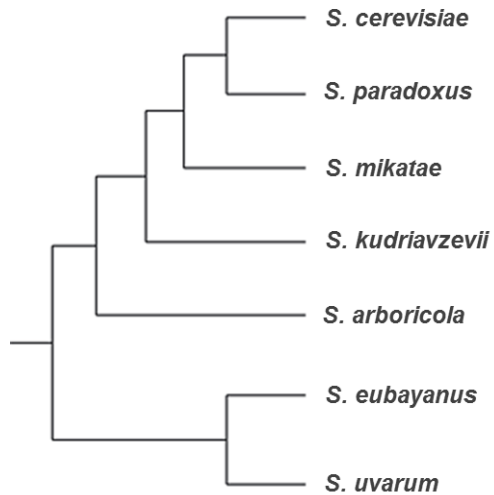


Figure 1: The *Saccharomyces sensu stricto* complex. Cladogram topography from (Almeida et al., 2014; Fig. 1a)

1.3. *Saccharomyces* life cycle

Saccharomyces cells naturally exist in two ploidies: haploids and diploids. Haploid cells are capable of mitotic division and can have one of the following two mating types: cells of the type “a” that produce a-pheromones or “ α ” cells that produce α -pheromones (Figure 2). The pheromones attract cells from the opposite type but cells are also capable of switching their mating type. This makes it possible that after mitosis one of the two clonal daughter cells can mate with its sister cell after changing the mating type. In general, *Saccharomyces* favors being diploid and so reproduces mainly by diploid mitosis. Under starvation diploid cells can enter meiosis (haploid cells die) and produce a tetrad of four resistant haploid spores. When conditions improve, spores germinate and often directly mate either their sister spores (inbreeding) or other haploid cells (outbreeding) to restore the diploid state (Herskowitz, 1988). Under optimal conditions, yeast cells can double their population every 100 minutes (Slater et al., 1977) but in reality growth rates vary strongly between different strains and environments (Wheals and Lord, 1992).

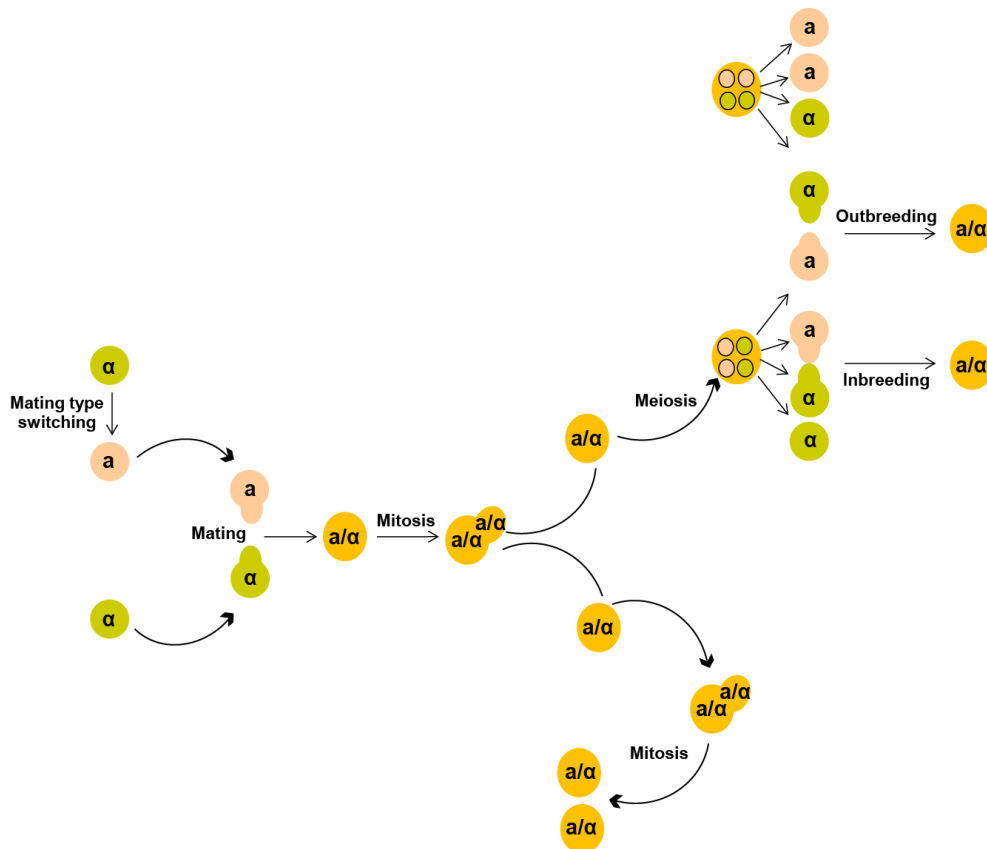


Figure 2: The life cycle of *Saccharomyces* spp.. A yeast cell can grow asexually by mitosis as either a haploid or a diploid, but spend the majority of their lives in the diploid phase. In stressful environments, diploids undergo meiosis, generating four haploid spores (tetrad). Each haploid yeast belongs to one of two mating types: “a” and “α”. Haploids of opposite mating type can mate together to produce a diploid. When a partner of the opposite mating type is not available, haploid cells can switch mating type to generate one.

2. *S. cerevisiae*: humanity's pet yeast

Saccharomyces yeasts especially *S. cerevisiae* have been used by humans all around the world for millennia to ferment a variety of edible and drinkable substrates. Its value lies in its metabolic pathway; the yeast consumes sugar and produces the fermentation by-products ethanol (used for making alcoholic beverages) and carbon dioxide (for raising bread). The oldest historical evidence of formal brewing in human’s history dates back to ~6000 BCE in ancient Babylonia as interpreted from a picture on an old piece of pottery (Hardwick, 1994). Precise models of a bakery and a brewery found in Egyptian tombs confirm that bread baking as well as brewing were well established 4000 years BCE (Spencer and Spencer, 1997). Microorganisms

other than *Saccharomyces* yeasts are also capable of fermentation and ethanol production so we cannot be sure that all present and historical examples of baking or brewing are associated with *Saccharomyces*. However, most non-*Saccharomyces* microbes only produce low concentrations of ethanol (Goddard, 2008). The oldest proven evidence of *S. cerevisiae* associated with humans comes from ribosomal DNA extracted from residues inside a wine jar from an ancient Egyptian tomb of King Scorpion I dated to 3000 BCE (Cavaliere et al., 2003). Under non-sterile conditions, sugar-rich solutions will usually be colonized by microbes capable of fermentation. By the end of this spontaneous fermentation processes, typically accompanied by increasing temperature and ethanol concentrations, *S. cerevisiae* usually dominates over other microorganisms (Fleet, 2003; Morrissey et al., 2004). While people still rely on the spontaneous fermentation of naturally occurring yeast species present on the grapes in some wineries, the traditional way of baking or brewing without using yeast starters is to take a small amount of good dough or beverage to start the next batch (Spencer and Spencer, 1997). Over time, the use of these starter cultures helped to select for improved yeasts by saving the “good” batch of dough, wine or beer for inoculating the next batch. So it is likely that the early fermentation processes by humans were the origin of selection for *S. cerevisiae* as “baker’s and brewer’s yeast”, making this yeast to a domesticated microorganism. Indeed, genome sequencing of *S. cerevisiae* strains all around the world from different fermentation environments like wine or sake as well as clinical (*S. cerevisiae* can cause fungal infections in immunocompromised patients (Aucott et al., 1990)) and wild strains show that the population structure of *S. cerevisiae* consists of only a few well-defined lineages. The influence of humans likely provided opportunities for cross-breeding between these lineages producing new combinations with mosaic genomes (Liti et al., 2009).

3. *S. cerevisiae*: the laboratory model organism

The ability of *S. cerevisiae* to ferment and produce alcoholic beverages also attracted the attention of scientists which was the start of collecting extensive knowledge about the yeast in several areas.

3.1. The early days of yeast research

Until the biochemical basis of the fermentation process was discovered fermentation was seen as a mysterious phenomenon. Using a primitive microscope Antonie van Leeuwenhoek was the first who described microorganisms in different samples as “very little animacules” (Barton and Northup, 2011). In 1680 he made the first recorded observation of yeast cells after he put a

drop of fermenting beer on a ground lens. He was a lens grinder, not a scientist and his skills in shaping lenses were so great that his observations were not confirmed by others and therefore not generally believed for a long time (reviewed in: Hardwick, 1994). In the early nineteenth century, even the existence of living microbes was a matter of debate (reviewed in: Barnett, 2003). In 1755 yeast was defined as “the ferment put into drink to make it work; and into bread, to lighten and swell it” in the English dictionary (Johnson 1755). Yeast was not seen as a living organism and the founder of modern chemistry Antoine Lavoisier described wine making as chemical reaction of “grape must = carbonic acid + alcohol” (reviewed in: Barnett, 1998). The original demonstration that yeasts are living organisms was made in 1836 by Charles Cagniard de Latour by microscopic observation of budding beer yeast and independently confirmed by Theodor Schwann, who recognized that chemical changes take place inside the living yeast cell, and by Friedrich Traugott Kützing (Rosenfeld, 2003). In 1859, Louis Pasteur discovered how yeast fermentation operates. He experimentally demonstrated that fermented beverages are a result of metabolic processes of living yeast transforming sugar into ethanol. In addition, he showed that only microorganisms are able to convert the sugar of grapes into alcohol (reviewed in: Barnett, 2000). After his publication the role of the living “germs” described early by Meyen (1838) with the name *Saccharomyces* (literally “fungi of the sugar”) became generally recognized and accepted (Martini, 1993). Around the same time the Danish brewer Jacob Christian Jacobsen founded the Carlsberg Brewery as well as the Carlsberg Laboratory. Emil Christian Hansen, facing the problems of microbial contamination during fermentation processes, developed a method to culture single colonies and he introduced solid medium cultivation (adapted from Robert Koch’s method for bacteria). In 1883 the Carlsberg Brewery started the industrial production of lager beer using one of Hansen’s yeast strains (reviewed in: Polaina 2002).

3.2. *S. cerevisiae*’s establishment as model organism

All these early efforts of scientists to explain the activity that is responsible for alcohol production was the start of *S. cerevisiae*’s second career: becoming one of the best studied model organisms in biology. In 1933 Øjvind Winge started to work in the Carlsberg Laboratory and as his first task he tried to recover stock cultures of yeast from the collection of Emil Christian Hansen and Albert Klöcker. The stocks were up to 46 years old and had been kept in liquid solution without being refrigerated and Winge was surprised to find that most could easily be revived (Winge and Hjort, 1935). It was the beginning of Winge’s contributions as “father of yeast genetics” (Szybalski, 2001) when he could show that yeast reproduces sexually and has a regular haplophase-diplophase cycle (Winge and Laustsen, 1937). A few years later he published in collaboration with Otto Laustsen the first case of Mendelian segregation in yeast what

opened the road for systematical studies of yeast genetics (Westergaard, 1964; Winge and Laustsen, 1937). Modern molecular biology using yeast was established with the demonstration that yeast can be transformed with foreign DNA in 1978 (Hinnen et al., 1978). In the same year a plasmid shuttle vector between the model bacterium *Escherichia coli* and *S. cerevisiae* was developed enabling cloning in yeast (Beggs, 1978). With becoming amenable to genetic engineering the yeast became extremely valuable for fundamental research as well as for industrial applications. It was the first host cell for a recombinant hepatitis B vaccine as well as for recombinant food-grade enzymes (reviewed in: Chambers and Pretorius, 2010). The easiness of genetic manipulation of yeast cells allows gene products from other eukaryotes to be characterized in the yeast system. In 1996, *S. cerevisiae* became the first eukaryotic organism whose genome was completely sequenced (Goffeau et al., 1996). This sequence information is extremely useful as a reference against sequences of higher organisms and those of a multitude of unicellular organisms, may be compared. Many of the biological principles discovered in budding yeast were found to be generally applicable to all eukaryotes. In addition to its advantages as a genetic model system, *S. cerevisiae* exhibits many traits suited to the study of evolutionary biology: its life history is quite simple, as it easily propagates and has a short generation time. These traits allow experiments to run for many generations with large populations that can be maintained in small spaces. In addition, the yeast can be frozen and stored for future comparison with evolved offspring. The diverse and fast succeeded breakthroughs and technological advances in molecular-, systems- and synthetic biology rarely happened and happen without *S. cerevisiae* being involved somehow.

4. The natural ecology of *Saccharomyces* yeast

Although we know the basic biology of *S. cerevisiae* very well (entering “*Saccharomyces*” under “topic” in Web of Science leads to 591,463 publication hits; for comparison: “*Caenorhabditis*”: 157,121, “*Drosophila*”: 598,479, “*Mus*”: 110,432; as of 28/6/2015), the natural ecology that shaped and still shapes the yeast’s genome is still largely unknown (Greig and Leu, 2009; “*Saccharomyces* AND ecology” leads to only 1850 hits in the Web of Science).

4.1. The problematic lack of ecology knowledge – Why should we care?

Although some researchers started ecological studies, most of our knowledge about *S. cerevisiae* is still based on studies of a few lab-domesticated isolates with mosaic genomes. Since the sequencing of its genome, yeast laboratories all around the world have collaborated to

knock out each gene, one-at-a-time, and test the phenotypes of each knockout (e.g. Entian et al., 1999; Giaever et al., 2002; Winzeler et al., 1999). Hughes et al., (2004) predicted optimistically that the date of determining the function of all gene products of an organism is near and the idea that this organism will be human's domesticated microorganism *S. cerevisiae*, seems comprehensible. An actual snapshot of the genome of *S. cerevisiae* (<http://www.yeastgenome.org/genomesnapshot>) leads to the positive result of 5121 verified genes but also 784 dubious and still 699 uncharacterized genes (as of 4/30/2015) which simply means that these knockout-mutants have no fitness cost or even small fitness benefits in all environments that have been tested. The function of many of these genes can very likely only be enlightened in a more natural and complex environment including competition with other microorganisms. This would definitely imply a better knowledge about the natural ecology of *Saccharomyces* to draw conclusions about natural populations. Remarkably, only about 20 % of all genes are essential for growth on rich glucose medium and an additional 15 % cause reduced growth when deleted (Giaever et al., 2002). Yeast gene knockouts are studied as single clones under laboratory conditions, but these conditions are unlikely to match the conditions encountered by yeast in the wild: gene functions in the lab may not reflect gene functions in the wild. Using more natural media of tree sap and oak infusions leads to a weakening of several mutants (Bell, 2010). This is particularly interesting as Jasnos et al. (2008) demonstrated that the negative impact of missing genes is smaller in stressful relative to favorable environments which has been discussed as a higher negative impact of any metabolic disturbances in a fast growing environment. What cannot be ignored is that a medium made out of natural material will not contain microorganisms after filter-sterilization but still a range of toxins, growth inhibitors and waste products preliminary produced by the diverse organisms from the natural environment. This additionally could explain a stronger effect of the deletions on yeast's fitness in such a medium. The natural ecology of *S. cerevisiae* is clearly far more complicated than the sterile sugar-rich media used in the laboratory. For a real understanding of an organism we need to be able to put it in its ecological and evolutionary background. With a stronger focus on its ecology, *S. cerevisiae* could also become a powerful model organism in ecological disciplines such as evolutionary and ecological genomics, population genetics, microbial biogeography and community ecology (Replansky et al., 2008). With the huge body of knowledge existing about *S. cerevisiae* in combination with the modern molecular techniques available today, we have the chance of an all-encompassing model organism useful in all biological disciplines in the future.

4.1.1. *One caveat: the domestication problem of S. cerevisiae*

Studying the natural ecology of an organism makes it necessary that this organism evolved in its natural environment without extreme disturbance from humankind as this can change the

whole picture we get. For *S. cerevisiae*, the long history as industrial and laboratory yeast makes this very difficult. If we go into a forest and see a dog we can pretty reliably say that this is not a wolf but its domesticated form, trying to do such a fast morphological assessment for an isolated *S. cerevisiae* strain is impossible and an intense genetic analysis would be necessary. The risk of vectored cross-contaminations makes it arguable if a *S. cerevisiae* strain isolated from any environment is truly “wild” as there is suspicion that industrial strains escape into the wild and wild strains enter the man-made fermentation environment. The fruit fly *Drosophila melanogaster*, which is a human commensal, is often found associated with vineyards (Keller, 2007) where commercial strains of *S. cerevisiae* showed to be omnipresent, coating and colonizing vineyard, cellar and equipment surfaces under specific circumstances (Ciani et al., 2004; Sabate et al., 2002; Vaughan-Martini and Martini, 1995). Flies may therefore vector commercial wine strains from industrial sources to the natural environment. However, data from spontaneous fermentations in New Zealand suggest that many of the *S. cerevisiae* involved, actually derive from the local environment (Goddard et al., 2010) rather than the other way around. Today we know that wild *S. cerevisiae* strains clearly exist. A detected DNA sequence diversity in five loci of diverse *S. cerevisiae* strains from various sources supported the hypothesis that actually wild populations comprise the origin of domesticated strains (Fay and Benavides, 2005). *S. cerevisiae* has also been isolated from primeval forests far away from human influence (Wang et al., 2012) and these strains are phylogenetically distinct from previously isolated *S. cerevisiae* strains and represent truly wild specimen. Even though wild *S. cerevisiae* exist, there is always a risk that any individuals isolated from a natural source simply escaped from human’s fermentation environments or hybridized with domesticated strains. This complicates data interpretation and compromises any observation that actually may apply to other conditions. The easiest way to avoid these problems is to study a close relative of *S. cerevisiae* that shares its many advantages without the problems associated with domestication (Johnson et al., 2004). In ecological studies it is therefore preferred to study the closest known relative of *S. cerevisiae*, *S. paradoxus* (Replansky et al., 2008). The two species are phenotypically and biochemically nearly indistinguishable, share almost the same profiles of assimilation and fermentation of organic compounds (Vaughan-Martini 1998) and exist in sympatry in different environments in nature (Naumov et al., 1998; Redžepović et al., 2002; Sampaio and Gonçalves, 2008; Sniegowski et al., 2002), but *S. paradoxus* has never been found associated with any domestication events.

4.2. The natural environment of *Saccharomyces* yeast

In contrast to the intensive research on *S. cerevisiae* in the laboratory and artificial fermentations, little attention has been paid to the yeast in its natural environment. The

identification of abiotic and biotic factors the yeast is challenged with in its natural life would bring us closer to a complete understanding of it. Perhaps the most important component missing from all laboratory environments tested is the presence of other interacting microorganisms. As no pure strain of organisms exists in a natural environment in complete isolation, every organism will not only respond to the chemical environment but will also be affected by a diversity of other species (Trudinger and Bubela, 1967). For studying natural microbial ecology and evolution it is important to realize that the studied organisms are imbedded in a complex natural community. Community structure evolves with time and its dynamics or stability depends on the interactions and interrelationships amongst populations and between single organisms and their adaptation to the environment. Such microbial interactions can drive evolution, adaptation, and speciation of community members. On the other hand evolutionary changes in members of a community can feed back to modify species interactions, community composition and ecological dynamics (Haloin and Strauss, 2008). Dissecting the interactions among community members is one essential component to an understanding of the properties of the community (Little et al., 2008).

Before such ecological studies can be realized, we need to know the natural habitat(s) of *Saccharomyces* to have a resource for easy isolation of the yeast in abundances which allow studying the natural diversity present outside and to have the possibility of characterizing the yeast's abiotic and biotic environment. Identifying the natural habitat sounds like a simple task but there is a lot debating between yeast researchers and different hypothesis exist with a strong focus of two very different habitats: fruits and oak trees.

4.2.1. *Saccharomyces: adapted to a fruit habitat?*

Emil Christian Hansen started the first ecological surveys of yeasts in 1881 and later wrote the theory that yeast cells are normal residents on the surface of sugary fruits. During winter, yeasts would survive in the soil after getting washed off by rain or along with fallen fruits. At the beginning of summer the cells will be transported back to the fruits by wind activity (reviewed in: Martini, 1993). This idea seems reasonable when we consider the fact that *S. cerevisiae* has been consistently used for making wine out of sugar rich grapes for thousands of years. Since these studies several facts about the yeast have been presented as evidences for the fruit-adaptation theory:

***S. cerevisiae* is the dominant microorganism in wine:** *S. cerevisiae* and other *Saccharomyces* yeasts have been used worldwide for making alcohol and indeed seem to be well adapted to liquid sugary environments. *S. cerevisiae* dominates over other microorganisms in the wine making process and most other natural grape inhabiting yeasts, although capable of

fermentation, are not able to ferment grape juice to completion (Contreras et al., 2014). This ability of *S. cerevisiae* as well as the ability to produce and tolerate high concentrations of alcohol are thought to be key adaptations to high sugar fermentation environments (reviewed in: Bauer and Pretorius, 2000).

***Saccharomyces*' special metabolic response to sugar (Crabtree effect):** *S. cerevisiae*'s specific and wasteful way of metabolizing available sugars is taken as evidence for the hypothesis that this yeast is adapted to high sugar conditions. Although in modern industrial fermentations yeast is added for speeding up the process and for quality insurance, *Saccharomyces* fermentation processes can happen spontaneously and the reason for that is that it can also occur under aerobic conditions. This surprising behavior is known as the Crabtree effect (Pronk et al., 1996) (named after the English biochemist Herbert Grace Crabtree). In contrast, Crabtree negative yeasts, such as *Kluyveromyces lactis* completely oxidize glucose to CO₂ through respiration under aerobic conditions (Lin and Li, 2011). Although fermentation of sugar by *S. cerevisiae* is roughly 8 - 9 times less metabolically efficient than aerobic respiration in terms of ATP production (Bakker et al., 2001; Pfeiffer and Morley, 2014), it potentially provides two selective benefits: i: Crabtree positive yeasts have an advantage in resource competition. Fermentation is rapid, allowing yeast to increase in number faster than respiring competitors (Pfeiffer et al., 2001). ii: Crabtree positive yeasts gain an advantage through interference competition. The by-products of fermentation are ethanol, which is toxic to many other microbes especially bacteria; small amounts of acid that change the pH; and heat which can be lethal to temperature-sensitive organisms (Goddard, 2008). The direct environment of the yeast gets poisoned by these products, inhibiting the growth of competitors. Furthermore, after exploiting all the available sugars, *S. cerevisiae* can then undergo a 'diauxic shift' and switch metabolic gears to use the accumulated ethanol as a substrate for aerobic respiration. The ability to make ethanol for later metabolic use is called the "make-accumulate-consume strategy" (Piškur et al., 2006). This means the yeast can recover some of the energy wasted by fermentation at the cost of 1 ATP, although most ethanol might simply disappear before it can be re-consumed (Thomson et al., 2005).

Evolution of the Crabtree effect coincided with the appearance of plant provided high sugar environments: The specific time point of the evolution of the Crabtree effect is thought to be another evidence that this trait evolved as an adaptation to high sugar environments. In *Saccharomyces* evolutionary history two important events are genetically responsible for evolving the Crabtree effect. The *Saccharomyces* lineage underwent a whole genome duplication approximately 100 million years ago (Mya) (after the divergence of *Saccharomyces* and the Crabtree negative *Kluyveromyces* lineage) (Figure 3) (Thomson et al., 2005). Whole genome duplication allowed rapid adaptation to new environments via two distinct

mechanisms. First, although *S. cerevisiae* lost 88 % of the redundant genes that were produced by this duplication, 457 genes were retained in its genome and most of these surviving genes are important for the sugar metabolism. They are thought to have been retained because they could be expressed at high levels due to their relative increased dosage (Kellis et al., 2004). Second, for other duplicated genes, the presence of two initially-identical gene copies allowed one copy to retain the original function and the second copy, free from functional constraints, could evolve new functions (neo-functionalization). It is assumed that another duplication event, that of the *ADH* gene, also contributed to the evolution of the Crabtree effect. *S. cerevisiae*'s genome encodes two *ADH* proteins: *ADH1* reduces pyruvate to ethanol and is expressed constitutively, and *ADH2* reconverts the ethanol to acetaldehyde and is only expressed when the sugar concentration inside of the yeast cell drops. After most sugars in the environment are depleted, the yeast cell switches to use ethanol as a carbon source using *ADH2* (diauxic shift). Interestingly *ADH2* did not result from the whole genome duplication event. Using a molecular clock the *ADH* duplication event can be dated back ~80 Mya and before this event, the ancient *ADH* was predominantly involved in generating ethanol instead of consuming it (Thomson et al., 2005). Selection for all of these events are thought to be driven by the increased availability of sugar in the environment caused by the proliferation of angiosperms (flowering plants) which became widespread in the natural world around the time the Crabtree effect may evolved (Lin and Li, 2011; Piškur et al., 2006; Thomson et al., 2005) The first angiosperms appeared in the fossil record 125 Mya years ago and were widespread by the time of the extinction of the dinosaurs (~65 Mya) (Moore et al., 2007). Researchers have proposed that the Crabtree effect evolved as an adaptation to exploit the new supply of sugar produced by these plants in nectar and fruits (Lin and Li, 2011; Piškur et al., 2006; Thomson et al., 2005).

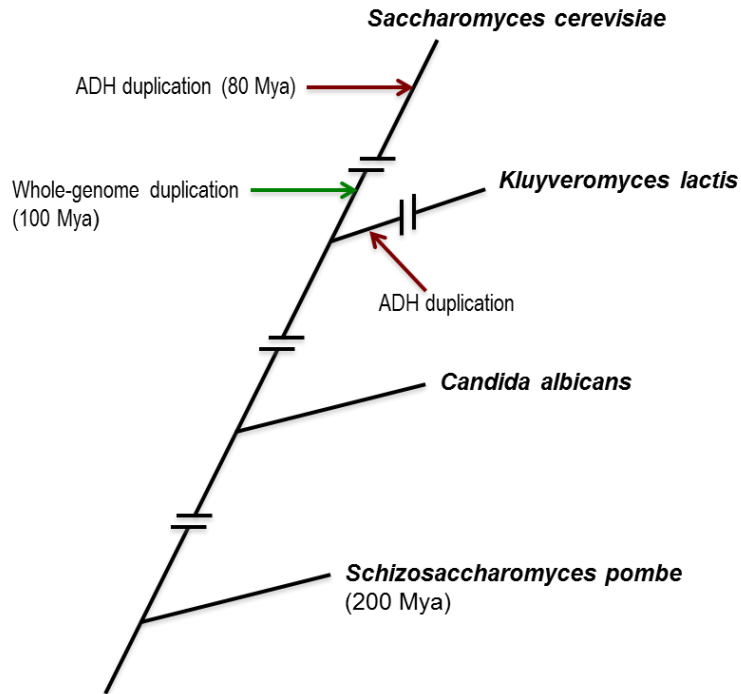


Figure 3: Evolution of the Crabtree effect. *Schizosaccharomyces pombe* separated from the *Saccharomyces–Kluveromyces* lineage at least 200 Mya ago, is capable of anaerobic fermentation and subsequent consumption of the ethanol for aerobic respiration, but not as its only carbon source. *S. pombe* evolved the Crabtree effect independently. *Candida albicans* and *K. lactis* are Crabtree negative yeasts. *K. lactis* can poorly ferment under anaerobic conditions but can efficiently use ethanol as its only carbon source, as a result of its own *ADH* duplication events. *C. albicans* still exhibit the original traits of the yeast progenitor: little accumulation of ethanol under aerobic conditions and strong dependence on the presence of oxygen (Information and picture modified from: Piškur et al., 2006).

Some discrepancies concerning the fruit adaptation hypothesis: There are also indicators that the *Saccharomyces*/fruit adaptation is not true. Competing hypotheses suggest that the Crabtree effect originated earlier than the appearance of the angiosperms in the evolution of Ascomycetes, and was not, therefore, an adaptation to fruit sugars, but was later lost in several lineages. Alternatively, the Crabtree effect originated independently in several lineages before angiosperms evolved. Finally, the Crabtree effect may have evolved over a long period of time, coinciding with the evolution of flowering plants, but is not necessarily an adaptation to them (Hagman et al., 2013). Likely due to the sugary environments in which the Crabtree effect plays an important role like the fermentation of grape juice (sugar content ~200 g/l (Sanz et al., 2004)) it was previously thought that high sugar conditions are necessary for the yeast to undergo aerobic fermentation. In fact, fermentation under aerobic conditions happens at a glucose concentration as low as 150 mg/l (Pfeiffer and Morley, 2014; Verduyn et al., 1984). Finally, it is important to mention that the fermentation environment of wine making --with a mass of smashed grapes building a pretty homogeneous environment with high heat and accumulating

ethanol-- is clearly pretty different from a fruit in the wild. So even if it seems logical that the wine yeast *S. cerevisiae* is adapted to live on grapes and other sugary environments, this is not necessarily true (reviewed in Kunkee et al 1970). Indeed, recent studies have demonstrated that *Saccharomyces* yeast are not as abundant on grapes or other sugar rich fruits as previously thought. When present on fruits, they are present in very small numbers and can only be detected using intensive enrichment isolation techniques (Vaughan-Martini and Martini, 1995). Only about one in 1000 intact grape berries have been estimated to carry *S. cerevisiae*, although this number can increase when the grapes get damaged (Mortimer and Polsinelli, 1999). This picture is supported by the modern molecular technique of pyrosequencing which finds *Saccharomyces* sequences in ripe vineyard grapes at a frequency of only one in 20 000 reads (Taylor et al., 2014).

4.2.2. *Saccharomyces*: adapted to oak habitat?

Wild *Saccharomyces* species (including wild *S. cerevisiae*) have consistently been detected in a very different habitat from vineyards; the surface of (and soil surrounding) hardwood trees, especially oaks (e.g. Charron et al., 2014; Glushakova et al., 2007; Johnson et al., 2004; Koufopanou et al., 2006; Sláviková et al., 2007; Sniegowski et al., 2002; Sylvester et al., 2015; Yurkov, 2005; Zhang et al., 2010).

Consistent isolation of wild *Saccharomyces* from oaks all around the world: The fact that *Saccharomyces* yeasts can be consistently isolated from oak surfaces at every season all around the world is an indicator that the yeast is strongly connected with this environment. The earliest isolation of *S. paradoxus* recorded in literature was 1914 from Russian oak exudates and later 1957 from the bark and surrounding soil of oak, as well as from soil surrounding pine (Yoneyama 1957). Ever since these original isolations, scientists have focused on oaks as source for *Saccharomyces* with a recent focus on the association between wild *Saccharomyces* and oak bark (Johnson et al., 2004; Koufopanou et al., 2006; Robinson et al., 2015; Sampaio and Gonçalves, 2008; Zhang et al., 2010). Nearly all isolates of *S. paradoxus* have come from oak. A recent survey of *S. paradoxus* available from culture collections found 81 % came from oak (Bozdag and Greig, 2014) and also *S. cerevisiae* can frequently be isolated from the oak environment (Naumov et al., 1998; Sampaio and Gonçalves, 2008; Sniegowski et al., 2002). In the Southern Hemisphere *Nothofagus* trees (southern beeches) inhabit the ecological niche of oaks, and cryotolerant *Saccharomyces* species can be found instead on these species (Libkind et al., 2011). In addition, although tree surfaces seem to be a nutrient poor habitat for the yeast that shows all these potential adaptations (e.g. Crabtree effect) to compete well in sugar rich

environments, the surface of oaks would feature one important advantage; it is a very constant habitat available all the year around and this is clearly not the case for fruits.

The yeast/oak association: a circular statement? The isolation success from oak samples varies considerably across different host trees, countries, sampling methods, sample masses and isolation protocols. Even with intensive enrichment, *Saccharomyces* is not detected in the majority of samples. The oak association story might have arisen through confirmation bias: original reports of *Saccharomyces* on oaks led other researchers focusing their attention on this environment, where more yeasts were found (at low levels), encouraging subsequent researchers to look, once again, at oaks when searching for new wild isolates. Wild yeasts are difficult to find, so it is understandable that researchers would not want to waste time looking for yeast on sources that had not been previously identified. Another famous example for such a misinterpretation is the model nematode *C. elegans* that has been found first in 1897 and was referred as being a soil nematode in literature since then. It took many years of work from *C. elegans* researchers grubbing through soils all over the world to realize that this is simply not the true natural source for the worm which is rather a colonizer of various microbe-rich habitats, particularly decaying fruit and plant material (Félix and Braendle, 2010).

4.2.3. *Saccharomyces adapted to both; fruit and oak habitat?*

Potentially both hypotheses are correct and *Saccharomyces* is adapted to the fruit as well as the oak habitat. As sugar rich habitats are not available all the year around *Saccharomyces* would need a place where it can stay in the meantime and bark might be used as “winter refuges” by the yeast. Knight and Goddard (2015) introduced the “fruit forest-reservoir hypothesis” suggesting that *S. cerevisiae* exists in a sporulated stage as a diffuse low abundance reservoir in different forest niches such as soil and tree bark, from which it is vectored to sugar rich fruits by insects, where it proliferates and in the end of the fruiting season some fraction of this population will return back to the forest environment. This hypothesis has actually some similarities with the already described (4.2.1.) earliest ecological hypothesis of Emil Christian Hansen that the yeast cells live on sugary fruits, will be washed down by rain, survive the winter in the soil and will be transported back by wind activity to the new fruits in the next season. The transportation by wind activity seems unlikely from our knowledge today but there are possibilities of cross-vectored habitat changes for *Saccharomyces*.

How *Saccharomyces* disperses between substrates remains poorly understood. These yeasts are not generally airborne and are therefore not assumed to be moved around by wind activity (Garijo et al., 2011; Mortimer and Polsinelli, 1999). They have been detected in the air of vineyards in some studies, but these observations were always associated with harvest (Adams,

1964) or the vinification process (Garijo et al., 2008; Ocón et al., 2013). Instead, animals seem to play a role in vectoring the yeast. Living cells have been isolated from bird cloacae and detected up to ~ 12 hours post-ingestion, leading to the hypothesis that the yeast could be disseminated during bird migration (Francesca et al., 2012). Humans also contribute to a wide yeast dispersal by shipping oak barrels around the world (Goddard et al., 2010). Insects may be the most ecologically important vectors of yeast. *S. cerevisiae* has been isolated from bees (Goddard et al., 2010) and in addition Stefanini et al. (2012) could demonstrate that social wasps act as vector and natural reservoir of *S. cerevisiae* during all seasons. *Drosophila* fruit flies are so far the most common insects associated with *Saccharomyces* yeasts (Ivannikova et al., 2006; Naumov et al., 2000; Phaff et al., 1956, Buser et al., 2014). *S. cerevisiae* spores, but not vegetative cells, can survive the digestive tract of *Drosophila* (Reuter et al. 2007). Consistent with that, *S. cerevisiae* cells produce several secondary metabolites that were proven to attract *Drosophila* flies (Becher et al., 2012; Buser et al., 2014; Christiaens et al., 2014) which can vector the yeast from one habitat to another making it possible that the yeast switches between environments.

4.2.4. *Saccharomyces: not adapted to any specific habitat*

It is possible that we might have to consider other hypotheses besides the classical niche adaptation theory, for example that *Saccharomyces* is not adapted to a specific niche but instead has evolved the general ability to inhabit many very different environments (Goddard and Greig 2015). The extreme focus on the fruit and oak environment for sampling *Saccharomyces* creates a wrong picture. Systematic sampling studies comparing many different environments are rare and often suffer from non-standardized sampling procedures. Comparisons between different studies are even more difficult as different sampling and enrichment methods are used. The standard *Saccharomyces* isolation protocols for sugar rich as well as low sugary plant surfaces are done using enrichment media, which means that a sample is placed into medium that favors aerobic or anaerobic fermentation by yeast and may sometimes be spiked with ethanol. This method works, but eliminates any information about the natural yeast's abundance in a sample. The enrichment culturing methods commonly used can likely lead to an over- or underestimation of *Saccharomyces* in specific environments. In some natural samples the possible occurrence of strong competitors which might out-compete single *Saccharomyces* cells in liquid enrichment could lead to an underestimation of the yeast's presence. In return, the lack of such competitors in other environments, might misleadingly overestimate the presence of *Saccharomyces*, which then can dominate the culture with a single cell inoculation. Thus, the association between the yeast and oaks or fruit may be an artefact caused by this bias and indeed, yeast may grow better elsewhere, or may even be found at low

levels on most surfaces. We might find *Saccharomyces* everywhere if we would only look. An unbiased, systematic survey of the abundance of wild yeast across a broader range of potential habitats would be very useful to identify biotic and abiotic factors important for the life history of *Saccharomyces* yeasts.

Scope of this thesis

The extensive existing knowledge about *S. cerevisiae* based on studies in the lab or in human-made fermentations is unrivaled. Combining genetic, ecological and evolutionary research gives us the attractive opportunity to create the first “general” model organism in biology: a complete, defined system, suitable for answering questions from any biological perspective (Replansky et al., 2008).

The aim of this thesis is to shed some light on the natural ecology of *Saccharomyces* yeast and to specifically search for the natural environment of the yeast.

Wild populations of *Saccharomyces* are widely considered to be associated with oak bark, a very different environment from wine fermentations. Besides the vineyard environment no other environment has been as extensively sampled as oak bark but the performance of *Saccharomyces* yeast in this environment remains uncharacterized. In **Chapter I** I show that a wild-isolated *S. paradoxus* strain grows well on a medium consisting of sterilized oak bark and water but is strongly inhibited by the presence of natural microbial competitors in unsterile oak medium. To identify candidate competitors, I sequenced the oak bacterial and fungal community and isolated a set of representative microbes and tested their effects on *S. paradoxus* growth in the natural medium under different conditions (spatial structure and temperature). I observed diverse effects ranging from direct killing to mutualism that were strongly influenced by temperature. This work has been published in *Molecular Ecology* (03/2015): Kowallik, V., Miller, E., and Greig, D. (2015). The interaction of *Saccharomyces paradoxus* with its natural competitors on oak bark. *Mol. Ecol.* 24, 1596–1610.

To understand microbial ecology and biodiversity one needs to consider both biotic and abiotic factors. In a natural environment almost all microbes exist in complex communities and will not only respond to the chemical environment but also have to deal with different abundances and diversities of other microorganisms. In **Chapter II**, I provide a more detailed experimental analysis of the interactions between *S. paradoxus* and two key oak bacteria: *Pseudomonas* and *Mucilaginibacter*. I found that the two bacteria undergo contraire interactions with the yeast; *Pseudomonas* completely dominates the interaction and actively kills *S. paradoxus*, but is not affected by the presence of the yeast nor by different temperatures. In contrast *Mucilaginibacter* promotes the growth of the yeast and this effect is only apparent in the complex, natural medium and is strongly dependent on different incubation temperatures.

Thus, not only the identity of competing microorganisms has a dramatic effect on the fitness of *S. paradoxus* but the interaction with these competitors in turn strongly depends on abiotic conditions.

In Chapter III, I show how our understanding of yeast metabolism depends upon the environment in which it is studied. The Crabtree effect, the peculiar behavior to ferment in the presence of oxygen is handled as being evidence that *Saccharomyces* yeasts are adapted to high sugar environments. Fermentation provides less energy than respiration does, but it provides energy faster, so when competition for a sugar resource occurs, natural selection may favor individuals that perform inefficient fermentation over those that use efficient respiration. An alternative hypothesis is that ethanol, heat and acids produced by fermentation interfere with competitors, reducing their fitness. Comparing a genetically modified Crabtree negative *S. cerevisiae* mutant strain with its Crabtree positive ancestor I show that the form of competition greatly affects the benefit of the Crabtree effect. The fermenting strain ends up having a lower yield in monoculture but a higher fitness when competing directly against the respiring type in sterile laboratory medium. This result is highly contingent on the medium the assays are conducted on, showing that the fermenter has surprisingly no benefit using the more natural grape juice medium. However, the advantage of the Crabtree effect was revealed in the presence of natural competitors where the fermenting strain exhibited much higher fitness than the obligate respirer. The dependence of the fitness consequences of the Crabtree effect on the presence of natural competitors indicates that fermentation by-products play a role in competing against other microorganisms. Indeed it shows how our interpretation of genetic and physiological differences between strains requires an understanding of the ecological context in which they arose.

While sequencing the oak bark microbiome revealed numerous bacterial and fungal species that can interact with *Saccharomyces*, it failed to identify any trace of the yeast itself. Furthermore, consistent with the available literature, I found very few *Saccharomyces* yeasts on oak bark using traditional sampling and enrichment culture techniques. Indeed, although differently reported in literature, existing evidence that *Saccharomyces* yeasts are specifically associated with oak trees is weak, and may have arisen simply from biased collection regimes. In **Chapter IV**, I did a systematic survey in a mixed forest in Northern Germany and did not only rely on enrichment presence/absence data but quantified the number of *Saccharomyces* cells showing that the yeast is present at much higher densities in the leaf litter surrounding these trees than on their bark. In addition the abundance of yeast decreased with distance from an

oak tree and comparing different tree genera revealed that oak trees are indeed the best habitat for *Saccharomyces*. Systematic, bi-monthly sampling over the course of one year gives further insight into abundance and distribution patterns of *Saccharomyces* and revealed tree-to-tree as well as seasonal variation.

The interaction of *Saccharomyces paradoxus* with its natural competitors on oak bark

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Abstract

The natural history of the model yeast *Saccharomyces cerevisiae* is poorly understood and confounded by domestication. In nature, *S. cerevisiae* and its undomesticated relative *S. paradoxus* are usually found on the bark of oak trees, a habitat very different from wine or other human fermentations. It is unclear whether the oak trees are really the primary habitat for wild yeast, or whether this apparent association is due to biased sampling. We use culturing and high-throughput environmental sequencing to show that *S. paradoxus* is a very rare member of the oak bark microbial community. We find that *S. paradoxus* can grow well on sterile medium made from oak bark, but that its growth is strongly suppressed when the other members of the community are present. We purified a set of twelve common fungal and bacterial species from the oak bark community and tested how each affected the growth of *S. paradoxus* in direct competition on oak bark medium at summer and winter temperatures, identifying both positive and negative interactions. One *Pseudomonas* species produces a diffusible toxin that suppresses *S. paradoxus* as effectively as either the whole set of twelve species together or the complete community present in nonsterilized oak medium. Conversely, one of the twelve species, *Mucilaginibacter sp.*, had the opposite effect and promoted *S. paradoxus* growth at low temperatures. We conclude that, in its natural oak tree habitat, *S. paradoxus* is a rare species whose success depends on the much more abundant microbial species surrounding it.

Keywords: bacteria, competition, ecology, fungi, natural history, *Saccharomyces*

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Introduction

The ability of *Saccharomyces cerevisiae* to transform grape juice into wine has made it an integral component of human culture, and fermentation can be considered an ancient form of biotechnology (Walker 1998). In more recent times, *S. cerevisiae* has also become one of the best-studied laboratory model organisms. In grape juice or in sugar-rich laboratory media, *Saccharomyces* yeasts ferment anaerobically, even when oxygen is available for aerobic respiration. This trait, known as the 'Crabtree effect', has two potential benefits: it allows more rapid (but less energetically efficient) growth than

aerobic respiration and it produces toxic ethanol which might inhibit natural competitors. Additionally, ethanol can later be consumed using aerobic respiration, and some of the energy wasted in fermentation can be recovered (Piskur *et al.* 2006; Goddard 2008). It has been proposed that the Crabtree effect evolved when fermentable fruit sugars became abundant following the radiation of angiosperms about 100 million years ago (Piskur *et al.* 2006). Initially, *S. cerevisiae* was thought to be a domesticated species, artificially selected by humans over the last 10 000 years to make alcoholic drinks and to raise bread (Vaughan-Martini & Martini 1995). However, recent phylogenetic analysis reveals the existence of a wild *S. cerevisiae* population in addition to clades associated with both grape wine and rice wine (Fay & Benavides 2005). Wild populations of

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2 V. KOWALLIK, E. MILLER and D. GREIG

S. cerevisiae have also been identified in primeval forests, far from human influence (Wang *et al.* 2012). Because of its use as a model organism, we know the basic biology of *S. cerevisiae* very well, but its natural history in the wild is almost entirely a mystery (Greig & Leu 2009).

Attempts to study *S. cerevisiae* in the wild are complicated by domestication. Whilst wild populations certainly exist, there is a risk that any individuals isolated from a natural source may have originated from human fermentations, or have recently interbred with domesticated strains. Researchers interested in the ecology and natural history of yeast therefore usually focus on the closest known relative of *S. cerevisiae*, *Saccharomyces paradoxus* (Replansky *et al.* 2008). The two species are phenotypically nearly indistinguishable 'sibling species' (Naumov 1987), sharing the same profiles of assimilation and fermentation of organic compounds (Vaughan-Martini & Martini 1998), suggesting that they evolved in similar environments, and indeed they can be found in sympatry in nature (Sniegowski *et al.* 2002; Sampaio & Gonçalves 2008). However, *S. paradoxus* is not domesticated and is not found in human alcoholic fermentations. *S. paradoxus* is therefore an ideal subject for natural studies, both for its own sake and for inferring the ecology and natural history of wild *S. cerevisiae*, which is expected to be similar (Replansky *et al.* 2008).

Oak trees are widely thought to be the principle natural habitat for *S. paradoxus*, *S. cerevisiae* and other *Saccharomyces* species (Sniegowski *et al.* 2002; Replansky *et al.* 2008; Sampaio & Gonçalves 2008). Given how well adapted *S. cerevisiae* is to growth at high density in sugar-rich fermenting grape juice, it is surprising that it lives on the relatively nutrient-poor surface of a tree, but nevertheless oaks are the main source of wild yeast for researchers. A recent survey of all *S. paradoxus* strains available from culture collections comprised 65 isolates from oak trees or from soil under oaks, 15 isolates from maple trees and none from other sources (Bozdag & Greig 2014). Despite this apparent association with oak, *Saccharomyces* is not detected in the majority of oak samples. Zhang *et al.* (2010) found *Saccharomyces* in only 24% of New Zealand oak samples. On oaks and surrounding soils in North America, 23% of samples contained *S. cerevisiae* or *S. paradoxus* (Sniegowski *et al.* 2002). *S. paradoxus* occurred in 0%, 10% and 28% of oak samples from southern England, depending on the tree and the sampling date (Koufopanou *et al.* 2006). Repeated sampling of 86 oak trees gave an overall success rate of 8%, but 70% of trees never yielded positive samples (Johnson *et al.* 2004). A survey of different Mediterranean oaks and closely related trees belonging to the *Fagaceae* family found *Saccharomyces* in over 70% of *Quercus pyrenaica* and *Quercus faginea*

samples, but the same isolation methods were successful on 45% of *Quercus garryana* samples from Canada and on just 8% of *Quercus robur* samples from Canada and Germany (Sampaio & Gonçalves 2008). A more recent survey of Canadian trees found *S. paradoxus* in 12% of samples coming from trees belonging to the *Fagaceae* family (mostly oak species) and in 4% of maple samples (Charron *et al.* 2014). The low and variable rates of detection of *S. paradoxus* on oak, as well as the contrast between oak and the artificial winemaking environment in which *S. cerevisiae* thrives, raise the possibility that oak is not the main habitat for *S. paradoxus*. We have successfully isolated *S. paradoxus* from oak, larch, beech and spruce trees in the same forest (V. Kowallik & D. Greig, unpublished data). But we know of only one published study that uses a standardized, well-described sampling protocol that allows the abundance of yeast on oak bark to be compared directly to other potential habitats: Sampaio & Gonçalves (2008) found that oaks and other closely related species in the family *Fagaceae* were about three times more likely to yield *Saccharomyces* than other tree species. An unbiased, systematic survey of the abundance of wild yeast across a broader range of potential habitats would be very useful, but many wild yeast researchers want isolates for genetic analysis and so are not motivated to quantify yeast abundance in different habitats, or to sample new habitats in which yeast may not be discovered. Further, the way in which yeast is typically isolated from environmental samples may give little indication of its true abundance.

Inferring the abundance of *S. paradoxus* on oak, or another potential habitat, from the proportion of samples in which it has been detected is problematic. Many authors only need examples of wild yeast for population genetic analysis and so do not report standardized sampling procedures that would permit comparisons between studies. But the main problem is the method by which yeast is isolated from the samples. Typically, an environmental sample, such as a piece of oak bark, is incubated for a few days in a sugar-rich liquid medium, which is sometimes spiked with ethanol. These 'enrichment culture' conditions, which approximate the winemaking process, can favour the growth of Crabtree-positive *Saccharomyces* over other microbial species, allowing the yeast to outcompete them and to dominate the culture so that it can easily be purified. However, the successful isolation of *Saccharomyces* from an enrichment culture gives no indication as to the number of *Saccharomyces* cells in the original sample: the *Saccharomyces* that takes over an enrichment culture may, in principle, be derived from a single yeast cell in the original sample, or they might already be the dominant microbe in the sample. Worse, the failure of an

enrichment culture to yield *Saccharomyces* cannot be taken to mean that it was absent from the sample: it is possible that it was present but was outcompeted by other species. Variation in the proportion of enrichment cultures that successfully yield *Saccharomyces* may therefore reflect variation in the microbial community, rather than in *Saccharomyces* abundance.

Given these uncertainties, we decided to investigate the perceived association between yeast and oak, comparing traditional culturing methods, which suffer from well-known biases (Kell *et al.* 1998) including those discussed above, to culture-free high-throughput environmental sequencing, which suffers from different set of potential biases (e.g. Polz & Cavanaugh 1998; DeSantis *et al.* 2005; Feinstein *et al.* 2009; Delmont *et al.* 2011). As far as we know, this is the first description of this well-known wild yeast habitat using environmental metagenomics, although another recent study used the method to examine the domestic *S. cerevisiae* community in its vineyard habitat (Taylor *et al.* 2014). We first surveyed local oak trees for *S. paradoxus* using an enrichment culture isolation method. We then tested the sensitivity and repeatability of the enrichment assay by attempting to re-isolate known numbers of oak-isolated cells that we 'spiked' into samples that had previously yielded no yeast. We used these data to estimate the average density of *S. paradoxus* on the oak trees we sampled. Finding the density to be low, and given apparent disparity between the winery and the oak environment, we tested how well wild *S. paradoxus* could grow on medium containing only oak bark extract. We found that it grew well in sterile monoculture, but was strongly inhibited by the natural microbial community on the bark. We determined the composition of this community by high-throughput sequencing the microbial metagenome of oak bark from trees containing *S. paradoxus* by enrichment culture. We found no *Saccharomyces* sequences among the samples, confirming both the very low frequency of *S. paradoxus* within the community and the effectiveness of enrichment culturing method for detecting. To investigate the influence of more common microbial species on the success of *S. paradoxus*, we competed a suite of representative members of the microbial community directly with *S. paradoxus* in both solid and liquid oak bark extract medium at summer (26 °C) and winter (5.5 °C) temperatures, finding both positive and negative interactions.

Materials and methods

Isolation of S. paradoxus from oak

Sampling Plön oaks. We sampled a set of 22 oak trees in Plön, northern Germany on 26–28 October 2010 and

on 13–14 January 2011 (see Table S1, Supporting information). We used two sampling methods: a sterilized increment hammer which removed ~4-mm-diameter plugs of bark, and sterile cotton buds moistened with sample solution (20% glycerol, 0.1% Tween 20) and rubbed against the bark in a ~4-mm-diameter spot. Samples were taken in groups of four in a 10 cm by 10 cm square; samples in groups of eight were taken directly above and below the vertices of this square. We resampled the same part of each tree on both occasions. Each bark sample (plugs, or the heads of the cotton buds) was vortexed and stored at –80 °C in 2 mL sample solution.

To make enrichment cultures, we first thawed and mixed each sample. A 100 µL of each sample was added to 900 µL of ME (malt extract medium: 5% malt extract, 0.4% lactic acid w/v) and to 900 µL YEPD (1% yeast extract, 2% peptone, 2% glucose). Enrichment cultures were incubated whilst shaking at room temperature for 2 days and tested for the presence of tetrad-forming yeast by spreading 5 µL onto sporulation agar (2% potassium acetate, 0.22% yeast extract, 0.05% glucose, 0.087% complete amino acid mix, 2.5% agar) and incubating for 2 days at room temperature before examining for tetrads using a microscope. Enrichment cultures containing tetrad-forming yeasts were purified by streaking to single colonies. Candidate colonies were tested to see whether they would mate with a haploid *S. paradoxus* tester strain [ho::KMX, lys5-], identifying them as members of the *Saccharomyces sensu stricto* group, and whether the resulting cross could produce viable meiotic spores, identifying them as *S. paradoxus*. This application of the biological species concept to yeast was originally developed by Naumov (1987) and successfully resolved the phenotypically indistinguishable *Saccharomyces sensu stricto* species (Naumov *et al.* 2000, 2010; for a current review of the *Saccharomyces sensu stricto* species see Boynton & Greig 2014).

Determining the sensitivity of ME enrichment culture assay on Plön oaks. We constructed oak bark enrichment cultures containing known numbers of *S. paradoxus* tetrads to test the sensitivity of our enrichment protocol. Tetrads are four haploid spores in an ascus and are built under starvation via meiosis by diploid cells. These spores are dormant and resistant, but germinate into metabolically active haploid gametes when returned to rich medium. As it is likely that we isolate *S. paradoxus* tetrads from oak bark, we decided to use tetrads in the sensitivity assay because other microbes present in the oak wash could outcompete *S. paradoxus* in the time the tetrads need to germinate.

We thawed and mixed together all the sample solutions from January 2011 that did not test positive for

4 V. KOWALLIK, E. MILLER and D. GREIG

S. paradoxus. We inoculated 120 900 μL ME cultures with 100 μL from this pool of previously negative sample solutions to create 'unspiked' cultures. We tested for the presence of *S. paradoxus* (see 1.1 above). Simultaneously, we tested 264 additional enrichment cultures that were initiated in the same way but which were also 'spiked' with *S. paradoxus*. To do this, we grew eleven strains of *S. paradoxus* (previously isolated from the oak tree: see 1.1 above) overnight in liquid YEPD. We serially diluted the cultures and plated onto YEPD agar plates to determine the density by colony counts. Simultaneously, we diluted to 10^{-6} and used 10 μL to inoculate each 'spiked' culture with the overnight culture, for 24 'spiked' cultures for each of the eleven *S. paradoxus* strains. This resulted in adding an average of 1.7 tetrads (range 1.3–2.8 cells) per spiked culture (see Table S1, Supporting information).

'Spiked' cultures that tested positive therefore contained *S. paradoxus* from either of two sources: from cells already present in the pool of previously negative sample solutions or from the diluted culture cells used to spike the samples. Using a Poisson distribution, we determined the probability that each enrichment culture was spiked with at least one cell. To determine the sensitivity of the assay, we used the following calculation:

a = observed proportion of 'spiked' enrichment cultures testing positive for *S. paradoxus*

b = probability of any enrichment culture testing positive if *S. paradoxus* cells present

c = Poisson-derived probability of a 'spiked' enrichment culture contains at least one cell of added *S. paradoxus*

d = probability that 'unspiked' enrichment culture contains *S. paradoxus*

$b*d$ is therefore the proportion of unspiked cultures that resulted in testing positive.

Proportion observed positive culture = Probability of 'spiked' culture containing at least 1 cell * Probability of detecting if cells are present + Probability of 'spiked' cultures not containing at least 1 cell * Probability of detecting if cells are present * probability that 'unspiked' cultures contain cells.

$$a = (c * b) + (1 - c) * (b * d)$$

Therefore, the probability of detecting any *S. paradoxus* cell, if present, is given by:

$$b = \{[a - (b * d)] / c\} + (b * d)$$

We also used a maximum-likelihood model as a second way to estimate the probability of detecting any *S. paradoxus* cell, if present (see Table S1 and Appendix S1, Supporting information).

Sampling Nehnten oaks. We focused on four oak trees (*Quercus robur*) in an old mixed oak/beech/spruce/larch forest in Nehnten, northern Germany for all the

following experiments on the oak microbial community. On 9 January 2013, we took 48 samples from each tree using wet cotton swabs but by putting the intact cotton swab directly in 1 mL ME. We streaked the ME samples onto sporulation medium and purified colonies. Because we intended to later use genetic and metagenomic sequencing methods to determine the microbial community (see 3.1 and 3.2, below) of these oak trees, individual *S. paradoxus* isolates were confirmed by Sanger sequencing the ITS region (see 3.1 below), rather than by mating to a tester strain as previously (1.1). There is good agreement between molecular and biological species definitions for *Saccharomyces sensu stricto* species (Naumov *et al.* 2000).

Growth of S. paradoxus on oak nutrients

We made oak bark infusion from the four local oak trees (above, section 1.3) as described by Belotte *et al.* (2003) for soil media preparation. Briefly, we placed 10 g samples of oak bark in sterile tea bags and incubated them in 150 mL sterile water for 24 h at room temperature. We sterilized oak bark infusion using one of three methods: autoclaving, filtration with a 0.22- μm filter and adding 0.4% G418 antibiotic (ENZO Life Sciences). To make oak bark infusion agar, we added 6% sterile agar for a final concentration of 1.5% agar.

For the growth study in sterile and unsterile conditions, we engineered strain Sp-Plön by inserting the KanMX4 cassette into both copies of its HO gene, thereby making it resistant to the G418 antibiotic (Goldstein & McCusker 1999). We grew Sp-Plön in ME overnight and determined the initial density through colony counts. A 30 μL of diluted culture (containing approximately 100 cells) was added to tubes containing 3 mL of the following 5 agar conditions: filter-sterilized oak bark infusion, heat-sterilized oak bark infusion, oak bark infusion with 0.4% G418, unsterilized oak bark infusion and YEPD. The inoculated tubes were incubated at room temperature for seven days. Growth on the surface of the agar in each tube was washed off with 1 mL H_2O , diluted and plated onto G418 agar plates (YEPD supplemented with 0.04% G418), which effectively suppress the growth of all microbes except the resistant strain Sp-Plön. We calculated the number of divisions Sp-Plön went through using the Malthusian parameter $\ln(\text{final number of cells}/\text{initial number of cells})/\ln(2)$ (Lenski *et al.* 1991).

Identification of oak micro-organisms

Identifying common oak micro-organisms by culturing. We serially diluted unsterile oak bark infusions from the four local oak trees (see 2, above), plating 100 μL of

10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} dilutions onto full-strength YEPD agar and 10% YEPD agar (0.1% yeast extract, 0.2% peptone, 0.2% glucose). Plates were incubated at room temperature and scored every second day for 10 days. We counted morphologically identical colonies and purified each morphotype by restreaking to new plates of the same nutrient concentration.

DNA was extracted from purified colonies using the MasterPure™ Yeast DNA Purification Kit (Epicentre) according to the manufacturer's instructions. Bacteria were identified by amplifying and Sanger sequencing the 16S region using Universal8f (AGAGTTTGA TCCTGGCTCAG) (Turner *et al.* 1999) and Universal1492r (ACGGCTACCTGTACGACTT) primers (Weisburg *et al.* 1991). Fungi were identified by amplifying and sequencing the ITS region using ITS1f (CTTGGTCATTTAGAGGAAGTAA) (Gardes & Bruns 1993) and ITS4 (TCCTCCGCTTATGATATGC) (White *et al.* 1990). Taxonomy was determined with NCBI BLAST search using Geneious Pro v. 6.1.6. Altogether, we sequenced 48 colonies from YEPD and 36 from 10% YEPD.

High-throughput sequencing of the oak microbiome. We used 454 sequencing of the 16S and ITS regions of microbial communities from four oak trees to determine species composition without culturing biases. The sampling scheme illustrated in Fig. 2 was devised to allow us to compare the microbial community in oak infusion with the total microbial bark community, to examine microbial variation within and between trees and to compare the sampling methods (small whole bark pieces and infusions made out of bark). We made a 300 mL oak infusion from each tree as described in section 2 with 20 g of oak bark. Infusion pellets were created by centrifugation. We also sampled four individual whole pieces of oak bark of approximately 1 g (~1 cm² external surface) from Tree 1 and one piece from each of the other three trees. We ground bark pieces and infusion pellets using a bead-beating machine (Precellys® Peqlab). DNA was extracted from all samples using the Soil DNA Kit from Omega Bio-Tek according to the manufacturer's protocol. The resulting ten DNA pellets were resuspended in 20 µl TE buffer and sent to LGC Genomics (GmbH, Berlin, Germany) for amplification of the fungal ITS (ITS1, 5.8S and ITS2) sequences using ITS1f (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATGATATGC) primers (Gardes & Bruns 1993; White *et al.* 1990) and of the bacterial 16S rRNA (V1 to V5) sequences using GM3 (AGAGTTT GATCMTGGC) and 926R (CCGTCAATTCMTTGGAGT TT) primers (Muyzer *et al.* 1995, 1996). PCR conditions were 30 cycles, 30 s 95 °C, 30 s 50 °C and 60 s 72 °C, performed with the polymerase Kapa2G Enzym (Kapa

Biosystems). The DNA from each sample was pooled and run on a 1/8 PicoTiterPlate on 454 GS FLX+ Titanium sequencer (Roche). A total of 127 694 ITS and 134 630 16S sequence reads were obtained.

16S sequences were analysed with the software package MOTHUR version 1.31.2 (Schloss *et al.* 2009). Raw reads were processed by trimming the primer sequences and any terminal sections with a mean quality score of 35 using a 50-bp sliding window. Resulting sequences <500 nucleotides were discarded. We removed homopolymers with greater than ten repeats using the trim.seqs command. Reads were randomly sequenced from both the forward and the reverse sites, so we used only the overlapping regions, reducing the average length of the sequences to ~330 bp. Sequences were then aligned to the comprehensive seed database from SILVA (Pruesse *et al.* 2007) downloaded on 29 April 2013. All potentially chimeric sequences were identified and removed with the motthur embedded de novo uchime function (Edgar *et al.* 2011). Sequence classification was performed using the motthur implementation of naive Bayesian classification based on the RDP Classifier (Wang *et al.* 2007) version 9, with a threshold bootstrap value of 70% for each taxonomic level. We removed all sequences classified as 'mitochondria', 'chloroplast', 'archaea', 'eukaryota' or 'unknown'. To allow fair comparisons of the microbial diversity to be made between the ten oak tree samples, we normalized the remaining sequences from every sample to 4000 sequences per sample. We created a distance matrix of aligned sequences and clustered them into operational taxonomic units (OTUs) at 97% sequence similarity using the average neighbour clustering algorithm. We analysed a total of 40 000 bacterial reads, from which 3604 OTUs were detected on 97% identity level.

ITS sequence reads were also quality processed using motthur. Reads were trimmed to a minimum length of 350 bp with the same parameters as described above. Chimeric sequences were detected and removed, and the sequence classification performed as described for the 16S sequences. We removed all sequences classified as 'chloroplast', 'archaea', 'bacteria' or 'unknown'. Each sample was normalized by subsampling to 4000 sequences per sample. For taxonomic classification, we used the same parameters as described for the 16S sequences and the 'dynamic' motthur release from 08.12.2013 of the UNITE database containing 40 679 representative sequences (RepS) and 2 441 reference sequences (RefS). All reference sequence sets represent a nonredundant version of all fungal rDNA ITS sequences in the current UNITE+INSD (International Nucleotide Sequence Databases) release of circa 350 000 sequences clustered approximately at the species level. All OTUs composed of two or more sequences are

6 V. KOWALLIK, E. MILLER and D. GREIG

referred to as species hypotheses (SHs) (Köljalg *et al.* 2013). The 'dynamic' representative sequence file contains varying threshold values. Additionally, we used the taxonomy file associated with each individual sequence of the UNITE database with minor reformatting. We did not align our ITS sequences to the database as our ITS sequences are too variable in length for a global alignment. We used instead the 'pairwise.seqs' command in mothur for the OTU-based analysis with ignoring the penalization of the sequence ends. We analysed a total of 40 000 fungal reads, from which 2881 OTUs were detected on 97% identity level.

We calculated Shannon's diversity indices and community similarities to investigate whether samples differ in microbial diversity according to sampling method and/or tree location. All statistical tests were performed with the statistical software R Version 0.98.977 (R Core Team 2012) based on OTUs at 97% similarity. We investigated whether sampling effort accounted for all OTUs present using intersample rarefaction curves produced using mothur with 1000 randomizations (Fig. S1A,B, Supporting information). Within-sample diversity (alpha diversity) was calculated using Shannon's index (Shannon & Weaver 1949) using the VEGAN package in R (Oksanen *et al.* 2013) (Fig. S2A,B, Supporting information). We verified that our data were normally distributed before testing for significant differences in alpha diversity using *t*-tests.

To determine whether the samples are more similar according to sampling method or source tree, we first calculated the Bray Curtis dissimilarity, a statistic used to quantify the community compositional differences between different sites. The Bray Curtis dissimilarity is ranged between 0 and 1, where 0 indicates the sites have the same composition (share all the species) and 1 indicates that the sites are completely different and do not share any species. To visualize and interpret these differences, we used the ordination technique nonmetric multidimensional scaling (NMDS), which uses rank orders based on the Bray Curtis dissimilarity, to collapse the multidimensional dissimilarity matrix into two arbitrary dimensions, so that it can be plotted. Bray Curtis dissimilarities were calculated using mothur, and NMDS plots were produced using VEGAN package in R. We further tested for significant differences ('location effect') between method, tree and interaction between method and tree using analysis of dissimilarity (ADONIS), implemented in VEGAN. ADONIS is a multidimensional analysis of variance on the Bray Curtis dissimilarity matrix.

Selection of oak microbes for competition experiments. We selected the twelve most common bacteria and fungi (six isolates each) isolated from the culturing assay (3.1,

above) to represent a simplified and experimentally tractable oak community for further competition experiments with *S. paradoxus*. We wanted to determine the abundance of these representative, culturable organisms within the oak bacterial and fungal microbiomes. This is not possible on the taxonomic 454 output because of the well-known problem of classifying a given sequence to the species level, especially for the bacterial 16S rRNA sequences (Fox *et al.* 1992), but also for many fungal ITS sequences (Schoch *et al.* 2012). So we determined the frequency of the specific ITS sequences of each our cultured species within the oak metagenome by building 'minor databases' containing our high-quality forward and reverse Sanger sequences and then searching these databases for the 454 16S or ITS sequences of the cultured species. We used the mothur implementation of naive Bayesian classification based on the RDP Classifier (Wang *et al.* 2007), with a threshold bootstrap value of 90% for the taxonomic level. We also searched the database of ITS reads for the presence of *Saccharomyces* sequences.

Competition experiments between S. paradoxus and representative oak micro-organisms

To test how different species within the oak bark microbiome affect the growth of *S. paradoxus*, we competed it directly against the twelve representative microbial species (see 3.3, above) on both liquid and solid oak infusion medium (see 2, above) at summer (26 °C) and winter (5.5 °C) temperatures.

The *S. paradoxus* strain we used, Sp-Nehmten, was isolated from one of the four focal trees (Tree 2) used for the initial oak infusion growth experiments (see 2, above) and for the analysis of the oak microbiome (see 3, above). We engineered this strain to be resistant to the antibiotic G418 by replacing both its homologous copies of the *HO* gene with the *KanMX4* cassette (Goldstein & McCusker 1999). For liquid experiments at 26 °C, all twelve representative microbes as well as strain Sp-Nehmten were separately grown in 2 mL filter-sterilized oak infusion at 26 °C for seven days with shaking. All cultures were diluted 10² and 15 µL of the Sp-Nehmten culture was then transferred into 12 new tubes each containing 2 mL filter-sterilized oak infusion, followed by 15 µL of one of each of the twelve microbes. Three further tubes were prepared as controls: one was inoculated with 15 µL of Sp-Nehmten alone, one was inoculated with 15 µL of Sp-Nehmten into unsterilized oak bark medium, and one was inoculated with 15 µL of Sp-Nehmten as well as 15 µL of a mix of all the twelve microbial species. Tubes were mixed, and a sample was serially diluted and plated onto G418 agar plates to yield single colonies, which

were counted to determine the initial number of *S. paradoxus* cells. Tubes were incubated at 26 °C for 4 days with shaking, and then a second sample was taken from each tube and plated on G418 as before to determine the change in density of Sp-Nehmten. For the experiment in liquid at 5.5 °C, the incubations were conducted with shaking for 20 days. For the experiments on solid, tubes containing 2 mL oak infusion combined with 0.5 mL 6% agar were used without shaking, washed and plated (see 2, above). All experiments were independently replicated three times.

Halo assays

We performed halo assays (inhibition assays) to visualize how patches of each of the twelve representative microbes affected the local growth of a lawn of Sp-Nehmten. We grew all representative oak microorganisms individually to saturation in oak infusion as described in 4, above. A 200 µL of an overnight culture of Sp-Nehmten was pelleted by microcentrifugation, resuspended in 1.9 mL of 0.75% soft agar at 45 °C and plated onto a petri dish of YEPD agar. Attempts to visualize haloes on solid oak medium did not work, presumably because the low nutrient level in oak medium does not support large enough colonies, so instead we also compared haloes on 10% YEPD agar, which presumably lies somewhere between laboratory YEPD medium and natural oak bark in its nutrient richness. We then placed 5 µL of each of the twelve cultures at equally spaced intervals around the plate and incubated at 26 °C and 5.5 °C. We examined the plate daily for evidence of growth interference on the Sp-Nehmten lawn.

Results

Isolation of S. paradoxus from oak

Plön oaks. Overall, 14 of 352 primary samples from 22 Plön oak trees yielded wild *S. paradoxus* yeast (Table S1, Supporting information). We found the increment hammer and swab methods each yielded seven isolates. The positive samples came from only 9 of the 22 trees, but we did not find significant differences among the trees in their probability of yielding isolates ($P = 0.0522$, Fisher's exact test on a 22×2 contingency table); however, given the low rate of positive samples, we had limited statistical power to detect tree-to-tree variation. Given how close the P -value was to being critical, it seems that a future survey with more samples per tree might reveal tree-to-tree variation in *S. paradoxus* abundance. YEPD yielded 10 positive samples compared to 4 positive samples in ME, but this difference was not significant ($P = 0.175$, Fisher's exact test on a 2×2

contingency table), again as expected given the low power of the test. We decided to use ME for the remainder of this study, as this is one of the standard media for *Saccharomyces* isolation enrichment culture (Naumov *et al.* 1992; Johnson *et al.* 2004) and better prevented growth of filamentous fungi.

Sensitivity of malt extract enrichment culture assay. The 120 'unspiked' enrichment cultures inoculated with 100 µL from a pool of oak bark washes that had not previously yielded *S. paradoxus* yielded 14 further isolates of *S. paradoxus* (11.7%, see Table S1, Supporting information). In contrast, *S. paradoxus* was re-isolated from 243 (92%) of the 264 'spiked' enrichment cultures. We calculated the sensitivity of the assay (e.g. the probability of detecting a spiked cell when present, averaged across the eleven strains used for spiking, see Methods 1.2), as approximately 1, that is we expect all tetrads present to be detected. Using the Poisson distribution, the average estimated sensitivity was 1.1 (range 0.88–1.26) and using the maximum-likelihood model the estimated average sensitivity was 1.39 (range 0.60–2.30). Probabilities higher than 1 can best be explained by error associated with estimating the proportion of cultures with at least 1 spiked yeast tetrad. Also, probabilities higher than 1 could have resulted if tetrads in the 'spiked' cultures formed colonies on solid medium at a lower frequency than germination and growth in the liquid enrichment cultures used in the sensitivity assays.

Nehmten oaks. We focused on four oak trees in Nehmten for the remaining experiments. We took 48 bark samples from each of the four trees. *S. paradoxus* was isolated from three of the samples from Tree 2, two samples from Tree 3 and none of the samples from trees 1 and 4. We found no significant variation among the four Nehmten oak trees in the proportion of positive samples ($P = 0.177$, Fisher's exact test on a 4×2 contingency table).

Growth of S. paradoxus on oak nutrients

Figure 1 shows how the natural microbiota in oak bark affected the growth of Sp-Plön, a wild *S. paradoxus* genetically modified to be resistant to the antibiotic G418. The average number of Sp-Plön cell doublings on nonsterilized oak bark infusion is 3.2, but this increases to 10.9 cell doublings when G418 is added, or when the medium is autoclaved (11.9 cell doublings) or filtered (13 cell doublings). These three sterilization treatments significantly improved growth compared to unsterile medium ($P = 0.0024$, pairwise Wilcoxon rank sum test with Bonferroni correction). Filtered medium supported significantly better growth than either autoclaved

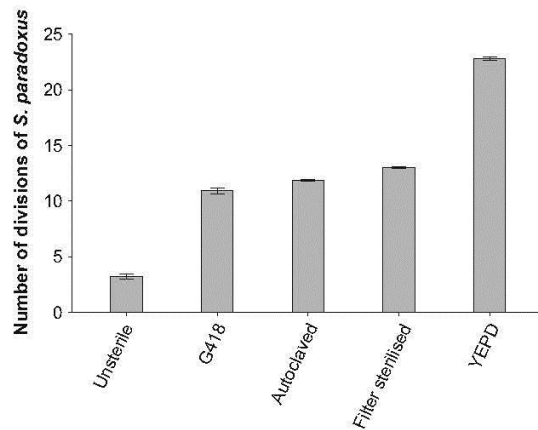


Fig. 1 The effects of sterilization on growth success of *Saccharomyces paradoxus*. Number of divisions of *S. paradoxus* on untreated, G418-amended, autoclaved and filtered solid oak infusion medium and in addition on solid high-sugar laboratory medium at room temperature after seven days. The plotted means are from nine measurements. Error bars indicate the standard errors of the means.

($P = 0.0024$) or G418-treated medium ($P = 0.0024$). Therefore, we used filtration for all further competition assays.

Identification of oak micro-organisms

Culturing. To determine a set of culturable microbial species to represent the oak microbiota, we plated unsterile oak infusion from the four local trees onto YEPD and 10% YEPD agar, purified the most common colony morphs by restreaking and genotyped them by sequencing their 16S rRNA (for bacteria) or ITS rRNA (for fungi) genes. We found the bacterial clones all contained one of 14 different 16S sequences, and the fungi all contained one of six different ITS sequences. Given the difficulty of classifying many microbes down to the species level using only 16S (Fox *et al.* 1992) or ITS (Schoch *et al.* 2012) sequences, we simply named each according to its genus (Fig. S1, Supporting information). We decided to use the six most frequently detected bacterial species (named as *Burkholderia*, *Sphingomonas*, *Massilia*, *Mucilaginitacter*, *Pseudomonas I* and *Pseudomonas II*) and all six fungal species (named as *Umbelopsis*, *Rhodotorula*, *Cryptococcus*, *Penicillium I*, *Penicillium II* and *Penicillium III*). Consistent with the low abundance of *S. paradoxus* determined from our enrichment isolation data, we did not find any *Saccharomyces* colonies during this screening.

High-throughput sequencing. We expected that culturing the oak bark microbiota would be highly biased, as

culture methods typically underestimate the size and diversity of microbial populations (Kell *et al.* 1998). We therefore used 454 sequencing of the unsterile oak bark infusion, and of whole oak pieces, from the four focal trees to better determine the microbial community.

Figure 2 shows how the trees were sampled and how the different samples varied in microbial composition. NMDS for both bacterial 16S (Fig. 2B; stress = 0.215; $R^2 = 0.785$) and fungal ITS (Fig. 2C; stress = 0.23; $R^2 = 0.817$) sequences showed that our samples clustered according to the method we used to sample the microbial community, rather than according to which tree the samples were taken from. Two-factor ADONIS supported this interpretation. For 16S sequences, we found a significant effect of the sampling method ($R^2 = 0.29$, DF = 1, P -value = 0.001), but not of tree ($R^2 = 0.11$, DF = 1, P -value = 0.283) or interaction between method and tree ($R^2 = 0.06$, DF = 1, P -value = 0.724). Likewise for fungal ITS sequences, we found a significant effect of the sampling method ($R^2 = 0.31$, DF = 1, P -value = 0.009), but not of tree ($R^2 = 0.07$, DF = 1, P -value = 0.665) or interaction between method and tree ($R^2 = 0.05$, DF = 1, P -value = 0.938). Rarefaction curves for species richness showed that our sampling did not approach saturation (Fig. S2, Supporting information). Wilcoxon rank sum test showed that average fungal (ITS) alpha diversity was significantly higher in infusions than in whole bark samples ($W = 24$, $P = 0.0095$), but no significant difference was found for the 16S alpha diversity ($W = 13$, $P = 0.914$) (Fig. S3A,B, Supporting information).

Further analysis was conducted by pooling the sequences from the four oak infusion samples. Figure S4 in the supplemental material represents the bacterial (A) and fungal (B) composition of the oak infusion microbiota. We could identify sequences of all 12 of the representative bacteria and fungi selected in the culturing experiments (above) and used in the competition experiments (below). The frequencies of these sequences in the 454 data set of the total microbial communities are shown in Fig. S4 (Supporting information). No sequences corresponding to *Saccharomyces* were found in any sample.

Competition experiments

The ecological interactions between the tested species and *S. paradoxus* strongly depended on temperature. Effects range from killing (*Pseudomonas I* at 5.5 °C), through complete suppression of growth (*Pseudomonas I* at 26 °C), through neutrality (*Penicillium II* at 26 °C), to promotion of growth (*Mucilaginitacter* at 5.5 °C) (Fig. 3). On solid oak bark infusion medium, most species had the same effect on *S. paradoxus* as they did in liquid,

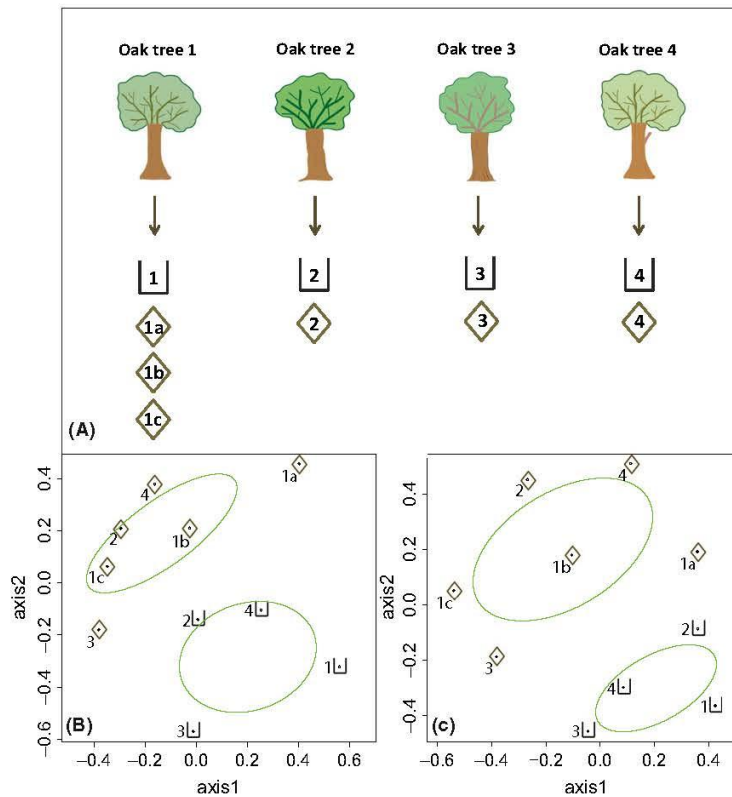


Fig. 2 Sampling scheme (A) From each of four oaks, one liquid infusion was prepared. In addition, three oak pieces were taken from one tree and one piece from each of the other oaks. DNA was extracted from all these samples, and the microbial community was determined by sequencing. Community differences at the 97% sequence OTU identity level were evaluated using NMSD of Bray-Curtis dissimilarities. This produced two-dimensional approximations of the distances between the points for bacterial 16S (B) and fungal ITS (C) sequences. Each point symbolizes a single oak sample's community, and the two symbols indicate the methods used (infusion and piece). The ellipses represent the standard deviation around the centroids of the respective isolation method in NMSD distance.

but overall the effects were less extreme (Fig. S5, Supporting information). An exception is *Pseudomonas* II, which inhibits the yeast much more strongly on solid than in liquid medium (Fig. 3, Fig. S5, Supporting information). Overall, there was a greater variance in effects among the different species at 5.5 °C than at 26 °C, and many organisms showed almost no effect on *S. paradoxus* growth at 26 °C.

Halo assays

To visualize effects that the twelve different microbes had on *S. paradoxus* growth, we performed halo assays on YEPD at 26 °C and 5.5 °C (Fig. 4). The patch containing *Pseudomonas* I produced a large halo in the surrounding lawn of *S. paradoxus* at 5.5 °C, and a smaller one at 26 °C, consistent with its effects on *S. paradoxus* growth in liquid oak infusion medium (Fig. 3). Consistent with its behaviour on solid oak infusion medium (Fig. S5, Supporting information), *Pseudomonas* II also produced a visible halo at 5.5 °C. No other clear halo effects could be seen. On 10% YEPD medium, the halos are hard to see at 5.5 °C because *S. paradoxus* is not growing much, but the halos are bigger compared to

YEPD and all competitors form visible colonies; similarly, the halo around *Pseudomonas* I at 5.5 °C is larger than at 26 °C (Fig. S6A,B, Supporting information).

Discussion

Saccharomyces paradoxus is present on oak bark at very low density

Our results confirm that enrichment culturing is a very sensitive method for detecting *S. paradoxus* on oak tree bark. Even though most of our experimental samples were spiked with an average of 1.7 cells of wild *S. paradoxus*, 92% of them scored positive – actually more than predicted, based on the Poisson distribution of spiked cells combined with the background rate of isolation of new strains. We can therefore confirm that the method is so sensitive that a sample testing negative is unlikely to contain *S. paradoxus*. Given this, we can estimate the average density of *S. paradoxus* cells on oak bark. We took 352 4-mm-diameter samples from the 22 Plön oak trees and used 5% of each to inoculate ME cultures, yielding 4 *S. paradoxus* isolates. Thus, the 221 mm² of oak bark we tested probably contained only 4 cells,

10 V. KOWALLIK, E. MILLER and D. GREIG

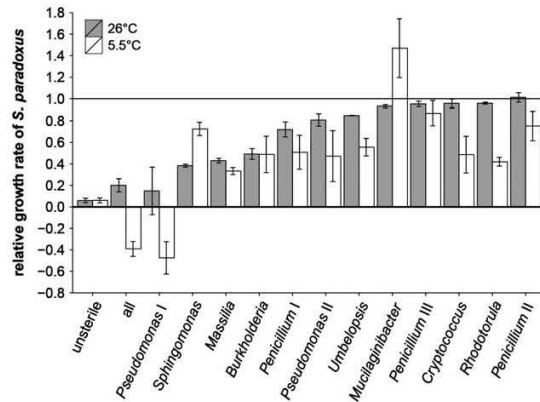


Fig. 3 Growth of *Saccharomyces paradoxus* relative to its growth in the absence of competition in liquid oak infusion medium. We used the initial and final G418 colony counts for each tube to calculate the average number of divisions Sp-Nehmten went through. We standardized each measure by dividing it by the number of divisions Sp-Nehmten went through when growing alone in the sterile control treatment of the same replicate block, to give a measure of the growth of Sp-Nehmten in each treatment relative to how well it can grow alone (the ratio of number of cell divisions; values >1 indicate that growth was promoted, values <1 indicate that growth was suppressed, values <0 indicate a net decline in cell numbers). The bars indicate the effect of the complete community ('unsterile'), the complete set of 12 representative microbes ('all') and the microbes tested individually. Error bars indicate the standard deviation across 3 replicates for each treatment.

suggesting that the density of *S. paradoxus* on the Plön trees is 1.81 cells per square centimetre of oak bark. Although *S. paradoxus* is very rare on oak compared to other microbial species, this low absolute density is nevertheless sufficient to support a substantial local population size. Whittaker & Woodwell (1967) estimate that there is at least 1 m² of tree bark above each square metre of forest floor, implying that the overall density

of cells is at least 2×10^{10} cells per square km of oak forest. The trees in our study were sampled in winter, and it is reasonable to expect that abundance of *S. paradoxus* changes seasonally, perhaps because nutrient availability on the bark changes, or because yeast migrate between oak bark and another habitat, or because yeast are consumed as a food source by insects. Our ability to wash *S. paradoxus* off oak bark into suspension also suggests that rain would carry the yeast from the tree to the leaf litter below. Any changes in seasonal abundance would provide valuable insights into the natural history of *S. paradoxus*. Our data did not show significant tree-to-tree variation in the probability of isolating *S. paradoxus*, either the 22 Plön oaks or the four Nehmten oaks, but the low proportion of positive samples offers us little statistical power to detect such variation.

Some important caveats should be considered. Our population density estimate assumes that the cells were efficiently washed off the bark into suspension. If they adhere to the bark, then the true density could be much higher and placing bark pieces directly into the enrichment medium would yield more isolates. Further, the eleven strains we used for spiking the samples to determine the enrichment culture sensitivity had previously been isolated from the same trees using ME and thus may have genotypes that are amenable to these enrichment conditions. It is possible that more *S. paradoxus* cells were present on the bark samples, but were not selected by the enrichment culture method, either because their genotypes do not give them high fitness in the malt extract enrichment conditions or because they are in the form of spores that do not germinate rapidly enough in the conditions. Our estimate of the *S. paradoxus* population density therefore applies only to nonadhering cells of the type that can be isolated in malt extract medium. Nevertheless, it is noteworthy that the density of such *S. paradoxus* cells on oak bark is this low, especially if oak bark is indeed the primary natural ecological niche of *S. paradoxus*.

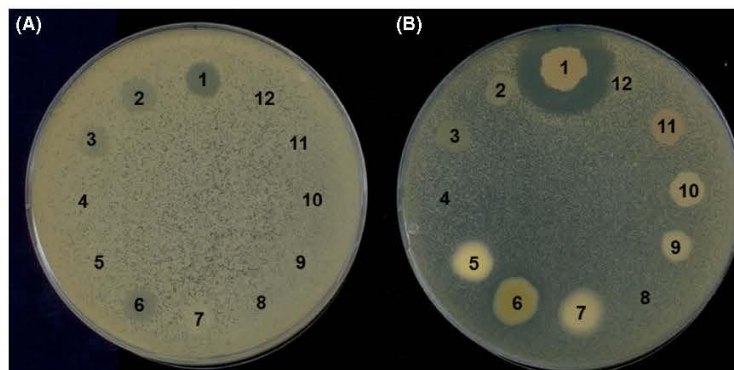


Fig. 4 Halo assays on YEPD at 26 °C (left) and 5.5 °C (right). The numbers indicate the different competitors. 1 = *Pseudomonas I*; 2 = *Sphingomonas*; 3 = *Massilia*; 4 = *Burkholderia*; 5 = *Penicillium I*; 6 = *Pseudomonas II*; 7 = *Umbelopsis*; 8 = *Mucilagibacter*; 9 = *Penicillium III*; 10 = *Cryptococcus*; 11 = *Rhodotorula*; 12 = *Penicillium II*.

The oak bark community

Both culturing and environmental sequencing are known to produce biased estimates of microbial community size and diversity. Microbes vary greatly in how well they grow in culture; indeed the majority of microbes in typical environmental samples cannot be cultured at all (Amann *et al.* 1995; Kell *et al.* 1998). Our enrichment cultures are a good demonstration of this phenomenon: the offspring of a single, spiked *S. paradoxus* strain dominates all the other microbial species initially present in environmental samples. Next generation sequencing provides some information independent of our ability to culture organisms, but there also exist some biases that can skew relative abundance information, such as DNA extraction bias (DeSantis *et al.* 2005; Feinstein *et al.* 2009; Delmont *et al.* 2011) and PCR bias (Polz & Cavanaugh 1998). Even DNA from dead organisms can be included in sequencing-based estimates of community compositions. Different operon copy numbers of ribosomal rRNA encoding genes lead to a biased abundance picture of organisms; for example, the 16S rRNA copies range from 1 to 15 depending on the bacterial species (Lee *et al.* 2009). Amplification bias according to the sequencing primers is recognized for bacteria (Engelbrekton *et al.* 2010; Schloss *et al.* 2011) as well as fungi. Some primers (e.g. ITS1-F, ITS1 and ITS5) show a bias towards amplification of basidiomycetes, whereas ITS2, ITS3 and ITS4, are biased towards ascomycetes (Bellemain *et al.* 2010). In our study, we tried to control some of these biases using different isolation methods from whole oak bark pieces and from oak bark infusion, and by sequencing in both directions.

Direct plating and culturing of oak bark infusion allowed us to identify 12 abundant microbial species with distinct colony morphologies to use for culture-based experiments (Fig. S1, Supporting information). A total of 454 sequencing confirmed that DNA from all these species except for *Cryptococcus* was present in the oak infusion metagenome (Fig. S4, Supporting information). Sequences from the 12 species were more common in DNA extracted from oak bark infusion than in DNA extracted from whole oak bark pieces, as expected, given that the species were cultured from infusion. Thus, sampling method affects the apparent community composition and indeed, we found significant differences in the sequence compositions between the whole oak bark pieces and the oak bark infusion, but not between one tree and another (Figs 2 and S3, Supporting information).

Although we analysed a total of 40 000 fungal reads, we did not find a single *Saccharomyces* sequence. This supports our enrichment culture results which show

that *S. paradoxus* is rare on oak tree bark and thus comprises only a tiny fraction of the total microbial community. Our results are comparable with the first study of the metagenome of vineyard grapes, which finds *Saccharomyces* sequences at a frequency of only one in 20 000 reads (Taylor *et al.* 2014). Considering how reliable winemaking is, it is remarkable to find that *Saccharomyces* species are so rare in both of their habitats, the natural oak tree habitat and even in the domestic winery habitat. Further, because we sequenced ground whole bark pieces as well as cells washed off into an infusion, we can be confident that we have not underestimated the abundance of *S. paradoxus* by missing cells that adhere to or are trapped within the oak bark matrix. The DNA we sequenced came from a total of 80 grams of bark processed by infusion and ~6 g processed by grinding whole bark. Our above estimates of 2.4 *S. paradoxus* cells per cm² of bark surface would suggest that the total sequenced sample should contain about 174 cells of *S. paradoxus*. However, the rarefaction curve (Fig. S2, Supporting information) clearly shows that we did not capture all the microbial sequence diversity within the sample. Greater sampling depth would be necessary to detect *S. paradoxus* reliably by environmental sequencing to determine its frequency within the total oak bark community. It is clear though that *S. paradoxus* is sparse on oak bark and a very rare member of the oak bark microbial community, at least in winter at this location in northern Germany.

Yeast growth on oak bark

The low density of *S. paradoxus* on oak bark prompts the question: can yeast actually grow there? Our results show that oak bark can indeed provide nutrients to support substantial growth of wild *S. paradoxus* (Fig. 1), strengthening the case that oak bark may be the natural niche for this species. Other authors have previously grown *S. cerevisiae* in sterilized oak infusion. Bell (2010) measured the effect on growth of systematic gene knockouts, and Giraldo-Perez & Goddard (2013) determined how a homing endonuclease affected growth rate and carrying capacity. As both authors measured growth using optical density, it is unclear how many cell divisions the medium supported, compared to normal laboratory media. However, oak infusion is clearly much poorer in nutrients than the standard laboratory medium YEPD. Glucose, sucrose and fructose are undetectably low in the bark of the oak *Quercus robur* (Sampaio & Gonçalves 2008), and *Saccharomyces* yeasts are not able to utilize cellulose directly as a carbon source (Van Rensburg *et al.* 1998). It is possible that *S. paradoxus* depends on the release of sugars from cellulose digestion by other members of oak bark communities,

12 V. KOWALLIK, E. MILLER and D. GREIG

and our observation that a *Mucilaginibacter* species promotes the growth of *S. paradoxus* is consistent with this. *Mucilaginibacter* is a member in the family *Sphingobacteriaceae*, and different members of this genus have the ability to hydrolyse organic matter such as xylan, pectin and laminarin (Pankratov *et al.* 2007; Madhaiyan *et al.* 2010; Han *et al.* 2012). *Mucilaginibacter* are also known to produce large amounts of extracellular polysaccharides containing the sugars glucose, galactose, mannose and rhamnose and may thus provide a carbon source for *Saccharomyces* (Urai *et al.* 2008). Whilst the *Mucilaginibacter* species can promote the growth of *S. paradoxus* on oak, most other species we tested (Fig. 3), as well as the community as a whole (Fig. 1), strongly inhibit its growth. One of the representative species we tested, a Pseudomonad, not only inhibited the growth of *S. paradoxus* but actively killed it by producing a toxin (Figs 3 and 4). Species of *Pseudomonas* are known to produce a wide range of different antifungal metabolites such as phenazines and pyrrolnitrin (Leisinger & Margraff 1979). Thus, even within the small number of species we tested, we discovered several forms of ecological interactions with yeast. We know that diversity within communities both promotes and depends upon a wide range of ecological interactions (Boddy & Wimpenny 1992). Some microbes may produce inhibitory peptides, proteins or glycoprotein, such as killer toxins, and enzymes that can lyse the cell walls of other species (Fleet 2003) or making new sugars available through digesting polysaccharides (Deak 2006). We found that the strength of both positive and negative ecological interactions between yeast and members of its community also depend on a simple abiotic factor, temperature, raising the possibility that seasonal changes as well as climatic conditions in different geographic regions could have significant impact on the abundance, range and life history of *S. paradoxus*.

Conclusions

Oak trees are widely considered to be the primary natural habitat for *S. paradoxus*, but it is possible that the special association between yeast and oak is actually an artefact due to various potential sampling biases. Here, we show that *S. paradoxus* is not only scarce on oak bark, but that it is also only a very rare member of the oak bark microbial community. Whilst we find that it can grow on nutrients present in oak bark, its growth is strongly suppressed by much more abundant microbial species. We do not know how the abundance of *S. paradoxus* on oak differs from place to place or from season to season, but it appears that the lifestyle of *S. paradoxus* in this habitat is very different from that of *S. cerevisiae* growing in fermenting wine must. Whilst *S. cerevisiae*

rapidly consumes abundant grape sugars, growing to massive density until it dominates the community in a near laboratory-like monoculture, *S. paradoxus* in its oak habitat must eke out a living as a rare scavenger, its fate subject to numerous superior species. Given the physiological similarity between the two 'sibling' species (Vaughan-Martini & Martini 1998), it is probable that *S. cerevisiae* has an evolutionary history similar to that of *S. paradoxus*: indeed, both species can be found on the same oak trees (Sniegowski *et al.* 2002; Sampaio & Gonçalves 2008). It is therefore reasonable to suppose that the results we present here for *S. paradoxus* also apply to wild *S. cerevisiae* populations inhabiting oak.

Whilst our growth assays using the natural yeast community are necessarily highly simplified, they have helped us to identify positive, negative and neutral interactions that can be modulated easily by temperature. We wish to encourage more ecologists to study yeast, and we hope that yeast will become a useful model system for experimental ecology. Although quantifying the natural habitat of *S. paradoxus* is a daunting task, there are great potential benefits, both to yeast geneticists who would wish to better understand the environment in which their species evolved and to ecologists who might seek a tractable and genetically well-characterized model system.

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ECOLOGY OF SACCHAROMYCES ON OAK 13

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14 V. KOWALLIK, E. MILLER and D. GREIG

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V.K. and D.G. conceived the study. E.M. did the sampling on oaks in Plön, engineering of Sp-Plön strain and determined the sensitivity of malt extract enrichment culture assay. V.K. did sampling in Nehnten, engineering of Sp-Nehnten strain, culturing, 454 sequence analysis, experiments and statistics. D. G. and V.K. wrote the manuscript. E.M. edited the manuscript.

Data accessibility

The sequences determined in this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under Accession no. SRP052992.

Mothur files; Taxonomic output and OTU tables for ITS and 16S sequences: Dryad doi:10.5061/dryad.c8n18.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 The relative abundance of bacteria [b] and fungi [f] colonies on YEPD and 10% YEPD cultured from oak infusions of four trees.

Fig. S2 (A): Rarefaction analysis of OTUs based on 97% identity between bacterial community diversity in oak infusion (blue) and on oak pieces (red).

Fig. S2 (B): Rarefaction analysis of OTUs based on 97% identity between fungal community diversity in oak infusion (blue) and on oak pieces (red).

Supporting Information Chapter I

Table S1: Raw sampling and sensitivity data of enrichment culture assay including Poisson distribution and maximum likelihood model results

Text S1: Maximum likelihood model description

Figures S6

Can be found online together with the publication:

(<http://onlinelibrary.wiley.com/doi/10.1111/mec.13120/supinfo>)

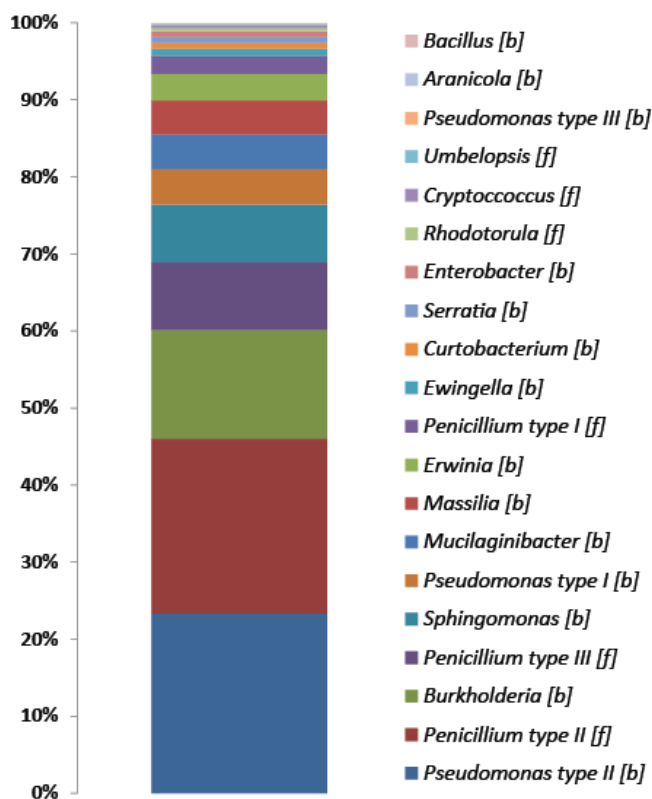


Figure S1: The relative abundance of bacteria [b] and fungi [f] colonies on YEPD cultured from oak infusions of four trees. Groups in bold were used for culture-based competition assays.

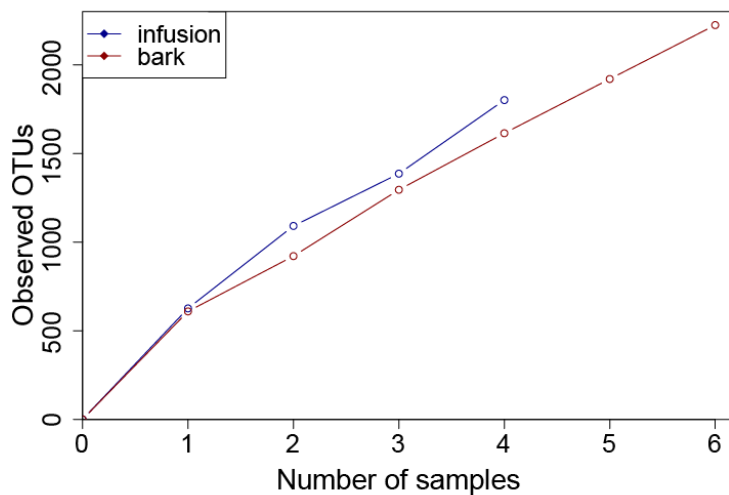


Figure S2A: Rarefaction analysis of OTUs based on 97% identity between bacterial community diversity in oak infusion (blue) and on oak pieces (red).

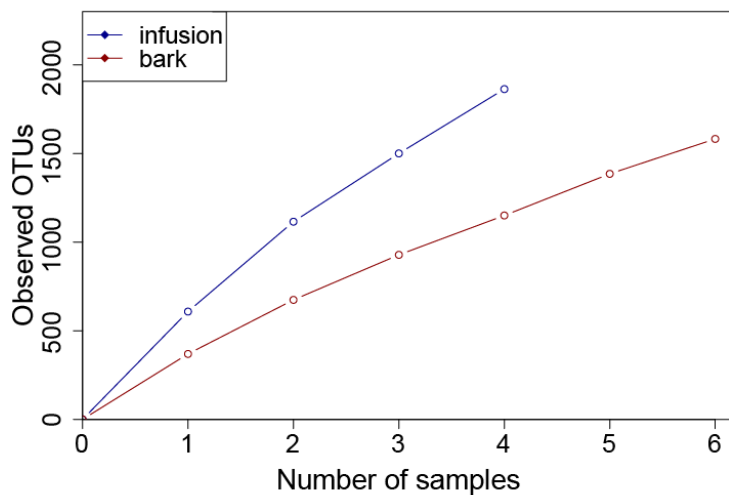
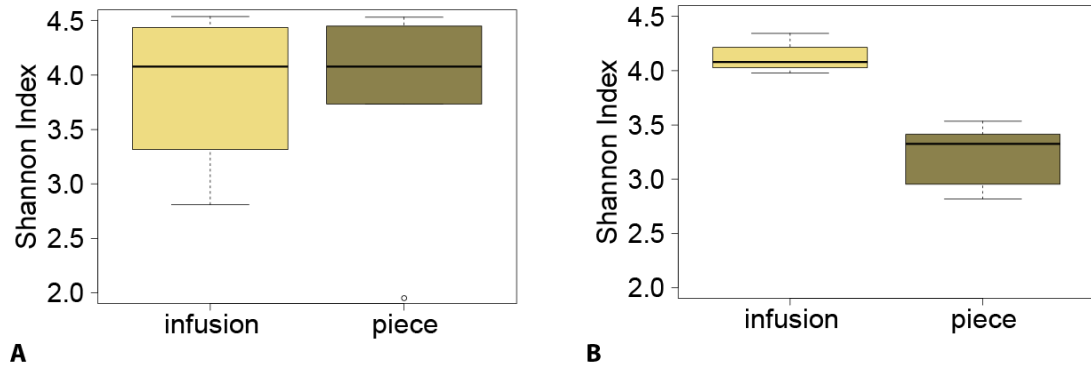
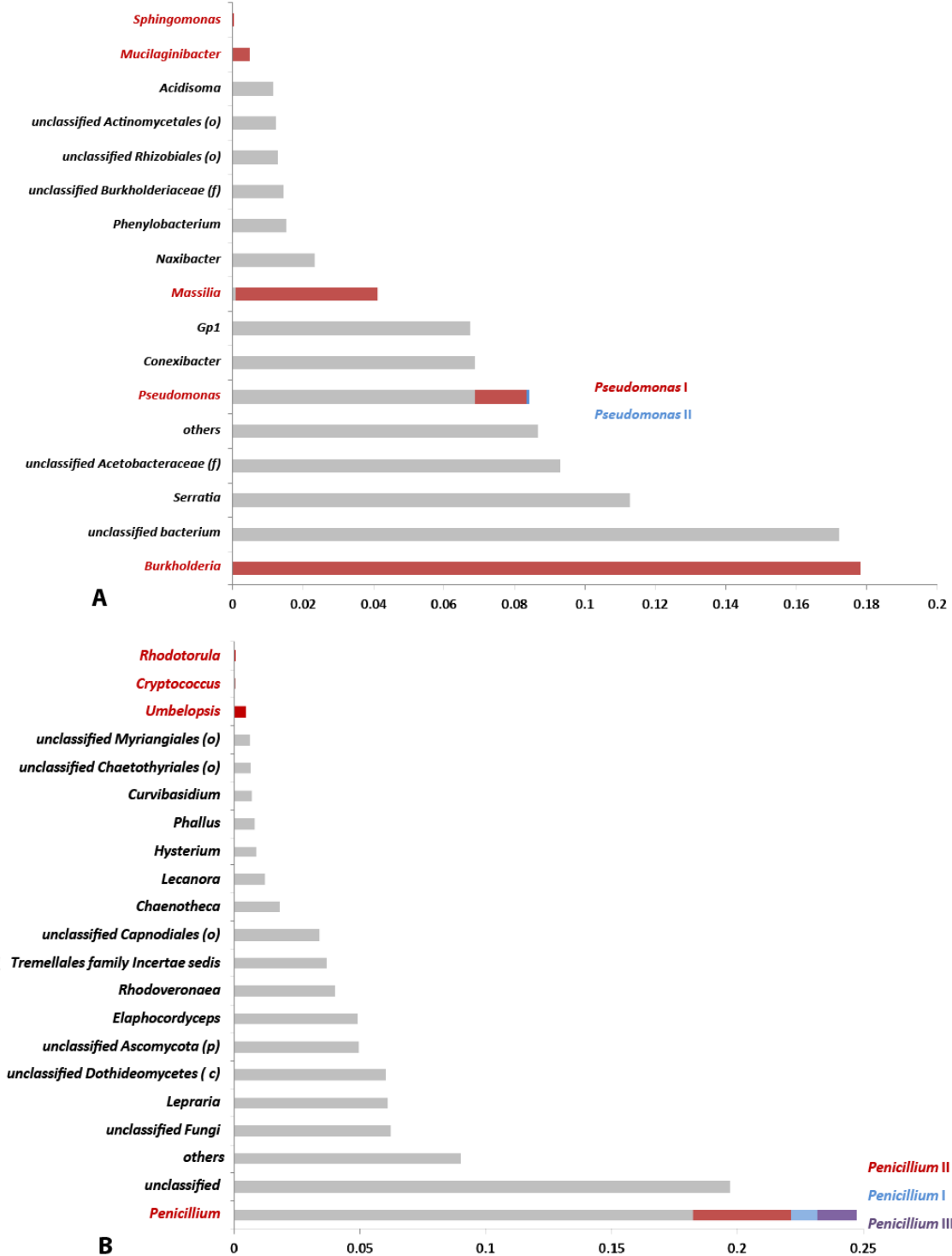


Figure S2B: Rarefaction analysis of OTUs based on 97% identity between fungal community diversity in oak infusion (blue) and on oak pieces (red).



Figures S3A+B: Alpha diversity based on OTUs at 97% similarity, with the Shannon index for 16S (A) and ITS (B) sequences.



Figures S4A+B: The taxonomic abundance on the genus level of fungi and bacteria in the four oak infusions. In red font are the cultured clones selected for competition experiments. Only genera with $\geq 0.5\%$ of the overall abundance are shown in grey bars. The colours within the bars represent the frequencies of the sequences of the 12 representative bacteria and fungi that we selected to represent the microbial community in culturing and competition experiments. Classification to genus was not always possible and so the last taxonomic level of classification is shown and indicated by p, c, o and f for phylum, class, order and family respectively.

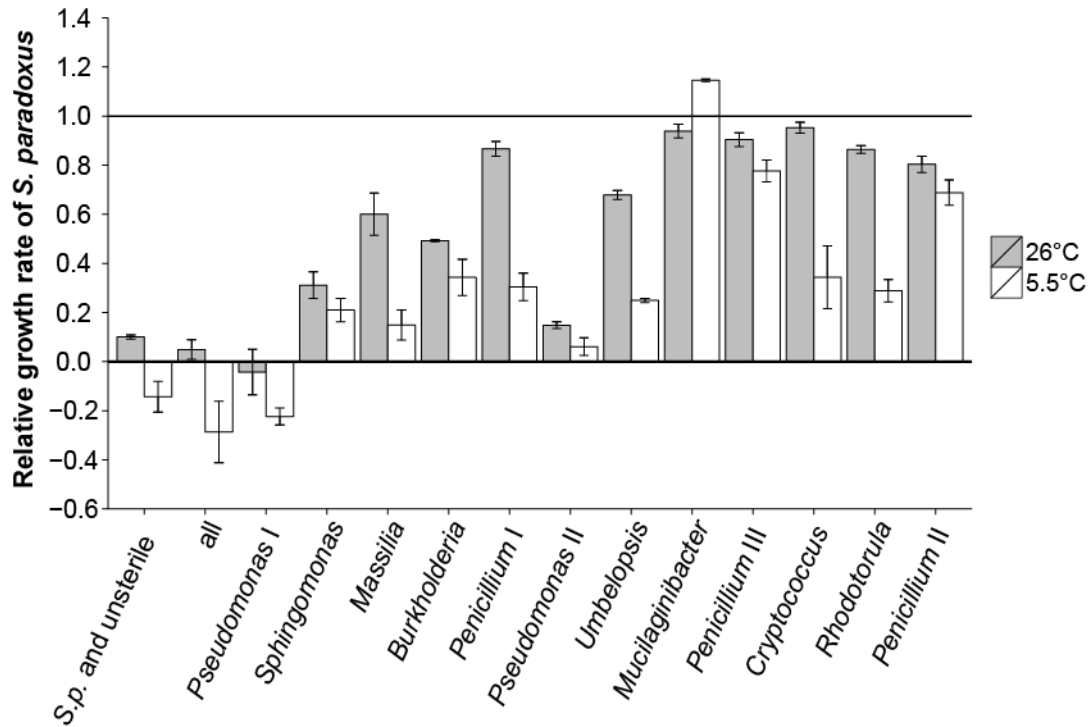


Figure S5: Growth of *S. paradoxus* relative to its growth in the absence of competition in solid oak infusion medium. We used the initial and final G418 colony counts for each tube to calculate the average number of divisions *Sp-Nehmten* went through. We standardized each measure by dividing it by the number of divisions *Sp-Nehmten* went through when growing alone in the sterile control treatment of the same replicate block, to give a measure of the growth of *Sp-Nehmten* in each treatment relative to how well it can grow alone (the ratio of number of cell divisions; values >1 indicate that growth was promoted, values <1 indicate that growth was suppress, values <0 indicate a net decline in cell numbers). The bars indicate the effect of the complete community (“unsterile”), the complete set of 12 representative microbes (“all”) and the microbes tested individually. Error bars indicate the standard deviation across 3 replicates for each treatment.

Chapter II Analysis of the interactions between *Saccharomyces paradoxus* and two oak bark bacteria (follow up study to Chapter I)

1. Introduction

1.1. Background

Although *S. cerevisiae* is one of the best known and widely used microorganisms in biological research, scientists have struggled to identify its natural habitat. The original hypothesis that grapes and other sugar rich fruits are covered in *Saccharomyces* yeast, could not be confirmed; *Saccharomyces* is rarely isolated from fruits (Taylor et al., 2014; Vaughan-Martini and Martini, 1995). Moreover, *S. cerevisiae* and its closest natural relative *S. paradoxus* have been most consistently isolated from the bark of hardwood trees and the soil surrounding these trees, mainly oaks (e.g. Charron et al., 2014; Sniegowski et al., 2002; Sylvester et al., 2015; Wang et al., 2012; Yurkov, 2005). Today, an association between wild *Saccharomyces* and oaks is generally accepted, with a stronger focus on bark than soil as the easiest place to isolate *Saccharomyces*. However, it is not clear if oak is the true ecological niche or if the yeasts are found there due to a bias in the sampling behavior of researchers.

In the first Chapter, I examined the microbial composition of oak bark and used a set of 12 microorganisms that I have isolated from this environment together with a *S. paradoxus* strain in competition experiments in liquid as well as on solid oak bark infusion medium at two temperatures (5.5 °C and 26 °C). Although I “only” investigated 12 microbial species, I was able to identify a wide range of interactions. Some microbes had no effect on the yeast whilst others inhibited or even killed *S. paradoxus* and one bacterium promoted the growth of the yeast at cold temperatures. One key factor influencing the outcome of the competitions was temperature; at cold temperature the observed effects, both antagonistic and growth-promoting, were stronger.

The two competitors producing the most interesting effects in the competition studies were:

- I) *Pseudomonas* I, which killed the yeast through an actively produced toxin
- II) *Mucilaginibacter*, which promoted the growth of the yeast at cold temperatures.

1.2. Microbial communities remain poorly understood.

Understanding the distribution and basic ecology of one of the most abundant and diverse groups of organisms on Earth is a crucial, and largely unsolved, issue in environmental research (Barton and Northup, 2011). In the twentieth century, work in microbiology was dominated by simplification. Microorganisms were isolated from complex communities, grown as isogenic lines, and their behavior studied in simple laboratory media. Although these studies have greatly improved our quality of life and understanding of the world, from dissection of the cell cycle to the development of vaccines, they have not brought us much closer to an understanding of natural microbial communities (Little et al., 2008). A driving factor in studying microbial diversity has been the development of tools and techniques for large scale nucleotide sequencing and analysis that is commonly referred to as genomics. The development of this field has allowed researchers to identify and quantify microorganisms in the environment opening up an entirely new world without the limitations imposed by difficulties in culturing. Only the smallest proportion of microorganisms is cultivable but now molecular phylogenetic analysis can be used to study taxonomic diversity in every environment. Moreover, new genomic methods also allow the analysis of specific genes that give not only an indication of the organisms present but the metabolic potential of an environment (Hall, 2007). However, most studies to date have been only descriptive and have focused on simply identifying and quantifying the species that make up microbial communities. All systems are more than a sum of their parts and species composition alone is not enough to predict the behaviors of the complex systems. This complexity of natural microbial communities demands that we also direct attention to it.

1.3. The paradox of high biodiversity within microbial communities

Microorganisms make up most of the biodiversity on Earth and by their omnipresence have a huge impact on the entire biosphere. They are the key players in the biogeochemical cycling of many important compounds in and between ecosystems, including oxygen, carbon, nitrogen, sulfur and methane. They decompose, mineralize, and recycle plant and animal biomass and release nutrients into the environment that are needed by other organisms and their presence is a precondition for the existence of plant and animal life (Meyer, 1994). The microbial world is immense and estimated at more than 10^{30} prokaryotic individuals (Whitman et al., 1998) - a number which does not include other microorganisms like molds or yeasts. These high numbers of individuals and the fact that microorganisms colonize all natural environments on Earth mean that microbes exhibit huge genetic diversity. Indeed, theoretical and empirical analysis of soil microbial diversity indicates that we can find 7000 different prokaryotic taxa at a total abundance of approximately 10^9 cells per cubic centimeter (Curtis et al., 2002; Torsvik et al., 1998). The maintenance of such high levels of diversity in a small soil sample requires an

explanation (Kassen and Rainey, 2004): what prevents the single best-adapted type from dominating? Imagining a very heterogeneous environment makes it easier to accept that this environment allows diverse organisms to co-exist, but we also find a high biodiversity in environments that seem more homogeneous like aquatic systems. The “paradox of the plankton” (Hutchinson, 1961) for example addresses the problem that phytoplankton species are limited by only a handful of resources (e.g., nitrogen, phosphorus, iron, light) but a single milliliter of seawater contains dozens of different phytoplankton species. Different models have been proposed to explain biodiversity but conventional approaches to microbiology have not supplied a satisfactory answer and the tendency of microbial ecologists to almost exclusively focus on the documentation of diversity has not helped to solve the problem.

Most natural environments are composed of several different microenvironments and when organisms evolve and adapt to particular sets of abiotic and biotic characteristics, this is called niche specialization and the resulting difference in patterns of resource utilization is called niche differentiation (Prosser and Nicol, 2012). One important theory, the niche exclusion principle (Hardin, 1960), states that one niche can support no more than one type of organism, whether it be a genotype or a species. So in principal, by generating diversifying selection, environmental heterogeneity could be a general explanation for biodiversity and the quantity of genetic variation in populations (reviewed in Kassen, 2002). Evolutionary theory predicts that in a spatially heterogeneous environment, selection favors the emergence of ecological specialists with a narrower niche width, meaning that different types are adapted to different niches. Ecological specialists trade off a competitive advantage in one niche against reduced competitive ability in another. At the other extreme, ecological generalists can survive in a broader range of environments and are more tolerant to environmental changes, but are not as competitive as specialists in specific environments (reviewed in Devictor et al., 2010). Niches vary in both abiotic and biotic factors; organisms living and growing in an environment can have remarkable influence on it. This is known as niche construction (or ecosystem engineering) and describes the case that organisms define, partly create or partly destroy their own niches through their metabolism and their activities. Such niche construction may regularly modify the abiotic and biotic sources of natural selection in environments (Odling-Smee et al., 1996). One example of niche construction is the wine making process, where *S. cerevisiae* modifies the environment to its own advantage by its fermentation activity (Goddard, 2008). It has also long been recognized that colonization order can determine community structure through a priority effect. Early arriving species have an advantage in their interactions with future colonists, for example due to an early increase in numbers and monopolization of available resources or by altering conditions in a way that positively or negatively impact a species arriving later (Connell and Slatyer, 1977; Shulman et al., 1983; Sutherland, 1974). Additionally, different forms of selection specifically preserve genetic diversity. Balancing selection in its simplest form arises

from a superiority in fitness of heterozygotes over homozygotes (Lewontin, 1974). Negative-frequency-dependent selection results in the highest fitness of a genotype when this is rare because resources are most abundant and in a low fitness when the genotype is common because resources are rare and competition intense (Smith, 1989).

The ideas discussed above indicate that adaptation to abiotic and biotic factors within and between niches drives diversity. However, some researchers think that adaptation plays only a minimal role. Neutral theory is a formal mathematical theory which suggests that biodiversity is controlled predominantly by neutral drift of species abundances (Bell, 2001; Hubbell, 2001; McGill, 2003) and this theory has generated approval but also controversy as it claims that many long studied mechanisms (such as niches) have little involvement in structuring communities (McGill, 2003). The paradox of biodiversity, how such high levels of diversity can be maintained in natural microbial communities, is also today still an unsolved issue and we need more than descriptive information to come to a solution.

1.4. The complexity of microbial communities and the interactions between members

The natural environment not only influences the survival of individual species, but also influences how different microorganisms interact with each other. The presence or absence of specific chemical compounds and the ability of the individual microorganisms to respond to their chemical environment are critical for growth and survival. As nothing in natural environments exists in complete isolation, every organism will be affected by the plethora of other organisms around them and therefore has to compete for resources (Trudinger and Bubela, 1967). The structure of a microbial community changes over time and its dynamics or stability depends on the interactions and interrelationships amongst populations and between single organisms and their adaptation to the environment. Such interactions can drive evolution, adaptation, and speciation of community members. On the other hand evolutionary changes in members of a community can also feedback to modify species interactions, community composition and ecological dynamics (Haloin and Strauss, 2008). Microbial interactions are complex and driven by the need for organisms maximizing the resources available to them in the immediate environment. With the growth of microbial communities, the demand for space and nutrients will also increase, resulting in the development of different strategies to persist and compete for resources. Microbe-microbe interactions can be intraspecific - between organisms of the same species, or interspecific - between organisms of different species. These interactions exist in a continuum from antagonistic to synergistic interactions (Little et al., 2008). Positive interactions are mutualism/symbiosis, in which both organisms derive benefit from one

another, and commensalism, in which one partner benefits from another one and this other partner is neither harmed nor benefits from the interaction. Neutralism is the association between microorganisms, where two different species occupy the same environment without affecting each other. Antagonistic interactions include amensalism, in which one organism adversely affects the other organism without being affected itself, and competition for one or more common resources. In addition there are interactions that are positive for one but negative for the other population like predation of one organism upon another or parasitism (Epstein, 2001). Data based on bacterial strains isolated from a common aquatic environment suggest that the typical interaction between co-evolved species will be competition rather than cooperation (Foster and Bell, 2012). Although the microbial life within complex communities still remains mysterious, humankind already benefit from several microbial substances. One of the most famous examples is penicillin, an antibiotic which saved thousands of lives since its discovery in 1928 (reviewed in: Demain and Sanchez, 2009) and which is nothing more than an antimicrobial compound produced by a mold to persist in a world full of competing microbes. A better understanding of microorganisms in their natural environment and their interactions with other community members will not only help us dissecting the complex microbiome around us but will help to answer how this complexity and biodiversity could evolve and persist.

1.5. Determining the mechanisms of the interactions between *S. paradoxus* and two oak bark bacteria

Dissecting the interconnected interactions among community members is one essential component to understanding the properties of the community (Little et al., 2008). Studying such ecological interactions between different community members make it essential to use a natural system and organisms that co-adapted in/to this environment. By studying the natural ecology of *Saccharomyces* yeast, I developed such a system. Assuming that *S. paradoxus* has adapted to live on oak bark, we can ask if the yeast has also evolved responses to two prominent oak bark bacteria: *Pseudomonas* and *Mucilaginibacter*.

In Chapter I, experiments revealed that *Pseudomonas* strongly inhibited *S. paradoxus* at 26 °C after seven days and actively killed the yeast at 5.5 °C after 20 days in both, solid and liquid oak infusion. This killing effect is not surprising as species of *Pseudomonas* are known to produce a wide range of different metabolites like phenazines and pyrrolnitrin which inhibit a variety of microorganisms, including fungi (reviewed in: Leisinger and Margraff, 1979). It is known that some bacteria increase toxin production in presence of competitors under nutrient limitation. It has been shown for example that the behavioral and genetic response of *Pseudomonas fluorescens* is determined by the identity of competing bacteria (Garbeva et al., 2011). An increase of phenazine virulence factor production by *Pseudomonas aeruginosa* can also be

affected by products secreted by other species. For example, farnesol - which is a quorum sensing molecule secreted by the fungus *Candida albicans* - increases phenazine levels in *P. aeruginosa* when the two species are cultured together (Cugini et al., 2010). New *P. aeruginosa* phenazines as well as phenazine modifications have been identified in the presence of fungi like *Aspergillus fumigatus* and *S. cerevisiae* (Gibson et al., 2009; Moree et al., 2012). So it could be possible that the presence of *S. paradoxus* triggers the anti-microbial compound production of the *Pseudomonas* spp. used in this study, which should come along with a cost for the bacterium.

The growth promoting effect of *Mucilaginibacter* spp. was only visible at 5.5 °C after 20 days and not at 26 °C after seven days. This temperature specificity seems interesting and diverse reasons could be responsible. It might be that the bacterium reacts very sensitively to cold temperatures, and is dying in the experiment at 5.5 °C, allowing the yeast to consume something it releases. It could also be the opposite and the bacterium is producing something beneficial for the yeast (e.g. amino acids), but this is reduced at warmer temperatures. *Mucilaginibacter* is a member of the family *Sphingobacteriaceae* and different members of this genus have the ability to hydrolyse organic matter such as xylan, pectin and laminarin (Han et al., 2012; Madhaiyan et al., 2010; Pankratov et al., 2007). It is not known if the bacterium will secrete simple sugars after breaking down the complex polysaccharides. *Mucilaginibacter* are also known to produce large amounts of extracellular polysaccharides containing the sugars glucose, galactose, mannose, and rhamnose (Urai et al., 2008), and may thus provide a carbon source for *Saccharomyces*. So in this specific two-organism competition scenario different effects on the bacterium would be possible. It could be that the bacterium is simply dying whilst competing against *S. paradoxus* (parasitism) but it might also be that the bacterium is not affected at all (commensalism) or even benefits from the presence of the yeast (mutualism).

The previous experiments in Chapter I focused exclusively onto the competitor's effect on *S. paradoxus* growth and this gives us no information about the yeast's effect on the competitors. Here, I further investigate the interaction between *S. paradoxus* and *Pseudomonas* I as well as between *S. paradoxus* and *Mucilaginibacter*.

1. The first point I wanted to address is if the interaction between the members are uni-directional (i.e. only from the competitor towards *S. paradoxus*) or bi-directional (also from *S. paradoxus* to the competitor).
2. As it is well known that the outcome of "social" microbial interactions can be influenced by the frequencies of the involved players (Li et al., 2015) I here tested whether there is any positive or negative frequency dependence by changing the initial frequencies of the bacterial competitors.

3. I was additionally interested in how the physical environment might affect maintenance of diversity and due to that I tested warm and cold temperatures.
4. For identifying the interaction's underlying mechanisms, I tested whether conditioned medium was sufficient to produce the effect of the other species on *S. paradoxus*. If conditioned medium would lead to the same effect compared to the presence of the living species, this would mean that it was likely produced by metabolic activity of the organism due to some diffusible factor or by the consumption of something in the medium. If not, it might indicate that the interacting species have to be physically present together (necessary cell-cell). I also tested combinations of the organisms to see if the previous presence of *S. paradoxus* is responsible for the production of a diffusible substance of a bacterium or if the positive effect of *Mucilaginibacter* can buffer the negative effect of *Pseudomonas* on *S. paradoxus*.

2. Methods

For all following experiments I used the transformed G418 resistant homozygote diploid *S. paradoxus* strain isolated from oak bark and co-isolated oak bark bacteria (Kowallik et al., 2015). The preparation of the oak medium, the introduction of the drug resistance into the wild *S. paradoxus* strain as well as the laboratory media for plating and counting colony forming units (cfu) is described in Chapter I.

2.1. Estimating the effects of the microbial species on each other in competition

As the aim of this chapter is to better understand the effects of the microbial species on each other, I used two calculations to get this information.

First, to see how both organisms behave when growing alone and in presence of the other species, I calculated the number of divisions (D) (Lenski et al., 1991) the organisms went through, as this will allow to directly compare the growth of the organisms in competition with their growth when being alone, as follows with $N(0)$ as the initial and $N(1)$ as the final cell number:

$$D = \frac{\ln\left(\frac{N(1)}{N(0)}\right)}{\ln(2)}$$

Second, I was interested in the effect of each organism's presence on the competing species and for this purpose I further calculated the growth rate of each organism in competition relative to growing alone by taking the number of divisions the microorganisms in competition (D_c) went through and dividing it by the number of divisions the organism went through when growing alone (D_a). This measurement describes the effect of the competing species on the organism's growth:

$$\text{Relative growth rate} = \frac{D_c}{D_a}$$

I calculated Wilcoxon Rank Sum tests in R on number of divisions to test if the presence of the other species has a significant influence on the competitor's growth. When I tested for an effect of the used different initial frequencies of the organisms, I calculated Wilcoxon Rank Sum tests on the log relative growth rate data. When I tested one treatment multiple times the resulted p -values were adjusted using the false discovery rate ("fdr") option in R (R-Team, 2015) to correct for multiple testing.

2.2. Characterizing the interaction between *S. paradoxus* and *Pseudomonas*

To study if there is a bi-directional interaction between *S. paradoxus* and *Pseudomonas*, I competed both organisms against each other in liquid oak infusion medium. The bacterium grows in monoculture roughly to a tenfold higher saturated cell density in the oak infusion compared to the yeast. For the yeast it might make a huge difference if one cell has to compete against one cell of *Pseudomonas* or against ten cells. To test if there is any effect due to the 10 times higher natural frequency of the bacterium or whether *Pseudomonas*' effect is independent of frequency, I used two dilutions of the bacterium in the experiments; a 10^{-1} (10 times more *Pseudomonas* than *S. paradoxus* cells, termed "natural ratio 1:10") as well as a 10^{-2} (same initial cell number as *S. paradoxus*, termed "standardized ratio 1:1") dilution. *S. paradoxus* and *Pseudomonas* were grown for three days in oak infusion at room temperature and from these cultures I prepared a 10^{-1} dilution of *S. paradoxus* and *Pseudomonas* as well as a 10^{-2} dilution of the bacterium. The *S. paradoxus* solution was equally mixed with either of the *Pseudomonas* dilution or with water as a control. For the bacterial controls both *Pseudomonas* dilutions were equally mixed with water. 20 μ l of either of these dilutions were given into 2 ml sterile oak infusion. Four replicates per control and competition treatments were incubated at 26 °C on a shaker for three days or at 5.5 °C for 20 days to test for an influence of temperature. I plated the treatments onto YEPD (1 % yeast extract, 2 % peptone, 2 % glucose, 2.5 % agar) with

added 0.4 % G418 antibiotic to estimate the cell number of the resistant *S. paradoxus* and with a much higher dilution onto YEPD to count the bacterial cells.

2.3. Competition of *S. paradoxus* with *Mucilaginibacter* using different initial frequencies

To further study the interaction between *S. paradoxus* and *Mucilaginibacter* I first tested if the initial frequency of the bacterium has a strong influence on the growth of both species in competition, or if the interaction is frequency-independent in a pilot study. I used both, the natural ratio with a 10 fold higher as well as the standardized ratio with an equal initial cell number of the bacterium compared to the yeast. Both organisms were grown for seven days to saturation in liquid oak infusion, then I prepared a 10^{-2} dilution of *S. paradoxus* and *Mucilaginibacter* (~10 times more *Mucilaginibacter* than *S. paradoxus* cells; termed “natural ratio 1:10”) as well as a 10^{-3} dilution of the bacterium (~same initial cell number as *S. paradoxus*; termed “standardized ratio 1:1”). The *S. paradoxus* dilution was equally mixed with *Mucilaginibacter* at the “natural ratio 1:10” as well as the “standardized ratio 1:1”. All dilutions were mixed equally with water, too, for the controls without competition. 50 μ l of each mixture was given into 2 ml sterile oak infusion. I prepared three replicates for each treatment and incubated them at 5.5 °C for 21 days as well as at room temperature (~22 °C) for 14 days to test for an effect of temperature. For a better understanding of what happens over time during the interaction I plated the treatments at six different time points (cold: 3, 8, 11, 14, 18, 21 unit; warm: 1, 2, 3, 8, 11, 14 unit) onto YEPD with added 0.4 % G418 to estimate the cell number of *S. paradoxus* and additionally onto 10 % YEPD to count the bacterial cells (which look very different from yeast cells and can certainly be distinguished).

2.4. Characterizing the interaction between *S. paradoxus* and *Mucilaginibacter* on a temperature gradient

As temperature had already shown to strongly influence the output of the competition I studied the interaction of *S. paradoxus* with *Mucilaginibacter* at a temperature range (5.5 °C, 13.5 °C, 17.5 °C, room temperature (~22 °C) and 30 °C) in liquid oak infusion. I also competed both organisms in standard laboratory medium (10 % YEPD) at 5.5 °C to test if we see the same interaction pattern under this controlled circumstances with glucose as sole carbon source. *S. paradoxus* as well as *Mucilaginibacter* were grown in oak infusion for seven days to saturation at room temperature. I prepared a 10^{-2} dilution of both organisms and mixed them equally for the competition treatments or mixed them with the same amount of water to get controls. I inoculated 2 ml new oak infusion with 50 μ l of the different solutions and let four replicates per

treatment incubate while shaking at the respective temperatures. For the lab medium competition three replicates per treatment were shaken at 5.5 °C. As described earlier, I plated and counted the experiments at several time points individually for each of the different temperature experiments and calculated and plotted the number of divisions as well as the relative growth rates (“effect of other species”) of both organisms in competition to their respective controls growing alone.

2.5. Conditioned media experiments

To test if any of the interactions reveal direct cell contact between *S. paradoxus* and the bacteria and if combinations of the previously grown organisms have different effects than the species alone, I grew the yeast in conditioned media. For this purpose all organisms were grown separately in 2 ml liquid oak infusion to saturation at room temperature and diluted 10^{-2} . I mixed all possible combinations of the three organisms together and added 50 µl of each microbe or a mixture of each two-organism combinations or of all three microbes into 2 ml new oak medium. As there will be always some evaporation of the liquid medium I also prepared additional 2 ml tubes of the sterile infusion and let all treatments incubate at 5.5 °C for 30 days. Afterwards I filtered all treatments using a 0.02 µm filter and added 20 µl of a 10^{-2} dilution of a saturated *S. paradoxus* in oak infusion back into four 2 ml replicates of each conditioned media treatment and let this grow again at 5.5 °C for 26 days. I plated and counted colony forming units onto YEPD plates and plotted the number of divisions *S. paradoxus* could go through in the different pre-used media or the sterile control medium.

3. Results

3.1. *Pseudomonas* dominates the interaction with *S. paradoxus*

Pseudomonas kills *S. paradoxus* but is not affected by the presence of the yeast (Figure 1). I competed *S. paradoxus* with *Pseudomonas* at two different starting frequencies: one representing the natural ratio where bacteria are more abundant than yeast (1 yeast: 10 bacteria) and one at a standardized ratio where both types were initially equally abundant (1 yeast: 1 bacterium). When competitions were carried out at 5.5 °C, I found that *S. paradoxus* grew much better when cultured alone compared to in competition with *Pseudomonas* at either the natural ratio (Wilcoxon Rank Sum test on numbers of divisions; $W = 16$, $p = 0.029$ after FDR correction) and the standardized ratio ($W = 16$, $p = 0.029$). A similar effect was observed at 26 °C

(natural ratio: $W = 16$, $p = 0.029$; standardized ratio: $W = 16$, $p = 0.029$; after FDR corrections). In contrast, the growth of *Pseudomonas* was unaffected by the presence of *S. paradoxus* when grown at 5.5 °C under either initial ratio. At 26 °C, I did observe a small but not significant effect of *S. paradoxus* on the growth of *Pseudomonas*: the bacterium underwent fewer divisions when competed against *S. paradoxus* at the natural ratio than when growing alone. Overall, competitions at the two different starting frequencies produced very similar results.

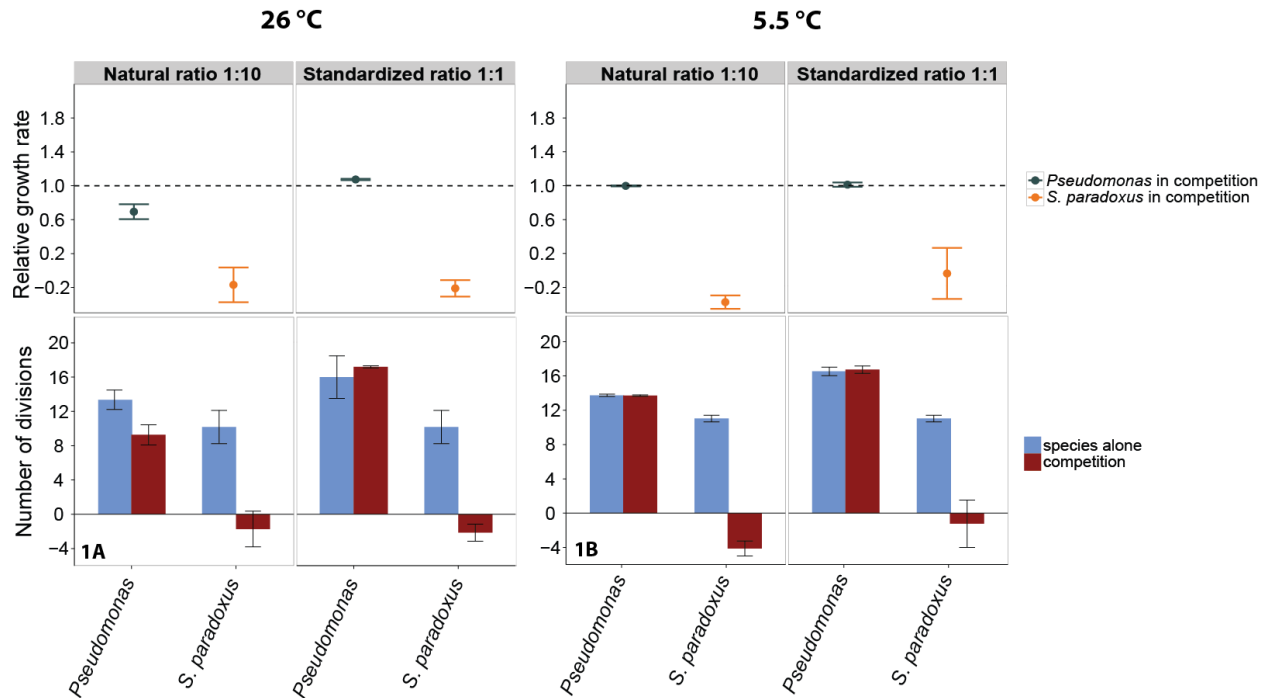


Figure 1A and B: Competition between *S. paradoxus* and *Pseudomonas* in oak infusion at 26 °C (1A) and 5.5 °C (1B) using two different initial frequencies of the bacterium. The bar graphs show the number of divisions each organism underwent when growing alone (blue) and in competition (red). The growth rate of each organism in competition relative to growing alone is shown in the upper graph. In the upper graphs, values = 1 indicate that *Pseudomonas* or *S. paradoxus* in competition with the other organism grow exactly as well as growing alone, values <1 indicate that the competing species inhibits growth, and values <0 indicate that the competing species kills the organism. For all graphs means and standard deviations are plotted.

3.2. *S. paradoxus* and *Mucilaginibacter* both benefit from the interaction

In oak medium *S. paradoxus* underwent more divisions in the presence of *Mucilaginibacter* than when growing alone (Figure 2). Thus, in contrast to *Pseudomonas* which actively killed yeast, *Mucilaginibacter* promoted yeast growth. The benefit of *Mucilaginibacter* to yeast growth was strongly dependent on temperature and most apparent at the two extreme temperatures 30 °C

and 5.5 °C with a fitness benefit of competition of ~ 80 % relative to growing alone. At the middle temperatures (12.5 °C and 17.5 °C) the growth promoting was much weaker. However, the benefit for yeast growth was most apparent over the first days at nearly all temperatures in oak medium, but persisted for much longer at 5.5 °C than at higher temperatures. During later growth, *S. paradoxus* grew at the same rate in the presence or absence of the bacterium. *Mucilaginibacter* was not negatively affected by the presence of the yeast in oak infusion and was almost consistently doing at least as well as growing alone. There has been even a small but not significant growth promoting on the bacterium in competition with *S. paradoxus* at the earliest time points at 30 °C. In laboratory medium at 5.5 °C there was no beneficial effect detectable and moreover the presence of *Mucilaginibacter* strongly inhibited *S. paradoxus* growth and in almost the same manner the presence of the yeast inhibited the growth of the bacterium. Detailed experiments of the interaction between *S. paradoxus* and *Mucilaginibacter* were carried out at the normal ratio, as initial time series pilot experiments at 5.5 °C and room temperature showed no difference between the normal ratio and the standardized ratio on the relative growth rate of both organisms in competition at any time point (Supplemental Table 1).

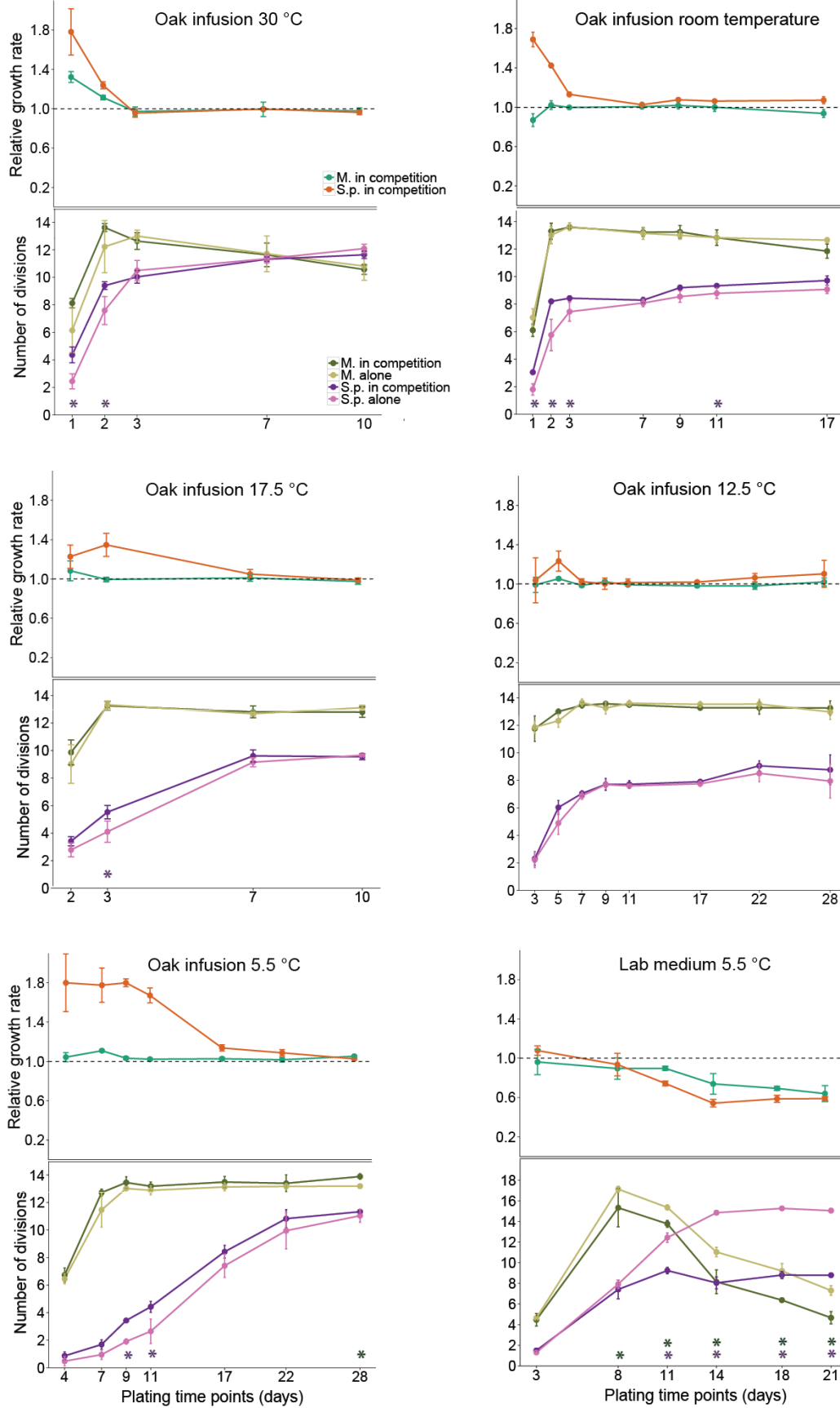


Figure 2: Competition experiments between *Mucilaginibacter* (M) and *S. paradoxus* (S.p.) at different temperatures. On the x-axis are the individual plating time points of every experimental run. Shown are the numbers of divisions each organism can go through growing alone (light green and light purple) and in competition (dark green and dark purple). The growth rate of both species in competition relatively to growing alone is shown in the upper graph; values = 1 indicates that *Mucilaginibacter* or *S. paradoxus* in competition with the other organism grow exactly as well as growing alone, <1 the competing species inhibits, >1 the competing species promotes the growth. For all graphs means and standard deviations are plotted. Stars above the x-axis indicate that at this plating time point *S. paradoxus* (purple) or *Mucilaginibacter* (green) in competition differ significantly in number of divisions (after Wilcoxon Rank Sum Tests) from the respective growing alone treatment.

3.3. Conditioned media has little effect on the growth of *S. paradoxus*

Although I expected conditioned oak infusion medium to differ substantially from sterile oak infusion medium, since the microorganisms should have used many resources and secreted metabolites prior to being filtered out, the effect of conditioned medium on the growth of *S. paradoxus* was significant but not strong (Figure 3). In all cases, except for medium previously containing *S. paradoxus* or *Mucilaginibacter*, the growth of *S. paradoxus* was significantly lower on conditioned medium than in sterile medium (Wilcoxon Rank Sum tests after FDR correction: *Pseudomonas*: $W = 16$, $p = 0.04$, *S. paradoxus* plus *Mucilaginibacter*: $W = 16$, $p = 0.04$, *S. paradoxus* plus *Pseudomonas*: $W = 16$, $p = 0.04$, *Mucilaginibacter* plus *Pseudomonas*: $W = 16$, $p = 0.04$, as well as *S. paradoxus* plus *Mucilaginibacter* plus *Pseudomonas*: $W = 16$, $p = 0.04$).

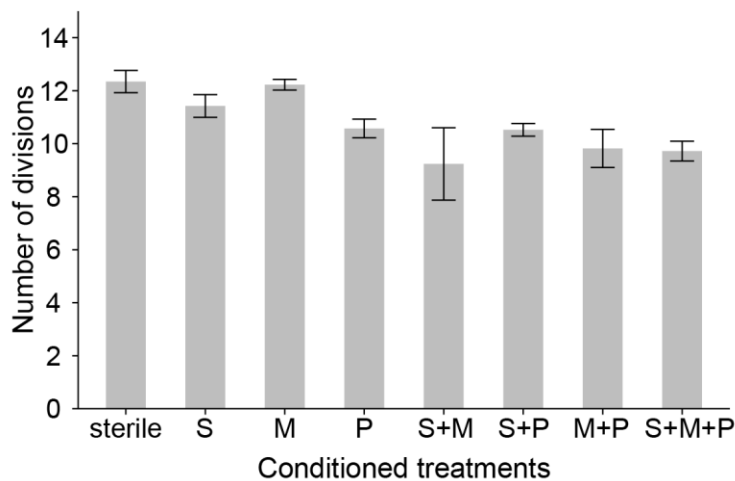


Figure 3: Mean number of divisions and standard deviation of *S. paradoxus* growing in conditioned oak media and in a sterile control. The organisms previously grown in the medium and filtered out are indicated with S = *S. paradoxus*, M = *Mucilaginibacter*, P = *Pseudomonas* I. The experiment was incubated at 5.5 °C for 26 days.

4. Discussion

The work done in this chapter aimed to dissect the complex interactions between *S. paradoxus* and two co-isolated bacteria in the natural oak bark infusion. The bacteria showed extreme and complete opposite effects on the growth of the yeast reflecting how biodiversity in an environment might help creating different niches which and that species interactions can therefore be strongly involved in high biodiversity.

4.1. *S. paradoxus* and *Pseudomonas* interaction is uni-directional with a resulting killing of the yeast

Pseudomonas killed *S. paradoxus* and was not affected by the presence of the yeast. The interaction is therefore unidirectional and strongly dominated by the bacterium, the yeast seems to be little more than “collateral damage” in this interaction. Under the experimental conditions I have chosen, it seems that the bacterium pays no fitness cost while competing against the yeast (Figure 1A and B). If *S. paradoxus* influences the bacterium in a way that it produces more or different antimicrobial compounds like it has been demonstrated for other *Pseudomonas* species in interaction with fungi (Cugini et al., 2010; Gibson et al., 2009; Moree et al., 2012) we would expect this to come along with a cost. Instead, the bacterium may consistently produce metabolic by-products that are harmful for the yeast. It remains possible that the fitness differences were too small to be observable or we may have missed the correct time point to detect any cost. If the *Pseudomonas* in competition grows, for example, slower compared to growing alone this might only be detectable in the beginning and not in the end when the organisms in both treatments reached saturation.

4.2. The interaction between *S. paradoxus* and *Pseudomonas* is not frequency-dependent nor strongly regulated by temperature

The interaction between *S. paradoxus* and *Pseudomonas* was not strongly influenced by the abiotic factor temperature: the bacterium killed the yeast at both cold and warm temperatures and additionally it did not matter if the initial frequency of the bacterium was higher or lower. At warm temperatures, the lower cell number of *Pseudomonas* at the standardized ratio killed the yeast as effectively as the higher cell number at the natural ratio showing that the bacterium is very efficient in its interference competition. At cold temperatures, *Pseudomonas* had a stronger killing effect on *S. paradoxus* when it was present at a higher initial concentration but it was still capable of killing yeast at lower initial concentrations. There was no significant effect of

the presence of yeast on *Pseudomonas*' growth. The reason why *Pseudomonas* in competition at high temperatures and high initial cell number ended up in some fewer numbers of divisions than growing alone might simply be that the bacterium reached saturation earlier in the high cell number treatment in which an additional second microbe (*S. paradoxus*) was present and consumed nutrients. It has been shown for *Pseudomonas fluorescens* that the viable cell number strongly decreases after reaching saturation in laboratory cultures (Aminov and Golovlev, 1986), and that was likely true in this case, as I did not see a similar effect in the respective standardized competition treatment.

4.3. The killing effect from *Pseudomonas* depends on the direct presence of the bacterium

Given the efficient killing of *S. paradoxus* by *Pseudomonas* when the species were grown together (Figure 1), I would have expected *Pseudomonas*-conditioned medium to also efficiently kill *S. paradoxus*. In contrast to this expectation, the killing effect of *Pseudomonas*-conditioned medium was small (Figure 3). Nor did conditioning medium with *Pseudomonas* together with yeast increase the toxicity beyond the level seen with *Pseudomonas* alone. Thus, a direct interaction between the yeast and this bacterium seems to be necessary for efficient killing. Mechanisms of bacterial toxicity that require direct cell-to-cell contact are well known. For example, the Type VI secretion system (T6SS) of *P. aeruginosa* injects at least three proteins (Tse1, Tse2, and Tse3) into the periplasmic space of bacterial competitors to kill them (Hood et al., 2010; Russell et al., 2011) and direct contact between cells is necessary for killing. A screen of different bacteria revealed that several bacterial species, including several *Pseudomonas* strains, harbor T6SS-encoded gene clusters in their genome (Boyer et al., 2009). This means that it is not unlikely that our *Pseudomonas* I strain exhibit such gene clusters and kills the yeast by a similar mechanism. From the halo assays in Chapter I it was shown that *Pseudomonas* produces a diffusible toxin which killed the yeast as there was a clear halo around the *Pseudomonas* colony meaning that no direct cell contact was necessary for the killing. It is likely that *S. paradoxus* is sensitive to more than one of the many antimicrobial compounds of *Pseudomonas*, meaning that a killing by a diffusible toxin and killing by cell contact are both possible. Other explanations would be that the bacterial metabolic by-products degrade after a short time and are not harmful to the yeast anymore or the filtering step somehow removed or changed compounds important for virulence.

4.4. The interaction between *S. paradoxus* and *Mucilaginibacter* benefits the yeast's growth

Competing *S. paradoxus* and *Mucilaginibacter* in oak infusion revealed a benefit on the yeast's growth. There are indications that the interaction is bi-directional in which both partners can benefit under certain conditions (mutualism). In oak infusion, across all temperatures, neither species grew worse in the presence of the other than when grown alone. This lack of antagonism indicates that each organism consumes different types of nutrients in the oak medium. Examining the interaction over time revealed that *S. paradoxus* benefits from the presence of *Mucilaginibacter* primarily through faster growth in the early stages (a head start) but the yeast did not ultimately reach higher cell numbers in the presence of the bacterium compared to the control. It would be very interesting to find out what chemically happens when *Mucilaginibacter* competes against *S. paradoxus* in the oak infusion. From literature it is known that bacteria of this genus are able to digest xylan, pectin and laminarin. Xylan, a hemicellulose, and pectin are both components of bark and wood (Timell, 1967). As most polysaccharides are too big to enter the cell, some bacteria secrete extracellular enzymes to hydrolyze such macromolecules in the environment. The hydrolyzed products are also available for competing organisms and this might be the case in our bacterium-yeast mutualism. Other bacteria assimilate the macromolecules in a similar way to the eukaryotic lysosome (Priest 1992). The mechanism by which *Mucilaginibacter* degrades complex sugars is currently not known. We also do not know if polysaccharides are actually present in the liquid infusion and, if they are, which type and in which concentration. Most polysaccharides are not water soluble and therefore may have been excluded from the infusion. Another explanation for the positive effect of the bacterium would be that it can degrade chemical compounds which are harmful to the yeast. This "detoxification" could explain the faster growth of yeast in the beginning of the experiments. An infusion made out of oak bark will contain toxins and secondary metabolites produced by the microorganisms naturally inhabiting the bark and growing for the 24 hours incubation time during medium preparation. In addition, oak bark contains several different bioactive secondary metabolites like tannins which can also inhibit diverse microorganisms (Andrenšek et al., 2004, Uddin and Rauf 2012). These products might negatively influence the yeast and the yeast would benefit from the presence of an organism that can somehow neutralize this.

4.5. The interaction of *S. paradoxus* and *Mucilaginibacter* is independent of the organism's frequency but strongly influenced by temperature

The growth promoting of *S. paradoxus* caused by *Mucilaginibacter* was strongly regulated by temperature but different initial frequencies of the bacterium did not lead to a different performance of the yeast nor the bacterium in competition. Using a five temperature gradient (5.5 °C, 12.5 °C, 17.5 °C, room temperature (~22 °C) and 30 °C) for the competition studies revealed that at all temperatures both organisms were doing at least as well as growing alone and even showed enhanced growth at specific temperatures. The yeast most strongly benefit from the bacterium at 5.5 °C and 30 °C, but a smaller benefit was observed at room temperature. Interestingly the effect of temperature on the growth benefit enjoyed by the yeast in the presence of bacteria was not directional, but instead was strongest at the two extreme temperatures (30 °C and 5.5 °C). The effect was smallest at intermediate temperatures (e.g. 12.5 °C). The reason why I exclusively detected the growth promoting effect of the yeast at 5.5 °C in Chapter I and why there was no detectable effect at 26 °C, is that I tested only one later time point at high temperature and therefore missed the relevant time point. The small beneficial effect by the yeast on *Mucilaginibacter* was a growth to a higher cell number in competition at 30 °C than alone. It seems that *Mucilaginibacter* is actually harmed by the high temperature as in general the bacterium reached higher cell numbers at lower temperatures, so the yeast might “buffer” the high temperature stress. Anyway, for a real interpretation it would be important to test higher temperatures on the interaction of *S. paradoxus* and *Mucilaginibacter* to see if the benefit for the bacterium increases.

4.6. No big effects on *S. paradoxus* growth using conditioned medium

Growing *S. paradoxus* in oak infusion, in which previously all three organisms of this study had been grown, alone or in all combinations showed a smaller effect onto the growth of the yeast than I would have expected (Figure 3). The difference to the sterile treatment, although significant, was surprisingly small since the conditioning organisms should have consumed most of the nutrients beforehand. The bacteria might consume different nutrient types but the previously grown *S. paradoxus* should have exploited the medium. We do not know if *S. paradoxus* respire or ferments in the oak infusion as aerobic fermentation can already occur at sugar concentrations as low as 150 mg/l (Pfeiffer and Morley, 2014). The produced ethanol could be later re-consumed by respiration in the conditioned medium by the yeast which would lead to a higher ATP output for the cell (Bakker et al., 2001; Pfeiffer and Morley, 2014). On the other hand the buffering of the harmful effect of temperature on *Mucilaginibacter* would not speak for a fermentation behavior as this would, even if very slightly, increase the temperature.

As we do not know the composition of the oak infusion, the chemistry might change over time especially when a growing microbe releases metabolic by-products into the medium. The oak infusion medium definitely seems to provide a much more stable environment than a simple medium with only one source of nutrients which we could already see in the competition study between *Mucilaginibacter* and *S. paradoxus* which was ran for long times (like 28 days at 12.5 °C) and still did not lead to a decrease of the organisms frequencies in the medium. Learning from these competition studies it would have been important to test the growth of the *S. paradoxus* in the conditioned *Mucilaginibacter* medium earlier, as the growth promoting effect was also visible in earlier time points. *S. paradoxus* and *Mucilaginibacter* previously grown together show to be more effective in exploiting the medium than both organisms alone, leading to fewer divisions of the after filtering added *S. paradoxus*. This result actually fits well with the general assumption that communities with a higher biodiversity yield a higher productivity (Lehman et al., 2000). Anyway, the three organism combination did not exploit the medium more than two organisms could, but this could actually be produced by the fact that *S. paradoxus* was killed in the presence of *Pseudomonas* while it could grow together with *Mucilaginibacter* and *Mucilaginibacter* could grow together with *Pseudomonas* (data not shown).

4.7. A more natural medium allowed us the detection of more complex interactions

Testing the interaction between *Mucilaginibacter* and *S. paradoxus* in laboratory medium containing glucose as sole carbon source eliminated any beneficial effect in competition and lead to pure resource competition with a decrease in the fitness of both competing organisms. *Mucilaginibacter* might produce something beneficial for the yeast (simple sugars, enzymes etc.) but this seems to only take place only in the natural medium. On the other hand, it is clear that this decrease in fitness in lab medium is produced by the fact that both organisms have to compete for the same resource (glucose) and so a potential benefit of excreted enzymes that break down more complex polysaccharides or other for the yeast beneficial substances would be lost. My results show that laboratory medium gives very different results compared to the undefined, complex oak medium which allows a higher diversity of organisms to co-exist and to even positively interacting with each other. This explains how important it is to test interactions in environments as natural as possible to detect a range of complex interactions. Further experiments would be necessary to really understand what is going on between the three microorganisms in the oak bark environment. The oak infusion is a great opportunity to test such interactions of microorganisms adapted to oak bark under relatively natural conditions. As

this medium is not defined, there is likely to be significant preparation-to-preparation variation. Moreover, long-term storage or freezing might also lead to a change of the chemistry of the medium. Variations that I detected repeating experiments in new oak infusion medium (data not shown) can be explained by that. It is important to mention that I never saw contradictory results, just variation in the strength of the effects. The prepared oak infusion is clearly not exactly the same as a piece of oak bark but it contains at least some of the same nutrients and metabolic products from naturally occurring microorganisms. However closely it mirrors oak bark, oak-infusion medium has allowed us to detect a more complex interaction that would have been concealed under pure lab conditions.

5. Conclusion

The closer analysis of the interactions between *S. paradoxus* and two co-isolated bacteria revealed contrary types of interactions. The *Pseudomonas* and *S. paradoxus* interaction is exclusively controlled by the bacterium which kills the yeast and neither the initial frequency of the bacteria nor the incubation temperature significantly influenced the interaction. The direct presence of *Pseudomonas* was necessary for killing the yeast. The interaction between *Mucilaginibacter* and *S. paradoxus* is beneficial for the yeast and might be even bi-directional. It is strongly dependent on the physical environment like temperature and complexity of the medium. Studying natural microbial interactions require the use of a medium as natural as possible; simple laboratory medium might not allow complex interactions. The two interactions described in this chapter show how the identity of organisms can alter the community composition and biodiversity. If we imagine that communities also change over time *Pseudomonas* might completely exclude *S. paradoxus* from places it is living in nature and might even be competitive enough to invade an already existing population of yeast. *Mucilaginibacter* might stabilize the existence of the yeast when present, helping it to grow faster and the yeast might protect the bacterium from high temperature stress. Determining if these interactions are the result of co-adaptation will require further work, but the studies described here provide an insight into the complexity of microbial interactions and how the presence of organisms can alter niches in an environment.

6. Supplemental Material

Supplemental Table 1: Test if the initial frequency has any influence on the relative growth rate of the organisms in competition. Compared are always the natural ratio 1:10 with the standardized ratio 1:1 treatments for *Mucilaginibacter* as well as *S. paradoxus* using Wilcoxon Ranked Sum tests on the relative growth rate data of the organisms in competition at 5.5 °C and room temperature for the six individual plating time points.

Time point	Temperature	<i>Mucilaginibacter</i>		<i>S. paradoxus</i>	
		W	p-value	W	p-value
Day 3	5.5 °C	8	0.2	5.5	0.83
Day 8	5.5 °C	4	1	5	1
Day 11	5.5 °C	0	0.1	3	0.7
Day 14	5.5 °C	4	1	3	0.7
Day 18	5.5 °C	2	0.4	5	1
Day 21	5.5 °C	5	1	5	1
Day 1	room temp.	8	0.2	2	0.8
Day 2	room temp.	5	0.4	3	0.7
Day 3	room temp.	5	1	4	1
Day 8	room temp.	6	0.7	4	1
Day 11	room temp.	6	0.7	5	1
Day 14	room temp.	9	0.1	6	0.7

Chapter III Is the Crabtree effect an adaptation to high sugar environments?

1. Introduction

All organisms need energy for growth, survival and reproduction and given the huge biodiversity present in nature we can find many different strategies used by organisms to achieve this goal. Not every organism is able to consume every energy source and some species have specialized in using specific nutrients in specific environments. *Saccharomyces cerevisiae*, due to its use in the wine making process, has long been thought to be such a specialist in high sugar fruit environments. Indeed some features of *Saccharomyces*' metabolism can be interpreted as adaptations to high sugar environments, but direct evidence is missing.

1.1. The aerobic and anaerobic metabolism of *Saccharomyces* spp.

In general simple sugars are converted in living cells to biochemical energy via two different pathways. In the presence of oxygen, most organisms (including most fungi) use cellular respiration to generate ATP. In the absence of oxygen some organisms can use fermentation for the same purpose. Yeasts can have different kinds of sugar metabolism, namely non-, facultative- or obligate-fermentative. Non-fermentative yeasts exclusively respire, whereas obligate fermentative yeasts exclusively ferment. Most yeast species identified including *Saccharomyces* yeast are facultative-fermentative, meaning that they can switch between respiration and fermentation as needed. The type of metabolism (fully respiratory, fully fermentative, or mixed respiratory-fermentative) depends on growth conditions, sugar type and concentration and oxygen availability (Rodrigues et al., 2006).

S. cerevisiae is able to consume several types of nutrients but glucose is its preferred carbon source. Glucose utilization requires the yeast to first sense the presence of this sugar in the environment and to then transport it across the plasma membrane into the cell. Glucose transport is regulated by a gene family of hexose transporters (*HXT*) which belong to a superfamily of monosaccharide facilitators. In *S. cerevisiae* this gene family consists of 20 members which have different substrate specificity and affinity, and are expressed under different, overlapping conditions. The identified genes are: *HXT1–HXT17*, *GAL2*, *SNF3* and *RGT2* (e.g. Bisson et al., 1993; Boles and Hollenberg, 1997; Kruckeberg, 1996; Özcan and Johnston,

1999). The transporters *Hxt1–Hxt4*, *Hxt6* and *Hxt7* are the major hexose transporters in yeast and can transport glucose, fructose and mannose (Reifenberger et al., 1995). All together 17 of the 20 *HXT* genes encode glucose transporters but under normal circumstances only six (*HXT1* and *HXT3–HXT7*) mediate uptake (Lin and Li, 2011). *RGT2* and *SNF3* encode proteins that function not as transporters but as sensors of extracellular glucose (Özcan and Johnston, 1999). In *S. cerevisiae* two sugar uptake systems are known: a constitutive, low-affinity system and a high-affinity system which is repressed by low concentrations of glucose (e.g. Bisson, 1988; Ramos et al., 1988; Serrano and Delafuente, 1974). Other *S. cerevisiae* genes mediate transport of other solutes and are homologous to the *HXT* family (Boles and Hollenberg, 1997). After sugar is transported into the cell, the yeast can respire or ferment to gain energy (Figure 1). Both types of metabolism can occur at the same time (respire-fermentative metabolism) in *S. cerevisiae*. Respiration as well as fermentation starts with glycolysis, which is a series of ten reactions converting each molecule of glucose to two molecules pyruvate in the cytoplasm of the cell. Glycolysis requires two molecules ATP to get started (one ATP is used to generate glucose 6-phosphate from glucose and later one ATP is used to generate a central intermediate 1,6-bisphosphate) but produces four ATP during the substrate-level phosphorylation step. The net reaction equation of glycolysis can be expressed as: $\text{Glucose} + 2 \text{P}_i + 2 \text{ADP} + 2 \text{NAD}^+ = 2 \text{pyruvate} + 2 \text{ATP} + 2 \text{NADH} + 2 \text{H}^+ + 2 \text{H}_2\text{O}$ (Piskur and Compagno, 2014). During aerobic respiration the two pyruvates will be oxidized to CO_2 via the formation of acetyl-CoA by the pyruvate dehydrogenase complex (PDC) followed by the citric acid cycle (tricarboxylic acid (TCA) cycle) and respiratory chain. The citric acid cycle is an eight-step process that oxidizes acetyl-CoA to CO_2 . One complete cycle yields a net energy gain of 3 NADH, 1 FADH_2 , and 1 GTP (which is subsequently used to produce ATP). The high energy electrons of NADH and FADH_2 are later transferred to a series of components comprising the electron transport chain which establishes a proton gradient across the inner membrane of the mitochondrion to synthesize ATP through oxidative phosphorylation. Finally the electrons are transferred to oxygen and water is formed. Oxygen's role as the final electron acceptor defines that the citric acid cycle and electron transport chain cannot proceed in absence of oxygen (Alberts et al. 1999). Compared to other eukaryotic cells *S. cerevisiae* produces relatively low levels of ATP during oxidative phosphorylation resulting from a mitochondrial external NADH dehydrogenase activity that does not pump protons (Bakker et al., 2001; Rodrigues et al., 2006). The entire ATP yield of respiration is 16 per molecule glucose: four ATP are from substrate level phosphorylation (two from glycolysis and two from GTP formed in the TCA cycle) and 12 from oxidative phosphorylation, 6 from each of the two pyruvate molecules (Bakker et al., 2001). When no oxygen is available the yeast cell undergoes fermentation to generate ATP. During fermentation the pyruvate produced by glycolysis is converted by recycling NADH (from glycolysis) to acetaldehyde by the enzyme pyruvate decarboxylase and subsequently to ethanol by alcohol

dehydrogenase 1 (*ADH1*). The ATP yield of alcoholic fermentation (2 ATP) is only 1/8th that of respiration (16 ATP) (Bakker et al., 2001). When the intracellular sugar concentration drops and oxygen is available, the yeast cell can enter a second growth phase using ethanol as carbon source. During this so called "diauxic shift" the ethanol is reconverted to acetaldehyde catalyzed by *ADH2* and then to acetyl-CoA which feeds into the citric acid cycle. To convert one molecule ethanol to acetyl-CoA one molecule ATP is required. Most alcohol produced by fermentation can likely not be consumed because it dissipates (Thomson et al., 2005).

In *S. cerevisiae*, different pathways can be activated or down regulated by the cell to satisfy its needs under different environmental conditions, reflecting different metabolic states. Carbon source and concentration and oxygen presence have the largest impact on this pathway regulation. The consumption of different carbon sources is differently regulated by using different metabolic pathways. Raffinose, glycerol, and ethanol are for example non-fermentable carbon sources. Fermentation rates of fermentable sugars (glucose, fructose, maltose, mannose and galactose) is dependent on sugar concentration (De Jong-Gubbels et al., 1995).

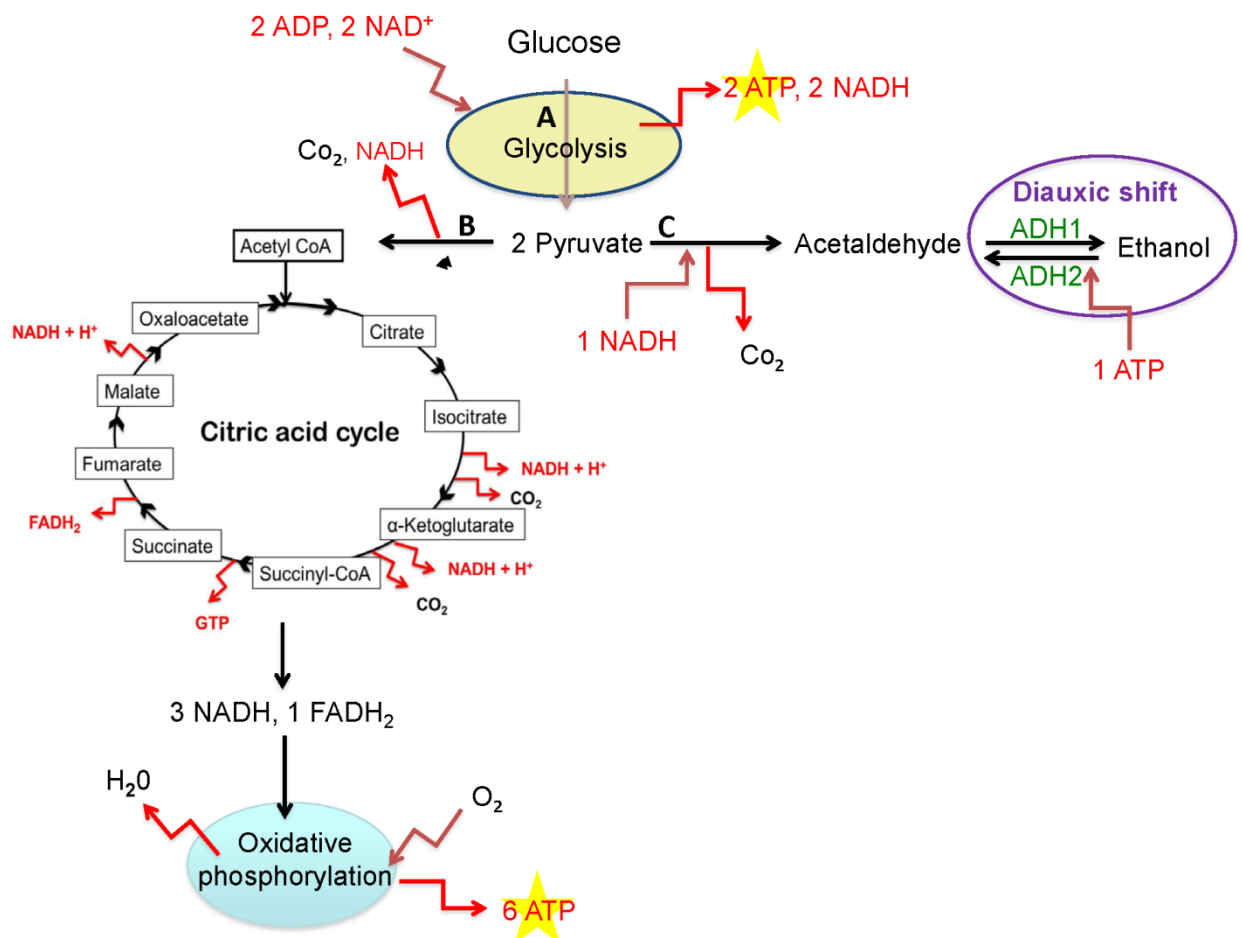


Figure 1: The metabolic pathway inside of *S. cerevisiae*. Glycolysis (A) produces 2 molecules of pyruvate and 2 molecules of ATP. Each pyruvate molecule can be used either **aerobically (B)** in the Citric acid cycle with following oxidative phosphorylation, or **anaerobically (C)**, i.e. fermentation. Aerobic respiration in yeast yields a further six molecules of ATP and one of GTP (which can be used to make ATP) per pyruvate molecule, so the total yield of ATP is 16 molecules ($2+(1+6)+(1+6)$) per molecule of glucose. No further ATP is yielded when pyruvate is converted to ethanol, but ethanol can later be converted to acetyl-CoA (at a cost of 1 molecule of ATP) which feeds into the citric acid cycle. The theoretical yield per molecule glucose, if initially fermented and then later if all ethanol is successfully converted to acetyl-CoA and respired aerobically, is 14 ($2+(6+1)+(6+1)-2$) molecules of ATP.

1.2. The Crabtree effect: an adaptation to high sugar environments?

Some microorganisms including *Saccharomyces* yeast show the surprising behavior of fermenting even though oxygen is available which is the Crabtree effect (Pronk et al., 1996) and not all yeast are capable of it. Non-fermentative yeast species that exclusively respire (like *Rhodotorula glutinis*), exclusively ferment (*Candida slooffii*), or have a mixed respiro-fermentative metabolism but will only ferment under anaerobic conditions are designated as Crabtree negative yeasts, whereas yeasts such as *S. cerevisiae* and *Schizosaccharomyces pombe*, which accumulate ethanol in the presence of oxygen, are called Crabtree positive yeasts (Rodrigues et al., 2006). *S. cerevisiae* and its close relatives are not the only yeasts that ferment under aerobic conditions but they seem to have specialized in this metabolic trait that includes fast growth, good ability to produce and consume ethanol as well as tolerance against several stresses such as high ethanol concentrations, low oxygen levels and high temperatures (Piškur et al., 2006). These yeasts do not only grow rapidly in high-sugar environments, but also prefer any fermentable carbon source to any carbon source that has to be metabolized by respiration (Zaman et al., 2008). Comparing the fermentative power of *S. cerevisiae* to six other yeasts naturally inhabiting fermenting grapes shows that no of the other yeasts is able to ferment fruit sugars to completion except for *S. cerevisiae* (Goddard, 2008).

Compared to aerobic respiration, fermentation produces low ATP and biomass yield, therefore it is wasteful and energetically expensive. The re-consumption (“diauxic shift”) of ethanol can compensate for some energy lost to inefficient fermentation. But some, if not most, ethanol will likely not re-enter the cell, and ethanol uptake has a cost of one ATP molecule per ethanol molecule (Thomson et al., 2005). Why would an organism evolve inefficient resource utilization that reduces its yield? In a scenario where an organism has exclusive access to a resource, the resource should be used as efficiently as possible. In reality microorganisms do not have exclusive access to resources as they do not live as monocultures and within microbial communities competition for space and nutrients will allow different (metabolic) strategies to persist (Little et al., 2008).

Although the Crabtree effect does not seem to be the best strategy on a first view, it has been suggested that it provides two benefits that outweigh its energetic inefficiency. Both benefits should only be apparent when Crabtree positive yeasts compete directly in the same local environment for resources with Crabtree negative competitors.

First, Crabtree positive yeasts have an **advantage under resource competition**. Fermentation is rapid, allowing yeast to increase in number faster than respiring competitors. Heterotrophic organisms (which require nutrition obtained by digesting organic compounds) face a trade-off between rate (moles of ATP per unit of time) and yield (moles of ATP per mole of substrate) of ATP production (Angulo-Brown et al., 1995; Stucki, 1980; Waddell et al., 1999). A yeast cell that respire sugar using a pathway with high yield and low rate will produce more ATP per sugar molecule, and therefore more offspring from a given amount of resource. This advantage will disappear when this yeast cell has to compete directly for a given amount of resource with cells that consume this resource rapidly and produce ATP at a higher rate but lower yield (Pfeiffer et al., 2001).

Second, Crabtree positive yeasts have an **advantage under interference competition**. Ethanol, a byproduct of fermentation, is toxic to many other microbes and may therefore decrease the abundance and diversity of competitors (Piškur et al., 2006; Thomson et al., 2005). In addition, fermentation produces esters (mainly acetate esters) (Saerens et al., 2008), different acids but in the highest amount acetic acid (Maiorella et al., 1983) that decrease the substrate pH, and heat which can be lethal to temperature sensitive organisms (Goddard, 2008). The direct environment of the yeast gets degraded by these products, inhibiting the growth of competitors. On the other hand, the fermentation-by-products ethanol and acetate in the environment are in high concentrations toxic to *Saccharomyces* itself. This and the lower yield relative to aerobic respiration seem to be the cost of fermentation (MacLean, 2007). The quality of changing an environment by fermentation in a way that directly or indirectly affects other organisms of the community can be called "niche construction" or "ecosystem engineering" (Goddard, 2008; Hastings et al., 2007; Jones et al., 1994). Furthermore, fermentation transforms the nutrients into a carbon source (ethanol) which cannot be consumed by most of the other microbes but can be consumed by the yeast itself. This strategy has been called the "make-accumulate-consume" strategy (Piškur et al., 2006) and can contribute to the advantage of fermentation.

1.3. Competition studies in the scientific literature and their interpretation problem

A small number of previous studies have compared Crabtree positive and Crabtree negative yeasts. *S. cerevisiae* usually dominates mixed cultivations with multiple other yeast species, but the reason for that seems to be very dependent on the identity of the competing yeasts. In well-mixed competitions with the Crabtree negative yeast *Kluyveromyces lactis*, *S. cerevisiae*'s fermentative metabolism with its rapid nutrient consumption and growth showed to be a profitable metabolic strategy (better resource competition) for competing for glucose in a well-mixed population. High glucose contents (like we find in grapes) showed to increase this fermentative advantage. In a spatially structured environment this benefit was not present as yeast cells did not grow in direct competition and could use the surrounding nutrients alone and efficiently, and the more efficient respiring *K. lactis* became dominant in the population (Bratulic, 2010). While a growth rate study in monocultures of commercial *S. cerevisiae* strains and six non-*Saccharomyces* wine yeasts in grape juice and standard laboratory medium revealed that *S. cerevisiae* had an advantage at high ethanol levels (only one of the other yeasts tolerated ethanol concentrations similar to *S. cerevisiae*), low levels of ethanol did not significantly poison the non-*Saccharomyces* yeasts and some appeared tolerant of reasonably high ethanol levels. The factor heat seemed to have a stronger effect on the yeast's growth than ethanol concentration and correlated with the decrease of the non-*Saccharomyces* and the increase in *S. cerevisiae* (Goddard, 2008). The same result was true in a different study showing that *Saccharomyces* niche construction via ethanol production did not provide a clear ecological advantage whereas a temperature rise gave *S. cerevisiae* a considerable advantage (Salvadó et al., 2011a). Studying mixed and unmixed growth assays of *S. cerevisiae* with the two *Hanseniaspora* yeast; *H. guilliermondii* and *H. uvarum* in synthetic grape juice medium showed that the non-*Saccharomyces* yeasts grew well for the first 1-3 days and then died off in *S. cerevisiae*'s presence regardless of the ethanol concentration. The initial cell densities of the yeasts in competition as well as toxic fermentation products (other than ethanol) of *S. cerevisiae* seemed to be responsible for the death of the *Hanseniaspora* yeasts (Pérez-Nevado et al., 2006). Oxygen played a key role for the competition output of the two non-*Saccharomyces* wine yeasts *Kluyveromyces thermotolerans* and *Torulospora debreuecki* in mixed cultures with *S. cerevisiae* revealing that the death of the non-*Saccharomyces* yeasts at low available oxygen conditions was not caused by toxic metabolites of *S. cerevisiae* but rather by the lack of oxygen (Holm Hansen et al., 2001). Furthermore cell-to-cell contact-mediated damaging mechanisms of *S. cerevisiae* seemed to play an additional role in their early death (Nissen and Arneborg, 2003). In a big study screening the competition between *S. cerevisiae* and 18 other Crabtree negative as well as positive yeast species it could be shown that *S. cerevisiae* dominated nearly all other

yeasts except for its sibling species *S. paradoxus* in grape juice and high sugar lab medium. High ethanol concentrations, low pH and poor nutrient conditions were shown to influence its fitness positively (Williams, 2014). Some more studies on the competitive dynamics in yeast communities were actually done during the wine fermentation process. This is a strongly artificial environment and often manipulated to the benefit of a good working fermentation and a good tasting end product, meaning a dominance of *S. cerevisiae* and this makes it not useful for any ecological studies. It seems not logic, if the Crabtree effect is the only competition advantage of *S. cerevisiae* that other Crabtree positive yeasts do not benefit in the same way. The results of the previously listed, controlled competition studies strongly vary and depend on the competitor yeast species. Competing *S. cerevisiae* against yeast species with a different evolutionary history and other costs and benefits of life history traits makes it hard interpreting the results. Differences in fitness might not necessarily be attributable to the Crabtree effect. For example competing the Crabtree negative *Candida utilis* against *S. cerevisiae* under glucose limited anaerobic conditions in a homogenous environment lead to the dominance of *S. cerevisiae* but anaerobic conditions have nothing to do with the Crabtree effect. Changing the conditions to aerobic ones lead to a dominance of the Crabtree negative yeast in mixed competition (Postma et al., 1989). The best and most elegant way of testing costs and benefits of the Crabtree effect would be to compare the same species differs only in this trait.

1.4. Use of a Crabtree positive and negative strain in our study

In this study I tested the relative fitness of a Crabtree positive *S. cerevisiae* wild-type yeast (strain KOY.PK2-1C83, the “fermenter” strain, although of course it can also respire) against its isogenic Crabtree negative mutant (strain KOY.TM6*P, the “respirer” strain, which can only ferment when oxygen is absent) (Elbing et al., 2004; Otterstedt et al., 2004). Both strains are *MATa* haploids meaning they can only reproduce by mitosis. The background of the Crabtree negative strain is a *Hxt*-null strain in which all known hexose transporters were deleted (*HXT1–17*, *GAL2*) as well as the three maltose transporters *AGT1*, *YDL247w* and *YJR160c*. This *Hxt*-null strain could not grow on glucose, fructose, mannose, sucrose or raffinose, very slowly on galactose, whereas the growth rates on maltose, ethanol or glycerol did not show to be decreased (Wieczorke et al., 1999). The reason for the better performance on glycerol, maltose and ethanol is that these nutrients are transported differently. The creation of the *Hxt*-null strain allowed the reintroduction of different *Hxts* to see how each transporter affects the sugar uptake metabolism of the yeast. One chimeric hexose transporter reintroduced into the engineered strain generated a Crabtree negative strain (“the respirer”). The sugar uptake of the respirer relies solely on the chimeric transporter, which is composed of parts of *Hxt1* (low-affinity transporter) and *Hxt7* (high-affinity transporter). The mutant strain respire sugar under

aerobic conditions resulting in higher biomass yields and a lower sugar consumption rate (glycolytic rate) than the wild-type strain ("the fermenter"). It also produces negligible amounts of ethanol, acid, and glycerol when grown on glucose in the presence of oxygen, compared to the fermenter (Table 1). On fructose, it produces more ethanol than it does on glucose, but still only half as much as the fermenter. The difference in ethanol yield between glucose and fructose for the respirer may be explained by different uptake kinetics for the two sugars (Henricsson et al., 2005). Sugar consumption and growth rate of the fermenter is much higher compared to the respirer.

Table 1: Reported metabolic differences of the respirer compared to its parental fermenter strain

	Respirer (compared to fermenter)	References
Ethanol production on glucose	10%	(Henricsson et al., 2005)
Ethanol production on fructose	50%	(Henricsson et al., 2005)
Acetic acid production	50%	(MacLean and Gudelj, 2006)
Glycolytic rate	25%	(Otterstedt et al., 2004)
Growth rate	70%	(Otterstedt et al., 2004)
Biomass yield	120%	(Otterstedt et al., 2004)

In theory a fermenting population that rapidly exploits nutrients will end up with a lower biomass yield but can easily invade a pure population of efficient respirers and outcompete it (Pfeiffer et al., 2001). This spread of selfish individuals to the detriment of the group as a whole is known as the "tragedy of the commons" and explains a situation in which individuals act independently according to pursuit personal gain contrary to the best interests of the whole group by depleting some common resource (Hardin, 1968; Lloyd, 1833). The individual using the more successful strategy is predicted to produce more offspring and therefore to spread. Such scientific theories are the development of mechanistic explanations based on underlying principles while relying on as few assumptions as possible. Thus they provide a unifying framework to explain empirical data and observations (reviewed in: Shou et al., 2015). In our system, the theoretical assumption under direct competition for limited resources would be to expect a benefit of the selfish strategy of faster resource consumption rates (the fermentation) (MacLean, 2007). Indeed, this theory is supported by experiments showing high fermenter fitness (on average +8 %) in direct competition in a chemostat (MacLean and Gudelj, 2006). On the other hand when both strains compete for local resource patches in a spatially structured environment the respirer can dominate (MacLean and Gudelj, 2006).

1.5. Project aim

The aim of this project was to test if the Crabtree effect in *S. cerevisiae* is really an adaptation to sugar rich fruit environments. By determining the costs and benefits of this trait using a Crabtree positive wild type *S. cerevisiae* and an isogenic Crabtree negative mutant strain I investigated whether there is a competitive benefit due to resource competition (i.e. higher rate of glucose utilization) or interference competition (i.e. production of ethanol/acids/heat that inhibit competitors). The fermenter's benefit of the faster growth rate (resource competition) can be expected to only be apparent when the Crabtree positive strain competes directly in the same local environment for resources with Crabtree negative competitors, as in monoculture the strain that uses the resource most efficiently and therefore grows to a greater density will be fitter. So I first tried to verify this basic assumption that the Crabtree positive yeast ends up with a lower yield in monoculture relative to the respirer but with a higher fitness when competing directly with the respirer in a local environment. I tested this in a controlled laboratory medium and in a natural medium (grape juice) and then tested the influence of added diverse microbial competitors on the fitness of both strains.

2. Methods

2.1. Transformation of drug resistance genes into yeast strains

In order to distinguish our manipulated yeast strains from each other and from other microbes that we are using as competitors in later experiments, we replaced *HO* (the gene responsible for switching the mating type) with the marker *KanMX4*, which confers resistance to the drug G418. This allowed us to eliminate all unmarked microbes by adding G418 to the culture medium to be able to count the colony forming units (cfu) of our *S. cerevisiae* strains on media plates. Furthermore, to distinguish the respirer from the fermenter in competition experiments, we replaced *URA3* with a *NAT* resistance cassette (confers resistance to the drug nourseothricin) in the respirer and with a *HYG* resistance cassette (confers resistance to the drug hygromycin) in the fermenter (Goldstein and McCusker, 1999).

Table 2: Genotype of the parental and resistant strains.

	Fermenter (<i>MATa MAL2-8c SUC2</i>)	Respirer (<i>MATa MAL2-8c SUC2</i> Integration into the cassette: <i>HXT7prom TM6*-HXT7term</i>)
Background		
First transformation	<i>ho::KMX</i>	<i>ho::KMX</i>
Second transformation	<i>ho::KMX ura3::HYG</i>	<i>ho::KMX ura3::NAT</i>

2.2. Experimental design

Being Crabtree positive is predicted to be most beneficial under direct competition due to a better resource competition. I therefore tested the fitness of the Crabtree positive fermenter relative to its isogenic Crabtree negative mutant strain under direct competition in the same culture (Strains together), and grown separately (Strains alone) (Figure 2 A and B) in a range of different liquid or solid media.

For inoculating solid media we first plated both *S. cerevisiae* strains onto YEPG plates (1 % yeast extract, 2 % peptone, 2 % glycerol, 2.5 % agar). Glycerol cannot be fermented by *Saccharomyces* so both strains are forced to respire aerobically in this medium. The plates were incubated for 2.5 days at 30 °C. Afterwards, the colony closest to a random dot marked on the backside of each plate was picked and diluted in 30 µl water (several colonies were mixed in one tube into the respective volume of water). 30 µl were used to inoculate culture tubes with loose caps to allow gas exchange and filled with 3 ml solid medium (agar surface in the tube ~1.5 cm²). For the direct competition treatment both strains were mixed to equal proportions in water and 60 µl were inoculated in the middle of the agar. For experiments in liquid media we grew both strains in the test medium to saturation and used 250 µl of each strain to inoculate 2.75 ml fresh medium for the monoculture treatments or we used 250 µl of an equal mix of both strains to inoculate 2.75 ml fresh medium for the direct competition treatments. I incubated all experiments at 30 °C with (liquid medium) or without (solid medium) shaking. Initial cell numbers for monocultures and direct competition treatments were estimated by diluting and plating samples onto YEPG plates. The numbers of colonies grown on plates were counted after three days of growth at 30 °C. For the direct competition treatments, the YEPG plates were replica plated onto NAT (YEPD + 0.01 % nourseothricin) and HYG (YEPD + 0.03 % hygromycin) plates for counting the colony forming units (cfu) of both strains as the respirer is resistant to

NAT and the fermenter to HYG. After reaching saturation (previously tested, data not shown), cultures were diluted and plated onto YEPG plates as described above. For the experiments on solid medium 1 ml sterile water was placed onto the agar in the tubes and these were vortexed to get all cells into solution. To understand how the strains respond to the different conditions, I calculated the number of divisions (D) (Lenski et al., 1991) for the fermenter (f) and the respirer (r) as follows with $N(0)$ as the initial and $N(1)$ as the final cell number:

$$D_{f,r} = \frac{\ln\left(\frac{N(1)_{f,r}}{N(0)_{f,r}}\right)}{\ln(2)}$$

For additionally getting information about the fitness of the fermenter relative to the respirer I estimated the relative yield (Y_f) by dividing the final cell number of the fermenter by the final cell number of the respirer in each test environment:

$$Y_f = \frac{N(1)_f}{N(1)_r}$$

Statistical analyses were performed in R using non-parametric Wilcoxon Rank Sum tests. To correct for multiple testing, the resulted p -values were adjusted using the false discovery rate (“fdr”) option of the R function `p.adjust()` (R-Team, 2015).

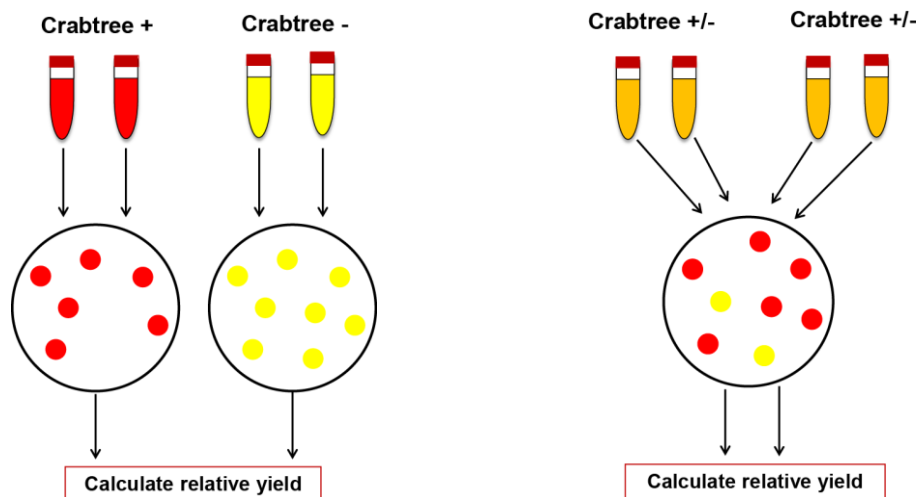


Fig. 2 A: Growth assay "Strains alone"; both strains are grown separately and plated out after incubation. The relative yield is calculated for the fermenter.

Fig. 2 B: Growth assay in direct competition "Strains together"; both strains are grown together and plated out after incubation. The relative yield is calculated for the fermenter..

2.3. Testing the effects of different media on the benefit of the Crabtree effect

To determine whether I can match the theoretical assumption of a better resource competition caused by the Crabtree effect I performed the “alone” and “together” fitness assays in a range of different media; the standard laboratory medium YEPD, Synthetic glucose medium, Synthetic fructose medium, the natural medium of grape juice as well as pure respiration medium.

The first step was to compare the *S. cerevisiae* wild type with the mutant strain on a resource which will be used in the same way by both strains. For this purpose I compared the respiration ability of both strains, forcing them to exclusively respire by using glycerol as solitary carbon source. I inoculated nine replicates per treatment using the solid artificial YEPG medium and incubated the experiment for eight days. I then tested the performance of both strains in liquid standard laboratory medium (YEPD) (1 % yeast extract, 2 % peptone, 2 % glucose) and incubated four replicates per treatment for four days. Liquid YEPD medium is far from being natural, it is a homogeneous continuously shaken environment and the nitrogen source for yeast growth comes from yeast extract (concentrates of the water soluble portion of autolyzed *S. cerevisiae* cells) and is therefore not a controlled nitrogen source. So I also prepared a solid synthetic glucose medium reported in the study of MacLean and Gudelj (2006) with the nitrogen source ammonium which is also the favored nitrogen source of the yeast (2 % glucose, 0.17 % yeast nitrogen base, 0.5 % ammonium sulfate, 0.002 % uracil and 1.6 % agar). Almost all previous studies on the two *S. cerevisiae* strains were done in glucose medium but the main sugar in many fruits is fructose and this might make a difference for the sugar uptake and fermentation or respiration behavior of the two strains. Due to this I prepared the same medium with fructose instead of glucose (2 % fructose, 0.17 % yeast nitrogen base, 0.5 % ammonium sulphate, 0.002 % uracil and 1.6 % agar) and incubated nine replicates per treatment and medium for four days. We were previously able to isolate *S. cerevisiae* from damaged grapes, even if in a low abundance (11 out 221 positive (5 %) Kowallik, Boynton, Greig unpublished) as well as various other grape microorganisms and therefore I used grape juice here to simulate a more natural fruit medium. As grape juice medium is a non-standardized environment and the results might therefore be more variable and as I was interested in a potential frequency-dependence of the fitness of the strains I ran several independent experiments.

To study the behavior of the strains in a homogeneous environment I prepared six experiments in liquid grape juice medium and to better reflect a grape I followed up doing five independent experiments on solid grape juice medium (2 % agar). As it could be previously shown that the initial frequency of the strains can indeed have an influence on the relative fitness of the respirer (MacLean and Gudelj, 2006) I tested this in the “strains together” treatments of the eleven grape juice experiments by determining the correlation coefficient between the relative

fitness data of the respirer in the end of the experiments with its initial yield relative to the fermenter.

2.4. Testing the effects of microbial competitors on the benefit of the Crabtree effect

To determine if the presence of natural microbial competitors affect the costs and benefits of the Crabtree effect in grape juice in terms of a better interference competition, I performed the fitness assays in the presence and absence of common members of the natural microbial community of grapes.

First, I took a competitor mix consisting of microorganisms we have isolated from damaged grapes: the three yeasts; *Wickerhamomyces* spp., *Kluyveromyces* spp., *Hanseniaspora* spp. and a *Bacillus* spp. bacterium. The *S. cerevisiae* strains as well as the competitors were grown onto solid grape juice to saturation, were washed off, equally mixed, diluted 10^{-1} and 20 μ l were used to inoculate 3 ml grape juice agar followed by 20 μ l of the respirer, or the fermenter, or a mixture of both. For the control treatments 20 μ l of the *S. cerevisiae* strains alone or in combination were inoculated. Five replicates per treatment were incubated at 30 °C for four days and then washed off with 1 ml water, diluted and plated onto G418 (YEPG supplemented with 0.02 % G418) plates to get rid of the competitors. The direct competition treatments were additionally replica plated onto NAT and HYG medium afterwards to estimate the *S. cerevisiae* strain frequencies.

To better understand the growth of each *Saccharomyces* strain in the presence of the natural competitors, I competed both the fermenter and respirer separately against the three grape microorganisms: *Wickerhamomyces* spp., *Kluyveromyces* spp. and *Bacillus* spp. I grew all organisms separately to saturation, diluted them and mixed the competitors with each of both *S. cerevisiae* strains in a ratio 1:5 and inoculated the mixtures for the competition treatments. I also inoculated tubes exclusively with the fermenter or respirer as controls. Four replicates per treatment were incubated at 30 °C for four days. All respirer and fermenter treatments in competition with one of the three competitors were plated onto G418.

3. Results

3.1. Being Crabtree positive is advantageous under direct competition in laboratory medium but surprisingly not in grape juice

Using laboratory medium lead exactly to the results predicted by theory; the fermenter had a lower yield in monoculture but a higher yield in direct competition relative to the respirer. In the more natural grape juice the fermenter was less fit in both competition scenarios.

Testing both strains on the respiration medium glycerol showed that the fermenter ended up with a higher yield in monoculture (6 times higher) as well as a much higher yield in direct competition (54 times higher) than the respiring mutant strain (Figure 3). In addition the respirer could undergo fewer divisions in direct competition in presence of the fermenter compared to growing alone (Wilcoxon test on number of divisions; $W = 30$, $p = 0.019$). Comparing the results from liquid standard glucose laboratory medium to liquid natural grape juice showed contradictory results. In the standard glucose lab medium the fermenter reached a lower yield in monoculture but a higher in direct competition relative to the respirer. In liquid grape juice the benefit of the Crabtree effect was similar compared to the one in liquid standard glucose medium in monoculture but greatly decreased under direct competition (Wilcoxon test on relative yield; $W = 24$, $p = 0.038$ after FDR correction), contrary to the theory that the Crabtree effect is an adaptation to fruit. There are many differences between standard laboratory medium and grape juice, so to understand which differences are important to these contradictory results I tested different factors. Using a solid synthetic glucose medium with added ammonium sulfate as nitrogen source showed no significant difference in relative yield compared to the standard glucose medium. Comparing solid and liquid grape juice medium also showed no significant difference but in both grape juice environments the fermenter was weaker than the respirer in direct competition. Substituting the fruit sugar fructose for the pure glucose in synthetic laboratory medium had little effect. The fermenter had no significantly different but slightly higher fitness relative to the respirer on fructose compared to glucose. In general the same behavior and the expected results could be observed for both strains in all three tested, fermentable laboratory media, only in grape juice medium the experiments ended up with a fitter respirer in direct competition and I cannot explain the reason for that with my data. When I tested if the initial relative yield of the respirer has an influence on the yeast's performance in direct competition in the eleven grape juice experiments I did not find a correlation between these two factors (Pearson's correlation coefficient: 0.079, $p = 0.83$) meaning that the relative fitness

of the strains is independent from their initial frequency.

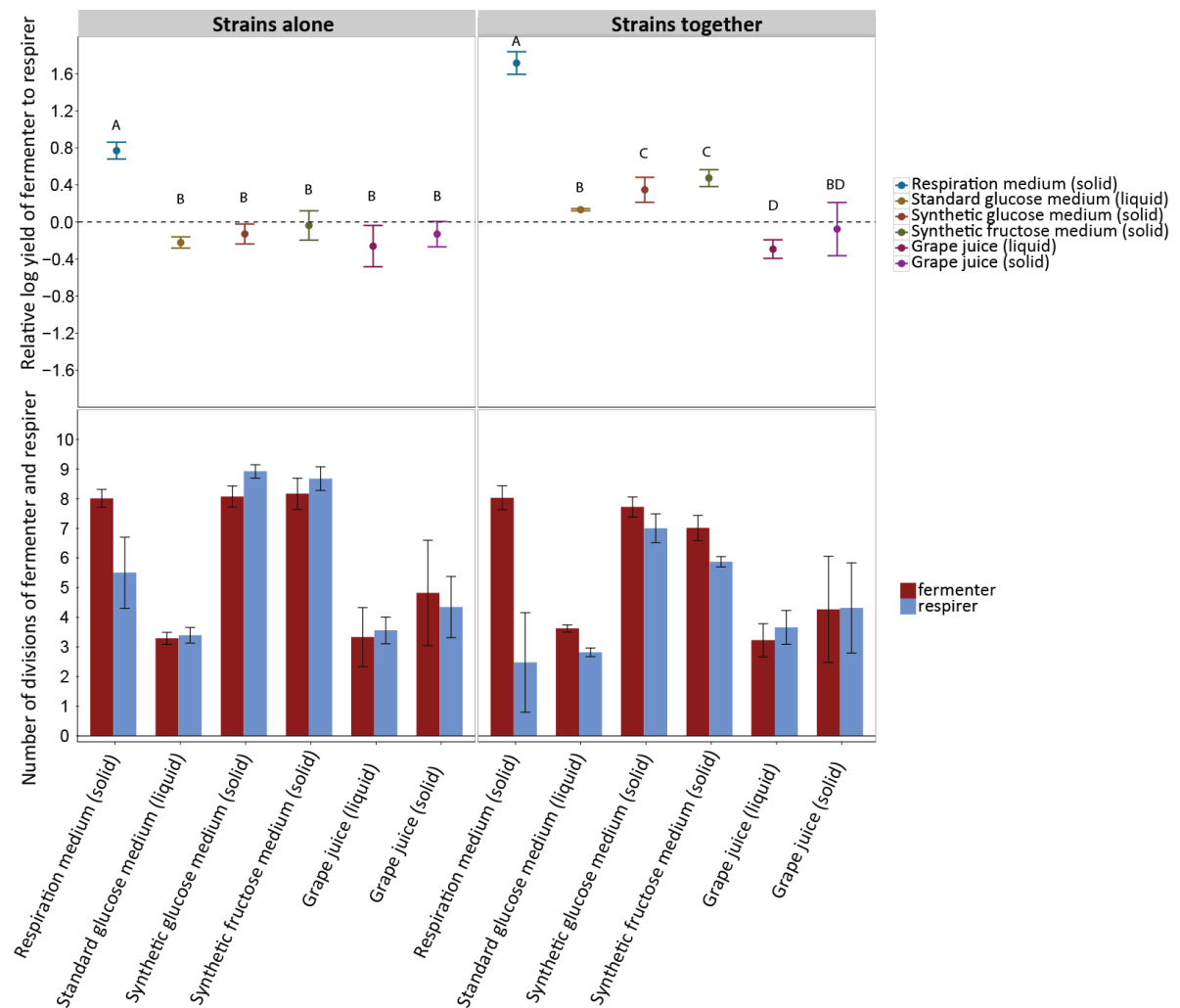


Figure 3: The fermenter and respirer alone (strains alone) or in direct competition (strains together) in different media: For the grape juice media independent experiments were pooled; liquid grape juice (means of six independent experiments pooled) and solid grape juice medium (means of five independent experiments pooled). The upper graphs show the relative log yield of the fermenter to the respirer. Values equal 0 mean that the fermenter has the exact same fitness as the respirer in the specific environment, >0 ; the fermenter is fitter, <0 ; the fermenter is less fit relatively to the respirer. The bar graphs show the number of divisions the fermenter and respirer went through in the different media. For all graphs means and standard deviations are plotted. As the experiments in the different media were done separately and at different time points (except for the synthetic glucose and fructose medium which were done as one experimental run) we cannot compare the numbers of divisions of the strains between different media as we started with different initial frequencies but we can compare the fermenter to the respirer within a medium. The relative log yield is independent of that as we grew the strains to saturation (upper graph) and therefore different media can be compared. Relative yield data points not sharing a letter are significantly different after pairwise Wilcoxon Rank Sum tests and FDR correction.

3.2. Adding natural competitors to grape juice increases the benefit of being Crabtree positive

Adding natural competitors greatly increased the benefit of the Crabtree effect, under both competition conditions, supporting the hypothesis that the Crabtree effect is an adaptation to fruit. However, this effect was strongly dependent on the competitor's identity.

Adding natural competitors to the experiments in grape juice showed that the fermenter had a fitness advantage relative to the respirer in three out of four competition cases (Figure 4). In competition with a mix of grape microorganisms (competitors and *S. cerevisiae* to equal proportions) the respirer's cell frequency was decreasing (it was dying) when growing alone (Wilcoxon Rank Sum tests on number of divisions; $W = 0$, $p = 0.057$ after FDR correction) and even stronger when grown together with the fermenter ($W = 0$, $p = 0.019$) compared to the control without added microbial competitors. The fermenter was not killed but inhibited in the alone ($W = 0$, $p = 0.0588$ after FDR correction) and together treatment ($W = 0$, $p = 0.016$). Although both strains were negatively affected in presence of the grape competitor mix a comparison of the fermenter's yield relative to the respirer shows that it reached a 1600 times higher yield alone and a 233 times higher yield in the "together" treatment. Competing the respirer and fermenter separately against single competitors gives more information about possible effects on the performance of both strains and shows that the output of competition strongly depended on the identity of the chosen competitor. The *Bacillus spp.* had no significant effect on the achieved number of divisions of the respirer nor the fermenter. Although not significant, it was visible that the presence of *Kluyveromyces spp.* inhibited the respirer ($W = 0$, $p = 0.057$ after FDR correction) but promoted the growth of the fermenter ($W = 16$, $p = 0.059$ after FDR correction). Similarly *Wickerhamomyces spp.* killed the respirer ($W = 0$, $p = 0.076$ after FDR correction) but promoted the growth of the fermenter ($W = 12$, $p = 0.066$ after FDR correction).

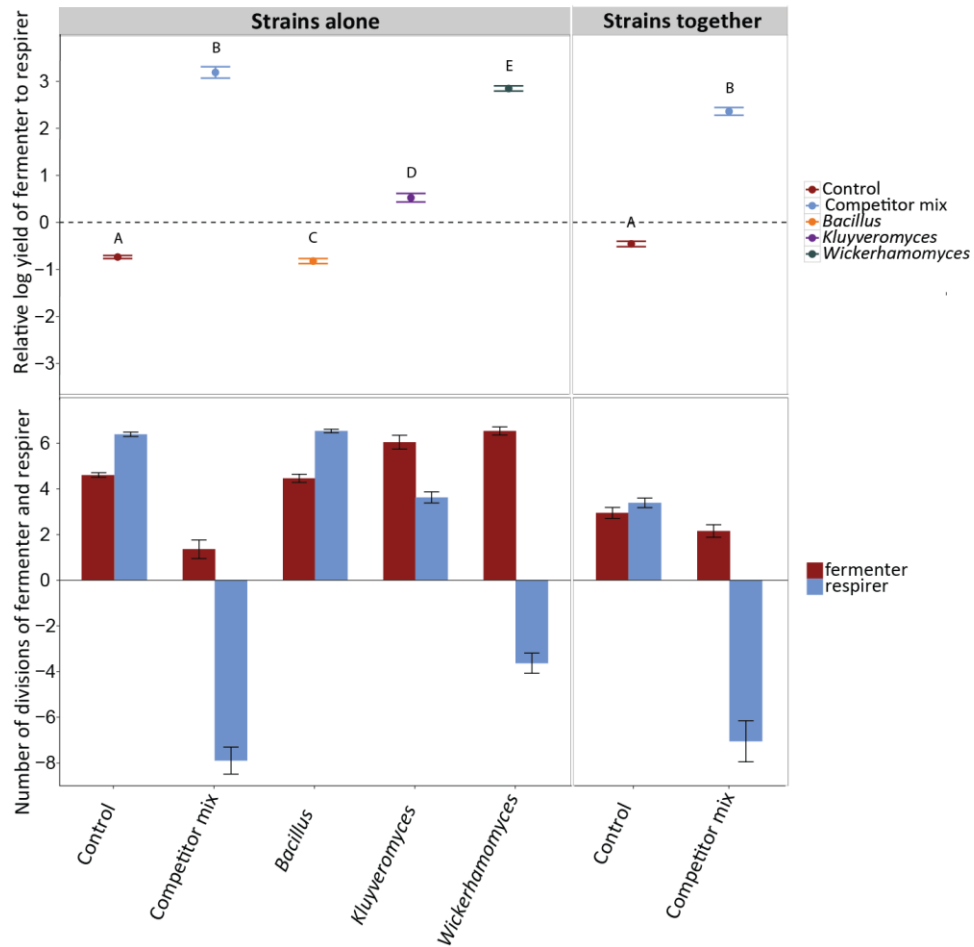


Figure 4: The fermenter and respirer in competition with microbial competitors alone (strains alone) or in the direct competition (strains together) on grape juice medium; control (no competing microbes added), competitor mix (*Bacillus*, *Wickerhamomyces*, *Kluyveromyces*, *Hanseniaspora*). The upper graphs show the relative log yield of the fermenter to the respirer. Values equal 0 mean that the fermenter has the exact same fitness as the respirer in the specific environment, >0; the fermenter is fitter, <0; the fermenter is less fit relatively to the respirer. The bar graphs show the number of divisions the fermenter and respirer went through in the different competition treatments. For all graphs means and standard deviations are plotted. Relative yield data points not sharing a letter are significantly different after pairwise Wilcoxon Rank Sum tests and FDR correction.

4. Discussion

The Crabtree effect is thought to be an adaptation to fruit, but almost all studies to date have used sterile laboratory medium without competitors and compared different yeast species with complete different evolutionary backgrounds. So far, there is no clear evidence that it is the Crabtree effect in *S. cerevisiae* which gives the yeast a competitive advantage over other species. I show that the form of competition, the chosen medium as well as the identity of added competitors greatly affect the benefit of the Crabtree effect.

4.1. Under respiration in non-fermentation medium, fermenter shows a better respiring efficiency than the respirer mutant strain

I found that the wildtype (fermenter) was fitter when forced to respire than the mutant (respirer) strain, both in monoculture and in direct competition (Figure 3). This result is different from early experiments using the *Hxt*-null strain with all hexose transporters deleted, showing that the mutant strain was not negatively affected on pure glycerol medium compared to its ancestor strain (Wieczorke et al., 1999). Indeed the major pathway of glycerol catabolism in *S. cerevisiae* is encoded by three genes which are not members of the hexose transporter family: *STL1*, *GUT1*, and *GUT2* and are therefore not deleted in the respirer (Swinnen et al., 2013). On the other hand there are indications that hexose transporters affect more than the transport of the specific sugars. A study demonstrated expression of *HXT5* during the exponential phase of cell growth, when cells were grown on ethanol or glycerol (Verwaal et al., 2002) indicating that it might be involved in the glycerol consumption. Additionally, taking a look into the *Saccharomyces* Genome Database (www.yeastgenome.org), where the collected information about every gene of *S. cerevisiae* is available, shows that the deletions of the single hexose transporter genes affect various fitness traits. Just to give some examples: deleting *Hxt4* and *Hxt8* lead to a decreased starvation resistance, *Hxt4* additionally to a decreased toxin resistance and decreased oxidative stress resistance, deleting *Hxt5* results in decreased nitrogen utilization. Also functional analysis of gene deletion strains on pure glycerol medium revealed differences in fitness (e.g. a higher fitness of *Hxt3* and a lower of *Hxt17*) relative to a wildtype control (Qian et al., 2012). These examples might indicate that the Crabtree negative mutant strain is affected in different ways by the gene deletions and therefore it is not surprising that its fitness is relatively lower compared to the unmodified strain. If the reason for the respirer's worse growth on glycerol compared to the fermenter is due to problems in terms of growing, surviving, glycerol transportation or during the respiration process cannot be answered. As the decreased fitness of the respirer was more pronounced in direct competition with the fermenter where it could

undergo fewer divisions than growing alone it seems that growth rate was actually more affected than yield. The fermenter showed the same growth in both competition environments on glycerol. These results as well as the documented effects from the hexose transporter deletions on the yeast indicate that the benefit of respiring compared to fermenting would naturally be even higher than it appears to be using the strains in this study.

4.2. In glucose medium the fermenter had an advantage in direct competition due to a better resource competition

In glucose medium the basic assumption that the fermenter ends up with a lower yield in monoculture but with a higher yield in direct competition relative to the respirer could be confirmed (Figure 3). The disadvantage in monoculture is caused by the inefficiency of fermentation compared to respiration but the rapidly growing fermenter had an advantage due to a better resource competition when competing directly for a local pool of resources.

4.3. In grape juice, fermenter is less fit relative to the respirer both alone and in direct competition

In the more natural grape juice medium the fermenter did not only end up with a lower yield in monoculture, but also with a lower fitness in direct competition relative to the respirer, meaning that there is a disadvantage being Crabtree positive in grape juice. This was an unexpected result concerning the faster sugar consumption rate of the fermenter and the already demonstrated higher fitness in direct competition in laboratory medium. The result in grape juice is more meaningful compared to the one in laboratory glucose medium in terms of answering if the Crabtree effect is an adaptation to fruit environments. Due to that I manipulated different factors which could explain the contradictory result by making the laboratory medium more similar to grape juice (Figure 3). I found that the composition of the medium; being liquid or solid did not have huge influence on the performance of the two strains. Nitrogen is a critically limiting resource for yeasts but replacing yeast extract with ammonium sulfate as a more controlled nitrogen source did not lead to a different result. Testing proline, which is besides arginine the most abundant amino acid in grape juice, as source of nitrogen, would be another possibility. Proline is the only amino acid which cannot be assimilated by the yeast during anaerobic fermentation as the enzyme proline oxidase is inhibited by anaerobic conditions (Panda, 2011) but this should actually not affect the yeast under my experimental conditions as oxygen was not limited and proline can be assimilated by aerobic respiration as well as aerobic fermentation (Hornsey, 2007). Most of the results and knowledge of the two *S. cerevisiae* strains is based on their behavior on artificial glucose

medium. Commercial grape juice has with more than 20 g per 100 ml (Sanz et al., 2004) a much higher sugar content than our standard laboratory medium with 2.5 g / 100 ml. In addition the laboratory medium contains glucose as sole carbon source but in grape juice the content of fructose is actually higher than the one of glucose (~11.4 g fructose and ~9.7 g glucose per 100 ml grape juice) and there is also a negligible amount of sucrose present (0.03 g / 100 ml grape juice) (Sanz et al., 2004). The already proven higher production of ethanol on fructose by the respirer (Henricsson et al., 2005) might already be an indicator that the mutant strain behaves differently consuming this carbon source. I experimentally showed that the high fructose content cannot explain the difference to the lab medium as the respirer was even less fit in the fructose compared to the glucose medium. I cannot exclude that a mixture of glucose and fructose in combination might have an influence onto the yeast's sugar uptake mechanisms. As grape juice medium is a non-standardized and more complex environment there might be several reasons why the relative fitness of the two strains do not match the ones obtained in laboratory medium.

4.4. Adding natural competitors to grape juice increases the fitness of the fermenter relative to the respirer

Although the fermenter was, relatively to the respirer, less fit in grape juice alone as well as together, adding a mix of grape microorganisms strongly increased the fitness of the fermenter in both competition environments. The respirer even died in presence of the same ratio of the competitor mix whereas the fermenter was inhibited likely due to simple resource competition by the competing microbes. With these first data we were not confident to declare that the fermentation by-products of the fermenter were the reason for its better performance in presence of microbial competitors. It could have been be that the respirer as it is a highly manipulated strain could not persist in the presence of other stress factors like different metabolic by-products from other microbes. If this is true it would be wrong to conclude that the difference in being Crabtree positive or negative was the reason for the good or bad performance of the strains. Competing both strains separately against three of the microbes helped to learn more about their individual response to the presence of the competitors. Indeed, depending on the microbial species, very different effects on the growth of the *S. cerevisiae* strains could be detected. *Bacillus* spp. had almost no effect on both strains and this was not caused by a bad performance of the bacterium in the used environment (I checked the growth and survival of all three competitors in the end of the experiment and all three could grow well). While *Kluyveromyces* spp. inhibited, and *Wickerhamomyces* spp. even killed the respirer the fermenter was positively affected by the presence of these two other yeast species. This is particularly interesting as it indicates that fermentation is potentially not only beneficial

for the yeast in terms of a resource or interference competition but the yeast might even benefit from the presence of some other microbes e.g. due to cross-feeding. The reason for the complete contraire effects of *Kluyveromyces* and *Wickerhamomyces* onto the fermenter and the respirer could be well explained by the strain's way of metabolizing the nutrients as they are identical except for the fact that one is fermenting and one is respiring. Cross feeding might indeed be one "fermentation supporting" fact when the competing microbes for example use the ethanol to produce something which benefits *S. cerevisiae*. The grape competitors I have chosen could be co-adapted with *S. cerevisiae* or other yeasts showing high fermentation activities and therefore potentially showed a cross-feeding interaction with the fermenter but not with the respirer. Such a co-adaptation would support the fruit adaptation hypothesis. Another possibility would be that something in the grape juice can be better consumed by respiration than by fermentation by *S. cerevisiae* which would explain the higher relative fitness of the respirer in sterile grape juice and in addition it could explain why the fermenter benefits from the presence of some other microbes when these would be able to pre-digest exactly these compounds. Anyway, this would not explain why the respirer suffered under competition with these microbes while the fermenter did not (or less) especially as the fermenter was less fit in sterile grape juice.

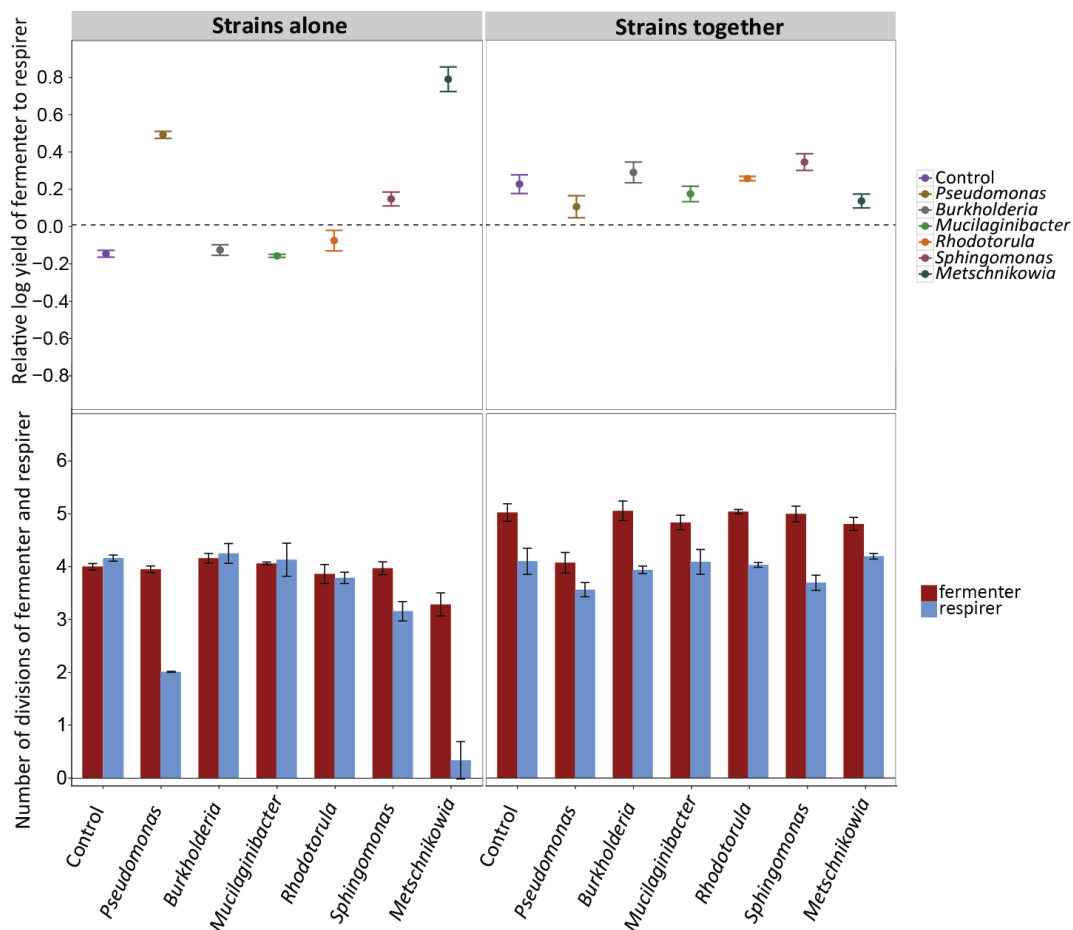
Explaining the results in the complex grape juice medium with added grape competitors which might have co-evolved with *S. cerevisiae* or at least with microorganisms that are fermenting in the fruit environment, turned out to be not that easy. For comparison I did a small follow up experiment in liquid standard glucose medium and competed both *S. cerevisiae* strains against five oak bark microorganisms (Kowallik et al., 2015) which should not be co-adapted to high sugar fermentations as well as one grape yeast (Supplemental Figure 1). When the strains competed alone against the microbes the fermenter was relatively unaffected and for the respirer there was high variation in the competition output ranging from strongly negative to no effects depending on the identity of the microbial competitor. Interestingly the most extreme negative effects on the respirer were wiped away in the presence of the fermenter in direct competition. As the strains are identical except for the hexose transporter differences and as in standard laboratory medium glucose is the sole carbon source the reason for this better performance of the respirer in direct competition must be the fermentation by-products of the fermenter and their effect on the other microbial species. This changes the picture of the efficient, "cooperating" respirer and the exploiting, "selfish" fermenter that exists in literature (MacLean, 2007) in the way that the respirer could be seen as "cheater" benefiting from the fermentation products of the fermenter but with the advantage of efficiently using the nutrients and having a higher yield when growing in monoculture or under spatially structured conditions. The data actually clearly demonstrate that the fermenter, no matter if it gave an advantage to the respirer, was always fitter in direct competition when other microorganisms were present.

Therefore the benefit of the fermenter in sugar rich environments is very likely caused by a better interference competition and therefore the Crabtree effect can indeed be an adaptation to the fruit environment.

5. Conclusion

This is the first study identifying the costs and benefits of the Crabtree effect in yeast by directly comparing Crabtree positive and negative *S. cerevisiae* strains in different environments. Crabtree positive yeasts gain a benefit from better resource competition when competing against Crabtree negative yeasts for a common pool of resources in laboratory medium. This result is dependent on the medium used: Crabtree positive yeasts are less fit than Crabtree negative yeasts in more complex grape juice medium when grown alone or together. Although the Crabtree positive yeast was less fit in sterile grape juice, it could outcompete the Crabtree negative strain in direct competition in grape juice with other microbes were present. This suggests that fermentation by-products play a role in competition against other microorganisms, and that the Crabtree effect might indeed be an adaptation to high sugar and microbial diverse fruit environments.

6. Supplemental material



Supplemental Figure 1: The respirer and fermenter in a control (sterile medium) and in competition with microbial competitors alone (strains alone) or in direct competition (strains together) in liquid standard laboratory medium. The upper graphs show the relative log yield of the fermenter to the respirer; values equal 0 mean the fermenter has the exact same fitness as the respirer in the specific environment, >0; the fermenter is fitter, <1; the fermenter is less fit relatively to the respirer. The bar graphs show the number of divisions the fermenter and respirer went through in the different competition treatments. For all treatments two independent replicates were prepared and incubated at 30 °C for four days. In the graphs means and standard deviations are plotted.

Chapter IV A systematic forest survey showing an association of *Saccharomyces* with oak leaf litter

1. Introduction

The fermentation ability of *Saccharomyces cerevisiae* in combination of human's passion for alcoholic beverages has made it an important component of human culture. In more recent times *S. cerevisiae* has also become one of the best-studied laboratory model organisms and was the first sequenced eukaryote (Goffeau et al., 1996). However its life in the wild remains mysterious (Greig and Leu, 2009). To fully interpret and understand the rich data generated by studying *S. cerevisiae* in the laboratory, it is important to better understand yeast natural history and to place the species in its ecological and evolutionary context. Connecting all the knowledge gained from discoveries made in the lab with a knowledge of the ecological and environmental conditions in which the species evolved, would be beneficial to biological research in many areas like evolutionary and ecological genomics, population genetics, microbial biogeography, community ecology and speciation (Replansky et al., 2008).

S. cerevisiae is readily and consistently found in artificial alcoholic fermentations. Many believe, therefore, that the ability of *S. cerevisiae* to dominate human-made sugar-rich fermentations indicates that its natural habitat is fruit. The unusual tendency of *Saccharomyces* yeast to prefer inefficient fermentation over more efficient respiration, even when oxygen is present (the Crabtree effect), is seen as an evolutionary adaptation to fruit (Piškur et al., 2006). But there is actually little direct evidence that *S. cerevisiae* is a specialist on fruit. Indeed the large number of places it can be found in low frequency suggest that it may be an ubiquitous niche-less generalist (Goddard and Greig, 2015).

Humans have been making alcoholic drinks, and therefore likely domesticated *S. cerevisiae*, for about 10,000 years (Vaughan-Martini and Martini, 1995). Whilst natural populations of *S. cerevisiae* certainly exist (Fay and Benavides, 2005; Wang et al., 2012), there is a risk that any individual found in a natural habitat may have recently escaped from a human fermentation or may have mixed ancestry. Researchers wishing to study wild yeast therefore often look instead at the closest known relative of *S. cerevisiae*, *Saccharomyces paradoxus* (Replansky et al., 2008). The two species are phenotypically and biochemically nearly indistinguishable, share almost the same profiles of assimilation and fermentation of organic compounds (Vaughan-Martini 1998)

and can exist in sympatry in natural habitats (Naumov et al., 1998; Sampaio and Gonçalves, 2008; Sniegowski et al., 2002), but *S. paradoxus* is not thought to be affected by domestication as it is not found in human fermentations.

Nearly all isolates of *S. paradoxus* have come from oak (*Quercus* spp.). A recent survey of *S. paradoxus* available from culture collections found 81 % came from oak, with rest recently isolated from the newly-identified North American habitat of maple trees (Bozdogan and Greig, 2014). The earliest isolation recorded in literature was 1914 from Russian oak exudates (Batshinskaya, 1914) and later 1957 from the bark and surrounding soil of oak, as well as from soil surrounding pine (Yoneyama 1957). Since this time we find a focus on oak tree bark as the dominant source of wild *Saccharomyces* strains (Charron et al., 2014; Johnson et al., 2004; Koufopanou et al., 2006; Sampaio and Gonçalves, 2008; Sniegowski et al., 2002; Sylvester et al., 2015; Wang et al., 2012; Zhang et al., 2010). In the Southern Hemisphere, *Nothofagus* trees (southern beeches) inhabit the ecological niche of oaks, and *Saccharomyces* species can be found instead on the surfaces of these trees (Libkind et al., 2011).

There are few studies comparing *Saccharomyces* isolation success among different potential habitats, and because different studies use different sampling methods, or do not standardize sampling at all, meta-analysis of different studies is not possible. A survey of Canadian trees found *S. paradoxus* in 12 % of samples coming from trees belonging to the Fagaceae family (mostly oak species) and in 4 % of maple samples (Charron et al., 2014) and another recent survey of different samples (soil, bark, leaves, acorns) from different tree species showed that *S. paradoxus* could be found in 16 % of all oak samples and that it had a significant association with *Quercus* (Sylvester et al., 2015). (Sampaio and Gonçalves, 2008) had so far the highest record of *Saccharomyces*-containing samples (over 70 % of *Quercus pyrenaica* and *Quercus faginea* bark samples in Portugal) and they took the samples in a standardized way that allows comparisons between tree species. They found that the bark of oaks and other closely related species in the family Fagaceae were about three times more likely to yield *Saccharomyces* than other tree species, but the overall sample size was not high enough to infer whether this apparent difference was statistically significant. In general, though, most oak samples do not yield any *Saccharomyces* and the results are in addition very varying across studies ranging from 8 % *Saccharomyces* isolation success in samples from Southern England (Johnson et al. 2004), Canada and Germany (Sampaio and Gonçalves 2008) over 23 % in samples from North America (Sniegowski et al. 2002) and 24 % from New Zealand (Zhang et al., 2010) to 70 % in samples from Portugal (Sampaio and Gonçalves 2008). Remarkably, almost all isolates of *S. paradoxus* and wild *S. cerevisiae* have come from enrichment cultures. This means that a sample, usually oak bark, was placed into a sugar-rich fermentable medium that is thought to favor the growth of *Saccharomyces*. Essentially, researchers are replicating the methods of ancient wine-makers.

Some such cultures produce a culture dominated by wild *Saccharomyces* but in most other microbes dominate. Therefore, the results give little indication of the initial numbers of *Saccharomyces* cells in the samples, as the ability of these yeasts to dominate in the enrichment culture may depend on the numbers initially present in a sample, the other microbes present in the same sample, and on the potentially variable ability of different *Saccharomyces* genotypes to compete in the enrichment medium. We recently tested the ability of enrichment culture to isolate single *S. paradoxus* cells spiked into oak bark samples that had previously tested negative for *S. paradoxus*. We found that enrichment culture could consistently isolate single cells, allowing us to estimate the density of cells on the surface of oak as less than 2 cells per square cm. Consistent with this low density, we did not find a single *Saccharomyces* sequence in 40 000 high-throughput ITS sequences from oak bark (Kowallik et al., 2015). The low abundance of *S. paradoxus* on oak bark leads us to question whether this really is its primary habitat.

Here I systematically quantify the abundance and distribution of *Saccharomyces* in a mixed forest in Nehnten, Northern Germany. First I sampled forest leaf litter along transects and discovered and found that samples closer to oak trees were more likely to contain yeast than samples taken from further away. Next, I compared leaf litter samples taken systematically from under four tree genera and found that oak samples were significantly more likely to contain yeast. By quantifying the number of yeast cells, I show that the density of yeast is much higher in oak leaf litter than on oak bark, suggesting that composing oak leaf litter is a better habitat for yeast than the surface of the oak tree. To determine the seasonal abundance and distribution of *Saccharomyces* in oak leaf litter, I sampled transects from six oak trees bi-monthly over one year, and quantified the cell numbers of *Saccharomyces* in the litter under each tree. The density of yeast in leaf litter is so high that individuals could be isolated directly from samples, without enrichment culture. Strains isolated by enrichment culture do not significantly out-compete strains isolated directly, when tested under enrichment culture conditions. This shows that enrichment culturing does not significantly under-sample the underlying leaf-litter population, and thus enrichment culturing can be used for *Saccharomyces* isolation. Additionally enrichment cultures of serially diluted samples can be used to enumerate populations in future studies. Altogether 636 *Saccharomyces* isolates were sequenced for this study and almost all could be identified as being *S. paradoxus* and three samples (0.47 %) as *S. cerevisiae*.

2. Methods

2.1. Leaf litter transects

Between July, 11, 2014 and September, 18, 2014, I sampled 18 transects in an old mixed forest in Nehmten, Northern Germany. Each transect was a straight line 20 meters long, starting at the trunk of an oak tree and ending at least 20 m away from the next nearest tree. Each 2 cm³ sample of compressed leaf litter was collected by using a sterile metal spatula to press leaf litter into the bottom of a 12 ml Falcon tube until it was filled to the 2 ml mark. I took three samples at five different points on each transect: 0 m (directly next to the trunk), 2 m, 5 m, 10 m, and 20 m. All 270 samples were taken immediately back to the lab and filled with a slightly modified version of Sniegowski's (2002) enrichment medium, PIM1 (0.3 % Yeast extract, 0.3 % Malt extract, 0.5 % Peptone, 1 % Sucrose, 8 % Ethanol, 0.001 % Chloramphenicol, and 0.52 % 1M HCL). To determine whether incubation temperature had any effect on isolation success, I incubated the samples from nine of the transects at 30 °C for ten days, and those from the other nine transects at 10 °C for 28 days, without shaking. I then streaked 25 µl of each tube onto plates made from Sniegowski's (2002) PIM2 (2 % methyl- α -D-glucopyranoside, 0.67 % Yeast Nitrogen Base w/o AA, 2 % agar, 0.1 % Antifoam (5 %) and 0.4 % 1M HCL). After incubation, the plates were examined for yeast colonies.

The method to identify candidate colonies as *Saccharomyces* was built of two steps. I first picked *Saccharomyces*-like colonies to sporulation agar (2 % potassium acetate, 0.22 % yeast extract, 0.05 % glucose, 0.087 % complete amino acid mix, 2.5 % agar) and incubated them for 3-5 days at room temperature before examining the cells with a microscope for the presence of characteristic tetrad ascospores. To determine how effective this characteristic was for identifying *Saccharomyces*, DNA was extracted from all 146 candidate colonies using the MasterPure™ Yeast DNA Purification Kit (Epicentre), and the ITS region was Sanger sequenced using ITS1(TCCGTAGGTGAACCTGCGG) (White et al., 1990). NCBI BLAST search using Geneious Pro v. 6.1.6. identified all candidate colonies as *S. paradoxus* and all these colonies formed tetrads. Additionally 21 yeast-like colonies that did not sporulate or formed very different spores compared to *Saccharomyces* have been sequenced to determine whether the ability to sporulate was a useful characteristic to identify *Saccharomyces*, or whether non-sporulating *Saccharomyces* might also exist. These 21 samples were identified as belonging to 7 other non-*Saccharomyces* yeast species. The perfect congruence between tetrad-formation and *Saccharomyces* identity persuaded us to use tetrad screening as pre-selection for the later parts of this study, greatly increasing the scale of experiments.

The data from the 18 transects were pooled and the effect of distance from an oak tree on *Saccharomyces* isolation probability was tested with a simple logistic regression model in R, with the additional factor “incubation temperature”. To test if the incubation temperature has a significant effect on *Saccharomyces* isolation success I tested the difference between the two regression lines using a Generalized Linear Model (GLM) on binary data in R.

2.2. Comparison of leaf litter under four tree genera

To test for a tree species specificity of *Saccharomyces*, I compared the four different main tree genera in the Nehnten forest in terms of isolation success of the yeast. In September 2014 I took one 2 cm³ sample of compressed leaf litter directly next to each of the trunks of 60 oaks (*Quercus* spp.), 60 beeches (*Fagus* spp.), 60 larches (*Larix* spp.) and 60 spruces (*Picea* spp.) and processed them as described under 2.1. above, except that all samples were incubated in PIM1 at 30 °C for 10 days. All sporulating yeast colonies were sequenced and again all pre-selected colonies belonged to *Saccharomyces*. As a control to test whether there might be missing *Saccharomyces* isolates that could not sporulate, I also tested the ITS sequences of 15 other candidate colonies that resembled *Saccharomyces* but did not form *Saccharomyces* like spores – all contained ITS sequences from other yeast species. I performed pairwise Fisher tests on the isolation success among all four tree types. To correct for multiple testing, the resulted *p*-values were adjusted using the false discovery rate (“*fdr*”) option of the R function *p.adjust()* (R-Team, 2015).

2.3. Enumeration of the *Saccharomyces* cell number in oak leaf litter and on oak bark

In January 2015 I collected three oak leaf litter samples and three oak bark samples each from six different oak trees. Each 2 cm³ sample of compressed oak leaf litter was collected as described under 2.1. above. Bark was sampled by cutting all the bark from a 10 cm by 5 cm patch at head-height using a sterile scalpel, and placing the bark pieces into a 50 ml Falcon tube (approximately 15 cm³ of loose bark pieces). All 36 samples were immediately taken to the laboratory, weighed, transferred into 50 ml Falcon tubes containing 20 ml sterile water and vigorously shaken for 1 hour to dislodge cells from the sampled bark or leaf litter material into suspension. The number of viable *Saccharomyces* cells was determined in each of these 36 samples using the most probable number (MPN) technique which is an estimation of organisms by noting growth in successive dilutions (McCrary, 1915). To do this, five independent serial dilutions of four steps were made. Each step consisted of a tenfold dilution from the original sample or the previous dilution. First, I transferred five independent 2.3 ml samples from every

20 ml original sample into five 15 ml falcon tubes. From these tubes I took out 200 μl and put this into 1 ml screw cap tubes (10^{-2} dilution of the original 20 ml sample). In addition I took 100 μl from the remaining 2.1 ml sample into 900 μl sterile water and transferred 200 μl of this into 1 ml tubes (10^{-3} dilution). A fourth serial dilution step was prepared and 200 μl transferred to 1 ml tubes. Finally the five 15 ml tubes with the 2 ml samples (10^{-1} dilution of original sample) as well as all 1 ml tubes were filled with liquid PIM1 medium, hermetically sealed and incubated at 30 °C for ten days and tested for the presence of *Saccharomyces* by tetrad-screening as described under 2.1. above, but without ITS sequence confirmation (as sequencing was impractical because of the large number of colonies generated during MPN assays). The most probable number of *Saccharomyces* cells in the suspensions was determined using a MPN table for five replicates per sample (Woodward, 1957). This was divided by the number of grams in a sample to determine the most probable number of cells per gram of leaf litter or oak bark material. I analyzed the Box-Cox transformed MPN data using a nested ANOVA with *sample type* (leaf litter or bark) nested in *tree*.

2.4. Comparison between direct-isolated and enrichment-isolated *S. paradoxus* strains

As the abundance of *S. paradoxus* turned out to be pretty high in oak leaf litter I was able to isolate strains directly by plate culturing, without previous enrichment culture step. I took additional leaf litter samples from six oak trees described under 2.3. above and put them in 20 ml water. For each sample, I plated 100 μl from the undiluted cell suspension and 100 μl from a 10^{-1} and 10^{-2} dilution, directly onto PIM1 agar plates (PIM1 with 2 % agar) and also put the same amount of each sample and dilution into liquid PIM1 medium. Solid as well as liquid samples were incubated at 30 °C. Strains from the liquid samples were isolated as described above. For the solid medium candidate colonies were identified as *Saccharomyces* by their ability to sporulate and by ITS sequencing as described under 2.1. above. I got 13 strain pairs and engineered one strain of each pair to be resistant to the antibiotic G418 by replacing both its homologous copies of the *HO* gene with the KanMX4 cassette (Goldstein and McCusker, 1999). After verifying that both homologous copies are replaced I ended up with nine strain pairs coming from nine different samples from three oak trees (five pairs from transect oak 1, three pairs from transect oak 2 and one pair from transect oak 5). I balanced the marking of the strains between the two isolation techniques and marked in five pairs the plate-cultured strain and in four pairs the liquid enriched strain. I grew all strains separately in liquid PIM1 at 30 °C and then equally mixed the respective strain pairs, diluted the mix 10^{-3} and inoculated five 15 ml screw cap falcon tubes containing 13 ml liquid PIM1 with 50 μl of each mix, closed the tubes tightly and incubated them at 30 °C for ten days . I also directly plated dilutions onto YEPD (1 %

yeast extract, 2 % peptone, 2 % glucose, 2.5 % agar) plates to get the initial cell number of the strains and incubated the plates at 30 °C for one day. The YEPD plates were replica-plated onto YEPD as well as G418 (YEPD supplemented with 0.02 % G418) plates for enumeration. After ten days I plated the experiment out to get the final cell number per strain. To calculate the relative fitness (W) of the liquid enriched and plate-cultured strains in direct competition, the ratio of Malthusian parameters (m) for the two strains was taken as a measure of their competitive fitness (Lenski et al., 1991). If $N(0)$ is the initial and $N(1)$ the final cell number, relative competitive fitness for a strain i when competing against another strain j can be expressed as:

$$W_{i,f} = \frac{\ln\left(\frac{N(1)_i}{N(0)_i}\right)}{\ln\left(\frac{N(1)_j}{N(0)_j}\right)}$$

To test if the culturing method has significant influence on the result I calculated ANOVA in R.

2.5. Over-the-year sampling study

For a better understanding of the unknown ecology of *Saccharomyces* I performed bimonthly sampling studies over one year to characterize changes in abundance and distribution of the yeast. In nature many factors are likely influencing the migration success, survival and/or proliferation of *Saccharomyces*. The different rates of decay of leaf litters from different oak trees might be very important, as well as temperature, the presence, abundance and metabolic activity of other microorganisms and feeding activity or potentially vectoring function of insects.

To test for changes over season in yeast distribution I sampled transects away from six oak trees. I took the samples as described above under 2.1. but this time one sample every meter (from direct to 20 meter distance). I changed between starting from the trunk going the 20 meters away and starting 20 meters away and going in tree direction to dilute the bias that I might introduce by walking the transects. I pre-screened candidate colonies as before (see 2.1.) by their ability to form tetrads, and confirmed all 348 tetrad-forming strains as *Saccharomyces* by ITS sequencing. I analyzed the distribution data with a Generalized Linear Mixed Model (GLMM) using the lme4 package in R (Bates et al., 2014). In this GLMM I tested distance with 21 levels (from direct to 20 m distance) as fixed effect and transects with six levels (the six trees samples) as well as season with seven levels (the seven time points sampled) as random effects. I also included testing for an interaction between season and distance to test for any changes in yeast distribution over the year, as well as for an interaction between distance and transect to see if some transects have a wider yeast distribution radius than others, as well as between tree and season to see whether trees affect the yeast distribution in response to season differently. I

used a binomial fit for the binary response variable “isolation success”. To evaluate the significance of fixed and random effects and of interactions between effects, alternative models without the variable or interaction of interest were compared to the full model using likelihood ratio tests. To test for overdispersion (the variance is larger than the mean), all variables were treated as fixed effects (converting to a GLM for this purpose), and the residual deviance of the model was divided by the number of degrees of freedom (which should approach ~1 in case of no overdispersion, >1 = overdispersion) (Crawley, 2012).

To additionally test for seasonal changes in abundance, I took three leaf litter samples at different sites directly under the trunk of each tree at the same time as I collected the samples for the transects. I estimated the MPN of *Saccharomyces* per leaf litter sample by identifying colonies as *Saccharomyces* by their ability to sporulate, as before (see 2.3.). I analyzed the Box-Cox transformed MPN data using a two-factor ANOVA with *transect* and *season* as factors.

Connecting the collected distribution and abundance data of *Saccharomyces* across the year I compared the seasonal variation in total positive samples found across each transect with the seasonal variation in absolute cell number under the trees for each sampling time point by a linear regression model in R.

To test if temperature has an effect on the distribution and abundance of *Saccharomyces* in this data set I determined correlation coefficients in R between the average temperature of the sampling months with the average MPN as well as the average numbers of isolated strains from all six transects per month.

3. Results

3.1. *Saccharomyces* isolation success decreases with increasing distance to an oak

The closer you are to an oak tree, the more likely you are to find *Saccharomyces*. Figure 1 shows that the proportion of samples containing *Saccharomyces* yeast is significantly negatively correlated with how far from an oak a leaf litter sample was taken, for samples incubated at 10 °C (Spearman’s rho = -0.713, $p < 0.001$) and at 30 °C (Spearman’s rho = -0.553, $p < 0.001$). There was no significant influence of temperature (GLM; $Z = 0.7$, $p = 0.48$), but distance was highly significant (GLM; $Z = -7.12$, $p < 0.001$).

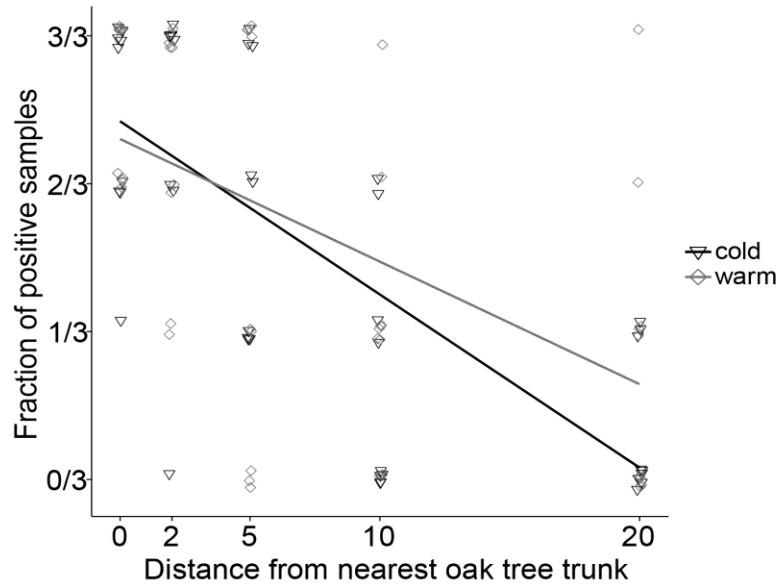


Figure 1: Relation between distance from oaks and the number of positive samples at 10 °C (cold) and 30 °C (warm) incubation temperature. The fraction of positive samples for nine transects for each incubation temperature are plotted. The solid lines are linear regression lines.

3.2. Oaks harbor most *Saccharomyces* isolates compared to other tree genera

Figure 2 shows that *Saccharomyces* was more strongly associated with oak than with the three other common tree types in the Nehnten forest. 53 out of the leaf litter samples taken under 60 oaks contained *Saccharomyces*, significantly more than the 42/60 positive larch samples, the 27/60 beech samples, and the 20/60 spruce samples (Pairwise Fisher's Exact tests; $p = 0.028$, $p < 0.001$, $p < 0.001$ after FDR correction, respectively).

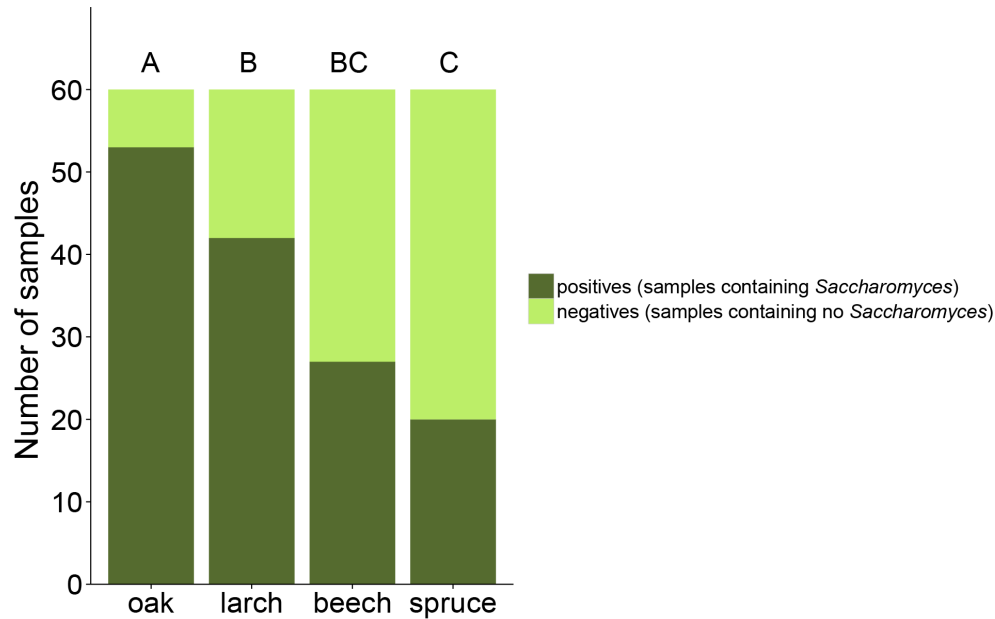


Figure 2: *Saccharomyces* found in 60 leaf litter samples of oak, larch, beech and spruce. Capital letters indicate significant differences in isolation success between different trees. Columns not sharing a letter are significantly different after Pairwise Fisher tests and FDR correction.

3.3. *Saccharomyces* is much more abundant in leaf litter than on bark

Saccharomyces cell density is higher in oak leaf litter compared to oak bark. The *Saccharomyces* cells / g in leaf litter range from 0 to 1319 cells with an average of 350 cells / g and in bark from 0 to a maximum of 70 cells with an average of 7 cells / g, and the two types of habitat differ significantly in the number of cells they support (Nested ANOVA; $F = 38.5$, $p < 0.001$) (Figure 3). The cell density also varies between the different trees (Nested ANOVA; $F = 13.54$, $p < 0.001$) and we find a significant interaction between sample and tree (Nested ANOVA; $F = 2.66$, $p = 0.048$) (Supplemental Figure 1).

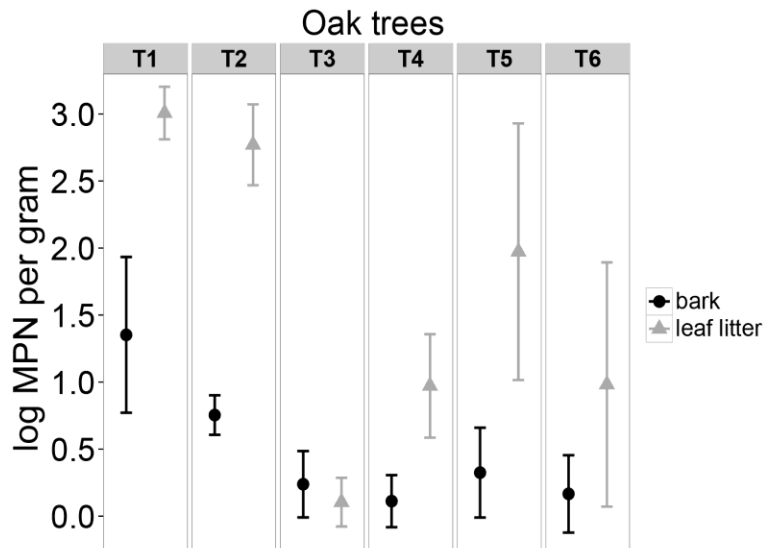


Figure 3A: The log most probable number (MPN) of 1 gram bark and leaf litter samples from each of six oak trees (T1-T6). The mean and standard deviation from each of three samples is plotted.

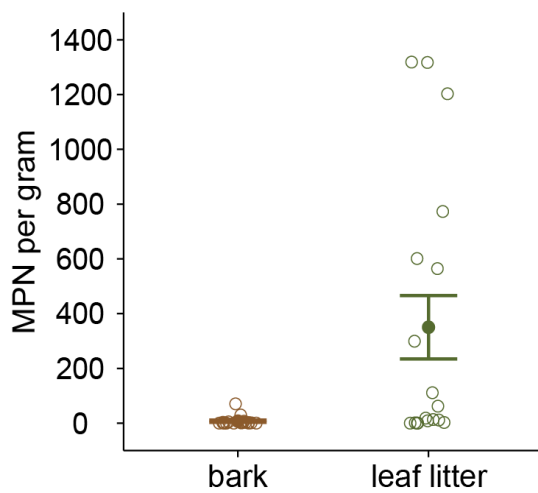


Figure 3B: The most probable number (MPN) of *Saccharomyces* in one gram of bark or leaf litter material from 18 samples (three samples from each of six oak trees). Plotted dark points are the means with added standard errors and the single data points are shown using open symbols.

3.4. No fitness difference between strains isolated with direct plating and enrichment isolation

The liquid enrichment method does not lead to studying artifacts but is a reasonable method to use for *Saccharomyces* isolation. The liquid cultured strains do not show to be consistently fitter in competition with the respective plate cultured strains in the liquid enrichment medium PIM1. Taking the average of the log relative fitness data of all nine liquid enriched strains in the competition gives: 0.027 and transforming this back to the normal relative fitness: 1.064. Statistical tests show there was no significant difference in strain fitness between the two methods (ANOVA: $F = 2.23$, $p = 0.139$).

3.5. *Saccharomyces* abundance and distribution varies between trees and over season

I determined how the distribution of *Saccharomyces* across six transects varied during the course of one year (Figures 4 and 5). A GLMM on binary data - using *distance* as fixed effect, and *tree* and *season* as random effects as well as interactions between the effects *distance* and *season* - explained a significant amount of variance in isolation success (Table 1). No overdispersion was found. Consistent with the earlier transect study (see 3. 1 above), distance from the nearest oak tree trunk (factor *distance*) had a significant effect on isolation success. Consistent with the tree-to-tree variation in cell number that I found in our bark and leaf litter comparison (see 3.3. above), I found that transects from different trees (factor *tree*) differed significantly in isolation success. And finally, I found that the month of the year (factor *season*) also affected isolation probability across the transects. There was no significant interaction between *distance* and *season*, nor between *distance* and *tree*, nor between *tree* and *season*.

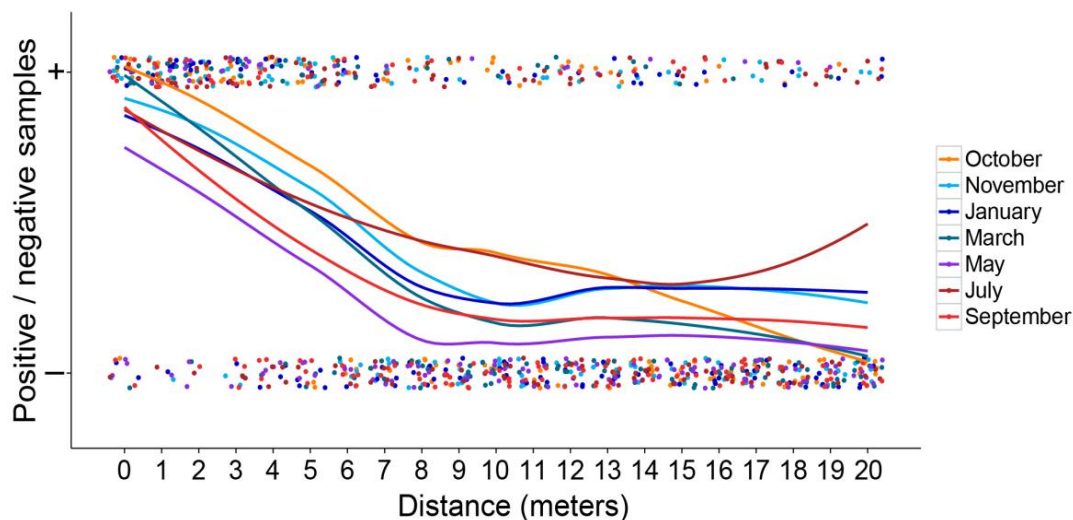


Figure 4: Average distribution of *Saccharomyces* isolates from all six transects at different months. The solid lines are local polynomial regression fittings.

Table 1: GLMMs on the effects of distance, tree and season (which month sampled) as well as on interactions between effects (characterized with :) on the isolation success of *S. paradoxus*. Akaike information criterion (AIC) describes the quality of fit of each model (higher AIC = information loss). To evaluate the significance of fixed and random effects and interactions, alternative models without the variable or interaction of interest were compared to the full model (bold) using likelihood ratio tests in R.

Effect tested	Fixed effects	Random effects	AIC	χ^2	d.f.	p
	D	T, S, D:T, T:S, D:S	876.1			
Distance (D):Tree(T)	D	T, S, D:S, T:S	877.6	3.528	1	0.06
Distance(D):Season(S)	D	T, S, T:S, D:T	876.83	2.767	1	0.096
Tree (T):Season (S)	D	T, S, D:S, D:T	874.28	0.214	1	0.643
Tree (T)	D	S, D:S	910.06	35.99	1	<0.001
Season (S)	D	T, D:S	881.53	7.4624	1	0.006
Distance (D)		T, S, D:S	968.73	94.664	1	<0.001

I also measured how the absolute number of *Saccharomyces* cells under the six oaks changed throughout the year (Figure 6). Consistent with the significant tree-to-tree variation in distributions across transects, different trees also varied in the abundance of *Saccharomyces* cells under them (ANOVA: $F = 54.1$; $p < 0.001$). However, the month (factor *season*) did not affect overall abundance of cells under the six trees (ANOVA: $F = 1.46$; $p = 0.203$), inconsistent with its affect on the transects (5). There is a significant interaction between *season* and *tree* (ANOVA: $F = 2.35$; $p = 0.0012$) which may be explained by different trees responding differently to the seasons.

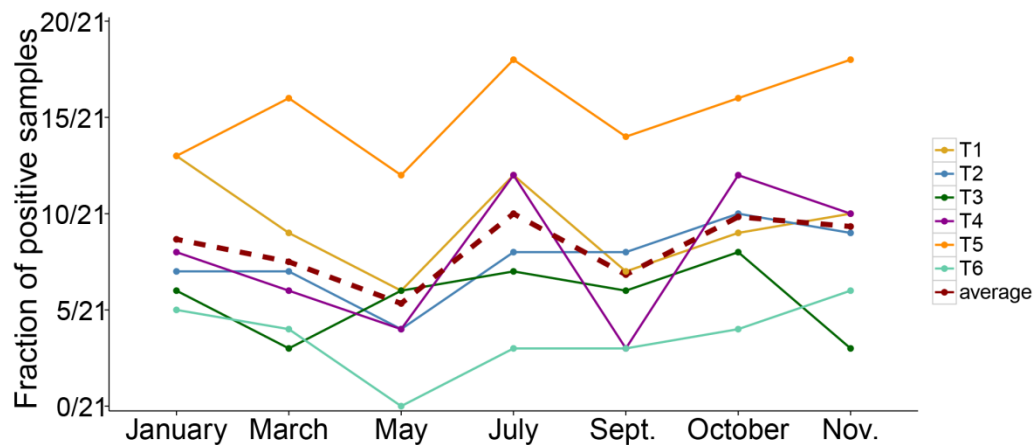


Figure 5: Fraction of positive samples of *Saccharomyces* per whole transect (T1-T6) over the year. The dashed red line shows the average of the six transects.

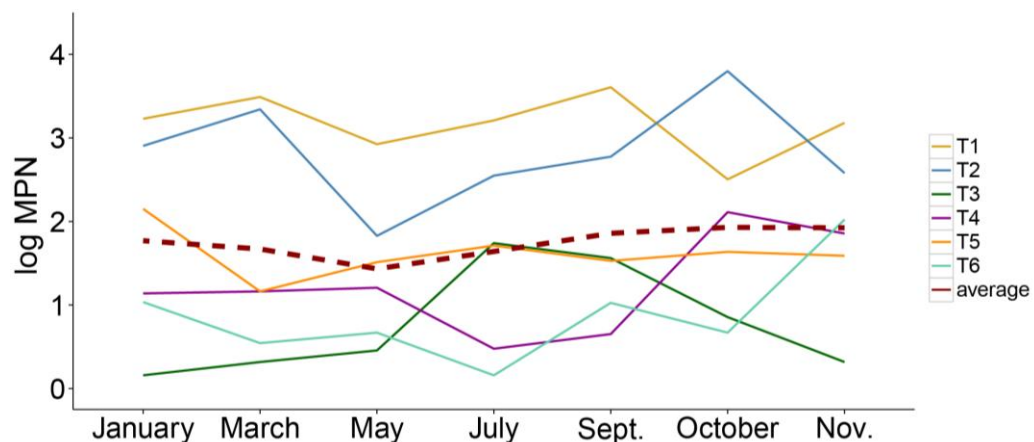


Figure 6: Log most probable number (MPN) of three samples per transect tree and month. The lines connect the mean of these three data points and the red dashed line represents the average MPN of all six transect trees per sampling time point.

Given these minor inconsistencies between the two forms of data (abundance and distribution), I compared the seasonal variation in total positive samples found across each transect (Figure 5) with the seasonal variation in absolute cell number under the trees (Figure 6), and found a significant positive correlation (Supplementary Figure 1, Pearson's $r = 0.343$, $p = 0.026$), giving confidence that these two independent methods accurately reflect the *Saccharomyces* in leaf litter. Testing the influence of temperature on my sampling data showed no significant influence on the abundance (Spearman's correlation coefficient $\rho = -0.036$, $p = 0.964$) nor the distribution (Spearman's correlation coefficient $\rho = 0.286$, $p = 0.556$) of *Saccharomyces*.

4. Discussion

4.1. Proximity to an oak tree predicts *Saccharomyces* abundance in leaf litter

Oak trees have long been thought to be the natural habitat for *S. paradoxus*, and for wild *S. cerevisiae*. Here we show that the proximity of an oak tree has a strong positive effect on *Saccharomyces*' occurrence in surrounding forest leaf litter. Sampling transects away from oaks shows that the isolation success declines dramatically with an increasing distance from the trunk, suggesting that the tree provides resources or conditions supporting the growth or survival of *Saccharomyces*. There are at least three reasons why oak trees might promote the abundance of yeast in their vicinity.

1. *Saccharomyces* yeast might grow on the surface of oaks, and disperse to nearby surroundings. This explanation is consistent with the long-established association of wild yeast with trees, especially oaks. Most *Saccharomyces* strains especially *S. paradoxus* strains have been isolated from tree surfaces, especially oak tree bark, or from oak tree exudates. It is therefore possible that cells grow on the surface of oak trees, either on the leaves or the bark, and are washed down the tree trunk by rain onto the ground.
2. A second possibility is that the leaves falling from trees provide nutrients to *Saccharomyces*, which is part of the community consuming resources from the decomposing leaf litter. Plant litter is composed of dead plant material such as leaves, bark, twigs and branches which have fallen to the ground, is humid, has several layers of different decaying organic material and new leaves will fall down every year "refreshing" the nutrient content. Leaf litter contains abundant complex polysaccharides derived from lignocellulose which cannot be utilized directly by *Saccharomyces*, but which are digested by extracellular enzymes from other fungi and

bacteria to yield simple sugars (Sinsabaugh and Linkins, 1990; Steffen et al., 2007) which *Saccharomyces* might consume. Consistent with the yeast being part of the decomposition community, I found that sterile medium made only from leaf litter and water supports *S. paradoxus* growth to over 10^5 cells per ml (unpublished data), and that a *Mucilaginibacter* spp. promotes the growth of *S. paradoxus* in oak bark medium (Kowallik et al., 2015).

3. A third possibility is that the major resource provided by the tree is not its surface, nor its dead leaves, but exudates from its root system. It was observed as long ago as 1904 (Hiltner, 1904) that the areas under trees harbored higher microbial densities, and this is due the influence of their roots on the surrounding soil (the rhizosphere). The rhizosphere extends from the surface of the root to a position in the soil that depends on the diffusion rate of exudates, the root's biochemistry and development but it is assumed that below the ground its extension is three orders of magnitude higher than the area occupied by the plant (López et al., 2012). In trees lots of the carbon fixed by photosynthesis will be translocated to the roots, depending on the species this is ~60 % (Ågren et al., 1980; Veen et al., 1989). The compounds present in tree root exudates include carbohydrates, amino-, organic-, phenolic- and fatty acids, vitamins as well as growth factors (Grayston et al., 1997). Complex microbial communities are supported by nutrients released from the root system (López et al., 2012), and there are many well-documented mutualisms between plants and the microbes that inhabit their rhizospheres. Beneficial microbial activities in the rhizosphere are for example nitrogen fixation and demineralization (Canbolat et al., 2005) as well as solubilization of phosphorus and carbohydrates (Kohler et al., 2006). But also the secretion of exopolysaccharides which will create new niches for other microorganisms (Haggag, 2007) or the production of secondary metabolites with antimicrobial activity are common microbial interactions in the rhizosphere. Fungi are key players in the rhizosphere and connect the actively absorbing rootlets with the surrounding soil which gave this interface the name mycorrhizosphere (Johansson et al., 2004).

4.2. *Saccharomyces* occurrence is decreasing with increasing distance to oak

The decrease of *Saccharomyces* occurrence in the leaf litter with an increasing distance to the oak trunk supports the yeast/oak association and all three hypotheses for the mechanisms by which oaks support yeast could theoretically explain this result. The effect of the tree has a relatively short range. Figure 4 shows that the *Saccharomyces* promoting region extents only about 8 m from the trunk and declines strongly afterwards. Leaf litter in a forest is relatively

protected against strong wind and we see a clear heterogeneous composition of the leaf litter. Close to an oak trunk oak leaves are dominating the litter and after ten meters distance the leaf litter will contain mainly needles or leaves from other tree species. This distance correlates with the range of the oak crowns which varied between ~5 - 10 meters (assessed while sampling the transects). This actually promotes the second hypothesis although we cannot exclude the third one as studies showed that the roots of *Quercus rubra* in forests terminate up to 17 meters from the base of the tree in a depth that varied between 5 - 50 cm (Lyford, 1980). As the roots will be much denser close by the tree a decrease in *Saccharomyces* abundance away from the trunk could also be well explained by an influence of the roots.

4.3. More *Saccharomyces* in leaf litter than on bark

The fact that I found far more *Saccharomyces* cells in leaf litter than on the trunk of the tree (Figure 3) strongly argues against hypothesis 1, the idea that yeast grow directly on the surface of the tree. *Saccharomyces* yeast have been isolated from soil surrounding oak trees several times (e.g. Nagornaya et al, 2003; Sniegowski et al., 2002; Sylvester et al., 2015) and *Saccharomyces* yeast could also be isolated from fresh oak leaves (Glushakova et al., 2007; Sláviková et al., 2007) but the isolation success was never remarkably high. This much lower isolation success from fresh leaves as well as surrounding soil in combination with the low cell number on oak bark suggests that the yeast actually proliferates in the decaying leaf litter. It is more likely that *Saccharomyces* found on oak tree bark migrated there from the leaf litter, perhaps splashed by rainwater spray or carried by animal vectors. It would be interesting to see whether the abundance of yeast on the tree trunk decreases with distance from the ground.

4.4. Seasonal effects on yeast abundance and distribution

The fall of leaves from the tree takes place once a year and in addition leaf litter is strongly exposed to seasonal influences like temperature, rain, sun etc. and so we expected a strong seasonal variation in yeast abundance if oak leaf litter itself is the primary resource that yeast exploit (hypothesis 2). There is indeed a seasonal effect on distribution along transects, suggesting that the zone influenced by a tree changes according to the season (Figure 4). But the effect is not as pronounced as we would expect, and no significant effect of season on overall abundance was detected (Figure 5). In contrast to leaf litter or tree surfaces, the root system seems not as exposed to seasonal changes and might provide a much more constant resource than the leaves themselves. But it could be demonstrated that root exudation is affected qualitatively as well as quantitatively by temperature and as most of the assimilated carbon in a plant derived from photosynthesis, changes in light clearly also modifies root

exudation (Grayston et al., 1997). The non-significance of season in the abundance data get more interesting on the second view as season has a significant interaction with tree. This could be best explained by the fact that leaf litter of each tree might decay differently depending on the quantity and quality of leaf litter, sun exposure etc. Bacteria and especially fungi are the active decomposing organisms responsible for litter decay and nutrient release by rapidly increasing in numbers which again depends on several seasonal, abiotic factors (Pandey et al., 2007; Voříšková et al., 2014).

4.5. Variation in yeast abundance within tree samples suggest patchy distribution

Additionally to the observed variance in *Saccharomyces* abundance between the transect trees we also see variation within the three samples per tree (Figure 3 A). It has to be taken into account that I sampled three different sites of a tree which also introduces lots of variation in natural factors. It is well known that resources are patchily distributed in soils (Hodge, 2006; Rennert, 2011) and the same is true for the microbial life which tends to live in aggregates and to form spots of activity (Ling et al., 2011; Nunan et al., 2003). The leaf litter abundance data confirm a spatial pattern of *Saccharomyces* in leaf litter as there is a pretty high variation within the three samples coming from one tree (Figure 3A). As *Saccharomyces* likely depends on other organisms to break down complex saccharides in the leaf litter it seems logic that we will find more *Saccharomyces* yeast close to a patch of organisms that are able to provide simple nutrients what would support the second hypothesis. But also the proximity of the nearest tree root which might provide nutrition could explain the patchy pattern of *Saccharomyces*.

I cannot finally say which of the three possible explanations is responsible for the nutrition of *Saccharomyces* coming from oaks. The data of this chapter certainly support the idea that *Saccharomyces* yeasts grow primarily on the ground, rather than on the bark surface, but the results do not strongly favor either root exudates or leaf litter as the influential factor. The three hypotheses are not mutually exclusive; indeed it is possible that *Saccharomyces* inhabits and proliferates in all these niches, and more. What I can say is that in almost all studies the isolation success from oak bark, fresh leaves or surrounding soil is much lower compared to my data from leaf litter. Anyway, without enough, standardized sampling data, which will not only study detection but also abundance of *Saccharomyces* yeasts in different natural environments, this question, is finally hard to answer.

4.6. Seasonal effects on *Saccharomyces* abundance and dispersion

For abundance and dispersion of an organism, season is an extremely important factor as several natural factors correlated with season like sun exposure, which will increase the temperature, moisture and frost, will have an influence on the microbial community composition. It has been shown that success of isolating *Saccharomyces* over a year in northeast America increased continually from April to August/September and decreased at the end of summer (Charron et al., 2014). Another recent study showed that *S. paradoxus* isolation frequency is associated with summer temperature, showing highest isolation rates at intermediate temperatures (Robinson et al., 2015). For my data I could not confirm a significant influence of temperature on the abundance nor the distribution of *Saccharomyces*. As there was a significant influence of season on yeast distribution and of an interaction between season and tree on yeast abundance, season seems to play a role but not necessarily exclusively due to the temperature in the season.

4.7. Oak provides a better habitat for *Saccharomyces* than larch, beech or spruce.

Consistent with both the historical association of wild yeast with oak trees, and a couple of other studies comparing oaks to other tree species, oaks were actually significantly better habitats than larches, beeches and spruces in. I was surprised to find that the second “best” habitat for *Saccharomyces* is leaf litter from larch, a conifer, and not beech, which has broad leaves like oaks. My design does not rule out the possibility that *Saccharomyces* grows only in association with oak litter, but can be isolated under other trees because leaf litter is slightly mixed in a mixed forest, however the high isolation success in the leaf litter of all trees suggest that yeast grows throughout the forest, but prefers oak litter. I do not know what makes oak special, but it is known that tree species can influence surrounding biogeochemistry through variation in the quantity and chemistry of their litter returned to soil which will have a huge impact on the identity, abundance and activities of diverse organisms (Reich et al., 2005). Different leaf litters encourage development of distinct microbial communities (Bray et al., 2012). There is also evidence that these microbial differences in litter, forest floors and soil result from influences of the nature of the litter itself (especially pH and base cation content of the litter and the leaf type) (Prescott and Grayston, 2013). Oak plant material provide high amounts of tannins and it is well known that these are toxic and bacteriostatic but also active against several fungi (Scalbert, 1991). The level of tannins in the leaves of *Quercus robur* varies over the year and increases from 0.5 per cent dry weight in April to about 5.0 per cent in September (Feeny, 1969) and these changes will clearly somehow influence the composition of the microbial community. Tannins are not the only microbe-active substances in oak leaves. The

bark and leaves of *Quercus robur* as well as other oak tree species have been used as traditional medicine for the treatment of various diseases such as chronic diarrhea or dysentery (Uddin and Rauf 2012). Indeed, studies show the presence of several different bioactive secondary metabolites being active against different bacterial pathogens (Andrenšek et al., 2004). In a survey testing the extract of *Quercus ilex* leaves 74 % of the tested bacteria (132 strains) showed to be resistant whereas the remaining 27 % coming from seven different bacteria genera including five species of *Pseudomonas* were susceptible (Güllüce et al., 2004). This is interesting as we found that two oak *Pseudomonas* strongly inhibit or even kill *S. paradoxus* (Kowallik et al., 2015). All these bioactive substances in oak material might, due to the inhibition of several other microbes, open a niche for *Saccharomyces* yeasts.

4.8. Strong tree-to-tree variation in harboring *Saccharomyces*

For the six transects there was a significant effect of tree, indicating that some trees support more yeast and others less (Figure 5). The abundance data also confirm that some oak trees are in general a better habitat for *Saccharomyces* than others, consistently over a whole year (Figure 6). That raises the interesting question about the reason for this tree-to-tree variation.

First, several location-correlated factors like wind, sun and rain exposure, surrounding vegetation, amount of leaf litter from other trees that mix up with the oak litter etc. can have a strong influence on the microbial community of the leaf litter and its decaying grade, and this would also influence the occurrence of *Saccharomyces*. Controlled field experiments showed that decomposer communities associated with different forest types affected the overall decomposition rate of the same leaf litter type (Wallenstein et al., 2010). It could also be shown that *S. paradoxus* isolation success is positively associated with trunk girth and older trees harbor more yeast (Robinson et al., 2015).

Second, a genetic difference between the oak trees can be the reason for the constant difference. Oak trees will already vary genetically within a species but oaks additionally hybridize between species. This is also known for the two most common and closely related oak species *Quercus robur* and *Quercus petraea* which have a wide sympatric distribution over Europe (Rushton, 1993). A study in a tropical forest on two oak species indicated that the oak host was significant in explaining some of the variation in ectomycorrhizal communities (Morris et al., 2009) another study on *Populus* trees showed under controlled experimental conditions that the genotype significantly influenced microbial community composition in the surrounding soils (Schweitzer et al., 2008). Genetic variation might also influence the chemical composition of the leaf material. Studies of the natural variation in the concentration of heartwood tannins for example determined that levels are highly variable between individual oak trees (Puech et al.,

1999). Also root exudation varies even between closely related tree species and changes qualitatively and quantitatively with plant age (Grayston et al., 1997). Very likely both factors; environmental as well as genetic factors will influence the leaf litter habitat.

4.9. *S. paradoxus* is the dominant *Saccharomyces* species in Nehmten

For this study 1536 leaf litter samples were collected and 636 tetrad-forming strains detected from which we sequenced the ITS regions (all the isolates from transects and tree-species comparisons) and confirm all 633 as being *S. paradoxus* and just three *S. cerevisiae*. The strains isolated from the abundance (MPN) experiments were not sequenced, but given that all 636 tetrad-forming strains were *Saccharomyces*, we believe that the MPN data gives an accurate measure of the abundance of this yeast in leaf litter. We also sequenced 36 strains with colonies that looked like *Saccharomyces* but which could not sporulate, hoping to find non-sporulating *Saccharomyces* isolates, but all were non-*Saccharomyces* genera (*Wickerhamomyces*, *Saccharomycodes*, *Debaryomyces*, *Cryptococcus*, *Torulaspora*, *Zygosaccharomyces*, *Hanseniaspora*, *Citeromyces*, *Metschnikowia*, *Candida*). Thus, we can conclude that enrichment sampling combined with tetrad-screening is a very efficient way to isolate *Saccharomyces*, and that *S. paradoxus* is by far the most dominant *Saccharomyces* species in this forest.

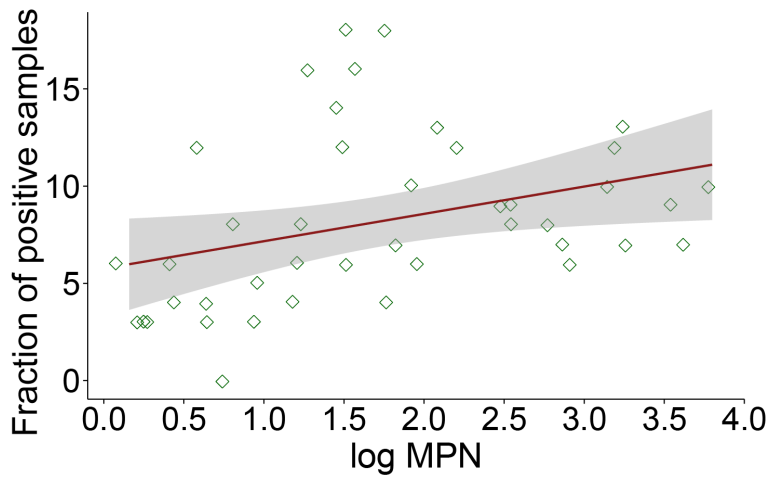
Other sampling studies have also shown that *S. paradoxus* is the main wild yeast species, with *S. cerevisiae* notably absent in samples from northern latitudes (Charron et al., 2014; Johnson et al., 2004; Sylvester et al., 2015). *S. cerevisiae* has a higher optimum and maximum growth temperature than *S. paradoxus* (Leducq et al., 2014; Salvadó et al., 2011b; Sweeney et al., 2004) and the locations where wild *S. cerevisiae* could be found are consistent with the geographic distribution of its optimal growth temperature and most *S. cerevisiae* strains isolated outside this range are human-associated strains (Robinson et al., 2015). I do not know if the *S. cerevisiae* strains in my study are human-associated or hybrid strains with a *S. cerevisiae* ITS sequence. Another possibility would be that *S. paradoxus* is doing better in the enrichment medium when directly competing in one sample against *S. cerevisiae* and in reality we would isolate more *S. cerevisiae* strains. However as also the plate cultured strains turned out to be exclusively *S. paradoxus* this yeast seems to be more frequent in Northern Germany. As there was no difference in fitness between the plate- and liquid cultured strains in the standard liquid enrichment medium we can rely on this method for getting *Saccharomyces* isolates although I cannot exclude that there will be a significant effect with an increased sample size. With the characterization of the high abundance of *Saccharomyces* in oak leaf litter it will be possible to

isolate many more *Saccharomyces* strains without liquid enrichment methods in future which will increase our knowledge on its natural diversity and ecology.

5. Conclusion

Saccharomyces is indeed associated with oak trees: its frequency strongly decreases with increasing distances from oaks and it shows a significant tree species specificity. The yeast is fifty times more abundant in oak leaf litter than on oak bark, indicating that it proliferates in litter. High *Saccharomyces* abundances in leaf litter should change the perspective for future sampling studies. Leaf litter provides a constant habitat for this yeast over the entire year with tree specific changes over the season. Tree individuals strongly differ in terms of *Saccharomyces* distribution and abundance: some trees were generally good habitats and others were generally bad habitats for the yeast. *S. paradoxus* is the most abundant *Saccharomyces* species in this Northern German mixed forest.

6. Supplemental Material



Supplementary Figure 1: Relation between fraction of positive samples for each of the six transects per each sampling month and the average log MPN for this tree at the same sampling time point. The solid line represents the linear regression with 95 % confidence interval.

7. Acknowledgements

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General Discussion

In this thesis, I describe studies of the natural ecology of *Saccharomyces* yeasts, with a special focus on the true wild habitat of the yeast.

As *Saccharomyces* yeasts - especially *S. cerevisiae* - have been used for such a long time in human history to produce alcoholic beverages from fruit, especially wine, many assume it is adapted to the fruit niche in nature. Since the 1950s, a very different environment has been discussed as the natural habitat of wild *Saccharomyces*: the surfaces of oak trees. Today, we still mainly find these two environments, sugar rich fruits especially grapes and oak surfaces especially bark, being referred to be the habitats of *S. cerevisiae* despite the striking differences between them

There are different scenarios possible:

1. *Saccharomyces* yeasts are adapted to sugar rich environments not to oak

On the first view the sugar rich environment seems more logical not only because *S. cerevisiae* is known to consistently dominate over all other microorganisms in the winemaking process but also because the yeast exhibits traits allowing it to compete very well in sugar rich environments. The Crabtree effect is purported to be one such trait, with proposed benefits in both resource competition and interference strategy. In the first Chapter I confirmed that being Crabtree positive is indeed advantageous in sugar rich environments, especially where *S. cerevisiae* has to compete against other microorganisms and therefore it may have evolved through better interference competition. This result seems to support the idea that the yeast is adapted to high sugar environments. On the other hand, except for the colonization of human-produced crushed fruit juices as part of alcohol production, *Saccharomyces* yeasts are rarely found on fruits (Martini, 1993; Mortimer and Polsinelli, 1999; Vaughan-Martini and Martini, 1995). Indeed, my own sampling of damaged grapes (which should be more likely to be colonized by *Saccharomyces* yeasts than intact grapes, Mortimer and Polsinelli, 1999) in an organic vineyard in Italy resulted in only eleven out of 221 samples containing *Saccharomyces* yeast (Kowallik, Boynton, Greig, unpublished). Furthermore, culture-independent pyrosequencing of ripe grapes revealed that 1 in 20 000 fungal reads belonged to *Saccharomyces* (Taylor et al., 2014). It seems that *S. cerevisiae* is abundant only in artificial human-made systems which might indicate that the yeast is in reality not adapted to sugar rich environments as a fruit is something completely different from a liquid, homogeneous and

warm environment. It might be that the fermentative lifestyle which provides benefit to *S. cerevisiae* over other microbes in the artificial crushed fruit environment did not evolve as an adaptation for something similar in nature but is instead an evolutionary spandrel (Goddard and Greig, 2015). A spandrel (which is originally an architectural concept) has been defined as a trait that exists as by-product of the evolution of some other trait, rather than being a direct product of natural selection (Gould and Lewontin, 1979). Anaerobic fermentation can occur in low-sugar environments (glucose concentration as low as 150mg/l, about 100-fold lower than grape juice, Pfeiffer and Morley, 2014). However, the amount of ethanol produced in these environments would be negligible; certainly not high enough to cause any harm to other microorganisms. Converting sugar into ethanol that can be consumed by the yeast itself but not by all other microorganisms seems like a good resource competition strategy. On the other hand several other yeasts like *Candida spp.*, *Metschnikowia spp.* and *Kluyveromyces spp.* (Kurtzman et al., 2011) as well as bacteria like *Pseudomonas fluorescens* (Yuan et al., 1998) which are all widely distributed in the environment are able to consume ethanol. In the presence of such microorganisms *Saccharomyces*' inefficient use of rare glucose under fermentation would provide an even higher competitive disadvantage compared to competitors that use glucose or the previously produced ethanol efficiently. A final potential benefit of anaerobic fermentation cannot be ruled out; small amounts of ethanol may also attract insect vectors (Buser et al., 2014) so it is at least possible that aerobic fermentation evolved as communication strategy to insect vectors.

2. *Saccharomyces* yeasts are adapted to oak and not fruit.

Alternatively, the yeast/oak association could indeed be the truth, and the dominance of *Saccharomyces* in artificial fermentations could be an evolutionary spandrel. Besides grapes there is no environment that has been as intensively sampled as the bark of oak trees and almost all wild *Saccharomyces* isolates in culture collections come from this environment. But even with the high sensitivity enrichment culture, *Saccharomyces* is not detected in the majority of samples. The repeated isolation of *Saccharomyces* from the oak environment might therefore be caused by a bias. Taking a look into the literature shows that the very early published isolations of wild *Saccharomyces* (from 1904 onwards) from oak surfaces were the starting point when scientists who wanted to study the genetics of wild *Saccharomyces* continued sampling oak trees with referring to the earlier publications, creating an incorrect picture about the yeast's association with oak. One fact supporting the hypothesis that *Saccharomyces* including *S. cerevisiae* lives on oak bark surfaces is that wild, undomesticated *S. cerevisiae* could be found in primeval forests far away from human influence (Wang et al., 2012) as well as on Mediterranean oaks (Almeida et al., 2015). In this thesis the results from first and fourth Chapter support the

hypothesis that *Saccharomyces* yeasts are associated with oaks as *S. paradoxus* as well as *S. cerevisiae* (data not shown) can use nutrients from oak bark and leaf litter to grow and *Saccharomyces* is present in a high abundance in oak leaf litter. The fact that the yeast occurrence declines with an increasing distance from oak trees and that I found significantly more *Saccharomyces* in samples from oak leaf litter than from other trees show that the yeast is not generally abundant in the leaf litter of a forest but associated with oaks.

3. *Saccharomyces* is a niche-less generalist

A third hypothesis would be that *Saccharomyces* is a niche-less generalist that can be found in many different environments (Goddard and Greig, 2015). This picture is supported by the fact that *Saccharomyces*, although mostly associated with oak trees or grapes in literature, has also been isolated from several different environments like the surfaces of different plants, soil or fruits. My data show that *Saccharomyces* is much more prevalent in oak leaf litter than other environments but I was able to isolate the yeast also from the leaf litter of other trees. When I sampled many different sugar rich and sugar poor environments the success in isolating *Saccharomyces* was normally not present (Table 1). As I was only able to find these yeasts on grapes, oak bark and in the leaf litter environments, it does not seem that *Saccharomyces* is really ubiquitous in the natural world although the culturing process might bias the detection of the yeast.

Table 1: Samples collected and tested via enrichment culturing for the presence of *Saccharomyces* yeast. The samples are not all taken in a standardized way, preventing direct comparison between environments. The data simply represent a screening of the environment for the presence of *Saccharomyces*.

	environment	samples taken	positives
High sugar environments	intact apples	106	0
	rotting apples	84	0
	apple flowers	35	0
	linden flowers	15	0
	apple compost	36	0
	rotten plums	20	0
	rotten mirabells	20	0
	elderberries	10	0
	oak leaves with honeydew	52	0
	damaged grapes (Italy)	221	11 (5%)
	Low sugar environments	soil around apple trees	22
leaves apple trees		45	0
bark apple trees		56	0
Leaves <i>Petasites</i> spp.		40	0
fresh oak leaves		34	0
oak bark		849	58 (6.8%)
oak leaf litter		60	53 (88%)
larch leaf litter		60	42 (70%)
beech leaf litter		60	27 (45%)
spruce leaf litter		60	20 (30%)

4. Combining the high sugar and oak hypothesis

It might also be true that *Saccharomyces* yeasts are really associated with both oak surfaces and sugar rich environments and there are at least four possibilities that might connect these different niches:

4.1. Oak exudates

One possibility to connect oaks with sugar rich environments are the oak exudates *Saccharomyces* has been isolated from (Bachinskaya 1914; Naumov et al., 1998). When oaks get wounded they produce a sugar rich sap which runs down the tree and it has been assumed that this sap provides high sugar conditions for *Saccharomyces*. This fact does not seem to influence *Saccharomyces* detection as first, as the isolation success from pure bark or surrounding soil is as good (or bad) as that from oak exudates (e.g. Sniegowski et al., 2002; Zhang et al., 2010) and second I only saw oaks producing sap twice and neither yielded *Saccharomyces* isolates. In other areas of the world like North America it seems much more common that oak trees are wounded

and produce this oak sap which might be due to different biotic factors compared to our areas like animals damaging the bark or different species of oak trees. In any case, the success of isolating *Saccharomyces* does not seem to depend on oaks being wounded or non-wounded.

4.2. Honeydew

Another sugar resource might be the honeydew produced by aphids. The aphid species *Tuberculooides annulatus* mainly colonizes oak trees (Heimbach, 1986). Aphids form a mutualistic interaction with ants and provide them a sugary solution, the honeydew, and in return the ants tend to and protect the aphids from predators and parasitoids (e.g. Addicott, 1979; Banks, 1962; Völkl, 1992), as well as from fungi which often hinder the growth of aphid populations (Pontin, 1960). Depending on the aphid species and on the host plant they feed on, the honeydew contains a mixture of sugars (xylose, glucose, fructose, sucrose, maltose, melezitose and raffinose), organic acids, amino acids and some lipids (e.g. Hussain et al., 1974; Mittler, 1958; Völkl et al., 1999). We hypothesized that this might be a niche of *Saccharomyces*. The yeast could benefit from rapid growth while fermenting and outcompeting other microbial competitors. It is a common picture to see many ants running up and down the oak trees and they could vector the yeast from the leaves onto the bark, which would explain why we can find *Saccharomyces* on oak bark. I tested more than 50 honeydew samples at different time points and did not detect a single *Saccharomyces* isolate. I realized that the leaves covered in honeydew soon get infected by black mold. These so called sooty molds consist of diverse species are known for this behavior (Hughes, 1976) and grow on sugar rich plant surfaces and produce a thin, superficial network of dense dark hyphae on honeydew (Faull et al., 2002; Hughes, 1976). Sooty molds inhibit other microbial species which normally colonize the phyllosphere (Chomnunti et al., 2014). Additionally a screen for yeast species colonizing honeydew on *Nothofagus* trees in New Zealand, which are known to provide a reservoir of wild *Saccharomyces* (Libkind et al., 2011) showed no *Saccharomyces* isolates on honeydew (Serjeant et al., 2008). Honeydew as a *Saccharomyces* habitat seems unlikely due to the above mentioned reasons but I cannot generalize about honeydew from every aphid species all around the world.

4.3. Proliferation on fruit and hibernating on tree surfaces

As sugar rich habitats are not available all the year around *Saccharomyces* would need a place where it can stay in the meantime. Knight and Goddard (2015) introduced the “fruit forest-reservoir hypothesis” which connects the fruit with the forest environment by suggesting that *S. cerevisiae* exists in a sporulated stage as a diffuse low abundance reservoir in different forest niches such as soil and tree bark, from which it is vectored to sugar rich fruits by insects, where

it proliferates and in the end of the fruiting season some fraction of this population will return back to the forest environment. The authors showed that *S. cerevisiae* sporulates on soil medium which is clearly not enough to verify the hypothesis but one basic requirement that it can be true. Consistent with this hypothesis is that *S. cerevisiae* produce several secondary metabolites that attract *Drosophila* flies (Becher et al., 2012; Buser et al., 2014; Christiaens et al., 2014) which can vector the yeast from one habitat to another. This hypothesis would make sense considering the low cell number of *Saccharomyces* on oak bark but the oak leaf litter samples can contain more than 10 000 cells per sample and this clearly looks like a proliferation of *Saccharomyces* in this environment.

4.4. *Wild grapevines*

The last hypothesis I would like to explore is the combination of grapes and oak. The grapes we use to make wine belong to *Vitis vinifera subsp. vinifera* (or *sativa*) which is a domesticated species. Nowadays, considerable attention is paid to elucidate the diversity of the wild grapevine genetic pool used for domestication and to identify the place and period of the original domestication. Most authors agree that a first domestication event happened in Caucasus ~6000 BC (Ekhvaia and Akhalkatsi, 2010). The wild form *Vitis vinifera subsp. sylvestris* still exists in Eurasia (in Europe it has been identified in France, Spain, Italy, Germany, Switzerland, Austria, as well as many other European countries) and North Africa (This et al., 2006). The lifestyle of wild vine is interesting as it climbs up tree trunks and oaks are dominant carrier trees (Ergül et al., 2011; Regner et al., 2015; Tiefenbrunner et al., 2005). The population size of wild vine has dramatically decreased due to invasive plant species and wild *Vitis* is a rare species today and might go extinct in the near future. Surprisingly nobody has checked which microorganisms are associated with the wild *Vitis*. The life of the wild grape plant might combine two habitats for *Saccharomyces*: sugar rich habitat of ripe grapes as well as bark and leaf litter of oak trees.

Another long lasting connection between grapes and oaks is the historically long use of oak barrels for wine storage. Although this is normally explained by the effect of oak barrels on the taste of the wine, the original reason might be the better prevention against spoilage. Oak wood barrels are a porous material and allow the wine to undergo “low oxidation conditions”, which increase the aroma of the wine. Tannins and volatile compounds like vanillin for example are extracted during storage and contribute to the final wine taste. To not lose these aromatic compounds after several uses of a barrel, roasted oak chips are added to the wine (Garde-Cerdán and Ancín-Azpilicueta, 2006). Due to its antimicrobial activity the oak material also prevents the wine from microbial contamination and it can thus be expected that oak barrels

have been adopted into wine making during history not only because of its flavor basis (Andrenšek et al., 2004). It is interesting to ask whether the matter of taste or the benefit of the oak barrel arose first. If the latter is true people started to associate the oak aromatic compounds with the “real wine taste” until the time point where nobody can reliably answer anymore why oak barrels are used except for the question of taste.

Finally I can conclude that my data clearly support the yeast/oak association but I cannot completely rule out any other hypothesis.

The natural ecology of *Saccharomyces* yeasts is far more complicated than the sterile sugar-rich media used in the laboratory. I have shown that wild *Saccharomyces* yeasts are associated with oak trees, but their performance depends not only on the chemical make-up of this environment, but also the presence of other microbes, as well as other abiotic factors and that these different factors exhibit complex interactions. Furthering our understanding of the natural ecology of *Saccharomyces* is necessary to transforming *S. cerevisiae* from a laboratory model, to a true model organism across fields of biology. Many very interesting questions still have to be answered before we have a complete picture about the ecology of *Saccharomyces* yeasts.

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Declaration of Author's Contribution

This thesis comprises of four chapters in form of published and unpublished material. Specific contributions for the chapters are detailed below.

Chapter I: Kowallik V., Miller E., Greig D. **The interaction of *Saccharomyces paradoxus* with its natural competitors on oak bark. *Molecular Ecology* (2015) 24, 1596–1610.**

V.K. conceived the study. E.M. did the sampling on oaks in Plön, engineering of Sp-Plön strain and determined the sensitivity of malt extract enrichment culture assay. V.K. did sampling in Nehmten, engineering of Sp-Nehmten strain, culturing, 454 sequence analysis, established the methods, performed experiments, did statistics. V.K. wrote the manuscript. E.M. edited the manuscript. D.G. supervised the project, read, and improved the paper.

Chapter II: Analyzing the interactions between *S. paradoxus* and two oak bark bacteria (follow up study to Chapter I).

V.K. conceived the study, did experiments, statistics and wrote the manuscript. D.G. supervised the project, read, and improved the manuscript.

Chapter III: Is the Crabtree effect an adaptation to high sugar environments?

V.K. and D.G. conceived the study. *S. cerevisiae* strains have been provided by the Cereduce company. V.K. did transformations of the strains, established the methods, performed experiments, did statics, and wrote the manuscript. D.G. supervised the project, read, and improved the manuscript.

Chapter IV: A systematic forest survey showing an association of *Saccharomyces* with oak leaf litter. (Will be submitted soon).

V.K. conceived the study, did field work, generated *Saccharomyces* strain library, established methods, did experiments, statistics and wrote the manuscript. D.G. supervised the project, read, and improved the manuscript.

I hereby declare that the information provided above is correct

Supervisor

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Affidavit

I herewith declare, that I prepared this thesis in agreement with the "Rules of Good Scientific Practice" of the German Research Foundation. Apart from my supervisor Dr. Duncan Greig's guidance, the content of this thesis and the experimental design is all my own work and I used no other tools or sources but the cited ones. The thesis has not been submitted either partially or wholly as part of a doctoral degree to another examining body, and no other materials are published or submitted for publication than indicated in the thesis.

Kiel, 14.12.2015

Vienna Kowallik