Accelerated Evolution of Lager Yeast Strains for Improved Flavour Profiles

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Doctor of Philosophy

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Declarations

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, with the following exceptions:

- GC-MS runs of volatiles compounds were carried out by Mr. Penchant Zhang, Dry Silvia Carlin and Prof. Urska Vrhovsek at the facilities of Fondazione Edmund Mach (FEM), in Italy.
- Library preparation of DNA for Whole Genome Sequencing. Carried out by Novogene.
- Library preparation of RNA for Whole RNA sequencing. Carried out by the sequencing facilities in the University of Manchester (UoM).
- Mapping of RNAseq reads against the combined genome of *S. cerevisiae* and *S.eubayanus* and the *S. pastorianus* reference genome CBS1483 was made by Dr Karsten Hookamp and Dr Fiona Mary Roche.
- Figure 6.3 was made by Georgia Thompson.

Roberto de la Cerda

Summary

S. pastorianus is an interspecific hybrid resulting from natural hybridization between *S. cerevisiae* and *S. eubayanus*. These two species belong to the *Saccharomyces* genus, a genus that encompasses different species related to fermentative processes. *S. pastorianus* strains carry out the fermentation of sugars and different nitrogen sources to produce a complex matrix, which is known as Lager beer. These strains can be divided into two groups based on their genomic architecture. Group I strains are allotriploid strains with a reduced S. cerevisiae genomic content while Group II strains are allotetraploid with more S. cerevisiae genomic content.

The aim of this work was to obtain evolved strains of *S. pastorianus* with improved flavour profiles. More specifically, we wanted to obtain strains with an increased flux towards the production of higher alcohols and esters derived from the catabolism of the aromatic amino acid phenylalanine, 2-phenylethanol and 2-phenylethyl acetate. These aromatic compounds are one of the most important in wort as they impart notes of roses and honey-like aromas.

To obtain strains with improved aromatic compounds, we decided to follow an accelerated evolution approach. Several methods are widely used, as chemical mutagenesis or UV mutagenesis, or hybridization between species. Chemical and UV mutagenesis normally induce single nucleotide changes in the strains. Hybridization is a potent tool but it implies some difficulty and it is time-consuming. Here, we used two different approaches that target the chaperone Hsp90p. This chaperone folds proteins that are involved in several processes, such as DNA repair. Previous studies have shown the potential of these two approaches, and cells exposed to high temperatures and to Radicicol have shown different chromosomal rearrangements.

For this experiment, two different strains were selected and submitted to high temperatures and Radicicol treatment. Then, a characterization of the mutants and small-scale fermentations were carried out. One Group I mutant and one Group II mutant were selected based on their overproduction of 2-phenylethanol and 2-phenylethyl acetate. Results also showed that mutants are overproducing higher alcohols derived from tyrosine and tryptophol, the other two aromatic amino acids.

To investigate the changes that high temperatures and Radicicol induced to the cells and to investigate the changes causing this phenotype in the mutant strains, we sequenced the genome of the two mutant strains together with the parental strains. Sequencing showed that mutant strains experienced chromosome loss, chromosome copy loss and chromosome rearrangements. Furthermore, single nucleotide polymorphisms were detected. Two nonsynonymous nucleotide changes were identified in Aro4p, an enzyme that catabolises the first step of the aromatic amino acid biosynthesis.

Then, we investigated the effects that these chromosomal changes and point mutations had in the strains in three different conditions: minimal medium without amino acids and small-scale fermentations in wort on Day 2 and 4. We detected an upregulation of *ARO9* and *ARO10*, two genes which products are involved in catabolism of aromatic amino acids. Both mutant strains show differences in transcriptomic regulation compared to their respective parental strains. Also, we reported gene dosage in Group II mutant. We investigated gene dosage in the parental strains and orthologue analysis has been carried out and gene dosage prevail in the cell. Interestingly, we detected a preference for *S. eubayanus* alleles in Group II parental strain. Analysis between the parental strains showed that despite the different genomic composition, metabolism of the strains goes towards the same direction and differences observed are due to copy chromosome changes.

"The roots of education are bitter, but the fruit is sweet."

Aristotle

"Science and art have that in common that everyday things seem to them new and attractive."

Friedrich Nietzsche

"Cross the Delta"

C2C – Delta

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Timouma, S., Balarezo-Cisneros, L.N., Pinto, J., de la Cerda, R., Bond, U., Schwartz, JM., Delneri, D., (2021). Transcriptional profile of the industrial hybrid *Saccharomyces pastorianus* reveals temperature-dependent allele expression bias and preferential orthologous protein assemblies. *Mol. Biol. Evol.* 2021;doi: 10.1093/molbev/msab282.

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Table of contents

List of figures

List of tables

Chapter 1

General introduction

Adapted from the published review:

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1.1. History of brewing

Beer is one of the most widely consumed alcoholic beverages over the world with over 1.91 billion hectolitres produced in 2019, (Statista). This alcoholic beverage has been produced and consumed throughout history as a part of different cultural events and social gatherings (Hornsey, 2003).

The first reports of beer brewing are found in Ancient Egypt, around 6000 BC. Attributed to being invented by Osiris, different types of beer, usually low- alcohol, were produced and drank daily. Beer was also used in religious rituals and as compensation for work (Hornsey, 2003).

Evidence of beer brewing and consumption around 4000 BC has been found in ancient Mesopotamia (current day Iran, Iraq, Syria, Turkey, and Kuwait). Various types of beer have been described in written evidence, such as in the Hymn to the Sumerian goddess of beer, Ninkasi (Hornsey, 2003). Also, in ancient Babylonia, written evidence was found in the Hammurabi Code, where beer laws were established related to beer purity (Nelson, 2014). Furthermore, there is physical evidence, such as a seal showing people drinking from vessels or vessels of different sizes used to brew in brewery-like house. Chemical evidence such as the presence of oxalate in bowls and vessels confirm that beer consumption was prevalent during this period (Michel et al., 1992).

Egypt transmitted the brewing techniques to the Greeks, despite the latter preferring wine over beer. Later, the Greeks transmitted the brewing techniques to the Romans (Nelson, 2005). Viticulture was preferred in the Mediterranean Basin while brewing was dominant in Central and Northern Europe, where the Celts were based. Several written evidence and materials related to barley malt fermentation have been found all over Europe (Nelson, 2014). These findings support the theory that Celts produced their own beer and imported wine from the Greeks (Rageot et al., 2019). Therefore, the brewing techniques used by Celts may have been the starting point of the brewing techniques of the British and German.

After the collapse of the Roman Empire, beer was relegated to monasteries. While primarily a cottage industry, the evolution of human agricultural practices led to an evolution and improvement of brewing practices. It was in Germany in 1516 when the Bavarian Duke Wilhelm IV established the Purity Law or *Reinheitsgebot* (Purity Law) to regulate commercial brewing. This law stated that only barley, hops and water could be used in Bavarian beers (Hornsey, 2012). The law also restricted brewing of beer to between St. Michael's Day (September 29) and St. George's Day (April 23) (Hornsey, 2012). The Purity Law does not mention yeast, which would not be discovered for over 300 years. Instead, the "gischt" or foamy material which appeared at the top of the brew was considered a by-product of the process. In order to ensure the consumption of beer throughout the year, beer was stored in cool dark caves (Pavsler & Buiatti, 2009). This method of refrigeration and storage is known as "lagering", and it produced a positive impact in the final beers as they were more stable.

Antonie van Leeuwenhoek, using the microscope, reported the existence of microorganisms, later identified as yeast and bacteria in 1680. Since then, several findings related to alcoholic fermentation and yeast have been achieved. The later work of J. H. van den Broek and Louis Pasteur independently affirmed the importance of yeasts in alcoholic fermentation (Barnett, 2000, 2003). Before the application of the Purity Law, beer was carried out at higher temperatures (15-26ºC), followed by a short maturation period and the yeast were observed to float on top of the vat at the end of the fermentation. This type of beer was known as ale beer. The application of the Purity Law and the implementation of lagering practices favoured the selection of the new yeasts. With brewing carried out during the cold months of the year (4-14ºC) and the beer then stored at cold temperatures, it was noticed that the yeast sank to the bottom of the vat, instead of floating on the top of the vat. This new yeast was referred to as lager yeast.

1.2. Brewing process

Each of the four main ingredients influences the quality and sensory properties of the beer (Wunderlich & Back, 2009). Barley malt is considered the main raw material in brewing and its liquid extract is called wort. Hops may be added during wort boiling, adding bitterness and flavour to beer and assists in beer microbial stability (Almaguer et al., 2014). The quality of the water is strictly controlled and physical factors such as pH

values and mineral concentrations (water hardness) influence the type of beer that can be produced. Finally, the yeasts convert wort fermentable sugars to ethanol and carbon dioxide, and through their complex biochemical pathways produce secondary metabolites that characterise the final aromatic profiles of beers (Hazelwood et al., 2008; Pires et al., 2014).

Wort production:

The brewing process has been extensively reviewed in book chapters and many journal articles (Alves et al., 2020; Bamforth, 2000; Briggs et al., 2004; Hughes, 2009; Wunderlich & Back, 2009). Briefly, brewing comprises of malting, milling, mashing, lautering, wort boiling (and whirlpooling), fermentation, maturation, and storage.
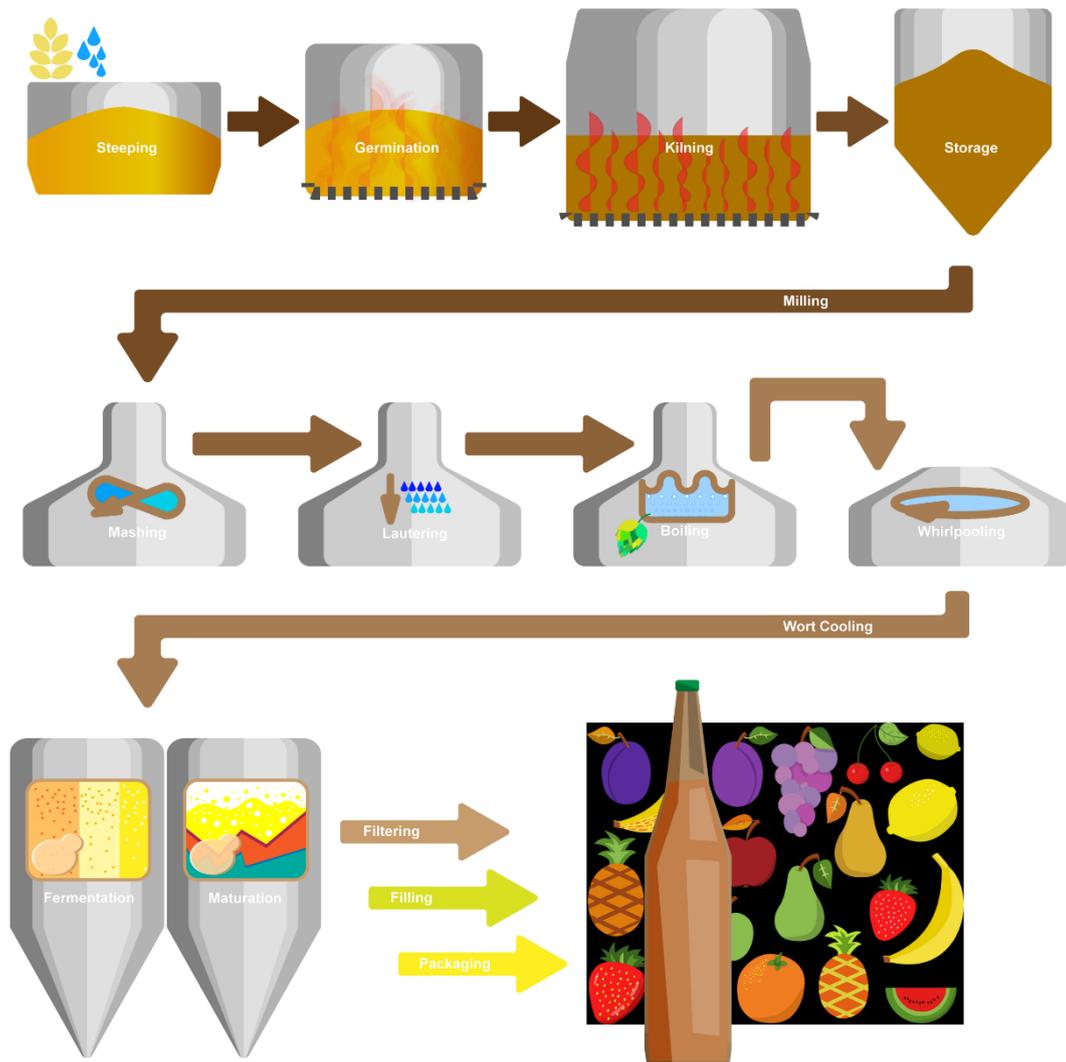
Figure 1.1. Schematic representation of the brewing process. Adapted from Lin et al. (2021).

Milling is a physical process to break down starch and protein, facilitating downstream enzyme modification in mashing. Malting and mashing break down starch and protein in barley to fermentable sugars and digestible nitrogen sources such as free amino acids and short peptides to ensure fermentation. Malting is a similar process to germination, and consists of steeping, germination, and kilning (Fincher, 2010). After water uptake in steeping, the plant hormone gibberellic acid triggers the expression of hydrolytic enzymes in the aleurone layer to degrade storage proteins and starch in the endosperm. The most important hydrolytic enzymes are α- and β-amylases, starch debranching enzymes and

endo- and exo-peptidases (Fincher, 2010). More than 40 endogenous endopeptidases have been identified (Jones & Budde, 2005). Malting is conducted for 3-6 days at temperatures between 12-18°C (Briggs et al., 2004). The process is completed by kilning – drying germinated barley at temperatures up to 80°C for pale malt-.This process is important to inactivate the enzymes, reduce the grain moisture to between 4-6%, and to produce colours and aromas (Briggs et al., 2004).

After malting, mashing is carried out. Mashing refers to a process of mixing ground malts (and other cereal grists) and water in a controlled ratio at a programmed temperature regime to extract sugars and nitrogen materials for fermentation(Briggs et al., 2004). Apart from sugars, nitrogenous materials in the wort are crucial for fermentation. Free amino nitrogen (FAN) mainly refers to amino acids, di- and tripeptides and ammonia that can be taken up by the yeasts (Hill & Stewart, 2019; Stewart et al., 2018). Both malting and mashing contribute to the release of FAN.

After mashing, the final wort is obtained by lautering. The grains sink to the bottom and forms a bed that will filter the liquid. The final wort will be boiled with hops. This step evaporates dimethyl sulphide (DMS) –(a product of S-methylmethionine from kilning (Briggs et al., 2004), deactivates enzymes, and sterilises the wort before fermentation. In summary, the final wort contains fermentable sugars including glucose, fructose, sucrose, maltose, and maltotriose, free amino nitrogen, and various nutrients for yeasts to function and propagate (**Table 1.1**).

Table 1.1. Typical sugars and nitrogen materials in 12ºP wort. Adapted from Lin et al. (2021)

Selected Components in Wort	g/L
Glucose and Fructose	9-12
Sucrose	4-5
Maltose	56-59
Maltotriose	14-17
Free amino nitrogen	0.10-0.25

The concentration of sugars from malt and other soluble content in wort can be measured by the Plato Gravity Scale and expressed in Degree Plato (°P) or as Degree Brix (°Bx), where 1°Bx is 1 g of sucrose in 100 g of solution. The composition of FAN, particularly

the amino acid profile in wort, directly affects flavour output after fermentation (He, Dong, Yin, Zhao, et al., 2014).

Fermentation by yeast:

Wort is cooled and inoculated with yeast strains. Normally, wort is inoculated with a pure culture of yeast strains, a practice that was introduced by Emil Christian Hansen in 1883 in the Carlsberg brewery (Boulton & Quain, 2008). A common practice after each fermentation is to harvest the yeast cells and prepare them for a next fermentation round (repitching). Yeast can be repitched up to 7-10 times after which viability is significantly affected. This is due to accumulation of damage derived from repetitive exposure of yeast to stress (Jenkins et al., 2018). Due to the lack of oxygen during the fermentation, the yeast cannot synthetise the necessary lipids and sterols required for membrane biosynthesis. To restore the permeability and integrity of the membrane, the harvested yeast is briefly aerated (White & Zainasheff, 2010).

The final amount of oxygen that is supplied at the beginning of the fermentation will determine the growth rate of the cells. In the presence of oxygen, the yeast prefers aerobic metabolism leading to cell growth and biomass production. Once the oxygen is depleted, the anaerobic metabolism starts, metabolising the nutrients in the wort and producing mainly ethanol and carbon dioxide. The yeast also produces a wide range of secondary metabolites (higher alcohols, esters, aldehydes) that are very important for the final beer flavour and stability (Lodolo et al., 2008). Wort is diluted down to a specific gravity of 10-12 (°Bx). In some cases, beer companies opt for high-gravity brewing whereby fermentation is carried out at a higher concentration of sugar (18-20° Bx). After the fermentation, the yeast is harvested, and the beer experiences a maturation step. After that, beer is filtered and bottled.

1.3. Yeast classification and the hybrid genomes of lager yeast.

Beer is a fermented beverage produced mainly by yeast classified to the genus *Saccharomyces* within family *Saccharomycetacea,* class *Saccharomycetes*, division *Ascomycota*. The classification of the genus, *Saccharomyces*, is under constant revision and has been quite controversial (Kurtzman & Robnett, 2003) with the number of species that comprises the genus changing over time (Libkind et al., 2011; Naseeb et al., 2017; Vaughan-Martini & Martini, 2011; Vaughan-Martinit & Martini, 1998).The original classification was based on different parameters such as morphological and physiological characteristics, such as characterization under the microscope, different ability to metabolise sugars or amino acids, or fermentation capacity. This type of classification had some disadvantages as these characteristics may vary between strains of the same species (Barnett, 1992).

With the development of molecular biology, new techniques emerged to classify species within the genus. One of the first molecular techniques used for the identification of *Saccharomyces* species was base composition (percentage of Guanine and Cytosine) and deoxyribonucleic acid (DNA) base sequence relatedness. The DNA renaturation method (Price et al., 1978; Seidler & Mandel, 1971), is a technique that measures the sequence identity of two different strains based on hybridization and spectrophotometry methods. This method reduced the number of species of this genus down to 4: *S. bayanus*, *S. cerevisiae*, *S. kluivery* and *S. pastorianus*.

Later, molecular techniques such as karyotyping (Naumov et al., 2001; Nguyen et al., 2000; Sheehan et al., 1991), restriction analysis of mitochondrial DNA (Guillamon et al., 1994), PCR fingerprinting of intron splice sites (de Barros Lopes et al., 1998) and Inter Transcribed Spacer (ITS) analysis (Guillamon et al., 1998) allowed to detect different polymorphisms between the *Saccharomyces* species and improved the classification. The development of whole genome sequencing techniques and the reduction in costs of DNA sequencing have paved the way for the sequencing of thousands of strains of *Saccharomyces* (Peter et al., 2018), thus greatly improving the classification and establishing the relationships between the species. Bioinformatic analysis of the sequences allows for the prediction of Open Read Frames (ORFs), chromosome rearrangements, Copy Number Variations (CNV) and other interesting characteristics related to phylogenetic analysis (Hess et al., 2020).

Such techniques led to the establishment of the strains that comprise the *Saccharomyces* genus today (Alsammar & Delneri, 2020). This genus contains 8 species, namely *S. arboricus, S. cerevisiae, S. eubayanus, S. jurei, S. kudriavzevii, S. mikatae, S. paradoxus* and *S. uvarum*, that can be found in different environments and possess different fermentative characteristics. Despite the genetic divergence at a nucleotide level between the different species within the genus, hybridization events have occurred between species leading to the formation of relevant industrial hybrids, such as *S. pastorianus* (S. *cerevisiae* x S. *eubayanus*), *S. bayanus* (*S. cerevisiae* x *S. uvarum*) and other hybrids between *S. cerevisiae* and *S. kudriavzevii* (Morales & Dujon, 2012). All the species belonging to this genus contain 16 chromosomes except for *S. pastorianus* and *S. arboricus* that contain 42-84 and 12 chromosomes respectively. (Borneman & Pretorius, 2015; Fischer et al., 2000; Wang & Bai, 2008).

S. cerevisiae was the first eukaryote that was sequenced (Goffeau et al., 1996). This species can be isolated in industrial environments such as wineries or breweries but also in wild environments like soil or leaves. *S. cerevisiae* strains are mesophilic and to have a great fermentative performance. These strains are widely used in alcoholic fermentation like beer, wine, sake. Also, they are used in bioethanol and cacao production, bread industry, etc. (Parapouli et al., 2020).

S. paradoxus is the closest strain to *S. cerevisiae,* with up to 90% sequence identity (Kellis et al., 2003). These strains can be isolated from wild environments, like exudate from oaks, soil, and insects such as *Drosophila*. (Naumov et al., 2000; Naumov et al., 1992; Naumov et al., 1998). This species is not recognized as a domesticated species but it has been found in several wineries (Orlic et al., 2007) and in African sorghum beer (Naumova et al., 2003).

S. mikatae is another yeast species that belongs to the *Saccharomyces* genus. It has 84% sequence identity to *S. cerevisiae* and genomic analysis has shown the presence of several translocations (Kellis et al., 2003). It is isolated from decayed leaves and soil in Japan (Naumov et al., 2000).

S. kudriavzevii has been isolated in Portugal and Japan, especially in decayed leaves and soil. This strain has been isolated as well in industrial environments in the form of triple

interspecific hybrids with *S. cerevisiae* and *S. uvarum* (Groth et al., 1999; Masneuf et al., 1998) or between *S. cerevisiae* only (Lopandic et al., 2007)*.* This species have shown a good ability to generate interspecific hybrids not only in industrial habitats but also in the laboratory (Gonzalez et al., 2007).

S. uvarum is a species that was previous classified as as *S. bayanus* or *S. bayanus var uvarum* (Pulvirenti et al., 2000). This species, along with *S. kudriavzevii,* are known to be the most cryotolerant in the genus *Saccharomyces* (Salvado et al., 2011).

S. jurei was the last member of this genus to be identified. Isolated from the tree *Quercus robur* (Naseeb et al., 2017)*,* this species has shown to have good ability to create hybrids with *S. cerevisiae* under laboratory conditions. These hybrids have been tested in beer fermentations, exhibiting a good fermentative performance and improved aromatic profile (Giannakou et al., 2021).

S. arboricola is one of the strains for which there is less information on its fermentation capacity. Isolated from tree bark, the hybrids obtained from this strain with other members of the *Saccharomyces* genus, are used in Sake fermentation (Wang & Bai, 2008; Winans et al., 2020).

S. eubayanus is a cold-adapted strain first isolated from galls of *Nothofagus* trees Patagonia, South America but later found in other wild environments in South and North America, Australasia and Asia, but only interspecific hybrids with *S. cerevisiae* have been found in Europe (Peris et al., 2016). This species shared 99.56% identity to the previously unclassified non-*S. cerevisiae* sub-genome of *S. pastorianus* (Libkind et al., 2011). At least two lineages and five sub-populations have been found in South America (Eizaguirre et al., 2018), and three different lineages have been found in Asia: West China and Sichuan, and Tibet (Bing et al., 2014). Genomic analysis has shown that the Holarctic lineage found in Tibet and North America is the closest wild predecessor (99.82%) to the non-*S. cerevisiae* sub-genome of the Lager brewing yeast (Langdon et al., 2020; Peris et al., 2016).

1.4. The lager yeasts, Saccharomyces pastorianus

Prior to the introduction of the Purity Law in the sixteenth century, fermentations at ambient temperatures using natural yeasts, ascribed as *S. cerevisiae*, produced a beer referred to as Ale. The introduction of the Purity law (Pavsler & Buiatti, 2009) and the new custom of lagering beer contributed to the emergence and domestication of a new interspecies hybrid yeast, *Saccharomyces pastorianus* (Gallone et al., 2016; Monerawela & Bond, 2017a). The hybrid yeasts contain genetic materials from at least two different strains of *S. cerevisiae* and the cryotolerant strain *S. eubayanus* (Dunn & Sherlock, 2008; Okuno et al., 2016). The combination of inherited traits, such as high fermentative capacity from *S. cerevisiae* and cryotolerance from *S. eubayanus* has resulted in a novel yeast better adapted to fermentation in a cool climate at temperatures between 8 – 14°C.

The heterogeneous *S. pastorianus* hybrids used for lager fermentations are classified into two distinct groups, the Saaz group (Hybrid Group I) and the Frohberg group (Hybrid Group II). Both groups originated from hybridisation events between different strains of *S. cerevisiae* and *S. eubayanus* (Dunn & Sherlock, 2008; Libkind et al., 2011). Group I strains are known to have a greater tolerance to lower temperatures and a poor utilisation of maltose and maltotriose, while Group II strains can use maltotriose as a carbon source (Gibson et al., 2013). The genomes of Group I strains have an approximate diploid *S. eubayanus* and a haploid *S. cerevisiae* DNA content. In contrast, Group II strains have an approximate 2n *S. cerevisiae* and 2n *S. eubayanus* DNA content (**Figure 1.2**). Furthermore, the chromosome copy number varies between strains of the same group with copy number of Group I strains ranging from 45 to 52 and Group II strains ranging from 42 to 84 (Monerawela & Bond, 2018).

Next Generation Sequencing and genomic analysis showed that the resultant hybrid strains not only possess chromosomes of both parental strains but also hybrid chromosomes with known recombination breakpoints (Bond et al., 2004) . Some of these breakpoints are located within coding regions, leading to a unique set of hybrid genes in the genus *Saccharomyces* (Hewitt et al., 2014; Monerawela & Bond, 2017b; Nakao et al., 2009; Okuno et al., 2016). In addition to inter-species hybrid chromosomes, translocations between chromosomes of the same sub-genome (Sc-Sc, Se-Se) are also observed.
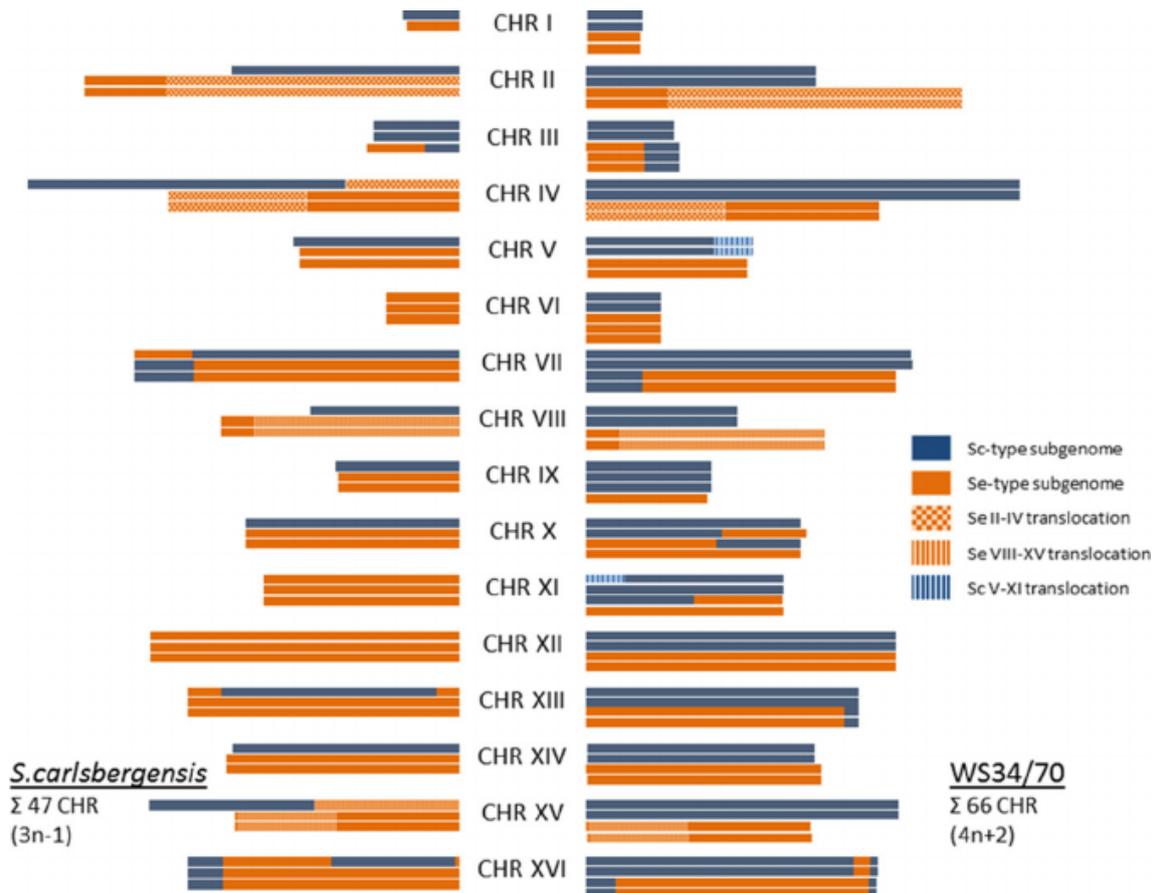
Figure 1.2. Representation of the genomes of Group I strain CBS1513 (left) and Group II strain WS 34/70 (right). Chromosomal rearrangements between *S. cerevisiae*-like (blue) and *S. eubayanu*s-like (orange) can be observed. Adapted from Walther et al. (2014).

Several scenarios have been proposed to account for the origin of *S. pastorianus* strains (**Figure 1.3**). Dunn and Sherlock originally hypothesised that both Group I and Group II arose from two independent hybridization events with Group I emerging from a hybridization of a haploid *S. cerevisiae* and a diploid *S. eubayanus*. Group II, in contrast, was hypothesised to have originated from a diploid *S. cerevisiae* and *S. eubayanus* strains (Dunn & Sherlock, 2008). Later, as more genome sequences became available, it was discovered that Group II yeasts contain two different *S. cerevisiae* sub-genomes that can be distinguished by Single Nucleotide Polymorphisms (SNPs), with one sub-genome shared with Group I strains. This, together with information that both groups share some common recombination events between the parental chromosomes, led to the hypothesis that both groups arose from at least two different sequential hybridisation events between

S. cerevisiae and *S. eubayanus*. The first hybridisation event may have occurred between a diploid *S. eubayanus* strain and a haploid *S. cerevisiae* "Ale", strain (Monerawela & Bond, 2017a; Monerawela et al., 2015). This progenitor hybrid gave rise to the Group I strains while Group II strains arose from a subsequent hybridisation with another *S. cerevisiae* strain. Both groups then evolved independently with Group I strains encountering a significant loss of the *S. cerevisiae* genome and both groups undergoing further recombination events between the sub-genomes (Monerawela & Bond, 2017a; Okuno et al., 2016; Salazar et al., 2019; Walther et al., 2014).
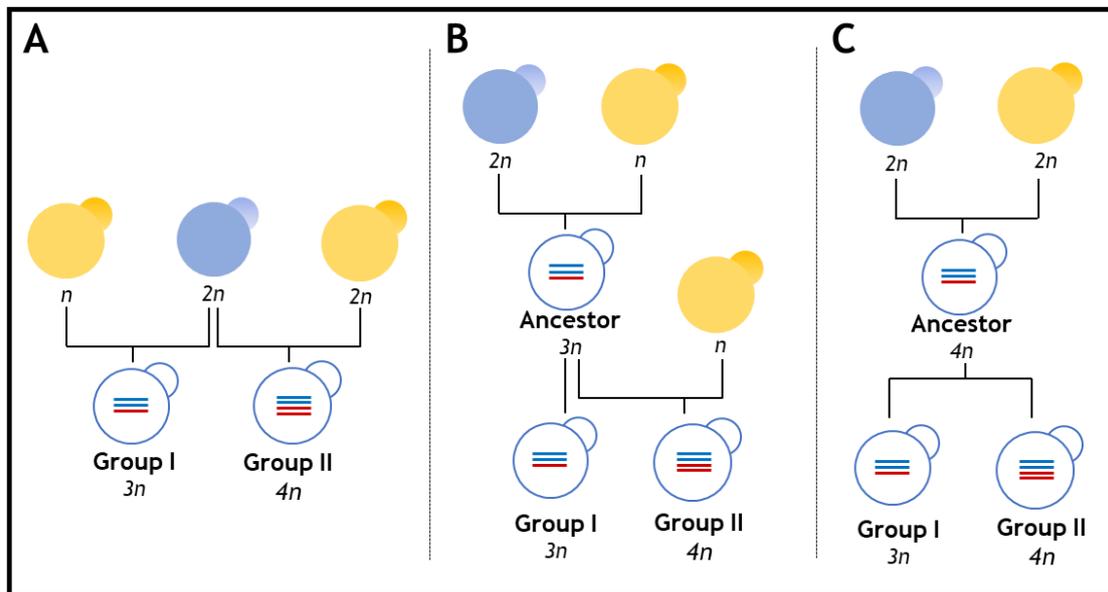
Figure 1.3. Several hypotheses of the hybridization events that originated S. pastorianus strains. In yellow: S. cerevisiae species, in blue: S. eubayanus species. In white, S. pastorianus species. **A**: two independent hybridization events as a possible origin of the strains (Dunn & Sherlock, 2008). **B**: One first hybridization event originated an ancestor of Group I strains. This ancestor would hybridise another S. cerevisiae strain, originating Group II strains (Okuno et al., 2016). **C**: One single hybridization event would gave an ancestor that would have undergone an evolution process and gave Group I and Group II strains (Okuno et al., 2016; Salazar et al., 2019).

The genome data is also consistent with a scenario in which both groups come from a single hybridisation event between a diploid *S. eubayanus* strain and a heterozygous *S. cerevisiae* diploid strain with Group I strains experiencing a selective loss of a significant proportion of the heterogeneous *S. cerevisiae* sub-genome due to different brewing conditions during the domestication process (Okuno et al., 2016; Salazar et al., 2019).

While the sequences of a large number of *S. pastorianus* genomes, 31 to date, are now deposited at the National Center for Biotechnology Information (NCBI), providing researchers with a vast amount of information on the genomes, just one genome, the Group II strain CBS1483 is fully annotated and assembled into chromosomes (Salazar et al., 2019). To date, a fully assembled and annotated genome of Group I strains has yet to be deposited at NCBI. The recently developed open-source tool for functional annotation of hybrid aneuploid genomes, HybridMine, will greatly aid in the annotation of *S. pastorianus* strains (Timouma et al., 2020).

1.5. Impact of yeast during beer production

Uptake of sugars and amino acids

Glucose and fructose are the first monosaccharides metabolised in wort. These sugars can be imported by facilitated diffusion via hexokinase or high-affinity transport via hexose transporters (Bisson & Fraenkel, 1983; Lang & Cirillo, 1987; Lewis & Bisson, 1991; Romano, 1982) (**Figure 1.4**).
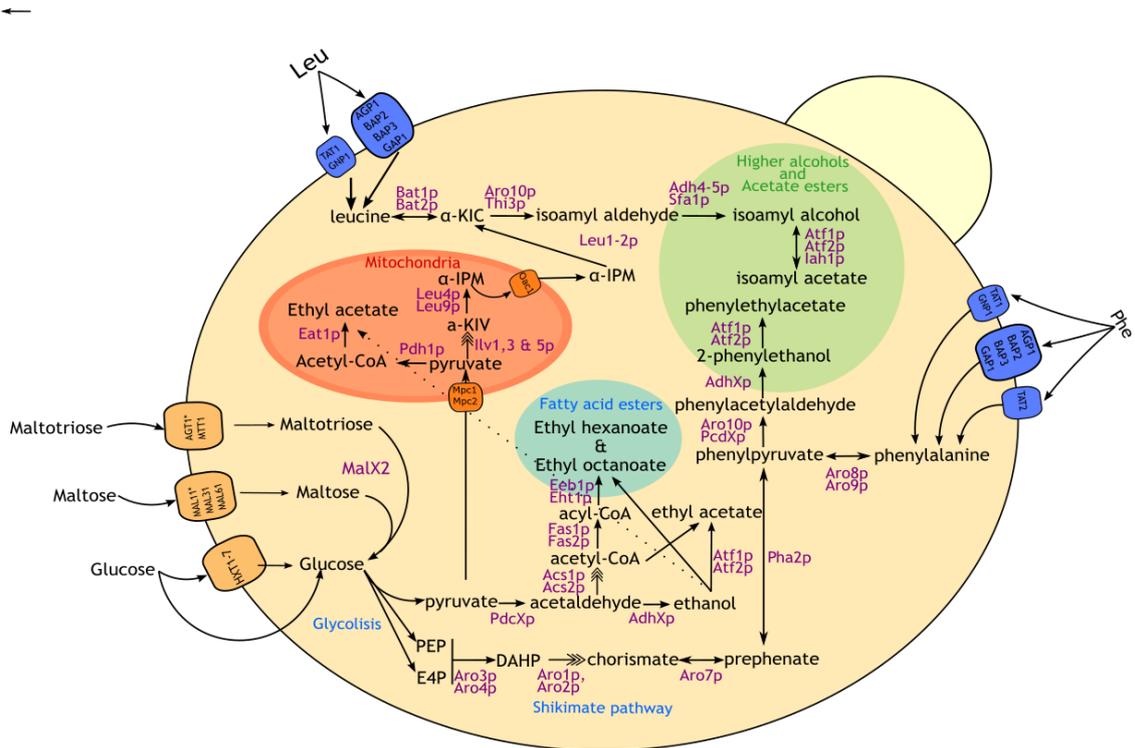
Figure 1.4 Overview of the main metabolic pathways involved in the synthesis of higher alcohol, acetate esters and medium-chain fatty acid esters in S. pastorianus**.** The major biochemical pathways for the production of higher alcohols and esters from branched-chain and aromatic amino acids are shown. The enzymes required for each step are shown in purple. The triple arrows indicate that several steps are omitted. The higher alcohol, 2-phenylethanol, is produced from the degradation of the imported phenylalanine or the degradation of phenylpyruvate from the Shikimate pathway. Isoamyl alcohol is produced from the degradation of imported leucine or from the transformation of pyruvate in the mitochondria. The fatty acid esters ethyl hexanoate and ethyl octanoate are derived from transesterification of medium chain fatty acids. The major transporters for amino acids are shown as are the transporters for the major sugars found in wort. PEP: Phosphoenolpyruvate, E4P: Erythrose-4-Phosphate, DAHP: 3- deoxy-D-arabino-heptulosonate-7-phosphate, α-KIV: α-ketoisovalerate, α-IPM: α-isopropylmalate and α-KIC: α-ketoisocaproate. Adapted from Lin et al. (2021)

When hexoses are present in wort, the synthesis of maltose and maltotriose transporters are inhibited (Federoff et al., 1983; Hu et al., 2000), but once these sugars are consumed, maltose is the next sugar to be used. At least three maltose permeases have been identified, namely *MAL*, *AGT1* and *MTT1/MTY1* (Jespersen et al., 1999; Salema-Oom et al., 2005; Vidgren & Londesborough, 2012). These genes are encoded by gene alleles from both parental strains. At least five Mal transporters are encoded by genes at five independent *MAL* loci (*MAL1-4* and *MAL6*). All these loci are located in subtelomeric regions, and each one contains three genes that are required for maltose metabolism.

MALX1 genes – X designating the locus number – encode for the maltose transporters (*MalT*). *MALX2* genes encode for α-glycosidases (*MalS*). *MALX3* genes encode for transcriptional activators that regulate the previous two genes (*MalR*) (Han et al., 1995). *MAL11*, also known as *AGT1*, is the only transporter of the 5 Mal loci that can import a wide range of sugars, including maltotriose. The *S. cerevisiae*-like copy of AGT1 is non-functional in *S. pastorianus* as it has an early stop codon within the open reading frame (Vidgren et al., 2009; Vidgren & Londesborough, 2012; Vidgren et al., 2005). Mtt1 is another transporter found in *S. pastorianus* strains. It is 90% similar to *MAL31* and 54% similar to *AGT1* (Salema-Oom et al., 2005). Therefore, *S. pastorianus* can import maltotriose by the transporter Se Agt1 and both alleles Sc and Se of Mtt1p. The uptake of this sugar differs between Group I and Group II strains and also between strains from the same group (Magalhaes et al., 2016; Vidgren et al., 2010).

Amino acid uptake from wort involves at least 16 amino acid permeases, 12 of which are constitutively expressed, while 4 are regulated via catabolite repression (Boulton & Quain, 2008; James & Stahl, 2014). The uptake occurs at the early stage of fermentation when ethanol levels are low. The process depends on the type of permeases, their specificity, competition for binding by amino acids, and feedback inhibition of certain permeases. Yeasts display preferences for different amino acids. Normally, aromatic and branched-chain amino acids are the last used by the strains as their catabolism produce the non-catabolizable higher alcohols. Other amino acids, such as serine, arginine, asparagine, alanine or aspartate are a preferred nitrogen sources (Hazelwood et al., 2008; Jones & Pierce, 1964; Lekkas et al., 2007; Ljungdahl & Daignan-Fornier, 2012; Stewart et al., 2018). In addition to importing amino acids from the wort, *Saccharomyces pastorianus* can synthetize de novo all amino acids.

Production of flavour compounds in *S. pastorianus*

Higher alcohols and their corresponding acetate esters are metabolites produced from the secondary metabolism of amino acids via the Ehrlich pathway (Hazelwood et al., 2008). It is the branched-chain (leucine, isoleucine, and valine), aromatic (phenylalanine, tryptophan, and tyrosine), and sulphur containing (methionine) amino acids that produce the major flavour compounds (Dickinson et al., 2000; Dickinson et al., 1998; Iraqui et al.,

1998). Each higher alcohol and acetate ester produces a distinctive aroma or flavour. For example, 2-phenylethanol and 2-phenylethyl acetate impart rose and honey notes, whereas isoamyl alcohol and acetate impart banana and fruity notes (Dzialo et al., 2017; Holt et al., 2019; Pires et al., 2014) .

The Ehrlich pathway consists of three steps (**Figure 1.4**) (Hazelwood et al., 2008; Pires et al., 2014). Firstly, the α-amino group of the amino acid is transferred to α-ketoglutarate in a transamination reaction, leading to an α-ketoacid intermediate. This is carried out by the transaminases Aro8p/9p and Bat1p/2p: the former mainly acts on phenylalanine, tryptophan, and tyrosine, while the latter acts on leucine, valine and isoleucine. Aro8p/Aro9p have broad substrate specificity and display activity towards leucine, methionine, and α-aminoadipate. Next, the α-ketoacid is converted to a fusel aldehyde in an irreversible decarboxylation step by decarboxylases Pdc 1p, 5p, 6p and Aro10p. Finally, the fusel aldehyde is reduced to a higher alcohol by two main classes of enzymes: alcohol dehydrogenases and aryl-alcohol dehydrogenases encoded by large multigene families (Dickinson et al., 2003; Nordling et al., 2002). The enzymes involved in the Ehrlich pathway vary in cellular localisation and substrate specificity (**Figure 1.4**). The catabolism of aromatic and branched-chain amino acids is regulated by Nitrogen Catabolite Repression (NCR) and by negative feedback inhibition by the terminal amino acid (Cooper, 2002; Hofman-Bang, 1999) on the first committed enzymatic step in the pathway.

Esters are an important class of aromas in beer. These molecules have a lower olfactive threshold than higher alcohols and generally have a better aromatic impact (Saison et al., 2009). There are two main groups of esters. Firstly, acetate esters are produced by the esterification of higher alcohols or ethanol with a molecule of coenzyme acetyl-CoA, catalysed by acetyltransferases. The reactions can occur in both the cytosol and mitochondria by Atf1p/Atf2p and Eat1p respectively (**Figure 1.4**) (Fujii et al., 1994; Kruis et al., 2017; Nagasawa et al., 1998). Secondly, medium-chain fatty acid esters are formed by Acyl-coenzymeA:ethanol O-acyltransferase, Eeb1p and Eht1p, whereby ethanol is added to medium chain fatty acids such as hexanoate and octanoate (Saerens et al., 2006).

17

The genes and proteins involved in the Ehrlich pathway have been well characterised in *S. cerevisiae* and it is known that other yeast species belonging to the *Saccharomyces sensu stricto* complex produce the same flavours but at different concentrations under the same fermentation conditions (Perez et al., 2021). The complex genome of *S. pastorianus,* with 2 sub-genomes from different *Saccharomyces* species, raises an interesting question about the participation of the sub-genomes in flavour production. Two previously discovered *S. pastorianus* unique genes, *LgATF1* and *LgATF2*, were later identified as being encoded by the *S. eubayanus* genome (Verstrepen et al., 2003; Yoshimoto et al., 1999). The high sequence identity between the sub-genomes in *S. pastorianus* leads to the possibility of trans-regulation of *S. cerevisiae* and *S. eubayanus* sub-genomes. Bolat et al, showed that in *S. pastorianus*, the deletion of the *S. eubayanus*-like copy of *ARO80*, the transcriptional activator that regulates amino acid biosynthetic genes, did not affect the expression of the *S. eubayanus ARO10* gene, indicating that there is a trans-regulation between sub-genomes, and that transcriptional factors may be interchangeable (Bolat et al., 2013). Trans-regulation of sub-genomes has also been studied in an interspecific hybrid of *S. cerevisiae* and *S. paradoxus* (Tirosh et al., 2009). The interspecific hybrid showed that trans effects exhibited condition-dependent regulation. Also, this trans-regulation may be due to a new environmental sensing obtained from a new genomic combination, or new interactions between cis-trans regulators. Trans-regulation may also occur through the formation of hybrid protein complexes consisting of proteins encoded by each sub-genome, or through competition for substrates and co-factors. Several studies have shown the formation of chimeric protein complexes in interspecific hybrids, (Dandage et al., 2021; Piatkowska et al., 2013) One such chimeric complex, Trp2p/Trp3p that catalyses the first step in tryptophan biosynthesis produced a fitness advantage in growth medium lacking tryptophan. These findings show that the proteome of interspecific hybrids can lead to new combinations of protein complexes that may impart a better adaptation to specific conditions. Currently, our knowledge of the effects of hybrid protein complexes on flavour profiles in *S. pastorianus* is limited, however in a recent study the presence of hybrid complexes as well as sub-genome-specific complexes in *S. pastorianus* (Timouma et al., 2021).

Several studies have measured the major flavour compounds produced by Group I and Group II lager yeasts under varying fermentation conditions in wort (Ekberg et al., 2013;

Gibson et al., 2013; Krogerus et al., 2015; Mertens et al., 2015; Walther et al., 2014). The aromatic profiles were shown to vary significantly depending on the fermentative conditions. Variables such as temperature, oxygen dissolved in the media, carbon source and concentration of sugar and FAN have been shown to impact the concentration of higher alcohols and esters in the beer (Dzialo et al., 2017). As part of a study to generate new interspecific hybrids, the flavour profiles of 7 Group I strains and 10 Group II strains were analysed (Mertens et al., 2015). Results of the parental strains reveals that Group II strains produce a better aromatic profile than Group I strains: esters such as ethyl acetate, isoamyl acetate, and phenyl acetate were produced at higher levels by Group II strains.

While it is difficult to ascertain the contribution of each sub-genome of *S. pastorianus* to the final aromatic profile in lager yeasts, lager-style beer fermentations have been carried out using different strains of the parent *S. eubayanus* (Mardones et al., 2020). These strains were shown to produce different aromatic profiles to *S. pastorianus*, suggesting that the complex hybrid genome may produce a more complex aroma and flavour profile.

1.6. Improvement of Lager yeast strains

Improvement of desirable traits in the yeast strains complement and enhance the alterations to the brewing process for today's consumer. Flavour profiles can be expanded or improved by altering amino acid metabolism in the lager yeast. One approach is to increase flux through the Ehrlich pathway to achieve higher concentration of higher alcohols and acetate esters. The biosynthesis of amino acids in yeast cells is regulated by feedback inhibition by the final product at the first irreversible reaction step in the biosynthetic pathway, the committed step. For the branched-chain amino acid, the committed steps are the first steps in leucine and isoleucine biosynthesis and are catalysed by the Leu4p and Leu9p for the former and Ilv1p for the latter (Holmberg & Petersen, 1988; Kohlhaw, 2003). For the aromatic amino acids, the committed step is the production of 3- deoxy-D-arabino-heptulosonate-7-phosphate by the enzymes Aro3p and Aro4p (**Figure 1.4**) (Braus, 1991). Mutants that disrupt the negative feedback regulation can be obtained by growing yeasts in the presence of analogues of the terminal amino acid. Strejc *et al* used the amino acid analogue 5,5,5-trifluoro-DL-leucine to increase flux

through the leucine biosynthetic pathway. The resultant mutant strains produced three times more isoamyl acetate (Strejc et al., 2013).

Classical mutagenesis, involving UV radiation and chemical mutagens such as ethyl methane sulfonate (EMS), methyl methane sulphonate (MMS), and N-methyl-N'-nitro-N-nitroso-guanidine (MNNG), have been used to isolate mutants with altered flavour profiles. This approach needs a robust selection method due to the high number of mutants produced and is complicated for polyploid strains such as *S. pastorianus* due to the high level of redundancy in the genome. Growth on the amino acid analogue, thiaisoleucine, was used as a selection following chemical mutagenesis to obtain lager yeast strains overproducing the flavour compound 2-methyl-butanol (Kielland-Brandt et al., 1979).

Mutants with reduced off flavours have also been produced. In one study, cells were treated with EMS and then exposed to chlorsulfuron, which inhibits acetohydroxy acid synthase, the enzyme responsible for α-acetolactate production. The selected strains had reduced levels of diacetyl, an off flavour with a smell of butter or butterscotch (Gibson et al., 2018). UV mutagenesis followed by selection in the presence of disulfiram and ethanol was used to reduce the production of acetaldehyde, a flavour that imparts notes of green apples, fresh cut grass and walnuts (Shen et al., 2014).

Hybridisation is a common technique that can be used to obtain new yeast strains with improved flavour profiles. *S. pastorianus* is a sterile hybrid and does not produce many viable spores, making it difficult to produce new strains by classic mating and sporulation. Several approaches such as spore-to-spore mating, rare mating and mass mating have been developed and new inter- and intraspecies hybrids have been generated (Steensels, Meersman, et al., 2014). New hybrids of *S. cerevisiae* and *S. eubayanus* were generated through spore-to-spore mating. The resultant hybrids not only showed a wide aromatic compound production but also a higher tolerance to higher temperatures. Some of the hybrids produced a better aromatic profile compared to both parental strains (Mertens et al., 2015).

Rare mating and mass mating between natural auxotrophs have also been used to create hybrids (Boeke et al., 1987; Krogerus et al., 2016; Krogerus et al., 2015; Toyn et al., 2000; Zaret & Sherman, 1985). Rare mating involves the generation of a hybrid from

strains of different ploidy, obtaining a final hybrid with a high ploidy that must be submitted to a stabilization process. In mass mating, spores from both parental strains are mixed, obtaining strains with a ploidy roughly of 2n. These techniques have been extensively reviewed by (Krogerus, Magalhaes, et al., 2017)

Another approach is directed evolution, whereby cells are grown for several generations (100 to 200 generations) under a specific selection pressure. This evolutionary method is usually coupled to a selection method. For example, strains with a reduced production of the off-flavour diacetyl were obtained after growth in the presence of chlorosulfuron, (Gibson et al., 2018) . Directed evolution has also been used to generate strains with improved thermotolerance, maltotriose utilization and ethanol tolerance (Gibson et al., 2020).

In one such study, an adapted strain produced a 5-fold uptake of maltotriose, resulting in higher alcohol production, improved fermentation attenuation and had no impact in aroma formation (Brickwedde et al., 2017). Directed evolution can be applied following UV mutagenesis. Using this approach, *S. eubayanus* strains capable of utilizing maltotriose were obtained. The new strain contained a novel chimeric MALT gene resulting from the recombination of MALT genes. The characterization of the novel gene showed high similarity to the MTY1 gene and enabled the utilization of this sugar in *S. eubayanus* strains (Brouwers et al., 2019).

High-gravity wort and very-high gravity wort are defined as fermentations with a higher content of fermentable sugars and increased FAN. Such fermentations have the potential to produce higher concentrations of ethanol, higher alcohols and esters. However, the high concentration of sugars increases osmotic pressure producing negative effects on the cells, such as increased cell volume, decreased cell viability and ethanol toxicity (Cahill et al., 2018; Casey et al., 1984). This can lead to stuck and sluggish fermentations and problems in repitching (Sigler et al., 2009). To overcome these problems, some strains have been generated by different techniques. James *et al,* evolved strains using EMS and growth in high-gravity wort at high temperatures (James et al., 2008). The selected strains produced higher concentrations of ethanol but additionally showed chromosome rearrangements, demonstrating that the plasticity of the hybrid genome of *S. pastorianus*

may play a key role in the adaptation to stresses like heat or high gravity wort. Similar approaches have generated strains with a better tolerance of ethanol and increase fermentation performance in this type of fermentation (Blieck et al., 2007; Huuskonen et al., 2010).

1.7. The effects and consequences of aneuploidy

Aneuploidy is the state of a cell that possesses an abnormal number of chromosomes. This aberrant state has been detected in a wide range of organisms like plants, yeast or even in humans. The lager yeasts are aneuploid in nature as a consequence of the hybridisation of the parental species, the subsequent loss of chromosomes and the gain of unique hybrid chromosomes. As stated before, this set of strains have a wide range of chromosomes.

In humans, the incidence of aneuploidy varies between cell types and can be detected in somatic and sexual chromosomes. The different number of copies are related to diseases/disorders, for example, Down syndrome (trisomy of chromosome 21) or Klinefelter syndrome (XXY), and also cancer (Ben-David & Amon, 2020; Hassold & Hunt, 2001). In plants, aneuploidy confers advantages, especially in crops. Aneuploidy has an impact in fungi and has been linked to antibiotic resistance in pathogenic fungi (Sionov et al., 2010).

Several studies have used yeast as a model to investigate aneuploidy. Yeast strains that are aneuploid possess a disadvantage in normal conditions compared to the respective euploid strain. Defects in cell cycle progression, changes in metabolism and redox homeostasis, altered RNA profile and proteotoxic stress are consequences of aneuploidy in yeast (Brennan et al., 2019; Dephoure et al., 2014; Sheltzer et al., 2011; Sheltzer et al., 2012; Thorburn et al., 2013; Torres et al., 2010; Torres et al., 2007). Extra copies of chromosomes can increase proteotoxic stress in cells due to an increase in protein synthesis. This increase in intracellular proteins led to protein misfolding and imbalance of cellular protein composition (Oromendia et al., 2012). Also, there is an imbalance of protein complexes. Stoichiometry of subunits of protein complexes may be altered also.

Several studies have linked the ability of cells to cope with proteotoxic stress with mutations in genes involved in protein degradation. A mutation that deactivates Ubp6p, a ubiquitin-specific protease, increases the proportion of proteins to be degraded. Also, the malfunction of Vps64p, a vacuole targeting factor, may help to deal with proteotoxic stress(Torres et al., 2010). In contrast, the correct function of Ubp3p, a ubiquitin-specific protease that cleaves Ub-protein fusions, may have a positive impact against proteotoxic stress (Dodgson et al., 2016). It has been reported that the overproduction of subunits of protein complexes could be regulated by the aggregation or/and degradation of the same subunits (Brennan et al., 2019).

RNA analysis of different aneuploid organisms have shown that there is an upregulation of genes related to stress. Furthermore, genes related to cell cycle and proliferation are downregulated. This study has shown that, independently of the chromosome copy number and organism, aneuploid organisms exhibit this pattern of expression (Sheltzer et al., 2012). Another study has shown that *SSD1*, RNA binding protein active in wild strains but found to be hypomorphic in laboratory strains, may be responsible for aneuploidy sensitivity (Hose et al., 2020). Ssd1p alters the physiology of mitochondrial physiology and intervenes in translational regulation.

Therefore, aneuploid species, indeed, may be able to buffer the effects of aneuploidy. It has been reported that, despite the negative effects induced by an aberrant karyotype, aneuploid strains may possess advantages in different stresses, for example, resistance to different antibiotics such as radicicol, high temperature or high pH. Relevant industrial traits have been linked to different aneuploidies (Gorter de Vries et al., 2017).

Yeast is a unicellular microorganism that is mostly haploid or diploid. This characteristic makes yeast interesting as a model for aneuploidy research. Aneuploidy can arise from the effects of stress on the cell. Pleiotropic stresses can be caused by different conditions, like for example high temperatures, presence of antibiotics or high pH. The pleiotropic stress may increase the genome instability and aneuploidy may arise as a consequence of this instability. It has been observed that both biotic and abiotic factors induce aneuploidy in the strains:

Hybridization. Several studies have produced hybrid strains for improved fermentative traits. The combination of different genomes (interspecifically) in one strain has a positive impact in flavour profiles, fermentative capacity or cryotolerance adaptation for example (Gallone et al., 2019; Garcia-Rios et al., 2018; Krogerus et al., 2016). This is due to the new combination of alleles and chromosome copy variations. Also, genomic instability has been reported after interspecific hybridization events. Strains generated by this method start with a high ploidy. These polyploid strains are normally submitted to several rounds of stabilization where the genome content gets reduced (Perez-Traves et al., 2014).

High temperature. High temperatures or Heat Shock Thermal Stress (HSTS) may induce chromosomal changes. It has been shown that high temperatures inhibit the Hsp90p, a chaperone. This inhibition inhibits the machinery to repair the DNA damage such as homologous recombination, non-homologous DNA end joining, and induces single and double strand breaks (Kantidze et al., 2016). Yeast strains submitted to high temperatures have experienced chromosome rearrangements and improved ethanol production (James et al., 2008; Jarosz & Lindquist, 2010).

Chemicals. It has been reported that several chemicals, such as hydrogen peroxide, sodium chloride and antibiotics like cycloheximide, tunicamycin, benomyl and radicicol (Chen et al., 2012), that cause different types of stresses in the cells may induce chromosome copy variations. Radicicol has been shown to be highly efficient as inducing aneuploidy Radicicol, known as monorden, belongs to the group of resorcylic acid lactones (RALs). This macrocyclic antifungal antibiotic is produced by endocytic fungi like *Pochonia chlamydosporia* or *Chaetomium chiversii*. This drug binds to Hsp90p, an evolutionary conserved chaperone which is involved in several processes like chromatin remodelling, DNA transcription, RNA processing, DNA replication, telomere maintenance, and DNA repair. Radicicol binds to the N- terminal domain of this chaperone and alters its function (Sharma et al., 1998). This drug and its analogues have been proposed as antitumor and anticancer treatments as Hsp90p is a chaperone that folds numerous oncoproteins.

It is uncertain why *S. pastorianus* maintained the extra chromosomes of both parental strains as it is believed that an impairment of chromosome copy can lead to problems in protein stoichiometry and inefficient metabolic energy use. Nevertheless, the

maintenance of the chromosomes could be advantageous to the strain. The effects of the chromosomal aneuploidy on the physiology of lager yeasts remains to be fully explored.

1.8. Aims and rationale of the research in this thesis

The beer market is a very competitive sector. Every year, billions of litres of beer are produced (Statista). Several types of beer are produced, therefore, differentiation between brands is an imperative of the industry. This may be obtained by modifying steps of the process, like for example increasing the time of the malting process, incorporating adjuncts to barley malt or to use different classes of hops. Another way is to improve the process by using different yeast strains. Several studies have improved the fermentative process, focusing especially on the overproduction of flavour compounds by yeast (Blieck et al., 2007; Gallone et al., 2019; Mertens et al., 2019; Mertens et al., 2015). While great strides have been made to understand the complexity of *S. pastorianus* genomes, much remains to be discovered, specifically in relation to flavour and aroma production during fermentation. The plasticity of the genomes and their aneuploidy may explain their adaptation and evolution in the brewing media (James et al., 2008; Rancati et al., 2008).

The aim of the project is to expand the aromatic flavour compound profile of *S. pastorianus* strains and specifically to overproduce the flavour compounds from the metabolism of phenylalanine. These higher alcohols not only are important in the brewing industry but also in the chemistry of food industry, as they are considered high-valuable products (Dzialo et al., 2017; Pires et al., 2014).

To achieve this goal, an accelerated evolution approach was employed to introduce mutations and copy number changes to the genome using the known aneuploid inducers, Heat Shock Thermal Stress (HSTS) or Radicicol. To select for mutants directly targeting the phenylalanine metabolic pathway, the surviving cells were plated on medium containing amino acid analogues of phenylalanine. The biosynthesis of amino acids in yeast cells is regulated by feedback inhibition by the final product at the first irreversible reaction step in the biosynthetic pathway, the committed step. For the aromatic amino acids, the committed step is the production of 3-deoxy-D-arabino-heptulosonate-7-phosphate by the enzymes Aro3p and Aro4p (**Figure 1.4**). It has been previously reported that amino acid analogues are toxic for different organisms. These analogues can be

incorporated into proteins, thus altering protein structure and enzymatic activity (Pine, 1978; Rodgers & Shiozawa, 2008). Growth inhibition by amino acid analogues can be reversed when the corresponding protein amino acid is added to the media. Also, the inhibition of growth is observed when the intracellular pool of amino acids is low. Therefore, amino acid analogues may compete with the intracellular proteinogenic amino acids (Norris & Lea, 1976; Richmond, 1976).

Previous studies have reported that mutants able to grow in the presence of amino acid analogues are defective in negative feedback regulation of committed steps, and thus have increased flux through the biosynthetic pathway, resulting in increased production of higher alcohols and esters produced from the amino acid (Cordente et al., 2018; Fukuda, Watanabe, & Asano, 2014; Fukuda, Watanabe, Asano, et al., 2014; Strejc et al., 2013). Therefore, this may confirm that amino acid analogues compete with proteinogenic amino acids, and mutants overproducing amino acids would overcome the growth inhibition by amino acid analogues.

The genomes and transcriptomes of selected mutants were characterised, and specific changes were correlated with alterations in the aromatic volatile profiles of the mutants.

Chapter 3 describes the mutagenesis carried out to obtain mutant lager yeast strains with increased production of the higher alcohol 2-phenylethanol and its corresponding ester, 2-phenylethyl ethanol. The mutagenesis strategy using Heat Shock Thermal Stress (HSTS) or radicicol treatment followed by selection using different amino acid analogues is described. To narrow down the number of mutants, a small-scale fermentation procedure, coupled with volatile aroma profiling, was devised. From this strategy, one Group I mutant and one Group II mutant were selected for more detailed analysis.

Chapter 4 describes the genome analysis of the selected mutants. Whole genome sequencing (WGS) of the two mutant strains and their parental strains are performed. Copy number variations and Single Nucleotide Polymorphism analysis identified several important changes in the mutants.

Chapter 5 shows the results of RNAseq experiments. The transcriptome of the mutant strains and their parents was examined under different physiological conditions, namely, growth in minimal medium without amino acids and under fermentation conditions in

wort. The chapter describes the important changes in gene expression that can account for the increased production of 2-phenylethanol and 2-phenylethyl ethanol in the mutant strains.

Chapter 6 describes the transcriptomic differences between Group I and Group II parental strains. The cells were grown in minimal medium without amino acids and under fermentative conditions. Although results show that gene composition and gene copy number are important in transcriptomic regulation, both Group I and Group II strains react similarly to the different conditions.

Chapter 7 provides a discussion of the combined results and a perspective on the implementation of the strategy employed in the thesis for the generation of yeast strains with improved flavour profiles.

Chapter 2

Material and Methods

2.1. Strains used in this study. Growth conditions.

The strains used in this study are listed in **Table 2.1**.

Table 2.1. Strains used in this study.

Strain	Description	Source
BY4741	*S. cerevisiae* S288C-derivative laboratory strain. Genotype: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0.	European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF), Frankfurt, Germany
PYCC 6148	*S. eubayanus* strain	Portuguese Yeast Culture Collection (PYCC, Lisbon, Portugal)
CBS1503	*S. pastorianus* strain, Group I	Collection de Levures d'Interet Biotechnologique, Paris, France
CBS1513	*S. pastorianus* strain, Group I	Collection de Levures d'Interet Biotechnologique, Paris, France
CBS1538	*S. pastorianus* strain, Group I	Collection de Levures d'Interet Biotechnologique, Paris, France
7012	*S. pastorianus* strain, Group II	Guinness Brewery, Dublin, Ireland
WS 34/70	*S. pastorianus* strain, Group II	Kindly supplied by Dr. Jurgen Wendland, Geisenheim Hoch Universitat, Germany

2.1. General media.

For propagation, strains were grown in 2% YPDM medium (10 g $\cdot$ L^{-1} yeast extract, 20 g $\cdot$ L^{-1} peptone, 10 g $\cdot$ L^{-1} glucose and 10 $\cdot$ L^{-1} maltose, supplemented with 20 g $\cdot$ L^{-1} of agar for solid plates) at 23-25ºC temperature with shaking (120 r.p.m). For wort fermentations, 4% YPDM was used, and cells were grown for two days. Cells were also grown in minimal medium, or YNB medium w/o amino acids and w/o ammonium salts supplemented with 10 g $\cdot$ L^{-1} of glucose and 10 g$\cdot$L^{-1} of maltose, and 5 g $\cdot$ L^{-1} of $(NH_4)_2SO_4$ as a nitrogen source and 1.7 g $\cdot$ L^{-1} of Yeast Nitrogen Base. The medium was supplemented with different concentrations of the phenylalanine amino acid analogues ρ-fluorophenylalanine (PFPA) and β-(2-thienyl)-DL-alanine (Sigma) or 20 mM of phenylalanine (Phe) (**Figure 2.1**). Synthetic Complete medium (SC) was used for the Radicicol treatment. SC contains 10 g $\cdot$ L^{-1} of glucose and 10 g $\cdot$ L^{-1} of maltose, 5 g $\cdot$ L^{-1} of $(NH_4)_2SO_4$, 1.7 g $\cdot$ L^{-1} of Yeast Nitrogen Base without amino acids and 2 g $\cdot$ L^{-1} of a mix of amino acids (**Table 2.2**).

Table 2.2. Concentrations of amino acids of the stock.

Reagent	Grams to add (g)
Adenine	0.5
Alanine	2
Arginine	2
Asparagine	2
Aspartic acid	2
Cysteine	2
Glutamine	2
Glutamic acid	2
Glycine	2
Histidine	2
Inositol	2
Isoleucine	2
Leucine	10
Lysine	2
Methionine	2
Phenylalanine	2
Proline	2
Serine	2
Threonine	2
Tryptophan	2
Tyrosine	2
Uracil	2
Valine	2
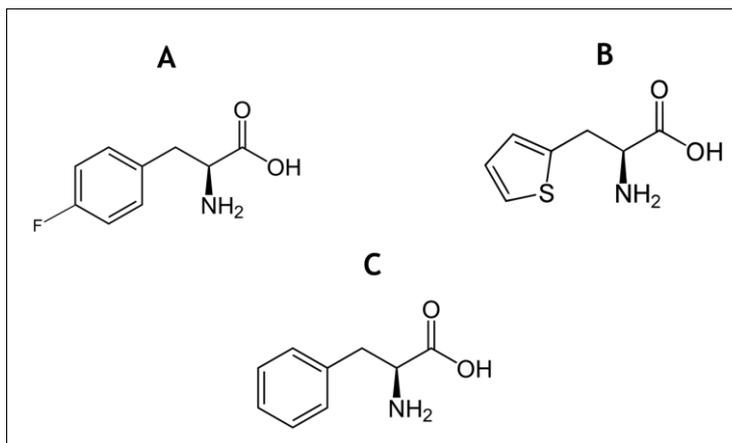
Figure 2.1. Amino acid analogues of phenylalanine used in this study. A: ρ-fluorophenylalanine, B: β-(2-thienyl)-DL-alanine. C: L-phenylalanine

2.2. Fermentative media.

Small-scale fermentations (10 and 20 mL) were carried out, in triplicate, in 10 and 12ºBx wort containing 1mM $ZnSO_4$ (Spraymalt, Brewferm, Amber 18EBC, Brouland, Belgium). Small-scale fermentations were carried out in 15 mL glass test tubes and in 30 mL falcon tubes. Cells were pitched at a cell density of 1.5×10^7 cells·mL^{-1}. The tubes were fitted with a water trap airlock attached to a bung and incubated at 13ºC at a 45° angle without shaking. The specific gravity of the wort was measured at the start of fermentation and at intervals throughout the fermentation using a refractometer (*HANNA*, Romania) after removing the cells by centrifugation.

Large-scale fermentations (2 L) were carried out in duplicate in 12ºBx wort in tall tubes with a volume capacity of 3 L with a water trap airlock. Samples were taken at intervals by passing tubing through the airlock.

Samples from small-scale and large-scale were taken as follows. 50 mL falcon tubes were prechilled at -20ºC. Then, 40 mL samples were centrifuged at 4000 r.p.m for 4 min at 4ºC to pellet the cells. Supernatants were transferred to prechilled 50 mL falcon tubes and tubes were frozen at -80ºC.

2.3. Correlation OD – number of cells.

Cells were grown overnight in 2% YPDM. Different suspensions of cells were prepared. The suspensions previously obtained were read at room temperature in 1 cm cuvettes with an optical path of 10 mm at 600 nm. Aliquots were placed in a Neubauer Chamber and were counted as previously described using the equation below (Green & Sambrook, 2019).

$$Cell\ number = \frac{cell\ count}{quadrant} \cdot df \cdot 10000$$

being df "dilution factor".

Correlation between number of cells and Optical Density (OD) were calculated. A linear regression was calculated in order to obtain an equation to use in future inoculations. The resulting equation is: y = 8657206x – 655427.
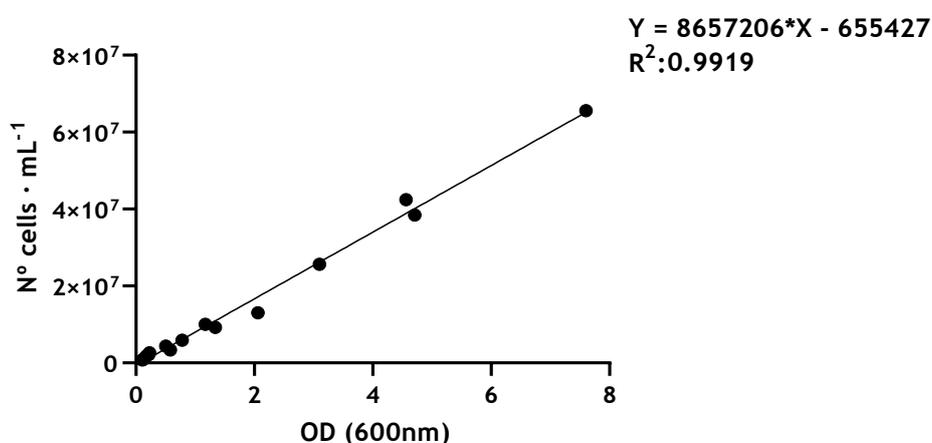
Figure 2.2. Linear regression of OD 600nm vs number of cells $\cdot$ mL^{-1}

2.4. Growth characterization and Minimal Inhibitory Concentration (MIC).

Growth was monitored by determining the optical density at 600nm in an Epoch 2 Microplate Spectrophotometer (Agilent BioTek). Measurements were taken every 2 h for three days. The 96-well plates with no shaking were incubated at 20ºC. Cells were inoculated at an OD$_{600}$ nm of approximately 0.1 in a volume of 0.25 mL of media (YPDM,

Minimal Medium, Minimal Medium containing amino acid analogues or Minimal Medium containing Phe). All the experiments were carried out in triplicate. Overall yeast growth was estimated as the area under the OD_{600} nm vs. time curve. The area under the curve was calculated on Prism.

The Minimal Inhibitory Concentration (MIC) of amino acid analogue PFPA was calculated as follows: cells were grown in 2% YPDM overnight and then were washed with sterile water before inoculating into 250 µL of Minimal Media with different concentrations of amino acid analogues in a 96 well-plate at an OD_{600} nm. Absorbance was measured at 600 nm every two hours for 50 h.

2.5. Determination of thermotolerance of the strains

10 ml cultures, inoculated at a concentration of 1.77 cells $\cdot$ mL^{-1}, were heated at different temperatures ranging from 45 to 55ºC for durations of 10 to 15 mins. The experiments were carried out in preheated glass tubes and in triplicate. After the heat shock, cells were allowed to recovery for 3-5 h. 100 µL aliquots were plated onto 2% YPDM agar plates and plates were incubated for 2 days at 25ºC. The number of colonies were counted to determine the rate of survival.

2.6. GC-MS analysis.

GC/MS analysis was carried out by our collaborators Mr Penghan Zhang, Dr. SilviaCarlin and Dr. Urska and Dr.Urska Vrhovsek at Fondazione Edmund Mach (FEM), Italy. Samples for analysis were shipped to our collaborators on dry ice.

2.6.1. Solid-Phase Micro Extraction.

2.5 mL of the samples were put in 20 mL vials and supplemented with 100 mg sodium chloride (final concentration 40 µg $\cdot$L^{-1}) and 25 µL of the 2-octanol as the internal standard (final concentration 200 µg $\cdot$L^{-1}). All samples were incubated for 10 min at 40°C, then the volatile compounds were collected on a divinylbenzene/carboxen/ polydimethylsiloxane fiber (DVB-CAR-PDMS) coating 50/30 µm, and 2-cm length SPME fibre purchased from Supelco (Sigma Aldrich, Milan, Italy) for 40 min.

2.6.2. GC-MS.

GC analysis was performed on a Trace GC Ultra gas chromatograph coupled with a TSQ Quantum Tandem mass spectrometer (Thermo Electron Corporation, USA), with adaptations as described in Ravasio et al. (2014). GC separation was performed on a 30 m VF wax capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 mm (Agilent; J&W; Folsom; CA, USA). The GC oven was kept at 40°C for 4 min and then increased by 6 °C/min to 250 °C and kept at the final temperature for 5 min. The injector and transfer line temperatures were kept at 250 °C as well. Helium was used as the carrier gas with a flow rate of 1.2 ml/min. The time for thermal desorption of analytes was 4 min. The MS detector was operated in full scan mode at 70 eV with a scan range from 35 to 350 m/z. Data analysis was performed using the software ThermoXcalibur (Version 2.2 SP1.48, Thermo scientific). Identification of compounds was based on comparison with a mass spectral database (NIST version 2.0) and with reference standards when available. The relative amount of each volatile was expressed as µg/L of 2-octanol (Ravasio, Davide, et al. "Adding Flavor to Beverages with Non-Conventional Yeasts." *Fermentation* 4.1 (2018): 15.).

2.6.3. Flavour profile.

In total 18 flavour compounds were chosen to represent the flavour profile for each sample. Among them, 10 compounds were measured quantitatively (acetaldehyde, ethyl acetate, isobutyl acetate, isobutanol, isopentyl acetate, isoamyl alcohol, ethyl hexanoate, ethyl octanoate, 2-phenylethanol, 4-vinylguaiacol, tyrosol, tryptophol). 6 compounds were measured semi-quantitative (decanoic acid, ethyl butyrate, methionol, hexanoic acid, octanoic acid, 2-phenylethylacetate).

2.7. Accelerated Evolution
2.7.1. Heat Shock.

For the HSTS, after 24h of incubation at 25ºC and 120 rpm in YPDM, 5 mL of cultures at $1 \cdot 10^7$ cell·mL^{-1} were heated at 45-55ºC for 10-15 minutes. After a period of recovery of 3-5 h, aliquots of 100 µL were plated onto Minimal media agar plates containing amino acid analogues.

2.7.2. Radicicol.

For Radicicol treatment, a modification of the method proposed by Chen G. et al was followed (Chen et al., 2012). Cells were grown in 2% YPDM to concentration of $1 \cdot 10^7$ cell·mL^{-1} and then washed inoculated in SC medium containing a final concentration of radicicol of 20 and 40 and 100 μg·mL^{-1} for 24 and 48h. After the treatment, aliquots of 100 μL were plated onto MM agar plates containing the minimum inhibitory concentration of the amino acid analogues.

2.8.2. Stabilization and selection of strains capable of growing in the presence of minimal requirements to grow.

The strains were re-streaked three-four times on agar plates containing the amino acid analogues to isolate stable-resistant mutants and reduce the pseudohyphal growth. After the stabilization process, all isolated colonies were plated on YNB plates w/o amino acids and w/o ammonium sulphate agar plates containing 20 mM of Phe as a sole nitrogen source to check that the growth in the presence of the analogue was not due to a defect in the transport of the amino acid analogue. Also, parental and mutant strains were grown in liquid in YNB with 20 mM of Phe, and growth was characterized.

2.9. Whole Genome Sequencing (WGS) and Sanger Sequencing.

2.9.1 DNA extraction.

DNA extraction was carried out following the protocol described by Querol et al. (1992) but with some modifications. Yeast cells were grown in an overnight culture of 5 ml of 2% YPDM. Cells were pelleted and washed with distilled water, spun down in a centrifuge. The pellet was resuspended in 500 μL of 0.9 M sorbitol and 0.1 M EDTA, pH 7.5 and transferred to 1.5 mL Eppendorf tubes. 30 μL of a solution of lyticase (1.5 mg · mL^{-1}) was added to the resuspended cells. Tubes were incubated for 20-40 min. After this, spheroplasts were centrifuged for 3 min at 12000 rpm and supernatant was discarded. The pellet was resuspended in 500 μL of 50 mM Tris HCl and 20 mM EDTA, pH 7.4. 13 μL of 10% SDS. 3 μL of RNAse A (10 mg · mL^{-1}) were added and were added and tubes were briefly vortexed. Samples were incubated for 65°C for 5 -10 min. 200 μL of 5M

potassium acetate were added and tubes were incubated on ice for 10 min. After this, tubes were centrifuged for 15 min at 12000 rpm and at 4ºC. Then, supernatant was transferred to a 1.5 mL Eppendorf tube containing 700 µL of 100% isopropanol. Tubes were incubated for 10 min at room temperature and then were centrifuged for 15 min at 12000 rpm, at room temperature. A round transparent pellet was obtained. This pellet was washed twice with 70% ethanol. After the wash steps, tubes were air-dried at room temperature, and DNA was resuspended in 50 µL of water.

A small aliquot (5 µL) was run on an agarose gel (1%) to check the size and integrity of the DNA and ensure that there was no RNA contamination. RNA-free DNA was measured using an Epoch 2 Microplate Spectrophotometer (Agilent BioTek) and diluted down to the concentration required for sequencing. The diluted samples were sent for sequencing (Novogene).

2.9.2. Mapping.

The original image data by high throughput sequencers were transformed to raw data (Raw reads) by CASAVA base calling. contaminated and low-quality reads were trimmed. The steps of quality control were applied as follows: 1) read pair was discarded if either one read contains adapter contamination. 2) read pair was discarded if more than 10% of nucleotides are uncertain in either one read. 3) read pair was discarded if the proportion of low-quality nucleotides is over 50% in either one read.

Quality of clean reads was analysed by FastQC (Babraham Institute). Then, reads were mapped against a reference genome using Bowtie2 (Langmead & Salzberg, 2012). For the WGS analysis, clean reads were mapped against the genome of *S. pastorianus* Group II strain CBS1483 (Assembly: ASM1102231v1) (Salazar et al., 2019). For the RNAseq analysis, clean reads were mapped against a custom genome that contains the reference genomes of *S. cerevisiae* strain S288C (Assembly R64) and *S. eubayanus* strain FM1318 (Assembly SEUB3.0) (Baker et al., 2015). The originated SAM files were transformed into BAM files using Samtools. Then, the BAM file was sorted, and an index was created

Statistic data such as coverage, percentage of reads mapped and unmapped was calculated using Samtools (Li et al., 2009).

2.9.3. Analysis of unmapped reads.

Unmapped reads were extracted from the final BAM file using Samtools (Li et al., 2009). Reads were sorted and then *de novo* assembled using SPAdes (Bankevich et al., 2012). Scaffolds were aligned against a customised database generated by joining *S. cerevisiae* and *S. eubayanus* reference genomes, both deposited on the NCBI (assemblies R64 and SEUB3.0, respectively). Open Reading Frames (ORFs) were generated using getorf (Rice et al., 2000) followed by a Blastp search in order to annotate them against a custom database with the annotation from the reference *S. cerevisiae* and *S. eubayanus* strains. Blast searches were made within the Blast+ environment in Python (Camacho et al., 2009).

2.9.3. Annotation.

For the WGS analysis, the annotation of CBS1483 made by Salazar et al. (2019) was used. Annotation of the unmapped reads was done using the blast+ environment on Python. A custom database was generated using the annotated protein sequences of *S. cerevisiae* and *S. eubayanus* reference strains.

2.9.4. CNV analysis.

Scripts were generated in Python to extract reads/windows size of 500 bp and then normalized by the size of the library (total number of reads). Then, a normalization factor was calculated, and each point was normalized to give an estimated copy number. Fold change between the mutant and parental strains was calculated.

2.9.5. SNP analysis.

Samtools were used in order to call SNP/InDel from BAM files. Then, SNPs with quality values lower than 20 and coverage less than 90 were discarded. ANNOVAR was used to annotate variants obtained from Samtools (Wang et al., 2010). A gene-based annotation file was generated using the genome of CBS1483 and a file containing the transcriptome of the same strain using the ANNOVAR tools.

2.9.6. Sanger sequencing.

SNPs were validated by Sanger sequencing. DNA was extracted as previously described and diluted using an Epoch 2 Microplate Spectrophotometer (Agilent BioTek). Primers were designed in order to amplify the promoter and the coding region of both alleles of *ARO4* (**Table 2.3**).

PCRs were carried out in order to amplify the sequence of interest using Phusion™ High-Fidelity DNA Polymerase (ThermoFisher scientific). PCR products were checked by Agarose Gel and 100 µL of PCR product were treated using QIAquick PCR Purification Kit (Qiagen) as manufacturer's protocol. Samples were diluted and sent for sequencing (Eurofins).

2.9.7. qPCR.

qPCR was used to confirm CNV found in the strains. DNA was extracted as previously stated. Specific primers were designed (**Table 2.3**) to discriminate between sub-genomes. All the primers were designed to amplify short products (70-250 bp) and with a Tm approximately of 60ºC. Primer efficiency was calculated and ranged from 90 to 110%. SYBR™ Green PCR Master Mix (Fisher) was used. For each primer pair a standard curve of 10-fold serially diluted DNA concentrations was included to calculate the primer efficiency. A melt curve was included each time a new primer pair was used in order to make sure the amplification of single bands.

2.9.8. Extraction of DNA embedded in agarose and Pulsed Field Gel Electrophoresis.

Chromosomal DNA were prepared by the method previously described by Nguyen and Gaillardin (1997). Chromosomes were separated in a CHEF DR-3 System (Biorad) using the following conditions. Plugs were inserted in wells formed in 1% agarose gel in 0.5% TVE buffer. Samples were electrophoresed for 30 h using a ramping programme from 40 s to 120 s at 6 V per cm, at 14ºC in 0.5% TBE buffer. Gels were stained with ethidium bromide for 30 min in a bath (2 ng/ml in water) and distained with distilled water before observation.

2.9.9. RNA extraction.

RNA extraction was extracted as previously described by Ares (2012) with some modifications. RNA was extracted from two different conditions. For cells grown in YNB medium, yeast cells were grown until they reached exponential phase (0.7-0.8 OD_{600} nm). For cells growing in wort medium, cells were collected on day 2 and day 4 of the fermentation. Cells were pelleted and resuspended 400 µL of buffer A (50 mM of sodium acetate (pH 5.2) and 10 mM of EDTA (pH 8.0)) were added. The solution was transferred to a 2 mL Eppendorf tube containing 100 µL of acid-washed glass beads. 40 µL of 10% SDS and 400 µL of a mix of Acidic Phenol:Chloroform:Isoamyl Alcohol (25:24:1) were added. The cells were incubated for 10 min at 65ºC on a thermoblock. Tubes were vortexed once a minute during the 10-min period, for 5-10 sec each time. Then, tubes were incubated for 5 min on ice. After the incubation, tubes were centrifuged at 14000 rpm for 8 min at 4ºC. Approximately 400-500 µL of supernatant was transferred to a 1.5 mL Eppendorf tube containing 400 µL of a mixture of PCA. Tubes were inverted 5-10 times and then centrifuged at 14000 rpm for 8 min at 4ºC. After the centrifugation step, 400 µL of supernatant was transferred to a new tube with 400 µL of chloroform. Tubes were mixed by inverting 5-10 times and then centrifuged again under the same conditions. Supernatant was transferred to a 1.5 mL Eppendorf tube. 50 µL of 3 M sodium acetate, pH 5.2 was added to the aqueous phase and then 100% cold ethanol was added up to 1.5 mL. Tubes were mixed by inverting and incubated 2-3 min at room temperature. Then, tubes were centrifuged at 14000 rpm for 8 min. Then, ethanol was removed, and the pellet was washed with ethanol 70% twice. Tubes were centrifuged at 14000 rpm for 3 min, ethanol was removed, and the pellets were air-dried for less than 5 min. RNA was resuspended in 50-100 µL of RNase-free water by pipetting cautiously. Total RNA was run on a 1% agarose gel to check the integrity of the RNA. After this, 10µg of total RNA was treated with 2.5 U of Recombinant DNase I (ROCHE), following the manufacturer's protocol. RNA was purified by another round of phenol/chloroform extraction, as previously described in this section.

The quality was checked using the Agilent 2100 Bionalayzer System (Agilent) to assess the integrity of the RNA for further analysis following the instructions of the manufacturers.

Table 2.3. Primers used in this study.

Gene	Sub-genome	Chr	Forward (5' --> 3')	Reverse (5' --> 3')
**CNV confirmation & RNAseq confirmation**
BAP2	*S. cerevisiae*	II	CAAAAGAGCCGAGGGTAGCA	CAGCAGGACCACCGTAATGT
ARO10	*S. eubayanus*	II	ACCATCCCATCAGAAACTCA	GGATATCACTCACTCCATAT
ARO80	*S. eubayanus*	II	AGACATTGGGCTGGAGTTCG	TGTGAGGGGCTGGTCTATCA
AGP1	*S. eubayanus*	III	CTCCTGCTCTTCGTCCCTGT	GCAGCAGCCACACTTGGACT
ARO10	*S. cerevisiae*	IV	ATTGTTGTCCATCGATACGA	GCCAGCTGATTCAACACTA
ARO80	*S. cerevisiae*	IV	AGTCGTTTCAACCCCTCGTC	AACGCCCTAGAGTTTCTGGC
BAP2	*S. eubayanus*	IV	AAACCTCGCCGGATTCCATT	TGGCCCACCGTAATGAAGAC
ACT1	*S. cerevisiae*	VI	CCATCCAAGCCGTTTTGTCC	TACCGGCAGATTCCAAACCC
ACT1	*S. eubayanus*	VI	TCCTTCCGTCTTGGGTTTGG	GGAGCCAAAGCGGTGATTTC
ATF2	*S. cerevisiae*	VII	GCGTAATGCGAACCTCCAAC	GTGCACCCTAACACCCTTCA
ATF2	*S. eubayanus*	VII	TGTCAGTAACCATTGCGGCT	AGGCTTGAATGAAGGGCGTT
ARO9	*S. cerevisiae*	VIII	TGAGCTACCCATTGCACGTT	CATTGAATCGTTGGACCCGC
BAT1	*S. cerevisiae*	VIII	AGGGTTTAGGTGTTGGCACT	CGCTCGTTGATGTCCCATTC
IAH1	*S. eubayanus*	VIII	GGTACAACTCCAAGTGGGCA	ACGCACATATCCGAGAGCTG
BAT2	*S. cerevisiae*	X	CCAACGTTCGACCCAGAAGA	GGGCGTAGTTTGCACCTAGT
BAT2	*S. eubayanus*	X	CCTTGCGTCCTACCACAGTT	TTCCTTGGTCAATGGTCCCG
YLL56C	*S. cerevisiae*	XII	CGGGTCATGAAGTTGTTGGC	TCCTTGAGATCACCGCGAAG
YLL56C	*S. eubayanus*	XIII	TCGTCATTGGCGTCTTCCTC	ACAGGCTACTGTGGCTATGC
ALG9	*S. cerevisiae*	XIV	CGCTATCTGTCCACTGGGTC	TCGAATGCGGTTCTGATGGT
ALG9	*S. eubayanus*	XIV	GGCATTGCAGATTGCGAACA	GCAACAGCAGATGGCAACAA
ATF1	*S. cerevisiae*	XV	ATTGCCGCTCACAACTACCA	TGTACCTCCGCGTCTTTTCC
BAT1	*S. eubayanus*	XV	GGTGACAAGAAGTTGGGTGC	TTGGTGAGACAACAGCAGCA
ARO9	*S. eubayanus*	XV	ACCAAAGACATGGTTGCCAG	GGTCTCATCGCAGAAGGTCT
**Sanger Sequencing**
ARO4_1			ATGGAAAACATTGTCATCG	CTAAATTTAGCGGTTAAATA
ARO4_2	*S. cerevisiae*	II	CTATATATCGTGTTAATTC	AACATCAGGGTCATTAATT
ARO4_3			GAAATTGTCAGATGAATT	CTCACAAACAACGTCATTG
ARO4_4			GGGTACCAACTACGACGCTAAG	TAGTTAATTAGTAAATAGC
ARO4_1			TCACGAGTTCGCTCACTCCCAAT	CATTTACGTTTGGAGTGCCAT
ARO4_2	*S. eubayanus*	II-IV	CTCTACCCTTGCAGTAGTA	ATTTGTCAAGTTGACGAACAACT
ARO4_3			CCTGGAAAAGCCAAGAACTACAG	GTTCACAGACGACATCGTTAACCT
ARO4_4			TTAATGATCGATTACTC	TTGTTAAGTAAGTAAATAGC

2.9.10. RNAseq Analysis

RNA sequencing was conducted on cDNA libraries using Illumina technology at the Centre for Biotechnology, University of Manchester (UoM). As reference for the mapping the *S. cerevis*iae and *S. eubayanus* genomes were used but with variations as present in the re-sequenced CBS1538 and WS 34/70 strains. For that, consensus Fasta sequences were generated from the WGS data mapped to *S. cerevis*iae and *S. eubayanus* through an in-house Perl script. To be able to transfer gene annotation from those genomes, insertions and deletions were ignored and only nucleotide replacements were taken into account (just over 88k for CBS1538 and 126k for WS 34/70). The paired RNA-Seq data were mapped against these strain-specific reference genomes using STAR. Multimapping reads are dealt with through the outSAMmultNmax parameter set to 1 and outMultimapperOrder set to 'random'. Additionally, only the highest quality alignments were kept by filtering with samtools (-q 255). The tool featureCounts was ran on the mapped data together with the gene annotation from the *S. cerevis*iae and *S. eubayanus* genome to aggregate reads counts. With the -B and the -P options set, only read counts that have both ends aligned within a distance of 50-600 basepairs were considered.

Read counts from the RNA mapping were loaded onto iDEP9.1 (Ge et al., 2018). Data were transformed for clustering and PCA by EdgeR using a minimum CPM of 0.5 in 1 library and adding a pseudocount of 4. PCA analysis was used to check the reproducibility of replicates. Outlier replicates were removed, and data reprocessed. Differentially Expressed Genes (DEG) were calculated using DESeq2 with a False Discovery Rate (FDR) cut-off of 0.05 and a minimum $\log_2$ fold change of ≥ 1 or ≤ -1. Enrichment of DEG was carried out using ClueGO (Bindea et al., 2009). Parameters used on ClueGo were set as follows. *Saccharomyces cerevisiae* S228C was used as a Load Maker List. KEGG Ontology and or KEGG COMPOUND ontology were used for the analysis with the following filters, p-value less than 0.05, minimum number of four genes per pathway, pathways containing more than 25% of the genes regulated, Kappa score was set at 0.4. A two-sided hypergeometric test and a Benjamini-Hochberg pV correction was applied.

S. *eubayanus*-like allele names were translated into *S. cerevisiae* gene names for ClueGo analysis. Venn diagrams were made using the online tool provided by Bioinformatics & Evolutionary Genomics (VIB / UGent; http://bioinformatics.psb.ugent.be/)

2.9.11. Statistics.

False discoveries rates for DEG and gene ontologies were determined using the Benjamini-Hochberg correction. Chi-squared tests were used to determine the enrichment of genes from sub-genomes under the different physiological conditions used.

Previous customised *S. cerevisiae - S. eubayanus* genome was used to map the reads using Hisat2 (Kim et al., 2015).

2.9.12. Differentially expressed genes (DEG) analysis and enrichment

Analysis of the reads and Log_2 fold change was calculated using iDEP (Ge et al., 2018). Enrichment of DEG was carried out using ClueGO, an app that works in the Cytoscape environment (Bindea et al., 2009; Shannon et al., 2003). Parameters used on ClueGo were set as follows. *Saccharomyces cerevisiae* S228C was used as a Load Maker List. KEGG Ontology was updated and used as only ontology for the analysis. KEGG COMPOUND ontology was excluded. Only pathways with pV less than 0.05 were shown. Pathways with a minimum number of four genes were chosen, and of these, pathways that contained more than 25% of the genes regulated, were shown. Kappa score was set at 0.4. A two-sided hypergeometric test and a Benjamini-Hochberg pV correction was applied.

S. eubayanus-like allele names were translated into gene names ClueGo only accepts gene IDs or gene names.

2.9.13. RT-PCR

For cDNA synthesis, 2 µg of DNA-free Total RNA was reverse transcribed into cDNA using SuperScript™ IV Reverse Transcriptase, according to the manufacturer's protocol. 1 µL of 50 µM random hexamers, 1µL of 10mM of dNTPmix and 2µg of DNA-free Total

RNA were placed in a PCR tube. Nuclease-free water was added up to 13µL. The mix was heated up at 65ºC for 5 min in order to anneal the primers to the template. Then, 4 µL of 5x SSIV Buffer, 1µL of 100 mM DTT, 1µL of RNase Inhibitor and 1 µL of SuperScript IV Reverse Transcriptase (200 U·µL^{-1}) were added to the previous mix. The new mix was incubated in a thermocycler under a program of three incubation steps. Firstly, at 23ºC for 10 min, followed for another incubation at 52.5 ºC for 10 min. To Inactivate the reaction, incubation at 80ºC for 10 min was carried out. cDNA was diluted 1:10 and then stored at -20ºC.

qPCR reactions contained approximately 6ng of cDNA, 5 pmol each primer (**Table 2.3**) and 5 µl of SYBR™ Green PCR Master Mix (Fisher) in a final volume of 10 µl. Reactions were run on a StepOne™ Real-Time PCR System (Applied Biosystems) for 40 cycles of: 15 seconds at 95°C; 30 seconds at 60°C; and 30 seconds at 72°C. *ACT1* was chosen based on the data obtained from the RNAseq experiment, as the expression of this gene was stable in all three conditions. Log$_2$ Fold change values were calculated using StepOne software v2.3 (Applied Biosystems).

Chapter 3

Characterization of parental strains, mutagenesis and mutant selection.

3.1. Introduction

Lager beer is a fermented beverage produced by the fermentation of the sugars present in wort by *Saccharomyces pastorianus*. This alcoholic beverage is one of the most produced and consumed types of beer worldwide (Pavsler & Buiatti, 2009). This market not only involves several international breweries but also medium and small companies. Under the Purity Law established in Germany in 1516, beer is made of three main ingredients: barley malt, hops and water (Hornsey, 2003). The application of this law led to a new type of beer, called Lager. Considered as a fermentation by-product, yeast is responsible for the fermentation of the combination of the three previous ingredients. This microorganism carries out the alcoholic fermentation, producing ethanol, CO_2 and other secondary metabolites that will affect the final aromatic profile and stability of the beer. The consequences of this law not only generated a new type of beer but also helped to domesticate a new yeast strain, *Saccharomyces pastorianus (Hornsey, 2003; Hornsey, 2012)*.

Examples of secondary metabolites are higher alcohols and acetate esters (Hazelwood et al., 2008). These aromatic compounds are derived from the catabolism of aromatic, branched-chain and sulphur-containing amino acids (Dzialo et al., 2017). Higher alcohols, along with acetate esters, impart fruity and floral aromas to the final aromatic profile of the beer. These compounds are important in the chemistry and food industry as they are used in perfumes, beverages or cosmetics (Etschmann et al., 2002).

Higher alcohols and acetate esters are not the only compounds that contribute to the final aromatic profile. Along with acetate esters, fatty-acid esters can be produced by yeast (Saerens et al., 2006). These compounds come from the esterification of Acyl-CoA molecules with ethanol. Depending on the length of the Acyl-CoA molecules, this type of esters imparts notes that are reminiscent of fruits, such as apple or tropical fruits.

As the competition within the lager beer market is important, brewers need to make their final product distinguishable and unique. Yeast can be a powerful tool to improve not only the fermentative process but also the final product. Several successful studies have achieved new strains belonging to the genus *Sacccharomyces* adapted to new environments or conditions. For example, new *S. pastorianus* strains have been obtained using different techniques, such as laboratory evolution, interspecific hybridization or by traditional

mutagenesis (Gibson et al., 2018; Krogerus et al., 2016; Krogerus et al., 2015). The aim of these studies was to target different steps of the brewing process, for example, to increase ethanol tolerance, to adapt the strains to high gravity fermentations or improve the flavour profile of low-alcohol fermentations. As stated before, the final organoleptic profile of beer is important, especially in lager beer as this type of beer is carried out at lower temperatures and low or no amounts of hops are added. Furthermore, wort is composed mainly of maltose. These factors are negatively correlated with the production of higher alcohols and esters (Dzialo et al., 2017).

To use yeast strains that overproduce aromatic compounds might be a useful solution to improve the aroma of lager beer. Several studies have shown that evolutionary strategies, coupled with a strong selection method, produce strains with a more active secondary metabolism (Strejc et al., 2013). Selection methods used to select strains may involve amino acid analogues. These amino acid analogues are similar to the proteinogenic amino acids but with a different chemical structure that make them toxic for normal strains. Previous studies have shown that strains able to grow in the presence of amino acid analogues overproduce higher alcohols and esters, depending on the analogue used (Cordente et al., 2018; Fukuda, Watanabe, & Asano, 2014; Fukuda, Watanabe, Asano, et al., 2014; Lee et al., 2018; Strejc et al., 2013). Several studies have proposed that phenylalanine amino acid analogues select for strains with overproduction of phenylalanine, as these toxic molecules select for strains with an impaired feedback regulation at the enzymatic level of ARO4p and ARO3p. These two enzymes, two 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthases, catabolise the first step of the aromatic amino acid biosynthesis in the Shikimate pathway. These two proteins are feedback-inhibited by the final amino acids. ARO4p is inhibited mainly by tyrosine and ARO3p by phenylalanine. Both of them are inhibited by all three aromatic amino acids, but at different rate/concentration (Cordente et al., 2018; Hartmann et al., 2003).

Here, we describe a method to obtain mutants of lager yeast strains that overproduce 2-phenyethanol and 2-pheylethyl acetate. Heat Shock Thermal Stress (HSTS) and Radicicol were used as mutagenic stresses. Both methods target the Hsp90, a chaperone involved in the correct folding of proteins involved in the repair of DNA damage (Chen et al., 2012; James et al., 2008). Inhibition of this chaperone induces gross chromosome rearrangements and an aneuploidy state in the cells. The effects of high temperature in lager yeast strains have been reported previously and the use of this abiotic stress induced chromosomal rearrangements

in the cells (James et al., 2008). Along with these results, Radicicol has induced aneuploidy in *S. cerevisiae* strains (Chen et al., 2012). This mutagenesis method, also referred as accelerated evolution, was coupled to a selection with amino acid analogues of phenylalanine. This evolution gave a set of mutant strains able to produce a better aromatic profile.

3.2. Results.

3.2.1. Characterization of the aromatic profile of parental strains.

In order to narrow down the number of strains to use, and to select one strain from each Group for mutagenesis, aromatic analysis by GC-MS was carried out on small-scale fermentations (**Figure 3.1**). Based on this analysis, the Group I strain CBS1538 was chosen as it displayed a better aromatic profile of the selected compounds over all the Group I strains analysed. Two Group II strains were used in this analysis, namely strain 7012 and WS 34/70. While 7012 displayed a better aromatic profile, the WS 34/70 strain was taken forward for mutagenesis as this strain is considered the prototypical Group II strain and has been widely studied and sequenced several times, with the data available at the NCBI database.
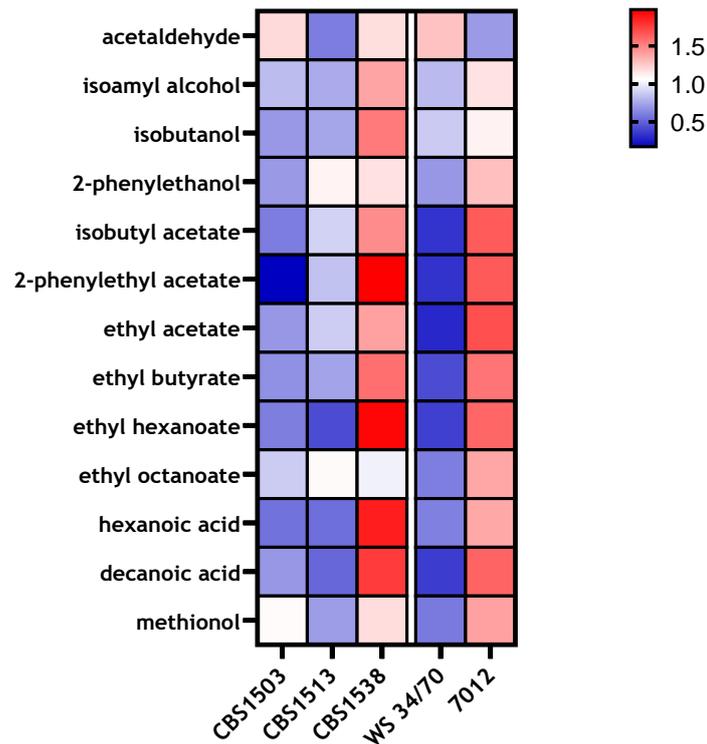
Figure 3.1. Characterization of the aromatic profile of the parental strains. The concentrations of the aromatic compounds were normalized by the average value of each group. Gap between columns divides the strains into Group I (left) and Group II (right).

3.2.1. Establishing the conditions for mutant selection

Experiments to determine the temperature and time length that kills 99.9% of the cells were conducted. Based on previous results, three temperatures and different times of incubation were tested (**Table 3.1**) (James et al., 2008). Cells were incubated in 2% YPDM for 2-3 h to recover from the high temperature stress. Both strains have different tolerance to high temperatures; CBS1538 showed a lower tolerance to high temperatures compared to WS 34/70. For Group I, the temperature used was less than 50ºC and for Group II the temperature was less than 55ºC.

Radicicol concentrations were set based on previous studies as radicicol did not induce any growth inhibition in the selected strains (Chen et al., 2012). Therefore, cells were treated with

3 different concentrations of Radicicol (20 µg/mL, 40 µg/mL and 100 µg/mL) and different times of incubation (24 or 48 h).

Table 3.1. Survival rate of the combinations of temperatures and time studied to get a survival rate less than 0.1%. Temperatures and incubation times tested for Group I strains (left) and Group II (right). Asterisk (*) means too many cells to count (> 400).

Temperature (ºC)	Time (min)	CFU	Death percentage
45	10	100%	0*
	12.5	100%	0*
	15	100%	0*
47.5	10	100000	99.33
	12.5	60000	99.6
	15	30000	99.8
50	10	4333	99.97
	12.5	0	100
	15	0	100
55	10	0	100
	12.5	0	100
	15	0	100

Temperature (ºC)	Time (min)	CFU	Death percentage
45	10	100%	0*
	12.5	100%	0*
	15	100%	0*
50	10	23500	99.84
	12.5	9333	99.93
	15	8000	99.95
55	10	4333	99.97
	12.5	2667	99.98
	15	2667	99.98

Two analogues of phenylamine, β-2-thienylalanine (B2TA) and ρ-fluorophenylalanine (PFPA) were used for selection of mutants following treatment with either heat shock or Radicicol. To determine the concentration of amino acid analogues required to inhibit cell growth, Minimal Inhibitory Concentration (MIC) was calculated based on growth parameters in 96-well plates (**data not shown**). The MIC for B2TA was 50 µg · mL^{-1} and for PFPA 150 µg · mL^{-1}. Based on these results, the concentrations used to isolate mutants were set higher to select mutants with a strong resistance and avoid false positive mutants. Therefore, final concentrations of 75 µg · mL^{-1} of B2TA and 400 µg · mL^{-1} of PFPA were used. Minimal medium was used as analogues did not have any inhibitory effect in 2% YPDM analogues.

3.2.2. Accelerated Evolution and screening.

To isolate *S. pastorianus* strains capable of overproducing 2-phenylethanol and 2-phenylethyl acetate, several rounds of mutagenesis were carried out. The selected parental strains were exposed to different high temperatures and to Radicicol under the conditions described in **Table 3.2**. Following treatment with heat or radicicol, the cells were plated on minimal medium containing 75 µg · mL^{-1} of B2TA and 400 µg · mL^{-1} of PFPA. A total of 96 mutants were isolated and were designated by a code based on the mutagenesis treatment and conditions. For example, treatment with 40 µg · mL^{-1} of radicicol for 24 h produced 9

clones of WS 34/70, designated as WS 9.1 - 9.9 and 13 clones of CBS1538, designated as CBS 9.1 – 9.13. Only mutants resistant to B2TA were found using heat shock.

Table 3.2. Number of mutants obtained after several rounds of evolution.

Code	Treatment	WS 34/70 B2TA	WS 34/70 PFPA	CBS1538 PFPA
1	Heat Shock 10' 50ºC	3	19	5
2	Heat Shock 10'-15' 45ºC	4	3	3
3	Heat Shock 12' 30" 55ºC	7	10	0
5	Radicicol 24h 100 µg/ml no recovery time	5	0	0
7	Radicicol <24h 100 µg/ml	0	7	0
8	Radicicol 24h 20 µg/ml	0	7	1
9	Radicicol 24h 40 µg/ml	0	9	13
10	Radicicol 48h 20 µg/ml	0	0	0
11	Radicicol 48h 40 µg/ml	0	0	1
		19	55	22

The isolation rate following heat shock treatment for WS 34/70 and CBS1538 were 1.86×10^{-7} and 1.08×10^{-7} respectively. Isolation rates for radicicol treatment were 7.66×10^{-7} for the WS 34/70 and 7.33×10^{-7} for the CBS1538. No clones were obtained if the parental strains were plated without any treatment. More mutants were obtained from the strain WS 34/70 as more rounds of evolution were carried out.

Mutants were submitted to 3 rounds of stabilization as pseudo-hyphal growth was evident in some clones (**Figure 3.2**). To reduce the number of cells experiencing pseudo-hyphal growth, colonies were restreaked on 2% YPDM agar plates, three-four times. In parallel, the colonies were restreaked on agar plates of minimum media containing the amino acid analogues at the established concentration to ensure that the resistance of the mutants was consistent.
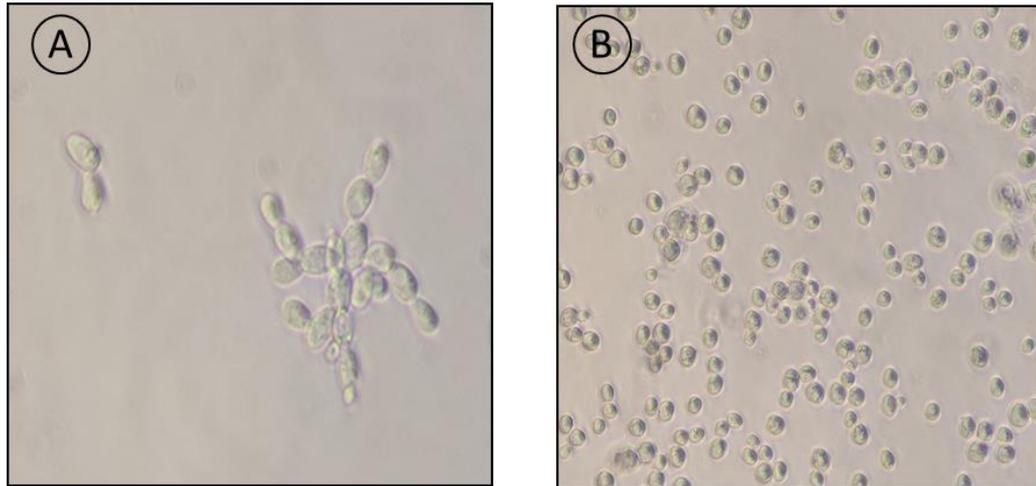
Figure 3.2. Morphology of the cells under the microscope after the evolution. Pseudo-hyphal growth experienced before the stabilization process (A) and single cell yeast after the stabilization process (B).

3.2.3.   Characterization of the mutants: GC-MS + Growth.

Twenty-two random mutants were selected for growth characterization and aromatic profile analysis in a small-scale fermentation. Firstly, the mutants were grown in minimal medium containing different concentration of the two amino acid analogues.

To discard any mutants that may have arisen from a defect on the amino acid permeases, mutants were grown in minimal medium without amino acids and without ammonium sulphate supplemented with 20mM of Phe as a sole nitrogen source. To investigate any new phenotypes associated with the mutants, the mutants were tested in minimal medium without amino acids, and 2% YPDM at 20 and 30ºC.

Strains resistant to the amino acid analogue B2TA are not able to grow or experienced a reduced growth in the presence of the second amino acid analogue used, PFPA (**Figure 3.3A**). In contrast, almost all the strains resistant to PFPA grew in the presence of B2TA. All the strains grew in minimal medium and in rich medium (**Figure 3.3B**). In minimal medium containing phenylalanine as sole nitrogen source, almost all the strains exhibit a similar growth rate compared to their respective parental strain, except two Group II mutants, WS 7.3 PFPA and WS 8.1 PFPA. These strains exhibited a reduced growth rate, indicating a sort of growth defect.

The same mutants show a decreased growth rate in 2% YPDM at 30ºC. In contrast, the thermotolerance of all the group I mutants is improved as all Group I mutants can grow better at 30ºC than the parental strain. Therefore, two strains out of 22 experience some growth defects.
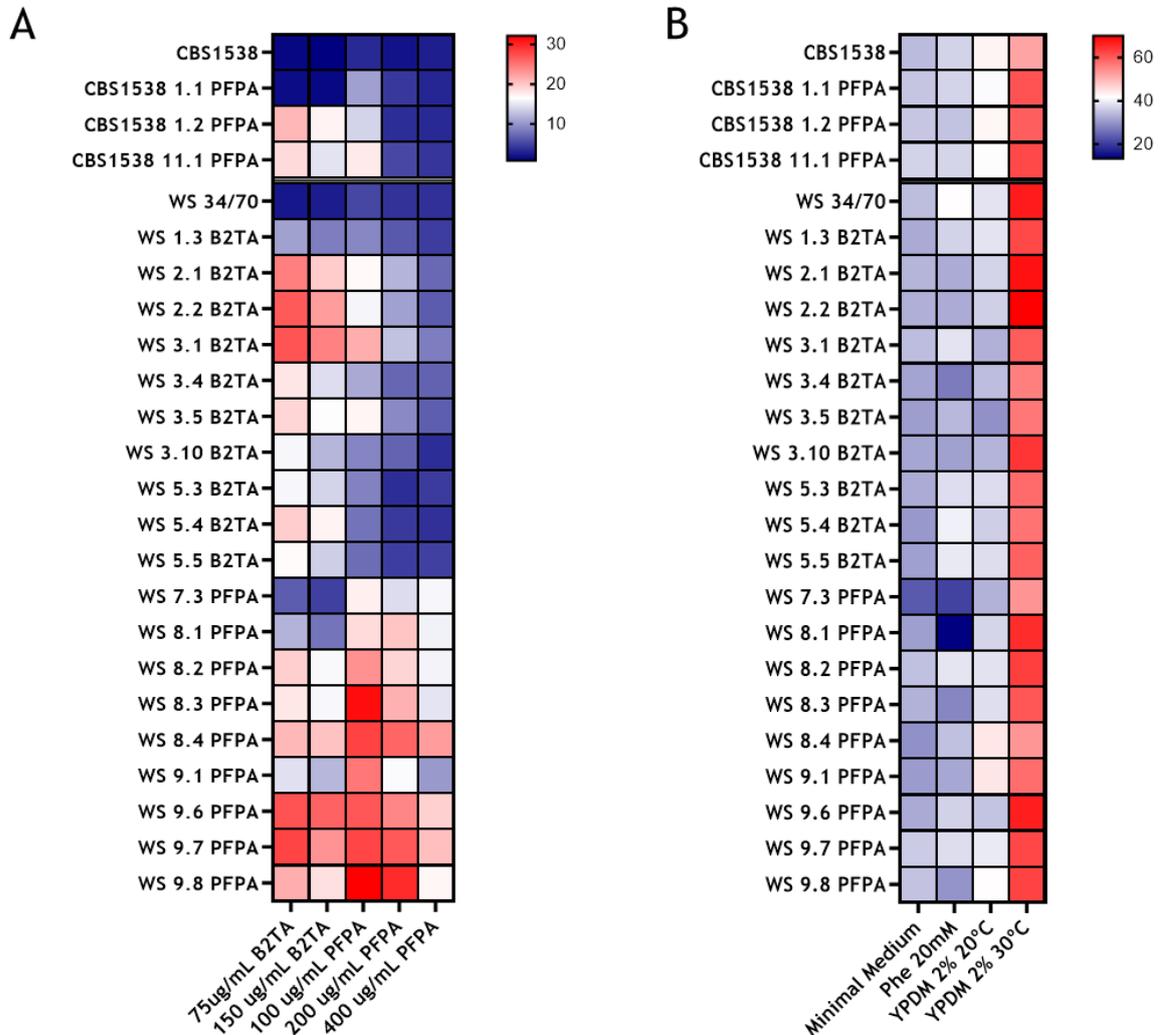
Figure 3.3. Characterization of the mutant and parental strains in different concentration of amino acid analogues (A) and in general media (B). Values correspond to the AUC during 68h of growth. Scale is based on the minimum and maximum AUC values observed.

Small-scale fermentations in 10º Bx wort were carried out. After 11 days of fermentation at 13ºC, wort samples were analysed by GC-MS to compare the aroma profiles of the mutants and to select for any with an increased production of 2-phenylethanol and 2-phenylethyl acetate (**Figure 3.4**). In total, 18 aromatic compounds were analysed, including higher

alcohols, acetate and fatty-acid esters, acids and off flavours like 4-vinylguaïacol and methionol. Of the original twenty-two mutants, aromatic profiles for 18 were obtained.
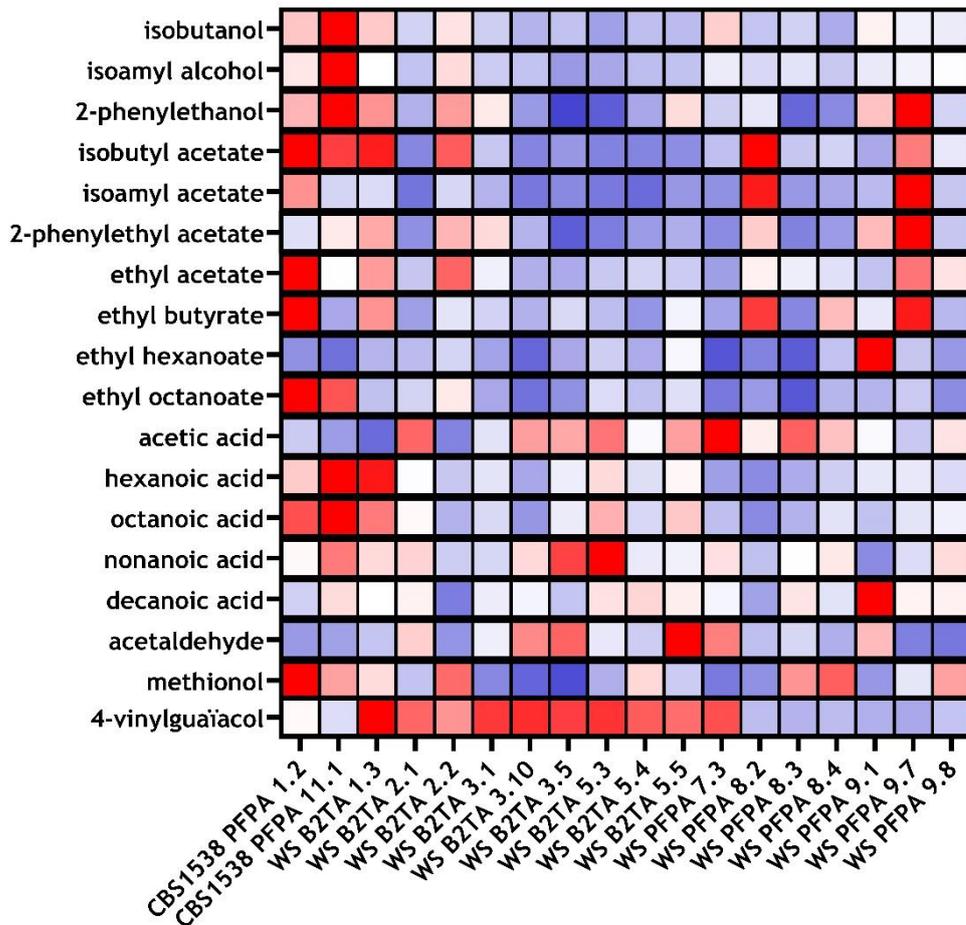
Figure 3.4. Characterization of the flavour profile of the mutants in small-scale fermentations. Values are normalized by the values of the respective parental strain. Each row has an independent colour scale, meaning red a higher fold change and dark blue lower fold change. White means same production as the respective parental strain (Fold change of one).

Overall, the aromatic profiles produced by the mutants are similar to that of the parental strains. All the mutants resistant to B2TA and one mutant resistant to PFPA, 7.3, experienced an overproduction of 4-vinylguaïcol. In total, 4 strains (11.1, 9.7, 1.3 and 2.2) produced more

than 1.5 x times 2-phenylethanol and 4 strains (1.3, 2.2, 9.1 and 9.7) produced more than 1.5 x times 2-phenyethyl acetate compared to the parental strains. Based on these findings, two strains, namely CBS1538 11.1 and WS 34/70 9.7, both obtained from radicicol treatment and selected on PFPA, that displayed increased production of 2-phenylethanol and 2-phenylethyl acetate were selected for further analysis.

3.2.4. Confirmation of the phenotype of the selected mutants (resistance to amino acid analogues and flavour profiles).

To confirm that the phenotype of resistance to PFPA was not transitory, mutants were successively plated in fresh agar plates with and without the amino acid analogue (**data not shown**). The resistance to the amino acid analogue was also characterized by the growth performance in liquid in 96-well plates (**Figure 3.5**).
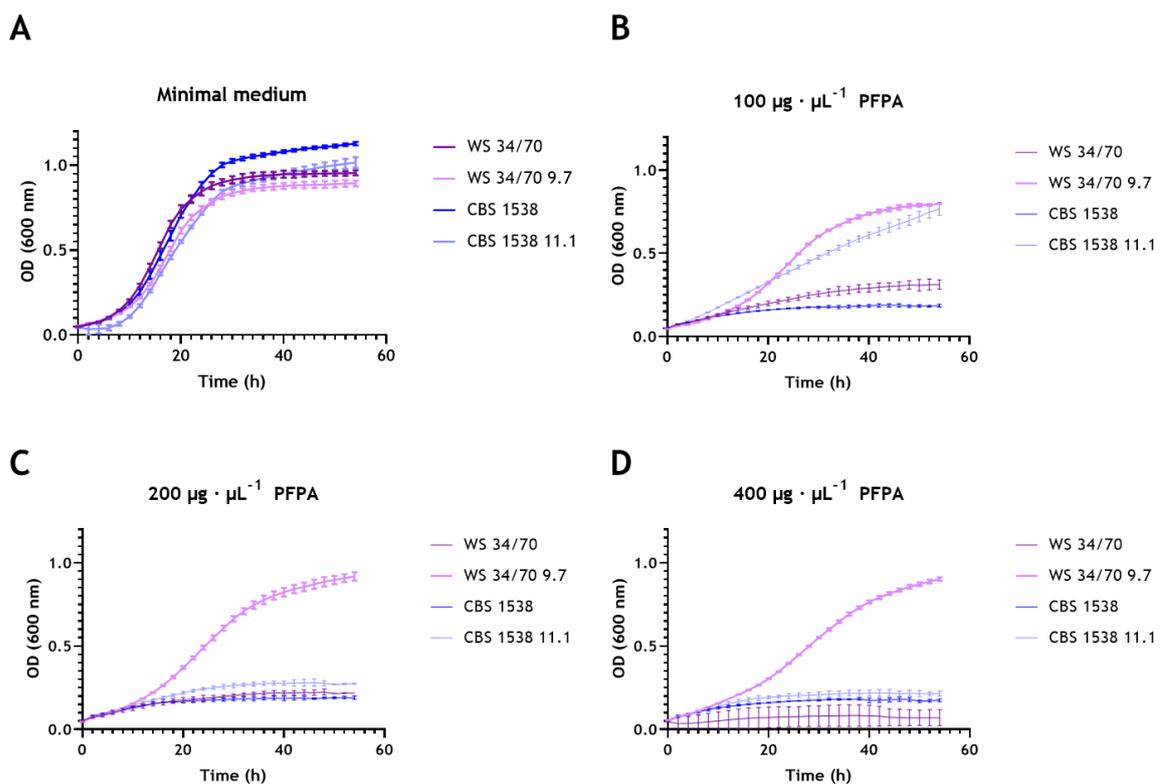
Figure 3.5. Characterization of the growth of the selected mutants in A) Minimal media without amino acid analogues, B) $100\,\mu g \cdot mL^{-1}$ of PFPA, C) $200\,\mu g \cdot mL^{-1}$ of PFPA and D) $400\,\mu g \cdot mL^{-1}$ of PFPA.

The growth in liquid in the presence of the amino acid analogues confirmed the resistance of the mutants to the amino acid analogues. Mutant 9.7 was resistant up to a concentration of $400\,\mu g \cdot mL^{-1}$ of PFPA while mutant 11.1 is more sensitive displaying a maximum resistance at $100\,\mu g \cdot mL^{-1}$ and performing a linear growth. It can also be observed that WS 34/70 is slightly more resistant to PFPA compared to CBS1538 (**Figure 3.5**).

Along with the growth characterization, the fermentation profiles of the mutant and wildtype strains were compared in small-scale (20 mL) and large-scale fermentations (3 L) fermentations conducted in 30 ml and 3 L tall tubes. Sugar consumption was measured every two days using a refractometer. The analysis of the sugar consumption revealed differences between the two parental strains. In both small- and large-scale fermentations, WS 34/70 ferments faster and reached a greater attenuation than the CBS1538 strain. In both small- and large-scale fermentations, the mutant 9.7 ferments faster that the wildtype strain and consumes more sugars compared to its wildtype strain, consuming 1°Bx more. In the tall tubes, the mutant 11.1 ferments slightly slower than the parent but there is no noticeable difference in the small tubes (**Figure 3.6** and **data not shown**)).
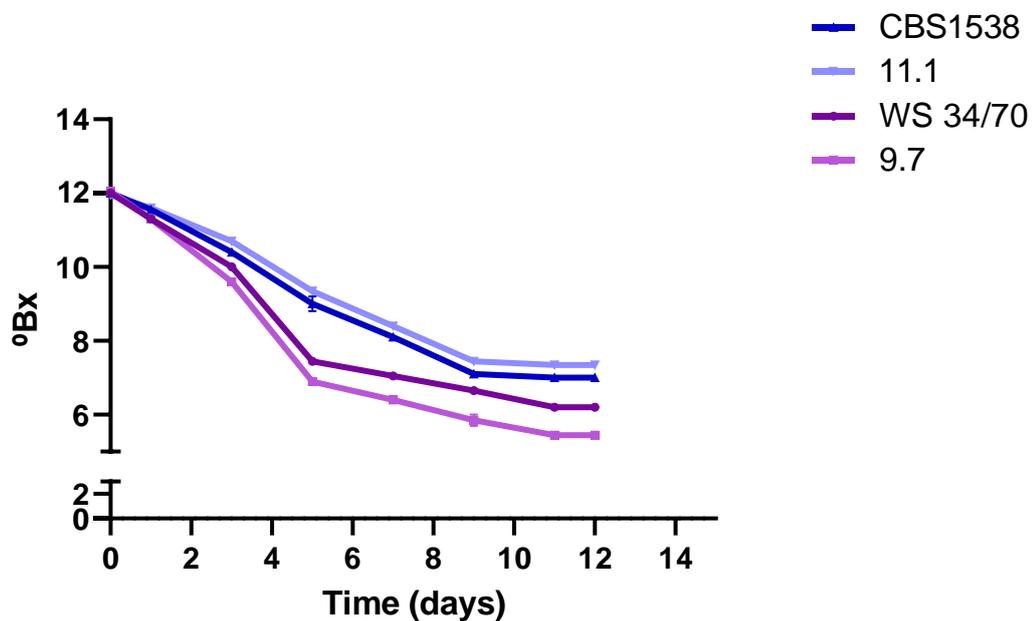
Figure 3.6. Sugar consumption of the mutants and parental strains in a large-scale fermentation of 12°Bx. Error bars correspond to two independent biological replicates.

To quantify the aromatic compounds produced in the mutants and the parental strains and to ensure that aromatic profiles observed in small scale fermentations were retained as the fermentations were scaled up, GC/MS analysis was repeated on small-scale (20 mL) and large-scale fermentations (3 L) fermentations conducted in parallel. Samples were frozen and sent to our collaborators in the Research and Innovation Centre, Edmund Mach Foundation S.Michele all'Adige, Italy. Analysis of the aromatic compounds was carried out using the GC-MS analytical method.

In total, eighteen aromatic compounds were analysed and quantified. Of these, hexanoic acid, octanoic acid, nonanoic acid and decanoic acid showed a high standard deviation with the Solid Phase Sample Extraction analysis. Therefore, these compounds are excluded from the analysis. The results for fourteen compounds are shown in **Table 3.3**.

The quantitative GC/MS analysis confirmed the increased production of 2-phenylethanol and 2-phenylethyl acetate in the mutants 9.7 and 11.1 with mutant 11.1 overproducing these compounds to a lower extent (**Figure 3.7A**). Additionally, increased levels of tyrosol and tryptophol, two higher alcohols derived from the aromatic amino acids tyrosine and tryptophan, respectively were observed in both mutants while mutant 9.7 also showed increased levels of isoamyl acetate and the off-flavour methionol (**Figure 3.7A**).

Comparing the different fermentation scales, almost all the compounds analysed had a lower concentration in the small-scale fermentations (**Table 3.3**). Although generally the relative concentrations of the volatiles were consistent between the small-scale and large-scale fermentations, acetaldehyde concentrations in the tall tubes were double that the observed in the small-scale fermentations, and isobutanol, that was produced at the same level compared to the parental strains.

4-vinylguaïacol is produced in a higher concentration in small tubes by CBS1538 and 11.1 compared to the Group II strains. The concentrations of each aromatic compound and the standard deviations between replicates are shown in Table 3.
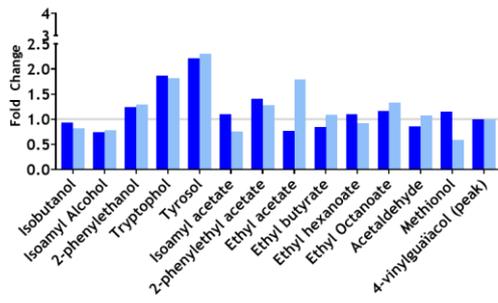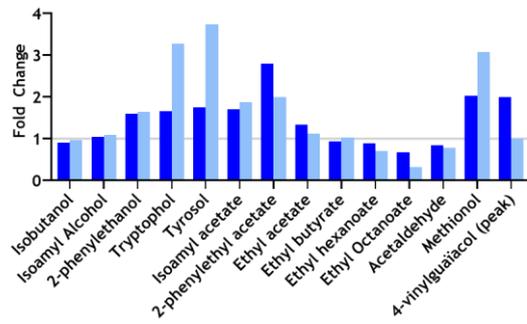
Figure 3.7. Fold change of the production of aromatic compounds by the mutant strains compared to their respective parental strains in small-scale fermentations, compared to large-scale fermentations. The value for the parental strain is set at 1.0. A.1) 11.1 vs CBS1538 and A.2) 9.7 vs WS 34/70. Dark blue: values from 3L fermentations, light blue: values from 20 mL fermentations.

Table 3.3. Volatile compounds produced at the end of fermentation of 3L (left) and 20 mL (right) of 12ºBx wort fermentations.

	Large-scale fermentation								Small-scale fermentation							
	CBS1538		11.1		WS 34/70		9.7		CBS1538		11.1		WS 34/70		9.7	
	mg · L⁻¹	St dev	mg · L⁻¹	St dev	mg · L⁻¹	St dev	mg · L⁻¹	St dev	mg · L⁻¹	St dev	mg · L⁻¹	St dev	mg · L⁻¹	St dev	mg · L⁻¹	St dev
Isobutanol	14.2	0.14	13.18	0.12	17.1	1.04	15.33	1.89	16.45	3.24	13.54	1.2	19.77	1.15	19.05	2.54
Isoamyl Alcohol	84.61	2.31	62.51	1.37	101.67	4.27	105.71	3.27	67	2.82	52.63	2.17	73.57	3.58	79.92	7.32
2-phenylethanol	56.32	4.98	69.87	12.75	48.64	0.9	77.88	5.26	18.73	1.35	24.23	2.42	24.92	1.9	40.83	0.72
Tryptophol	0.29	0.09	0.54	0.02	0.49	0.04	0.81	0.09	0.28	0.07	0.51	0.09	0.11	0.02	0.36	0.13
Tyrosol	15.43	1.8	34.05	0.5	25.33	1.18	44.15	2.09	10.83	0.62	24.91	1.69	7.29	1.14	27.17	2.59
Isoamyl acetate	1.43	0.31	1.58	0.39	1.91	0.08	3.24	0.4	0.16	0.07	0.12	0.02	0.31	0.09	0.58	0.19
2-phenylethyl acetate	0.99	0.02	1.39	0.17	0.87	0.04	2.43	0.27	0.18	0	0.23	0.02	0.17	0	0.34	0.03
Ethyl acetate	13.87	0.73	10.58	0.26	18.83	1.64	25	1.29	6.92	1.39	12.43	0.31	11.14	0.54	12.45	1.13
Ethyl butyrate	3.97	0.27	3.35	0.14	2.2	0.08	2.04	0.03	1.71	0.23	1.85	0.08	1.65	0.03	1.68	0.1
Ethyl hexanoate	0.64	0.03	0.7	0.02	0.26	0.01	0.23	0	0.12	0.01	0.11	0.01	0.1	0.01	0.07	0.01
Ethyl Octanoate	1.31	0.08	1.53	0.05	1.17	0.14	0.78	0.02	0.06	0.01	0.08	0.01	0.31	0.05	0.1	0.03
Acetaldehyde	10.82	0.54	9.33	0.38	12.63	0.42	10.68	1.14	26.21	2.1	28.28	2.66	25.21	2.78	19.73	5.29
Methionol	0.33	0.02	0.38	0.05	1.12	0	2.26	0.18	0.27	0.05	0.16	0.02	0.29	0.04	0.89	0.15
4-vinylguaïacol (peak)	0.09	0.01	0.09	0.01	0.02	0	0.04	0.02	0.01	0	0.01	0	0.01	0	0.01	0

3.3. Discussion.

3.3.1. *S. pastorianus* **strains produce different aromatic profiles.**

All five parental strains analysed displayed different aromatic profiles in 10º Bx small-scale fermentations. The production of different aromatic compounds is different between strains and between groups. CBS1503, also known as *Saccharomyces monacensis,* is the strain that displayed the poorest aromatic profile. Acetaldehyde is the only aromatic compound that is produced in a higher concentration by this strain. This compound is catalogued as an off flavour as it imparts a "green aroma" or notes similar to paint (Hughes & Baxter, 2007). Overall, CBS1538 presented a higher production of the compounds analysed.

Of the two Group II strains analysed, 7012 is the strain that produces more concentration of aromatic compounds. Also, it displays a better aromatic profile compared to the Group I strains. However, Group II strain WS 34/70 was selected despite its plain aromatic profile as several genomes are deposited on the NCBI and it is a strain widely used in lager beer fermentations (Gibson et al., 2013; Okuno et al., 2016; Walther et al., 2014).

3.3.2. **Both parental and mutant GI and GII strains have different tolerance to high temperatures.**

The experiments to determine the Heat Shock Temperature shows different results between Group I and Group II parental strains. In both cases, Group II WS 34/70 can tolerate higher temperatures, such as 50 or 55 ℃. Temperatures of more than 47.5℃ seem to be lethal for CBS1538.

Both strains tolerate 45℃ for short time periods. This temperature is considered high for the strains, but both strains form viable colonies after the Heat Shock at that temperature. The temperature sensitivity of CBS1538 was also evident during the characterisation of the mutants with CBS1538 growing much slower than WS 34/70 in 2% YPDM at 30℃.

The difference in temperature tolerance of the two strains may be due to the genetic background of the strains as both belong to two different groups of *S. pastorianus* strains. WS 34/70 is an allotetraploid strain with higher content of *S. cerevisiae*-like (Sc-like) sub-genome. CBS1538, is an allotriploid strain, with a reduced Sc-like genome compared to WS 34/70 (Okuno et al., 2016).

S. eubayanus is known to be cryotolerant while *S. cerevisiae* is characterized to grow at higher temperatures (mesotolerant) (Bing et al., 2014; Gamero et al., 2013; Gibson et al., 2013). It is not well understood what the relationship is between the ability to grow better at different temperatures and the genome composition, but a wide range of cellular targets have been studied. Several studies carried out in wine yeast have linked the ability to grow at high or low temperatures with the lipid composition of the cell membrane. Lower temperatures influence the flexibility of the membrane, making it more rigid (Beltran et al., 2006; Krogerus, Seppanen-Laakso, et al., 2017). Also, other studies have linked the temperature adaptation to changes in the genome, specifically to genes located to sub-telomeric regions (Garcia-Rios et al., 2017). Genes like *ADH3* and *GUT2* have been linked to cold temperature adaptation in *S. cerevisiae* and *S. kudriavzevii* (Paget et al., 2014).

3.3.3. Mutants display different tolerance to amino acid analogues and high temperatures.

The evolution strategy used here produced mutants that were able to grow in the presence of toxic amino acid analogues. Based on the growth parameters, strains resistant to growth in the presence of B2TA were not able to grow in the presence of PFPA. However, almost all the mutants resistant to PFPA were also resistant to B2TA. This may indicate that both amino acid analogues have different targets in the cells, and that PFPA could have a stronger impact than B2TA. Similar results have been reported. Cordente et al. (2018) used ρ-fluorophenylalanine and *o*-fluorophenylalanine to isolate wine yeast strains. Both amino acid analogues selected for mutants that overproduced 2-phenylethanol and 2-phenyethyl acetate. The mutants isolated with PFPA showed a higher overproduction of these two aromatic compounds. It has previously been described that different amino acid analogues have different degrees of toxicity in *E. coli*. Of the amino acid analogues tested, ρ-fluorophenylalanine was more toxic than *o*-fluorophenylalanine, and the latter is more toxic than *m*-fluorophenylalanine (Pine, 1978).

Several studies have isolated mutants with impaired feedback inhibition in sake, wine and lager yeast (Cordente et al., 2018; Fukuda, Watanabe, & Asano, 2014; Fukuda, Watanabe, Asano, et al., 2014; Lee et al., 2018). Analysis of the sequences of *ARO4* showed non-

synonymous mutations that are located along the peptide sequence. Also, other mutations located in other genes of the Shikimate pathway have been found, like the ones located in *TYR1*, a prephenate dehydrogenase involved in tyrosine biosynthesis. The study hypothesized that mutations in Tyr1p may give an isoform with a reduced activity, therefore diverting the flux to the biosynthesis of the two other aromatic amino acids (Cordente et al., 2018).

Furthermore, as previously reported, there is no correlation between the resistance to the amino acid analogue and the overproduction of aromatic compounds derived from amino acid catabolism (Cordente et al., 2018). For example, mutant 9.7 has a good resistance to the amino acid analogues B2TA and PFPA. Mutant 8.4, for example, is resistant to the amino acid analogues at a same rate as mutant 9.7, but its flavour profile is very similar to the wildtype WS 34/70. In contrast, mutant 1.3 is not very resistant but its flavour profile is more complex compared to the one produced by mutant 8.4.

All the selected mutants were tested in different physiological conditions. They were grown in minimal medium in the absence of amino acids to discard possible defects in the nitrogen metabolism. They were also grown in minimum medium but supplemented with phenylalanine as a sole nitrogen source. The reason for this was to check if cells were resistant to the amino acid analogue due to a defect in amino acid permeases. Also, the growth in 2% YPDM was checked to see possible growth defects in a complex medium. This condition was also tested but at a higher temperature, 30ºC to see if there is any growth advantage at that temperature.

All but three of the mutants (WS 3.4, WS 7.3 and WS 8.1) grew well in all the conditions tested. The AUC values for these mutants were lower than the parental strain in almost all the conditions. There is no mutant strain able to grow better than their respective parental strain when phenylalanine is the only nitrogen source in the medium.

Interestingly, Group I mutants experienced a higher growth rate in 2% YPDM at 30ºC compared to the wildtype. This may indicate that thermotolerance of the strains have improved after evolution.

3.3.4. Flavour profiles of the mutants are similar to the parental strain.

Amino acid analogues were used to select strains with an increased flux through the Ehrlich pathway. To induce mutations, two approaches that target Hsp90p were used. High temperatures are known to inhibit Hsp90p. This chaperone is responsible for folding proteins that are involved in DNA repair processes. The inhibition of the repair machinery of the cell induces single and double strand breaks. Previous studies have used high temperatures to obtain evolved strains. The result of these high temperatures (45ºC-55ºC) showed that high temperatures applied to *S. pastorianus* strains induced gross chromosomal rearrangements. As a result, mutants were able to ferment 19ºBx wort (James et al., 2008).

Radicicol is an antibiotic that binds to the N-terminal of Hsp90p and modifies the structure of this chaperone. This inhibition has been studied in yeast and results show aberrant chromosome number in the mutants treated with radicicol (Chen et al., 2012; Sharma et al., 1998).

Both amino acid analogues used here are analogues of the aromatic amino acid phenylalanine. Several studies have proposed that phenylalanine amino acid analogues select for strains with overproduction of phenylalanine, as these toxic molecules select for strains with an impaired feedback regulation at the enzymatic level of Aro4p and Aro3p. These two enzymes, two 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthases, catabolise the first step of the aromatic amino acid biosynthesis. These two proteins are feedback-inhibited by the final amino acids. Aro4p is inhibited mainly by tyrosine and Aro3p by phenylalanine. Both of them are inhibited by all three aromatic amino acids, but at different rate/concentrations. The accelerated evolution not only gave mutants overproducing the aromatic compounds 2-phenylethanol and 2-phenylethyl acetate, but also alcohols derived from tyrosine and tryptophan. Furthermore, the accelerated evolution has conferred mutant 9.7 the ability to consume more sugars from wort. Wort is a complex media whose main sugar is maltose. Maltotriose is the second most abundant sugar (Briggs et al., 2004). Then, glucose and fructose constitute around 10% of the sugars in wort. The first sugars to be consumed are the hexoses as these monosaccharides can be uptaken by passive diffusion or by permeases and inhibit the metabolism of more complex sugars (Bisson & Fraenkel, 1983; Romano, 1982). Maltose is easily digested once these hexoses are consumed. Maltotriose is a trisaccharide and its fermentation by yeast is important as it is the second

most abundant sugar in wort. In *S. pastorianus* strains, this ability varies between Group I and Group II strains. Previous studies have shown that Group II strains are able to import and metabolise this sugar while three out of eight strains belonging to Group I are able to metabolise it (Ekberg et al., 2013; Magalhaes et al., 2016; Vidgren & Londesborough, 2012; Vidgren et al., 2005). This is due to the different presence of different *MAL* loci and other sugar transporters, like *AGT1* and *MTT1*.

Further analysis will be needed to understand this higher consumption of sugars by the mutant strain 9.7.

3.3.5. Mutants are stable and maintain the phenotype.

We observed that the new mutants experienced, initially, an aberrant growth characterised by the absence of cytokinesis. Stabilization of the mutants showed a drastic decrease of these pseudo-hyphal cells and an increase of single budding cells. This aberrant cell morphology is observed in cells under stress (Gancedo, 2001; Zaragoza & Gancedo, 2000).

The characterization of the growth of the mutant strains shows that the resistance to the amino acid analogue is stable. Nevertheless, the growth in the presence of the amino acid analogues experiences a longer lag phase and a decrease of cell density/AUC compared to the growth in minimal medium.

Small-scale and large-scale fermentations of the selected mutants were carried out to check that the flavour profile of the strains was consistent. The aromatic profile was analysed by GC-MS. Both mutants are overproducing 2-phenyl ethanol and 2-phenylethyl acetate, but mutant 11.1 at a lower extent. Two other higher alcohols that come from the catabolism of the aromatic amino acids tyrosine and tryptophan were analysed. Both strains overproduce these two higher alcohols. Tyrosol is an antioxidant found in high concentrations in olive oil (Di Benedetto et al., 2007). This higher alcohol has become attractive as it can be transformed into hydroxityrosol, a more potent antioxidant and anticarcinogenic that is found in olive oil, wine and beer (Rebollo-Romero et al., 2020). Overproduction of this compound may be beneficial for health, but it might modify the final organoleptic profile of beer as it has been reported that high concentrations of tyrosol in wine changes the colour to yellow and it increases the

bitterness. While tyrosol has a positive impact in health, tryptophol is linked to cell apoptosis in humans. The role of this higher alcohol and its derivatives in yeast are linked to the quorum sensing signalling and they might have antibiotic effect (Palmieri & Petrini, 2019). Tryptophol, together with tyrosol, may increase bitterness and astringency and may change the colour of the final product due to an increase of phenolic compounds (Andrewes et al., 2003; Cordente et al., 2018; Soejima et al., 2012).

Moreover, mutant 9.7 produces more isoamyl acetate and ethyl acetate too, and it reaches a higher attenuation of wort sugars. These results go along with the results reported by (Cordente et al., 2018). This is beneficial as isoamyl acetate imparts notes of banana and ethyl acetate have a solvent-like and sweet aroma (Lin et al., 2021).

The mutant 9.7 also produces more methionol, a metabolite that is produced by the catabolism of the amino acid methionine via modifications of the Ehrlich pathway (Perpete et al., 2006). This sulphur-containing off-flavour imparts notes that are reminiscent of vegetables, soupy-like (Hill & Smith, 2000).

3.4. Conclusion.

Accelerated evolution coupled to a selection strategy using amino acid analogues was applied. This strategy was succesful as mutants able to grow in the presence of the toxic amino acid analogues were produced. The isolation of mutants was followed by a stabilization step and characterization in different media. Analysis of flavour profile of 22 random mutants allowed us to select two strains overproducing 2-phenylethanol and 2-phenylethyl acetate. Along with these higher alcohol and its ester, strains were able to overproduce tyrosol and tryptophol, two higher alcohols derived from the aromatic amino acids tyrosine and tryptophan. This may indicate that the strains have overcome the negative feedback regulation at Aro3/Aro4p enzymatic level.

Further Whole Genome Sequencing (WGS) and RNAseq analysis in different conditions will be carried out to investigate the consequences of Radiciciol in the cell.

Chapter 4

Whole Genome Sequencing

4.1. Introduction.

Yeast species belonging to the *Saccharomyces* genus are microorganisms that can be found in a wide range of environments, from wild ecosystems to industrial environments (Alsammar & Delneri, 2020). For example, *S. eubayanus* can be found in wild environments solely while other species such as *S. cerevisiae* and *S. kudriavzevii* can be found in both wild and man-made environments such as wineries (Bing et al., 2014; Libkind et al., 2011). However, *S. pastorianus* strains can be found in human made environments exclusively, such as breweries. The genus *Saccharomyces* compromises a set of species with good fermentative traits that makes them perfect to participate in fermentative processes, such as beer, wine, bakery, or more biotechnologically focused, like bioethanol or cacao production (Menezes et al., 2016; van Zyl et al., 2007). The versatility in fermentative environments for species belonging to the *Saccharomyces* genus has spurred researchers to decipher the genomic basis responsible for the fermentative traits and to use this information to improve the fermentative process.

Since the first sequencing of *S. cerevisiae* (Goffeau et al., 1996), a growing number of new sequencing rounds of *Saccharomyces* species have been carried out (Peter et al., 2018). The evolution of sequencing techniques led to easier, faster and cheaper sequencing runs. Sequencing can give information about the genome of the organisms such as genome sequence, Single Nucleotide Polymorphisms (SNPs) or Copy Number Variation (CNV). Also, information related to evolutionary events can be obtained and phylogenetic analysis can be carried out. Chromosomal rearrangements like translocations, inversions and introgressions can be detected (Hess et al., 2020; Peter et al., 2018). The information gleaned from sequencing analysis can be helpful to identify and link phenotypes to genome composition.

It was in 2011 when *S. eubayanus* was first isolated from Patagonia forest (Libkind et al., 2011). Following isolation of *S. eubayanus* in other geolocations, sequencing analysis showed that there are different lineages located in Tibet, China and the U.S.A.. The phylogenetic analysis of all these lineages showed that Tibetan lineage is the closest parent strain of *S. pastorianus* (Bing et al., 2014).

The genome of *S. pastorianus* results from interspecific hybridization between *S. cerevisiae* and *S. eubayanus* species. As new genomes of *S. pastorianus* strains were released, different hypothesis about the hybridization event between these two strains arose (Okuno et al., 2016; Salazar et al., 2019; Walther et al., 2014). Firstly, two independent hybridization events as a possible origin of the strains was suggested (Dunn & Sherlock, 2008). Secondly, one hybridization event originated from an ancestor of Group I strains. This ancestor would have hybridised with another *S. cerevisiae* strain, originating Group II strains (Monerawela & Bond, 2018; Monerawela et al., 2015; Okuno et al., 2016; Walther et al., 2014). Thirdly, a unique hybridization event between a diploid *S. eubayanus* and a heterozygous diploid *S. cerevisiae* strain has been proposed. This event would led to an ancestor that would have undergone an evolution process, giving Group I and Group II strains (Okuno et al., 2016; Salazar et al., 2019). These three hypotheses are supported by the evidence found in the genome sequences such as hybrid/chimeric chromosomes and SNPs. The hybrid chromosomes have recombined by break points, some of which are located in coding regions, thus generating a set of hybrid genes that are unique in the *S. pastorianus* (Monerawela & Bond, 2017a, 2017b, 2018). The birth of *S. pastorianus* generated two different groups of strains based on the genomic content. Group I has less DNA content of *S. cerevisiae* and chromosome copy numbers range from 31 to 47. In contrast, Group II strains contain more genomic content of *S. cerevisiae* and chromosome copy numbers range from 32 to 75. Genomic DNA sequences of strains within these groups have been released, such as the Group I CBS1503 (known as *S. monacensis*), CBS1513 (known as *S. carlsbergensis*) and CBS1538 (Walther et al., 2014), and Group II strains WS 34/70 (Okuno et al., 2016; Walther et al., 2014) and CBS1483 (Salazar et al., 2019; van den Broek et al., 2015). Other isolates of WS 34/70 have been sequenced. The estimation of chromosome ploidy confirms the variability in chromosome copy between different isolates (van den Broek et al., 2015). Of all the Group I and Group II strains, only CBS1483 has been fully assembled and annotated (Salazar et al., 2019).

Fermentation processes have been carried out for more than 6000 years and throughout this time, human beings have influenced the domestication of yeast strains (Baker et al., 2015; Gallone et al., 2016; Libkind et al., 2011). The fermentative process has been improved through modifying different steps of the brewing process (Hornsey, 2003).

Another approach to improving the fermentative process involves improving or evolving yeast strains. Such new strains have improved different targets of beer brewing, such as fermentation in high-gravity wort or improved flavour profiles (Gibson et al., 2020; Huuskonen et al., 2010; Strejc et al., 2013)

Yeast are microorganisms with a complex metabolism able to produce ethanol and secondary metabolites that impart aromas (Dzialo et al., 2017; Ljungdahl & Daignan-Fornier, 2012). These aromas impart notes of fruity and floral aroma but also off-flavours like sulphur aromas. Several approaches have been used to improve the aromatic profile, such as generation of new interspecific hybrids, chemical mutagenesis or adaptative evolution (Gibson et al., 2020; Gibson et al., 2017; Gibson et al., 2018; James et al., 2008; Krogerus et al., 2016; Krogerus et al., 2015; Strejc et al., 2013).

The previous chapter described the isolation of two novel *S. pastorianus* strains through treatment with Radicicol followed by selection in media containing amino acid analogues of phenylalanine. Characterization of these two strains showed overproduction of 2-phenylethanol and 2-phenylethyl acetate, and also tyrosol and tryptophol. This chapter describes studies undertaken to investigate the genomic changes found in the mutants and to link them with the observed phenotypes of the two evolved strains.

4.2. Results.

4.2.1. Mapping of mutants and parental strains.

Two selected mutants from the previous accelerated evolution experiment, Group I mutant 11.1 and Group II mutant 9.7, together with their respective wildtypes, CBS1538 and WS 34/70, were sequenced to investigate the effects that Radicicol might have induced in the mutant cells.

DNA libraries of 150 bp fragments were prepared for each strain and sequenced using Illumina Technology (Novogene). Adapters of the reads were removed and reads with low quality were filtered out (Martin, 2011). Quality of the reads was calculated using FastQC (Babraham Institute). After this, clean reads were ready to be mapped. As *S. pastorianus* strains have a complex genome, reads were mapped against the already sequenced genome of *S. pastorianus*, deposited on the NCBI database (assembly: ASM1102231v1). This Group II strain, CBS1483, has been fully assembled into

chromosomes and annotated (**Table 4.1**), and it constitutes the reference genome of *S. pastorianus* strains (Salazar et al., 2019). The genome of this strain contains 15 Sc-like and 16 Se-like chromosomes. This strain does not contain a full Sc-like chromosome III nor a full Se-like chromosome III as these two chromosomes are recombined by the Matα locus (Monerawela & Bond, 2017b; van den Broek et al., 2015). Therefore, this strain has a hybrid chromosome III and fragments of approximately 190 Kb of the Sc-like chromosome III and another fragment of approximately 200 Kb of the Se-like chromosome III are missing.

Table 4.1. Genome assembly and annotation data for CBS1483.

Protein	10760
tRNA	552
Total	11312
Nº chromosomes	31
Length (Mb)	22.34
%GC	39.07

Bowtie2 was used to map the clean reads against the reference genome (**Table 4.2**). Overall, the mapping rate was higher than 96.0%, with just less than 3.2% of reads that did not map. Average coverage of all the positions of the genome, including positions where no reads were mapped, differs within strains but in each case was more than 100x.

Table 4.2. Number of clean reads, mapping rate and annotation of mutant and WT strains against the CBS1483 reference genome.

		CBS1538	11.1	WS 34/70	9.7
QC analysis	Total sequences (pair-end)	17670578	19002686	18533969	14023125
	%GC	39	38	37	38
Mapping	Reads mapped:	96.15%	96.11%	97.33%	97.38%
	Average coverage:	220,84x	236,70x	233.96x	144.60x
Annotation	Total number of genes:	7115	7115	11173	10991
	Mapped reads:	6959 98%	6959 98%	11029 98.7%	10847 98.7%
	Unmapped reads:	156 2%	156 2%	144 1.3%	144 1.3%

S. pastorianus strains have been reported to be widely aneuploid (Monerawela & Bond, 2018). This set of strains have different ploidy between Group I and Group II strains and also within strains of the same group (Bond et al., 2004; Monerawela & Bond, 2018; Okuno et al., 2016; van den Broek et al., 2015). Both CBS1538 and WS 34/70 have been sequenced previously and the analysis showed that CBS1538 has a complete Se-like chromosome III and WS 34/70 has a complete Sc-like chromosome III (Okuno et al., 2016; Walther et al., 2014).

In order to check the presence of whole Sc-like and Se-like chromosomes III in the parental strains and in the mutants, unmapped reads were analysed. Unmapped reads were extracted using Samtools and *de novo* assembled using SPAdes (Bankevich et al., 2012). Scaffolds were aligned against a customised database generated by joining *S. cerevisiae* and *S. eubayanus* reference genomes (**Table 4.3**), both deposited on the NCBI (assemblies R64 and SEUB3.0, respectively). Open Reading Frames (ORFs) were generated using getorf (Rice et al., 2000) followed by a Blastp search in order to annotate them against a custom database with the annotation from the reference *S. cerevisiae* and *S. eubayanus* strains (**Table 4.3**). Blast searches were made within the Blast+ environment in Python (Camacho et al., 2009).

The analysis of unmapped reads revealed the presence of the fragment of Sc-like chromosome III in WS 34/70 and mutant 9.7. The same analysis was carried out in the Group I strains, showing the fragment of the Se-like chromosome III missing in CBS1483.

The ORFs that were detected in this analysis were added to the list of ORFs of CBS1483 (**Table S 4.1**).

Table 4.3. Genome assembly and annotation of *S. cerevisiae* and *S. eubayanus* genomes.

	S. cerevisiae	*S. eubayanus*	**Total**
Protein	6016	5303	11319
rRNA	14	2	16
tRNA	299	302	601
Other RNA	117	1	118
Total	6464	5608	12072
Nº chromosomes	16	16	32
Length (Mb)	12.16	11.54	23.7
%GC	38.21	39.88	

As CBS1483 has a different chromosome architecture compared to the Group I strain CBS1538 and Group II strain WS 34/70, reads were mapped against the combination of *S. cerevisiae* and *S. eubayanus* genomes (**Table 4.4**). The results obtained from the two independent mapping and annotation rounds showed similar mapping rate, being slightly less when the custom genome was used. Furthermore, a similar number of ORFs were detected.

Table 4.4. Number of clean reads, mapping rate and annotation of mutant and WT strains against the customized genome.

		CBS1538		11.1		WS 34/70		9.7	
QC analysis	Total sequences (pair-end)	35341156		38005372		37067938		28028520	
	%GC	39		38		37		38	
Mapping	Reads mapped:	93.17%		92.90%		93.30%		95.82%	
	Average coverage:	206.47x		221,17x		216.58x		168.31x	
Annotation	Total number of genes:	6965		6974		11502		11250	
	Sc-like	1505	22%	1510	22%	6122	53%	6129	54%
	Se-like	5460	78%	5464	48%	5380	47%	5121	46%

4.2.2. Chromosome copy number.

Chromosome Copy Number (CCN) was calculated to analyse the Chromosome Number Variations (CNV) of the mutant strains. These analyses were carried out to investigate the effects of Radicicol in the cells as it was reported before that Radicicol induces aneuploidy (Chen et al., 2012). To calculate the CCN, Samtools and customised Python scripts were used. This analysis was, firstly, carried out using the *S. pastorianus* reference genome CBS1483 (**Table S 4.1**). Due to the different chromosome rearrangements present in CBS1483 compared to both Group I and Group II strains, we decided to map the reads to the customised genome that contains the genomes of both *S. cerevisiae* and *S. eubayanus* strains, deposited on the NCBI (**Figure 4.1**).
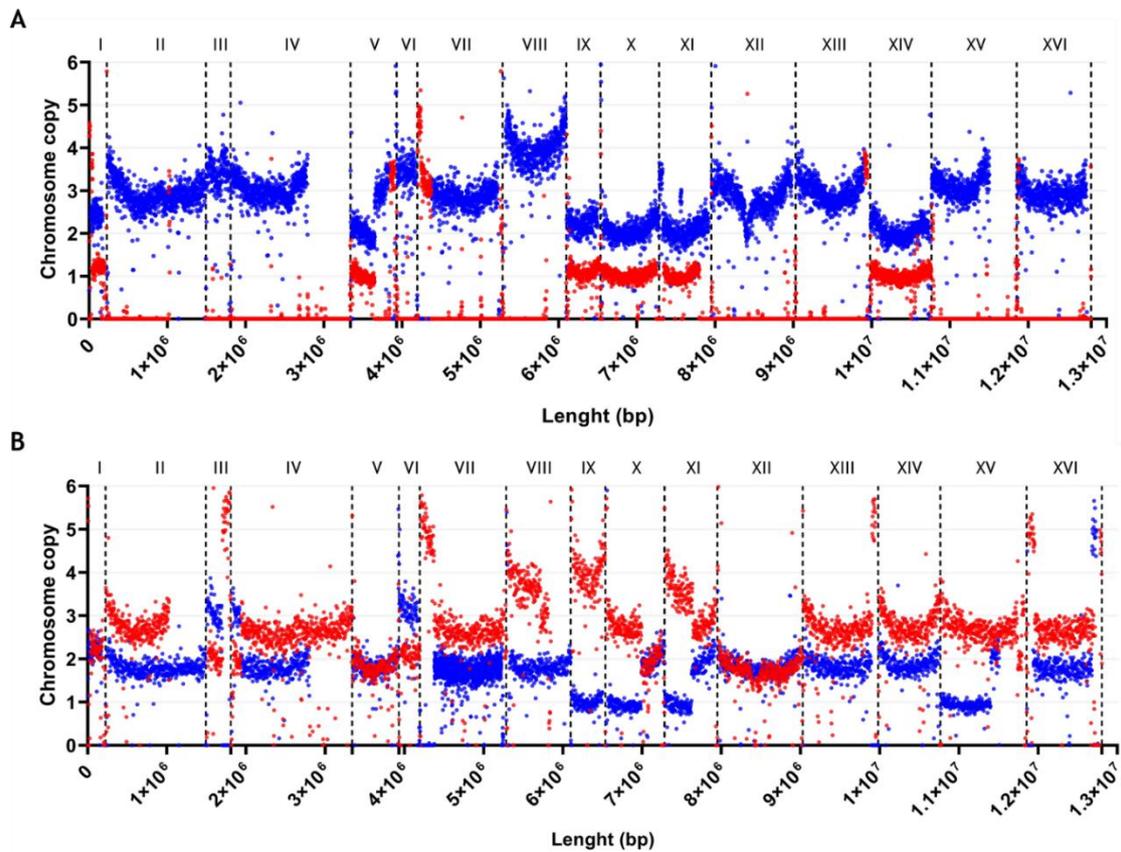
Figure 4.1. Chromosome copy number based on sequence coverage mapped against parental genomes. In red, *S. cerevisiae* sub-genome, in blue, *S. eubayanus* sub-genome. A) Group I strain CBS1538, B) Group II strain WS 34/70. *S. eubayanus* strain has two different translocations that can be observed in *S. pastorianus* strains. Translocations can be seen between chromosomes II and IV, and between chromosomes VIII and XV.

The CCN analysis shows differences between Group I and Group II parental strains and it confirms the variability of copy number in different *S. pastorianus* strains as CBS1538 has 51 chromosomes and mostly all of them are Se-like chromosomes. Only Sc-like chromosomes I, V, IX X, XI and XIV are present in this strain. In contrast, WS 34/70 has approximately 79 chromosomes. Both Sc-like and Se-like sub-genomes are present in this strain. Consequently, copy number varies depending on the strains. CBS1538 has a reduced gene copy number (6965) compared to WS 34/70 (11502), and this gene copy number is mostly Se-like (**Table 4.4**).

The custom genome does not contain any chromosome recombinations, other than two chromosome translocations found in *S. eubayanus*. These two translocations are observed

between chromosomes II and IV and chromosomes VIII and XV, in *S. eubayanus* (Baker et al., 2015). Apart from this translocation, different chromosome rearrangements can be observed in both strains, such as hybrid chromosome V in CBS1538 and chromosome XI in WS 34/70 (**Figure 4.1**).

Interestingly, a new recombination point has been detected in WS 34/70. This break point is located in chromosome IV, specifically in the *RPL35A* locus (YDL191W). The alignment of both alleles of *RPL35A* shows an overall identity of 71.8%. Nevertheless, after the nucleotide position 508, the identity/similarity scales up to 95.9%. The presence of hybrid chromosome, ScVIII/SeVIII, recombining at YHR165C, that was previously identified in the WS34/70 strain and in another Group II strain 7012 by our group (Bond et al., 2004; Monerawela & Bond, 2017b) but which is not documented in other published sequences (Okuno et al., 2016; Wendland, 2014) was reconfirmed. Other possible rearrangements have been detected (**Figure 4.1**). There is a change in coverage that starts in an intergenic region and finishes at the *RAD17* locus (YOR368W). Another similar change has been detected at the *FRE2* locus (YKL220C), on chromosome XI (**Figure 4.1**). A possible chromosome rearrangement has been observed in Group I strain CBS1538. It is located in chromosome VII, in the *KAP144* locus.

4.2.3. Chromosome Copy Variations between mutant and wildtype strains.

Once the chromosome copy number was established, the fold change between chromosomes of the mutant strains and their respective parental strains was calculated using the custom genome (**Figure 4.2**) and CBS1483 as reference genomes (**Figure S 4.1**).

Radicicol induced aneuploidy and chromosome loss in the mutant strains with the mutant strain 9.7 experiencing the most changes (**Figure 4.2**). Three different types of chromosomal changes can be observed in the mutants. Firstly, chromosome copy changes can be observed in both strains: mutant strain 11.1 has lost a copy of Se-like chromosome V and has gained one Sc-like chromosome V copy (**Figure 4.3A**). This may be interpreted as a gain of a copy of hybrid chromosome V. There is also a reduction in copy number on Se-like chromosome XV (**Figure 4.2B**).

The second type of change is the complete loss of a parental type chromosome as observed in mutant 9.7, which has lost the Se-like chromosome XI. As this strain has a hybrid Sc-Se-like chromosome XI, mutant 9.7 has lost 336Kb of Se-like DNA, losing 166 specific Se-like genes (**Figure 4.3B**). Thirdly, loss of a fragment of a chromosome was observed in mutant 9.7. Here, the Se-like chromosomes XIII and XVI have lost part of their structure (**Figure 4.3C**). Analysis of the sequence of these two chromosomes and alignment of the regions shows that both chromosomes contain a region with high similarity (EMBOSS water) in an intergenic region. The search of this common region against the NCBI nr database shows that this common region contains a Long Terminal Repeat (LTR) similar to Tsu4 (**Table 4.5**). Thus, the copy number changes can be interpreted as the formation of a new reciprocal translocation between XIII and XVI. This new recombination may have led to a new chromosome with approximately 367kb of Se-like chromosome XIII and 350 kb of Se-like chromosome XVI.

Table 4.5. Recombination points detected in both parental and mutant strains. In grey, recombination point detected in the strain. In white, recombination point not found.

CBS1538	11.1	WS 34/70	9.7	
grey	grey	white	white	I - ACS1
white	white	grey	grey	III - MATα
white	white	grey	grey	IV - RPL35A
grey	grey	white	white	V - *CHD1*
grey	grey	white	white	V - *RPS24A*
grey	grey	white	white	VII - *KAP144*
grey	grey	grey	grey	VII - *XRN1*
grey	grey	grey	grey	VII - *ZUO1*
white	white	grey	grey	VIII - *PRP8*
white	white	grey	grey	X - *TDH2*
white	white	grey	grey	XI - *PRI2*
grey	grey	white	white	XI - *TOR1*
grey	grey	white	white	XI - *VMA5*
white	white	grey	grey	XII - *VIP1*
grey	grey	white	white	XIII - *FKS3*
white	white	white	grey	XIII - Tsu4-like
white	white	grey	grey	XIII - *YME2*
white	white	grey	grey	XVI - *GPH1*
white	white	grey	grey	XVI - *HSP82*
white	white	grey	grey	XVI - *QCR2*
white	white	white	grey	XVI - Tsu4-like

The copy number changes in mutant 9.7 were confirmed by qPCR of genomic DNA (**Figure 4.4A**). Selected chromosomes were analysed, confirming the new copy number of the strain. PCR with a positive control were carried out in order to check the absence of Se-like chromosome XI in 9.7. Three different pair of primers that amplified three different regions of Se-like chromosome XI were designed. PCR showed that only positive controls amplified in mutant 9.7 and that the three pairs of primers and the positive controls amplified in WS 34/70 (**data not shown**).

Along with qPCR, a short CHEF gel run was carried out (**Figure 4.4B**). The short electrophoretic run shows new bands that may indicate the presence of new chromosomes.

The main effect of radicicol in the mutant strains is the reduction of copies of chromosomes. In the mutant 9.7, the most affected strain, there is a significant loss of Se-

like chromosomes. For example, Se-like chromosomes II-IV, IV-II, V, VII and XIV are present in one copy.
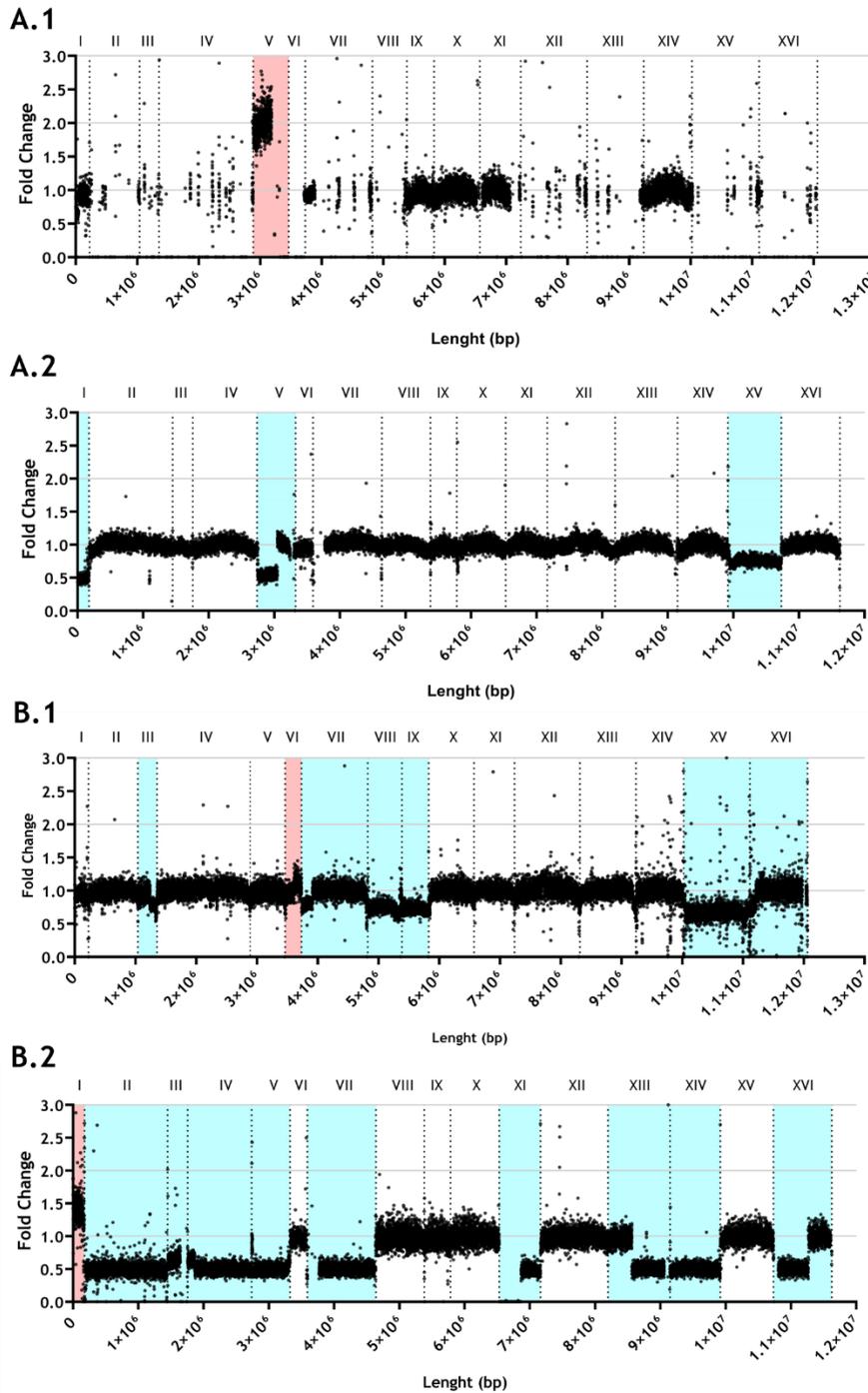
Figure 4.2. Fold change of chromosome copy number of mutants compared to their respective parental strains. In light red: chromosome gain, in light blue: chromosome loss. **A**: 11.1 compared to CBS1538. **A.1**.: *S. cerevisiae*-sub-genome, **A.2**.: *S. eubayanus* sub-

genome, **B**: 9.7 comapred to WS 34/70. **B.1**.: *S. cerevisiae* sub-genome, **B.2**.: *S. eubayanus* sub-genome.
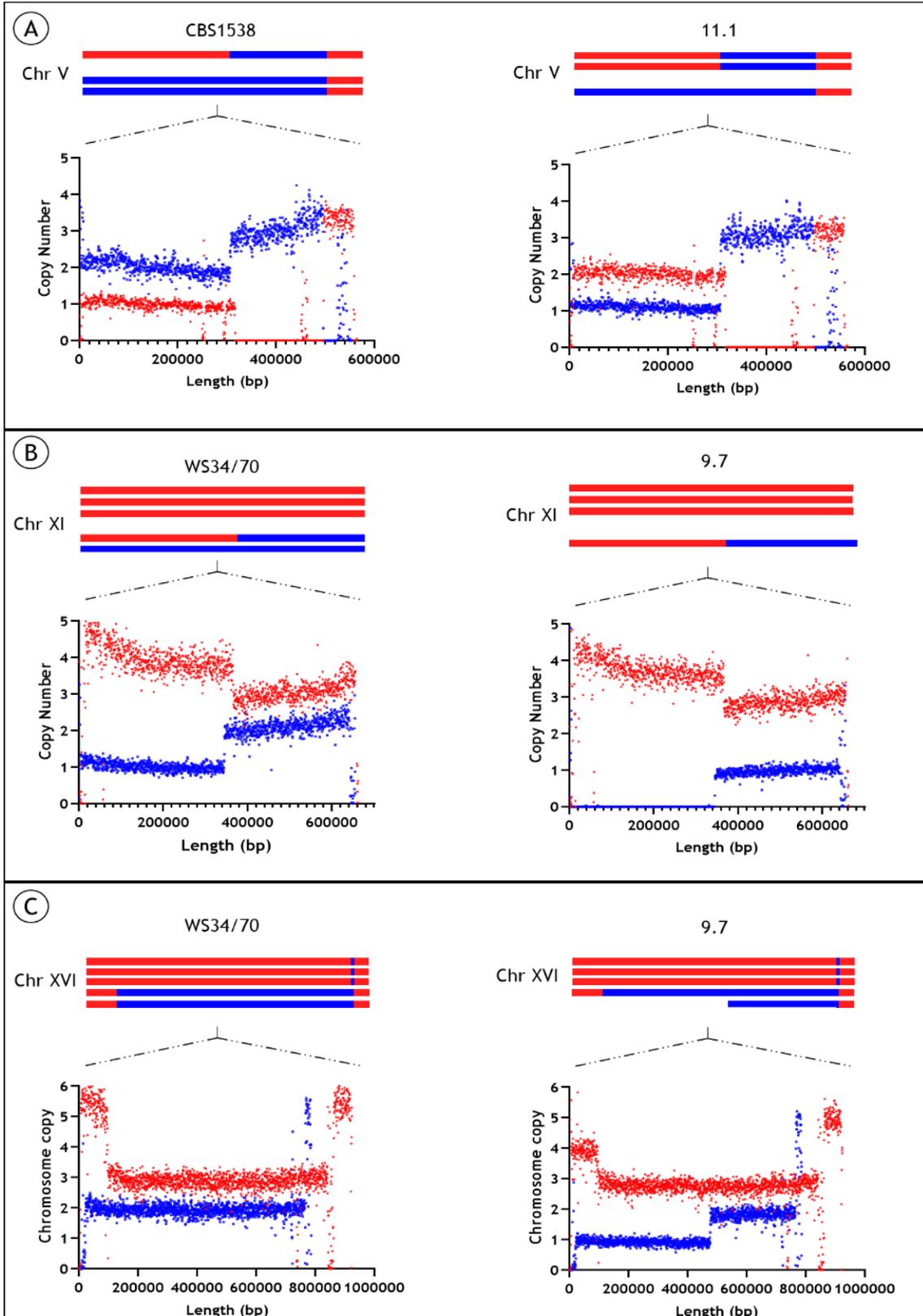
Figure 4.3. Main chromosome changes observed in both mutant strains after Radicicol treatment. **A**: formation of new hybrid chromosomes, **B**: chromosome loss, and **C**: segmental aneuploidy or reciprocal translocation.
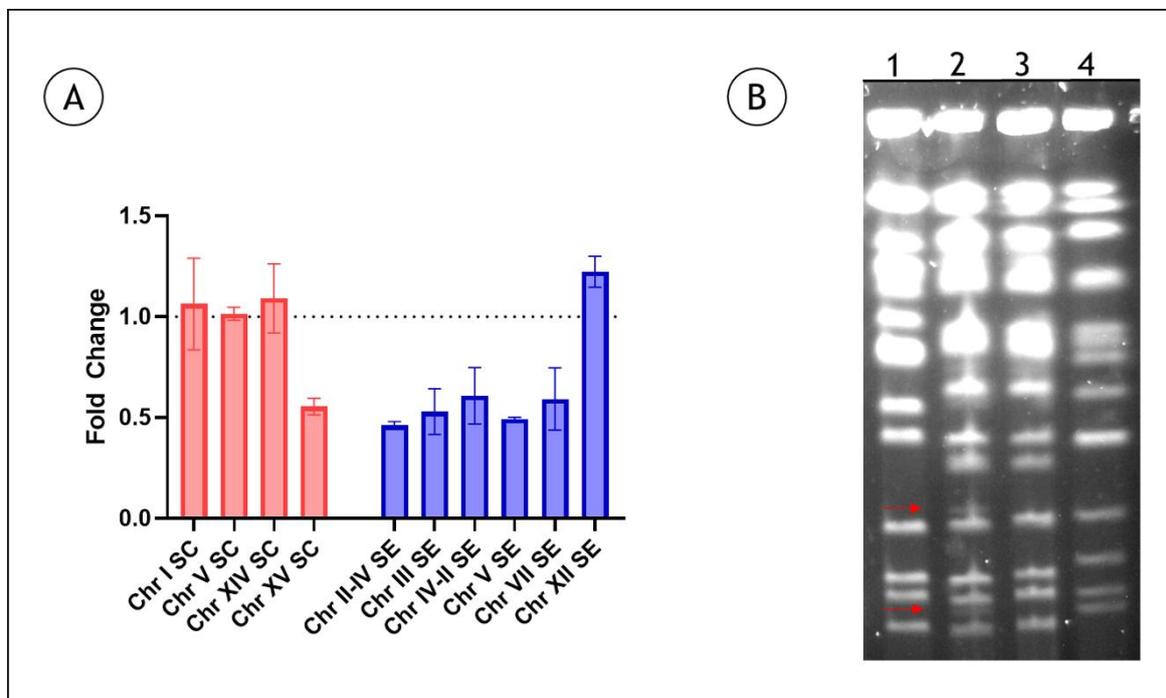
Figure 4.4. **A**: Quantification of the copy number ratio of chromosomes Sc-like chromosomes I, V, XIV, and XV, and Se-like chromosomes II-IV, III, IV-II, V, VII and XI of 9.7 compared to the Group II parental strain. Data is presented as average and standard deviation of 3 independent reactions. **B**: Short CHEF gel run, from 1 to 4: 1.- *S. cerevisiae*, 2.- Mutant 9.7, 3.- WS 34/70, 4.- *S. eubayanus.*

4.2.4. Analysis of allelic variation and gene copy number.

The CNV results show that the new strains differ in chromosome copy number compared to the parental strains. To investigate additional genomic changes in the mutant strains, an SNPs analysis was carried out. Together with the CNV analysis, SNPs analysis is useful in identifying new phenotype of the strains. For that, Samtools was used to call variants and ANNOVAR was used to annotate the new variants. SNPs with a quality value less than 20 and a value of coverage less than 90 were discarded. The SNPs analysis was carried out using the assembly and annotation of CBS1483 due to the more similar

annotation between the strains used in this study and higher number of annotated paralogues that belong to sugar metabolism, like *HXT* genes and *MAL* loci.

The Group I mutant strain 11.1 showed 134 new allelic variants, of which 21 (15.67%) produce a nonsynonymous change in coding regions. Group II mutant, 9.7, has a higher number of SNPs, at 562 in total of which 105 (18.68%) are nonsynonymous changes in coding regions (**Figure 4.5**).
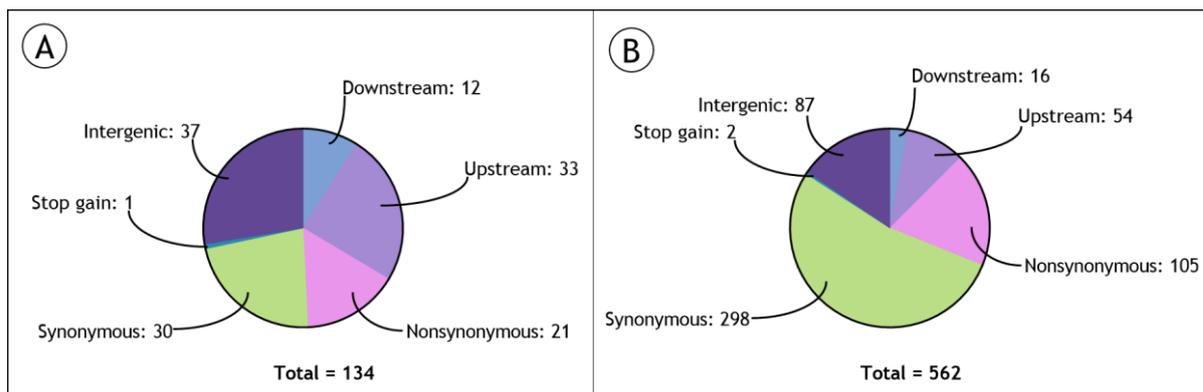
Figure 4.5. SNPs found in both mutant strains. A: Group I mutant 11.1, B: Group II mutant 9.7.

The analysis of nonsynonymous changes in coding regions revealed two mutations located in genes that are involved in the Shikimate Pathway. Both strains contain a mutation in *ARO4*, a 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase that catalyse the first step of the Shikimate pathway. Mutant 11.1 contains a mutation (D22Y) in Se-like *ARO4* while mutant 9.7 has a mutation in the Sc-like allele (S195F). Both mutations give a nonsynonymous change in two different amino acid positions of the protein. The mutation 584C>T in *ARO4* of mutant 9.7 was checked by Sanger sequencing. Specific primers were designed to discriminate between the Sc-like and Se-like alleles of *ARO4*. The promoter and the whole gene were sequenced in fragments of approximately 800bp. The sequencing shows that the *ARO4* Sc-like S195F mutation was present, and that the promoter nor the rest of the gene did not have any mutation.

Other SNPs were found in both mutant strains, but none was related to phenylalanine metabolism other than *ARO4*. Enrichment of the SNPs was carried out (**Table 4.6**). Other SNPs not included in the enrichment analysis but that may be interesting are the ones

found in *MAL31_1*, *MAL33_1* and *AGP1*. *MAL31* and *MAL 33* belong to the same *MAL* locus, and *MAL31* encodes for a maltose transporter and *MAL33* codifies for a transcriptional activator involved in maltose metabolism. *AGP1* is a low-affinity amino acid permease.

Table 4.6. Categories of the nonsynonymous SNPs found in mutants 11.1 and 9.7.

KEGG ID	11.1	9.7
Galactose metabolism		*IMA2*
Fatty acid elongation		*ELO2*
Purine metabolism		*ADE12, APA1*
Alanine, aspartate and glutamate metabolism		*ADE12*
Valine, leucine and isoleucine biosynthesis		*ILV6, LEU2*
Histidine metabolism		*HIS4*
Phenylalanine, tyrosine and tryptophan biosynthesis	*ARO4*	*ARO4*
Phosphonate and phosphinate metabolism		*EPT1*
Starch and sucrose metabolism		*IMA2*
Inositol phosphate metabolism		*INP51*
Glycerophospholipid metabolism		*EPT1, PLB1*
Ether lipid metabolism		*EPT1*
Pyruvate metabolism		*MLS1*
Glyoxylate and dicarboxylate metabolism		*MLS1*
Butanoate metabolism		*ILV6*
C5-Branched dibasic acid metabolism		*ILV6, LEU2*
Thiamine metabolism	*THI20*	*NFS1, THI21*
Pantothenate and CoA biosynthesis		*ILV6*
Sulfur metabolism		*APA1*
Biosynthesis of unsaturated fatty acids		*ELO2*
ABC transporters		*PDR11*
Ribosome biogenesis in eukaryotes	*RIA1*	*REX2*
RNA degradation		*PAT1*
MAPK signaling pathway		*SSK22*
Phosphatidylinositol signaling system		*INP51*
Cell cycle	*WHI5*	
Sulfur relay system		*NFS1*
Protein processing in endoplasmic reticulum		*PDI1*
Endocytosis		*SNX4*

This study focuses on the SNPs found in *ARO4* due to the function of this gene to the biosynthesis of aromatic amino acids. To investigate the structure of the new enzymes, the isoforms of *ARO4* were examined using AlphaFold in the colab environment of

Google Drive (Jumper et al., 2021). The *in silico* analysis of the new allelic variants did show slight differences between the new isoform and the wildtype protein, especially in mutant 11.1 (Figure 5A). The model of the new isoform shows that mutation D22Y partially perturbs the formation of a short two-stranded β-sheet located in the tail of the protein. In the new Sc-like isoform, the amino acid change has not induced major changes despite the change from a serine (polar) to a phenylalanine (nonpolar) (**Figure 4.6**).
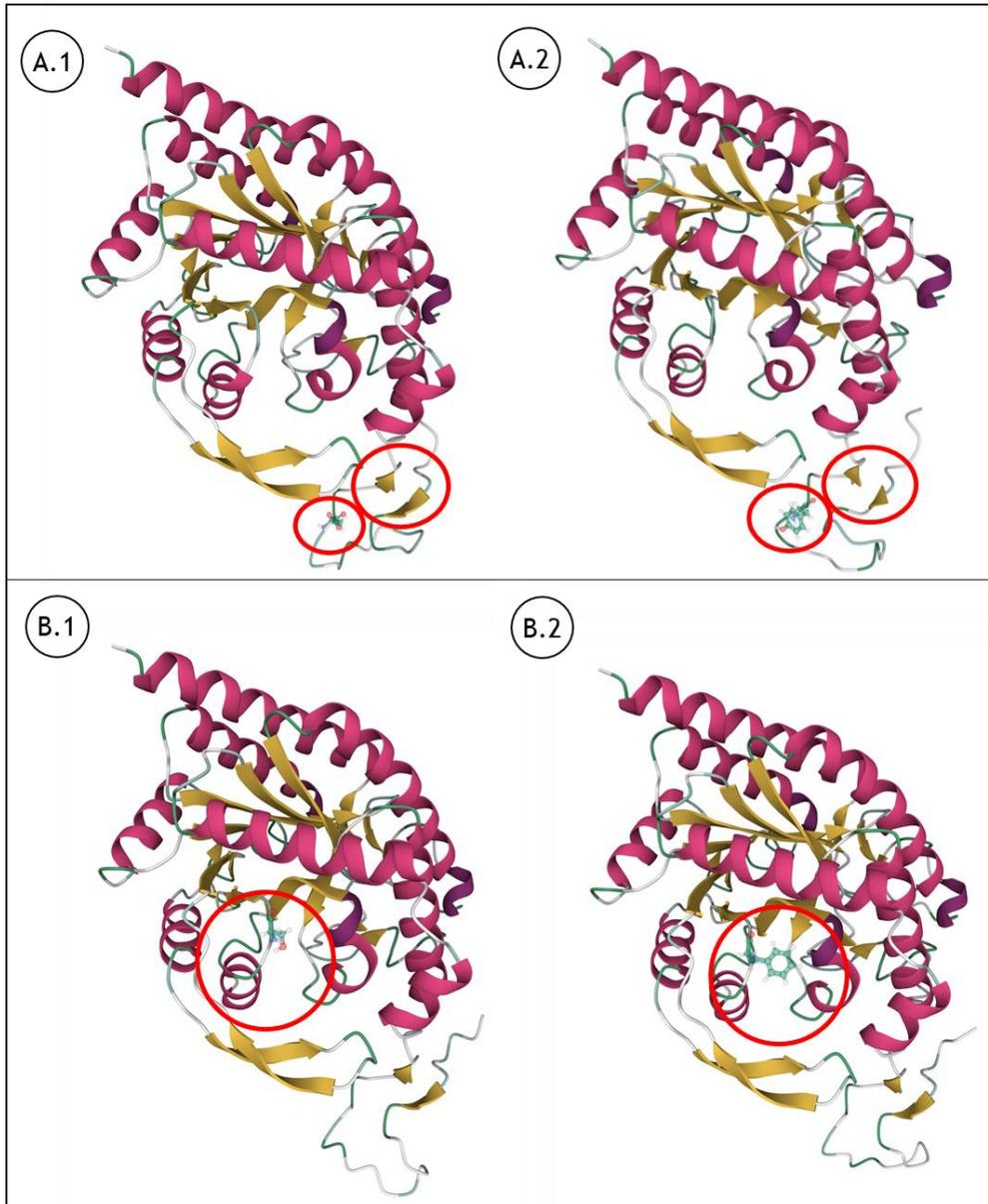
Figure 4.6. Protein tertiary structure of Aro4p. **A.1**: Se-like Aro4p wildtype, **A.2**: Se-like Aro4p D22Y, **B.1**: Sc-like Aro4p wildtype, **B.2**: Sc-like Aro4p S195Y

Once the chromosome copy number and allelic variant analyses were completed, the gene copy number for genes involved in the amino acid metabolism was calculated.

The coverage of all the genes was extracted and normalized using Samtools and custom Python scripts. This analysis reveals that genes that are involved in the Shikimate Pathway and aromatic amino acid catabolism are present in different copy number between Group I and Group II parental strains.

The mutants do not show extra copies of the *ARO4* genes but have a different mutant to wildtype allele frequency. Mutant 9.7 has two copies of the new allele and one of the wildtypes while mutant 11.1 has only one copy of the new allele and two of the wildtype. Copy number differences in genes in the Shikimate and aromatic amino acid catabolism pathways are observed in the mutant strains. Se-like alleles of the genes involved in tryptophan biosynthesis are present in lower copy in mutant 9.7 (9.7: *ARO3_1*, *ARO4_2*, *ARO2_2*, *PHA2_2*, *TRP2_2*, *TRP3_2*, *TRP4_2*, *TRP5_2*, *TYR1_2*, *ARO9_1, ARO8*) (**Table 4.7**).

The deletion of the Se-like chromosome XI means that the mutant 9.7 is missing 167 Se-like alleles. All of them have corresponding Sc-like orthologues, therefore the strain is not missing any gene. An example of Se-like alleles that are missing in the mutant strain is *TRP3*, a Indole-3-glycerol-phosphate synthase. This enzyme forms a bifunctional hetero-oligomeric anthranilate synthase:indole-3-glycerol phosphate synthase enzyme complex with *TRP2*.

Table 4.7. Estimated gene copy number of the genes that participate in the Shikimate pathway and aromatic amino acid catabolism. Hyphen means that genes have two different allelic variants. The number before the hyphen means number of copies of the

wildtype gene and number after the hyphen number of copies of the new alleles. "_1" means Sc-like and "_2" means Se-like.

Gene	Chromosome	CBS1538	11.1	WSWT	9.7
ARO3_1	ScIV	0	0	3	2
ARO3_2	SeII-SeIV	3	3	2	2
ARO4_1	ScII	0	0	3	2-1
ARO4_2	SeIV-SeII	3	1-2	2	1
ARO1_1	ScIV	0	0	3	3
ARO1_2	SeII-SeIV	3	3	2	2
ARO2_1	ScVII	0	0	3	3
ARO2_2	SeVI	3	3	2	1
ARO7_1	ScXVI	0	0	3	3
ARO7_2	SeXVI	3	3	2	2
PHA2_1	ScXIV	1	1	4	4
PHA2_2	SeXIV	2	2	2	1
TRP2_1	ScV	0	0	2	2
TRP2_2	SeV	3	3	2	1
TRP3_1	ScXI	0	0	4	4
TRP3_2	SeXI	3	3	1	0
TRP4_1	ScIV	0	0	3	3
TRP4_2	SeII	3	3	2	1
TRP5_1	ScVII	0	0	3	3
TRP5_2	SeVII	3	3	2	1
TYR1_1	ScII	0	0	3	3
TYR1_2	SeIV	3	3	2	1
ARO8	SeVII-ScVII	3	3	5	4
ARO9_1	ScVII	0	0	4	3
ARO9_2	SeXV-SeVIII	3	3	1	1
ARO10_1	ScIV	0	0	3	3
ARO10_2	SeII-SeIV	3	3	2	2
ARO80_1	ScIV	0	0	3	3
ARO80_2	SeII-SeIV	3	3	2	2

4.3. Discussion.

4.3.1. Assembly of parental strains using a reference genome.

De novo assembly of *S. pastorianus* strains is a difficult task due to presence of two similar sub-genomes in the same cell, the presence of high chromosome copy numbers and the presence of hybrid chromosomes. Here, we followed two different approaches to map the reads obtained after sequencing by Illumina. Firstly, we used the already sequenced, assembled and annotated CBS1483 Group II strain as the reference genome as it contains almost all the Sc-like and Se-like chromosomes. However, this strain has different hybrid chromosomes compared to the strains used in this study, so it is more difficult to understand the chromosome rearrangements present in CBS1538 and WS 34/70.

Secondly, we used a customised genome as a reference genome which does not contain any hybrid chromosomes. For that, the genomes of *S. cerevisiae* and *S. eubayanus*, for which assembly and annotation files can be found on NCBI database, were used. This allowed an easier detection of recombination breakpoints and an easier interpretation of the chromosomes in both Group I and Group II strains.

CCN was calculated for the parental and mutant strains using the custom genome. The mapping rate was more than 93% and coverage of the genome was sufficient. The coverage allowed the estimation of the CCN and gene copy number of all the strains.

The strain CBS1538 used in this study is an allotriploid strain that contains a reduced Sc-like sub-genome. This data agrees with the results obtained in previous studies (Okuno et al., 2016). Okuno et al. (2016) also revealed chromosome content differences between the strains within Group I strains. Two sub-groups were identified based on the *S. cerevisiae* sub-genome. It is not clear why CBS1538 maintains this reduced set of Sc-like chromosomes, but it may indicate that these Sc-like chromosome copies confer an advantage to the strain.

It has been previously reported that even different isolates of WS 34/70 have different CCN (van den Broek et al., 2015). The WS 34/70 strain used in this study has a different estimated ploidy compared with the same strain used in the study carried out by van den Broek et al. (2015) or isolates analysed on other previous studies (Okuno et al., 2016;

Walther et al., 2014). Furthermore, the coverage of the Group II parental strain WS 34/70 indicated that chromosome IV has recombined by the RPL35A locus.

These changes in CCN and hybrid chromosomes, not only between isolates, but also between different strains of *S. pastorianus*, may indicate that *S. pastorianus* strains are still evolving, and that this complex genome is unstable. The presence of two sub-genomes with high similarity shows that recombination within orthologs may be an advantage for these strains. The recombination between orthologs might be helpful for the strains to avoid chromosome loss and deal with aneuploidy. It has been reported that aneuploidy in cells might be useful to adapt to different stresses (Gorter de Vries et al., 2017). Furthermore, these recombinations lead to the generation of new allelic variants that might be advantageous for the strains.

4.3.2. Radicicol induced aneuploidy in the mutants.

Here, we described the first results of the effects of Radicicol in aneuploid hybrid *S. pastorianus* strains. Sequencing of the strains revealed that Radicicol induced changes in the genome. Chromosome instability after Radicicol treatment was reported previously (Chen et al., 2012). Chen *et al* tested this antibiotic in a diploid *S. cerevisiae* strain and the exposure to this antibiotic showed that resistant strains to Radicicol displayed a gain of copy number for chromosome XV. This chromosome contains two genes that may be linked to this resistance to this antibiotic: *STI1* and *PDR5*. *STI1*, also called *HSP90*, encodes for a conserved chaperone and it is the main target of Radicicol. The inhibition of this chaperone induces chromosome instability as this chaperone folds proteins involved in DNA repair and kinetocore formation. *PDR5* is a plasma membrane ATP-binding cassette (ABC) transporter, and it might be involved in the detoxification of Radicicol. Therefore, another copy of chromosome XV may increase the copy of these two genes, thus, increasing the protein level of Hsp90p and Pdr5p.

In this study, as previously described in chapter 3, Radicicol did not induce any growth inhibition in *S. pastorianus* strains. This might be due to the higher copy of chromosome XV in both strains compared to the diploid strain used by Chen et al. (2012). CBS1538 has three Se-like copies and WS 34/70 has three Sc-like copies and one Se-like copies. Surprisingly, the mutant 9.7 has less copies of *STI1*. Gene copy number analysis shows that mutant 9.7 has one copy less of the Sc-like and the Se-like *STI1*.

Chromosomal copy number was calculated, and results show different types of aneuploidy. Firstly, different degrees of aneuploidy have been detected in both mutants 11.1 and 9.7. Secondly, there is a complete loss of chromosome XI in 9.7. Finally, the data suggests the formation of new hybrid chromosomes and a possible chromosome rearrangement, or by default, segmental aneuploidy in mutant 9.7.

There is not a common pattern on chromosome loss nor chromosome gain after the Radicicol treatment. Mutant 9.7 shows a high degree of aneuploidy after the evolution and a greater resistance to the antibiotic Radicicol compared to mutant 11.1. It is not well understood how strains can cope with aneuploid genomes. Aneuploidy has been detected in other organisms and the effect that it produces is different between them. In humans, aneuploidy has negative consequences mainly, and diseases and disorders are observed in individuals with aberrant karyotype (Ben-David & Amon, 2020; Hassold & Hunt, 2001). In yeast, negative effects have been reported like an increase in proteotoxic stress, reduced fitness or delay in growth (Brennan et al., 2019; Dephoure et al., 2014; Sheltzer et al., 2011; Sheltzer et al., 2012; Thorburn et al., 2013; Torres et al., 2010; Torres et al., 2007). Nevertheless, aneuploidy has been proposed as an evolutionary pathway to adapt to different stresses. Several industrial traits are connected to extra chromosome copies. Therefore, aneuploid strains may be able to deal with an aberrant number of chromosomes and maintain them as they might give a different advantage to the strains (Gilchrist & Stelkens, 2019; Gorter de Vries et al., 2017).

The mutant 9.7 has loss a Se-like chromosome XI, and only hybrid chromosome XI and Sc-like chromosome XI remain in the cell. This loss entails a reduction in the total number of alleles. Examples of these genes are *TRP3*. Trp3p forms a complex with Trp2p, and both catalyse the first step of tryptophan biosynthesis. Now that Se-like *TRP3* is no longer in the genome of mutant 9.7, the probability of the formation of chimeric complexes between Sc and Se-like alleles may increase. Previously results show that a hybrid Trp2p/Trp3p complex may have a positive effect in cell fitness (Piatkowska et al., 2013) and recent results have hypothesised that protein complexes in *S. pastorianus* strains can formed by using Sc-like or Se-like subunits (Timouma et al., 2021). In mutant 9.7, Se-

like *TRP2* is present in one copy. Therefore, it may be interesting to study whether the Trp2p-Trp3p complex is chimeric or uni-specific.

Interestingly, a possible chromosome rearrangement has been detected in the Group II mutant 9.7. Analysis of the sequence have shown that areas where the chromosomes Se-like XIII and XVI broke does not contain any ORFs. Alignment analysis has shown a region with high similarity and further analysis of this region showed the presence of a sequence highly similar to the LTR Tsu4 transposon. The LRT Tsu4 have been detected in *S. uvarum* and in the hybrid *S. bayanus*. Moreover, this LTR has been detected in other species of the genus *Saccharomyces*, such as *S. paradoxus*, and it has been hypothesised that this species acquired this LTR by Horizontal Transposon Transfer (Bergman, 2018; Drouin et al., 2021). The LTR-like sequence detected in Group II *S. pastorianus* strains is located on chromosomes SeXIII and SeXVI, and both chromosomes experienced a loss of a segment by this LTR-like sequence. This suggests that these two chromosomes might have recombined by the LTR-like Tsu4. Recombination mediated by transposons have been observed before in *S. cerevisiae* and in other eukaryote species, like *Drosophila* (Kim et al., 1998; Lim & Simmons, 1994; Mieczkowski et al., 2006). These repetitive elements might reshape the chromosome structure and might be recombination hotspots involved in genome instability and evolution.

An alternative hypothesis for the loss of segments of chromosomes XIII and XVI is segmental aneuploidy, which has been previously observed in different organisms. For example, different human cell types like oocytes, embryos and blastocyst experience segmental aneuploidy (Escriba et al., 2019). Loss of chromosomes and generation of new ones is a process that is strictly controlled in eukaryotes and is rarely observed. In *Candida glabrata*, a yeast that can be a pathogen to humans, generation of new chromosomes have been observed. Karyotyping of different isolates have shown the different size of chromosomes due to chromosome recombination and segmental duplications. As a consequence, new chromosomes were observed (Polakova et al., 2009).

The short CHEF gel run shows new bands. These new bands may indicate that recombination between chromosomes have occurred and/or new chromosomes have been generated. The CHEF run indicates new bands with a lower intensity (less copies). Further analysis is needed to understand the segmental aneuploidy observed in mutant 9.7 as it could be a new chromosomal recombination.

4.3.3. Mutants display new allelic variants.

SNPs have been identified in both strains. These nucleotide changes have been detected along the genome. These mutations can be observed in exonic regions, upstream or downstream of these coding regions. Both synonymous and non-synonymous changes were detected.

Several mutations that induce a nonsynonymous change in proteins are found in both mutant strains. Mutations in *ARO4*, an enzyme that catalyse the first step of the Shikimate pathway. Aro4p condensates erythrose-4-phosphate and phosphoenolpyruvate (PEP) into 3-deoxy-D-arabino-heptulosonate-7- phosphate (DAHP) were found in both mutants. This β/α barrel enzyme is regulated by feedback inhibition of the final product of the pathway, the aromatic amino acids phenylalanine, tyrosine and tryptophan. Mainly, Aro4p is inhibited by tyrosine, but also higher concentrations of the other two aromatic amino acids can inhibit the enzymatic activity. The mutations were found in the *ARO4* Sc-like copy in mutant 9.7 and in the *ARO4* Se-like copy in mutant 11.1. The nucleotide changes produce amino acidic changes in the protein. In both mutants, apolar amino acids are introduced in the respective enzymes. In silico analysis shows subtle differences in the variants. The new amino acid substitutions S195F in Sc-like Aro4p in mutant 9.7 and D22Y in Se-like Aro4p in mutant 11.1 may have an impact in negative feedback regulation of the respective enzymes. Both mutations exchange amino acids with different structures, from polar to apolar. The presence of aromatic rings as side chains may change the conformation of the enzyme. The analysis shows that the aromatic rings occupy space that might be important for the negative feedback regulation. Further analysis is needed.

89

These mutations have been previously described as mutations that make the enzyme insensitive to the feedback inhibition by the amino acids (Cordente et al., 2018; Hartmann et al., 2003). Cordente et al. (2018) successfully isolated mutants overproducing 2-phenylethanol and 2-phenylethyl acetate using amino acid analogues in the *S. cerevisiae* wine strain, AWRI796. Different mutations in Aro4p showed different production of 2-phenylethanol and 2-phenylethyl acetate. The present study reports the same mutations reported by Cordente et al. (2018), D22Y in mutant 11.1 and S195F in mutant 9.7. This may indicate that this specific change is responsible for the insensitivity of Aro4p against the feedback regulation and that other amino acid in the same position might not make Aro4p insensitive to the negative feedback regulation. Furthermore, the S195F mutation has been detected in heterozygosity. We tried to introduce this mutation in the haploid laboratory strain BY4741, but it was not successful. This could mean that this mutation could be lethal in homozygosis.

Also, other mutations that release Aro4p from the negative feedback inhibitory regulation have been reported (Hartmann et al., 2003). The study carried out by Hartmann et al. (2003) has discovered 13 different residues that are linked to a reduced feedback inhibition of the enzyme, such as D22G and S195P. The first residue is located in the N-terminal tail of the enzyme, near to a β strand that interacts with other β sheets. The second residue is located in the connector region between the half-sites of the regular barrel. The mutation detected in mutant 11.1 is located in the Se-like Aro4p. This, together with the high similarity between Sc-like and Se-like Aro4p (97.8%) may indicate that Se-like Aro4p has conserved the same amino acid positions that are involved in the negative feedback regulation in Aro4p.

Other nonsynonymous mutations have been detected in the mutants. In order to understand these new mutations, further analysis have to be done. Results from Chapter 3 shows that mutant 9.7 overproduces isoamyl acetate, and also, this strain consumes more sugars compared to WS 34/70. Nonsynonymous mutations have been detected in *LEU2* and *MAL*. To correlate these nucleotide changes, further analysis needs to be done.

4.4. Conclusions.

Analysis of the genome of the strains showed interesting findings. The complex genome of *S. pastorianus* strains is still evolving. We detected this in the different chromosome copy number of the isolates of the strains use in this study. Furthermore, new recombination breaks were found in the parental strain WS 34/70. The mutant strains obtained after the treatment with Radicicol confirmed the plasticity of the genome. This antibiotic induced instability in the cell and, therefore, chromosome copy changes were detected. Also, different SNPs were found in both mutant strains. All these changes may be linked to the overproduction of higher alcohols derived from aromatic amino acid catabolism.

4.5. Supplemental figures and tables

Table S 4.1. Estimated Chromosome copy number and gene copy number of the mutant strains and their respective wildtypes. Hyphen indicates a recombination point.

Chromosome	Size (Mb)	Estimated Chromosome copy number				Estimated gene copy number			
		WS 34/70	9.7	CBS1538	11.1	WS 34/70	9.7	CBS1538	11.1
ScI	0.21	2	2	1	1	96	96	90	90
ScII	0.81	3	3	0	0	394	394	9	9
ScIV	1.48	3	3	0	0	742	742	2	2
ScV	0.59	2-3	2-3	0	0	268	268	174	176
ScVI	0.26	3	3	1-0-3	2-0-3	122	122	2	2
ScVII	0.86	3	3	0	0	435	435	0	0
ScVIII	0.55	4	3	0	0	268	267	1	1
ScIX	0.43	3	2	1	1	203	203	196	196
ScX-SeX	0.77	2-2	2-2	1	1	365	365	360	361
ScXI	0.66	2	2	1	1	321	321	224	224
ScXII	1.14	3	3	0	0	469	468	4	3
ScXIII	0.87	3	3	0	0	430	430	1	1
ScXIV	0.78	2	2	1	1	389	389	385	385
ScXV-ScXI	1.06	3	2	0	0	508	506	2	2
ScXVI	0.93	3	3	Just telomeres		455	455	39	38
SeI	0.18	2	3	2	1	78	79	70	68
SeII-SeIV	1.28	2	1	3	3	621	621	621	621
SeIII-ScIII	0.31	3	2	3	3	149	149	100	100
SeIV-SeII	1	3-2	2-1	3	3	489	486	489	490
SeV	0.58	2	1	2-3-0	2-3-0	279	278	258	258
SeVI	0.27	3	3	3	3	125	125	125	125
SeVII-ScVII	1.05	2	1	3	2	516	513	516	516
SeVIII-SeXV	0.81	2	2	4	4	392	392	394	394
SeIX	0.41	1	1	2	2	203	203	202	202
SeX-ScX	0.7	1-2	1-2	2	2	335	332	329	329
SeXI	0.66	1-2	0-1	2	2	309	144	310	310
SeXII	1.04	2	2	3	3	473	473	474	474
SeXIII-ScXIII	0.97	2	2-1	3	3	470	468	456	456
SeXIV	0.77	2	1	2	2	377	376	376	376
SeXV-SeVIII	0.75	1-2	1-2	3	3	368	368	371	371
SeXVI	0.79	2	1-2	3	3	380	379	379	379
						11029	10847	6959	6959
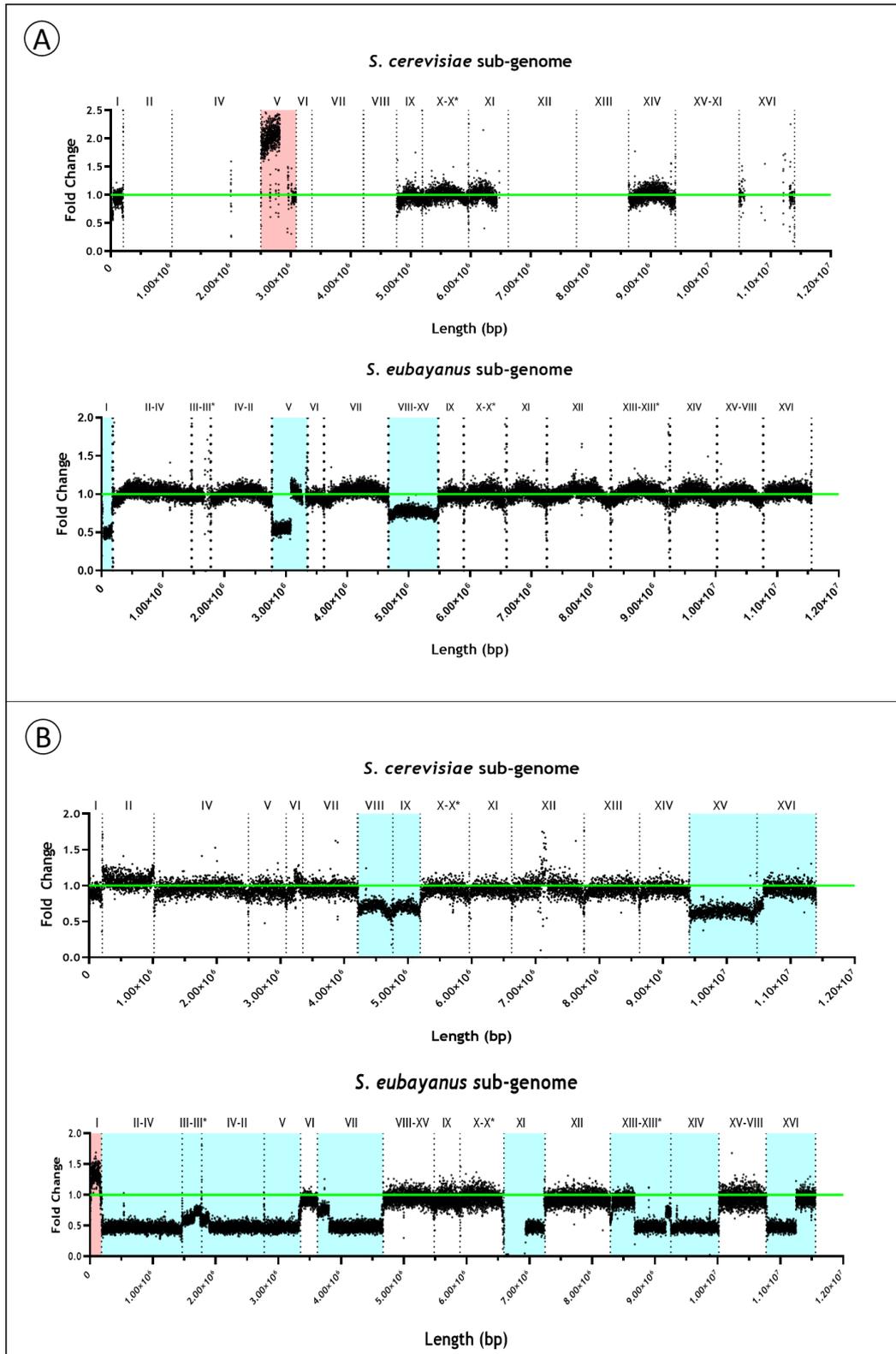
Figure S 4.1. Fold change of chromosome copy number of mutants compared to their respective parental strains. In light red: chromosome gain, in light blue: chromosome

loss. A: 11.1 compared to CBS1538, B: 9.7 vs WS 34/70. Astherisc means chimeric chromosomes (Sc-Se or Se-Sc).

Chapter 5

Analysis of the transcriptome of the mutants

5.1. Introduction.

Brewing is the process where wort, liquid rich in sugars and nitrogen nutrients, is transformed into beer. Wort is a medium that contains a wide range of sugars, such as maltose, the main sugar present in wort, maltotriose or monosaccharides like glucose and fructose (Briggs et al., 2004). During the fermentation process, yeast transform these sugars into ethanol and CO_2. Depending on the brewing process, beer can be classified into two main groups. "Ale" style, which its fermentation is carried out by *S. cerevisiae* strains (also known as top-fermenters), is produced at temperatures that ranges from 15ºC to 26ºC. "Lager" style uses *S. pastorianus* strains to carry out the fermentation. This type of beer is brewed under lower temperatures that range from 4ºC to 14ºC (Pavsler & Buiatti, 2009). The yeast used in this process sink into the bottom of the vat, receiving the name of bottom-fermented strains.

During the process of lager brewing, yeast transforms part of the sugars and nitrogen compounds into flavour molecules that give the characteristic crisp and clean flavour that characterise this type of beer (Dzialo et al., 2017; Lin et al., 2021). Yeasts are able to produce different flavour profiles, and of these, higher alcohols and esters are the most important ones (Hazelwood et al., 2008). Higher alcohols are secondary metabolites obtained from the secondary metabolism of yeast. These molecules come from the catabolism of aromatic, branched-chain and sulphur-containing amino acids. The side chain of these amino acid cannot be diverted to the central carbon metabolism. Therefore, they are converted into higher alcohols via the Ehrlich pathway. This pathway has three steps that transform the amino acids into higher alcohols. The first step involves a transamination of the amino acid to produce a molecule of an α-keto acid and a molecule of glutamate that will divert to the nitrogen metabolism. The second step involves a decarboxylation of the α-keto acid to produce a fusel aldehyde. Then, depending on the redox state of the cell, this fusel aldehyde may be oxidised to a fusel acid, or more conveniently, reduced to a higher alcohol (Hazelwood et al., 2008; Pires et al., 2014).

Each of the nine possible amino acids that are catabolised via the Ehrlich pathway produce a different higher alcohol, which imparts a characteristic flavour. Not all of these higher alcohols impart positive notes, such as pineapple, roses, banana or honey-like

aromas. Some of them, specifically the ones that come from sulphur-containing amino acids impart off flavours (Dzialo et al., 2017).

These higher alcohols can be transformed into acetate esters via a condensation reaction between higher alcohols and a molecule of Acetyl-CoA (Holt et al., 2019; Lin et al., 2021; Verstrepen et al., 2003; Yoshimoto et al., 1999).

Several enzymes can catabolise the same step in the Ehrlich pathway. For example, the first steps of the pathway are catalysed by enzymes with a higher affinity for the different families of amino acids that are subjected to this pathway. For example, transaminases Aro8p and Aro9p, and decarboxylase Aro10p have a higher affinity for aromatic amino acids, while transaminases Bap1p and Bap2p have a higher affinity for branched-chain amino acids (Dickinson et al., 2000; Vuralhan et al., 2003).

Nitrogen metabolism is a complex net of reactions that is highly regulated. Transcriptional factors, sensors, permeases and negative feedback at enzymatic level are some of the tools that yeast have to control the nitrogen metabolism. Depending on the media composition, the source of amino acids may differ. When the amino acids are present in the media, yeast are able to import them via permeases and directly catabolise them. If the media does not contain amino acids but another nitrogen source, such as ammonium sulphate, cells are forced to *de novo* synthesis amino acids. Then, amino acid biosynthesis is regulated by the General Amino Acid Control (GAAC) superpathway (Ljungdahl & Daignan-Fornier, 2012).

The aim of this chapter is to investigate the transcriptome of two mutant strains that overproduce the aromatic compounds 2-phenylethanol and 2-phenylethyl ester in minimal medium without amino acids and in fermentative conditions, specifically on Day 2 and Day 4 of small-scale fermentations in wort. These two strains were previously obtained by accelerated evolution approaches that involved Radicicol as mutagenic agent. The exposure to this antibiotic generated a set of chromosome rearrangements and non-synonymous changes in the strains. Therefore, we aim to investigate the consequences of these changes in the strains and to study their transcriptome compared to their respective parental strains in the same conditions.

5.2. Results.

5.2.1. Mapping RNAseq reads to the reference genome.

Total RNA was extracted for two mutant strains and their respective parental strains in three different conditions: minimal medium without amino acids, and fermentation in 10ºBx wort fermentations on Day 2 and Day 4. Samples of total RNA were submitted for sequencing. After sequencing, raw reads were trimmed, and a quality filter was applied to remove those reads with not enough quality. The mapping was done against the *de novo* sequenced genomes that were mapped to the combination of *S. cerevisiae* and *S. eubayanus* reference genomes, deposited on the NCBI. *S. eubayanus* genome is not fully annotated as the annotation does not contain some important genes that were interesting for this study, for example *ARO9*. Furthermore, different alleles of genes involved in sugar metabolism like the *HXT* family, or the *MAL* loci are extensively annotated in *S. pastorianus.* Therefore, it was decided to map the reads against the reference genome of the Group II strain CBS1483. Chromosome copy number and gene copy number of the strains used in this study were calculated in the previous chapter from *de novo* sequencing round. Alleles of the Open Reading Frames (ORFs) detected in CBS1483 are notated with a "_1" after the name of a gene if it is an Sc-like allele and with a "_2" if it is a Se-like allele. Other alleles may contain other numbers than "_1" or "_2", such as "_3" or "_4". This means that extra copies have been detected in this strain, and the assignation to their respective sub-genome will be done based on the position of the ORF on the specific chromosome.

The mapping of the RNAseq reads confirmed the different chromosome composition of the Group I strains as no reads were detected in the absent Sc-like chromosomes II, IV, VIII, XII, XIII, XV and XVI. Furthermore, it was confirmed the chromosome loss of Se-like chromosome XI detected previously in mutant 9.7 (**Figure S 5.1**). In addition, appears that two of the replica samples of 9.7 may have recombined at the *XRN1* locus, located in chromosome VII. As a result, a fragment of the chromosome has been lost in two replicates of mutant 9.7, specifically sample 2 in wort Day 2 and sample 1 in Wort Day 4 Analysis of the raw reads of the replicates of all the strains analysed show a clear separation between Group I and Group II strains being the first principal component of 88% variance (**Figure S 5.2**).

A total number of 6635 transcripts were detected for Group I strains, of which 1430 are Sc-like and 5205 are Se-like. For Group II strains, total number of transcripts of 10477 were detected, of which 5212 are Sc-like alleles, 5265 are Se-like. The mutant 9.7 has 163 transcripts less due to the loss of Se-like chromosome XI.

5.2.2. Analysis of Differentially Expressed Genes.

The analysis of the mutant strains compared to the wildtype strains revealed changes in the transcriptome of the mutant strains. Only alleles with a Log_2 fold change value of $\leq$-1 or 1$\geq$ and with a P_{adj} value less than 0.05 were investigated (**Figure 5.1**). Of these two mutant strains, more Differentially Expressed Genes (DEGs) were observed in Group II mutant. Mutant 11.1 shows a very similar transcriptome compared to CBS1538. The analysis of the DEGs, more DEGs are upregulated in all three conditions tested (**Figure 5.1A**). In contrast, mutant 9.7 has more DEGs compared to the Group I mutant, and of these, the majority are downregulated. Minimal medium is the condition with more DEGs in 9.7 (n =1013) (**Figure 5.1B**).
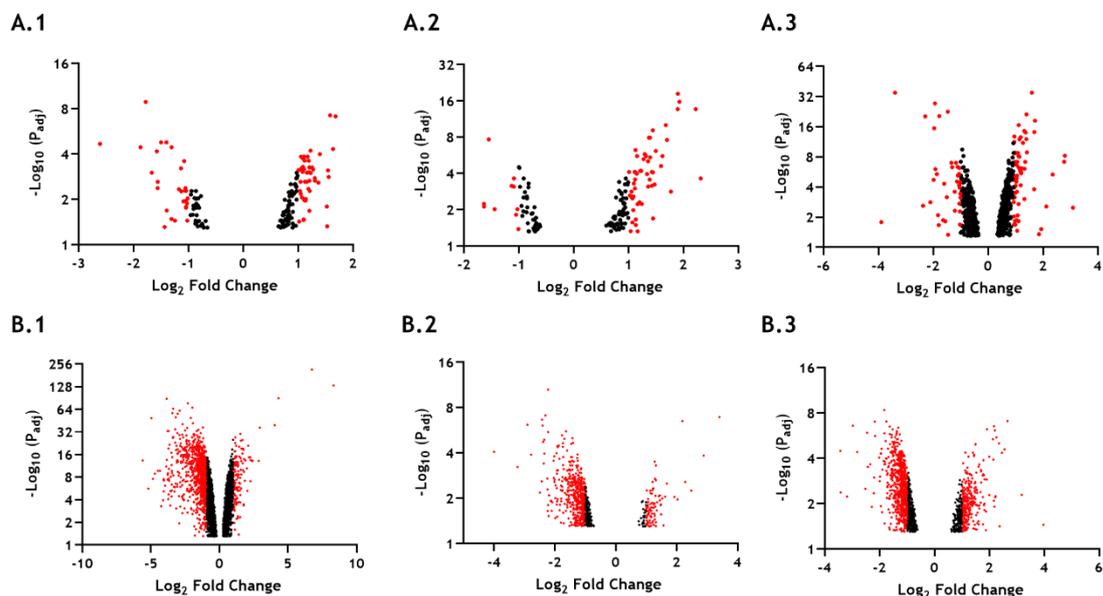
Figure 5.1. Statistically significant differentially expressed genes. In black, alleles that are significant but with a Log_2 fold change between -1 and 1. In red, alleles with a Log_2 fold change $\geq$1 or less than $\leq$-1. A) mutant 11.1 compared to its parental strain CBS1538, B) mutant 9.7 compared to its parental strain WS 34/70. "A.1" means in Minimal media, "A.2" means in wort Day 2, and "A.3" means in wort Day 4.

The *S. pastorianus* mutant strains obtained previously presented changes in chromosome copy number. Mutant 11.1 experienced a chromosome copy gain of Sc-like chromosome V, and chromosome copy loss of Se-like chromosomes I, V and XV. Mutant 9.7 experienced a higher chromosome loss rate, with loss of copies of Sc-like chromosomes VIII, XI, XV and Se-like chromosomes II-IV, III, IV-II, V, VII, XI, XIII, XIV and XVI-VIII. Only one chromosome experienced a chromosome gain, Se -like chromosome I. Hence, analysis of the proportion of Sc-like and Se-like differentially regulated alleles was carried out. The analysis of the proportion of the differentially expressed alleles showed that the differences in the transcriptome are mainly due to changes in the Se-like pool of alleles. All three conditions show a different number of DEGs and, also, the Sc:Se proportion varies within condition (**Table 5.1**). Ratios higher than one (">1") indicates that the majority of DEGs are Se-like. Ratios lower than one ("<1") indicates a higher usage of Sc-like alleles.

The analysis of the DEGs of mutant 11.1 shows a reduced number of Sc-like alleles are differentially regulated. The majority of DEGs are classified as Se-like alleles. However, mutant 9.7 shows different Se:Sc proportion of alleles between the conditions. In cells grown in minimal medium, both Sc-like and Se-like upregulated alleles are approximately the same, while mainly downregulated alleles correspond to Se-like alleles. In wort Day 2, there are twice as many Sc-like alleles upregulated compared to Se-like alleles, while the downregulated alleles are mainly Se-like alleles. In wort Day 4, there is an increase in Sc-like upregulated alleles and in Se-like downregulated alleles.

Table 5.1. Ratio of *S. eubayanus* (Se) and *S. cerevisiae* (Sc) differentially expressed genes (Log$_2$ fold change ≥ 1 or ≤ -1.0) and median of Log$_2$ fold change under the three conditions tested.

		Min		Wort Day 2		Wort Day 4	
Strain		**Sc**	**Se**	**Sc**	**Se**	**Sc**	**Se**
11.1 Up	Ratio Se:Sc	2.54		12.25		6.83	
	Median	1.13	1.17	1.22	1.28	1.15	1.20
11.1 Down	Ratio Se:Sc	13.00		4.00		3.11	
	Median	-1.25	-1.21	-1.34	-1.09	-1.46	-1.42
9.7 Up	Ratio Se:Sc	1.18		0.55		0.25	
	Median	1.3	1.18	1.18	1.21	1.21	1.27
9.7 Down	Ratio Se:Sc	2.13		16.09		16.28	
	Median	-1.56	-1.47	-1.41	-1.37	-1.24	-1.28

Further analysis of DEGs showed a low number of pairs of alleles with a discordant regulation, where one allele of a gene is upregulated, and the other allele of the gene is downregulated. In mutant 11.1, no discordant regulation has been found. Mutant 9.7 shows only two pairs of discordantly regulated alleles, *GAL7* in minimal medium and *MTH1* in wort Day 4.

To investigate the higher number of downregulated genes and the higher proportion of downregulated Se-like alleles in mutant 9.7 (**Table 5.1**), expression profiles and estimated copy of alleles were investigated. The copy number for each Sc-like and Se-like alleles was calculated from the sequence coverage from the *de novo* genome sequencing. On day 2, the highest number of downregulated DEG can be observed when ratio between gene copy number of WS 34/70 and mutant 9.7 is 2:1 (n =434) (**Figure 5.2A**). As the Sc:Se ratio decreases, the average of the Log$_2$ Fold change of the genes decreases (**Figure 5.2B**). The analysis of the majority of the downregulated genes correspond to the chromosomes that experienced copy loss. As previously stated, this strain has less copies of Se-like chromosomes II-IV, III-III, IV-II, VII-VII, XI, XIII, XIV and XVI. It can be observed that the upregulated genes of mutant 9.7 correspond to the chromosome copy gain of Se-

like chromosome I, corresponding to highest ratio (3:5). Gene dosage can be observed in the other two conditions tested in 9.7 (**data not shown**).
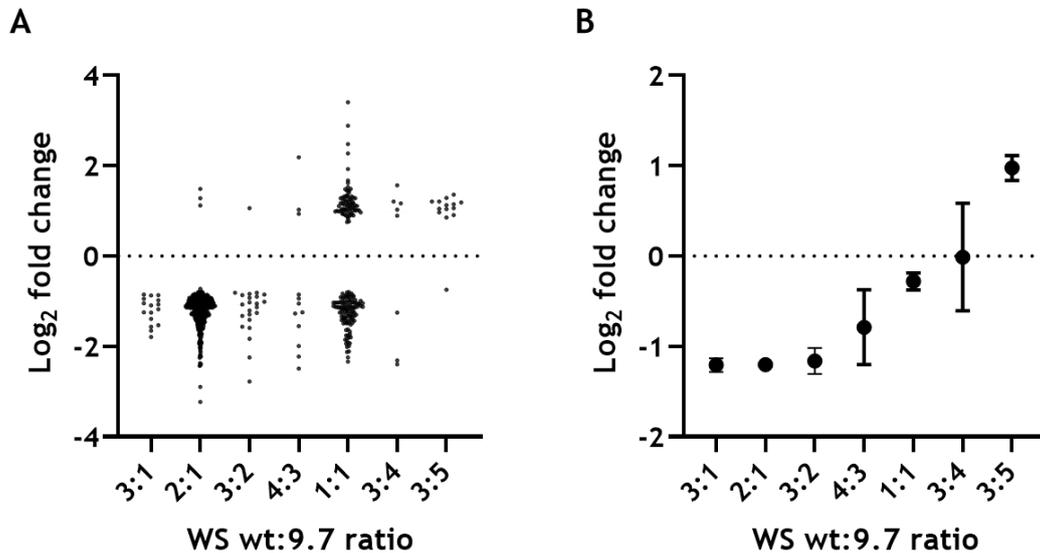
Figure 5.2. Analysis of the gene copy number on differentially regulated alleles in mutant 9.7. **A)** Grouping of differentially expressed alleles based on the different gene copy ratio. **B)** Average Log$_2$ fold change values of alleles with different gene copy ratios.

As the new strains acquired a new phenotype after the evolution, analysis of commonly upregulated genes was carried out to investigate possible differentially regulated genes that are specifically regulated in the mutants. Analysis of the commonly regulated genes showed that 11.1 has two common upregulated alleles in all three conditions. These two upregulated genes correspond to a hypothetical gene and to a Sc-like *MEP2*, an ammonium permease. There are no commonly downregulated genes in all three conditions (**Figure 5.3A**).

In contrast, 9.7 has four alleles that are commonly upregulated in all three conditions and eighty-three commonly downregulated terms in all three conditions (**Figure 5.3B**). The four upregulated terms are both Sc-like and Se-like alleles of *ARO9*, Sc-like *MET32* and Se-like *DEP1_2*. Of the downregulated genes, there is only one pair of alleles commonly downregulated in all three conditions, *SNO3*.
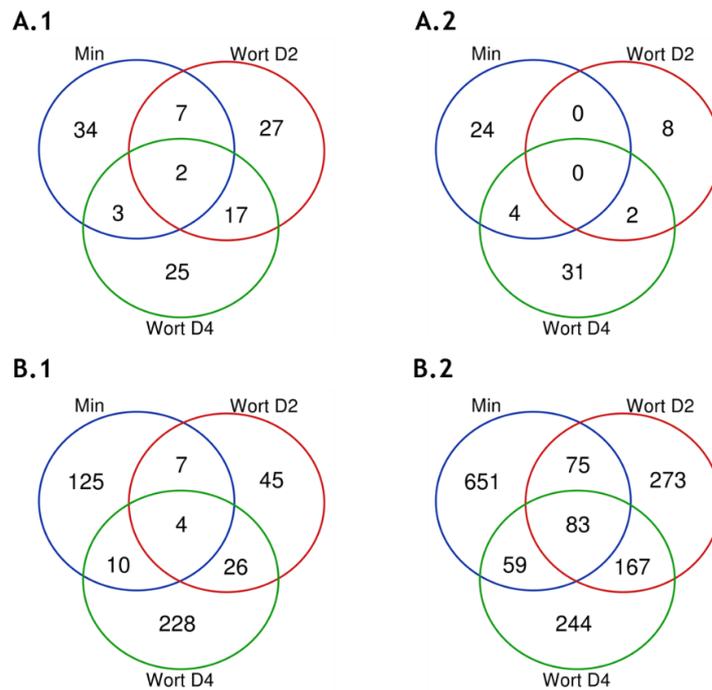
Figure 5.3. Comparison of commonly differentially regulated genes. **A**) Venn diagrams showing the **A.1**) upregulated and **A.2**) downregulated common genes in mutant11.1 in the three conditions tested. **B**) Venn diagrams showing the **B.1**) upregulated and **B.2**) downregulated common genes in mutant 9.7 in the three conditions tested.

5.2.3. Gene ontology and pathway enrichment

Analysis of the gene ontology was carried out for all the upregulated and downregulated terms. Due to the reduced DEGs found in mutant 11.1, no enrichment was found. As mutant 9.7 has more DEGs, different terms were obtained. The number of upregulated terms is reduced due to the low number of upregulated DEGs. Only one category, sulphur metabolism, is upregulated in minimal medium. Genes such as *MET3*, *MET5*, *MET14* and *MET5* are upregulated in minimal medium. There is no enrichment of upregulated terms on Day 2 nor Day 4. In contrast, downregulated terms gave a higher number of categories, especially in minimal medium (**Figure 5.4**). The enrichment shows that carbon metabolism, fatty acid metabolism and nitrogen metabolism are downregulated in minimal medium. On Day 2, mutant 9.7 have a similar transcriptome regulation compared to WS 34/70, with just one downregulated category, ABC transporters. On Day 4, fatty acid metabolism, together with tyrosine and histidine metabolism, are downregulated in mutant 9.7.

As the results of the enrichment analysis is limited to the total number of DEGs in both mutant strains, expression of genes of individual pathways was investigated.
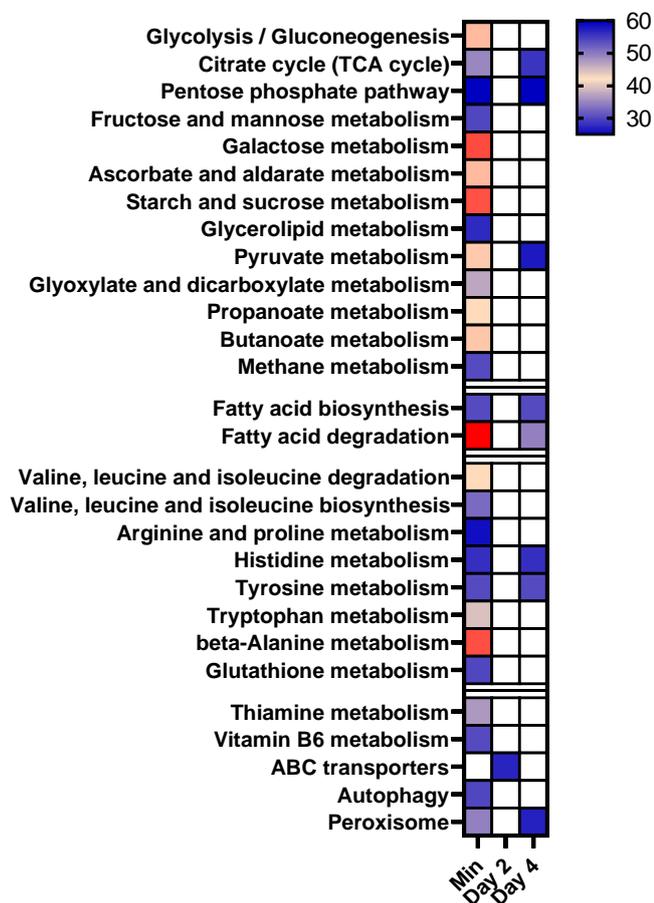
Figure 5.4. Gene ontology analysis of differentially expressed genes in Group II mutant strain. Genes displaying a Log$_2$ fold change ≤1 are shown as no enrichment has been detected for genes with a Log$_2$ fold change value ≥1. The heatmap shows the percentage of genes of each pathway that are differentially regulated. White shows no enrichment.

The Ehrlich pathway was investigated as this pathway is involved in the catabolism of aromatic, branched-chain and sulphur-containing amino acids. Previous analysis showed that mutants overproduce 2-phenylethanol, 2-phenylethyl acetate and tyrosol and tryptophol. All these compounds are synthetised from the catabolism of aromatic amino acids. The analysis shows upregulation of alleles, especially in Group II mutant (**Figure 5.5**). Se-like *ARO9* is upregulated in mutant 11.1 on Day 4 while mutant 9.7 has both Sc-

like and Se-like alleles of *ARO9* in all three conditions tested. Moreover, this strain has upregulated the Sc- like allele of *ARO10* in minimal medium and on Day 2, while the Se-like allele is only upregulated in minimal medium. Analysis of other three decarboxylases, *PDC1*, *PDC5* and *PDC6*, shows no differentially regulation of the first decarboxylase but upregulation of both Sc and Se-like alleles of *PDC5* and *PDC6* was observed in Minimal medium.

Analysis of the enzymes that transform the fusel aldehydes to higher alcohols were investigated, and no significant differences were observed in both strains nor for any conditions. The analysis of the aldolases that catabolise the oxidation step were investigated too, and no significant regulation was observed.
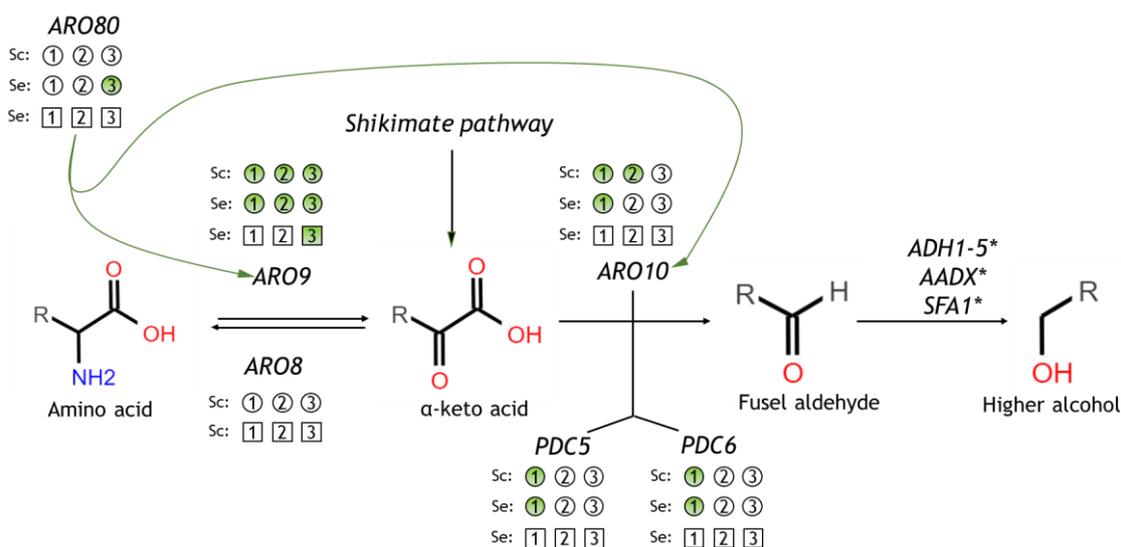
Figure 5.5. Analysis of the alleles of the genes involved in the Ehrlich pathway. Squares correspond to alleles of the Group I mutant 11.1, Circles correspond to the alleles of Group II mutant. In green, upregulated alleles. In white: no significant differentially expressed alleles were detected. Asterisks (*) mean that no significant differentially expressed alleles were detected for the enzymes involved in the reduction step of the pathway. AADX refers to different aryl-alcohol dehydrogenases *AAD3*, *AAD4*, *AAD6*, *AAD10*, *AAD14*, *AAD15*, *AAD16*. Green lines represent activation of *ARO9* and *ARO10* by transcriptional activator *ARO80*.

Both alleles of *ARO9* and *ARO10* have a different expression pattern depending on the strain and on the condition (**Table 5.2**). Mutant 11.1 does not show statistically significant expression of *ARO9* nor *ARO10* alleles in all the conditions, except in wort on Day 4, where the Se-like allele of *ARO9* has a $\log_2$ fold change of 1.21.

Mutant 9.7 has a different regulation of these two genes. The highest expression of the transaminase *ARO9* has been seen in Minimal medium by mutant 9.7, with Log$_2$ fold change values of 6.75 for Sc-like allele and 4.03 for the Se-like allele. In wort medium, the expression values of *ARO9* decrease down to 2.19 for the Sc-like allele and 2.28 for the Se-like allele on Day 2. On Day 4, the expression values slightly decrease compared to wort Day 2. *ARO10* alleles show the highest expression in minimal medium, with values of 8.30 for the Sc-like allele and 4.30 for the Se-like value. In wort on Day 2, Sc-like *ARO10* is overexpressed while the Se-like allele does not have a significant expression. In wort on Day 4, no expression of alleles of *ARO10* was detected.

Table 5.2. Average Log$_2$ Fold change values of both Sc-like (_1) and Se-like (_2) alleles of *ARO9* and *ARO10* in minimal medium, Wort Day 2 and Wort Day 4. Hyphen ("-") denotes that expression is not statistically significant. "n.p" means that the allele is not present in the mutant.

	11.1			9.7		
	Minimal medium	**Wort Day 2**	**Wort Day 4**	**Minimal medium**	**Wort Day 2**	**Wort Day 4**
ARO9_1	n.p.	n.p.	n.p.	6.75	2.19	2.01
ARO9_2	-	-	1.21	4.03	2.28	2.13
ARO10_1	n.p.	n.p.	n.p.	8.32	3.41	-
ARO10_2	-	-	-	4.30	-	-

In order to validate the expression levels obtained from the RNAseq experiment, RT-PCR of *ARO9* and *ARO10* alleles in minimal medium and wort Day 4 was carried out (**Figure 5.6**). For that, specific primers able to discriminate between alleles were designed. Results show concordance between the results obtained from the RNAseq experiment and the RT-PCR.
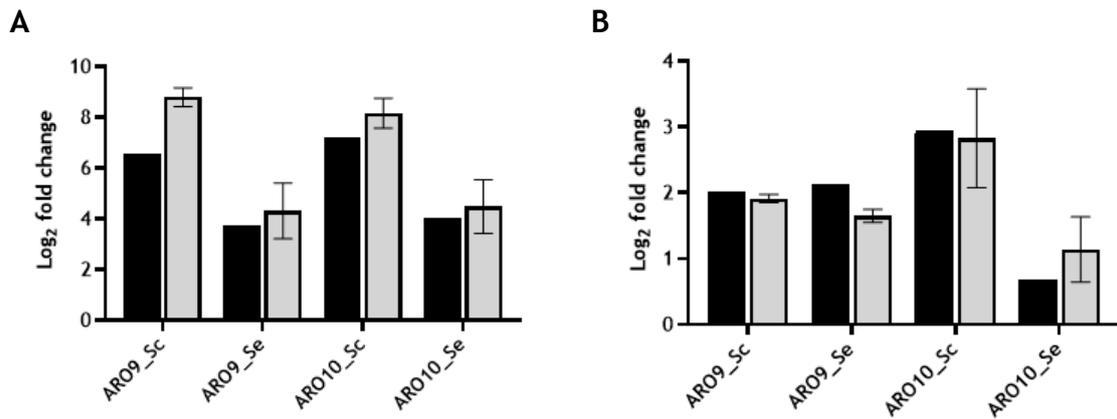
Figure 5.6. Validation of Sc-like and Se-like alleles of *ARO9* and *ARO10* by RT-PCR in **A)** Minimal medium and **B)** Wort Day 4. In red, RNAseq values of corresponding alleles, in blue: average of three biological replicates. *ACT1_1* was validated based on the results of RNAseq and was used as control gene.

Analysis of the genes encoding for proteins in the Shikimate pathway and *MAL* loci were investigated (**Figure 5.7**). All differentially expressed alleles with a P_{adj} value <0.05 were considered for the analysis. The analysis of the Shikimate pathway shows no differentially regulated alleles in the strain 11.1 while slight differences can be observed in 9.7 (**Figure 5.7A**). In minimal medium, mutant 9.7 shows an upregulation of genes involved in aromatic amino acid biosynthesis, but none of the genes have a Log_2 fold change ≥1. Only two alleles, *ARO2_2* and *ARO4_2* are downregulated. On Day 2, regulation of the Shikimate pathway does not contain any DEG while on Day 4, *ARO2_2* and *ARO4_2* are downregulated, with Log_2 Fold values ≤1.

MAL loci were examined for both strains. Mutant 11.1 did not show any divergent regulation of the genes of the MAL loci, however there were differences in expression of MAL genes in mutant 9.7 (**Figure 5.7B**). This strain is able to metabolise more sugars in wort and achieve a lower final °Bx value compared to its parental strain WS 34/70 (data not shown). In Minimal media, more differentially regulated *MAL* genes are observed, but all of them are downregulated. *MAL* genes such as *MAL11_3*, *MAL31_1* and *MAL31_3* have the lowest expression values of the differentially regulated genes. On Day 2 and on Day 4, no differences were detected between 9.7 and its parental strain.
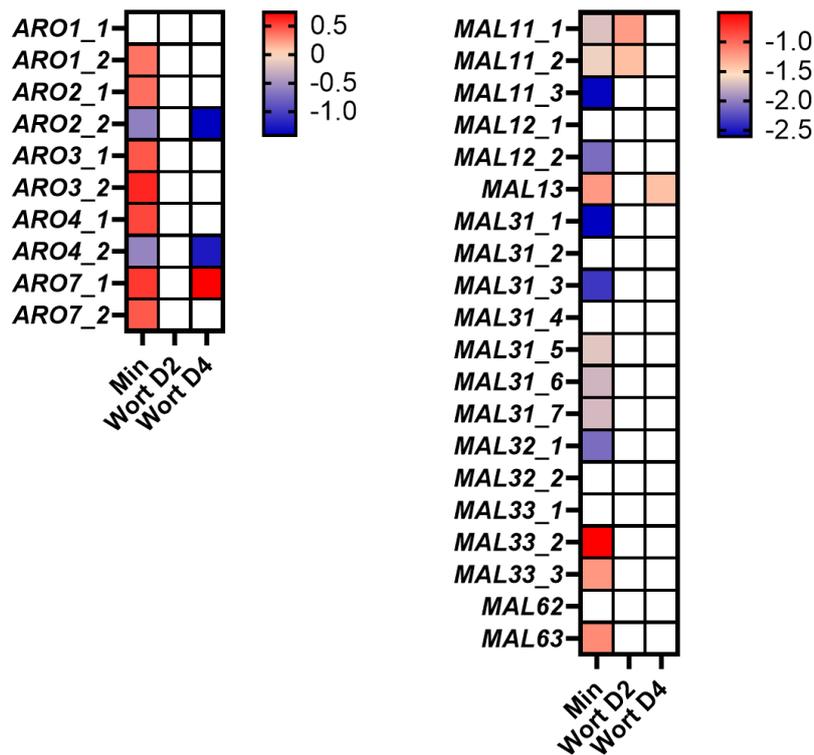
Figure 5.7. Differentially expressed genes involved in (**A**) the Shikimate pathway and (**B**) genes located in the *MAL* loci.

Furthermore, expression levels of permeases/transporters involved in nitrogen transport was examined. 32 transporters/permeases, that are classified into different clusters, have shown different regulation in the mutants compared to their respective parental strains (**Figure 5.8**). Group I mutant 11.1 strain shows an upregulation of *MEP2_1* in all conditions, as previously showed. Interestingly, this mutant does not show any downregulation of nitrogen permeases/transporters in all three conditions, only upregulated permeases/transporters have been detected.

However, mutant 9.7 have a more diverse regulation of these transporters. Some of the transporters have a different regulation in Minimal media and some of the transporters are commonly regulated in wort on Day 2 and Day 4. For instance, *MEP1_1, MEP1_2* and *MEP2* transporters, and *GNP1_1* and *GNP1_2* are all upregulated in minimal medium.

Commonly regulated transporters on both Day 2 and Day 4 have been detected, such as downregulation of *TAT1*, *CAN1*, *HNM1_2*, and *FEN2*, and upregulation of *MMP1*. These transporters are differentially regulated in wort but not in minimal medium.

The mutant strain 9.7 shows more upregulated permeases in minimal medium compared to wort conditions, where the majority of permeases are downregulated compared to the parental strain. Only *GAP1* is upregulated on Day 4.
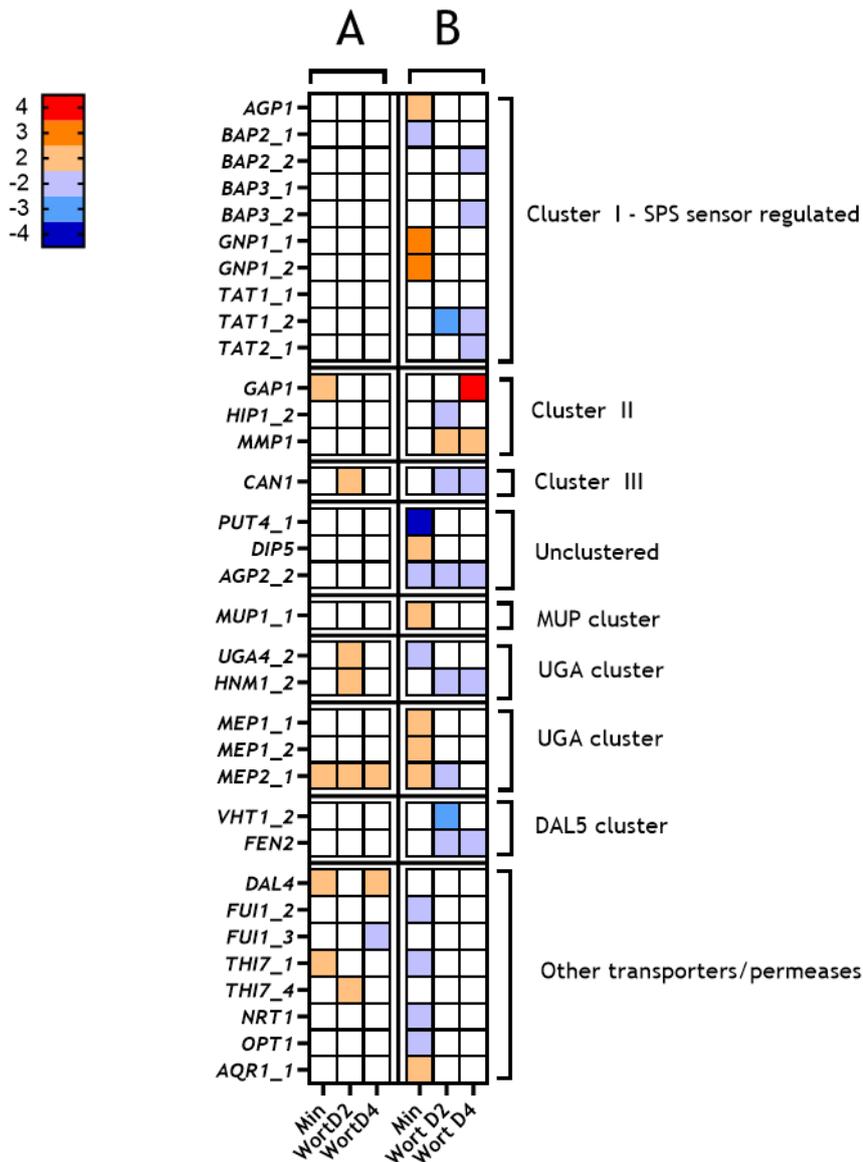
Figure 5.8. Differentially regulated nitrogen permeases and transporters in A) mutant 11.1 and B) mutant 9.7. Permeases were grouped into clusters based on their regulation.

Due to the chromosome loss of Se-like chromosome XI in mutant 9.7, 163 Se-like alleles are missing in this mutant strain. Analysis of these 163 alleles showed that complementary Sc-like alleles are present in the strain. Analysis of the pathways affected was investigated (**Table 5.3**). 72 categories are affected. Of these, central carbon metabolism, fatty acid metabolism, amino acid metabolism, vitamin metabolism, RNA and DNA regulation and cell degradation pathways such as autophagy are affected. Some of the alleles missing codify for subunits that form protein complexes with other subunits, such as *TRP3*.

Table 5.3. Genes missing in 9.7 due to loss of Se-like chromosome XI. KEGG terms affected due this loss.

KEEG pathway	Term	% Associated Genes	Associated Genes Found
KEGG:00010	Glycolysis / Gluconeogenesis	7.27	FBA1, GPM1, PCK1, PGM1
KEGG:00020	Citrate cycle (TCA cycle)	12.90	MDH1, PCK1, SDH1, SDH3
KEGG:00030	Pentose phosphate pathway	10.71	FBA1, PGM1, PRS1
KEGG:00040	Pentose and glucuronate interconversions	9.09	UGP1
KEGG:00051	Fructose and mannose metabolism	4.35	FBA1
KEGG:00052	Galactose metabolism	8.33	PGM1, UGP1
KEGG:00061	Fatty acid biosynthesis	15.38	FAS1, OAR1
KEGG:00100	Steroid biosynthesis	11.11	TGL1, TGL4
KEGG:00190	Oxidative phosphorylation	6.58	ACP1, ATP7, SDH1, SDH3, VMA5
KEGG:00220	Arginine biosynthesis	5.88	AAT1
KEGG:00230	Purine metabolism	7.02	MET14, PGM1, PRS1, YNK1
KEGG:00240	Pyrimidine metabolism	10.34	URA1, URA6, YNK1
KEGG:00250	Alanine, aspartate and glutamate metabolism	6.25	AAT1, GFA1
KEGG:00260	Glycine, serine and threonine metabolism	3.33	GPM1
KEGG:00270	Cysteine and methionine metabolism	4.65	AAT1, MDH1
KEGG:00270	Cysteine and methionine metabolism	4.65	AAT1, MDH1
KEGG:00330	Arginine and proline metabolism	8.70	AAT1, SPE1
KEGG:00350	Tyrosine metabolism	7.69	AAT1
KEGG:00360	Phenylalanine metabolism	11.11	AAT1
KEGG:00400	Phenylalanine, tyrosine and tryptophan biosynthesis	11.76	AAT1, TRP3
KEGG:00410	beta-Alanine metabolism	7.69	FOX2
KEGG:00480	Glutathione metabolism	8.70	OXP1, SPE1
KEGG:00500	Starch and sucrose metabolism	4.88	PGM1, UGP1
KEGG:00520	Amino sugar and nucleotide sugar metabolism	11.76	GFA1, MCR1, PGM1, UGP1
KEGG:00561	Glycerolipid metabolism	9.38	GPT2, TGL4, YJU3
KEGG:00562	Inositol phosphate metabolism	4.76	SAC1
KEGG:00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	4.00	MCD4
KEGG:00564	Glycerophospholipid metabolism	7.50	GPT2, SPO14, TGL4
KEGG:00565	Ether lipid metabolism	25.00	SPO14, TGL4
KEGG:00590	Arachidonic acid metabolism	33.33	TGL4
KEGG:00592	alpha-Linolenic acid metabolism	25.00	TGL4
KEGG:00600	Sphingolipid metabolism	14.29	LAC1, YSR3
KEGG:00620	Pyruvate metabolism	5.77	MAE1, MDH1, PCK1
KEGG:00630	Glyoxylate and dicarboxylate metabolism	3.45	MDH1
KEGG:00630	Glyoxylate and dicarboxylate metabolism	3.45	MDH1
KEGG:00640	Propanoate metabolism	7.14	FOX2
KEGG:00670	One carbon pool by folate	6.67	MTD1
KEGG:00680	Methane metabolism	7.69	FBA1, GPM1
KEGG:00770	Pantothenate and CoA biosynthesis	4.35	CAB3
KEGG:00780	Biotin metabolism	16.67	OAR1
KEGG:00790	Folate biosynthesis	9.09	RMA1
KEGG:00860	Porphyrin and chlorophyll metabolism	11.76	CYT2, MET1
KEGG:00900	Terpenoid backbone biosynthesis	5.26	RAM2
KEGG:00920	Sulfur metabolism	6.67	MET14
KEGG:00970	Aminoacyl-tRNA biosynthesis	0.30	MST1
KEGG:02010	ABC transporters	11.11	PXA2, STE6
KEGG:03008	Ribosome biogenesis in eukaryotes	1.11	MTR2
KEGG:03010	Ribosome	1.60	MRP17, RPL14B, RPL17B
KEGG:03013	RNA transport	6.32	GCN3, LOS1, MLP1, MTR2, TIF1, TRZ1
KEGG:03015	mRNA surveillance pathway	10.42	HBS1, MPE1, MTR2, PAP1, SWD2
KEGG:03018	RNA degradation	1.56	PAN3
KEGG:03020	RNA polymerase	6.67	RPC25, RPC37
KEGG:03022	Basal transcription factors	9.38	TFA1, TFA2, TOA2
KEGG:03030	DNA replication	6.45	PRI2, RAD27
KEGG:03040	Spliceosome	2.44	PRP16, PRP40
KEGG:03050	Proteasome	2.78	RPT1
KEGG:03410	Base excision repair	11.11	APN1, RAD27
KEGG:03450	Non-homologous end-joining	10.00	RAD27
KEGG:04011	MAPK signaling pathway	3.51	HSL1, KDX1, MSN4, STE3
KEGG:04070	Phosphatidylinositol signaling system	4.35	SAC1
KEGG:04111	Cell cycle	1.54	CDC16, HSL1
KEGG:04113	Meiosis	3.01	CDC16, MSN4, TOR2, TPK3
KEGG:04120	Ubiquitin mediated proteolysis	5.88	CDC16, UBA1, UFD4
KEGG:04130	SNARE interactions in vesicular transport	5.00	YKT6
KEGG:04136	Autophagy	4.35	TOR2
KEGG:04138	Autophagy	7.78	ARC19, AVT3, ELM1, MSN4, TOR2, TPK3, YKT6
KEGG:04139	Mitophagy	2.50	TOR2
KEGG:04141	Protein processing in endoplasmic reticulum	2.15	DOA1, LHS1
KEGG:04144	Endocytosis	8.86	ARC19, CAP1, DID2, SMY1, SPO14, VPS24, YPT52
KEGG:04145	Phagosome	8.57	DYN1, VMA5, YPT52
KEGG:04146	Peroxisome	5.00	PEX1, PXA2
KEGG:04213	Longevity regulating pathway	7.89	MSN4, TOR2, TPK3

5.3. Discussion.

5.3.1. Mapping of the reads.

The mapping of the reads was carried out using the two reference genomes used to map the DNA reads in the previous chapter. The first genome used was the one obtained from joining the two genomes of the known ancestors of *S. pastorianus*, *S. cerevisiae* and *S. eubayanus*. The second genome used as a reference was the genome of the *S. pastorianus* Group II strain CBS1483. The latter genome was chosen as it contains a more detailed annotation of *HXT* transporters, *MAL* loci and other important genes related to the production of aromatic compounds from the catabolism of aromatic amino acids, like *ARO9*. The annotation of *S. eubayanus* does not contain information about *ARO9*, although Blast analysis shows that the gene is present in the genome of this strain.

The mapping confirmed the absence of the Sc-like chromosomes II, IV, VI, VII, VIII, XII, XIII, XV and XVI in the Group I strain. It also confirmed the chromosome loss of the Group II mutant chromosome Se-like XI. Interestingly, two different samples of mutant 9.7 experienced a chromosome rearrangement during the experiment. Of three independent biological replicates, two experienced a chromosome rearrangement in chromosome Se-like VII, at the *XRN1* recombination point. This chromosome rearrangement led to the loss of a fragment of Se-like chromosome VII, and no reads were detected after the *XRN1* recombination point. This confirms the complexity of the genomes of *S. pastorianus* strains and that natural chromosome rearrangements can occur in these strains by recombination hotspots. As it was previously confirmed, different isolates of the strain WS 34/70 of *S. pastorianus* have different chromosome copy number (van den Broek et al., 2015). In fact, we confirmed this genome plasticity in the previous chapter as the isolate of the strain WS 34/70 used in this study not only had different chromosome copy number, but also new recombination points compared to previous studies (Okuno et al., 2016; Walther et al., 2014).

5.3.2. Analysis of the transcriptome of the mutants.

The transcriptome of the mutants was analysed in three different conditions: Minimal medium, wort on Day 2 and wort on Day 4. The conditions were chosen as we aimed to investigate the transcriptome changes of the mutants in these three different conditions.

The first medium was chosen as it contains ammonium sulphate as sole nitrogen source, and no amino acids are present in the medium. This would force the expression of amino acid biosynthetic pathways. The other two conditions were chosen to investigate the metabolism of the strains under fermentative conditions.

The mutant strains showed differences compared to the respective parental strains. Group I mutant 11.1 has a lower number of DEGs compared to mutant 9.7. The ratio between Sc-like and Se-like shows that the differences observed are due to the different expression of Se-like alleles. This is expected as this strain has a reduced content of the Sc-like sub-genome.

The Group II mutant 9.7 has a more divergent regulation of the gene expression. Higher numbers of DEGs have been observed. Analysis of the proportion of Sc-like and Se-like ratios of DEGs shows that a higher number of Sc-like alleles are upregulated compared to Se-like alleles on Day 2 and Day 4. In minimal medium, approximately half of the upregulated terms are Sc-like. However, the ratio of Se:Sc downregulated alleles is different. More Se-like alleles are downregulated in all three conditions, especially on Day 2 and Day 4.

Further analysis of the DEGs and chromosome copy number showed that expression is linked to gene copy number. Analysis of the Log_2 Fold change values of the DEGs and the chromosome copy number shows a high number of downregulated genes when the gene ratio of WS 34/70:9.7 is less than 1:1. However, when the ratio is more than 1:1, DEGs show a positive Log_2 Fold change value. This mutant strain, 9.7, has lost several chromosomes, specifically a reduction on copies of *S. eubayanus* sub-genome, and chromosome copy increase has been detected for Se-like chromosome I. Therefore, gene dosage differences have occurred.

Analysis of commonly regulated terms shows few terms that are regulated in all three conditions. This analysis may indicate that overexpression of the genes may be a genuine trait of the strains. In the case of the mutant 11.1, *MEP2*, a permease involved in ammonium uptake, is commonly upregulated in all three conditions.

Mutant 9.7 also shows upregulated terms in all three conditions. Both alleles of *ARO9*, a transaminase involved in the catabolism of aromatic amino acids, is upregulated in all

three conditions. Furthermore, *DEP1_2* and *MET32_1* are upregulated. *DEP1p* is a Component of the Rpd3L histone deacetylase complex, and it is a transcriptional modulator that regulates different targets such as structural phospholipid biosynthesis genes and metabolically unrelated genes, as well as maintenance of telomeres, mating efficiency, and sporulation. *MET32* is a Zinc-finger DNA-binding transcription factor that is involved in transcriptional regulation of the methionine biosynthetic genes (Blaiseau et al., 1997).

5.3.3. Gene ontology and pathway analysis.

Enrichment analysis was carried out to investigate if there were metabolic pathways that were differentially regulated in both strains. Due to the low number of DEGs, no enrichment was found in the strain 11.1. For the Group II mutant 9.7, enrichment analysis was calculated as more DEGs were detected. Due to the low number of upregulated DEGs of 9.7, just one upregulated KEGG term was found in one out of the three conditions tested. Sulphur metabolism was upregulated in minimal medium. This might be linked to the upregulation of *MET32* as this zinc-finger transcription factor is involved in methionine biosynthesis. Previous results obtained in Chapter 2 shows an overproduction of methionol, the corresponding higher alcohol of methionine.

Much more downregulated terms are found in the enrichment analysis. In minimal medium, mutant 9.7 seems to have the carbon metabolism, fatty acid metabolism and nitrogen metabolism downregulated. On Day 2, differences between mutant 9.7 and its parental strain are lower, with just downregulation of ABC transporters. Day 4 seems to be similar to the parental strain, but fatty acid metabolism is downregulated, along with TCA cycle and histidine and tyrosine metabolism.

Because of the reduced number of DEGs detected for Group I strains, enrichment analysis by ClueGo did not give relevant information. Therefore, analysis of individual pathways was carried out to link the observed phenotype in both mutant strains.

The Ehrlich pathway was firstly analysed for the reason that genome analysis identified nucleotide changes in *ARO4* alleles. These mutations make the enzymes insensitive to the negative feedback inhibition by the final amino acid and thus, mutant strains overproduce higher alcohols derived from the Shikimate pathway. Overproduction of aromatic

compounds such as 2-phenylethanol, 2-phenylethyl acetate and tyrosol and tryptophol have been detected in both strains.

The regulation of the nitrogen metabolism is a very complex network with different regulatory steps. Aromatic amino acids are synthetised via the Shikimate pathway. This pathway is controlled by GCN4p, a basic leucine-zipper (bZIP) transcriptional activator of amino acid biosynthetic genes (Hope & Struhl, 1987). This activator responds to amino acid starvation and binds to promoters to activate genes involved in amino acid biosynthesis. Gcn4p is rapidly degraded by the ubiquitin pathway during conditions of non-starvation, but its half-life dramatically increases during amino acid starvation (Meimoun et al., 2000).

The catabolism of aromatic amino acids is regulated by Aro80p, a Zinc finger transcriptional activator of the Zn_2Cys_6 family (Lee & Hahn, 2013). This transcriptional factor is constitutively binding the promoter of genes involved in the catabolism of aromatic amino acids. It activates the transcription of Aro9p and Aro10p.

Analysis of transaminases involved in aromatic amino acid catabolism showed that *ARO9* is highly upregulated in the strain 9.7 in all conditions tested. However, mutant 11.1 shows upregulation of this transaminase but to a different extent, and only on Day 4. Transaminase Aro8p, involved mainly in the production of aromatic amino acids are not differentially regulated. This may indicate that the flux goes towards the production of aromatic compounds. This was confirmed by the overexpression of *ARO10*, especially in 9.7 strain. *ARO10* is highly upregulated in minimal medium. Furthermore, other two decarboxylases that catalyse the same step, are upregulated in minimal medium.

Our hypothesis is that in minimal medium, as there is amino acid starvation, Gcn4p activates the biosynthesis pathways of amino acids. Therefore, Aro4p is synthetized. As Aro4p has mutations that make the enzyme insensitive to negative feedback regulation by the final aromatic amino acid produced, the production of intermediaries of aromatic amino acids rises. Then, overexpression of both alleles of *ARO9* and *ARO10* may indicate that aromatic amino acids are being produced in excess, and therefore, the flux of these amino acid intermediaries goes towards the Ehrlich pathway.

The overexpression of *ARO9* is observed in all three conditions, but at different levels. It is in minimal medium where the highest expression values are shown as expected. Therefore, this may indicate that optimum media to overproduce flavour compounds might be medium that does not contain aromatic amino acids, as these higher alcohols and esters are high-valuable products in industry (Etschmann et al., 2002). Further analysis might be need.

Upregulation of *ARO80* was not observed, except for in mutant 9.7 on Day 4. The upregulation of *ARO80* is not expected as this Zinc finger transcriptional activator is constitutively binding the promoters of genes involved in amino acid catabolism and activation is mediated by Aro80p and GATA factors (Lee & Hahn, 2013).

Mutant 11.1 does not show a significant overexpression of *ARO9* in Minimal media on Day 2. The higher expression starts on Day 4. This may indicate that the expression of *ARO9* may increase as the fermentation continues. This has been reported previously. Expression of two transaminases, *BAT1* and *BAT2* was measured under fermentative conditions in wheat beer (He, Dong, Yin, Chen, et al., 2014). Both Sc-like and Se-like of the transaminases are used in a different rate, but expression of alleles of these transaminases increases as the fermentation continues. Nevertheless, further analysis might be done to try to understand the phenotype of this strain.

The analysis of the *MAL* loci was investigated as previous results showed that mutant strain 9.7 consumes more sugars from wort. Furthermore, mutations in *MAL31_1* and *MAL33_1* were previously detected in this strain. The analysis of the *MAL* loci showed that Group I mutant did not have any differential regulation of genes located in these loci. Group II mutant 9.7 shows all of the differentially regulated *MAL* genes have a downregulated expression in all three conditions compared to its respective parental strain.

Analysis of nitrogen transporters and permeases showed that. The mutant strains show differential expression of the permeases involved in the exchange of nitrogen compounds with the media. The analysis of common upregulated alleles showed that 11.1 is overexpressing a permease, *MEP2_1*, in all three conditions. MEP2p is a permease that is involved in NH_4^+ import and retrieval of excreted ammonium (Boeckstaens et al., 2007).

In minimal medium, mutant 9.7 has a different regulation of permeases compared to WS 34/70. Here, *MEP1* and *MEP2*, two permeases with different capacities and affinity for importing ammonium, and *GNP1*, a broad specificity amino acid permease are overexpressed (Marini et al., 1994; Zhu et al., 1996). The upregulation of *MEP1* and *MEP2* by mutant 9.7 may indicate that this strain is able to import NH_4^+ at higher rates that its parental strain. The higher import rate of NH_4^+ may be beneficial for the mutant strains. NH_4^+ is incorporated into glutamate and this amino acid participates in the Shikimate pathway. Aro8p catalyses the transamination reaction of the α-keto acid to produce amino acids as it transfers the amino group from glutamate to α-keto acid. The analysis of the Ehrlich pathway shows that the flux goes towards the overproduction of aromatic compounds and previous data has validated the overproduction of aromatic compounds derived from the catabolism of these amino acids. Nevertheless, the mutants may be overproducing the amino acids and secrete them to the medium. This may be the case of mutant 11.1, as this strain is overproducing 2-phenylethanol at a lower extent. Further analysis to analyse this possible phenotype is needed.

Analysis of the Se-like alleles that are missing in mutant 9.7 shows that the strain is missing 167 alleles. The strain has not loss any pathway, as corresponding Sc-like alleles are present in the strain. Several pathways are affected but further analysis is needed to understand how the new stoichiometry rate of subunits works in the mutant 9.7. An interesting case is the Trp2-Trp3p complex. This complex forms the bifunctional hetero-oligomeric anthranilate synthase:indole-3-glycerol phosphate synthase enzyme complex with Trp2p. It has been previously discussed that chimeric complexes can be formed by different subunits in interspecific hybrids (Dandage et al., 2021; Piatkowska et al., 2013; Timouma et al., 2021). Some of the new chimeric complexes may be beneficial, as previously reported, but further analysis is needed.

5.4. Conclusions.

RNAseq analysis gives a big picture of the transcriptome of the strains in a specific moment. Here, we used this technology to understand transcriptomic changes that two mutant strains possess after a round of evolution and posterior isolation using amino acid analogues. Previous results have confirmed that these two strains overproduce aromatic compounds derived from the catabolism of aromatic amino acids, 2-phenylethanol and 2-

phenylethyl acetate, tyrosol and tryptophol. Whole Genome Sequencing of the strain has revealed different chromosome rearrangements and nucleotide changes. We observed overexpression of *ARO9* and *ARO10*, indicating that an increased flux through the Ehrlich pathway is present. An intracellular increase of aromatic amino acid activates the expression of *ARO9* and *ARO10* but not the expression of other genes in the pathway, such as *ADH1-5*. Group I mutant is overproducing higher alcohols, specially tyrosol and tryptophol, but it does not show overproduction of *ARO9* and *ARO10* at the levels of 9.7. Instead, *MEP2_1* is overexpressed in all three conditions which may increase the uptake of ammonium, a nitrogen source, by the cell and thus drive in a positive direction the synthesis of amino acids, their higher alcohols and esters.

5.5. Supplemental figures
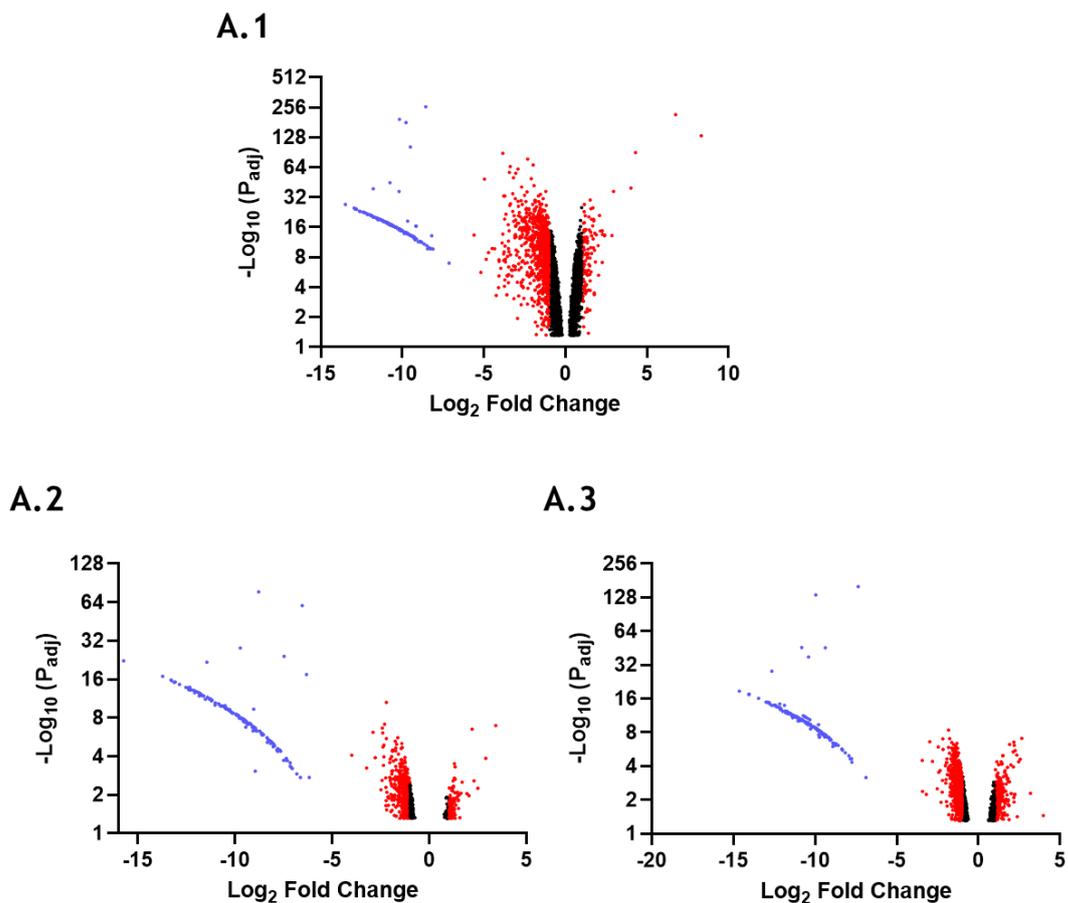
Figure S 5.1. Confirmation of the absence of Se-like chromosome XI in 9.7. In blue: no expression of the Se-like alleles missing in 9.7. Therefore, alleles were removed as they constitute outliers and might affect the analysis of the DEGs. In black, alleles that are

significant but with a Log$_2$ fold change between -1 and 1. In red, alleles with a Log$_2$ fold change ≥1 or less than ≤-1. "**A.1**" means in Minimal media, "**A.2**" means in wort Day 2, and "**A.3**" means in wort Day 4.
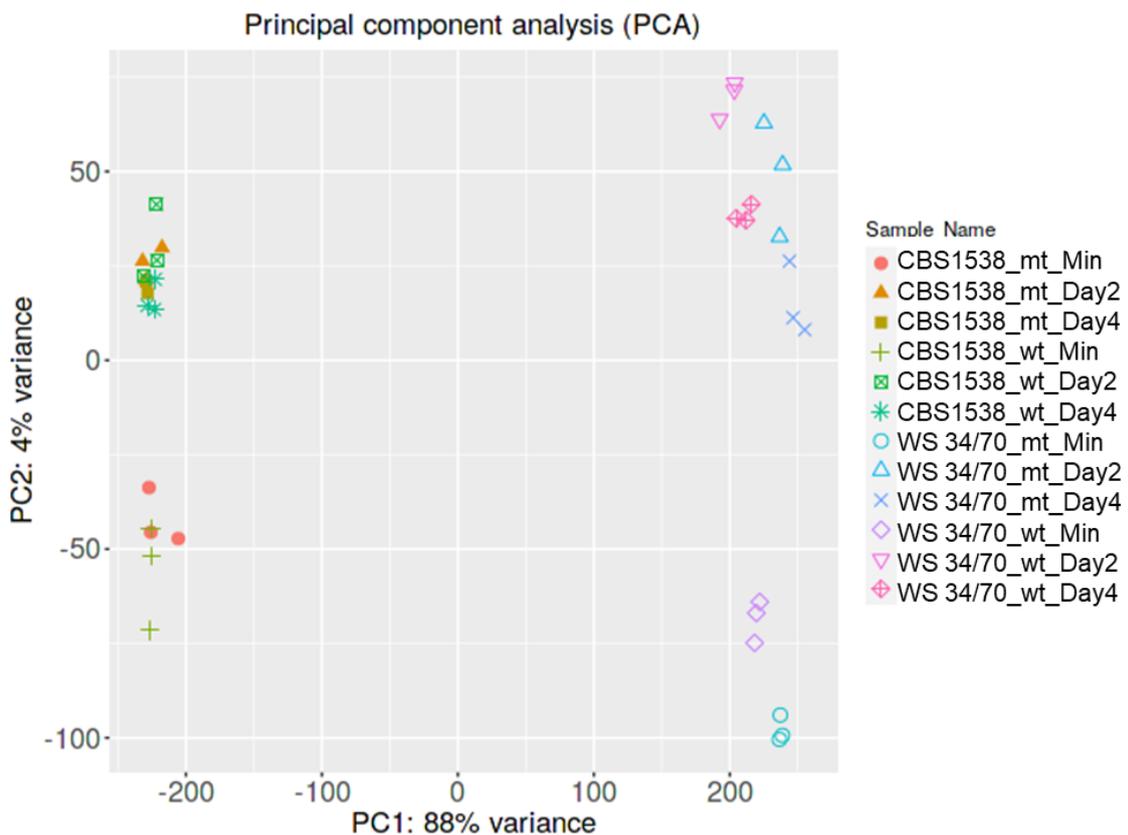
Figure S 5.2. Principal Component Analysis (PCA) of all the samples used in this study. Principal Component 1 (PC1) shows an 88% of variance between Group I and Group II strains. Less variability can be observed between mutant 11.1 and CBS1538.

Chapter 6

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Analysis of the transcriptome of Group I and Group II parental strains

The data showed in this chapter will be soon sent for publishing under the title:

Gene expression profiles of *Saccharomyces pastorianus* Group I and II strains are influenced by the aneuploid nature of their genomes.

The following researchers have been involved in this work:

Roberto de la Cerda García-Caro, Karsten Hokamp, Fiona Roche, Georgia Thompson, Soukaina Timouma, Daniela Delneri and Ursula Bond.

6.1. Introduction

The yeasts used in lager beer production have long been recognised as being unique and distinct from those used for making ales (Hornsey, 2003). In 1870, in recognition of their unique physiological qualities, this group of yeast were given a taxonomical classification of *Saccharomyces pastorianus* by Max Reess (Barnett, 2000).After more than a century of genetic studies, we now know that *S. pastorianus* strains are natural hybrids of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* (Dunn & Sherlock, 2008; Monerawela & Bond, 2017a). While *S. cerevisiae* has long been associated with beer production, especially for ales, *S. eubayanus* was only discovered in Patagonia, South America, in 2011 (Libkind et al., 2011). While Patagonia is considered to be the primary radiation source of *S. eubayanus*, natural isolates have also been found in North America, China, Tibet and New Zealand but to date, no isolates have been identified in Europe (Bing et al., 2014; Eizaguirre et al., 2018; Langdon et al., 2020; Peris et al., 2016). The hybridisation events that generated the current strains of *S. pastorianus* are estimated to have occurred some 500-600 years ago following the introduction of *S. eubayanus* into Central Europe from China or Tibet, most likely along trade routes such as the Silk Road. An inherited property of cryotolerance from the *S. eubayanus* genome allows *S. pastorianus* strains to ferment at temperatures as low as 7-13ºC (Krogerus, Seppanen-Laakso, et al., 2017). This property together with the robust fermentation kinetics inherited from the *S. cerevisiae* genome created yeast hybrid strains capable of producing a crisp clean tasting lager that is now the most favoured alcohol-containing beverage.

Genome analysis of *S. pastorianus* strains identified two distinct types, based on genome content and chromosome composition (Bond et al., 2004; Dunn & Sherlock, 2008; Kodama et al., 2006; Nakao et al., 2009; Walther et al., 2014; Wendland, 2014). Group I, or Saaz strains, are typically triploid in DNA content, retaining all the parental *S. eubayanus* chromosomes but have lost many *S. cerevisiae* chromosomes. This group includes several strains isolated from breweries in Bavaria (Germany) and Bohemia (Czechia) and includes the strains CBS1538, CBS1513, and CBS1503 that were originally isolated in The Carlsberg Laboratory by Emil Christian Hansen in the late 19th century (Barnett & Lichtenthaler, 2001). The Group II, or Frohberg strains, include isolates from Dutch, Danish and North American breweries and are mainly tetraploid in

DNA content, containing approximately 2n *S. cerevisiae* and 2n *S. eubayanus* genome content. Both groups display chromosomal aneuploidy with chromosome numbers ranging from one to six (Monerawela & Bond, 2018; Salazar et al., 2019). In addition to the parental chromosomes, *S. pastorianus* strains contain several hybrid chromosomes containing both *S. cerevisiae* and *S. eubayanus* genes that resulted from recombination, at precise locations, between the parental chromosomes (Bond et al., 2004; Dunn & Sherlock, 2008; Hewitt et al., 2014; Monerawela & Bond, 2017b). Some of the recombination breakpoints are located within coding regions, creating a set of hybrid genes unique to lager yeasts.

Based on genome analysis, the current understanding of the origin and evolution of *S. pastorianus* strains is that both Group I and II strains evolved from a common hybridisation event between *S. eubayanus* and *S. cerevisiae* strains to generate a progenitor hybrid. Subsequently, this progenitor strain underwent a second hybridisation event with a second *S. cerevisiae* strain to generate Group II strains (Bond et al., 2004; Dunn & Sherlock, 2008; Monerawela et al., 2015). This model is supported by shared recombination sites on hybrid chromosomes, evidence of Single Nucleotide Polymorphisms (SNPs) in the *S. cerevisiae* genome of Group II strains and differences in telomeric regions in Group I and II strains (Monerawela et al., 2015). The genome data is also consistent with a scenario in which both groups come from a single hybridisation event between a diploid *S. eubayanus* strain and a heterozygous *S. cerevisiae* diploid strain, with Group I strains experiencing a selective loss of a significant proportion of the heterogeneous *S. cerevisiae* genome (Okuno et al., 2016; Salazar et al., 2019; Wendland, 2014). Both groups then evolved independently with each undergoing further recombination events between the sub-genomes (Hewitt et al., 2014; Monerawela & Bond, 2017a; Okuno et al., 2016; Salazar et al., 2019). Both groups have subsequently diverged to create distinct sub-groups, each with their own unique physiological and biological properties. Fermentation analysis of Group I and II strains reveals that each group produces distinctive aroma and flavour profiles (Gibson et al., 2013).

The complex genome of *S. pastorianus*, containing orthologous alleles emanating from different parental chromosomes, poses interesting questions regarding gene regulation and its impact on the fermentation properties of the strains. Superimposed on the presence of orthologous alleles are complexities of gene dosage due to the aneuploid nature of the

genomes. Aneuploidy has been shown to influence gene expression patterns in eukaryotic cells (Pavelka et al., 2010; Ried et al., 2019; Sheltzer et al., 2012; Torres et al., 2007). The presence of gene orthologues from two different parental *Saccharomyces* species, together with copy number differences between the orthologues has the potential to affect the cellular proteome and specifically the stoichiometry of *S. cerevisiae* and *S. eubayanus* proteins within protein complexes (Piatkowska et al., 2013). Furthermore, gene copy number differences between the Group I and Group II strains may lead to differences in cellular physiology and thus the fermentation properties of the two types of *S. pastorianus*.

Previous transcriptome analyses of *S. pastorianus* strains under fermentation conditions were limited by technology and mainly focussed on the analysis of *S. cerevisiae* genes (James et al., 2003; James et al., 2002; Olesen et al., 2002; Smart, 2007). Many of the transcriptome studies predated the discovery of *S. eubayanus* as a contributing parent to *S. pastorianus* and advances in RNA sequencing technologies (Horinouchi et al., 2010). Several more recent studies focussed on the analysis of sub-sets of genes with specific roles in fermentation or in specific physiological conditions such as cold storage or responses to temperature (Somani et al., 2019; Timouma et al., 2021; Xu et al., 2014). Important findings regarding the gene expression of genes involved in maltose utilisation, carbohydrate metabolism, glycerol mobilisation, anaerobiosis, and protein biosynthesis have emerged from these studies, providing a road map for a more detailed transcriptome analysis.

Here, we analysed the transcriptomes of the Group I CBS1538 and the Group II WS 34/70 *S. pastorianus* strains and specifically examined the contribution of the two sub-genomes to the gene expression patterns under fermentations conditions. Using *de novo* genome sequencing of these strains, we related gene expression patterns to gene copy numbers. We show that the relative expression of *S. cerevisiae* and *S. eubayanus* orthologues is positively correlated to gene copy number in the Group II strain. Gene copy number plays a smaller role in gene expression patterns in the Group I strain. Despite the reduced *S. cerevisiae* content in the Group I strain, *S. cerevisiae* orthologues contribute significantly to biochemical pathways upregulated during fermentation, specifically to amino acid metabolism. We did not observe evidence of gene dosage compensation in the *S.*

pastorianus strains. Finally, comparison of the transcript patterns in the Group I and II strains identified both common and unique gene expression patterns during fermentation.

6.2. Results.

6.2.1. Fermentation profiles of Group I and II strains.

Two *S. pastorianus* strains, CBS1538 and WS 34/70, representative of Group I and Group II strains respectively, were chosen for analysis. Both strains displayed similar fermentation profiles in 10% wort up to Day 4 but thereafter, strain WS 34/70 fermented faster than strain CBS1538 and reached a lower final attenuation (**Figure 6.1A**). At the end of fermentations in 3L tall tubes, the two strains produced similar volatile compound profiles with the exception that ethyl butyrate and methionol levels were higher in WS 34/70, and ethyl hexanoate was produced in higher levels in strain CBS1538 (**Figure 6.1B**). Similar volatile profiles were obtained in small scale (15 mL) fermentations, the scale used for RNA extractions, with the exception that higher levels of acetaldehyde were observed in both strains (data not shown).
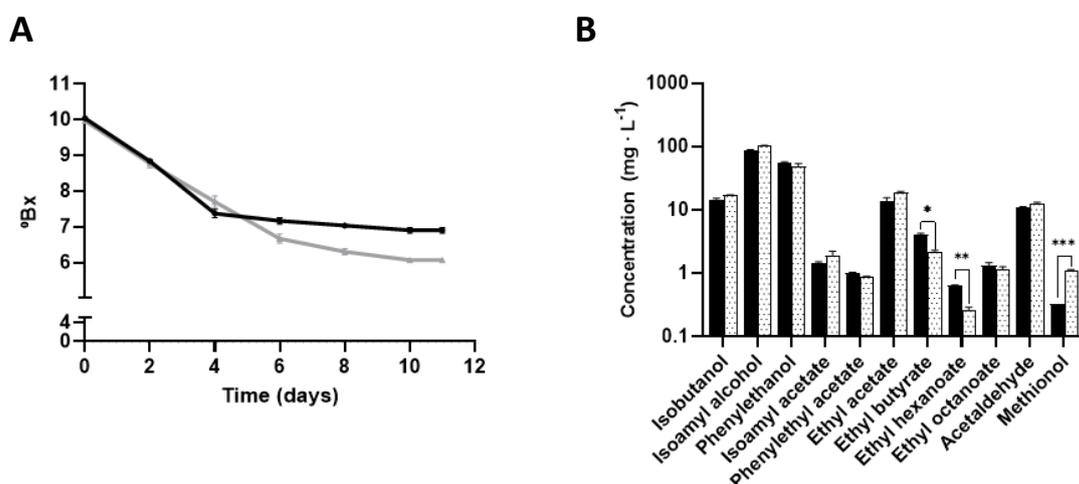
Figure 6.1. Fermentation profiles of the Group I CBS1538 and Group II WS 34/70 strains. **A.** Fermentations were carried out in 10% Wort at 13°C in 15 mL cylindrical tubes. Sugar consumption was measured with a Brixometer. CBS1538: grey line, WS 34/70, black line. Error bars represent the standard deviations from the mean of triplicate fermentations. **B.** Volatile profiles of compounds present in wort at the end of

fermentations. Fermentations were carried out in 10% Wort at 13°C in 3L tall tubes. Error bars represent the standard deviations from the mean of duplicate fermentations. P values are indicated as: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

6.2.2. Chromosome composition of strains CBS1538 and WS 34/70.

To compare the transcriptomes of Group I and Group II lager yeasts, RNA was extracted from strains CBS1538 and WS 34/70 on Day 2 and Day 4 of small-scale fermentations in 10% wort. These time points were chosen as previous studies have shown that this is the period of maximum metabolism during the fermentation (James et al., 2003). RNA was also extracted from the same strains grown in minimal medium without amino acids to provide a baseline for comparison. To map the transcripts, the genomes of CBS1538 and WS 34/70 isolates used for RNA extraction were sequenced *de novo* and annotated using data from the annotated and fully assembled reference genome *S. pastorianus* 1483 (Group II strain) as well as the parental reference genomes *S. cerevisiae* and *S. eubayanus* (**Figure 6.2**). Both approaches yielded highly similar results, however since the *S. pastorianus* 1483 genome lacked some information for *S. cerevisiae* genes on chromosomes III and VII, the data from mapping to the parental genomes was used for this analysis. Information from genome sequencing of the two strains as well as transcript mapping confirmed the absence of *S. cerevisiae* chromosomes II, III, IV, IV, VII, VIII, XII, XIII, XV and XVI in CBS1538 (**Figure 6.2A**). Furthermore, we observed alterations in the copy number of chromosomes and differences in hybrid chromosomes to the previously reported chromosome content of both strains (Okuno et al., 2016) (**Figure 6.3**). The estimated chromosome copy numbers were 48 and 76 for CBS1538 and WS 34/70 respectively.

A
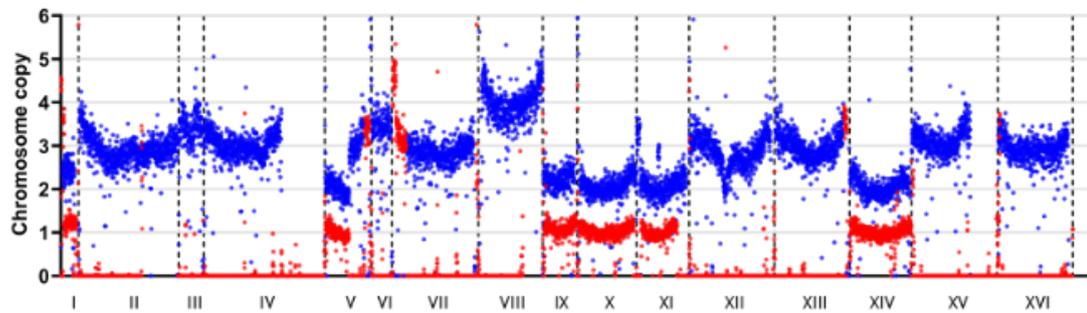
B
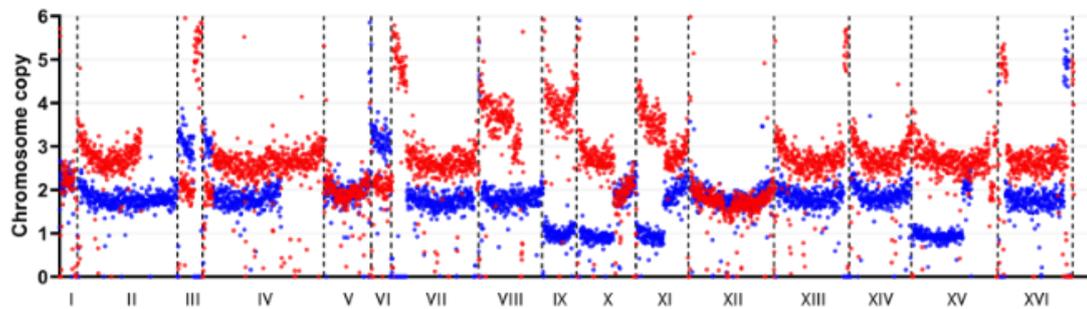
Figure 6.2 Chromosome copy number of CBS1538 (**A**) and WS 34/70 (**B**) genomes. Estimated copy number and chromosome types for *S. eubayanus* (blue) and *S. cerevisiae* (red) isolates from de novo sequencing mapped to the parental genomes. The start of each chromosome is shown by a vertical dotted line. Asterisk "*", denotes translocations between *S. eubayanus* chromosomes II/IV, IV/II, VIII/XV, XV/VIII present in both strains. Hybrid chromosomes are evident from the change in copy number within a chromosome.
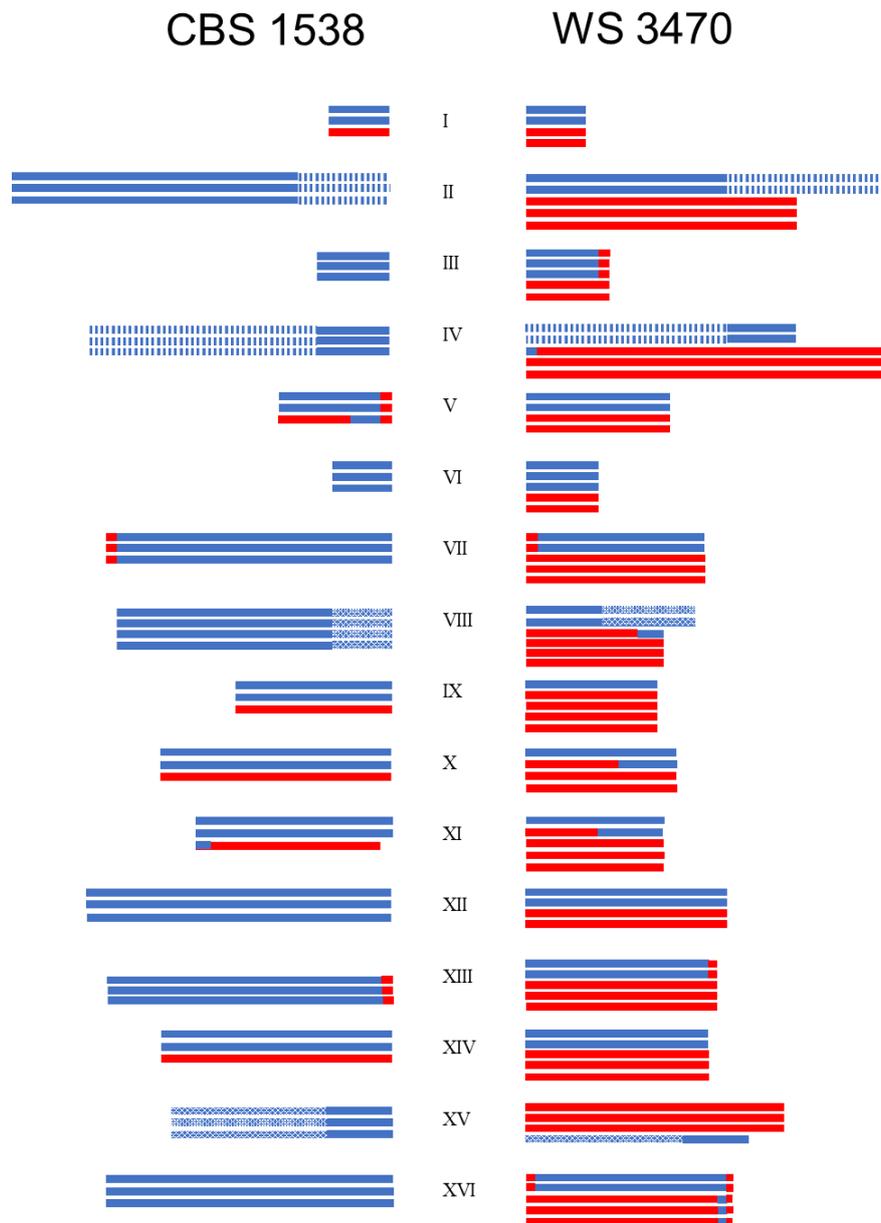
Figure 6.3. Estimated types and copy number of *S. cerevisiae* (red) and *S. eubayanus* (blue) chromosomes in CBS1538 and WS 34/70. Different translocations and recombinations can be observed. Blue fragments with horizontal white lines show recombination between Se-like chromosomes II and IV. Blue fragments with white dots show translocations between Se-like chromosomes VIII and XV. Both translocations are detected in *S. eubayanus* strains, indicating that these translocations occurred before the hybridization events that led to the *S. pastorianus* hybrid strains.

6.2.3. Gene expression profiles of Group I and II strains during fermentation.

Transcripts were either assigned as *S. cerevisiae* (Sc) or *S. eubayanus* (Se) based on sequence identity and chromosome assignment. A total of 11335 transcripts/Open Reading Frames (ORFs) were detected for the WS 34/70 strain of which 5083 were assigned as *S. eub*ayanus and 6252 as *S. cerevisiae* (ratio Se:Sc = 0.82:1). For the Group I strain, a total of 6742 transcripts were detected, 5163 were *S. eubayanus* and 1635 were *S. cerevisiae* (ratio Se:Sc = 3.2:1). We examined the differential gene expression of transcripts mapped to each strain in three different experimental conditions (Minimal medium without amino acids, wort Day 2 and wort Day 4). In the WS 34/70 strain, the greatest number of genes displaying differential gene expression was detected in the comparison of cells grown in minimal medium and wort on Day 2 where 42% of transcripts were differentially expressed, while 28% of genes were differentially expressed between growth in minimal medium and in wort on Day 4. A much lower level of differential expression was detected between cells grown in wort on Days 2 and 4 where just 14% of genes were differentially expressed. The pattern of differential genes expression in CBS1538 was similar to that observed with the WS 34/70 strain with 32% of transcripts differentially expressed between growth in minimal medium and wort on Day 2, 23% between growth in minimal medium and wort on Day 4 and 16% between growth in wort on Days 2 and 4.

As *S. pastorianus* contains both *S. cerevisiae* and *S. eubayanus* sub-genomes, we examined the differential gene expression patterns for *S. cerevisiae* or *S. eubayanus* alleles under the three different experimental conditions. In the WS 34/70 strain, *S. eubayanus* genes are significantly over-represented in the differentially upregulated gene pool under all three conditions but were expressed at the expected ratio in the differentially downregulated gene pool (**Table 6.1**). Interestingly, on Day 2 relative to Day 4 in wort, there are twice as many Se alleles upregulated compared to Sc alleles. In the CBS1538 strain, Se alleles are overrepresented in both the upregulated and downregulated gene pools in cells in wort on Day 2 relative to Day 4 and in wort on Day 2 relative to minimal medium.

Table 6.1. Ratio of *S. cerevisiae* (Sc) and *S. eubayanus* (Se) in differentially up-regulated and down-regulated transcript pools (log2-fold change ≥ 1 or ≤ -1) and median log2-fold changes under the three conditions in CBS1538 and WS 34/70. Ratios of differentially expressed Se vs Sc genes with significant differences from the expected are shown with an asterisk, p≤0.001**, p≤0.05*.

Strain		D2 vs Min Sc	D2 vs Min Se	D4 vs Min Sc	D4 vs Min Se	D2 vs D4 Sc	D2 vs D4 Se
WS 34/70 Up	Ratio Se:Sc	1.06**		0.96**		1.93**	
	Median	1.75	1.86	1.65	1.6	1.23	1.29
WS 34/70 Down	Ratio Se:Sc	0.77		0.77		0.81	
	Median	-1.67	-1.67	-1.47	-1.42	-1.31	-1.34
CBS 1538 Up	Ratio Se:Sc	3.76*		2.97		4.97**	
	Median	1.62	1.61	1.53	1.54	1.25	1.31
CBS 1538 Down	Ratio Se:Sc	4.29**		3.85		4.01*	
	Median	-1.71	-1.64	-1.44	-1.41	-1.41	-1.38

The median $\log_2$-fold change for *S. cerevisiae* and *S. eubayanus* alleles in both the WS 34/70 and CBS1538 strains are extremely consistent under all conditions indicating that both Group I and Group II strains, and their Se and Sc sub-genomes, respond similarly to the physiological conditions. The median $\log_2$-fold change and the range of DEG on Days 2 and 4 wort was less than that observed for differential expression between Minimum medium and wort on Days 2 and 4 for both strains (**Table 6.1** and **data not shown**).

We next examined the relationship of DEG under the three conditions in the two strains. As might be expected, there is a significant overlap in the genes differentially expressed between growth in minimal medium and in wort on Days 2 and 4 in both the Group I and Group II strains (**Figure 6.4**). While fewer in number, there is a significant overlap in the genes differentially expressed in wort Day 2 relative to Day 4 and in wort Day 2 relative to minimal medium. On the other hand, there are no genes differentially expressed that are specific to wort Day 2 relative to Day 4 and to wort Day 4 relative to minimal medium in WS 34/70 and just one gene in this category in CBS1538.
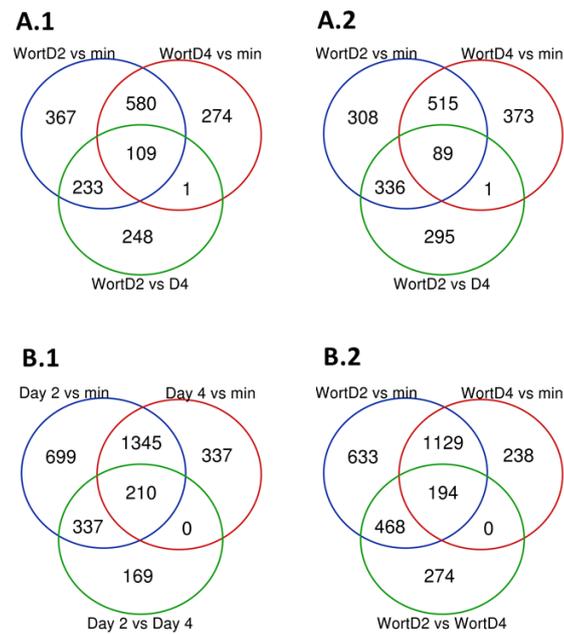
Figure 6.4. Venn diagrams showing the relationship of differentially upregulated and downregulated transcript pools under the three conditions shown. Commonly regulated genes in **A)** Group I strain CBS1538, and **B)** WS 34/70. Upregulated terms are distinguished by a "1" after the letter and downregulated terms by a "2".

Only a small number of genes are commonly upregulated and downregulated respectively in all three conditions in the Group I and the Group II strains (**Figure 6.4**). The commonly upregulated gene pool in WS 34/70 (n=210), contains 23 pairs of Sc and Se orthologous alleles, including, *ADH1, ADH5, PGK1, GPH1, HXK1*, *ENO1*, that are central to carbohydrate metabolism and *ILV6,* required for branched chain amino acid biosynthesis. Likewise, both Sc and Se alleles of *LEU4*, *LYS1* and *SPD1* are upregulated in all three conditions in CBS1538. Orthologous gene pairs are also observed in the commonly downregulated gene in both strains.

6.2.4. Gene ontologies enriched in Group I and II strains under fermentation conditions.

Gene ontology analysis of the differentially expressed genes under the three conditions in the Group I and Group II strains revealed an enrichment in both carbohydrate and amino acid metabolism on Day 2 of fermentation in the upregulated gene pool (**Figure 6.5**A). Genes required for the utilisation of all major sugars including sucrose, fructose, galactose as well as pentose sugars along with the genes required for glycolysis and

pyruvate metabolism are all upregulated in wort relative to Minimum medium and on Day 2 relative to Day 4 in the Group II strain. Except for genes involved in starch and sucrose metabolism, the observed gene set for carbohydrate metabolism in the Group II strain were not enriched in the Group I strain. Genes for the biosynthesis and metabolism of amino acids are enriched during the fermentation in wort in both Group I and Group II strains. In addition to the enrichment of genes for these major metabolic activities in the two strains, we observed differences in specific gene ontologies between the two strains, with genes involved in glycerolipid metabolism, porphyrin metabolism, ABC transporters and longevity upregulated in the Group II strain and not the Group I strain.
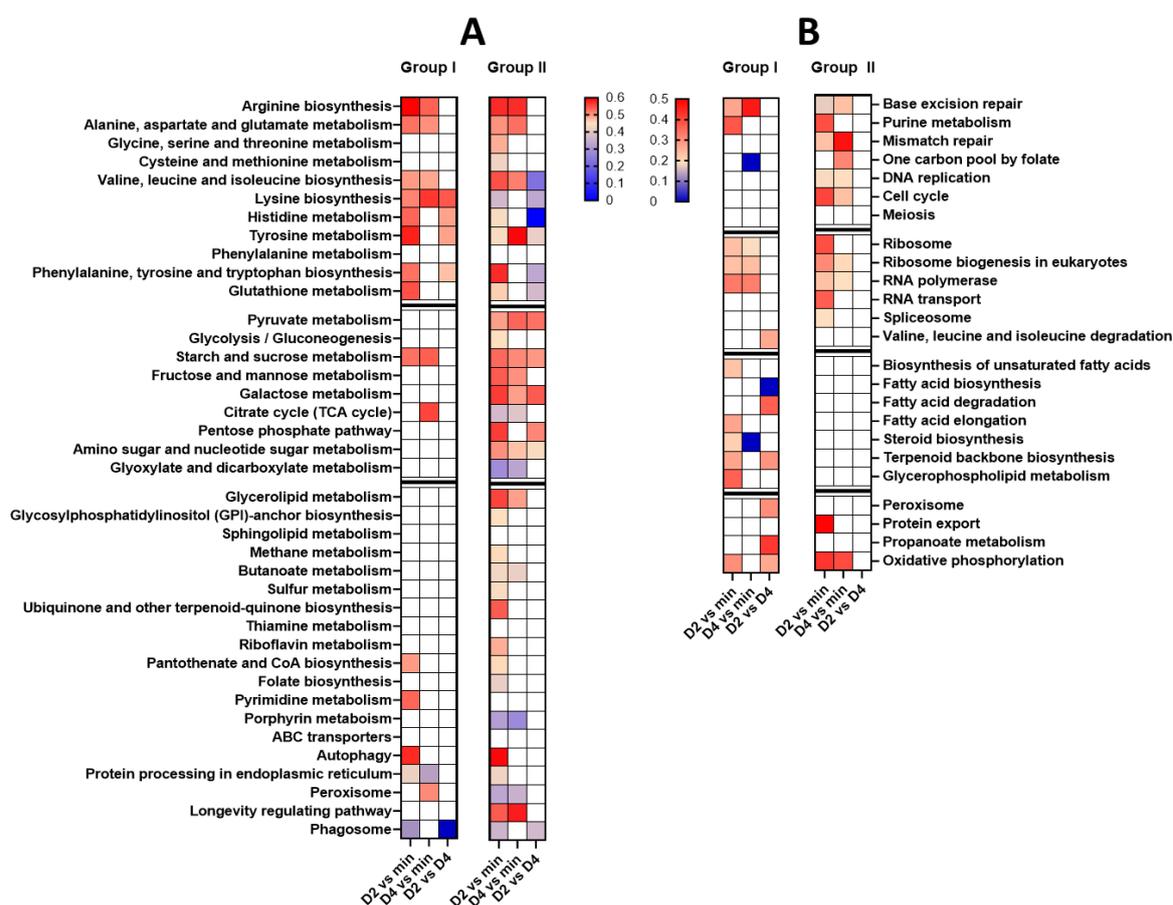
Figure 6.5. Gene ontology analysis of differentially expressed genes in Group I and Group II strains. Genes displaying a Log$_2$ fold change ≥ 1 (**A**) or $\leq$ -1 (**B**) were examined for enriched gene ontologies using ClueGo. Graph shows deviations from the expected ratio of Sc to Se alleles; expected ratio is buff, > expected gradient buff to red, < expected gradient buff to blue. Blank cells mean no enrichment. Group I expected ratio Sc:Se =0.2:0.8, Group II, Sc:Se = 0.55:0.45 as shown in the colour legend.

Genes involved in nucleic acid metabolism, DNA repair and DNA replication are downregulated under fermentation conditions and on Day 2 relative to Day 4 in wort (**Figure 6.5B**). Likewise, genes involved in major anabolic pathways such as protein synthesis including genes required for ribosome biogenesis and rRNA, mRNA and tRNA processing are downregulated in wort and on Day 2 relative to Day 4. Interestingly, a set of genes involved in fatty acid, glycerophospholipid and steroid metabolism, required for the production of membrane components, are uniquely downregulated in the Group I strain.

We examined the contribution of the Sc and Se sub-genomes to these enriched pathways. Se and Sc alleles were observed to contribute at the expected Se:Sc ratio to the differentially regulated gene pool (**Figure 6.5A, B**) or in a higher proportion than expected (**Figure 6.5A**, **B**). Se alleles contribute in a higher proportion than expected on Days 2 and 4 of fermentation (**Figure 6.5A**). Conversely, we observed that Sc alleles contribute in a greater proportion than expected to amino acid metabolism in the Group I strain under all three conditions (**Figure 6.5A**). For some specific pathways, we observed that Sc or Se alleles accounted for all the differentially regulated genes, for example Se alleles are used exclusively in histidine metabolism on Day 2 relative to Day 4 in the Group II strain and to phagosome associated genes in the Group I strain in wort on Day 2 relative to Day 4 (**Figure 6.5A**).

For the downregulated genes, we observe that Sc alleles contribute to gene ontologies at the expected ratio, or in some pathways, in a higher proportion than expected from the ratio of Sc:Se for the whole genome in both strains while Se alleles predominate in just three categories, namely one carbon pool by folate, fatty acid and steroid biosynthesis in the Group I strain (**Figure 6.5B**).

6.2.5.   The effect of gene dosage on gene expression profiles.

As *S. pastorianus* strains are aneuploid in nature and have different copy numbers of *S. cerevisiae* and *S. eubayanus* chromosomes, we were interested to determine if gene expression profiles were influenced by the copy number of Sc and Se alleles. The copy number for each Sc and Se gene was calculated from the sequence coverage from the *de*

novo genome sequencing. The differential gene expression of Sc and Se alleles within each strain was determined for the three experimental conditions and was correlated to the gene copy number (**Figure 6.6**). Sc and Se alleles are present in varying ratios in the Group II strain. The analysis of some 1555 Sc and Se orthologues indicates that the differential expression of Sc and Se alleles was positively correlated to the copy number (**Figure 6.6A, B**). As the Sc copy number increases, so too does the levels of Sc transcripts and vice versa for Se alleles. At a ratio of Sc:Se, 1:2 and 3:4, Se transcripts predominate while at ratios, 3:1, 4:1 and 5:1 Sc alleles predominate. Interestingly, at a 1:1 ratio, Se alleles predominate in the Group II strain while at 3:2, 2:1 there is almost equal levels of both transcripts. The relationship between differential gene expression of Sc and Se orthologues and gene copy number is observed in both parental and hybrid chromosomes as exemplified in **Figure 6.6C** for chromosome X. The WS 34/70 strain has four copies of chromosome X, one Sc, one Se and two hybrid chromosomes (Sc/Se) with the recombination point occurring at the gene *THD2* (YJR009C) (**Figure 6.6**). The ratio of Sc:Se to the left of *TDH2* is 3:1 and to the right 1:1 (**Figure 6.6C**). For genes to the left of *TDH2*, expression of Sc alleles predominates due to the higher Sc copy number while to the right, Se alleles predominate despite the equal copy number (**Figure 6.6C**). The correlation of copy number to orthologue ratio was observed in all three experimental conditions (**data not shown**).

The analysis of the gene expression of Sc and Se orthologues in the Group I strain presented a different scenario. Firstly, due to the reduction of the Sc genome content in the Group I strain, the number of orthologues is much smaller (n=237). Secondly, the variations in copy numbers are much smaller, with the majority of orthologues present in a ratio of Sc:Se, 1:2 (**Figure 6.6D**). Surprisingly, we see the opposite expression pattern to what was observed in Group II; at the Sc:Se 1:2 ratio, the levels of Sc alleles predominate over the Se alleles. There are just two alleles present in a 2:3 and one at a 2:1 ratio and there is no apparent correlation in the differential expression of these alleles with the Sc:Se ratio of copy number (**Figure 6.6D**).
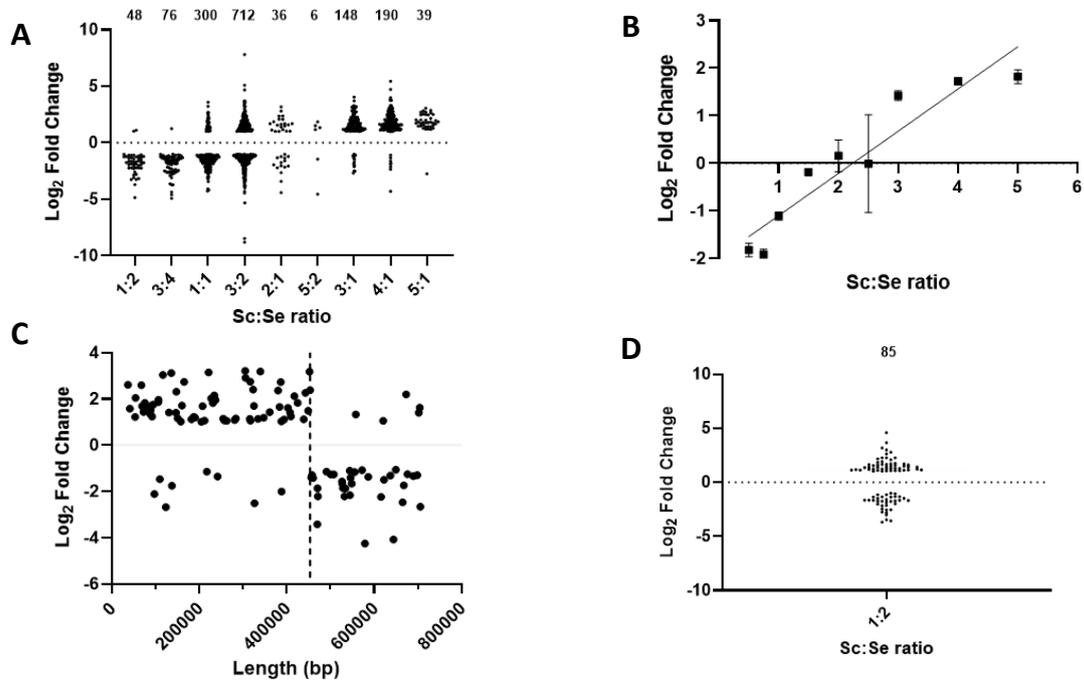
Figure 6.6. Effect of gene copy number on gene expression patterns. The differential gene expression of Sc and Se orthologues were grouped according to the ratio of gene copy number in WS 34/70 (**A**). Data for Day 2 in wort is shown. The number of paired orthologues at each copy number ratio is shown above the graphs. **B.** Correlation of differential expression of Sc:Se gene orthologues in WS 34/70 with copy number ratio. The median of Log_2 fold changes in expression between Sc and Se orthologues is plotted against copy number ratio. Error bars represent the standard error of the mean. **C.** Log_2-fold gene expression difference in Sc and Se orthologues on chromosome X in WS 34/70. The dotted line marks the recombination site (*THD2*) on hybrid chromosomes. Sc:Se ratio to left of vertical dotted line is 3:1 and after 1:1. **D.** The differential gene expression of Sc and Se orthologues were grouped according to the ratio of gene copy number in CBS1538.

6.2.6. Consolidated transcriptomes of Group I and Group II strains.

To directly compare the transcript landscapes in the Group I and II strains, we generated a consolidated transcriptome for each strain by obtaining the sum of allele transcripts encoded for each gene. We compared the expression of this gene set in the Group I and II strains under the three different experimental conditions (**Figure 6.7**). There is a high

degree of overlap between the differentially expressed gene pool in both strains suggesting that both strains respond similarly to the environmental conditions (**Figure 6.7A**). Growth in Minimal medium invoked the greatest condition-specific differential gene expression between the two strains but a substantial number of genes were also specifically expressed in wort on Day 2 and Day 4 respectively. An analysis of gene ontologies associated with the differentially expressed gene set reveals differences between the Group I and II strains (**Figure 6.7C**). Firstly, we observed that genes associated with ribosome biosynthesis are enriched on Day 2 and 4 in wort in the Group II strains while metabolism of certain amino acid, pentose phosphate pathway and oxidative phosphorylation are also upregulated on Day 2 in this strain. Genes associated with DNA repair and protein processing and transport are upregulated in minimal medium. Conversely, genes associated with membrane biosynthesis, sugar metabolism and amino acid metabolism are upregulated in minimal medium in the Group I strain and a subset of these genes are also upregulated on Day 4 in wort. Surprisingly, there are no gene ontologies enriched on Day 2 in the Group I strain (**Figure 6.7C**).

As the gene copy number for the sum of gene orthologue transcripts still varied between Group I and II strains and was shown to influence gene expression profiles within a strain, we looked to see if gene copy number influences the differential gene expression observed between the Group I and Group II strain. To do this, we compared the Log_2 fold changes between gene expression in the two strains to the ratio of the total gene copy number (sum of all Se and Sc alleles) for each gene. The data for gene expression on Day 2 in wort is shown in **Figure 6.7B**. There is a significant correlation between Log_2 fold change and ratio of total gene copy number between the two strains. This correlation was consistent across all three experimental conditions (**data not shown**). Thus, as observed for gene expression within a strain, gene dosage plays a more significant role in the differential expression of genes between the Group I and Group II strains.
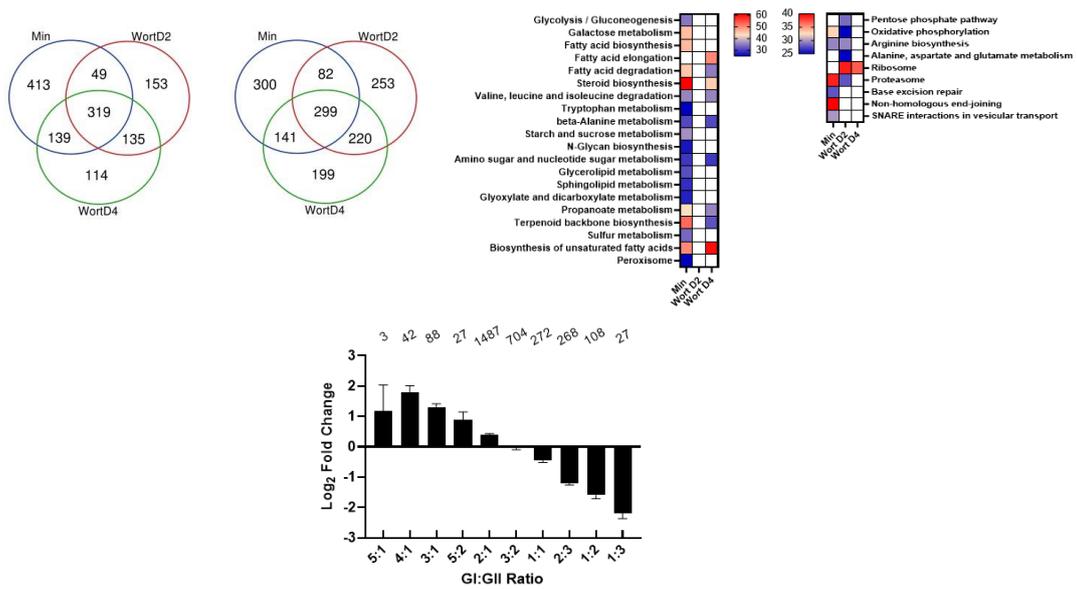
Figure 6.7. Comparison of gene expression in Group I and Group II strains. Transcript reads for Sc and Se alleles were summed in CBS1538 and WS 34/70 respectively to create a combined transcriptome. Gene expression profiles in the three conditions, growth in minimal medium, in wort on Day 2 and in wort on Day 4 were compared between the two strains. The data for Day 2 in wort are shown. **A.** Venn diagram of genes upregulated in the Group I and Group II strains. **B.** Gene ontologies enriched in the Group I and Group II strains respectively. White squares, no enrichment, enriched ontologies with the shading represents the percentage of associated genes for each ontology group with the minimum set at 25%. **C.** Log$_2$-fold differences in gene expression between Group I and II strains were grouped according to the ratio of Sc:Se gene copy number. The number of genes in each ratio category is shown above the graphs. Data for Day 2 in wort is shown. The error bars represent the standard error of the mean Log$_2$-fold change in gene expression between Group I and II strains.

6.1. Discussion.

The origin and evolution of lager yeasts are widely debated, and several hypotheses have been proposed to account for the divergence in chromosome composition and copy number between the Group I and II strains (Monerawela et al., 2015; Okuno et al., 2016; Salazar et al., 2019). The major distinction between the two groups is the loss of a significant portion of the *S. cerevisiae* sub-genome through the loss of whole chromosomes in the Group I strains. In addition, differences in chromosome composition, copy number and the number and type of hybrid chromosomes exist between strains within each group. Despite these differences, strains from both groups ferment sugars and produce aromatic beers, with individual strains displaying variations in the final aromatic volatile profile (Gibson et al., 2013). A major difference between Group I and II strains lies in the ability to ferment maltotriose. While all Group II strains can uptake and ferment maltotriose, only a subset of Group I strains have the necessary transporters able to import this trisaccharide (Magalhaes et al., 2016; Vidgren & Londesborough, 2012; Vidgren et al., 2005). The Group I strain used in this study, CBS1538, does not ferment maltotriose. We confirm here the superior fermentation rates of the Group II strains and show that the two strains produce unique flavour profiles. The two strains consume sugars at the same rate up to Day 4 and thereafter the Group II strain fermented at a faster rate. WS 34/70 produces more isoamyl acetate than CBS1538, surpassing olfactory thresholds. This ester comes from the esterification of the higher alcohol isoamyl alcohol and imparts banana-like aromas, a desirable compound in beer. The WS 34/70 also produced higher levels of ethyl butyrate while in contrast, CBS1538 overproduces ethyl hexanoate. Both esters contribute to the tropical fruit aroma in beer (Holt et al., 2019; Lin et al., 2021).

We were interested in understanding how the two sub-genomes, present in the strains, contribute to the overall transcriptome during fermentation and furthermore what are the consequences for the reduction in the *S. cerevisiae* sub-genome to the gene expression patterns between the Group I and II strains. The complex genomes of lager yeasts pose several challenges to the analysis of the transcriptomes of these strains. At present, just one *S. pastorianus* Group II genome, that of strain CBS1483, has been fully annotated and assembled into chromosomes and this serves as the reference genome for *S.*

pastorianus at NCBI (Salazar et al., 2019), although up to 16 annotated genomes are also available https://www.ncbi.nlm.nih.gov/genome/browse/#!/eukaryotes/342/. Due to differences in chromosome, gene copy numbers, and chromosomal rearrangements, the reference genome may not be the ideal genome for transcript mapping, for example, it differs from the two strains, WS 34/70 and CBS 1538, used in this study, as it lacks hybrid chromosomes III and VII. There are also chromosome copy number differences between the strains used here and the reference strain.

To avoid such issues, we re-sequenced the WS 34/70 and CBS1538 strains and used the combination of the parental strains *S. cerevisiae* and *S. eubayanus* genomes to map the transcriptomes. Using this approach, we identified 11335 Open Reading Frames in WS 34/70 strain and 6805 in the CBS1538 strain. The copy number for each annotated gene was determined from the sequence coverage depth from *de novo* sequencing.

We noted some differences in the chromosome composition of both WS 34/70 and CBS1538 to the published data (Okuno et al., 2016; van den Broek et al., 2015; Wendland, 2014). Specifically, we noted one extra copy of chromosomes Sc III, VII, XIV, and XVI in relation to the most recently published WS 34/70 sequence (Okuno et al., 2016). Additionally, we reconfirm the presence of a hybrid chromosome, ScVIII/SeVIII, recombining at YHR165C, that was previously identified in the WS 34/70 strain and in another Group II strain 7012 by our group (Bond et al., 2004; Monerawela & Bond, 2017b) but which is not documented in other published sequences (Okuno et al., 2016). For the CBS1538, we noted just two copy number differences, an extra copy of both SeVI and SeVIII. Chromosome copy number differences are also noted between published genomes for WS 34/70 (Okuno et al., 2016; van den Broek et al., 2015; Wendland, 2014). The differences in copy numbers between studies may arise from differences in methods used to determine sequence coverage depth or may reflect genuine differences between strain isolates. The lager yeast strains emerged just some 500-600 years ago and thus may still be experiencing genomic flux in this early stage of evolution. We previously showed that the genomes of lager yeasts are dynamic and can undergo chromosome rearrangements to produce new hybrid chromosomes as well as chromosome copy number changes following exposure to stress such as high temperatures (James et al., 2008) . Interestingly, recombination events at YHR165C on chromosome VIII were particularly sensitive to heat stress (James et al., 2008).

Furthermore, fermentations carried out in high specific gravity wort (22°P) and at an ambient temperature of 20°C, which is higher than that used for routine fermentations (13°C), led to chromosome copy number changes in a single round of fermentation (James et al., 2008). Thus, differences in propagation and culturing of *S. pastorianus* strains may contribute to the differences observed between strain isolates.

The analysis of the gene expression patterns reveals that the Group I and Group II strains respond similarly to the physiological conditions imposed with both strains showing similar $\log_2$ fold changes under all conditions. Interestingly, we observed that the Sc and Se sub-genomes are differentially utilised under the different physiological conditions with Se alleles contributing significantly to gene expression on Day 2 of fermentation in both strains and on Day 4 in the Group II strain.

Gene ontology analysis revealed the upregulation of pathways associated with carbohydrate and amino acid metabolism on Day 2 of fermentation although there was less enrichment of genes associated with sugar metabolism in the Group I strain. The upregulation of carbohydrate and amino acid metabolism in the early stages of fermentation is consistent with what is known about the overall metabolic activity of yeast during fermentation. Cells undergo 1-2 doublings during the first three days of fermentation and thereafter, cell numbers remain unchanged or slightly decrease. The lack of representation of genes associated with carbohydrate metabolism in the Group I strain, with the exception of those associated with starch and sucrose metabolism, distinguishes the two strains and appears to reflect differences in metabolic activity. As overall sugar consumption is similar in the two strains on Day 2, as evidenced in Fig.1, it is possible genes associated with carbohydrate metabolism in the Group I strains are induced but did not reach the cut off threshold for analysis. It does not appear that sugar metabolism is slowed down in this strain as otherwise we may have expected to see such genes upregulated on Day 4. The observed differences in carbohydrate metabolism may reflect the previously noted differences in the types and copy numbers of genes encoding for maltose and maltotriose transporters between the two strains (Magalhaes et al., 2016; Vidgren & Londesborough, 2012; Vidgren et al., 2005).

The upregulation of genes associated with amino acid metabolism is significant as the secondary metabolites associated with flavour such as higher alcohols and esters are

produced from the catabolism of amino acids. The gene ontology also revealed differences in metabolism between the two strains. For example, genes associated with methane, butanoate and sulphur metabolism, which can also contribute to flavour profiles, are upregulated in Group II but not in Group I.

Gene ontology analysis confirm the over representation of Se alleles in the upregulated genes in the Group II strain and additionally identified specific pathways where either Sc or Se alleles are exclusively enriched. We observed that Se alleles are exclusively enriched in histidine metabolism while conversely, Sc alleles exclusively contribute to the enrichment of genes associated with phenylalanine, tyrosine and tryptophan biosynthesis in the Group II strain.

Considering the reduced Sc content in the Group I strain, it is surprising that Sc alleles appear to be over-represented in genes enriched in several pathways. Specifically, we see Sc alleles exclusively contributing to Arginine and Lysine biosynthesis and Tyrosine metabolism. We also observed that Sc alleles are more likely to be downregulated than Se alleles in both Group I and Group II strains; however specific pathways here also contain exclusively Se alleles.

Previous studies in the haploid *S. cerevisiae* showed that the steady state levels of mRNA transcripts are tightly controlled to maintain homeostasis. Disruption of mRNA turnover, for example by deletion of the major 5' to 3' exonuclease *XRN1*, is compensated by changes to RNA polymerase II transcription to restore homeostasis (Haimovich et al., 2013; Sun et al., 2013). Conversely, increased transcription rates resulting from increased gene copy number lead to compensatory changes in mRNA turnover to preserve the expected steady state levels of mRNAs (Osley & Hereford, 1981). However, a recent analysis of laboratory and wild *S. cerevisiae* strains revealed no evidence of dosage compensation and instead described a direct correlation between gene copy number and gene expression levels (Torres et al., 2016).

Here we show that the aneuploid nature of the *S. pastorianus* genomes directly contributes to the gene expression patterns during fermentation. We observed a direct correlation between orthologue copy number and the levels of steady state levels of orthologue transcripts in the Group II strain: as the copy number of Sc or Se genes increases so too does the associated levels of transcripts. Interestingly, when the orthologue gene copy

number is 1:1, we observed that the Se alleles showed higher levels of transcripts than the Sc alleles in all conditions tested. Surprisingly, the pattern of usage of orthologues is reversed in the Group I strain. While there is less variation in the ratios of Sc:Se genes with most orthologues present in a 1:2 ratio, here we observed that Sc orthologues produce higher levels of transcripts than the Se counterpart although there is a greater variation in the distribution of gene expression patterns amongst orthologues at this ratio in the Group I strain. We did not observe any evidence of dosage compensation in the lager yeasts. This may indicate that transcription of the two sub-genomes is independent, however previous studies indicated that trans-regulation between transcription of the two sub-genomes may occur in *S. pastorianus* as deletion of the *S. eubayanus*-like copy of *ARO80*, the transcriptional activator that regulates amino acid biosynthetic genes, did not affect the expression of the *S. eubayanus ARO10* gene (Bolat et al., 2013). Our current knowledge of the interplay between the two sub-genomes is limited and further studies are required.

The *S. eubayanus* parent of the lager yeasts is a cryotolerant strain and thus it might be hypothesised that Se alleles will be favoured during the fermentations which are carried out at 13°C. While we did observe a greater contribution of Se alleles to the transcriptome in the Group II strain during fermentation conditions, it is not universal, and this contribution is tempered by gene copy number effects.

To compare the overall steady state levels of protein encoding transcripts in the Group I and II strains, we created combined transcriptomes for each strain by summing the Sc and Se transcripts that encode for the same protein. Using this approach, we observed that overall transcription patterns were remarkably similar between the two strains indicating that despite the significant differences at a genome level and pathways of evolution, the two lager yeast groups display similar transcription patterns in fermentation conditions. Nevertheless, differences in transcription patterns were evident. Consistent with what was observed for the differential expression of Sc and Se alleles within a strain, the gene expression patterns in either strain was significantly influenced by gene copy number.

Thus, our analysis of the expression of gene orthologues within a strain and between strains indicates that the aneuploid genomes of the lager yeasts create complex patterns of gene expression during fermentation and that gene copy number plays a crucial role in the gene expression patterns both within a strain and between strains.

Chapter 7

General discussion and future perspectives

Yeast are microorganisms with a very important relevance in the biotechnology sector. Different yeast species, such the ones that belong to the genus *Saccharomyces*, have participated in different biotechnological processes throughout history. Strains of these species participating in these processes, such as wine and beer production, or bakery, have been domesticated by humankind as these processes have been evolved and improved (Gallone et al., 2016). Different yeast species participate in food biotechnological processes. This microorganism is not only important in this kind of process. The complex metabolism of yeast, the versatility of the strains, small genome and eukaryote nature has made them a good model organism and a good cell factory (Nielsen, 2019).

Specially, yeast belonging to the *Saccharomyces* genus have been the most widely used species. This genus comprises a set of strains related to fermentative processes, such brewing, wine and bakery (Bamforth, 2000; Cordente et al., 2012). These species have a high similarity at the nucleotide level, but different phenotypes can be observed, such as the good fermentative trait of *S. cerevisiae* strains or the cryotolerance of *S. eubayanus*, *S. uvarum* and *S. kudriavzevii* species (Alsammar & Delneri, 2020; Gamero et al., 2013; Libkind et al., 2011; Paget et al., 2014). The high similarity between these species have made possible the generation of interspecific hybrids. These hybrids have been found in man-made environment and resulted from natural hybridization events.

Domestication of these hybrids has generated yeast strains only found in fermentative environments, such as the hybrids found in cider and wine production (Gorter de Vries et al., 2019; Masneuf et al., 1998). *S. pastorianus*, interspecific hybrids between *S. cerevisiae* and *S. eubayanus* species, are only found in man-made environments such as breweries. Generation of hybrids has been extensively studied and implemented at a laboratory level, and it has shown that generation of interspecific hybrids coupled to a selection system is a great tool to produce new strains that may combine traits of both parental strains (Gallone et al., 2019; Garcia-Rios et al., 2018; Hebly et al., 2015).

S. pastorianus is a natural interspecific hybrid used in the Lager beer production. These strains are categorized into two groups based on their genome composition. Group I are allotriploid strains that contain less *S. cerevisiae* genome, and Group II contain allotetraploid strains that have full *S. cerevisiae* and *S. eubayanus* genomes. These strains

are characterized as aneuploid strains, which means that strains have an aberrant karyotype.

To improve the fermentative process is crucial in a competitive sector, especially with the development of the craft beer industry (Statista). Therefore, specialization of the final product is important. Yeast have been characterised and improved at a laboratory level. Several techniques have been used in order to improve different traits of the strains. Genetically Modified Organism (GMO) techniques are straightforward and efficient techniques that allow genomic edition, but strict regulation of GMOs does not allow the use of these modified strains in the agricultural and food processes (Cebollero et al., 2007; Pérez-Torrado et al., 2015; Steensels, Snoek, et al., 2014). Therefore, genetic improvement needs to be done using techniques not catalogued as GMO techniques. Several mutagenic techniques have been described and extensively used, such as chemical and physical mutagenesis, hybridization and laboratory evolution. These techniques have proved successful and evolved strains have been obtained (Cordente et al., 2018; Gamero et al., 2013; Garcia-Rios et al., 2018; Gibson et al., 2020; Gibson et al., 2017; Gibson et al., 2018; Krogerus et al., 2016; Krogerus et al., 2015; Strejc et al., 2013).

The main objective of this project was to generate *S. pastorianus* strains able to overproduce 2-phenylethanol and 2-phenylethyl acetate, two aromatic compounds derived from the catabolism of the aromatic amino acid phenylalanine. For that, we applied two methods that act differently from others such as chemical mutagenesis or UV radiation. These latter two methods mainly produce nucleotide changes, while the chosen methods may induce chromosome changes (Kielland-Brandt et al., 1979). High temperatures are known to induce gross chromosomal rearrangements as *HSP90*, a chaperone that folds proteins involved in repair DNA processes, is inhibited. This inhibition leads to gross chromosomal rearrangements, mainly due single and double strand breaks (Kantidze et al., 2016; Velichko et al., 2012). Radicicol is an antibiotic that induces changes in chromosome copy number by also targeting *HSP90* (Chen et al., 2012). These methods were tested as we wanted to investigate the possible effects that inhibition of *HSP90* may have in complex genomes as *S. pastorianus* strains. As these strains contain two sub-genomes with a high similarity between them (≈80%), we wanted to see if Heat Shock or Radicicol could force chromosome rearrangements or changes in copy number and simulate an accelerated evolution in the cell.

The mutagenesis method was coupled to a selection method that used amino acid analogues. These analogues of amino acid are toxic amino acids that select for strains with an impaired regulation of amino acid biosynthesis. Two amino acid analogues of phenylalanine were used in this study to shed some light on the regulation of the metabolism of *S. pastorianus* strains. Furthermore, we wanted to establish a new method to obtain *S. pastorianus* mutants with improved flavour profiles. It has been previously shown that *S. cerevisiae* mutants resistant to this analogue overproduce 2-phenylethanol and 2-phenylethyl acetate. Other amino acid analogues such as 5,5,5-trifluoro-DL-leucine have been used and mutants overproducing isoamyl alcohol and isoamyl acetate were successfully obtained (Oba et al., 2006; Strejc et al., 2013).

Firstly, small-scale fermentations using different Group I and Group II were carried out to select strains based on their aromatic profile. Results showed differences between groups and within strains of the same group. A Group I strain, CBS1538, and a Group II strain WS 34/70 were submitted to mutagenesis following a selection using amino acid analogues of phenylalanine. After the evolution, 96 mutants were obtained, and of these, 22 random mutants were characterized. Initially, the mutant strains displayed a pseudohyphal growth. This phenotype is observed in cells that are under stress, such as amino acid deprivation under. This phenotype is characterised by a different regulation of cell morphology, cytokinesis and adhesion (Gancedo, 2001; Zaragoza & Gancedo, 2000). This phenotype was transitory as cells experienced a round morphology under the microscope after the stabilization step. This may indicate that Heat Shock and Radicicol may induce genome instability and this destabilization results in morphological changes.

Once the mutant strains did not experience pseudohyphal growth, a characterization in different media was carried out. Characterization of growth in the presence of two different analogues of phenylalanine and in general medium showed that both Heat Shock and Radicicol were effective in mutant generation resistant to amino acid analogues. Only two mutant strains showed some growth defects out of 22.

Analysis of the aromatic profile of small-scale fermentations were carried out and the analysis showed that overproduction of the two target compounds was divergent. It has been observed that resistance to the amino acid analogue is not linearly correlated to the overproduction of aromatic compounds. This may indicate that Heat Shock and Radicicol

may induce different gross chromosomal rearrangements or/and SNPs that may confer resistance to the amino acid analogue but not related to an overproduction of the amino acids or intermediaries of these, and therefore, an increase of flavour compounds.

Two mutant strains that overproduced 2-phenylethanol and 2-phenylethyl acetate were selected. Both strains were obtained using the same concentration of Radicicol (40 µg/mL) but different incubation times. Mutant 11.1 was incubated 48 h and mutant 9.7 was incubated for 24 h.

Small-scale and large-scale fermentations were carried out to confirm the phenotype of the strains. Group I mutant 11.1 overproduced 2-phenylethanol 1.24x times and 2-phenylethyl acetate 1.40x times. The Group II mutant 9.7 overproduced 2-phenylethanol 1.60x times and 2-phenylethyl acetate 2.80x times. Two other higher alcohols obtained from the catabolism of aromatic amino acids tyrosol and tryptophol were also analysed, and both strains were overproducing these two higher alcohols. Mutant 11.1 overproduced tyrosol and tryptophol 2.20x and 1.86x times respectively. Mutant 9.7 overproduced tyrosol and tryptophol 1.74x times and 1.65x times. Furthermore, 9.7 overproduced isoamyl acetate 1.70x times. High concentrations of tyrosol might be beneficial as this compound, together with hydroxytyrosol are antioxidants (Di Benedetto et al., 2007; Rebollo-Romero et al., 2020). However, high concentrations might have an impact on the aromatic and visual profile of beer, as previously reported (Cordente et al., 2018). Tryptophol is related to genotoxicity and sleepiness at higher concentrations but concentrations found naturally in beer are not a concern (Palmieri & Petrini, 2019).

Also, this strain overproduces methionol, to a final concentration of 2.26 mg $\cdot$ L^{-1}. It is an off flavour with notes of raw potatoes (Ferreira & Guido, 2018), but these flavours sometimes are appreciated by the consumers. For instance, in red wine, contamination by *Bretannomyces* produces off flavours from ferulic acid and *p*-coumaric acid, such as 4-ethylphenol and 4-ethylguaïacol. Depending on the consumer's taste, it may be beneficial as it adds complexity to the flavour profile (Suárez et al., 2007).

Further research on the impact of the overproducing higher alcohols and esters on the final organoleptic profile and health might be interesting, as increasing the level of antioxidants in beer might increase distinctiveness of beer.

Furthermore, it has been observed that mutant 9.7 consumes more sugars from wort. This is an interesting trait of this strain that is interesting to explore. Further analysis of the sugar profile after fermentation would be interesting.

Sequencing of both mutants and parental strains was carried out to link the phenotype of the strains to the genome composition. Due to the complexity of the genome of *S. pastorianus* strains, de novo sequencing data was mapped against a reference genome and not *de novo* assembled. Two different reference genomes were used: the combination of *S. cerevisiae* and *S. eubayanus* genome and the genome of the *S. pastorianus* Group II strain CBS1483. Both strategies gave similar information related to chromosome copy number and gene copy number, but subtle differences were reported. The combination of genomes allowed us to have a clearer view of chromosomal rearrangements as this combination of genomes does not contain any hybrid chromosome, only the one found between *S. eubayanus* chromosome VIII and XV (Baker et al., 2015). However, the annotation of this genome is incomplete as some genes such as *ARO9* are not annotated in *S. eubayanus* despite its presence in the genome. The genome of CBS1483 has more accurate annotation but the chromosomal architecture makes it more difficult to visualise the chromosome rearrangements of both parental and mutant strains.

Sequencing of the parental strains showed that Group II strain WS 34/70 has a different chromosome copy number and different chromosome break points compared to previous results. For example, we firstly described the recombination by *RPL35A* in WS 34/70 and we detected the recombination between Se-like chromosomes VIII and XV at *PRP8*. This confirms the plasticity and instability of these strains and might confirm that *S. pastorianus* strains are still evolving. The chromosome copy number of the strain also showed that different isolates of *S. pastorianus* strains have different chromosome copy (Okuno et al., 2016; van den Broek et al., 2015; Walther et al., 2014).

Sequencing of the mutant strains showed different chromosomal rearrangements and point mutations. Analysis of the chromosome copy number based on the coverage revealed a different chromosomal architecture in the mutant strains. Mutant 11.1 suffered some chromosome copy losses, where Se-like chromosomes I, V and XV have lost copies while Sc-like chromosome V has gained one copy. Mutant 9.7 experienced different chromosome copy loss, chromosome loss and chromosome rearrangements.

Interestingly, we here hypothesise that Se-like chromosomes XIII and XVI might have been recombined by a region similar to a retrotransposon with high similarity to the *S. bayanus* transposon Tsu4.

The presence of two similar sub-genomes and high chromosome copy might be advantageous to the *S. pastorianus* strains. It can be observed in Group I strains. This group can be divided into two sub-groups based on the Sc-like content of the strains. CBS1513 has twelve Sc-like chromosomes while CBS1538 has six Sc-like chromosomes. Therefore, the maintenance of these chromosomes might indicate that the strains are still evolving, and that the maintenance of the specific chromosomes may give an advantage to the strains. Also, the high homology between sub-genomes allowed a recombination between two chromosomes, avoiding chromosome loss.

Previous results showed that haploid *S. cerevisiae* resistant strains had a disomy of chromosome XV as two important genes are involved in this tolerance. These genes are the *HSP82* chaperone and *PDR5*, a pleiotropic drug pump. The selected strains in this study experienced different chromosome alterations but none related to copy gain of chromosome XV. Due to the higher copies of both Sc-like and Se-like chromosome XV of both parental strains, *S. pastorianus* might have a higher resistance to Radicicol. When evolution was carried out, inhibition by Radicicol was measured but after incubation of cells with higher concentrations of Radicicol (100 μg/mL), no inhibition was observed. Nevertheless, the results obtained from this study indicate that even small concentrations of Radicicol are enough to induce chromosome instability, and therefore, chromosome rearrangements.

Together with new chromosomal structure in both mutant strains, we detected several Single Nucleotide Polymorphisms (SNPs). Analysis of point mutations revealed mutations along the genome. But we decided to focus on the ones located in coding regions. Two nonsynonymous mutations were analysed, as a mutation in *ARO4* Se-like was detected in mutant 11.1 (D22Y) and one mutation was detected in Sc-like *ARO4* in mutant 9.7 (S195Y). The product of this gene catabolises the first step of the Shikimate

pathway, along with *ARO3*. This step is controlled at an enzymatic level at these enzymes. Both *ARO3* and *ARO4* are inhibited by the final aromatic amino acids. Previous published results showed that key amino acidic positions are involved in the sensitivity against the negative feedback regulation in Aro4p (Hartmann et al., 2003). Mutations of these amino acids make the enzyme Aro4p insensitive to the inhibition caused by aromatic amino acids. Overproduction of 2-phenylethanol and 2-phenylethyl acetate together with tyrosol and tryptophol confirms that mutations in *ARO4* make the enzyme insensitive to the final amino acids and that volatile compounds are overproduced. The mutation found in mutant 9.7 was previously described by Cordente et al. (2018). Here, in this study, a mutation found in an Se-like *ARO4* is described for the first time. Nevertheless, this mutation, D22Y, was described before but in *S. cerevisiae* gene. This means that Se-like *ARO4* might have the same amino acid positions involved in the negative feedback as Sc-like *ARO4*. *In silico* analysis of Sc-like and Se-like ARO4 shows that the primary structure is very similar, with a 97.8% similarity and that the tertiary structure is almost identical.

Mutant 9.7 has loss the *TRP3* Se-like allele. Results from the aromatic profile showed overproduction of both tyrosol and tryptophol. This may suggest that this copy loss does not affect the tryptophan metabolism and further analysis might be interesting to investigate chimeric complexes such as Trp2p/Trp3p. Mutant 9.7 is an ideal strain to investigate this as different gene copy number of Sc-like and Se-like alleles of *TRP2* and *TRP3* with the absence of Se-like *TRP3* will show either specific, chimeric complex or presence of both specific of chimeric complexes at the same time.

As the strains have a different copy number of the new allelic variant of *ARO4* and composition of the genome is different, different degree of overproduction of aromatic compounds cannot be linked to the different mutation. Previously results shows that the different mutations located in *ARO4* may confer different degree of resistance to the negative feedback regulation (Cordente et al., 2018). Mutant 11.1 has only two copies of the wildtype allele and one of the mutant alleles. Mutant 9.7 has two copies of the mutant allele and one of the wildtype alleles, and two of the Se-like *ARO4*. Attempts to introduce the mutation found in mutant 9.7 into the haploid laboratory strain BY4741, was not successful. Previous results have found the S195Y mutation in heterozygosity, as we did. It may indicate that this mutation might not be advantageous in homozygosis as it could be lethal for the laboratory strain, but further analysis might be needed.

Other SNPs were found in the strains, some of them in genes involved in maltose metabolism such as *MAL31* and *MAL33*, leucine metabolism (*LEU2*) and other genes. These SNPs might be linked to the phenotype of mutant 9.7. This strain is able to consume 1ºBx more of sugars and this might be linked to SNPs found in the *MAL* genes. This strain overproduces isoamyl alcohol also, which may be linked to the point mutation found in *LEU2*, but further analysis of these new allelic variants is needed.

Therefore, the whole genome sequencing analysis showed chromosome rearrangements, copy loss and gain, and SNPs. Previous results from Chen et al. (2012) does not show if Radicicol induced SNPs in the strains. We investigated if spontaneous mutants resistant to amino acid analogues could be isolated. Non-treated parental strains were plated onto plates containing the amino acid analogues and no colonies were isolated after five days. Then, this question arises: are the SNPs induced by Radicicol or are the cells experiencing mutations once they are on agar plates containing amino acid analogues? It is possible that unstable cells suffered some sort of polymerase errors during Radicicol treatment or just after when cells were first plated and were experiencing pseudohyphal growth. Then, future work needs to be done to unveil whether Radicicol induces SNPs.

Once the genomic analysis was done, we investigated what the changes in the transcriptome of the new strains are in three different conditions, in minimal medium without amino acids, and under fermentative conditions in small-scale fermentations on Day 2 and Day 4. Furthermore, this analysis was also done between the parental strains to investigate how different *S. pastorianus* strains behave. Analysis of the differences in the transcriptomes between the Group I strain CBS1538 and Group II WS 34/70 showed that genome composition plays a key role in regulation. Here, were report that gene copy number rules the transcription of RNA as genes with higher copy numbers have a higher expression. This has been observed with the intra-analysis of Sc:Se orthologs in WS 34/70. As allele copy increases, expression increases. Interestingly, when Sc:Se ratio is 1:1, expression of Se-like alleles is higher than expected. This may be due to the cryotolerance of *S. eubayanus* as it is known as a cryotolerant species. Probably, Se-like alleles have a different regulation with advantage in low temperatures, such the ones used in these experiments (20ºC in minimal medium and 13ºC in wort fermentations). It has been previously reported that Se-like alleles predominate over Sc-like alleles in cold temperatures (Timouma et al., 2021), but this is the first study that links expression of

different alleles to allele copy number in *S. pastorianus*. Furthermore, we compared Group I transcriptome against Group II mutant using a combined transcriptome for each strain by summing the Sc-like and Se-like transcripts that encoded for the same protein. The enrichment analysis showed that even despite the differences in genome composition, both strains show similar transcriptome patterns.

The analysis of the mutant strains has shown that both strains have some genes that are differentially regulated compared to their respective parental strains.

The chromosome rearrangements, together with the SNPs, induced enough changes to cause a different transcriptional regulation of the mutant strains. The most significative changes have been observed in mutant 9.7. This mutant shows an upregulation of *ARO9* and *ARO10*, both genes involved in the catabolism of aromatic amino acids. It is true that mutant 11.1 has a mutation in *ARO4* too but data shows that *ARO9* and *ARO10* are not differentially expressed, only *ARO9* is upregulated in wort on Day 4. It may indicate that, indeed, different gene copy number of the new allelic variant of *ARO4* may be involved in the different overproduction of 2-phenylethanol and 2-phenylethyl ethanol (**Figure 7.1**). Further analysis must be carried out to investigate if different mutations of *ARO4* produce a different degree of insensitivity against the negative feedback regulation or if different mutations or copy number are behind these differences. But what it is interesting is the higher upregulation of *ARO9* and *ARO10* in mutant 9.7. These genes are highly upregulated in minimal medium and our hypothesis is that this medium forces the expression of *ARO4* due to the lack of preferred nitrogen sources. Then, overproduction of aromatic amino acids, or their intermediaries, are overproduced. Therefore, it might be interesting to investigate overproduction of this higher alcohol and ester in this type of medium as an alternative to the toxic chemical synthesis of 2-phenylethanol (Etschmann et al., 2002).
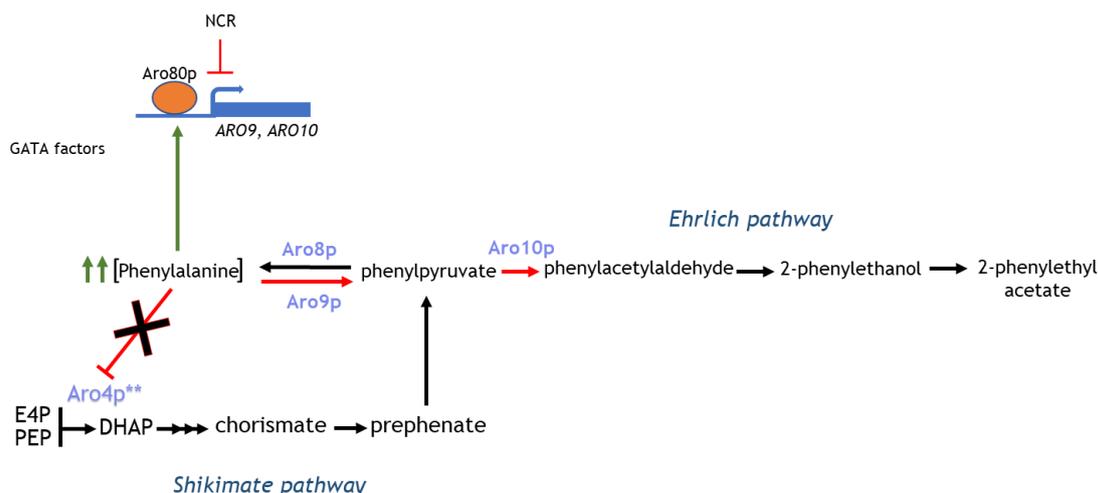
Figure 7.1. Hypothesis of the regulation of *ARO9* and *ARO10* in mutant 9.7 in minimal medium. Mutation in Aro4p makes this enzyme insensitive to the feedback inhibition by the aromatic amino acids tyrosine and phenylalanine. This increase in concentration positively promotes regulation of the transcription factor Aro80p to then upregulate *ARO9* and *ARO10*. Aro80p constitutively binds to the promoter of *ARO9* and *ARO10*, and it is regulated by the Nitrogen Catabolite Repression (NCR).

Although mutant 9.7 overproduces aromatic compounds that positively impact the flavour profile in wort fermentations, this mutant overproduces methionol. RNAseq analysis showed that this mutant overexpresses MET32, a Zinc-finger DNA-binding transcription factor involved in methionine metabolism. Further investigation should be done to study a correlation of MET32 with the overproduction of methionol in this strain.

Analysis of nitrogen permeases was carried out. Different regulation of ammonium permeases might be due to the increased flux from the Shikimate pathway towards the Ehrlich pathway. Also, it could be due to the selection medium. It is possible that the combination of minimal medium and the amino acid analogue can select for strains with an improved metabolism of NH_4^+. Both Group I and Group II mutants had ammonium permeases upregulated. Further analysis needs to be done to investigate whether that upregulation of ammonium permeases is due to overproduction of amino acids (or their intermediaries) or due to selective pressure.

Finally, the data in this thesis suggests that Radicicol as a potent mutagenic reagent to obtain complex polyploid strains with improved traits. This chemical might be used to obtain evolved strains with higher sugar consumption, flavour profiles or to improve the thermotolerance of the strains.

To sum up, we here encourage the usage of Radicicol as a mutagenic reagent in *S. pastorianus* strains. This method coupled to a strong selection method might be useful to obtain strains with improved traits. *S. pastorianus* strains are natural hybrids that might be exploited, and treatment with Radicicol may save time-consuming steps as hybrid generation involves several steps such as searching for natural auxotrophs and/or mating.

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