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Dominance of wine *Saccharomyces cerevisiae* strains over *S. kudriavzevii* in industrial fermentation competitions is related to an acceleration of nutrient uptake and utilization

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Significance Statement

This article is an important contribution to decipher the molecular mechanisms involved in the adaptation to respond to ecological interactions among closely related yeasts species, of interest to understand their growth and survival in fermentation environments.

Summary

Grape must is a sugar-rich habitat for a complex microbiota which is replaced by *Saccharomyces cerevisiae* during the first fermentation stages. Interest on yeast competitive interactions has recently been propelled due to the use of alternative yeasts in the wine industry. The main issue resides in the persistence of these yeasts due to the specific competitive activity of *S. cerevisiae*. To gather deeper knowledge of the molecular mechanisms involved, we performed a comparative transcriptomic analysis during fermentation carried out by a wine *S. cerevisiae* strain and a strain representative of the cryophilic *S. kudriavzevii*, which exhibits high genetic and physiological similarities to *S. cerevisiae*, but also differences of biotechnological interest. In this study, we report that transcriptomic response to the presence of a competitor is stronger in *S. cerevisiae* than in *S. kudriavzevii*. Our results demonstrate that a wine *S. cerevisiae* industrial strain accelerates nutrient uptake and utilization to outcompete the co-inoculated yeast, and that this process requires cell-to-cell contact to occur. Finally, we propose that this competitive phenotype evolved recently, during the adaptation of *S. cerevisiae* to man-manipulated fermentative environments, since a *S. cerevisiae* wild strain with North America oak bark origin showed a remarkable low response to competition.

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Keywords: ecological competition, molecular adaptation, *Saccharomyces* yeasts, wine fermentation, transcriptomic response, cell-to-cell contact.

Introduction

In most natural environments, a vast diversity of microorganisms coexists and compete for space and resources. In many aspects, microbial habitats resemble ecological battlegrounds where microorganisms fight until domination or utter destruction of the opponent. Grape must is sugar-rich habitat for a complex microbiota of yeasts and bacteria that are usually replaced by just one or a few *Saccharomyces cerevisiae* strains after the first stages of wine fermentation (Querol *et al.*, 1994; Fleet, 2003). In this paper, we understand the concept of dominance as the phenomenon that is observed in mixed microbial populations when one individual (strain) is outnumbered by another (Pérez-Torrado *et al.*, 2017).

Competitive interactions between *S. cerevisiae* and other naturally present microorganisms in wine must, mostly non-*Saccharomyces* yeast, have been the subject of diverse studies (Fleet, 2003; Bagheri *et al.*, 2016; Ciani *et al.*, 2016). This interest has recently been propelled due to the fact that, in the last years, the use of alternative yeasts in winemaking has become a widespread trend to respond to the new demands of the wine industry (Jolly *et al.*, 2014; Pérez-Torrado *et al.*, 2017). These demands come from, first, the effect of global warming on vines, which produces an uncoupling of sugar content and phenolic maturity in grapes resulting in higher ethanol yields; and two, an increasing market demanding wines with lower ethanol content and with diverse flavours and aroma.

S. cerevisiae yeasts are characterized by their high capability to ferment simple sugars into ethanol even in the presence of oxygen, Crabtree (1928) effect. Although alcohol fermentation is energetically much less efficient than aerobic respiration, it provides with a

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selective advantage to these yeasts to outcompete other microorganisms: sugar resources are consumed faster and the ethanol produced during fermentation (Goddard, 2008), as well as higher levels of heat and CO_2, can be harmful or less tolerated by their competitors (Piskur and Langkjaer, 2004; Piškur *et al.*, 2006; Conant and Wolfe, 2007; Merico *et al.*, 2007; Hagman *et al.*, 2013; Williams *et al.*, 2015). Also, nitrogen source consumption and biomass production are more efficient in *S. cerevisiae* (Monteiro and Bisson, 1991; Andorrà *et al.*, 2012). Therefore, a more efficient nutrient uptake seems to be one of the most important factors for *S. cerevisiae* dominance.

Strikingly, some studies suggested a secondary or irrelevant role for ethanol as a selective factor, and pointed to other factors as determinant of the competition outcome. Some authors proposed a relevant role to the production and release of toxic peptides by *S. cerevisiae*, such as those derived from glyceraldehyde 3-phosphate dehydrogenase (GAPD) (Pérez-Nevado *et al.*, 2006; Albergaria *et al.*, 2010; Branco *et al.*, 2014). Temperature, as already mentioned, has also been proven to be highly influential on competitions (Goddard, 2008; Arroyo-López *et al.*, 2011). In fact, fermentations under low temperature conditions can benefit the competition capability of cryophilic *Saccharomyces* yeasts, such as *S. eubayanus*, *S. kudriavzevii* and *S. uvarum,* which can coexist with *S. cerevisiae* until the end of mixed–culture fermentations at low temperatures (Alonso-del-Real, *et al.*, 2017).

Strains belonging to these three cryophilic *Saccharomyces* species were already proposed as promising starters for wine fermentations (Arroyo-López *et al.*, 2010; Peris *et al.*, 2016; Alonso-del-Real *et al.*, 2017; Henriques *et al.*, 2018). They exhibit physiological properties that are especially relevant during the winemaking process, such as their good performance in fermentations at low temperatures, resulting in wines with lower alcohol and higher glycerol contents (Varela *et al.*, 2016; Pérez-Torrado *et al.*, 2017), as well as the production of larger

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and diverse amounts of aromatic compounds (Gamero *et al.*, 2013; Stribny *et al.*, 2015). *S. kudriavzevii*, as a member of the *Saccharomyces* genus, exhibits a higher genetic and, physiological similarity with *S. cerevisiae*, as well as species-specific differences (Arroyo-López *et al.*, 2010; Gamero *et al.*, 2013; Stribny *et al.*, 2015, 2016; Peris *et al.*, 2016), including a better cold adaptation (Tronchoni *et al.*, 2014). In previous studies, we also characterized *S. kudriavzevii* behaviour during competition with *S. cerevisiae* (Arroyo-López *et al.*, 2011; Alonso-del-Real, Lairón-Peris, *et al.*, 2017). However, the main problem of their use, as occurs with most alternative yeast, resides in their implantation and persistence during wine fermentations. Alonso del Real *et al.* (2017) showed that *S. cerevisiae* is not affected by most temperature conditions when competing with *S. kudriavzevii* during fermentation, except at very low temperatures, i.e. 8º C. It is interesting to note that low temperature fermentations, in which *S. kudriavzevii* coexist with *S. cerevisiae* in high proportions (close to 50%), produce wines containing less ethanol and higher amounts of glycerol than wine fermentations conducted only by *S. cerevisiae*, however, higher temperatures result in domination of the culture by *S. cerevisiae*, with very low proportion of *S. kudriavzevii*.

Factors of presumable relevance in the domination phenomenon are cell-to-cell contacts and interactions, as assessed in previous studies (Nissen and Arneborg, 2003; Renault *et al.*, 2013; Kemsawasd *et al.*, 2015; Wang *et al.*, 2015). Moreover, quorum sensing mediated mechanisms have been proposed as taking place during competition (Rivero *et al.*, 2015). In fact, competitor cells have to be in close proximity for an effective response to competition (Arneborg *et al.*, 2005; Pérez-Torrado *et al.*, 2017). However, little information is available about the recognition mechanisms and specific responses of *Saccharomyces* yeasts to the presence of a competitor. This information could be of especial relevance to understand yeast

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interactions during wine fermentation because they potentially affect yeast metabolism and growth, and thus alter the final characteristics and quality of wine.

Transcriptomic analyses have the potential to unveil the cell response to competition at the molecular level. The gene expression program of *S. cerevisiae* during wine fermentation has been profiled in previous studies (Rossignol *et al.*, 2003; Zuzuarregui *et al.*, 2006; Novo *et al.*, 2013; Barbosa *et al.*, 2015; Mendes *et al.*, 2017). Previous studies using this approach regarding competition focused in the interactions among *S. cerevisiae* and bacteria or far distant non-*Saccharomyces* yeasts. More recently, the use of RNAseq allowed to study differential expression not only in *S. cerevisiae*, but also in the co-inoculated competitor *Torulaspora delbrueckii* yeast, observing an activation of both growth and carbon metabolism, which seemed to occur earlier in *S. cerevisiae* (Tronchoni *et al.*, 2017). These authors observed expression activation of genes related to sugar and nitrogen metabolism under aerobic conditions when *S. cerevisiae* was cultured with other non-*Saccharomyces* yeast (Curiel *et al.*, 2017).

In the present work, we performed a transcriptomic analysis during wine fermentations co-inoculated with a wine *S. cerevisiae* strain and a strain representative of the closely related species *S. kudriavzevii* to shed light into the molecular mechanisms involved in the interaction between these two species that could be responsible of the dominance of *S. cerevisiae* in fermentations. Also, we included a *S. cerevisiae* strain isolated from oak tree bark in North America in order to check for this trait to be linked to the origin of a given population.

Results

Analysis of differential gene expression during competition between *S. cerevisiae* and *S. kudriavzevii*

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The aim of this work is to elucidate the molecular response behind the dominance of *S. cerevisiae* when competing against the yeast of potential industrial interest *S. kudriavzevii* during wine fermentations. A transcriptome analysis of the wine yeast *S. cerevisiae* T73 in fermentation at 12 °C and 20 °C, with and without the presence of a *Saccharomyces* wild yeast, *S. kudriavzevii* CR85, was performed. Samples were collected at three different fermentation stages: early exponential phase (EEP), late exponential phase (LEP) and stationary phase (SP). These three time points at the very beginning of fermentation were selected based on previous results that showed that after these stages cell populations remained stable at the same proportions (**Figure S1**). In addition, the same experiment, but this time using a *S. cerevisiae* strain isolated from oak bark, *S. cerevisiae* strain YPS128 and *S. kudriavzevii* CR85, was performed with the goal of elucidating whether the competition effect is associated at species or strain levels.

The defined variables in the differential expression analysis were *time*, *temperature*, *culture* (single or co-inoculated) and *species*. PCA of our samples showed that 59% of variance corresponded to PCA component 1, which could be practically identified with the variable *species* itself (**Figure S2**). Even when samples were clustered just according to genes exclusively affected by the variable *culture,* first branch unequivocally separated *S. cerevisiae* and *S. kudriavzevii* samples (**Figure S3).** Although gene expression differences among *Saccharomyces* species are very interesting, the main objective of the present study is to determine the effect of the species competition on gene expression. For this reason, we decided to keep all of the available genes for each species. Thus, two *species*-specific datasets were used for the subsequent analyses.

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A PCA of the *S. cerevisiae* dataset showed that samples grouped mainly according to the variable *time*, meaning that the phase of fermentation was the main factor for sample variance. The variable *culture* accounted for less sample variance, that is, for lower levels of differential expression (**Figure 1**). In the case of *S. kudriavzevii*, there was an overlap among the different fermentation stages; 20°C LEP and 12°C LEP samples cluster with 20°C EEP samples, and 20°C SP monoculture samples, respectively. In comparison to *S. kudriavzevii, S. cerevisiae's* variable *culture* seems to provide a higher sample variance. Differential gene expression analysis between mono and co-culture was carried out by contrast analysis for each species at each temperature and time point to avoid masking effect of these variables.

Interestingly, *S. cerevisiae* showed a stronger transcriptional response to competition during the EEP, higher at 12 °C than at 20 °C (**Figure. 2a**). This response decreases during the following stages at both temperatures, but faster at 20ºC. On the contrary, *S. kudriavzevii* presented during EEP a clearly higher response at 20ºC than at 12ºC, but, as the fermentation progresses, the number of differentially expressed genes increase at both temperatures, becoming very similar during LEP and identical at SP. These results are in agreement with the growth dynamics exhibited by both species in co-cultures compared to monocultures. *S. kudriavzevii* cell density was severely affected at SP, whilst *S. cerevisiae* remained practically unaltered at this stage (**Figure 2b**).

A first general overview of the differential expression analysis highlights a higher expression remodelling in *S. cerevisiae* T73 at EEP, which points to a detection of the competitor at the first stages of fermentation, and a response that might be more efficient in *S. cerevisiae* under these conditions. In the subsequent stages, the unsuccessful *S. kudriavzevii* showed a

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noticeable stress response due to its difficulties in competition culture, which is assessed below.

a) Differential expression in *S. cerevisiae* at EEP during competition

Gene expression modulation in response to competition was already noticeable at EEP. *S. cerevisiae* showed greater differential gene expression between monoculture and co-culture samples at 12°C than at 20°C (**Figure 2a**). At 20°C, 680 genes were overexpressed in co-culture, and 658 genes were repressed (p-value < 0.05, **Table S2**). At 12°C, of the 3518 differentially expressed genes in co-culture, 1874 were overexpressed and 1644 repressed (**Table S2**). Assessing the function of those genes by functional categories enrichment analysis, processes related to metabolism and cell growth were found at both temperatures (**Table S1**). With respect to repressed genes in co-culture, at 12°C we found many categories related to transcription, ribosome synthesis and translation (**Table S1**).

We found 198 upregulated and 79 downregulated genes in common at both temperatures, which were designated as culture-dependent (and temperature-independent) genes at this stage of fermentation. These genes were also classified into functional categories and clustered according to their expression level for *S. cerevisiae* at EEP (**Figure 3a)**. One of the most relevant categories is gene expression regulation, with a high number of genes involved in histone modification and nucleosome regulation, and the basal transcription factor CDC39, which is indicative of a deep transcriptome reprogramming. In addition, genes involved in mRNA splicing are also present, as well as some genes related to cytoplasmic ribosomes and translation regulation. Among the repressed genes, those encoding mitochondrial ribosomal proteins are the most represented class. This agrees with the finding of upregulated genes related to respiration. This change in metabolism is coupled with an upregulation of mitosis and cell cycle progression, and the repression of telomere maintenance genes, which points to

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a faster cell proliferation. In addition, multiple stress response genes were also upregulated, especially those involved in oxidative stress and heavy metal detoxification. Also, glutathione seemed to be synthetized at EEP as well as a relevant set of ABC transporters involved in multidrug detoxification are also overexpressed.

Genes involved in mating pheromone response, as well as some meiosis activating genes, were also overexpressed (**Figure 3a**). Genes involved in endocytosis, protein trafficking, protein degradation, and UPR response were among the upregulated genes. The overexpression of *GAT1* and *APG1*, genes encoding general amino acid transporters, amino acid biosynthesis and TOR signalling pathway genes, are considered as indicative of an acceleration of the nitrogen uptake and metabolism. In addition, iron and zinc uptake and homeostasis genes were also overexpressed. As for carbon metabolism, glycerol, ergosterol, long fatty acid, pentose phosphate pathway and acetate synthesis seemed to be favoured, with an important role of plasma membrane regulation.

To check which transcription factors were regulating gene expression during yeast competition, we used the *contrast* function of *DESeq2* package to generate an expression dataset similar to the previous one, but considering both temperatures. We manually assigned *p-value* 0 to the culture-dependent genes set and *p-value* 1 to the rest of genes. Then, this dataset was loaded into *PheNetic* (De Maeyer *et al.*, 2015) web tool, which uses publicly available interactomics data to create networks from a given expression dataset, revealing possible master regulators and cellular processes relevant for the sample. In this case, the analysis determined Cin5p (*YOR028C*), Phd1p (*YKL043W*) and Spt23p (*YKL020C*) as the central transcription factors, which are involved in response to external stimulus and are known to recruit the general repressor Tup1p to certain promoters (Hanlon *et al.*, 2011) (**Fig. 3b**). Other transcription factors known to be involved in external stimulus response were

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Yrm1p (*YOR172W*) and Cbf1p (*YJR060W*). In addition, Abf1p (*YKL112W*), involved in vesicle trafficking; Sda1p (*YOR344C*), required for cytoskeleton organization and ribosome biogenesis; and Ihf1p (*YLR223C*), which regulates ribosomal genes transcription and is regulated by TOR signalling pathway, constituted the main nodes in the interaction network.

The typical gene expression along the fermentation for the differentially expressed genes at EEP is the dissipation of this response in the subsequent stages, as in *MIP1* (**Figure 3C**). However, we could find only one gene, *HSP30,* that was kept downregulated in the co-culture with respect to the single culture, especially at 12 °C (**Figure 3C**).

b) Differential expression in *S. cerevisiae* at LEP and SP during competition

At LEP, we found important differences with respect to temperature and at the species level. Regarding the number of differentially expressed genes, *S. cerevisiae* showed 29 at 20 °C and 1388 at 12 °C (**Figure 2**). No significant enriched functional categories were obtained for the 20 °C condition. However, at 12°C, there was a clear response of membrane and cell wall remodelling. (**Table S1**). Also, several genes involved in iron homeostasis were overexpressed in co-culture (**Table S2**). 55 differentially expressed genes were found at SP, which are involved in meiotic phase entrance, translation repression, and response to DNA replication stress (**Table S2**).

c) Differential Expression in *S. kudriavzevii* during competition

Comparatively to *S. cerevisiae, S. kudriavzevii* showed at EEP a lower response to the presence of *S. cerevisiae*, with 75 and 980 hits at 12°C and 20°C, respectively. Differential gene expression increased dramatically in the next stages as can be appreciated in figure 2. At LEP, repressed genes at 20°C and 12°C arose to 1749 and 1043, respectively. Finally, a huge remodelling of expression in *S. kudriavzevii* took place at SP in the co-cultures with ~2,500

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hits for both temperatures. All the genes and enriched GO terms can be explored in the supplementary material of this paper (**Tables S1 and S3**). However, we included a summary of the main enriched functional categories for *S. kudriavzevii* in Table 1. At EEP, there is already a slight response to the presence of *S. cerevisiae*. But, in concordance with the higher number of genes, the response becomes much higher in the next stages, with genes and related to stress response, nutrient homeostasis, and metabolism remodelling.

d) Identification of transcription factors responsible of the differential gene expression during competition

Datasets of differentially expressed genes for every time point, temperature and strain were analysed with *Phenetics* (**Table 2**). At a first glimpse, we could observe the logical lack of central transcription factors for *S. kudriavzevii* during EEP at 12 °C and for *S. cerevisiae* during LEP and SP at 20 °C given the low number of genes in these datasets. Nonetheless, Cin5p, Phd1p and its paralog Sok2p, Mga1p, and Msn4p appeared as the most common factors for all the conditions.

Nutrient consumption during competition

One of the most important results of the comparative transcriptome analysis is that competition favours the expression of genes related to nutrient uptake and cell division, which, in the case of the wine *S. cerevisiae* strain, occur from the first stages of the co-inoculated fermentation. To determine whether nutrient uptake is actually playing a key role in the imposition of *S. cerevisiae* or not, we compared the consumption profiles of nitrogen and carbon sources in single vs. co-inoculated fermentations.

a) Nitrogen uptake

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Nitrogen source concentrations present in the medium (amino acids and ammonium) were measured by HPLC after 12 hours and 24 hours in the single and co-inoculated fermentations at 20 °C. After 12 hours, differences in consumption are almost inexistent among the samples, however, *S. cerevisiae* had consumed after the first 24h (LEP) a larger amount of most nitrogen sources in the medium than S. *kudriavzevii* with the clear exception of tryptophan (Figure S4, Figure 4). An interesting outcome of this analysis is the different pattern of nitrogen source preferences exhibited by the wine *S. cerevisiae* and the wild *S. kudriavzevii*. This way, there are clearly significant differences in the consumption of histidine, which is consumed by *S. cerevisiae* but not by *S. kudriavzevii*, tryptophan, one of the preferred amino acids for *S. kudriavzevii* but one of the less consumed by *S. cerevisiae*, and ammonium which is more preferable for *S. cerevisiae* than for *S. kudriavzevii*. Interestingly, nitrogen source consumption in co-inoculated cultures showed a very similar profile to that exhibited by *S. cerevisiae* in single cultures for all sources, including those that are differentially preferred. This is indicative that wine *S. cerevisiae* determines the amino acid uptake pattern because is faster consuming those nutrients present in the medium. This allows *S. cerevisiae* to outcompete *S. kudriavzevii* and dominate wine fermentations.

b) Sugar consumption

In a previous study (Tronchoni *et al.*, 2009), we observed a different patterns of fructose and glucose consumption during fermentation in different *Saccharomyces* strains, including the two strains used in this study. Therefore, fructose and glucose concentrations were measured by HPLC along fermentation to determine if the carbon source uptake rate is also accelerated during competence. Consumption kinetics of these compounds at 12 °C and 20 °C fits to a non-linear model (**Figure 5**). In co-inoculated fermentations at 12°C, fructose consumption was clearly faster than in monocultures, which was statistically verified by the time necessary

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to consume 90% of the corresponding carbon source (**Table 3**). Although differences are not statistically significant in the case of glucose consumption at 12ºC, a similar trend is observed. However, at 20 °C there were no differences in the fructose consumption between *S. cerevisiae* monoculture and the competition, but the difference is significant with respect to the single culture of *S. kudriavzevii*. Again, although differences are not statistically significant with respect to glucose consumption at 12ºC, consumption in the single culture of *S. cerevisiae* and in the competition are identical and different from consumption in *S. kudriavzevii* monoculture. *S. kudriavzevii* gene expression did not suffer major changes at this stage, so this sugar consumption acceleration was more likely due to *S. cerevisiae* activity.

Importance of cell contact in competitive fitness

As important part of the competitive response mechanism, we wanted to check whether direct cell-to-cell contact is necessary to trigger this process, we performed a set of fermentations in which a dialysis membrane was used to compartmentalize the cultures to avoid cell-to-cell contact. The *intrinsic growth rate* (*r*) parameter was calculated as a metric for fitness. Interestingly, whereas fermentations in which competitors were separated by membranes showed very similar fitness, co-cultures in the same compartment presented significantly lower values than the single cultures, with p-values of $2.32E^{-4}$ for *S. kudriavzevii* and $2.01E^{-3}$ for *S. cerevisiae* (**Figure 6**, *Sk_co_contact* and *Sc_co_contact* bars). This effect was clearer in *S. kudriavzevii*, agreeing with the stress response observed in competition with *S. cerevisiae T73*. These results indicate that cell-to-cell contact is a necessary, or at least important, condition for wine yeast to overcome their competitors.

Is the response to competition with *S. kudriavzevii* similar in wine and in wild *S. cerevisiae* strains?

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To elucidate whether the response to competition is identical or different between wine and wild *S. cerevisiae* strains, we performed a similar study of the response to competition between *S. kudriavzevii* and a wild *S. cerevisiae* strain, YPS128, isolated from a Pennsylvanian Oak tree (Sniegowski *et al.*, 2002). All fermentations were conducted at 20ºC, a temperature at which both strains coexist (Alonso-del-Real *et al.*, 2017).

Regarding the dynamics of differential expression between *S. cerevisiae* YPS128 in co-cultures with respect to monocultures, LEP was the only phase when *S. cerevisiae* YPS128 showed a certain level of differential gene expression, with 65 overexpressed genes and 2 repressed genes for cultures in competition (**Figure 7a, Table S4**). This suggests that faster nutrient uptake did not take place in co-fermentation using a wild strain of *S. cerevisiae* as we had observed with the wine strain T73, which points to an important adaptation to fermentation by *S. cerevisiae* wine strains. This was confirmed by the HPLC analysis on sugar composition during the competition (**Table 3**), and agrees with the lack of growth rate acceleration in co-cultures during competition (**Figure 7b**), in contrast to the acceleration observed in T73 co-cultures (**Figure 2b**).

On the contrary, *S. kudriavzevii* level of response follows a similar pattern to that observed in its co-culture with the wine strain T73 (**Figure 7a**). At EEP, overexpressed genes were related to sporulation, and others had a variety of functions such as glucose transport or nitrogen assimilation utilization (**Table S6**). There was an important gene expression regulation at LEP. The 494 overexpressed genes generated enriched GO terms *ammonium transport*, *fatty acid metabolic process*, *response to stress*, *protein refolding*, (**Table S5**). In addition, the MIPS categories *metabolism of nonprotein amino acids*, *oxidative stress response*, *C4-dicarboxylate transport* and *cell periphery* were found (**Table S5**). For the 213 repressed genes, every GO term enrichment result is related to vesicle transport, such as *ER to Golgi*

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transport or *membrane* (**Table S5**). Interestingly, a situation of stress and metabolism remodelling was taking place at this stage, despite the slight differential transcriptome regulation of *S. cerevisiae* YPS128. Finally, at SP stage, a huge transcriptome regulation change was observed, with around 1,500 overexpressed genes and 1,200 repressed genes in co-culture (**Table S6**). Involved processes showed nutrient limitation and metabolic profile remodelling. Processes that appeared to be diminished are *mitosis*, *cell cycle*, *mitochondrial translation*, *protein transport* and *ribosomal proteins* (**Table S5**). Thus, nutrients uptake and homeostasis together with response to toxicity seemed to be the main cell functions supported, reflecting a harsh situation for *S. kudriavzevii* in co-culture with respect to single culture.

Our results are compatible with a situation in which *S. cerevisiae* YPS128 did not change its behaviour during competition, and hence, is not able to reduce *S. kudriavzevii* up to the same extent as the industrial strain.

Furthermore, we showed above that cell-to-cell contact is important in the competition between *S. kudriavzevii* and a wine *S. cerevisiae* strain. However, when we assessed the performance of *S. cerevisiae* YPS128 in a compartmentalized fermentation, no significant differences were observed either in *S. cerevisiae* or in *S. kudriavzevii* fitness when cell contact is allowed or not (**Figure 8,** S*k_co_contact* and *Sc_co_contact* bars). This result suggests that efficient competitive response in wine fermentation is a strain dependent trait in *S. cerevisiae*, and likely specific of the highly competitive wine yeasts. It also agrees with the lower differential gene expression in the wild strain compared to the wine one.

Discussion

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Crabtree effect is a common to all *Saccharomyces* species ecological strategy and could explain how *Saccharomyces* yeasts could outcompete bacteria and non-*Saccharomyces* yeasts, but not how the ancestor of wine *S. cerevisiae* successfully occupied and outcompeted other *Saccharomyces* yeasts in the new ecological niche found in the crushed grape berries gathered by humans to produce the first fermented beverages. The simplest answer is that these yeasts have since then been exposed to selective pressures due to fluctuating stresses occurring during wine fermentation, such as osmotic stress due to high sugar concentrations, anaerobic stress, acid stress, nutrient limitations, ethanol toxicity or sulphite toxicity (Querol *et al.*, 2003). As a result of this unaware domestication, wine *S. cerevisiae* yeasts are better adapted to this environment than other *Saccharomyces* yeasts (Arroyo-López *et al.*, 2010; Navarro-Tapia *et al.*, 2016). This is supported by the fact that wine *S. cerevisiae* yeasts exhibit differential adaptive traits (Marsit and Dequin, 2015) and conform a genetically differentiated population (Fay and Benavides, 2005; Liti *et al.*, 2009; Almeida *et al.*, 2015).

In the last years, several studies tried to dissect in more detail yeast competition by using bottom-up approaches based on co-culturing different strain combinations in the laboratory, mainly wine *S. cerevisiae* and non-*Saccharomyces* yeasts due to their winemaking applications. This way, different possible, and up to some point, compatible mechanisms or phenotypes relevant for competitive interactions between *S. cerevisiae* and non-*Saccharomyces* have been identified. Although, in some studies, cell-to-cell contact seemed to be unimportant in the competitive phenomenon, which would depend mostly on nutrient depletion or toxic metabolite release (Wang *et al.*, 2015), others indicated that interactions were clearly dependent on a cell-to-cell contact or a close proximity of the competitors (Nissen and Arneborg, 2003; Renault *et al.*, 2013). In some studies, cell-to-cell contacts mediated a killer effect of the constitutive accumulation of GADP-derived peptides in the cell

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wall of *S. cerevisiae*, which affects viability of non-*Saccharomyces* yeasts (Kemsawasd *et al.*, 2015; Branco *et al.*, 2018). Also, cell proximity was also required for a sulphite-sensitive *S. cerevisiae* strain to be affected by the toxic effect of the sulphite efflux produced by a tolerant strain (Pérez-Torrado *et al.*, 2017). In some cases, competition can be passive, such as the constitutive production of toxic compounds (Branco *et al.*, 2016), but in other is regulated as a specific response to the presence of competitors. Active response to competitors can be mediated by indirect effector molecules (quorum-sensing signalling) or by cell-to-cell contact. As an example of the former, Rivero *et al* (2015) proposed that the detection of a competitor *S. cerevisiae* strain by a winery dominant *S. cerevisiae* strain was mediated by the altruistic autolysis and release of Hsp12p, which acts as a quorum sensing signal to stimulate killer activity and auto protection, encoded by the *PAU* genes. As an example of the latter, Perrone *et al.* (2013) suggested that dominant response of a strain only occurs when there is a detection of the competitor mediated by cell-to-cell contact.

In the present study, we also reported how a wine *S. cerevisiae* strain was able to dominate the fermentation niche after the detection of the competitor mediated by cell-to-cell contact. As a response to competition, T73 extensively reprogrammed gene expression, which lead to a more efficient nutrient consumption and apparent growth anticipation. This behaviour had been previously observed in the case of competitions against bacteria and non-*Saccharomyces* yeast (Tronchoni *et al.*, 2017). This seems to be linked to the modification of the plasma membrane composition. Ergosterol modifies the fluidity of the yeast membrane, which allows a more efficient activity of membrane transporters and increases tolerance to ethanol, characteristics related with a higher fermentation performance. According to the transcription factors found to be most likely the central nodes in this genetic acclimation, stress like response also seems to take place during competition. However, once *S. cerevisiae*

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411 accelerated nutrient uptake and accumulation in the first fermentation stages, the expression
412 remodelling response decreased in the subsequent periods. Interestingly, *HSP30* was found to
413 be the only gene downregulated in the three different time points. Hsp30p is a chaperone
414 involved in the correct folding of certain membrane proteins, among which Pmp1p is one of
415 the most important. Pmp1p is a basic element in intracellular pH regulation and is directly
416 involved in processes such as stress response (Dong *et al.*, 2017) and aging. Pmp1p
417 accumulates in the plasmatic membrane after every budding event, and its accumulation
418 determines cell aging by impeding further cell divisions (Henderson *et al.*, 2014). Thus, the
419 repression of HSP30 expression would imply defects in Pmp1p folding, which could
420 constitute a mechanism for cell division deregulation.

421 *S. kudriavzevii* also exhibits a response to competition in which nutrients uptake seems to be
422 important. High affinity sugar transporters were overexpressed in a moment when sugar was
423 still at elevated concentration in the medium, as well as the oligopeptide transporter coding
424 gene *OPT1*, which have been recently identified among the upregulated genes of wine *S.*
425 *cerevisiae* in co-culture with *Oenococcus oeni* (Rossouw *et al.*, 2012). Oligopeptides
426 transporters activation could be a mechanism for nitrogen resources increased acquisition
427 (Marsit *et al.*, 2016). Moreover, the nodes obtained for the control of gene expression in
428 response to competition at 20 °C are very similar to those found for *S. cerevisiae*, pointing to
429 a similar response which would be temperature dependent. However, this response in *S.*
430 *kudriavzevii* is delayed and weaker than in *S. cerevisiae,* which acquires and accumulates
431 nutrients in a faster way, and hence, this response could be activated as a consequence of the
432 progressive reduction of nutrients available in the medium, especially limiting nitrogen
433 sources, rather than by the presence of a competitor. In fact, when *S. kudriavzevii* detected the

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nutrient depletion caused by *S. cerevisiae*, increasingly triggers stress response mechanisms to cope with it in the later stages of fermentation.

Regarding the regulation of the expression, we identified several central transcription factors present in most conditions. The most frequently found was *CIN5*. Cin5p belongs to the Yap protein family, and is involved in protein degradation (Sollner *et al.*, 2009), salt tolerance (Ni et al., 2009), and diverse stress response (Nevitt *et al.*, 2004). Noteworthy, its paralog Yap6p, also involved in salt tolerance (Mendizabal *et al.*, 1998) is present among the central factors in *S. kudriavzevii* during SP at 20 °C. Phd1p and Sok2p regulate pseudohyphal growth in opposite ways, being Sok2p a repressor of the enhancer Phd1p. Swi5p, another of the transcription regulators found, is also involved in this process (Pan and Heitman, 2000). Pseudohyphal growth occurs under nitrogen limitation conditions, mediated by the heterodimers Tec1p and Ste12p (Gavrias *et al.*, 1996), also present in our analysis. Cin5p, Yap6p, and Phd1p regulate expression under changing environmental conditions, such as stress by nutrient limitation, by recruiting the transcription repressor Tup1p (Hanlon *et al.*, 2011). Mga1p has also been related to heat shock response and pseudohyphal growth. The general stress response transcription factors Msn2p and Msn4p were also present in almost all datasets indicating cells are responding to stress.

For the present study, we used a wine *S. cerevisiae* strain because the main goal was to understand the mechanisms that allow a wine strain to outcompete strains from another *Saccharomyces* species not present in wine. In fact, the wine strain T73 was selected for commercialization as a dry yeast due to its good performance during wine fermentation and is widely used at industrial level (Querol *et al.*, 1992). However, despite other *S. cerevisiae* strains isolated from diverse fermentative and wild environment are variable with respect to fermentation capability, osmotic and ethanol tolerances, they generally show better

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458 characteristics than strains from other *Saccharomyces* species (Arroyo-López *et al.*, 2010)
459 and, depending on the fermentation temperature, they can outcompete them (Alonso-del-Real,
460 *et al.*, 2017). Here, we report two different competitive phenotypes in *S. cerevisiae*. We
461 hypothesize that the wine strain exhibits a strong response including enhanced nutrient uptake
462 abilities based on an active conditional response to the presence of the competitor *S.*
463 *kudriavzevii.* These results are congruent with the observation mentioned above that *S.*
464 *cerevisiae* T73 response to competition consisted on a deep gene expression remodelling
465 which would switch the cells into a more actively nutrient uptaking state. On the contrary, the
466 wild isolate YPS128 showed a passive constitutive response to the same competitor. These
467 results are of especial relevance from an evolutionary point of view because they indicate that
468 wine strains acquired new active mechanisms of response to competition during their
469 adaptation to fermentation environments, such as the general acceleration of nutrient uptake
470 and accumulation during competition. This mechanisms is compatible with the acquisition of
471 other specific mechanisms based on the production of toxic compounds (Pérez-Torrado, *et al.*,
472 2017). For example, sulphite production is used by sulphite-tolerant strains, a trait that has
473 appeared at least twice in wine S. cerevisiae strains (Pérez-Ortín *et al.*, 2002; Zimmer *et al.*,
474 2014).

475 As mentioned, the active response of the wine *S. cerevisiae* strain to competition depends of a
476 direct contact or a close proximity to the competitor *S. kudriavzevii*. However, additional
477 research is required to unveil the mechanisms triggering this response. The recent description
478 of the *S. cerevisiae* pangenome based on 1,011 genomes (Peter *et al.*, 2018) reported 2,856
479 variable (present/absent) ORFs, being *cell-cell interaction* one of the most enriched functional
480 categories. Indeed, some of the genes we found as differentially expressed in competition,
481 which functions remain unknown, could be specifically involved in microbial interactions.

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Among them, the *FLO* gene family could be a clear candidate as the main function of these genes is self-recognition and flocculation interaction with other cells (Goossens *et al.*, 2015). In fact, a study in which *FLO1*, *FLO5, FLO9* and *FLO10* expression was controlled in co-cultures of *S. cerevisiae* and several non-*Saccharomyces* yeast, aggregation-flocculation and yeast competitive fitness varied depending on the competitor species and the overexpressed flocculin, which implies a species- or strain-specific mechanism of cell-to-cell interaction (Rossouw *et al.*, 2015). Whatever the role of the *FLO* gene family is, the recognition mechanism involved in the competition interactions is not only species dependent, but also strain dependent, as the transcriptomic response in the wine *S. cerevisiae* T73 is completely different to that observed in the wild *S. cerevisiae* YPS128.

Transcriptomic analyses are broadly considered a good first approach to understanding the state of a given cell population or its response to a stimulus. In fact, its use is becoming wider in the study of the physiology of *Saccharomyces* from industrial or other origins, especially the species *Saccharomyces cerevisiae* (Carvalho-Netto *et al.*, 2015; Sardi *et al.*, 2016; Nielsen *et al.*, 2017; Yang *et al.*, 2017; Zhang *et al.*, 2018). However different authors had demonstrated a notable lack of correlation with proteomics or metabolomics data that cannot be diminished (Gygi *et al.*, 1999; Chen *et al.*, 2002; Pascal *et al.*, 2008; Ghazalpour *et al.*, 2011; Yeung, 2011). Thus, we tried to confirm the most relevant features of the extensive response observed by physiological or metabolic experiences. Moreover, we intend to conduct further research to obtain a more accurate and informed prediction on the nature of interactions in the wine microbiota; *i. e.* whether the recognition of a strange species could depend on cell wall proteins physical interactions. This can have many important implications in the management and design of the inoculation process to improve wine fermentations according to the producer's and consumer's demands.

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Experimental Procedures

Yeast Strains

Three different *Saccharomyces* strains were used in our experiments. We chose a commercial strain, T73 (Lalvin T73 from Lallemand Montreal, Canada), as a typical representative of a wine *S. cerevisiae* yeast. We also included YPS128, isolated from Pennsylvania woodlands, as a representative of a wild *S. cerevisiae* strain. Finally, for *S. kudriavzevii*, we chose strain CR85, a wild isolate from oak tree bark in Agudo, Ciudad Real, Spain, characterized by being closely related to the parent of the wine hybrids *S. cerevisiae x S. kudriavzevii* and by its good performance in microvinification (Peris *et al.*, 2016). Complete genome sequences are available for these three strains (unpublished results from our laboratory and GenBank BioProject ref. PRJEB7245 and PRJNA480800).

Synthetic Must Fermentation

Synthetic must (SM, Rossignol *et al.*, 2003) was used in microvinification experiments, with 100 g/L glucose and 100 g/L fructose. GPY medium (2% glucose, 2% peptone, 1% yeast extract) was used for overnight growth of precultures.

Fermentations were performed with single cultures of *S. cerevisiae* T73, *S. cerevisiae* YPS128, and *S. kudriavzevii* CR85 and cocultures of equal proportions of *S. cerevisiae* T73 and *S. kudriavzevii* CR85, and *S. cerevisiae* YPS128 and *S. kudriavzevii* CR85. All fermentations were performed in triplicate in 250 mL flasks with screw caps that contained 200 mL of SM.

Overnight precultures were grown in GPY medium at 25◦°C and used to inoculate synthetic musts with an initial concentration of 10^6 cells/mL. Fermentations were incubated at two temperatures (12 °C and 20 °C) with agitation at 100 rpm.

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RNA Sequencing

Sample collection for gene expression profiling was done at three different fermentation times: early exponential phase (EEP), late exponential phase (LEP) and stationary phase (SP). These correspond to 24, 90 and 135 h, respectively, for 12 °C fermentations and to 12, 24 and 50 h, respectively, for 20 °C fermentations. Cells where centrifuged and stored at 80 °C. RNA isolation was performed with the High Pure RNA Isolation kit (Roche Applied Science, Germany). After oligo (dT) mRNA purification, RNAseq libraries were generated with the TruSeq Stranded mRNA Library Preparation Kit (Illumina, CA, USA). A pool of the libraries from the samples of the single *S. cerevisiae* T73 and *S. kudriavzevii* CR85 fermentations, and the *S. cerevisiae* T73 / *S. kudriavzevii* CR85 co-cultures was sequenced on a NextSeq Sequencing System from Illumina (2 x 150 bp). Another pool including the single *S. cerevisiae* YPS128 culture and the *S. cerevisiae* YPS128 / *S. kudriavzevii* CR85 co-culture was also sequenced on a separate batch. All raw reads have been deposited under the BioProject PRJNA487511.

Pair end and read length sequencing allowed to effectively separate sequences coming from genomes with high identity. The large amount of reads that were generated in the process, granted obtaining enough data from the less represented transcriptomes in competitions.

RNAseq and Differential Gene Expression Analysis

Sequence reads from the *S. cerevisiae* T73 and *S. kudriavzevii* CR85 experiment were mapped to a combined reference of both genomes using Bowtie2 v. 2.2.9 (Langmead, 2013). Similarly, sequences from *S. cerevisiae* YPS128 and *S. kudriavzevii* CR85 experiment were mapped to a combined reference of those two genomes. The genomes of *S. cerevisiae* T73 and *S. kudriavzevii* CR85 were previously sequenced and annotated in our laboratory (M. Morard, unpublished), and contained 6009 and 5537 genes, respectively, according to the

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RATT tool (Otto *et al.*, 2011), refined by manual editing. Of them, 5414 genes were orthologous in both species. The reference genome sequence of *S. cerevisiae* YPS128 was obtained from Liti *et. al* (2009), but the annotation was revised in our laboratory. Read counts for each gene were obtained using HTSeq-Count (HTSeq-0.6.1p1, -m intersection-nonempty) (Anders *et al.*, 2015). We obtained on average 13.5 million reads per strain and sample, with a range of 2.2 to 28.5 million after removing one expression outlier replicate of the sample *S. cerevisiae* T73 / *S. kudriavzevii* CR85, 12 C, EEP. We observed a median of 1034 reads per gene across all 118 samples.

Differential gene expression was estimated by using the R package *DESeq2* (Love *et al.*, 2014), based on fitting genes to a generalized linear model (GLM) to obtain maximum-likelihood estimates for the log fold changes (LFCs), to then acquire *maximum a posteriori values* from a second GLM round, which correspond to the final LFC values. Then, Wald tests were performed for differential expression by contrasting two groups, mono- vs. co-cultures at each sampling time and temperature in most of the cases, as detailed in the Results section. Subsequently, Wald test p-values were adjusted for multiple testing, using the approach of Benjamini and Hochberg (1995). The resulting adjusted p-values were used as our reference p-values in the Results section.

Functional enrichment analyses were performed with the web tool *FunSpec* (Robinson *et al.*, 2002), specially designed for yeast datasets. This tool calculates the probability that a *Gene Ontology* (GO) or *Munich Information Centre for Protein Sequences* (MIPS) term is enriched in a given list of genes using the hypergeometric distribution. A Bonferroni correction was applied to compensate the problem of multiple comparisons.

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For principal component analyses (PCA), a variance stabilizing transformation dataset from the $\log_2$fold scale normalized data given by *DESeq2* was used. This function, included in the same R package, allows a more efficient clustering of samples into groups.

The expression matrix for heatmap building was obtained with the function *getVarianceStabilizedData* from the *DESeq2* package. Data was scaled to study the variation of each gene in the given set of samples. The *war.D* clustering method for Euclidean distance matrices was performed. We summarize the main biological processes with a reasonable number of categories that included all the analysed genes.

HPLC Analysis and data treatment

Amino acids and ammonium were determined by High Performance Liquid Chromatography (HPLC, Thermo Scientific Dioned ultimate 3000 series, Waltham, MA, USA). Separation was made in a Thermo Scientific Accucore C18 column (4.6mm*150mm particle size 2.6um) following the method described in Gómez-Alonso *et al.* (2007).

Glucose and fructose concentrations along the fermentation were determined by HPLC (Thermo Fisher Scientific,Waltham, MA, USA) using a refraction index detector and a HyperREZTM XP Carbohydrate H + 8µm column (Thermo Fisher Scientific) equipped with a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific). Samples were appropriately diluted, filtered through a 0.22-µm nylon filter (Symta, Madrid, Spain), and injected in two technical replicates. The analysis conditions were: eluent, 1.5mM of H2SO4; 0.6ml min-1 flux and an oven temperature of 50◦C.

Glucose and fructose utilization by yeasts during fermentation were fitted by means of the three following mathematical equations as in (Tronchoni *et al.*, 2009):

1. A linear decay function:

$$Y = S_0 - K * t$$

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Where Y is the percentage of glucose or fructose still present in must, t is the time (hours), S_0 is the value of interception in the origin, and K is the kinetic constant.

2. An exponential decay function:

$$Y = D + S * e^{-K * t}$$

Where Y is the percentage of glucose or fructose still present in must, t is the time, D is a specific value when t tends to infinity, S is the estimated value of change, and K is the kinetic constant.

3. A sigmoid or altered Gompertz decay function:

$$Y = A + C * e^{-e^{(K * (t - M))}}$$

Where Y is the percentage of glucose or fructose still present in must, t is the time, A is the lower asymptote when t tends to infinity, K is the kinetic constant, C is the distance between the upper and lower asymptote, and M is the time when the inflection point is obtained.

Equations were fitted by means of linear and nonlinear regression procedures with the R function *nls* (R core team, 2018), minimizing the sum of squares of the difference between the experimental data and the fitted model. Fit adequacy was checked by the proportion of variance explained by the model (R^2) respect to the experimental data. For each yeast and temperature, the three equations were tested, but only the function with the highest R^2 was chosen. Subsequently, these equations were used to calculate the time necessary to consume 90% of the initial sugar concentration present in must (*t90*).

Compartmentalized fermentations

Dialysis tubes (VISKING® dialysis tubing RC diameter 28 mm, cut-off MWCO = 12.000-14.000, SERVA Electrophoresis GmbH, Germany) were used to create an inner compartment

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of 20 mL of synthetic must located inside 250-mL capacity screw cap bottles with 180 mL of SM (outer compartment), as described elsewhere (Wang *et al.*, 2015). This way, metabolite and other solute can be exchanged between two yeast populations, inoculated and cultured in the separated compartments. The different fermentation inoculation patterns are shown in **Table 4**. Each compartment was inoculated to reach an initial concentration of 10^6 cells/mL of each strain. Bottles were incubated at 20 ºC and 100 rpm. Cell viability was measured by plating into GPY-agar plates at different time points up to 60 hours of fermentation. After that, cell deposition in the bottom of the membrane prevented us from obtaining decent reproducibility among replicates. In the case of fermentations with both cell types in contact in contact, two technical replicates were done; one of them incubated at a non-selective temperature (25 ºC), and the other at a selective temperature (37 ºC) at which only *S. cerevisiae* can grow. This way, selective temperature cultures can be used to determine the CFU for *S. cerevisiae*, and subtracting this value from the total CFU obtained in the non-selective plates, we can estimate CFU for *S. kudriavzevii* CR85.

To measure fitness, we calculated the intrinsic growth rate (r) using the exponential growth equation (Williams *et al.*, 2015):

$$N_t = N_0 * e^{r * t}$$

Where N_t is final cell density (CFU/mL), N_0 is initial cell density (CFU/mL), and t is time in hours. Fermentation time 60 hours was used to estimate the intrinsic growth rate because it showed the lowest deviation among replicates.

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References

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

Almeida, P., Barbosa, R., Zalar, P., Imanishi, Y., Shimizu, K., Turchetti, B., et al. (2015) A population genomics insight into the Mediterranean origins of wine yeast domestication. *Mol. Ecol.* **24**: 5412–27.

Alonso-del-Real, J., Contreras-Ruiz, A., Castiglioni, G.L., Barrio, E., and Querol, A. (2017) The Use of Mixed Populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to Reduce Ethanol Content in Wine: Limited Aeration, Inoculum Proportions, and Sequential Inoculation. *Front. Microbiol.* **8**:.

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

Anders, S., Pyl, P.T., and Huber, W. (2015) HTSeq-A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**: 166–169.

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

Arneborg, N., Siegumfeldt, H., Andersen, G.H., Nissen, P., Daria, V.R., Rodrigo, P.J., and Glückstad, J. (2005) Interactive optical trapping shows that confinement is a determinant of growth in a mixed yeast culture. *FEMS Microbiol. Lett.* **245**: 155–159.

Arroyo-López, F.N., Pérez-Torrado, R., Querol, A., and Barrio, E. (2010) Modulation of the glycerol and ethanol syntheses in the yeast *Saccharomyces kudriavzevii* differs from that exhibited by *Saccharomyces cerevisiae* and their hybrid. *Food Microbiol.* **27**: 628–637.

Arroyo-López, F.N., Pérez-Través, L., Querol, A., and Barrio, E. (2011) Exclusion of *Saccharomyces kudriavzevii* from a wine model system mediated by *Saccharomyces cerevisiae*. *Yeast* **28**: 423–435.

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

Bagheri, B., Bauer, F.F., and Setati, M.E. (2016) The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. *South African J. Enol. Vitic.* **36**:.

For Peer Review Only

683 Barbosa, C., García-Martínez, J., Pérez-Ortín, J.E., and Mendes-Ferreira, A. (2015)
684 Comparative transcriptomic analysis reveals similarities and dissimilarities in
685 *Saccharomyces cerevisiae* wine strains response to nitrogen availability. *PLoS One* **10**:.

686 Benjamini, Y. and Hochberg, Y. (1995) Controlling the False Discovery Rate: A Practical and
687 Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B* **57**: 289–300.

688 Branco, P., Albergaria, H., Arneborg, N., and Prista, C. (2018) Effect of GAPDH-derived
689 antimicrobial peptides on sensitive yeasts cells: membrane permeability, intracellular pH
690 and H+-influx/-efflux rates. *FEMS Yeast Res.*

691 Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M.G., et al.
692 (2014) Identification of novel GAPDH-derived antimicrobial peptides secreted by
693 *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol.*
694 *Biotechnol.* **98**: 843–853.

695 Branco, P., Francisco, D., Monteiro, M., Almeida, M.G., Caldeira, J., Arneborg, N., et al.
696 (2016) Antimicrobial properties and death-inducing mechanisms of saccharomycin, a
697 biocide secreted by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **101**: 159–
698 171.

699 Carvalho-Netto, O. V, Carazzolle, M.F., Mofatto, L.S., Teixeira, P.J., Noronha, M.F.,
700 Calderón, L. AL, et al. (2015) *Saccharomyces cerevisiae* transcriptional reprograming
701 due to bacterial contamination during industrial scale bioethanol production. *Microb.*
702 *Cell Fact.* **14**: 13.

703 Chen, G., Gharib, T.G., Huang, C.-C., Taylor, J.M.G., Misek, D.E., Kardia, S.L.R., et al.
704 (2002) Discordant protein and mRNA expression in lung adenocarcinomas. *Mol. Cell.*
705 *Proteomics* **1**: 304–13.

706 Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., and Romano, P. (2016) Yeast
707 Interactions in Inoculated Wine Fermentation. *Front. Microbiol.* **7**: 555.

708 Conant, G.C. and Wolfe, K.H. (2007) Increased glycolytic flux as an outcome of whole-
709 genome duplication in yeast. *Mol. Syst. Biol.* **3**: 129.

710 Crabtree, H.G. (1928) The carbohydrate metabolism of certain pathological overgrowths.
711 *Biochem. J.* **22**: 1289–98.

712 Curiel, J.A., Morales, P., Gonzalez, R., and Tronchoni, J. (2017) Different Non-
713 *Saccharomyces* Yeast Species Stimulate Nutrient Consumption in *S. cerevisiae* Mixed
714 Cultures. *Front. Microbiol.* **8**: 1–9.

715 Dong, Y., Hu, J., Fan, L., and Chen, Q. (2017) RNA-Seq-based transcriptomic and
716 metabolomic analysis reveal stress responses and programmed cell death induced by
717 acetic acid in *Saccharomyces cerevisiae*. *Sci. Rep.* **7**: 1–16.

718 Fay, J.C. and Benavides, J.A. (2005) Evidence for Domesticated and Wild Populations of
719 *Saccharomyces cerevisiae*. *PLoS Genet.* **1**: e5.

720 Fleet, G.H. (2003) Yeast interactions and wine flavour. *Int. J. Food Microbiol.* **86**: 11–22.

For Peer Review Only

721 Gamero, A., Tronchoni, J., Querol, A., and Belloch, C. (2013) Production of aroma
722 compounds by cryotolerant *Saccharomyces* species and hybrids at low and moderate
723 fermentation temperatures. *J. Appl. Microbiol.* **114**: 1405–1414.

724 Gavrias, V., Andrianopoulos, A., Gimeno, C.J., and Timberlake, W.E. (1996) *Saccharomyces*
725 *cerevisiae* TEC1 is required for pseudohyphal growth. *Mol. Microbiol.* **19**: 1255–1263.

726 Ghazalpour, A., Bennett, B., Petyuk, V.A., Orozco, L., Hagopian, R., Mungrue, I.N., et al.
727 (2011) Comparative Analysis of Proteome and Transcriptome Variation in Mouse. *PLoS*
728 *Genet.* **7**: e1001393.

729 Goddard, M.R. (2008) Quantifying the complexities of *Saccharomyces cerevisiae*'s
730 ecosystem engineering via fermentation. *Ecology* **89**: 2077–2082.

731 Gómez-Alonso*, S., Hermosín-Gutiérrez, I., and García-Romero†, E. (2007) Simultaneous
732 HPLC Analysis of Biogenic Amines, Amino Acids, and Ammonium Ion as Aminoenone
733 Derivatives in Wine and Beer Samples. *J. Agric. Food Chem* **55**: 608–613.

734 Goossens, K.V.Y., Ielasi, F.S., Nookaew, I., Stals, I., Alonso-Sarduy, L., Daenen, L., et al.
735 (2015) Molecular mechanism of flocculation self-recognition in yeast and its role in
736 mating and survival. *MBio* **6**: e00427-15.

737 Gygi, S.P., Rochon, Y., Franza, B.R., and Aebersold, R. (1999) Correlation between protein
738 and mRNA abundance in yeast. *Mol. Cell. Biol.* **19**: 1720–30.

739 Hagman, A., Säll, T., Compagno, C., and Piskur, J. (2013) Yeast "Make-Accumulate-
740 Consume" Life Strategy Evolved as a Multi-Step Process That Predates the Whole
741 Genome Duplication. *PLoS One* **8**: e68734.

742 Hanlon, S.E., Rizzo, J.M., Tatomer, D.C., Lieb, J.D., Buck, M.J., and Lustig, A.J. (2011) The
743 Stress Response Factors *Yap6*, *Cin5*, *Phd1*, and *Skn7* Direct Targeting of the Conserved
744 Co-Repressor *Tup1-Ssn6* in S. cerevisiae.

745 Henderson, K.A., Hughes, A.L., and Gottschling, D.E. (2014) Mother-daughter asymmetry of
746 pH underlies aging and rejuvenation in yeast. *Elife* **3**:.

747 Henriques, D., Alonso-del-Real, J., Querol, A., and Balsa-Canto, E. (2018) *Saccharomyces*
748 *cerevisiae* and *S. kudriavzevii* Synthetic Wine Fermentation Performance Dissected by
749 Predictive Modeling. *Front. Microbiol.* **9**: 88.

750 Jolly, N.P., Varela, C., and Pretorius, I.S. (2014) Not your ordinary yeast: Non-
751 *Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* **14**: 215–237.

752 Kemsawasd, V., Branco, P., Almeida, M.G., Caldeira, J., Albergaria, H., and Arneborg, N.
753 (2015) Cell-to-cell contact and antimicrobial peptides play a combined role in the death
754 of *Lachanchea thermotolerans* during mixed-culture alcoholic fermentation with
755 *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **362**: 1–8.

756 Langmead (2013) Fast gapped-read alignment with Bowtie 2. **9**: 357–359.

757 Liti, G., Carter, D.M., Moses, A.M., Warringer, J., Parts, L., James, S.A., et al. (2009)
758 Population genomics of domestic and wild yeasts. *Nature* **458**: 337–41.

For Peer Review Only

759 Love, M.I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and
760 dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**: 550.

761 De Maeyer, D., Weytjens, B., Renkens, J., De Raedt, L., and Marchal, K. (2015) PheNetic:
762 Network-based interpretation of molecular profiling data. *Nucleic Acids Res.* **43**: W244–
763 W250.

764 Marsit, S. and Dequin, S. (2015) Diversity and adaptive evolution of *Saccharomyces* wine
765 yeast: a review. *FEMS Yeast Res.* **15**: fov067.

766 Marsit, S., Sanchez, I., Galeote, V., and Dequin, S. (2016) Horizontally acquired oligopeptide
767 transporters favour adaptation of *Saccharomyces cerevisiae* wine yeast to oenological
768 environment. *Environ. Microbiol.* **18**: 1148–1161.

769 Mendes, I., Sanchez, I., Franco-Duarte, R., Camarasa, C., Schuller, D., Dequin, S., and Sousa,
770 M.J. (2017) Integrating transcriptomics and metabolomics for the analysis of the aroma
771 profiles of *Saccharomyces cerevisiae* strains from diverse origins. *BMC Genomics* **18**:
772 455.

773 Mendizabal, I., Rios, G., Mulet, J.M., Serrano, R., and de Larrinoa, I.F. (1998) Yeast putative
774 transcription factors involved in salt tolerance. *FEBS Lett.* **425**: 323–328.

775 Merico, A., Sulo, P., Piškur, J., and Compagno, C. (2007) Fermentative lifestyle in yeasts
776 belonging to the *Saccharomyces* complex. *FEBS J.* **274**: 976–989.

777 Monteiro, F.F.. and Bisson, L.F. (1991) Biological Assay of Nitrogen Content of Grape Juice
778 and Prediction of Sluggish Fermentations American Society of Enologists.

779 Navarro-Tapia, E., Nana, R.K., Querol, A., and Pérez-Torrado, R. (2016) Ethanol Cellular
780 Defense Induce Unfolded Protein Response in Yeast. *Front. Microbiol.* **7**:.

781 Nevitt, T., Pereira, J., and Rodrigues-Pousada, C. (2004) YAP4 gene expression is induced in
782 response to several forms of stress in *Saccharomyces cerevisiae*. *Yeast* **21**: 1365–1374.

783 Nielsen, J.C., Senne de Oliveira Lino, F., Rasmussen, T.G., Thykær, J., Workman, C.T., and
784 Basso, T.O. (2017) Industrial antifoam agents impair ethanol fermentation and induce
785 stress responses in yeast cells. *Appl. Microbiol. Biotechnol.* **101**: 8237–8248.

786 Nissen, P. and Arneborg, N. (2003) Characterization of early deaths of non-*Saccharomyces*
787 yeasts in mixed cultures with *Saccharomyces cerevisiae*. *Arch. Microbiol.* **180**: 257–263.

788 Novo, M., Mangado, A., Quirós, M., Morales, P., Salvadó, Z., and Gonzalez, R. (2013)
789 Genome-wide study of the adaptation of *Saccharomyces cerevisiae* to the early stages of
790 wine fermentation. *PLoS One* **8**: e74086.

791 Otto, T.D., Dillon, G.P., Degrave, W.S., and Berriman, M. (2011) RATT: Rapid Annotation
792 Transfer Tool. *Nucleic Acids Res.* **39**: e57.

793 Pan, X. and Heitman, J. (2000) *Sok2* regulates yeast pseudohyphal differentiation via a
794 transcription factor cascade that regulates cell-cell adhesion. *Mol. Cell. Biol.* **20**: 8364–
795 72.

For Peer Review Only

Pascal, L.E., True, L.D., Campbell, D.S., Deutsch, E.W., Risk, M., Coleman, I.M., et al. (2008) Correlation of mRNA and protein levels: cell type-specific gene expression of cluster designation antigens in the prostate. *BMC Genomics* **9**: 246.

Pérez-Nevado, F., Albergaria, H., Hogg, T., and Girio, F. (2006) Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **108**: 336–45.

Pérez-Ortín, J.E., Querol, A., Puig, S., and Barrio, E. (2002) Molecular characterization of a chromosomal rearrangement involved in the adaptive evolution of yeast strains. *Genome Res.* **12**: 1533–9.

Pérez-Torrado, R., Barrio, E., and Querol, A. (2017) Alternative yeasts for winemaking: *Saccharomyces* non- cerevisiae and its hybrids. *Crit. Rev. Food Sci. Nutr.* **31**: 1–11.

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

Peris, D., Pérez-Través, L., Belloch, C., and Querol, A. (2016) Enological characterization of Spanish *Saccharomyces kudriavzevii* strains, one of the closest relatives to parental strains of winemaking and brewing *Saccharomyces cerevisiae* x *S. kudriavzevii* hybrids. *Food Microbiol.* **53**: 31–40.

Perrone, B., Giacosa, S., Rolle, L., Cocolin, L., and Rantsiou, K. (2013) Investigation of the dominance behavior of *Saccharomyces cerevisiae* strains during wine fermentation. *Int. J. Food Microbiol.* **165**: 156–162.

Peter, J., De Chiara, M., Friedrich, A., Yue, J.-X., Pflieger, D., Bergstrom, A., et al. (2018) Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. *Nat.*

Piskur, J. and Langkjaer, R.B. (2004) Yeast genome sequencing: the power of comparative genomics. *Mol. Microbiol.* **53**: 381–9.

Piškur, J., Rozpedowska, E., Polakova, S., Merico, A., and Compagno, C. (2006) How did *Saccharomyces* evolve to become a good brewer? *Trends Genet.* **22**: 183–186.

Querol, A., Barrio, E., and Ramón, D. (1994) Population dynamics of natural *Saccharomyces* strains during wine fermentation. *Int. J. Food Microbiol.* **21**: 315–23.

Querol, A., Fernández-Espinar, M.T., del Olmo, M. lí, and Barrio, E. (2003) Adaptive evolution of wine yeast. *Int. J. Food Microbiol.* **86**: 3–10.

Querol, A., Huerta, T., Barrio, E., and Ramon, D. (1992) Dry Yeast Strain For Use in Fermentation of Alicante Wines: Selection and DNA Patterns. *J. Food Sci.* **57**: 183–185.

R Core Team (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

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

For Peer Review Only

Rivero, D., Berná, L., Stefanini, I., Baruffini, E., Bergerat, A., Csikász-Nagy, A., et al. (2015) Hsp12p and *PAU* genes are involved in ecological interactions between natural yeast strains. *Environ. Microbiol.* **17**: 3069–3081.

Robinson, M.D., Grigull, J., Mohammad, N., and Hughes, T.R. (2002) FunSpec: a web-based cluster interpreter for yeast. **3**: 35.

Rossignol, T., Dulau, L., Julien, A., and Blondin, B. (2003) Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast* **20**: 1369–1385.

Rossouw, D., Bagheri, B., Setati, M.E., and Bauer, F.F. (2015) Co-Flocculation of Yeast Species, a New Mechanism to Govern Population Dynamics in Microbial Ecosystems. *PLoS One* **10**: e0136249.

Rossouw, D., Du Toit, M., and Bauer, F.F. (2012) The impact of co-inoculation with Oenococcus oeni on the trancriptome of *Saccharomyces cerevisiae* and on the flavour-active metabolite profiles during fermentation in synthetic must. *Food Microbiol.* **29**: 121–131.

Sardi, M., Rovinskiy, N., Zhang, Y., and Gasch, A.P. (2016) Leveraging Genetic-Background Effects in *Saccharomyces cerevisiae* To Improve Lignocellulosic Hydrolysate Tolerance. *Appl. Environ. Microbiol.* **82**: 5838–49.

Sniegowski, P.D., Dombrowski, P.G., and Fingerman, E. (2002) *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. *FEMS Yeast Res.* **1**: 299–306.

Sollner, S., Schober, M., Wagner, A., Prem, A., Lorkova, L., Palfey, B.A., et al. (2009) Quinone reductase acts as a redox switch of the 20S yeast proteasome. *EMBO Rep.* **10**: 65–70.

Stribny, J., Gamero, A., Pérez-Torrado, R., and Querol, A. (2015) *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* differ from *Saccharomyces cerevisiae* during the production of aroma-active higher alcohols and acetate esters using their amino acidic precursors. *Int. J. Food Microbiol.* **205**: 41–6.

Stribny, J., Romagnoli, G., Pérez-Torrado, R., Daran, J.-M., and Querol, A. (2016) Characterisation of the broad substrate specificity 2-keto acid decarboxylase Aro10p of *Saccharomyces kudriavzevii* and its implication in aroma development. *Microb. Cell Fact.* **15**: 51.

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

Tronchoni, J., Gamero, A., Arroyo-López, F.N., Barrio, E., and Querol, A. (2009) Differences in the glucose and fructose consumption profiles in diverse *Saccharomyces* wine species and their hybrids during grape juice fermentation. *Int. J. Food Microbiol.* **134**: 237–43.

For Peer Review Only

873 Tronchoni, J., Medina, V., Guillamón, J.M., Querol, A., and Pérez-Torrado, R. (2014)
874 Transcriptomics of cryophilic *Saccharomyces kudriavzevii* reveals the key role of gene
875 translation efficiency in cold stress adaptations. *BMC Genomics* **15**: 432.

876 Varela, C., Sengler, F., Solomon, M., and Curtin, C. (2016) Volatile flavour profile of
877 reduced alcohol wines fermented with the non-conventional yeast species *Metschnikowia*
878 *pulcherrima* and *Saccharomyces uvarum*. *Food Chem.* **209**: 57–64.

879 Wang, C., Mas, A., and Esteve-Zarzoso, B. (2015) Interaction between *Hanseniaspora*
880 *uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation. *Int. J. Food*
881 *Microbiol.* **206**: 67–74.

882 Williams, K.M., Liu, P., and Fay, J.C. (2015) Evolution of ecological dominance of yeast
883 species in high-sugar environments. *Evolution* **69**: 2079–93.

884 Yang, J.-R., Maclean, C.J., Park, C., Zhao, H., and Zhang, J. (2017) Intra and Interspecific
885 Variations of Gene Expression Levels in Yeast Are Largely Neutral: (Nei Lecture,
886 SMBE 2016, Gold Coast). *Mol. Biol. Evol.* **34**: 2125–2139.

887 Yeung, E.S. (2011) Genome-wide correlation between mRNA and protein in a single cell.
888 *Angew. Chem. Int. Ed. Engl.* **50**: 583–5.

889 Zhang, W., Li, Y., Chen, Y., Xu, S., Du, G., Shi, H., et al. (2018) Complete genome sequence
890 and analysis of the industrial *Saccharomyces cerevisiae* strain N85 used in Chinese rice
891 wine production. *DNA Res.* **25**: 297.

892 Zimmer, A., Durand, C., Loira, N., Durrens, P., Sherman, D.J., and Marullo, P. (2014) QTL
893 Dissection of Lag Phase in Wine Fermentation Reveals a New Translocation
894 Responsible for *Saccharomyces cerevisiae* Adaptation to Sulfite. *PLoS One* **9**: e86298.

895 Zuzuarregui, A., Monteoliva, L., Gil, C., and del Olmo, M. lí (2006) Transcriptomic and
896 proteomic approach for understanding the molecular basis of adaptation of
897 *Saccharomyces cerevisiae* to wine fermentation. *Appl. Environ. Microbiol.* **72**: 836–47.

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Tables

Table 1. Summary of the main GO terms and genes differentially expressed by *S. kudriavzevii* CR85 in competition with *S. cerevisiae* T73

		12 °C	20 °C
EEP	**UP**	sexual reproduction (*SPS18, FUS2, GAT3, CCH1, DON1, MSH5, 322YSW1*), stress response (*RSB1, FAA1, SLH1, FRT2*), nutrient homeostasis and transport (*IZH4, MLS1, HFA1, FAT3, TRK1, YIL166C, SUC2, PUS6, ZRT1, AGX1, ARG5, MPH2, HXT2*)	structural constituen of cell wall, plasma membrane, regulation of C-compound and carbohydrate, stress response, sugar transport, pseudohyphal growth and sporulation (*STE7, GPA2, DFG5, PTP2*), methionine transport and synthesis genes (*MUP3, MET6, SAM3*), iron ion uptake and homeostasis (*FRE3, FIT2, FIT3, TIR1, TIR3, FET4*), and aroma synthesis (*ATF1, ARO10*)
	DOWN	glucose catabolism,	translation, cell cycle
LEP	**UP**	Ergosterol biosynthetic process, aerobic respiration, integral to membrane response, membrane oxidative stress, sporulation or pseudohyphal growth (*IME1, RIM4, RIM11, SPO24, MSC7, YNL194C, SLZ1,ADY2, FMP45, DIG2, HYP2, RCK1, AQY1*), nutrient limitation (*GIS1, ADR1, SIP2, MRS4, ICY1, PUT1, HMX1, HXT2 ,HXT7 and GAL11*), response to drugs (*PMP3, PDR1, YOR1, FYV4, PDR5, CIN5*), aromatic compounds synthesis (ARO80), general stress response (*MSN4*)	
	DOWN	nucleotide binding	
SP	**UP**	ribosome biosynthesis, transcription, DNA-dependent, metal ion binding, ergosterol biosynthesis process, iron ion homeosthasis, sequence specific DNA binding	methionine biosynthesis process, transmembrane transport, oxidation-reduction process, sulfate assimilation, cysteine biosynthesis process, iron ion homeosthasis, zinc ion binding, fatty acid metabolic process, ergosterol biosynthetic process, sequence specific DNA binding
	DOWN	mitocondrial translation, proteolysis, oxidative stress respirtation, trehalose biosynthetic process and protein refolding	translation, mitosis, mitochondrial translation, proteasomal ubiquitin-independent, trehalose biosynthetic process and protein refolding

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905 **Table 2. Summary of nodal transcriptions factors in gene interaction networks obtained**
906 **with *Phenetics***

	Temperature	EEP	LEP	SP
S. cerevisiae	12 °C	Cin5p, Mga1p, Msn4p, Phd1p, Swi5p, Tos8p	Cin5p, Hmo1p, Msn4p, Ste12p, Sip4p, Tos8p, Yox1p,	Met28p, Met32p, Tec1p, Thi2p, Tos8p, Sok2p
	20 °C	Aft1p, Hap1p, Mga2p, Msn2p, Msn4p, Phd1p, Spt23p, Tye7p, Ume5p		
S. kudriavzevii	12 °C		Adr1p, Cin5p, Hap1p, Met32p, , Mga1p, Phd1p, Sok2p, Yap5p	Cin5p, Hap4p, Met32p, Mga1p, Msn4p, Phd1p, Ste12p, Sok2p, Tos8p, Yap6p
	20 °C	Cin5p, Hap1p, Hap4p, Mga1p, Msn4p, Sok2p	Cin5p, Msn4p, Nrg1p, Sip4p, Sok2p, Swi5p	Cin5p, Mig1p, Phd1p, Sok2p, Yap5p, Yap6p

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Table 3. Time (h) to consume 90% of glucose and fructose initially present in the media (*t90*). Values are given as mean ± standard deviation of three biological replicates and two HPLC detection runs. An ANOVA analysis was carried out.

Temperature	Sugar	t90 $_{T73}$	t90 $_{YPS128}$	t90 $_{CR85}$	t90$_{T73-CR85}$	t90$_{YPS128-CR85}$
12 °C	Glucose	371.1 ± 10.3^{b}	382.6 ± 20.1^{b}	316.0 ± 4.0^{a,b}	255.2 ± 61.2^{a}	361.0 ± 12.7^{b}
	Fructose	601.1 ± 11.9^{c}	623.5 ± 16.6^{c}	486.0 ± 59.1^{b}	346.3 ± 70.5^{a}	548.6 ± 8.4^{b,c}
20 °C	Glucose	214.6 ± 16.2^{a}	191.4 ± 8.1^{a}	280.1 ± 0.9^{b}	220.1 ±4.5^{a}	211.9 ± 23.5^{a}
	Fructose	368.5 ± 13.2^{a,b}	342.9 ± 10.9^{a}	446.0 ± 8.0^{c}	387.7 ± 14.0^{b}	339.8 ± 25.0^{a}

The values followed by different superindexes in the same row are significantly different according to the Tukey HSD test (α = 0.05).

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918 **Table 4. Summary of compartmentalized fermentations performed.**

	MONOCULTURE			SEPARATED				IN CONTACT	
Inner compartment	ScT73	ScYPS128	SkCR85	ScT73	SkCR85	ScYPS128	SkCR85	ScT73, SkCR85	ScYPS128, SkCR85
Outer compartment	ScT73	ScYPS128	SkCR85	SkCR85	ScT73	SkCR85	ScYPS128	ScT73, SkCR85	ScYPS128, SkCR85

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Figures

Fig 1. Principal component analysis of differential gene expression for *S. cerevisiae* and *S. kudriavzevii*

Fig 2. (A) Number of differentially expressed genes in competition for each species at every temperature and phase of fermentation**. (B)** Growth curves of single (continuous curves) and mixed cultures (dashed curves) at 20 °C. Samples were taken at 15 h, 24 h, 39 h and 60 h. Values are mean of three to six replicates. Error bars represent standard deviation.

Fig 3. (A) Hierarchical clustering of culture-dependent genes during EEP divided into functional categories. **(B)** Genetic interactions of culture-dependent genes given by Phenetics (network size = 100). **(C)** Expression profile (Norm. expr.) of genes *MIP1* and *HSP30*. Variance stabilizing transformation of the dispersion estimates dataset was used to reduce dependence of the variance on the mean.

Fig 4. **Increment of percentage of consumption of different nitrogen compounds between 24 hours and 12 hours in fermentation at 20ºC**. Values are the mean of three replicates. Error bars represent standard deviations. ANOVA analysis and Tuckey test were performed for significance evaluation (* = p-value < 0.05, ** = p-value < 0.01).

Fig 5. Sugars consumption profiles during fermentations with single cultures of *S. cerevisiae* and *S. kudriavzevii* and co-cultures. Curves are the representation of sugars concentrations data fitted to different models (R2 > 0.9).

Fig 6. Fitness after 60 hours in compartmentalized fermentations. Intrinsic growth rate (r) was calculated for inner and outer compartment in fermentations with only one of the yeast in both compartments (*Sc_mono* and *Sk_mono*), the two yeast separated in different compartments (*Sc_co_no.contact* and *Sk_co_no.contact*), and the two yeast mixed in both compartments (*Sc_co_contact* and *Sk_co_contact*). Values are mean of six replicates (both from inner and outer compartment taken together), and error bars represent standard deviation. ANOVA analysis and Tuckey test were performed for significance evaluation (** = p-value < 0.01, *** = p-value < 0.001).

Fig 7. (**A**) Number of differentially expressed genes in competition. (**B**) Growth curves of single (*Sc*, *Sk*) and mixed cultures (*Sc_co*, *Sk_co*) at 20 °C. Samples were taken at 15 h, 24 h, 39 h and 60 h. Values are mean of three to six replicates. Error bars represent standard deviation.

Fig 8. Fitness after 60 hours in compartmentalized fermentations. *Intrinsic growth rate* (*r*) was calculated for inner and outer compartment in fermentations with only one of the yeast in both compartments (*Sc_mono* and *Sk_mono*), the two yeast separated in different compartments (*Sc_co_no.contact* and *Sk_co_no.contact*), and the two yeast mixed in both compartments (*Sc_co_contact* and *Sk_co_contact*). Values are mean of six replicates (both from inner and outer compartment taken together), and error bars represent standard deviation. An ANOVA analysis and a Tuckey test were performed for significance evaluation; however, differences were not significant in any comparison.

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961 **Supporting information**

962 **Table S1: Enriched functional terms obtained from the differentially expressed genes of**
963 ***S. cerevisiae* T73 at EEP during competition**

964 **Table S2: Differentially expressed genes of *S. cerevisiae* T73 during competition**

965 **Table S3: Differentially expressed genes of *S. kudriavzevii* CR85 during competition with**
966 ***S. cerevisiae* T73**

967 **Table S4: Differentially expressed genes of *S. cerevisiae* YPS128 during competition**

968 **Table S5: Enriched functional terms obtained from the differentially expressed genes of**
969 ***S. kudriavzevii* CR85 at EEP during competition with *S. cerevisiae* YPS128**

970 **Table S6: Differentially expressed genes of *S. kudriavzevii* CR85 at EEP during**
971 **competition with *S. cerevisiae* YPS128**

972 **Figure S1: Growth curves of S. cerevisiae T73 and S. kudriavzevii throughout all the**
973 **fermentation at 12 °C and 20 °C.** Cell number was measured by cell counting**.** Values are
974 mean of three replicates**.**

975 **Fifure S2: Principal component analysis of differential gene expression for *S. cerevisiae***
976 **and *S. kudriavzevii***

977 **Figure S3: Hierarchical clustering of genes affected by the variable *culture*.** Euclidean
978 distance matrix obtained from the expression matrix was used for clustering with *ward.D*
979 method.

980 **Figure S4: Percentage of consumption of different nitrogen compounds at 8 hours (A)**

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981 **and 24 hours (B) in fermentation at 20ºC**. Values are the mean of three replicates. Error
982 bars represent standard deviations.

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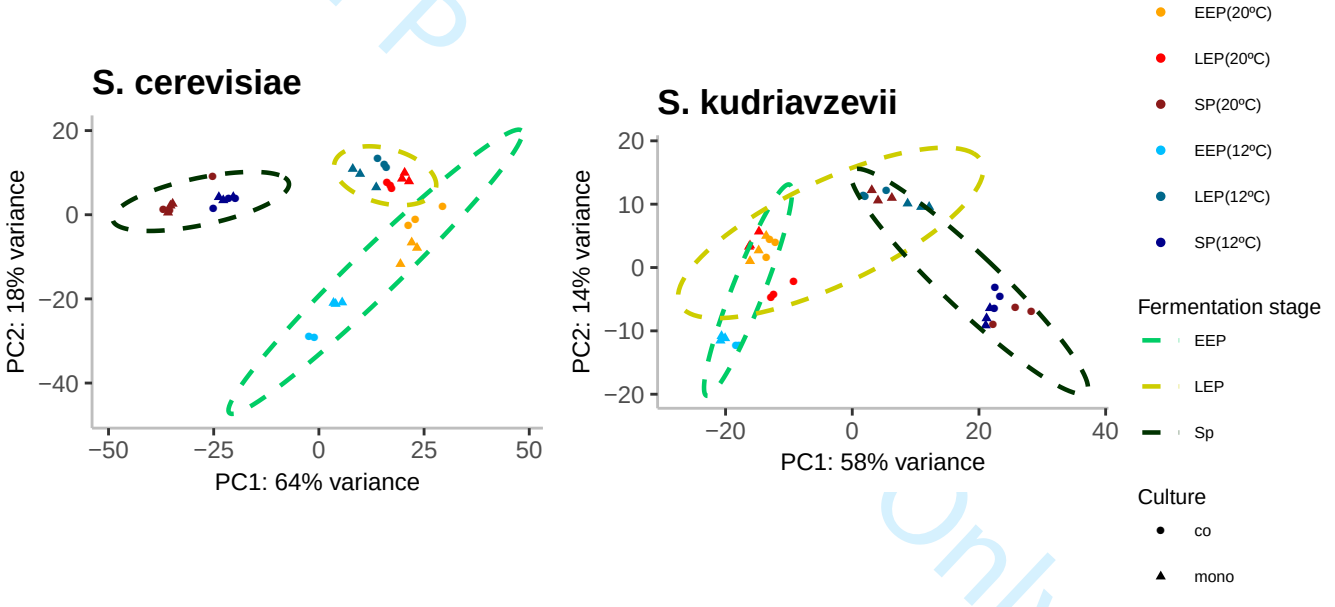

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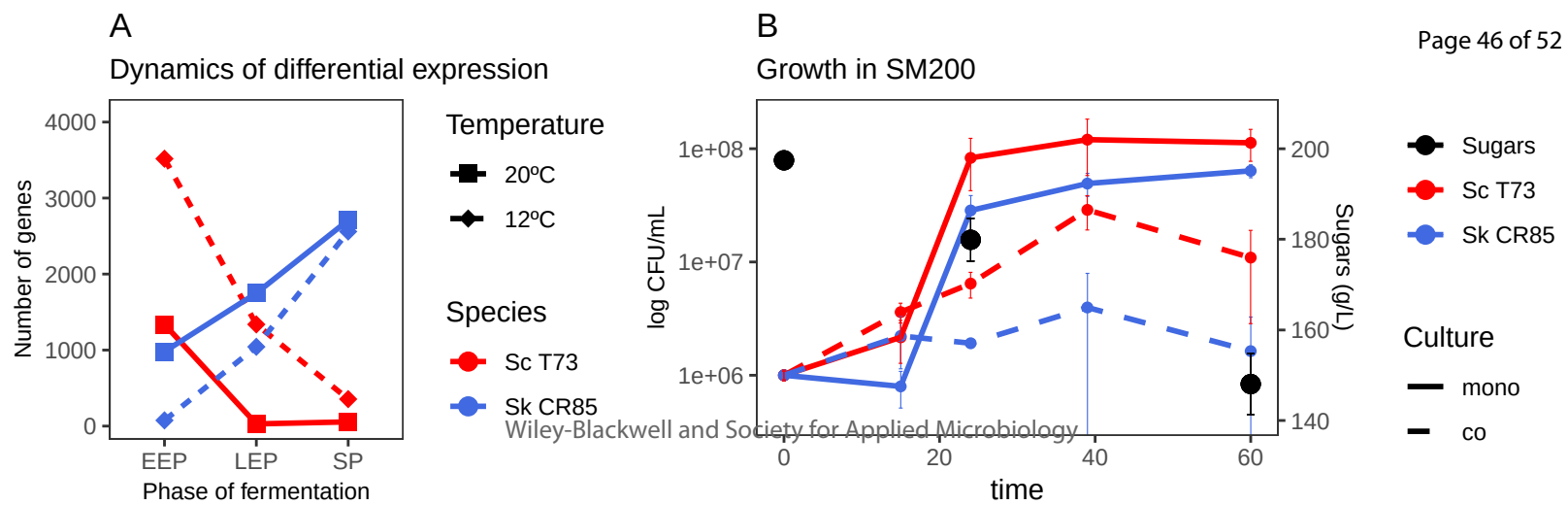

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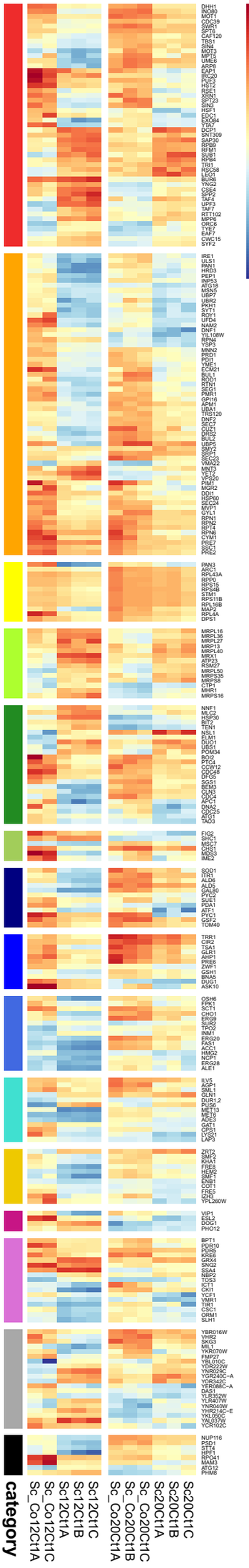
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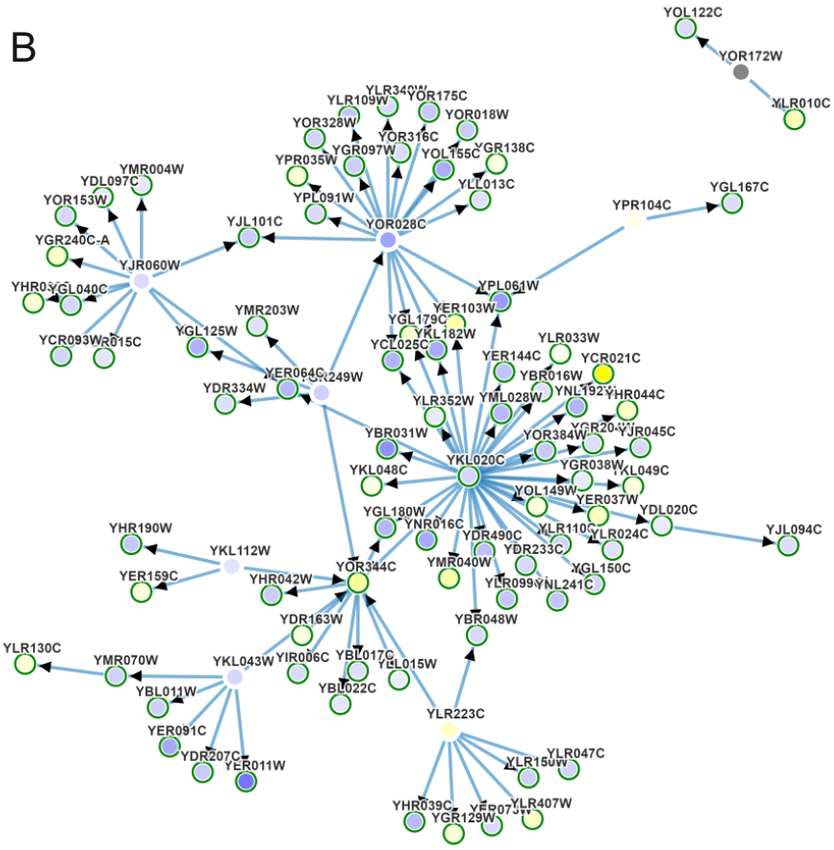
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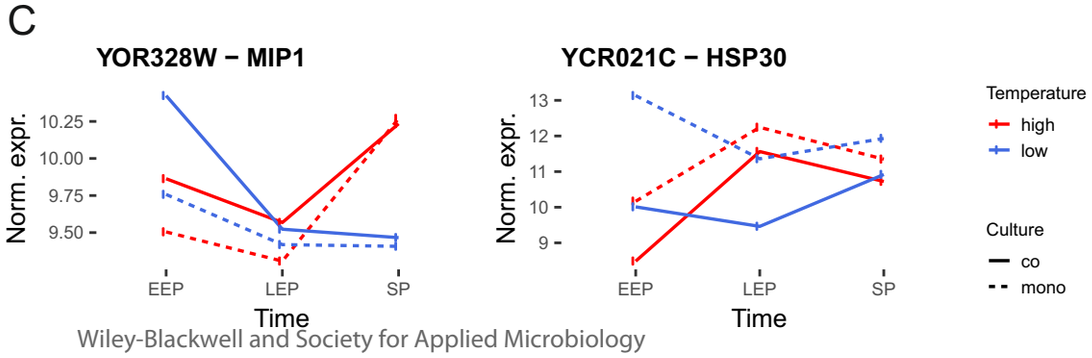
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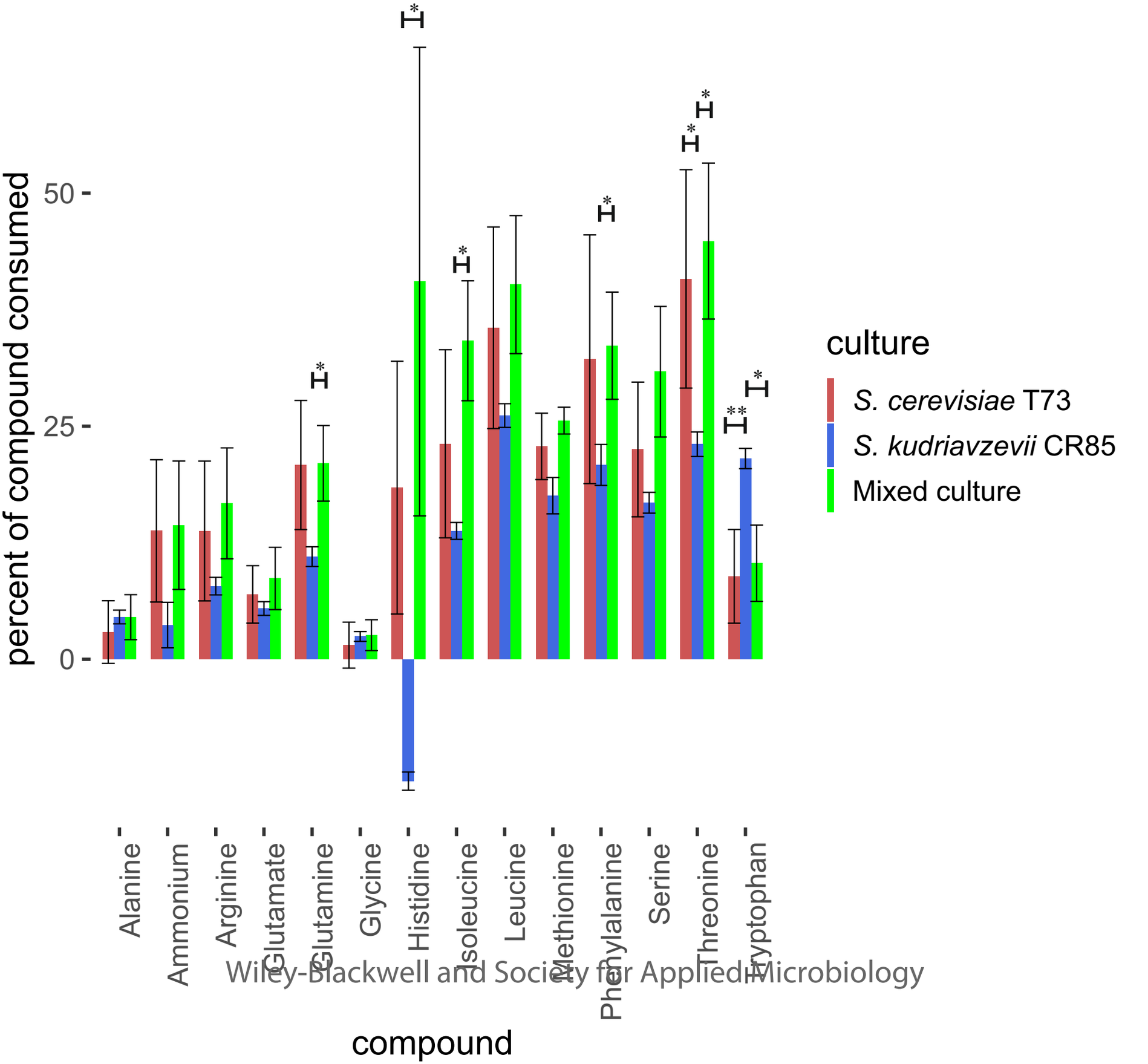

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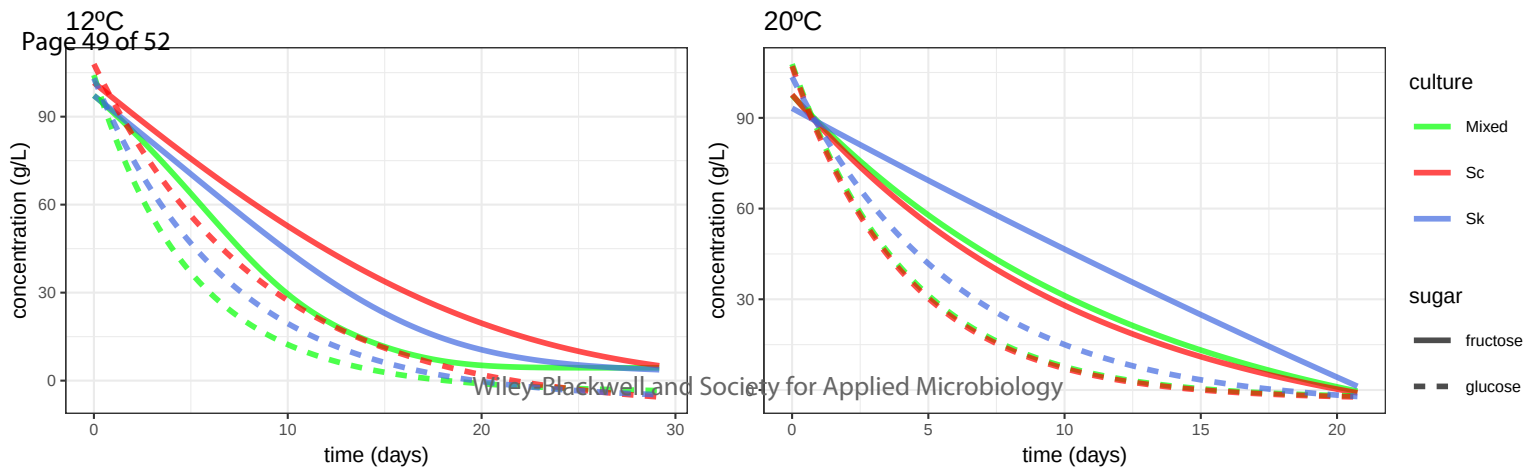
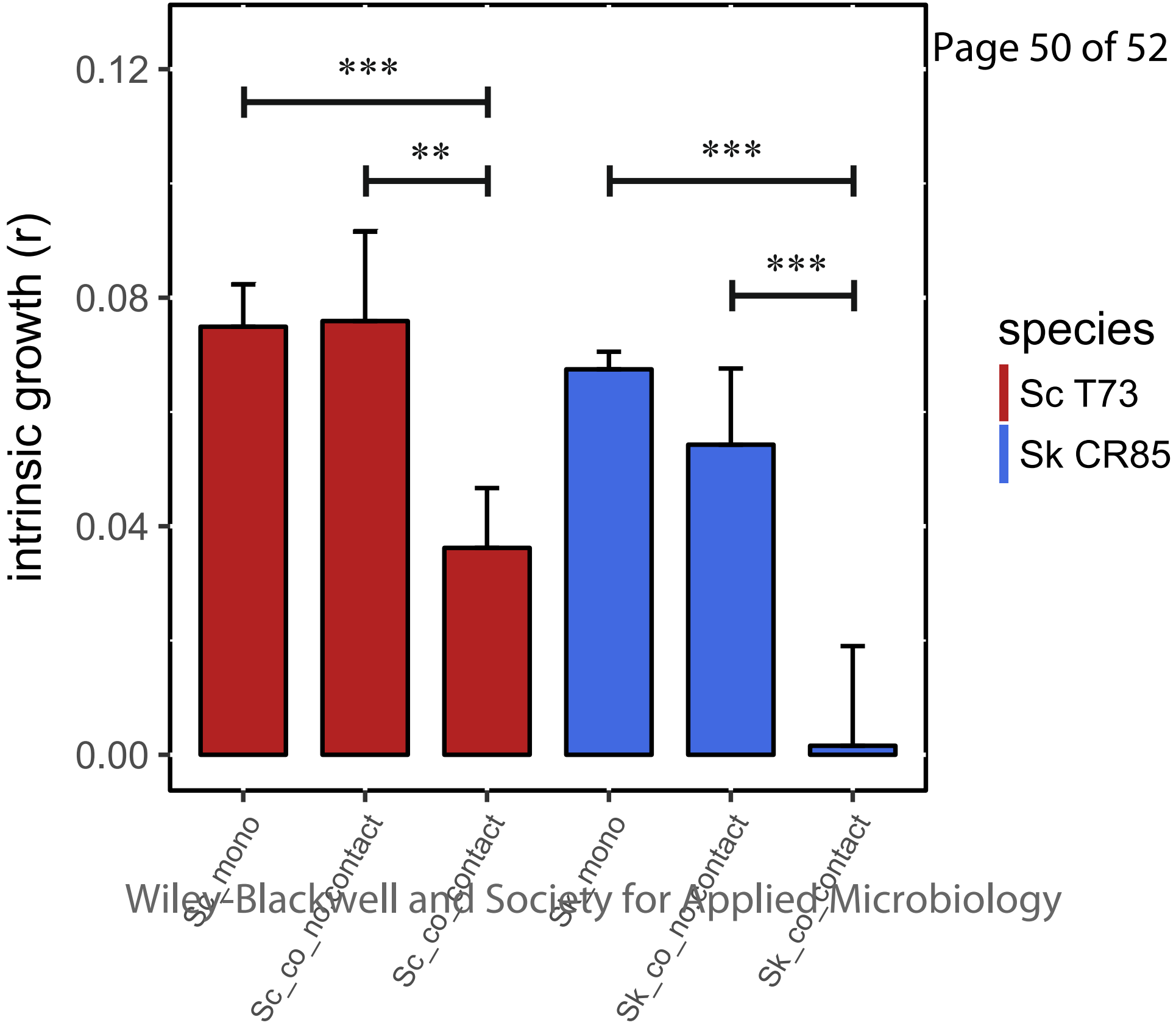


Wiley-Blackwell and Society for Applied Microbiology


0.12
0.08
0.04
0.00
intrinsic growth (r)
***
**
***
***
species
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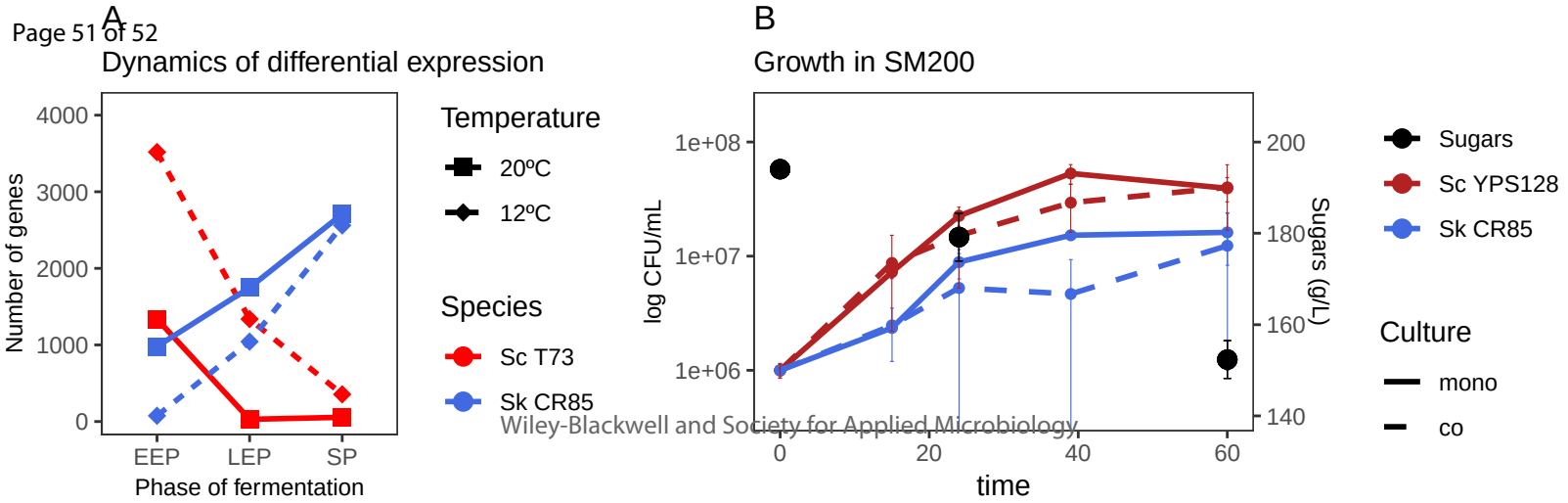
Sk CR85
Sc_mono
Sc_co_noncontact
Sc_co_contact
Sk_mono
Sk_co_noncontact
Sk_co_contact



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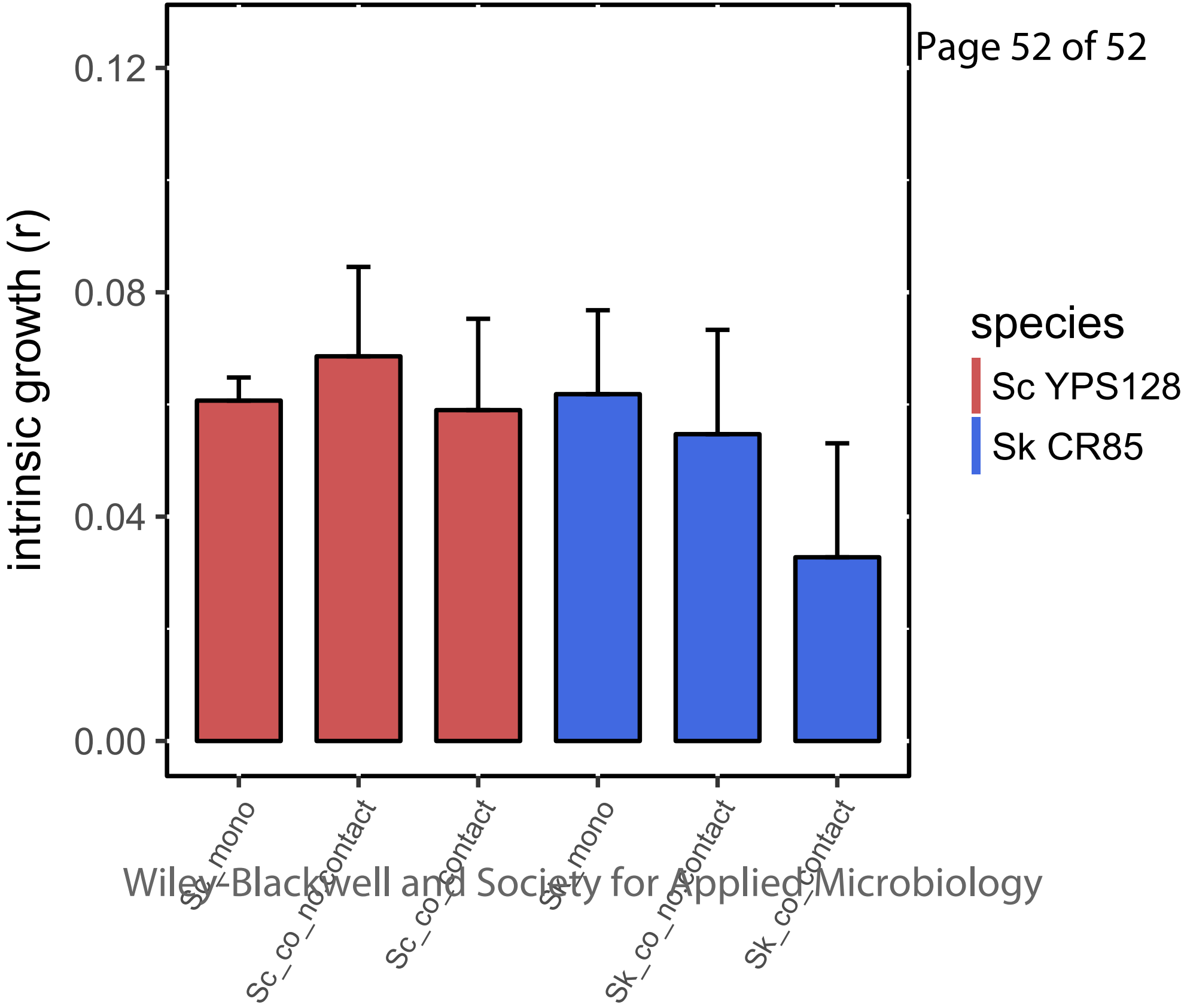
intrinsic growth (r)
0.12
0.08
0.04
0.00
species
Sc YPS128
Sk CR85
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Sc_co_noncontact
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Sk_mono
Sk_co_noncontact
Sk_co_contact