Aroma production and fermentation performance of *S. cerevisiae* x *S. kudriavzevii* natural hybrids under cold oenological conditions.

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Abstract

This work aims to describe the wine fermentation characteristics of 23 natural *S. cerevisiae x S. kudriavzevii* hybrid yeasts related to fermentative environments isolated from different regions and their significance for the aroma spectra of the produced wines. Fermentations were performed at 12 °C in artificial must, and *S. cerevisiae* and *S. kudriavzevii* pure species strains were used for comparison purposes. We determined the relevant kinetic parameters of fermentation, the concentration of the main metabolites and the main aroma-related compounds produced after fermentation. The results revealed that some strains that show well-rounded characteristics could be profitable yeast starters for low-temperature fermentation in winemaking, such as wine hybrid SPG172 but, surprisingly, also beer hybrid CECT11002, adding the efficient fermentative kinetics to the high production of aroma-related compounds. In addition, a novel metabolic correlation between fermentation performance and aroma production is described.

Keywords: Wine yeast, *S. cerevisiae*, *S. kudriavzevii*, natural hybrids, aroma, cryotolerance.

1. Introduction

It is well-known that fermentations to produce alcoholic beverages like wine, beer or cider are dominated by *Saccharomyces cerevisiae* species. However, in the last two decades the intense development of molecular biology techniques and next-generation sequencing technologies has significantly improved our understanding of important role of other

Saccharomyces yeasts. Nowadays we know that many of the yeast strains used for centuries are interspecific hybrids (Krogerus et al., 2017; Pérez-Torrado et al., 2018). A hybrid is a new lineage that comes about by bringing together two diverged genomes (Marcet-Houben and Gabaldón, 2015). The existence of natural interspecific hybrids in yeasts was first indicated by the genetic early characterisation of *Saccharomyces carlsbergensis* in the Carlsberg Laboratory in Copenhagen, which showed that the lager yeast genome included genetic material from *S. cerevisiae* and a non-*S. cerevisiae* yeast (Nilsson-Tillgren et al., 1981). The combination of two genomes with different genetic features may be advantageous for survival under unfavourable conditions or for the colonisation of new environmental niches (Belloch et al., 2008) by enhancing genetic flexibility and promoting adaptive change (Greig, 2002). With the genus *Saccharomyces*, one of the most interesting mechanisms of adaptation to industrial processes is the formation of interspecific hybrids (Lopes et al., 2002). Natural *S. cerevisiae x S. kudriavzevii* hybrids have been found to be associated with fermentation processes in different areas of Europe, regions with oceanic and continental climates, such as England, Belgium, Germany, French Brittany and Alsace in France, Switzerland and Austria (González et al., 2008). *S. cerevisiae x S. kudriavzevii* hybrids exhibit good fermentative capabilities at low temperatures, and produce wines with smaller alcohol quantities and larger glycerol quantities, which can be very useful for solving challenges in the winemaking industry, such as the necessity to enhance the aroma profile (Pérez-Torrado et al., 2018; Peris et al., 2018; Querol et al., 2018; Tronchoni et al., 2017).

The *S. kudriavzevii* species has been described as a good higher alcohols producer (Gamero et al., 2014; Stribny et al., 2015). *S. cerevisiae x S. kudriavzevii* hybrids produce wine with

65 different aroma profiles compared to species *S. cerevisiae* and *S. kudriavzevii* by increasing
66 secondary aroma synthesis, including higher alcohols, acetate esters and ethyl esters, these
67 being the main aromatic compounds that contribute to floral and fruity aromas (Gamero et
68 al., 2014). Peris et al. (2012) carried out experiments to evaluate the genome composition
69 of a set of wine and brewing *S. cerevisiae x S. kudriavzevii* natural hybrids of diverse
70 origins. They also constructed a maximum parsimony tree based in the presence/absence of
71 full chromosomes and chromosome regions showing two main groups: Group I (W46, 441,
72 W27, and SPG 16-91 as well as brewing strains CECT 11003, and CECT11004); Group II
73 (HA 1841, HA 1842, VIN7, and SOY3). However, wine fermentation performance, aroma
74 profile and differences in wine composition among hybrids with different genome
75 compositions and origin were not evaluated.

76 This study focused on the performance of *S. cerevisiae x S. kudriavzevii* natural hybrids of
77 diverse origins in cold wine fermentations in synthetic must, conditions most of these
78 strains were isolated. We selected a low temperature (-12 ºC) were kinetic differences
79 among *S. cerevisiae* and *S. kudriavzevii* in must fermentations were more evident (Arroyo-
80 López et al., 2009; Alonso-Del-Real et al. 2017). We determined the relevant kinetic
81 parameters of fermentations, the concentration of the main metabolites, and the main
82 aroma-related compounds produced after the fermentation. Our results revealed that some
83 strains could be profitable yeast starters for low-temperature fermentation in winemaking.
84 We also found a novel correlation between fermentation performance and aroma
85 production.

86

2. Materials and Methods

2.1. Strains and media

The yeast strains used in this study corresponded to 28 natural hybrids, *S. cerevisiae* and *S. kudriavzevii*. Their references, sources of isolation and geographical origins are listed in Table 1. Yeast cells were maintained and grown in YPD medium (2% glucose, 2% Bacto peptone, and 1% Yeast extract).

2.2. Microvinifications

We used glass bottles of 250 mL with a two-piece airlock (a little plastic device used in making fermented beverages that allows carbon dioxide to escape from the fermenter without letting any new air in, which cuts down on any possible bacterium contamination) and a drilled rubber stopper. Glass bottles were filled with 200 ml of MS300 synthetic must (100 g/L glucose, 100 g/L fructose, 6 g/L citric acid, 6 g/L malic acid, mineral salts, vitamins, anaerobic growth factors, 300 mg/L assimilable nitrogen) to simulate standard grape juice (Bely et al., 2003) at 12°C with agitation (150 rpm) in triplicate. The assimilable nitrogen (ammoniacal nitrogen and α-amino nitrogen) was provided by a mixture of 19 amino acids (612.6 mg/L L-proline, 505.3 mg/L L-glutamine, 374.4 mg/L L-arginine, 179.3 mg/L L-tryptophan, 145.3 mg/L L-alanine, 120.4 mg/L L-glutamic acid, 78.5 mg/L L-serine, 759.2 mg/L L-threonine, 48.4 mg/L L-leucine, 44.5 mg/L L-aspartic acid, 44.5 mg/L L-valine, 37.9 mg/L L-phenylalanine, 32.7 mg/L L-isoleucine, 32.7 mg/L L-histidine, 31.4 mg/L L-methionine, 18.3 mg/L L-tyrosine, 18.3 mg/L L-glycine, 17.0 mg/L L-lysine, and 13.1 mg/L L-cysteine) corresponding to 180 mg nitrogen and 460

mg/L ammonium chloride (corresponding to 120 mg nitrogen). Synthetic must was dosed in glass bottles and inoculated with yeast cells to reach an optical density (OD) of 0.3.

2.3. Growth parameters determination

Cultures were monitored continuously to check if the valves worked correctly. Bottles were weighted twice a day and weight loss was determined. Before curve fitting, the weight loss data were mathematically transformed into a % of consumed sugar (CS) according to Pérez-Través et al. (2014). Data were plotted against the time to obtain the sugar consumption curves, which were adjusted to the modified Gompertz equation to calculate the maximum consumption rate (m) and lag phase (λ) for each strain (Arroyo-López et al. 2009). The kinetic parameters were calculated by directly fitting measurements *versus* time to the reparameterised Gompertz equation:

$$y = D * \exp\{-\exp[((m * e)/D) * (\lambda - t)) + 1]\}$$

where $y = \ln(CSt/CS0)$, CS0 is the initial CS and CSt is the CS at time t; $D = \ln(CSt/ CS0)$ is the asymptotic maximum, m is the maximum consumption rate (h^{-1}), and λ is the lag phase period (h). The Gompetz equation was fitted to data points by the non-linear regression module of the STATISTICA 7.0 software package, and by minimising the sum of the squares of the difference between the experimental data and the fitted model. Fit adequacy was estimated by the proportion of variance explained by the model (R2) compared to the experimental data.

2.4. Glycerol, residual sugars and ethanol determination

The glucose, fructose, glycerol, and ethanol at the end point of microvinifications were determined by HPLC (Thermo Fisher Scientific, Waltham, MA, USA) using a refractive index detector and a HyperREZ ™ XP Carbohydrate H + 8 μm column (Thermo Fisher Scientific), equipped with HyperREZTM XP Carbohydrate protection (Thermo Fisher Scientific). Samples were diluted 5 times, filtered through a 0.22 mm nylon filter (Symta, Madrid, Spain) and injected in duplicate. The analysis conditions were: eluent, 1.5 mM H_2SO_4; flow of 0.6 ml min^{-1} and the stove temperature was 50 °C.

2.5. Quantification of volatile compounds

Samples were taken for analysing higher alcohols and esters at the end point of triplicate (biological replicates) fermentations. The extraction of volatile compounds and gas chromatography were carried out following the protocol of Rojas et al., (2001). Extraction was performed using headspace sampling by means of solid phase microextraction (SPME) with polydimethylsiloxane fibres (PDMS) (Supelco, SigmaeAldrich, Barcelona, Spain). The volatile compounds were separated by gas chromatography in a Thermo TRACE ULTRA Chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) with a flame ionisation detector (FID) using an HP INNOWAX 30 mx 0.25 mm capillary column coated with a 0.25 μm layer of cross-linked polyethylene glycol (Agilent Technologies Inc.). Helium was the carrier gas (flow of 1 ml min^{-1}). The programmed oven temperature was: 5 min at 60°C, 5°C per min up to 190°C, 20°C per minute up to 250° C and 2 min at 250°C. The temperature detector ran at 280°C and the temperature injector at 220°C under undivided conditions. A chromatography signal was recorded by an HP Vectra QS/16S

Detector. The internal standard was 2-heptanone (0.05% w/v). The retention times of the eluted peaks were compared with those of higher alcohols and standard commercial esters. Concentrations were quantified in mg mL^{-1} by the calibration of standards graphs (R2 value> 0.99). We could not detect significant amounts of n-propanol and amyl alcohol in our fermentations with this method.

2.6. Statistical analysis

Growth curve fitting, Student *t*-tests and ANOVA's were performed with the STATISTICA 7.0 software**.**

3. Results

The fermentation capacity of the natural hybrids isolated from different environments and geographic regions was evaluated. This evaluation was made under conditions that simulated wine fermentation and in synthetic must at low temperature (12°C) and with controlled agitation. *S. cerevisiae* T73 and *S. kudriavzevii* CR85 were used as the control strains. Supplementary Table 1 shows the values obtained for the most relevant kinetic parameters of the fermentation, while Supplementary Table 2 indicates the concentrations of the main produced metabolites after fermentation, including glucose, fructose, ethanol and glycerol.

3.1. Sugar consumption

All the strains, except for *S. cerevisiae* T73, consumed all the sugars present in the synthetic wort (Supplementary Table 2). Figure 1 shows the sugar consumption by grouping the hybrids according to their region and source of isolation (wine, beer or non-alcoholic) to better visualise the differences. As previously mentioned, all the hybrids were able to ferment well at low temperature, as did the hybrids isolated from beers or hospital patients and a nutritional supplement. Moreover, these graphs suggest that the lag phase of most of the wine hybrids was shorter compared to that of non-wine hybrids, which indicates better adaptation to the tested growth conditions. To better analyse the sugar consumption profiles in more detail, we compared other kinetic parameters as the time required to consume 50% (t50) or 95% (t95) of total sugars, the maximum consumption rate (m) and the lag phase (λ).

3.2. Time required to consume 50% of the total sugars

By considering t50, it was possible to analyse the strains better adapted to the first fermentation phase, whose lag phase was shorter. It is noteworthy in Figure 2A that the majority of hybrids consumed 50% of the sugars present in the synthetic must more quickly than the control strains of *S. cerevisiae* T73 as a representative of wine yeast and CR85 of *S. kudriavzevii* as a representative of a better adapted species to grow at low temperatures. Figure 2 also indicates the fastest hybrids in the first fermentation part, and those that needed more time and the significantly different strains when compared with one another (ANOVA, turkey $p<0.05$) are coloured green and red, respectively, as in the following graphs in this study. A central group with the highest number of strains showing values not significantly different than the average was stablished. Then, the strains not belonging to these central groups are considered significantly different. As observed in this Figure,

193 hybrid SPG172 was the first to consume 50% of the sugars, followed by W46, W27,
194 HA1841 and SPG14-91. All these wine hybrids were isolated from the same Swiss
195 geographical region, except for strain HA1841, which was isolated from Austria. The
196 hybrid strains that presented the most problems are AMH, CECT1388, PB7 and VIN7,
197 which are grouped together with *S. cerevisiae* and *S. kudriavzevii* parental strains.

198 **3.3. Time required to consume 95% of the total sugars**

199 Parameter t95 accounted for the time needed to consider fermentation to be practically
200 finished. The fermentation carried out by the control strain of *S. cerevisiae* T73 was
201 completed after 573 h, whereas the strain of *S. kudriavzevii* CR85 required an average time
202 of 499 h to complete fermentation (Figure 2B). Again, the fastest strains were HA1841,
203 W27, SPG172, W46, SPG14-91 and IF6, mostly of a vinous origin and from the same
204 isolation region in Switzerland, except for strain HA1841 that is Austrian.

205 **3.4. Maximum sugar consumption rate**

206 This growth parameter is shown in Figure 2C, where the strains with the highest m are
207 indicated in green and those with the lowest values are denoted in red. According to our
208 results, the fastest strains were the hybrids of wine origin HA1841 and SPG172, whose m
209 values equalled 0.635 and 0.609 g L^{-1} h^{-1}, respectively. The maximum m was 2.29-fold
210 higher for the fastest strain (HA1841) than for the slowest one (CECT1388). The strains
211 isolated from the environments not related to the fermentation of alcoholic beverages
212 MR25 and IF6 gave values of 0.36 and 0.50 g L^{-1} h^{-1}, respectively. We observed that strain
213 *S. cerevisiae* T73 was among the strains with the lowest m, together with hybrids AMH and

PB7, which were also isolated from wine environments. Therefore, no clear relationship between the source of isolation and the behaviour of this parameter could be established.

3.5. Lag phase

Regarding this parameter, known as lag phase (λ), our results (Figure 2D) indicated that λ was shorter for most of the natural hybrids than for strain *S. kudriavzevii* CR85. On average, the lag phase for *S. kudriavzevii* CR85 was 84.5 h, but the average adaptation time for hybrid SPG441 was 21.7 h; that is, 3.89-fold longer for the reference strain than for the natural hybrid. Hybrids HA1835 and VIN7 needed longer times for adaptation, whereas the lag phase of hybrids SPG441, SPG14-91, W46, CECT11003 and SPG172 was faster.

3.6. Metabolite production

The concentrations of glucose, fructose, glycerol and ethanol were measured by HPLC, as described in the Materials and Methods section, and are shown in Supplementary Table 2. Glycerol production in relation to the parental *S. kudriavzevii* is noteworthy (Figure 2E). Contrarily to what was expected, no significant differences were observed, except for natural hybrid VIN7 that produced a glycerol concentration of 7.3 g L^{-1}, which was even higher than the control of *S. kudriavzevii* (CR85) that produced 7.0 g L^{-1}, and both were higher glycerol synthesis strains (in green) (Figure 2E). The other strains produced smaller quantities with concentrations ranging from 4.1 g L^{-1} to 5.7 g L^{-1}, whose differences were not statistically significant. The control of *S. cerevisiae* T73 produced around 4.6 g L^{-1} of glycerol, an intermediate production.

Another important parameter for hybrids is ethanol production. As we described in the Introduction, one of the characteristics of fermenting with the *S. kudriavzevii* species is a lower ethanol yield compared to *S. cerevisiae*. As shown in Figure 2F, significant differences were found in the ethanol content produced by hybrid W27 compared to the control of *S. kudriavzevii* (CR85), which also showed low ethanol levels with 11.04% and 11.39% of ethanol, respectively. In contrast, hybrids SPG319 and CECT1990 generated higher ethanol production. As expected, the production of the other hybrids between *S. cerevisiae* (T73) and *S. kudriavzevii* (CR85) was intermediate.

We have also evaluated the presence of organic acids as acetic, malic, tartaric, citric, succinic and lactic acid (Supplementary Table 3) after wine fermentations of all the strains. Hybrid strain VIN7 was the strain showing significantly higher levels of acetic acid production where as IF6, CECT1990, CECT11011, HA1841 and CECT11002 where the strains that presented the highest levels of tartaric, citric, malic, succinic and lactic acid, respectively. Strain W27 was characterized by presenting the lowest levels of succinic and lactic acid whereas SPG16-91 showed the lowest levels of citric and malic acids. SPG14-91 produced the lowest levels of acetic acid and CECT11004 fermentation presented the lowest levels of tartaric acid.

3.7. Aroma production

Another interesting parameter in winemaking is the production of aroma compounds. We studied the differences in the synthesis of the aroma-related compounds produced between the different hybrids by focusing on higher alcohols and esters. It should be notice that aroma compounds that depend on the presence of a grape precursors are not studied in this

work since fermentations are performed in a synthetic must. The samples taken at the end of the fermentation were analysed by gas chromatography, as described in the Materials and Methods section. The results (Table 2) indicate the compounds where the highest concentration (in bold) was obtained and were significant differences were observed (superscript letters). Here we can see that strain CECT11002 produces the highest concentration of isoamyl acetate (banana and pear aroma), 2-phenyl-ethanol acetate (fruit and flower aroma) and 2 phenyl ethanol (flowers, and roses in particular). The clinical hybrid IF6 stands out for isobutanol acetate production (undesired excess aroma, reminiscent of hydrocarbons), as do SPG172 for ethyl hexanoate production (apple); SPG319 for isobutanol and isoamyl alcohol production (enamel, undesired), and HA1841 for ethyl acetate production (undesired, glue). The critical role of these strains in the perception of wine aroma was confirmed by the significant odour active values shown in the specific mentioned aroma compounds (Supplementary Table 4).

We also evaluated the production of higher alcohols and esters in all. In this case, we observed that the strain that produced the higher concentration of higher alcohols was SPG319. This was because it produces high concentrations of two undesired alcohols, i.e. isobutanol and isoamyl alcohol. The highest ester-producing strain was HA1841 but, as in the previous case, it produced an undesired aroma, ethyl acetate.

Considering all the data, the hybrids with significant differences in the synthesis of aromas that contributed positively to overall wine aroma were CECT 11002 for producing isoamyl acetate, 2-phenyl-ethanol acetate and 2-phenyl ethanol, and SPG172 for producing ethyl hexanoate. No correlation between the aromatic profile and the beer or wine origin was

observed because both the strains that showed significant differences corresponded to a beer and a wine isolate.

3.8. Overall fermentation performance and aroma profile

To gain an overview of yeast performance during the wine fermentations at 12ºC, we compared the different hybrids and pure species strains, and all parameters determined in this work, by hierarchically clustering all the data (Figure 3). As expected, the results showed that the strains with high sugar consumption rates presented low t50, t95 and λ, and *vice versa*. The cluster showed that strains were divided into four main groups: group B (formed mainly by wine hybrids) showed good fermentative kinetic parameters (m, t50, t95 and λ) and high aroma compound production. The Group A (formed beer and wine hybrids mainly) showed intermediate levels of kinetic parameters and aroma production and Group C (formed by the pure species strains and four wine hybrids) displayed bad fermentative kinetic parameters and low aroma compound accumulation. Finally, group D is a mosaic group harbouring strains with extreme levels, for example strain W27, that resembles strains from group B but shows the highest levels of ethanol, or for example the strain SPG16-91, that resembles strains from group A but presents the lowest levels of malic and citric acids. These results suggested a correlation between fast sugar consumption and high aroma compound accumulation as the significant correlation (0.69) observed between m and total esters (Supplementary Figure 1).

4. Discussion

The use of hybrids in industrial fermentation has acquired much attention in the last years due to the interest of generate new artificial hybrids with new characteristics of interest and without generating GMO's. But also interesting natural hybrids can be found isolated in industrial environments with adapted properties (Pérez-Torrado et al., 2018). A remarkable case is the *S. cerevisiae* x *S. kudriavzevii* hybrids because their cryophilic nature can be of special interest for cold wine fermentations (Belloch et al., 2008; Tronchoni et al., 2017; Querol et al., 2018). Wine fermentations at low temperatures (10–15 °C) are used to retain flavor volatiles and enhance aromatic complexity, especially white and rosé (Torija et al., 2003; Beltran et al., 2008). In this study, we focused on the oenological characterisation of the *S. cerevisiae* x *S. kudriavzevii* hybrids by analysing the fermentation kinetics, residual sugars, the main metabolites and the main aroma compounds produced. The results indicate that some of these hybrid strains display the best performance during low-temperature wine fermentations, and are serious candidates to be used as starters in certain fermentations, especially cold fermented white wines where this fermentation type is used (Molina et al., 2007). On top of this, hybrids CECT11002 and SPG172 stand out because of the good aromatic profile of the produced wines.

Cold wine fermentations are especially problematic conditions for yeast performance (Bisson, 1999). Besides the stressful environment of a wine fermentation, where high osmotic pressure at the first part continues with nitrogen exhaustion and ethanol stress, low temperature produces a strong impact on the yeast growth and metabolism (Aguilera et al., 2007). Wine *S. cerevisiae* strains are well adapted to wine fermentations and *S. cerevisiae* strains are adapted to cold fermentations but any of them are well adapted to both conditions at the same time (Belloch et al., 2008; Pérez-Torrado et al., 2018). In this work

we have confirmed these previous observations since hybrids were the first to consume 50% of the sugars present in the synthetic must compared with the control strains and show higher sugar consumption rates, specially a group of strains isolated in wine environments in Switzerland and Austria.

Aroma production by hybrids *S. cerevisiae* × *S. kudriavzevii* W27 and HA1841 was previously investigated in oenological conditions (González et al. 2007; Gangl et al. 2009). These studies showed that aroma production profile of these hybrids at low fermentation temperature was similar to that of *S. kudriavzevii*. Other study found a similar trend for W27, AMH, HA1841 and VIN7 for higher alcohols production that were comparable to those of *S. kudriavzevii* at 12°C (Gamero et al., 2013). This work has confirmed those results but, more interestingly, has discovered that other hybrids, not previously studied in oenological conditions, show high aroma production compared to the parental strains as CECT11002 and SPG172.

It was not possible to establish a relationship between the source of isolation and the kinetic behaviour. However, it is noteworthy that the two wine strains with low consumption rates AMH and PB7 present special genetic characteristics. PB7 is a tetraploid hybrid isolated from north Spain (León), and AMH (from Germany) has the smallest content of the *S. kudriavzevii* genome compared to all the other hybrids (Peris et al., 2012). This suggest that these strains could be genetically instable as occurs with the VIN7 hybrid (unpublished results), that also shows bad fermentation kinetic properties. It should be noted that strains *S. cerevisiae* T73 and *S. kudriavzevii* CR85 were among those with the worse kinetic parameters, together with hybrids AMH and PB7. This indicate a better adaptation to low-temperature wine fermentation of the *S. cerevisiae* × *S. kudriavzevii* hybrids. This results

supports the concept of hybrid vigour or heterosis that results from a complex interaction between two genomes, increasing diversity of gene alleles and reducing the impact of deleterious alleles (Shapira et al., 2014).

An interesting and unexpected positive correlation was found between the fast sugar consumption profile and the amount of esters produced during wine fermentation through the Ehrlich pathway. The formation of these compounds is related to the redox balance within yeast cells (Margalit, 2004; Pretorius and Lambrechts, 2000). Thus, one possible explanation is that the slow sugar-consuming strains have more time to diversify the redox balance with other compensating pathways that attract less metabolic flux than the Ehrlich pathway, such as the pentose phosphate pathway (Cadiere et al., 2011). Specific metabolomic studies will be required to test this hypothesis and to unveil the metabolic basis of this discovery.

Funding

G. Ortiz-Tovar was supported by CONACYT doctoral scholarship 176060. This work was supported by grants AGL2015-67504-C3-3-R from the Spanish Government and FEDER to EB.

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458 **Figure legends**

Figure 1 The sugar consumption kinetics during synthetic must microfermentations at low temperature of *S. cerevisiae* T73 (a), *S. kudriavzevii* CR85 (a) and 23 natural hybrids from several environments and different parts of the world. Austrian wine (b), Belgian beer (c), Swiss wine (d and e), German, English and New Zealand beers (f), German, South African and Spanish wines (g), non-alcoholic origin (h). Modelization from biological triplicates consumption data was performed fitting measurements *versus* time to the reparametrized Gompertz equation. Colours of the different strains were chosen randomly.

Figure 2 Kinetic and metabolic parameters presented by 23 natural hybrids and by *S. cerevisiae* T73 and *S. kudriavzevii* CR85 strains during synthetic must microfermentations at low temperature. The time elapsed to consume 50% of the sugars present in the synthetic must (t50) (panel **A**), the time elapsed to consume 95% of the sugars (t95) (panel **B**), the maximum consumption sugar rate (m) (panel **C**), the lag phase (λ) (panel **D**), the glycerol production (panel **E**) and the ethanol production (panel **F**) are shown. The significantly different strains, when compared with one another (ANOVA, turkey $p<0.05$), are coloured green and red, respectively. A central group with the highest number of strains showing values not significantly different than the average was created. Then, the strains not belonging to these central groups are considered significantly different.

Figure 3. Heat map of the complete dataset obtained in this work for each strain. Hierarchical clustering (average linkage with Spearman's rank correlation for distance measurements) was used to group the strains and compounds with the online tool Heatmapper, scaled for each parameter. Strains were grouped into three groups (A, B, C,

D) according to the different behaviours displayed during cold wine fermentations. Note that these groups are different to previous groups described by Peris et al. (2012) based on genomic composition. The wine isolated hybrid names are labelled purple and the beer isolated hybrids are labelled green. The pure species controls are shown in bold.

Supplementary table 1. Parameters related to the sugar consumption curve of natural hybrids.

Supplementary Table 2 Concentrations of the main metabolites at the end of fermentation.

Supplementary Table 3. Perception threshold and odour active values of the main aroma compounds at the end of the wine fermentation.

Supplementary Figure 1. Correlation between values of m and total esters obtained in cold microvinifications with all strains.

495 **Table 1.** List and characteristics of the strains used in microvinifications at 12°C.

Strain	Species	Country of origin	Source of isolation
HA 1835[(1)]	Sc x Sk	Austria	Wine
HA 1837[(1)]	Sc x Sk	Austria	Wine
HA 1841[(1)]	Sc x Sk	Austria	Wine
HA 1842[(1)]	Sc x Sk	Austria	Wine
VIN7[(2)]	Sc x Sk	South Africa	Wine
W27[(3)]	Sc x Sk	Switzerland	Wine
W46[(3)]	Sc x Sk	Switzerland	Wine
SPG 14-91[(4)]	Sc x Sk	Switzerland	Wine
SPG 16-91[(4)]	Sc x Sk	Switzerland	Wine
SPG 126[(4)]	Sc x Sk	Switzerland	Wine
SPG 172[(4)]	Sc x Sk	Switzerland	Wine
SPG 319[(4)]	Sc x Sk	Switzerland	Wine
SPG 441[(4)]	Sc x Sk.	Switzerland	Wine
AMH[(3)]	Sc x Sk	Germany	Wine
PB7[(5)]	Sc x Sk	Spain	Wine
CECT 1388	Sc x Sk	England	Beer
CECT 1990	Sc x Sk	Germany	Beer
CECT 11002	Sc x Sk	Belgium	Beer
CECT 11003	Sc x Sk	Belgium	Beer
CECT 11004	Sc x Sk	Belgium	Beer
CECT 11011	Sc x Sk	New Zealand	Beer

MR25[6]	Sc x Sk	Spain	Respiratory tract
IF6[6]	Sc x Sk	Spain	Dietary complement
T73[3]	Sc	Spain	Wine
CR85[6]	Sk	Spain	Oak bark

Sc: *Saccharomyces cerevisiae*; Sk: *Saccharomyces kudriavzevii*; [1] Ksenija Lopandic (Austrian Centre of Biological Resources and Applied Mycology, Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Vienna, Austria); [2] Anchor Wine Yeasts; [3] Lallemand Bio and; [4] Jürg Gafner (Swiss Federal Research Station for Fruit-Growing, Viticulture and Horticulture, Wädenswil, Switzerland); [5]José Manuel Álvarez Pérez (Vine and Wine Research Institute, University of León, Ponverrada, Spain); [6] from our laboratory; CECT (Spanish Type Culture Collection).

510 **Table 2 Concentrations of the main aroma compounds at the end of fermentation (mg/l)**

Strain	Ethyl acetate	Isobutanol acetate	Isobutanol	Isoamyl acetate	Isoamyl alcohol	Ethyl hexanoate	2-phenyl-ethanol acetate	Benzylic alcohol	2-phenylethanol	Total higher alcohols	Total esters
CECT11002	114±12^{c-f}	nd	15±0.3^{a}	**5.2±0.39^{g}**	192±3.$^{a-e}$	0.34±0.38^{a}	**2.8±0.2^{i}**	13.6±0.^{a}	**88.6±1^{cd}**	310±12^{a-d}	122±12^{d-g}
CECT11004	72±6^{a-c}	0.21±0.07^{a-d}	47±2.2^{d-h}	1.2±0.35^{a-d}	197±27^{a-e}	0.4±0.06a.$^{}$	0.2±0.1^{a-d}	16±4.1^{a}	26.9±4$^{a.b}$	286±30^{a-d}	74±6^{a-d}
W46	84±21^{a-d}	nd	53±1.4^{f-j}	1.6±0.24^{b-e}	188±3^{a-e}	0.4±0.04$^{a.b}$	0.7±0.01^{d-h}	15±0.9^{a}	45±5^{a-d}	301±2^{a-d}	87±22^{a-e}
CR85	57±6$^{a.b}$	0.08±0.01$^{a.b}$	44±5^{c-h}	0.5±0.09^{a}	175±16^{a-d}	0.33±0.01^{a}	0.1±0.01$^{a.b}$	14±2.3^{a}	27±4^{a-c}	260±28^{a-d}	58±7$^{a.b}$
MR25	83.1±8^{a-d}	nd	17±1.9$^{a.b}$	1.6±0.15^{c-e}	194±26^{a-e}	0.54±0.08^{a-d}	0.5±0.06^{a-h}	15±2.5^{a}	74±13$^{c.d}$	299±42^{a-d}	86±8^{a-e}
IF6	115±8^{c-f}	**0.49±0.29^{d}**	89±12^{k}	1.9±0.3^{d-f}	198±31^{a-e}	0.4±0.03$^{a.b}$	0.95±0.33$^{g.h}$	16±0.4^{a}	48±2^{a-d}	352±44^{b-d}	118±9^{d-g}
AMH	61±11$^{a.b}$	nd	42±0.4^{c-g}	0.64±0.08^{a-c}	176±9.6^{a-d}	0.5±0.07^{a-d}	nd	18±6.2^{a}	30±2^{a-c}	266±18^{a-d}	62±12^{a-c}
W27	122±0.$^{2d-f}$	0.33±0.09^{b-d}	59±5.2^{g-j}	2±0.03^{d-f}	200±8.6^{a-e}	0.57±0.01^{a-d}	0.9±0.33^{f-h}	16±1.7^{a}	73±48^{b-d}	348±64^{b-d}	126±1^{e-g}
CECT11003	80.6±3^{a-d}	0.22±0.01^{a-d}	58±5.4^{g-j}	1.6±0.2^{c-e}	239±8.5^{b-e}	0.4±0.06$^{a.b}$	0.5±0.23^{a-h}	14±1.2^{a}	58±1^{a-d}	368±36^{c-d}	83±4^{a-e}
CECT1990	117±2^{c-f}	0.25±0.05^{a-d}	41±0.1^{c-g}	1.8±0.09^{d-f}	172±2^{a-d}	0.9±0$^{d.e}$	0.7±0.07^{c-h}	16±1.1^{a}	57±1^{a-d}	286±0.5^{a-d}	120±2^{d-g}
CECT1388	81±6.5^{a-d}	0.14±0.01$^{a.b}$	29±1.8^{a-c}	1.6±0.09^{b-e}	215±11^{a-e}	0.6±0.07^{a-d}	0.3±0.02^{a-e}	14±0.8^{a}	54±0.2^{a-d}	311±13^{a-d}	83±7^{a-e}
CECT11011	97±5^{a-e}	0.36±0.07^{b-d}	19.4±1$^{a.b}$	2±0.06^{d-f}	136±4.6^{a}	0.6±0.03^{a-d}	0.6±0.17^{b-h}	18±1.6^{a}	25±3$^{a.b}$	198±4.5^{a}	101±4^{a-f}
PB7	77±5^{a-d}	0.1±0.03$^{a.b}$	38±8.8^{c-f}	0.5±0.12$^{a.b}$	168.6±26^{a-d}	0.5±0.07^{a-c}	0.13±0^{a-c}	14.5±2^{a}	29±10^{a-c}	249±47^{a-c}	78±5^{a-e}
VIN7	145±30$^{e.f}$	0.2±0.03^{a-d}	31±2.3^{a-d}	2.1±0.2^{d-f}	155±5^{a}	0.4±0.05$^{a.b}$	0.66±0.21^{b-h}	11±0^{a}	31±6^{a-c}	228±1$^{a.b}$	149±31$^{f.g}$
SPG126	87±10^{a-d}	0.17±0.1^{a-c}	63±8.9$^{i.j}$	1.6±0.2^{c-e}	244±8$^{d.e}$	0.4±0.1$^{a.b}$	0.42±0.12^{a-g}	17±1.6^{a}	43±12^{a-d}	367±48^{b-d}	89±11^{a-e}
T73	56±26^{a}	nd	35±7^{b-e}	0.7±0.3^{a-c}	162±16^{a-c}	0.5±0.05^{a-d}	0.13±0^{a-c}	10±8.8^{a}	23±2^{a}	230±12$^{a.b}$	57±26^{a}
SPG172	105±6^{b-f}	0.23±0.1^{a-d}	51±4^{e-i}	2.8±0.4^{f}	206±12^{a-e}	**1.2±0.08^{e}**	0.4±0.06^{a-f}	14±1.5^{a}	27±7.6^{a-c}	298±25^{a-d}	109±5^{c-g}
HA1842	115±4.$^{5c-f}$	0.18±0.04^{a-c}	52±3^{e-i}	2±0.09^{d-f}	213± 20^{a-e}	0.75±0.03^{b-d}	0.4±0.07^{a-g}	17±1.7^{a}	27±4.6^{a-c}	309±30^{a-d}	119±5^{d-g}
SPG319	86±19^{a-d}	0.15±0.04^{a-c}	**69±3^{j}**	2.1±0.4^{d-f}	**268±3^{e}**	0.4±0.02$^{a.b}$	0.57±0.21^{a-h}	18±3^{a}	42±12^{a-d}	**397±22^{d}**	89±19^{a-e}
HA1841	**148±14^{f}**	0.23±0^{a-d}	48±3^{d-i}	2.4±0.2$^{e.f}$	162±8^{a-c}	0.8±0.15^{c-e}	0.9±0.03^{e-h}	18±1^{a}	42±6^{a-d}	265±6^{a-d}	**153±15^{g}**
SPG16-91	74±4^{a-d}	0.09±0.02$^{a.b}$	56±2^{g-j}	1.3±0.4^{a-e}	239±29^{c-e}	0.4±0.08$^{a.b}$	0.4±0.15^{a-g}	17±2.4^{a}	44±5^{a-d}	356±34^{b-d}	77±5^{a-e}
HA1837	95±7^{a-d}	0.12±0.01$^{a.b}$	45±2^{c-i}	1.8±0.3^{d-f}	195±16^{a-e}	0.7±0.05^{a-d}	0.5±0.12^{a-h}	13±2^{a}	35±8^{a-c}	287±28^{a-d}	98±8^{a-e}
HA1835	86±9^{a-d}	0.15±0.01^{a-c}	39±2^{c-f}	1.6±0.04^{c-e}	161±13$^{a.b}$	0.7±0.03^{a-d}	0.5±0.1^{a-h}	8±5.5^{a}	38±14^{a-c}	246±20^{a-c}	89±9^{a-e}
SPG14-91	103±6^{a-f}	0.44±0.16$^{c.d}$	62±5^{h-j}	2.9±0.7^{f}	242±35^{b-e}	0.5±0.02^{a-d}	1±0.24^{h}	9±0^{a}	48±13^{a-d}	361±53^{b-d}	108±7^{b-g}
SPG441	77±25^{a-d}	0.26±0.06^{a-d}	46±1^{c-i}	1.2±0.4^{a-d}	157±0.9^{a}	0.3±0.15^{a}	0.4±0.04^{a-g}	19±1^{a}	28±2^{a-c}	250±1^{a-c}	80±26^{a-e}

511 Superscript letters indicate the significant homogeneous group obtained by a one-way ANOVA

512 analysis (Tukey test, n=2, p-value <0.05). nd = not detected by gas chromatography. Bold values

513 indicate the highest value.

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