THE PRODUCTION OF AROMATIC ALCOHOLS IN NON-*SACCHAROMYCES* WINE YEAST IS MODULATED BY NUTRIENT AVAILABILITY

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ABSTRACT

Aromatic alcohols (tryptophol, phenylethanol, tyrosol) positively contribute to organoleptic characteristics of wines, and are also described as bioactive compounds and *quorum sensing* molecules. These alcohols are produced by yeast during alcoholic fermentation via the Erhlich pathway, although in non-*Saccharomyces* this production has been poorly studied. We studied how different wine yeast species modulate the synthesis patterns of aromatic alcohol production depending on glucose, nitrogen and aromatic amino acid availability. Nitrogen limitation strongly promoted the production of aromatic alcohols in all strains, whereas low glucose generally inhibited it. Increased aromatic amino acid concentrations stimulated the production of aromatic alcohols in all of the strains and conditions tested. Thus, there was a clear association between the nutrient conditions and production of aromatic alcohols in most of the wine yeast species analysed. Additionally, the synthesis pattern of these alcohols has been evaluated for the first time in *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Starmellera bacillaris*.

Keywords: Phenylethanol, Tyrosol, Tryptophol, Alcoholic fermentation, Erhlich pathway

1. INTRODUCTION

S. cerevisiae is a model microorganism used in many industrial applications due to its ability to synthesize ethanol from diverse sugar sources. For this reason, this budding yeast is commonly employed in several technological processes such as brewing, baking and winemaking. In the latter, alcoholic fermentation involves the succession and coexistence of a large diversity of yeast genera and species. At the beginning of fermentation, non-*Saccharomyces* yeast populations are high, and genera such as *Hanseniaspora, Issatchenkia, Starmerella (sym. Candida), Torulaspora, Metschnikowia* are commonly found. During fermentation, non-*Saccharomyces* species are replaced by *S. cerevisiae* due to its superior performance during this process and its ability to produce high ethanol concentrations (Fleet, 2003; Heard and Fleet, 1988). However, several studies recently confirmed the positive contributions of these non-*Saccharomyces* yeasts to wine: they are able to produce certain additional aromatic compounds that improve the flavour and bouquet (Fleet, 2003; Jolly et al., 2014; Lleixà et al., 2016). Although ethanol is the major by-product of alcoholic fermentation, yeasts also produce other alcohols such as aromatic alcohols derived from the aromatic amino acids that are present in grapes. Tyrosine (Tyr), tryptophan (Trp) and 2-phenylalanine (Phe) are converted to tyrosol (TyrOH), tryptophol (TrpOH) and 2-phenylethanol (PheOH), respectively, via yeast metabolism through the Ehrlich pathway, particularly under nitrogen-limiting conditions (Ehrlich, 1907; Hazelwood et al., 2008). Those alcohols are largely used as additives in foods and beverages, and PheOH specifically is widely used in the cosmetics industry due to its rose-like scent (Fabre et al., 1998). Fusel alcohols positively influence the flavour and bouquet of wines and are also of interest due to their potential bioactivity for humans. TyrOH and TrpOH have been described as an antioxidant and a sleep inducer, respectively (Cornford et al., 1981;

53 Giovannini et al., 1999). Moreover, recent studies in yeasts have demonstrated that
54 these three aromatic alcohols are involved in growth regulation (Avbelj et al., 2015;
55 Zupan et al., 2013) and are suggested to be quorum sensing molecules (Dickinson,
56 1996; Lorenz et al., 2000). Many microbes use quorum-sensing communication to
57 transmit information about population density and environmental conditions (Bassler,
58 2002; Fuqua et al., 1994); in yeast, filamentous growth has also been associated with
59 these quorum-sensing molecules (Hornby et al., 2001; Kruppa, 2009; Sprague and
60 Winans, 2006).

61 In some yeast species, such as *S. cerevisiae*, the aromatic alcohols secreted into the
62 extracellular medium are recognized by other cells and modulate their behaviour
63 accordingly. Moreover, as mentioned above, nitrogen-limiting conditions increase the
64 production of aromatic alcohols, leading to stronger filamentous growth in *S. cerevisiae*.
65 In a recent study (González et al., 2017), we studied the behaviour of a collection of *S.*
66 *cerevisiae* strains in the context of filamentous growth in the presence of different
67 alcohols and observed higher effects for ethanol than for aromatic alcohols in terms of
68 its ability to promote stronger filamentous growth under nitrogen-limiting conditions.
69 On the other hand, studies performed with non-*Saccharomyces* yeasts, such as
70 *Hanseniaspora uvarum*, *Pichia kudriavzevii* and *Pichia fabianii*, have reported that
71 these yeasts also demonstrate changes in their growth mode under nutrient-limited
72 conditions (nitrogen or carbon) or other stress factors (Pu et al., 2014; van Rijswijck et
73 al., 2015). Therefore, the finding that aromatic alcohols exert different responses on
74 morphogenesis in a yeast-dependent manner shows that these molecular signals may be
75 species-specific (Chen and Fink, 2006).

76 The aim of this study was to evaluate the production of aromatic alcohols (TyrOH,
77 TrpOH and PheOH) by four wild, non-*Saccharomyces* wine strains in different media

(containing different concentrations of nitrogen and carbon) and compare them to a "model" *S. cerevisiae* wine strain.

2. MATERIALS AND METHODS

2.1 Yeast strains and inoculum preparation

The following wine yeast species were used in this study: commercial *Saccharomyces cerevisiae* QA23 (Lallemand, Canada) and four wild non-*Saccharomyce*s isolates from the winemaking Priorat region of Spain, specifically Hv4 (CECT 1313O, *Hanseniaspora uvarum*), Cz4 (CECT 13129, *Starmerella bacillaris* (sym. *Candida zemplinina*)), Mpp (CECT 13131, *Metschnikowia pulcherrima*) and Tdp (CECT 13135, *Torulaspora delbrueckii*) (Padilla et al., 2016). Yeast strains were taken from stocks preserved at -80 °C in glycerol and grown on YPD plates (2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) agar) for 48-72 h at 28 °C. Then, cells were cultured for 24 h in 50 mL of YPD medium at 28 °C and 120 rpm and transferred into 250 mL of fresh minimal medium (1X Yeast Nitrogen Base without amino acids or ammonia, 2% (w/v) glucose, 10 mM $(NH_4)_2SO_4$) and cultured for 2 days at 28 °C and 120 rpm.

2.2 Fermentation conditions

Fermentation was performed in three different media based on synthetic must (Beltran et al., 2004), containing modified sugar and nitrogen concentrations: standard synthetic must (SM: 200 g/L sugars -100g/L glucose and 100g/L fructose- and 300 mg of N/L for yeast assimilable nitrogen (YAN)), low glucose must (LGM: 20 g of glucose/L and 300 mg of N/L for YAN) and low nitrogen must (LNM: 200 g of sugars/L -100g/L glucose and 100g/L fructose- and 100 mg of N/L for YAN) (Table 1). Fermentations were initiated by inoculating precultures in minimal medium to obtain an initial population of

$2x10^6$ cells/mL. To study the production of aromatic alcohols (TyrOH, TrpOH and PheOH) in each must, aromatic amino acids (Tyr, Trp and Phe), which are precursors of those alcohols, were added at two concentrations, standard concentration based on the regular concentrations of aromatic amino acids (1x) (Beltran et al., 2004), and five-fold increased (5x), see Table 1. The increased concentrations of aromatic amino acids occurred at the expense of the remaining amino acids to maintain the same final concentration of nitrogen. Fermentations were performed in triplicate at 28 °C with continuous orbital shaking (120 rpm). Cell populations were evaluated daily by measuring the optical density (OD_{600nm}), and 1 mL of wine was stored to quantify the extracellular concentrations of TyrOH, TrpOH and PheOH by UHPLC after five days of fermentation for LGM and after seven days for SM and LNM.

Another set of alcoholic fermentations in SM and 5x aromatic amino acids was performed to associate the synthesis and secretion of aromatic alcohols with growth phases. Then, intracellular and extracellular concentrations of aromatic alcohols were analysed during the first 48 h (0 h, 12 h, 24 h, 48 h).

2.3 Aromatic alcohol analysis

Aromatic alcohols were detected and quantified by performing liquid chromatography triple quadrupole mass spectrometry (LCQqQ). For intracellular samples, prior extraction using boiling buffered ethanol (Gonzalez et al., 1997) was necessary. Briefly, a volume of culture corresponding to 10^7 cells was centrifuged at 5000 rpm for 10 min, and 1 mL of boiling absolute ethanol buffered with 2 mL of 1 M Hepes (pH 7.5) was added directly into the tube. Subsequently, the mix was incubated for 3 min at 80 °C, and the supernatant was evaporated at 45 °C using a SpeedVac (Thermo Fisher Scientific, USA). The residue was resuspended in 1 mL of sterile MilliQ water. Then,

100 µL of this extract or of the extracellular sample was diluted 1:10 in MeOH (≥ 99.7% (LC-MS), Sigma Aldrich, USA), centrifuged at 15000 rpm and maintained at 4ºC for 10 min. Samples were then serially diluted in MeOH to a final dilution of 1:100 for intracellular samples and 1:1000 for extracellular samples, and analysed by LCQqQ.

A 1290 UHPLC Series Liquid Chromatograph coupled to a 6490 QqQ/MS (Agilent Technologies, Palo Alto, USA) was used to evaluate TyrOH, TrpOH and PheOH. The chromatographic column was an Xbridge Shield RP18 (150 x 2.1 mm i.d., 3.5-µm particle size) (Waters). The mobile phases were 0.2% acetic acid (≥ 99.7%, Sigma-Aldrich, USA) in water (MilliQ system, Millipore) (solvent A) and ACN (solvent B). The flow rate was 0.6 mL/min. To validate our quantitative method, calibration curves, linearity, precision, accuracy and the limits of detection and quantification were determined by analysing serial standard dilutions prepared in ultrapure LC-MS water and pooled samples spiked with standard solutions. The obtained validation parameters for the method for TyrOH (≥ 99% Sigma-Aldrich, USA), PheOH (≥ 98%, Sigma-Aldrich, USA) and TrpOH (≥ 99%, Sigma-Aldrich, USA), permitted the quantification of studied compounds in extract samples.

2.4 Aromatic amino acid analysis

Final concentrations of Tyr, Phe and Trp were determined using an Agilent 1100 Series High-performance Liquid Chromatograph (Agilent Technologies, Germany) (Gómez-Alonso et al., 2007). Fifty microlitres from each sample was used in a derivatization reaction with diethyl ethoxymethylenemalonate (DEEMM). Separation was performed in an ACE HPLC column (C18-HL) with a particle size of 5 µm (250 x 4.6 mm) that was controlled thermostatically at 20 °C. Two eluents were used: eluent A contained 25 mM acetate buffer (pH= 5.8) with 0.02% sodium azide, and eluent B contained an 80:20

mixture of acetonitrile and methanol. The flow rate was 0.9 ml/min. The concentration of each compound was calculated using internal (L-2-aminoadipic acids, 1 g/l) and external standards.

2.5 Statistical analysis

Three biological replicates were performed for each experiment. Tukey's test was used to generate p-values. To better understand how different measured parameters influenced the production of aromatic alcohols, Principal Component Analysis (PCA) was performed using XLSTAT Software.

3. RESULTS

3.1 Production of aromatic alcohols by different wine yeast species

In addition to aromatic alcohols, residual sugar and maximal growth were determined at the end of fermentation (Table 2). In LGM, all sugars were consumed by yeast. At high sugar concentrations (SM and LNM), only *S. cerevisiae* was able to consume all sugars present in the must, followed by *T. delbrueckii*. High sugar concentrations were present at the end of the process for the other three species, particularly under nitrogen restriction (LNM). In general, the increase in aromatic alcohol precursors affected the maximal growth and sugar consumption.

In SM, all strains tested were able to produce the three aromatic alcohols, although *S. bacillaris* produced much lower levels (Fig 1A). Under the 1x condition, *S. cerevisiae* QA23 exhibited the maximum production of aromatic alcohols, together with the highest growth and total consumption of sugars (Table 2). TrpOH was the primary aromatic alcohol produced, followed by PheOH and TyrOH. Notably, synthetic must contains higher concentrations of Trp compared to Phe or Tyr (see Table 1). On the other hand, non-*Saccharomyces* species synthetized lower levels of aromatic alcohols;

specifically, *S. bacillaris* produced the lowest concentrations (below 5 µM TyrOH and PheOH, and no detectable TrpOH). Among non-*Saccharomyces* species, *T. delbrueckii* presented the highest growth and sugar consumption (although more than 20 g/l residual sugars was present after seven days of fermentation), *S. bacillaris* demonstrated the lowest growth, and *M. pulcherrima* demonstrated the lowest sugar consumption

Higher concentrations of aromatic amino acids in the media (5x) resulted in higher production of aromatic alcohols, despite the lack of proportional increases in alcohols with increases in precursors (Fig 1A). As observed in 1x condition, TrpOH was the major aromatic alcohol produced with the exception of *S. bacillaris,* which synthetized more PheOH, but at much lower levels. Moreover, although the differences were not statistically significant, the presence of higher concentrations of aromatic amino acids affected cell growth in all species: growth was lower for *S. cerevisiae*, *H. uvarum*, and *T. delbrueckii* but higher for *S. bacillaris* and *M. pulcherrima* (Table 2).

To better understand how different variables affected the strains, PCA was performed (Fig1B). This PCA analysis accounted for 90.17% of the variance: all variables were primarily explained by component F1 (74.86%). Triplicates appeared together in the biplot, and PCA clearly separated *S. cerevisiae* and *T. delbrueckii* from the other species, which grouped together, primarily due to their superior growth and higher consumption of sugars. In those strains, samples from media containing different aromatic amino acid contents grouped separately based on the higher production of aromatic alcohols under the 5x condition. Two additional PCA were performed using only the data from the 1x and 5x conditions (Fig S1). This PCA explains 92.23% and 91.7% of the variance under the 1x and 5x conditions, respectively. PCA generated three clusters: *S. cerevisiae, T. delbrueckii*, and the rest of the non-*Saccharomyces* strains.

3.2 Synthesis of aromatic alcohols under low nitrogen conditions

To analyse the effects of nitrogen concentration on the synthesis of aromatic alcohols, fermentations were carried out using low nitrogen synthetic must (100 mg of N/L) at two different concentrations of aromatic amino acids (1x and 5x).

In LNM, all strains grew poorly and, in general, fermented less sugar than in SM (Table 2), with the exception of *S. cerevisiae*, which consumed practically all sugars present in the media despite nitrogen limitation. As in SM, TrpOH was the main fusel alcohol synthesized, except in *S. bacillaris*. This strain did not secrete TrpOH under 1x condition and produced the other aromatic alcohols at only very low concentrations, despite consuming almost all of the precursors. The second aromatic alcohol most frequently secreted by non-*Saccharomyces* species was TyrOH, unlike *Saccharomyces*, which secreted PheOH (Fig 2).

When the precursors of these aromatic alcohols were five-fold higher, growth and sugar consumption during fermentation were not significantly affected, with the exception of Mpp. In this case, higher concentrations of aromatic amino acids in the medium resulted in a clear increase in growth, and sugar consumption was doubled in comparison to 1x condition. In terms of the production of aromatic alcohols, a significant increase in their concentrations was observed after the addition of precursors. However, TrpOH was the most relevant because the concentration of this alcohol was between 6 and 24 times greater than that under the 1x condition. In both cases, aromatic amino acids were practically exhausted, but production rates were higher under the 5x condition (Table 3 and Supplementary Table S1 for 5X).

PCA (Fig 2B) indicated a variance of 87.29% (F1: 56.53%; F2:27.76%), and the variables were primarily explained by component 1. *S. cerevisiae* was clearly separated

from non-*Saccharomyces* species largely due to its ability to generate a higher biomass and its elevated sugar consumption. Moreover, within this strain, samples from media containing different aromatic amino acid contents grouped separately due to the higher production of TrpOH and PheOH in 5x medium. In general, non-*Saccharomyces* species were also separated based on the concentration of aromatic amino acids (1x and 5x), primarily due to the higher amounts of TrpOH and TyrOH under the 5x condition, with the exception of the *S. bacillaris* Cz4 strain, which presented similar concentrations under both conditions.

LNM medium contained the same amount of sugars as SM, but the concentrations of YAN and aromatic amino acids were three-fold lower. Despite these lower amounts of precursors, the production of aromatic alcohols was similar in both media, indicating a higher yield of alcohol in LNM. Thus, the ratio between each aromatic amino acid and its respective aromatic alcohol was calculated (Table 3). For all strains, with the exception of *M. pulcherrima*, this rate of transformation was significantly higher in LNM than in SM, explaining why the final concentration of aromatic alcohols was similar despite the presence of fewer precursors. In *S. cerevisiae* QA23 grown in LNM under the 1x condition, all Tyr and Phe present in the medium were converted into their corresponding alcohols (ratios of approximately 1). In non-*Saccharomyces* species, the largest increase in this ratio occurred for TrpOH and TyrOH, corresponding to the higher production of these two aromatic alcohols by non-*Saccharomyces* species as opposed to *S. cerevisiae*, which synthesized more PheOH than TyrOH. When a PCA was performed using the data from SM and LNM, 90.21% of the variance was explained (F1: 50.20%, F2: 40.01%), being the amount of aromatic amino acid consumed and the ratio Tyr/TyrOH mainly explained by F1, and the rest of variables by F2. The uptake of aromatic amino acids was negatively correlated with alcohol

transformation ratios, confirming that nitrogen limitation resulted in higher aromatic alcohol production ratios. Due to the differences in aromatic amino acid uptake, as well as growth and sugar consumption, the PCA clearly separated both media, and the latter two variables, together with aromatic alcohol production, were responsible for *Saccharomyces* forming a separate cluster versus non-*Saccharomyces* species.

3.3 Synthesis of aromatic alcohols at low sugar conditions

To analyse the effects of sugar concentration on aromatic alcohol synthesis, the same experiment was performed using a medium containing the same nitrogen concentration as SM but with low glucose content (20 g/l). All species depleted the sugars in LGM media (Table 2), and all were able to produce the three aromatic alcohols (Fig 3) but at much lower concentrations than those obtained with SM or LNM. Increased amounts of precursors (5x condition) also induced a significant increase in aromatic alcohol production for all species, particularly *T. delbrueckii*, which produced almost no aromatic alcohols under the 1x condition but exhibited higher synthesis of aromatic alcohols under the 5x condition, as well as significantly better growth (Table 2). Interestingly, in this medium, *T. delbrueckii* produced PheOH as the main aromatic alcohol, and *M. pulcherrima* produced the highest amount of TrpOH under either aromatic amino acid condition, even higher than *S. cerevisiae*. Notably, *M. pulcherrima* reached the highest cell density in this low glucose media, primarily under the 5x condition. Once again, *S. bacillaris* produced very low amounts of aromatic alcohols and only produced TrpOH under the 5x condition, despite exhibiting poor growth (Fig 3; Table 2).

PCA accounted for a variance of 71.02% (F1: 49.30%, F2: 21.72%) (Fig S2). All factors were primarily explained by the F1 component, with the exception of sugar

consumed, which was associated with F2. In general, the 1x and 5x conditions clustered separately, but in LGM, *S. cerevisiae* was not separated from non-*Saccharomyces* species. Instead, *M. pulcherrima* grouped separately from the others due to its high growth and aromatic alcohol production, while *S. bacillaris* under the 5x condition was in the other cluster.

3.4 Synthesis and secretion of aromatic alcohols during fermentation

To study the synthesis and secretion of aromatic alcohols in different species during alcoholic fermentation and growth, fermentations in SM medium with 5x aromatic amino acids were repeated, and samples were obtained to analyse the intracellular and extracellular concentrations of these molecules during the first 48 h (Fig 4). Initially, cells contained basal intracellular concentrations of fusel alcohols (from preculture growth), and then fusel alcohols increased considerably at 12 h, matching the early exponential phase. Then, during the period between the end of the exponential phase and the beginning of stationary phase, the intracellular concentration of aromatic alcohols decreased drastically, particularly for TrpOH and PheOH, likely due to their secretion into external media. Once in stationary phase, the intracellular levels of those alcohols maintained basal concentrations, similar to those observed at the beginning of the process. This decrease in intracellular levels coincided with the increase in aromatic alcohols observed in the extracellular medium, which slowed down when the cells were in mid-stationary phase. This profile was observed in *S. cerevisiae* (Fig 4A), *H. uvarum* (Fig 4B) and *M. pulcherrima* (Fig 4D). However, in *T. delbrueckii*, PheOH demonstrated a peak at 12 h as in the other species, but TrpOH peaked at 24 h (Fig 4E), likely due to the low population of this strain ($1x10^6$ cells/mL) in early exponential phase compared to the other strains (> $5x10^6$ cells/mL) in mid-exponential phase. Therefore, the synthesis of PheOH coincided with early exponential phase, and that of

297 TrpOH coincided with the mid-late exponential phase. Due to this delay in growth and
298 thus aromatic alcohol synthesis, accumulation in the extracellular medium was also
299 delayed, resulting in the detection of very low amounts of aromatic alcohols at 24 h
300 outside the cells. Finally, in *S. bacillaris* (Fig 4C), the intracellular accumulation of
301 aromatic alcohols was very low (as expected given the low concentrations detected in
302 the extracellular medium) and delayed due to the longer lag phase of this strain
303 compared with the other strains. Moreover, in this strain, PheOH was the major
304 aromatic alcohol that accumulated both inside and outside of the cell, unlike the other
305 species.

306 **4. DISCUSSION**

307 In this study, we analysed the synthesis of aromatic alcohols (TrpOH, PheOH and
308 TyrOH), which may act as quorum-sensing molecules, by different wine yeast species
309 during alcoholic fermentation. Additionally, we studied how the concentrations of
310 aromatic amino acids, yeast assimilable nitrogen and sugar affected the production of
311 these aromatic alcohols.

312 *S. cerevisiae* QA23 produced higher concentrations of aromatic alcohols, and its
313 production yield was highest in must with nitrogen restriction. These findings agree
314 with previous studies that showed an inverse correlation between initial nitrogen
315 concentration and the production of fusel alcohols (Beltran et al., 2005; Carrau et al.,
316 2008; Jiménez-Martí and del Olmo, 2008; Mouret et al., 2014), indicating that the closer
317 the nitrogen concentration is to growth-limiting levels, the higher the yield of fusel
318 alcohols. Indeed, the Ehrlich pathway may be activated to provide nitrogen from amino
319 acids such as the aromatic amino acids, which are limited for protein synthesis and
320 growth. Thus, higher levels of these alcohols may be a signal to modulate the

regulation of yeast growth (Mas et al., 2014). Indeed, Chen and Fink (2006) reported that the production of these autosignaling alcohols is regulated by nitrogen; specifically, they are activated under nitrogen-poor conditions. On the other hand, the same authors stated that aromatic alcohol production is not affected by low concentrations of glucose; however, according to our results (Fig 3 and Table S2), *S. cerevisiae* produced significantly lower concentrations of fusel alcohols in LGM than in SM, despite the same total nitrogen content in both media. There are different potential explanations for this low production. First, it may be related to low cell density (the OD_{600nm} value in LGM was 4.80 ± 0. 37 *vs.* 11.47 ± 1.32 and 7.64 ± 1.66 in SM and LNM, respectively). Additionally, cells at low density required lower protein synthesis, which may explain why cells consume fewer aromatic amino acids. Recent studies have associated the production of these alcohols with cell density, suggesting that high population density stimulates the synthesis of aromatic alcohols (Avbelj et al., 2015; Chen and Fink, 2006; Sprague and Winans, 2006; Wuster and Babu, 2009). However, in the case of low nitrogen (LNM), the production yield (with respect to the amount of precursor consumed) was higher despite a lower cell density than that in SM, indicating that cell density is not the sole variable that determines the synthesis of these alcohols. Second, the more rapid depletion of glucose in LGM may also be responsible for the low amounts of aromatic alcohols produced, as explained (Espinosa Vidal et al., 2014). In their experiments, these authors observed that the accumulation of isoamyl alcohol halted after glucose exhaustion, despite the presence of residual leucine, its precursor, suggesting that leucine degradation through the Ehrlich pathway was strongly dependent on the cytosolic availability of NADH and/or of the amino acceptor *α*-ketoglutarate. This was confirmed in glucose pulse experiments in the stationary phase that re-established the synthesis of the higher alcohol. Finally, differences in respiro-

fermentative metabolism may also be partly responsible for this low accumulation; in *C. albicans*, the production of aromatic alcohols is higher under anaerobic growth than aerobic growth (Ghosh et al., 2008). In LNM and SM, the metabolism of *S. cerevisiae* is anaerobic due to the Crabtree effect; however, in LGM, a medium containing significantly lower amounts of sugar, *S. cerevisiae* metabolism is partially aerobic, which may result in decreased aromatic alcohol synthesis.

Aromatic alcohols are quantitatively the largest group of volatile components, and their presence, particularly phenylethanol, is essential for overall flavour quality. The Erhlich pathway is active in other yeast such as *Kluyveromyces marxianus* and *Yarrowia lipolytica* (Celińska et al., 2013; Fabre et al., 1998). Additionally, other non-conventional wine yeast, such as *H. uvarum*, *Zygosaccharomyces bailii*, *T. pretoriensis* (Zupan et al., 2013), *S. bacillaris*, *M. pulcherrima* and *T. delbrueckii*, which we reported here, are able to produce aromatic alcohols. However, in all cases, non-*Saccharomyces* species produce lower quantities than *S. cerevisiae*, indicating that the Erhlich pathway may not be as active in non-*Saccharomyces* species as in *Saccharomyces* under nitrogen-limiting conditions.

TrpOH was the major aromatic alcohol synthesized by most species. This may be attributable to the fact that Trp was present in higher concentrations than the other two aromatic amino acids in the media; however, the production yield of TrpOH was also higher for most species. Additionally, although Trp is a poor source of nitrogen, cells uptake this amino acid early during fermentation (Beltran et al., 2005; Henschke and Jiranek, 1993), which may favour the synthesis of TrpOH. The increase in precursors resulted in a general increase in the production of aromatic alcohols, which was consistent with previous studies (Ghosh et al., 2008; Gori et al., 2011), although the increases in alcohols were not truly proportional to the increases in precursors. Crepin et

al. (2017) claimed that modulation of the production of targeted fermentative aromas was achieved by modifying the availability of exogenous amino acids; the authors reported a low contribution of the carbon skeletons of consumed amino acids to the production of volatile compounds. However, these authors studied the synthesis of higher alcohols derived from branched chain amino acids, which primarily originate from sugar catabolism (Crépin et al., 2017), whereas aromatic alcohols are synthesized when their precursors are added to the medium (Ehrlich, 1907; Webb and Ingraham, 1963). Moreover, in *S. cerevisiae*, certain reactions in the aromatic amino acid biosynthesis pathway from glucose are subject to feedback inhibition (Helmstaedt et al., 2005). On the other hand, in *Saccharomyces*, the increase in alcohol precursors resulted in a lower cell density, which may be related to the high content of Trp in this medium, as the growth *of S. cerevisiae* is negatively affected when Trp is used as a unique nitrogen source (Gutiérrez et al., 2013).

The production of aromatic alcohols has been previously studied in other wine yeast species, such as *H. uvarum*, *S. bacillaris* (Sym *C. zemplinina*), *T. pretoriensis* and *Z. bailii*, in a medium similar to SM (Zupan et al., 2013). However, this production has not yet been studied in other yeast species of increasing interest for wine fermentation. Among the three aromatic alcohols, phenylethanol has been the most studied in fermentations, both in single and in coinoculated fermentations, due to its positive influence in the aroma (Belda et al., 2017; Benito et al., 2015; Gobbi et al., 2013; Parapouli et al., 2010; Sadoudi et al., 2012). In general, non-*Saccharomyces* species synthesize lower amounts of aromatic alcohols than *S. cerevisiae*. However, in different studies that have used coinoculation between non-*Saccharomyces* species and *S. cerevisiae* in fermentation, the resulting wines presented a higher contents of aromatic alcohols (Azzolini et al., 2015; Varela et al., 2016, Belda et al., 2017). The higher

synthesis of these alcohols in *S. cerevisiae* may occur because non-*Saccharomyces* species differ from *S. cerevisiae* in the distribution of metabolic flux during fermentation and therefore differ in ethanol production, biomass synthesis, and by-product formation (Ciani et al., 2000; Magyar and Tóth, 2011; Milanovic et al., 2012; Tofalo et al., 2012).

Moreover, these species experience an elevated contribution of respiration to their metabolism; this is the case even for *T. delbrueckii,* which has been described as Crabtree-positive (Alves-Araujo et al., 2007). Indeed, Quirós et al. (2014) investigated the extent of respiration-fermentative metabolism in different yeast strains and reported that some species commonly found in oenological environments, such as *M. pulcherrima, Starmerella bombicola* and *T. delbrueckii*, respire between 40-100% of consumed sugar under suitable aeration conditions. Thus, an elevated respiratory metabolism may underlie lower aromatic alcohol production, as described for *C. albicans* (Ghosh et al., 2008). In fact, in this study, *C. albicans* cells grown anaerobically at 30 °C produced roughly twice as much PheOH, TrpOH and TyrOH as they do under aerobic conditions. As in *Saccharomyces*, the higher production of aromatic alcohols was detected under nitrogen-limiting conditions in non-*Saccharomyces* species. Another interesting aspect of our study is the finding that *S. bacillaris* produces all three aromatic alcohols, although at very low concentrations, unlike the observations of Zupan et al. (2013); one explanation for this may be the lower detection limits used in our method. Under the conditions used by Zupan et al. (2013), no aromatic alcohol synthesis was detected during alcoholic fermentation by *S. bacillaris*. *S. bacillaris* belonged to the *Candida* genus until 2012 (Duarte et al., 2012), and although it is currently included in another genus, it continues to share many features with *Candida*. Low levels of aromatic alcohols were secreted by *S. bacillaris*,

but these were similar to the concentrations detected in *C. albicans* in SD medium (3 µM TyrOH (Chen et al., 2004)). Regarding *M. pulcherrima*, it is interesting that the synthesis of aromatic alcohols appeared to be favoured under low glucose condition. Furthermore, this species grew and underwent significantly better fermentation when the aromatic amino acid contents were increased, unlike the other species. In a recent study performed using single nitrogen sources, Kemsawasd et al. (2015) observed that aromatic amino acids did not support well neither growth or fermentation performance in the species tested that included *M. pulcherrima*, although another strain was used. After *S. cerevisiae*, *T. delbrueckii* presents a better oenological profile; higher concentrations of aromatic alcohols in SM medium were also observed for other non-*Saccharomyces* yeasts. *T. delbrueckii* synthesized the fewest aromatic alcohols, with the exception of *S. bacillaris*, in other media containing low nitrogen and low glucose. Therefore, *T. delbrueckii* appears to be affected by nutrient limitation. Moreover, in LGM, the profile of accumulated aromatic alcohols changed, and PheOH was the major alcohol formed by *T. delbrueckii*.

When the intracellular synthesis of these aromatic alcohols was studied during SM fermentation, similar profiles were observed for *S. cerevisiae*, *H. uvarum* and *M. pulcherrima*. These yeasts synthesized aromatic alcohols during the early exponential phase, when the yeast population was increasing and the need for nitrogen was higher; therefore, the deamination and transamination of amino acids were essential steps to fulfil the nitrogen demands of cells. The transamination reaction, which is the first step in the Ehrlich pathway, is followed by decarboxylation, which is thermodynamically favoured (Henry et al., 2007). This decarboxylation pulls transamination toward complete utilization of the nitrogen-donating amino acid, resulting in the formation of the fusel alcohol or fusel acid, depending on the redox state of the cell (Hazelwood et

al., 2008). We have observed that cells secrete those aromatic alcohols into the medium during the shift from exponential to stationary phase, when mechanisms required for the starvation period are induced, which is consistent with previous studies (Chen and Fink, 2006; Gori et al., 2011; Zupan et al., 2013). The secretion of these molecules at the end of the exponential phase indicates a connection to cell density, suggesting that aromatic alcohols are potential QS molecules in these species, as previously suggested for *C. albicans*, *S. cerevisiae* and *D. hansenii* (Chen et al., 2004; Chen and Fink, 2006; Gori et al., 2011). According to Fabre et al. (1998), if Phe is added after the exponential growth phase, no bioconversion to PheOH is observed during stationary phase in *Kluyveromyces marxianus*. Compared to other yeasts investigated in this study, *T. delbrueckii* alone reached its highest levels of PheOH and TrpOH at two different times. PheOH may exert an auto-stimulatory effect on TrpOH production, as synergies between both alcohols have been observed in *S. cerevisiae* (Chen and Fink, 2006). Based on this result, *T. delbrueckii* undertakes growth phase-dependent differential regulation to synthesize these aromatic alcohols. This delay in synthesis, together with a low population during the first 12 h, also resulted in a delay in the extracellular secretion of the aromatic alcohols. In the case of *S. bacillaris*, very low intracellular accumulation and extracellular secretion were related to the deficient growth of this species.

In this study, we have demonstrated that an increase in precursor levels resulted in the higher accumulation of the resulting alcohols; therefore, these compounds may be increased under oenological conditions. Moreover, aromatic alcohols have been reported to possess quorum-sensing activity, and their effects, together with ethanol, on *S. cerevisiae* morphology have been thoroughly described (Chen and Fink, 2006; González et al., 2017). However, there is limited knowledge of the effects of these QS

molecules in non-*Saccharomyces* wine yeasts. Further studies investigating the roles of those aromatic alcohols in non-*Saccharomyces* yeast species will be necessary to understand yeast interactions and quorum-sensing mechanisms in wine yeasts.

5. CONCLUSIONS

To summarize, we show that, in addition to the well-studied *S. cerevisae,* the Erhlich pathway is active in other yeasts that produce aromatic alcohols during alcoholic fermentation, although their regulation appears to be somewhat different than that of *S. cerevisiae*. *S. bacillaris* was the lowest producer of aromatic alcohols, whereas *S. cerevisiae* was the highest producer. Carbon and nitrogen availability as well as precursors influence the production of these alcohols; in particular, nitrogen depletion induced notable levels of aromatic alcohols. To date, the synthesis of these molecules by wine yeasts demonstrates high oenological potential, as *M. pulcherrima* and *T. delbrueckii* had not previously been evaluated. Here, these two species were able to synthesize considerable amounts of alcohols, which may regulate their growth. Aromatic alcohols also have many important biotechnological applications, and relevant concentrations of these compounds positively affect wine. In this study, we demonstrated that varying nutrient concentrations in must result in adjustments to the synthesis patterns of aromatic alcohols for all yeast species studied.

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FIGURE LEGENDS

Figure 1. Production of aromatic alcohols by different yeast species in standard must (SM). (A) Concentrations of aromatic alcohols obtained in fermentations performed in SM (300 mg/l nitrogen and 200 g/l sugars, and with different concentrations of aromatic alcohol precursors: regular concentration (1x) and five-fold increased (5x)). Statistical analysis was performed by comparing 1x against 5x conditions for each species; asterisks denote p-values < 0.05. **(B)** Bi-plots depicting Principal Component Analysis (PCA) with the following variables: aromatic alcohol concentrations, maximal cell density and consumed sugar during fermentation.

Figure 2: Production of aromatic alcohols by different yeast species under nitrogen limitation (LNM). (A) Concentrations of aromatic alcohols obtained in fermentations performed in LNM (100 mg/l nitrogen and 200 g/l sugars, and with different concentrations of aromatic alcohol precursors: regular concentration (1x) and five-fold increased (5x). (B) Statistical analysis was performed by comparing 1x against 5x conditions for each species; asterisks denote p-values < 0.05.

Figure 3. Production of aromatic alcohols by different yeast species in low glucose must (LGM). (A) Concentrations of aromatic alcohols obtained in fermentations performed in LGM (300 mg/l nitrogen and 20 g/l sugars, and with different concentrations of aromatic alcohol precursors: regular concentration (1x) and five-fold increased (5x)). Statistical analysis was performed by comparing 1x against 5x conditions for each species; asterisks denote p-values < 0.05.

Figure 4. Monitoring the intra- and extracellular production of aromatic alcohols and cell populations during fermentation with different yeast species. Fermentations were carried out for 48 h at 28 °C in standard must (SM) supplemented with a five-fold increase in aromatic amino acids (5X). **(A)** *S. cerevisiae* QA23, **(B)** *H.*

uvarum Hu4, **(C)** *S. bacillaris* Cz4 **(D)***M. pulcherrima* Mpp, and **(E)** *T. delbrueckii* Tdp. Aromatic alcohol concentrations are expressed as the average of biological triplicates.

728 **Table 1: Reducing sugar, nitrogen and aromatic amino acid contents of the**

729 **different synthetic musts used in this study.**

			Nitrogen concentration					
			Sugar concentration (g/L)		Aromatic amino acid (g/l)			Yeast assimilable nitrogen (YAN-mg nitrogen/L)
Synthetic Must		[Aromatic amino acid]	Glucose	Fructose	Tyrosine	Tryptophan	Phenylalanine	aaa + NH_4Cl
Synthetic Must (standard)	**(SM)**	*1x*	100	100	0.020	0.174	0.038	300
		5x	100	100	0.098	0.871	0.189	300
Synthetic Low Glucose Must	**(LGM)**	*1x*	20	0	0.020	0.174	0.038	300
		5x	20	0	0.098	0.871	0.189	300
Synthetic Low Nitrogen Must	**(LNM)**	*1x*	100	100	0.007	0.058	0.013	100
		5x	100	100	0.033	0.290	0.063	100

730

Table 2: Maximal growth and sugar consumption achieved by each yeast species and in each medium. The results are expressed as the average; the standard deviation (SD) was calculated from three biological replicates. Statistical significance was calculated by comparing three different musts within the same strain; letters indicate significant differences (p-value < 0.05).

Yeast strain	Must	[Aromatic amino acid]	Maximal growth		Consumed Sugar	
			600nm	SD	g/l	SD
S. cerevisiae QA23	SM	*1x*	11.47^{c}	1.32	199.95^{b}	0.00
		5x	9.22^{c}	1.01	194.60^{b}	1.60
	LNM	*1x*	7.64^{b}	1.66	198.58^{b}	1.00
		5x	7.25^{b}	0.97	197.26^{b}	1.75
	LGM	*1x*	4.80^{a}	0.37	19.97^{a}	0.04
		5x	4.20^{a}	0.31	19.49^{a}	0.35
H. uvarum Hu4	SM	*1x*	4.72^{c}	0.14	145.40^{c}	10.23
		5x	4.33^{c}	0.24	136.75^{c}	3.42
	LNM	*1x*	1.95^{b}	0.38	78.43^{b}	6.45
		5x	2.29^{b}	0.36	81.55^{b}	6.00
	LGM	*1x*	3.89^{a}	0.46	19.93^{a}	0.11
		5x	3.76^{a}	0.12	19.90^{a}	0.00
S. bacillaris Cz4	SM	*1x*	3.65^{a}	0.33	123.66^{c}	3.49
		5x	4.44^{c}	0.25	127.40^{c}	2.59
	LNM	*1x*	2.45^{b}	0.35	71.85^{b}	2.36
		5x	2.71^{b}	0.32	81.35^{b}	8.61
	LGM	*1x*	3.74^{a}	0.16	19.913^{a}	0.12
		5x	2.38^{b}	0.39	19.653^{a}	0.26
M. pulcherrima Mp4	SM	*1x*	3.67^{b}	1.08	106.18^{d}	0.06
		5x	6.57^{c}	0.65	100.20^{d}	7.80
	LNM	*1x*	3.36^{b}	0.38	37.66^{b}	4.88
		5x	5.36^{a}	1.02	70.39^{c}	15.29
	LGM	*1x*	4.49^{a}	0.56	19.99^{a}	0.00
		5x	5.72^{a}	0.20	19.82^{a}	0.26
T. delbrueckii Tdp	SM	*1x*	10.31^{c}	0.40	176.34^{c}	2.72
		5x	8.35^{c}	1.58	177.82^{c}	2.00
	LNM	*1x*	5.95^{b}	0.66	107.81^{b}	6.03
		5x	5.00^{b}	0.79	93.03^{b}	10.89
	LGM	*1x*	3.06^{a}	0.50	19.99^{a}	0.00
		5x	4.28^{d}	0.08	19.95^{a}	0.04

737 **Table 3. Production yield of aromatic alcohols in SM and LNM.** The yield was
738 calculated as the ratio between the concentration obtained for each aromatic alcohol and
739 the concentration consumed of its precursor. The results are expressed as the average;
740 the standard deviation was determined by taking into account three biological replicates.
741 In all cases, comparisons are within the same species; letters indicate significant
742 differences (p-value < 0.05).

743

		[TyrOH] / [Tyr]		[PheOH] / [Phe]		[TrpOH] / [Trp]	
Yeast strain	**Must**	**(µM/µM)**	**SD**	**(µM/µM)**	**SD**	**(µM/µM)**	**SD**
S. cerevisae QA23	SM	0.50[a]	0.19	0.33[a]	0.09	0.34[a]	0.08
	LNM	1.01[b]	0.18	1.03[b]	0.06	0.50[b]	0.05
H. uvarum Hu4	SM	0.20[a]	0.07	0.06[a]	0.00	0.05[a]	0.03
	LNM	0.72[b]	0.14	0.10[b]	0.03	0.23[b]	0.05
S. bacillaris Cz4	SM	0.04[a]	0.01	0.01[a]	0.00	*ND*	-
	LNM	0.19[b]	0.02	0.07[b]	0.00	*ND*	-
M. pulcherrima Mpp	SM	0.09[a]	0.04	0.09[a]	0.01	0.03[a]	0.02
	LNM	0.13[b]	0.01	0.09[a]	0.01	0.04[a]	0.00
T. delbrueckii Tdp	SM	0.17[a]	0.04	0.06[a]	0.02	0.03[a]	0.02
	LNM	0.71[b]	0.26	0.25[b]	0.04	0.19[b]	0.00

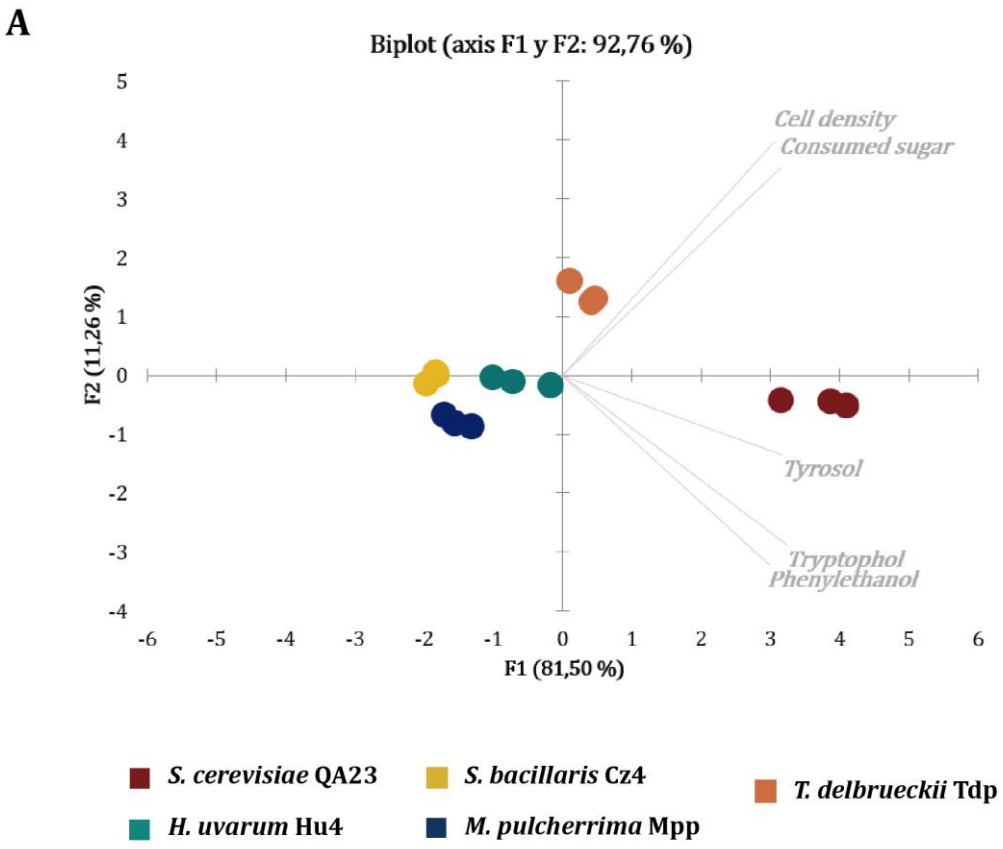
744

SUPPORTING INFORMATION

Figure S1: Bi-plots depicting Principal Component Analysis (PCA) with the following variables: aromatic alcohol concentrations, maximal cell density and consumed sugar during fermentation in SM 1X (A) and 5x (B) of aromatic amino acid content.

Figure S2: Bi-plots depicting Principal Component Analysis (PCA) with the following variables: aromatic alcohol concentrations, maximal cell density and consumed sugar during fermentation in LGM.

754 Figure_S1



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757 Figure_S2

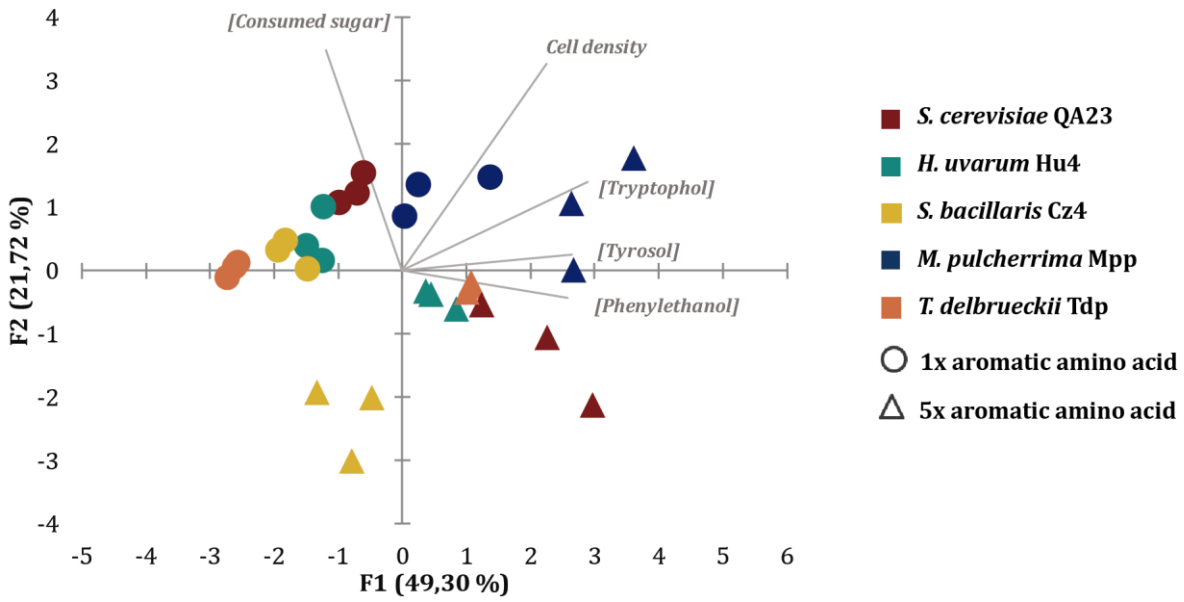

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Table S1: Production yield of aromatic alcohols in SM and LNM at 5x of aromatic amino acid content. The yield was calculated as the ratio between the concentration obtained for each aromatic alcohol and the concentration consumed of its precursor. The results are expressed as the average; the standard deviation was determined by taking into account three biological replicates. In all cases, comparisons are within the same species; letters indicate significant differences (p-value < 0.05), aaa; aromatic amino acid.

			[TyrOH]/[Tyr]		**[PheOH]/[Phe]**		**[TrpOH]/[Trp]**	
Yeast strain	**Must**	**[aaa]**	(µM/µM)	**SD**	(µM/µM)	**SD**	(µM/µM)	**SD**
S. cerevisae QA23	SM	*5x*	0.62[a]	0.15	0.04[a]	0.01	1.05[a]	0.08
	LNM	*5x*	0.37[b]	0.07	0.50[b]	0.13	0.70[b]	0.10
H. uvarum Hu4	SM	*5x*	0.24[a]	0.02	0.01[a]	0.00	0.44[a]	0.03
	LNM	*5x*	0.59[b]	0.09	0.09[a]	0.01	0.53[b]	0.05
S. bacillaris Cz4	SM	*5x*	0.05[a]	0.02	0.01[a]	0.00	0.01[a]	0.00
	LNM	*5x*	0.07[a]	0.03	0.04[a]	0.01	0.01[a]	0.01
M. pulcherrima Mpp	SM	*5x*	0.08[a]	0.04	0.02[a]	0.00	0.48[a]	0.05
	LNM	*5x*	0.40[b]	0.04	0.06[a]	0.02	0.29[b]	0.07

T. delbrueckii Tdp	SM	*5x*	0.26[a]	0.14	0.04[a]	0.01	0.95[a]	0.80
	LNM	*5x*	0.21[a]	0.04	0.11[b]	0.01	0.23[b]	0.09

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Table S2: Production yield of aromatic alcohols in LGM. The yield was calculated as the ratio between the concentration obtained for each aromatic alcohol and the concentration consumed of its precursor. The results are expressed as the average; the standard deviation was determined by taking into account two biological replicates, aaa; aromatic amino acid.

		[TyrOH]/[Tyr]		**[PheOH]/[Phe]**		**[TrpOH]/[Trp]**	
Yeast strain	**[aaa]**	(µM/µM)	**SD**	(µM/µM)	**SD**	(µM/µM)	**SD**
S. cerevisae QA23	*1x*	0.16	0.15	0.14	0.01	0.09	0.00
	5x	0.15	0.00	0.09	0.04	0.03	0.00
H. uvarum Hu4	*1x*	0.13	0.00	0.03	0.00	0.02	0.00
	5x	0.41	0.01	0.08	0.00	0.16	0.01
S. bacillaris Cz4	*1x*	0.04	0.00	0.00	0.00	0.01	0.00
	5x	0.03	0.01	0.00	0.00	0.00	0.00
M. pulcherrima Mpp	*1x*	0.09	0.00	0.11	0.12	0.09	0.01
	5x	0.05	0.02	0.26	0.00	0.00	0.00
T. delbrueckii Tdp	*1x*	0.00	0.00	0.00	0.00	0.00	0.00
	5x	0.03	0.01	0.03	0.01	0.01	0.00