Unveiling the Power of Sterols: Optimizing Wine Fermentation with Strategic management

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Unveiling the Power of Adding Sterols in Wine: Optimizing Alcoholic Fermentation with Strategic Management

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ABSTRACT

Excessive grape must clarification can result in sluggish alcoholic fermentation and sometimes alcoholic stuck fermentation, because of the lack of sterols for the yeasts growth. To avoid this risk, addition of sterols (ergosterol or phytosterols) can be performed, resulting in higher *Saccharomyces cerevisiae* viability and shorter fermentation duration. However, no dedicated study was implemented to evaluate the efficiency of different strategies of sterol management (considering sterol type, added concentration and timing of addition) during wine fermentation. So, first, to evaluate whether the response of wine yeast strains to sterol nutrition was similar according to the type and the concentration of the sterol present in the initial must, it was studied the response of a set of 10 *S. cerevisiae* strains in a synthetic grape must with low, medium and high concentrations of ergosterol or phytosterols. Then, the impact of the timing of sterol addition was evaluated on 2 *S. cerevisiae* strains with opposite behaviours. This work confirmed previous results concerning the role of ergosterol and phytosterols but also revealed new findings in this field. At first, it was confirmed that ergosterol played an important role in improving the maintenance of viable cells towards the end of fermentation, particularly in sterols-limited situations, while phytosterols demonstrated an ability to reduce acetate and glycerol production. But, in a second part, our study sheds new light on the beneficial impact of sterol addition on amino acid assimilation in yeast, leading to an increase in maximum fermentation rate, biomass production and percentage of viable cells. However, the main novelty of this research work concerns the timing of ergosterol addition. This addition at the start of fermentation in a phytosterols-free synthetic must enabled faster fermentations, as well as higher fermentative aroma synthesis, compared to addition during stationary phase Even if the impact of ergosterol additions were relatively similar for both strains tested, notable differences were found concerning amino acid assimilation and biomass production, suggesting differences in the regulation of nitrogen metabolism between both strains. These findings provide new insights into our understanding of sterol role in enological fermentation. It offers a basis for both the development of innovative strategies for sterol management and the selection of wine yeast strains under sterol starvation.

Keywords: wine yeast, sterol management, nitrogen-sterol balance, yeast phenotype diversity, fermentative aromas.

1. Introduction

Sterols are a part of the yeast lipidome and are responsible for the maintenance of yeast cell membrane integrity and optimal functionality (Aguilar et al., 2010; Klug and Daum, 2014). During wine fermentation, they promote yeast growth and metabolism and ensure a good viability at the end of fermentation, avoiding sluggish and stuck fermentations (Casalta et al., 2019, 2013; Duc et al., 2017; Ochando et al., 2017; Rosenfeld et al., 2003).

Ergosterol is the final product in the yeast sterol synthesis pathway and corresponds to 90% of the total content of sterols for *Saccharomyces cerevisiae* strains (Ejsing et al., 2009). Its production requires oxygen (at least 7nM dissolved oxygen), as the enzymes involved in the synthesis of ergosterol and its precursors are oxygen-dependent (Jordá and Puig, 2020). Under anaerobiosis, *S. cerevisiae* strains can assimilate phytosterols from the grape must, thanks to the ABC transporters (ATP-binding cassettes) Aus1p and Pdr11p (Jacquier and Schneiter, 2012; Li and Prinz, 2004; Tesnière et al., 2021). In grape berries, β-sitosterol is the major phytosterol (around 90% of the total sterol content), followed by stigmasterol and campesterol (Tumanov et al., 2015). The comparison between both sterol types and their roles during wine fermentation was recently reviewed in Girardi Piva et al. (2022b).

Grape must clarification is a critical step employed before white wine fermentation to decrease the synthesis of undesirable aldehydes and herbaceous alcohols in the final product, by removing solid particles rich in sterols (Karagiannis and Lanaridis, 2002; Ma et al., 2020). However, excessive clarification results in a low sterol content, which leads to high yeast cell death, limits biomass production, and leads to incomplete alcoholic fermentation (Casalta et al., 2019, 2016; Ochando et al., 2017; Rodríguez-Vargas et al., 2007; Sablayrolles and Barre, 1986; Waldbauer et al., 2011).

Oxygen addition allows to compensate for the lack of phytosterols in grape must, by allowing the synthesis of ergosterol and its precursors by yeasts (Fornairon-Bonnefond et al., 2001; Julien et al., 2000; Ochando et al., 2017; Sablayrolles et al., 1996). Another possibility is the addition of grape solid particles containing phytosterols at the beginning of fermentation (Casalta et al., 2013, 2012). Inactive dry yeasts can also be added during rehydration of active dry yeasts to provide ergosterol (Belviso et al., 2004; Soubeyrand et al., 2005).

In a recent study, we showed that, under sterol starvation, sterol type affects fermentation kinetics along with biological and Central Carbon Metabolism parameters (Girardi Piva et al., 2022a).

We therefore wonder if the type of sterol could also affect these same parameters with higher doses of sterols, until reaching a concentration where sterols are no longer the limiting nutrient.

To answer this question, different strategies of addition of sterol were tested in the present research work. In a first step, we evaluated the effect of sterol supplementation at the start of the fermentation process (sterol dose and type, ergosterol versus phytosterols) on fermentation kinetics, cell viability and synthesis of Central Carbon Metabolism (CCM) metabolites for a set of 10 *S. cerevisiae* wine strains. In this part, three different sterol contents were tested: a low content (1.0 mg/L) mimicking sterol limitation, as in the case of excessive grape must clarification; an intermediate content similar to many classical enological fermentations (2.5 mg/L) and a higher sterol concentration (4.0 mg/L), in which the nitrogen-sterol balance enables a complete assimilation of nitrogen.

Then, in a second step, the impact of the timing of sterol addition in a synthetic must lacking sterols was evaluated. Ergosterol was thus added either at the beginning of fermentation or at the beginning of the stationary phase. This specific study was performed for 2 strains displaying opposite fermentation profiles and sensibility to sterol type. Impacts of these sterol additions were tested on fermentation kinetics, biomass production, viability and some key metabolites, such as acetate, glycerol, succinate and fermentative aromas.

2. Materials and methods

2.1 Strains

A set of 10 *Saccharomyces cerevisiae* wine yeast strains numbered L2, L3, L4, L6, L8, L10, L16, L17, L18 and L21 (identical to those tested in Girardi Piva et al., 2022a) were used for sterol type and dose experiments. L6 and L16 are the fastest strains, L2, L3, L4, L8 and L17 the strains with intermediate fermentation time and L10, L18 and L21 the slowest strains (Girardi Piva et al., 2022a). Two of these strains with opposite phenotypes were used to study the impact of sterol addition at different timings: L6 (strain more resistant to sterol starvation and less susceptible to sterol type) and L10 (strain less resistant to sterol starvation and more susceptible to sterol type). All strains were obtained as active dried yeasts from Lallemand Oenology (Blagnac, France). Fermenters were inoculated with 0.05 g/L of active dried yeast, previously rehydrated for 20 minutes at 37°C in a glucose solution (50 g/L).

2.2 Experimental fermentations

Experimental fermentations were performed in a synthetic must (SM), which mimics a grape must, following the protocol described by Bely et al. (1990). This synthetic must (SM 400) contained 400 mg/L of assimilable nitrogen, with a ratio (m/m) of 72% assimilable amino acids and 28% ammonium (NH_4Cl) and 200 g/L of sugars (50% glucose and 50% fructose). The pH was adjusted to 3.3.

A purified phytosterol complex, containing mainly β-sitosterol (≥ 70 %) (85451, Sigma-Aldrich) was used to prepare the phytosterol solution, while the ergosterol solution was prepared with synthetic ergosterol (E6510, Sigma-Aldrich). Two sterol stock solutions with 15 g/L of sterols (either phytosterols or ergosterol) containing Tween 80® and ethanol (1:1, *v*/*v*) were prepared and then diluted with ethanol to obtain a final solution of 1.5 g/L sterols before addition to the synthetic must (Casalta et al., 2019).

2.2.1 Sterol dose and type

Three concentrations of sterols (ergosterol or phytosterols) in the SM 400 were tested: 1.0 mg/L, 2.5 mg/L and 4.0 mg/L (Table 1) to evaluate the impact of sterol dose.

All fermentations were performed in 300 mL fermenters filled with 250 mL of the corresponding medium. Fermenter medium deaeration was performed before sterol addition by bubbling pure argon for 20 minutes to ensure anaerobic conditions. Moreover, fermenters were fitted with fermentation locks to maintain anaerobiosis (Rollero et al., 2015). All fermentations were performed in biological triplicates (total of 180 fermenters).

Fermenters (300 ml) were placed on magnetic stirring plates (260 rpm) at 24°C. In addition, fermentation kinetics were followed via an internally developed control software dedicated to the study of alcoholic fermentation with a temperature control system and automatic weighing. This task was performed with a robotic arm (Lab Services, Breda, Netherlands), as described in Girardi Piva et al., 2022a). It allowed monitoring the amount of produced CO_2 (in g/L) and the fermentation rate (in g CO_2/Lh).

2.2.2 Timing of sterol addition

For the evaluation of the timing of sterol addition, 1.2 L fermenters were filled with 1.0 L of MS400. Anaerobiosis was ensured by pure argon bubbled during 30 minutes and fermentation locks. Afterwards, 1.0 mg/L of phytosterols were added to all fermenters to mimic excessive clarified grape musts. Moreover, 3.0 mg/L of ergosterol were added at the beginning of fermentation (T0) or during stationary phase (T30, corresponding to 30 g/L of released CO_2) to switch to a condition where nitrogen was the limiting nutrient (Table 1). Both modalities were compared with a control without ergosterol addition. All fermentation conditions were performed in biological triplicates (a total of 18 fermentations).

1.2 L fermenters were placed on scales with magnetic stirring plates (260 rpm). A lamp system and a temperature sensor for each fermenter allowed maintaining temperature at 24°C (Sablayrolles et al., 1987). Fermentation kinetics were followed with automatic weighing every 20 minutes.

2.3 Sample preparation

Two samplings were done during both experiments. The first sampling was done at 85% of fermentation progress, and the sample was divided in two: the first fraction was used for yeast cell viability determination and cell counting; the second one was centrifuged for 10 min at 3000 rpm at 4°C and the corresponding supernatant was stored at -20°C until nitrogen content analysis. The second sample was collected at the end of fermentation. The centrifuged supernatant (10 min at 3000 rpm at 4°C) was stored at -20°C to quantify central carbon metabolism (CCM) metabolites. For the study of the impact of the timing of ergosterol addition, samples were collected at 85% of fermentation progress and the supernatant was stored at -20°C before centrifugation until CCM metabolites and fermentative aromas analysis.

2.4 Analytical methods

2.4.1 Cell viability

Cell viability was determined by flow cytometry using an Accuri® C6 cytometer (Accuri, BD Biosciences) with propidium iodide (IP) as marker, as described by (Delobel et al., 2012). Viability was determined as the percentage of intact and fragile cells among all cells.

2.4.2 Cell counting

Samples were diluted 1600-fold with Isoton II® (Beckman-Coulter). After sonication (30 seconds, 10W), cells were counted with a Coulter Z2 electronic counter (Coulter Multisizer3, Beckman Coulter) fitted with a 100-µm aperture probe.

2.4.3 Nitrogen

The assimilated nitrogen content (ammonium and amino acids) was determined at 85% of fermentation progress. The ammonium (NH_4) concentration was determined enzymatically (Boehringer Mannheim, Mannheim, Germany). Its percentage was calculated as follows (Eq. 1):

$$\%\ \text{Assimilated}\ \mathrm{NH_4}\ 85\% = (\,[\mathrm{NH_4}]\text{must} - [\mathrm{NH_4}]85\%)\,/[\mathrm{NH_4}]\text{must}$$

The free amino acid (AA) content was determined by cation exchange chromatography with post-column ninhydrin derivatization (Biochrom 30, Biochrom), as described by Crépin et al. (2012). The percentage of assimilated amino acid content was determined as follows (Eq. 2):

$$\%\ \text{Assimilated AA}\ 85\% = (\,[\mathrm{AA}]\text{must} - [\mathrm{AA}]85\%\,)/[\mathrm{AA}]\text{must}$$

2.4.4 Determination of CCM metabolites and residual sugars

Acetate, glycerol, succinate and residual sugars concentrations were determined by high-performance liquid chromatography (HPLC 1290 Infinity, Agilent Technologies, Santa Clara, CA, USA) with a Phenomenex Rezex ROA column (Agilent Technologies, Santa Clara, CA, USA) at 60°C, as described by Rollero et al. (2015).

2.4.5 Fermentative aroma analysis

The volatile compounds analysis was performed by gas chromatography and mass spectrometry, as described by Rollero et al. (2015). First, the volatile compounds were extracted with dichloromethane. Then, the concentration in fermentative aromas (higher alcohols, acetate esters, ethyl esters and acids) was measured via GC/MS in SIM mode using a DB-WAX GC column. Thirty-three compounds were quantified using internal deuterated standards.

2.5 Fermentation progress and variables coding

The fermentation progress corresponds to the ratio between the final CO_2 production and the amount of CO_2 produced at a specific time, which is proportional to the amount of sugars consumed. For both conditions tested, 85% of fermentation progress corresponded to 80 g/L of produced CO_2. Similarly, 33% of fermentation progress corresponded to 30 g/L of produced CO_2.

Some variables were coded to simplify results presentation: tCO_2_x corresponded to the time to release "x" grams of CO_2; tCO_2_End corresponded to the time to achieve the end of fermentation; Vmax to the maximum fermentation rate.

2.6 Statistical Analysis

Statistical analyses were performed with R software version 3.6.2 (R Development Core, 2019). To describe the variability of the data set, PCA was performed with the package FactoMineR (v2.3).

A three-way Anova was performed for the sterol dose experiment. Strain, sterol type and sterol dose were the factors evaluated using aov function with a statistical significance level of 5%, following the model below (Eq. 3):

$$y = \beta_0 + \beta_1 Strain + \beta_2 Sterol + \beta_3 Dose + \beta_{12} Strain{:}\,Sterol + \beta_{13} Strain{:}\,Dose + \beta_{23} Sterol{:}\,Dose + \epsilon$$

Where β_0 is the intercept term, β_i the linear coefficients (i = 1, 2, and 3), β_{ij} the interaction coefficients (i = 1, 2, and 3; j = 1, 2, and 3) and ϵ are independent N(0, σ^2) error terms. Hypotheses were checked and the normality of residual distributions and homogeneity of variance were evaluated with standard diagnostic graphs.

3. Results

3.1. Impact of sterol dose and type

The impact of sterol type and concentration on fermentation kinetics was evaluated for 10 *S. cerevisiae* strains that all performed differently, according to sterol dose and type.

3.1.1 Overview of the impact of sterol dose and sterol type

A PCA was performed to provide an overview on the global variation on the dataset and highlight those that contributed the most to this variation. Most variables describing fermentation kinetic, biomass, and CCM metabolites highly differentiated the individuals tested, except for succinate production. As shown in Fig. 1, Dim 1 and Dim 2 explained 78% of total variation. Dim 1 was associated mostly with kinetics variables, biomass and cell viability and Dim 2 with CCM metabolites (in particular glycerol).

We can observe that the maximum fermentation rate (Vmax), biomass and the amount of amino acid (assimilated_AA) were all positively correlated but inversely correlated with the beginning of fermentation (tCO_2_30). Moreover, viability was correlated with these biological variables and the maximum fermentation rate, and inversely correlated with variables describing fermentation duration (production of CO_2 at 85% of sugar consumption or at the end of fermentation, tCO_2_80 and tCO_2_End respectively). Interestingly, a faster fermentation start was associated with shorter fermentations, and appeared linked to better biological parameters maintenance. Acetate content was correlated with tCO_2_30, while succinate was correlated with biological variables.

The effect of sterol dose and type, two categorical variables, could be observed from the different colours applied to individuals in the scatterplot (Fig. 1B). Fermentations performed with a low sterol content (1.0 mg/L of either ergosterol and phytosterols: E1.0 and P1.0, respectively) can easily be distinguished in the left part of Fig. 1B, suggesting a sterol dose effect. Indeed, these fermentations started slowly with a delayed end. This was associated with large amounts of residual amino acids, a low biomass content and a low viability at 85% of fermentation progress. By contrast, fermentations performed with a high sterol content (4.0 mg/L of ergosterol and phytosterols: E4.0 and P4.0, respectively) were completed earlier, displayed a higher Vmax and a higher viability at the end of fermentation. Interestingly, fermentations performed with 2.5 mg/L of sterols (E2.5 and P2.5: ergosterol and phytosterols, respectively) were closer to fermentations performed with 4.0 mg/L of sterols. Nevertheless, this distance varied according to the strain, indicating a major strain effect.

Regarding sterol type, denoted in pink and blue for phytosterols and ergosterol, respectively, it is quite clear that fermentations conducted with phytosterols are mainly located in the lower part of Fig. 1B, whereas fermentations performed with ergosterol (in blue) are located in the upper part of the scatterplot, which suggests an effect of sterol type.

We can observe specific behaviours of strains, such as for L10, L18 and L21, that were the most susceptible to sterol limitation and that completed fermentation faster when the fermentation medium contained ergosterol instead of phytosterols. In contrast to these 3 strains, L4 and L6 maintained higher viability at the 3 doses tested and were less impacted by the sterol type.

To better evaluate each parameter influence (dose and type of sterol and strain) and the interaction between them, an analysis of variance (ANOVA) was performed. Table 2 shows the significance of the different factors and their interactions.

Sterol type had a significant or very significant effect on all variables tested, except for the quantity of assimilated amino acids (assimilated_AA). This means that, independently of sterol dose, the sterol type (ergosterol compared to phytosterols) impacted kinetics parameters, CCM metabolites and almost all biological variables. In addition, dose and strain effects were highly significant for all variables (p-value < 10^{-3}).

Interestingly, significant interaction effects were noted between the sterol type and the strain (p-value < 1 x 10^{-3} for viability, biomass, assimilated_AA, tCO_2_80, and CCM metabolites; < 1 x 10^{-2} to Vmax and < 5 x 10^{-2} to tCO_2_30), as well as for sterol dose and strain (p-value < 1 x 10^{-3} for all variables tested). This suggests that strains do not respond to the sterol type and content available in the synthetic must in a similar manner. In addition, very significant interactions were also detected between sterol type and their content for viability, time to complete 85% of fermentation (tCO_2_80), and succinate content. Moreover, a P-value < 1.8 x 10^{-4} was observed for acetate. This shows that the differences in the response to the sterol type varied according to the content in the media for these variables.

3.1.2 Impact of sterol dose and sterol type in fermentation kinetics, biological and CCM variables

To better understand the significant impact of sterol dose and type on wine fermentation evidenced with ANOVA, boxplots were drawn individually for kinetics, biological and CCM variables. Moreover, barplots by strain, sterol type and dose were performed to observe the behaviour of each strain.

3.1.2.1 Kinetics variables

In agreement with PCA results, a higher sterol content in the fermentation medium provoked an increase in the maximum fermentation rate (Fig. 2A). A striking result was the wider dispersion of Vmax with phytosterols, which indicates a more variable strain response to this sterol type, in comparison to ergosterol.

Higher sterol concentrations led to shorter fermentation time, as indicated by the time required to ferment 85% of total sugars (Fig. 2B), in comparison to fermentations performed with only 1.0 mg/L of sterols. However, the increase in fermentation speed obtained by the addition of sterol was not a linear function of the sterol content. Indeed, increasing the sterol content of the synthetic must from 2.5 to 4.0 mg/L did not show any significant increase in fermentation progress, as measured for tCO_2_80 for L2, L3, L4, L10, L17 and L21 for both types of sterols, L16 and L6 with phytosterols and L8 with ergosterol. By contrast, when the sterol content was increased from 1.0 mg/L to 2.5 mg/L, L21 was able to reduce its time to release 80 g/L of CO_2 of 82h and 63 h with phytosterols and ergosterol, respectively. In opposition to fermentations performed with higher concentrations of sterols, we can observe a higher variability of the time required to ferment 85% of the sugar content at 1.0 mg/L.

Finally, the presence of ergosterol in the fermentation medium led to a reduction of fermentation time, as measured by tCO_2_80, in comparison to phytosterols: L10, L18 and L21 at 1.0 mg/L, L8 at 2.5 mg/L and L16 at 4.0 mg/L.

3.1.2.2 Biological variables

Following Vmax results, biomass, assimilated amino acids and viability increased with higher sterol concentrations. In addition, differences between ergosterol and phytosterols were bigger at 1.0 mg/L than at 4.0 mg/L and 2.5 mg/L for biomass (Fig. 3A). Moreover, strains were not impacted by sterol dose in the same way for this biological parameter. For example, biomass increased of 63% when sterol concentration raised from 1.0 mg/L to 4.0 mg/L for L3, while this difference was of 154 % for L18.

We can observe that assimilable amino acids consumption was lower than 68% for all strains at 1.0 mg/L of sterols, confirming that sterols were the limiting nutrient at this level (Fig. 3B). As expected, there was no longer any sterol limitation at 4.0 mg/L of sterols, as this concentration allowed all strains to consume all ammonium (data not shown) and almost 100% of amino acids in the synthetic grape must. Interestingly, strains L2 and L17 were already able to assimilate all amino acids with 2.5 mg/L of sterols. Moreover, some strains were susceptible to the sterol type for the consumption of amino acids: L21 (at 2.5 mg/L), L3 (at 1.0 and 2.5 mg/L) and L8 (at 2.5 mg/L).

Higher sterol concentrations increased viability (Fig. 3C): for example, the percentage of living cells of strain L10 increased by 69% with phytosterols and 35% with ergosterol, when comparing the extreme doses of sterols tested. However, no differences were found for viability between 2.5 and 4.0 mg/L with either sterols for L6 and with ergosterol for L4. At 4.0 mg/L of sterols, viability increased considerably for all strains. Moreover, a wider dispersion of viability between strains was found at 1.0 mg/L of sterols: between 40% and 65% with ergosterol and between 20% and 65% with phytosterols.

Strains L3, L4, L10, L17 and L18 showed better viability with ergosterol compared to phytosterols at 1.0 mg/L and strains L3 and L21 at 2.5 mg/L. However, viability was higher with phytosterols for L2 and L16 at 2.5 mg/L of sterols and for L10, L21 and L16 at 4.0 mg/L. Finally, strains L6 and L8 were not susceptible to sterol type for the percentage of living cells for all sterol doses.

3.1.2.3 Central carbon metabolism variables

The amount of acetate synthesized was inversely correlated to the sterol dose (Supplementary Fig. 1A), in agreement with Ochando et al. (2017) and Deroite et al. (2018) studies. A remarkable result was that phytosterols nutrition led to a lower acetate production than ergosterol at all sterol doses for all strains. L4 was the strain that synthetized more acetate, while L2 produced low acetate concentrations. Despite a significant effect of sterol dose for strains in general, no significant differences were found neither for L16 and L17 between E2 and E4, nor for L2 and L8 between P2 and P4. Interestingly, the amount of acetate synthesized with 2.5 mg/L of phytosterols was equivalent to the amount produced with 4.0 mg/L of ergosterol for all strains, save L16.

Supplementary Fig. 1B shows variations in glycerol content depending of sterol type. Indeed, a significantly higher production of glycerol was observed in presence of ergosterol than phytosterols. Regarding sterol dose, despite a clear glycerol increase with sterol dose for the strain L4 and at a lesser level for L6, no significant differences were observed for the other strains.

Regarding succinate (Supplementary Fig. 1C), we observed a higher value for this metabolite with ergosterol, compared to phytosterols, when sterol concentration was equal to 4.0 mg/L for almost all strains (strains L2, L8, L10, L16, L17, L18 and L21).

Furthermore, residual sugars (data not shown) were less than 3.0 g/L for all strains, independently of wine fermentation conditions tested (except L21, which was not able to complete fermentation with 1.0 mg/L of sterols), which means that almost all strains were able to achieve complete fermentation with either sterols, regardless of sterol dose.

3.2. Impact of the timing of sterol addition

To study the timing of sterol addition required to restore a normal fermentation, we compared the response of two strains with extreme behaviours: strain L10, which was the most impacted by sterol starvation and sterol type, and strain L6, that showed little change according to sterol type. Two stages were chosen for the addition of 3.0 mg/L of ergosterol to the synthetic must: at the beginning of fermentation or entry into stationary phase (at 33% of fermentation progress). The fermentations that received sterols were compared to a control without sterol addition, mimicking excessively

clarified grape musts. Fermentation kinetic parameters, as well as biological and CCM metabolites and fermentative aromas were measured.

3.2.1 Fermentation kinetics

Fermentation kinetics in Supplementary Fig. 2 confirm that both strains hardly completed fermentation under sterol starvation, in particular L10 that presented a sluggish fermentation (400h to finish fermentation). As expected, ergosterol addition resulted in a shorter fermentation time for both strains, independently of the time of addition. Finally, such addition allowed L10 to have fermentation duration closer to L6.

Interestingly, fermentation times were 20 h and 10 h shorter when addition was done at the beginning of the fermentation for L6 and L10, respectively, compared to stationary phase. A remarkable result was a temporary increase of fermentation rate after ergosterol addition at 33% of fermentation progress for both strains (mainly for L10).

Analysis of variance showed a strain effect and a modality effect for all variables tested, except for residual sugars (data not shown). Thus, a Tukey test was performed on these variables combined with bar charts.

3.2.2 Biological and central carbon metabolism variables

Under sterol starvation condition (control), Fig. 4A shows that L6 assimilated 58% and L10 54% of amino acids from the synthetic grape must. As expected, both strains were able to assimilate all amino acids content when ergosterol was added at the beginning of the fermentation (T0). A striking result was that strains did not respond in the same way when ergosterol was added during stationary phase (T30). This allowed L10 to assimilate 27% more amino acids than the control, while no significant difference in amino acid consumption was found for L6 between T30 and control.

Regarding biomass (Fig. 4B), ergosterol addition at T0 enhanced cell growth (x 2 for both strains), while different situations could be detected for L10 and L6 when the addition was performed at T30: more biomass was produced for L10, compared to the control (more 2.0 x 10^7 cells/mL), while the biomass content was not impacted for L6. As expected, we observed a viability increase due to the supplementation of ergosterol for both *S. cerevisiae* strains, in particular for L10 that showed more than 73% of viable cells after ergosterol addition and 16% without it (Fig. 4C). For L6, cell viability was only increased by 21% when ergosterol was added. Interestingly, we observed a small increase in viability (9 % more) for L10 when ergosterol was added at the start of fermentation, compared to the stationary phase. On the other hand, L6 was not susceptible to the timing of ergosterol addition in terms of viability.

CCM metabolites were also impacted by the timing of ergosterol addition (Fig. 4D, 4E and 4F). Interestingly, ergosterol addition during stationary phase led to an increase in acetate production for both strains (0.9 g/L of acetate for L6 and 0.7 g/L for L10). However, ergosterol addition at the start of fermentation decreased acetate content with a total of 0.4 g/L acetate for both L6 and L10. In addition, higher concentrations of glycerol were also observed for both strains when ergosterol was added at T30 (7.6 g/L of glycerol for L6 and 5.9 for L10). However, the lowest glycerol concentration was found at T0 for L6 (6.5 g/L), while for L10 it was found in the control (4.9 g/L).

Comparing both strains, L6 produced more than 1.0 g/L of succinate and L10 less than 0.8 g/L. L6 and L10 showed the same tendency in terms of succinate content regarding the timing of ergosterol addition. At T0, ergosterol addition resulted in increased succinate: over 0.3 g/L for L6 and more than 0.1 g/L of succinate for L10, compared to the control. Interestingly, the addition at T30 had the opposite effect and less succinate was quantified than the other 2 modalities (T0 and control): 0.8 for L10 and 0.6 for L6.

Less than 3.0 g/L of sugars were found at the end of fermentation for both strains, showing that they were able to complete fermentation independently of the modality tested (data not shown). Thus, the timing of ergosterol addition had no impact on the amount of residual sugars for L6, nor for L10.

3.2.3 Fermentative aromas

The fermentative aromas for the study of the impact of timing of sterol addition were evaluated (Supplementary Table 1). The most significant fermentative aromas are presented in Fig. 5A. Dim 1 accounted for 47% of the variation and was particularly related to acids (dodecanoic, decanoic and isobutyric acids) and ethyl esters (ethyl hexanoate and ethyl butanoate), while Dim 2 accounted for 37 % of the variation and was mostly related to acetate esters.

The timing of ergosterol addition impacted the synthesis of fermentative aromas for both strains. The addition of ergosterol increased aroma synthesis, compared to the control condition. The most striking novelty was a higher production of fermentative aromas when ergosterol was added at the beginning of fermentation. It is very interesting to notice that, despite ergosterol addition, each strain conserved its own fermentative aroma profile, as Seguinot et al. (2018) have shown when nitrogen additions were performed. L10 mostly synthesized acids, the higher alcohols methionol and isobutanol and ethyl hexanoate and ethyl butanoate. Regarding L6, propanol (a nitrogen marker) and acetate esters were principally produced, such as isoamyl acetate and 2 phenylethyl acetate.

4. Discussion

In this study, our primary objective was to investigate the response of wine yeast strains to different types and concentrations of sterol supplementation during alcoholic fermentation. We assessed how these variations in sterol nutrition influenced fermentative kinetics, biological factors (cell count and viability) and metabolite production. Furthermore, we investigated the influence of the timing of ergosterol addition, particularly when sterol availability was limited, on the same variables as well as on aroma production.

4.1 Sterol dose and type

Strains were faced with two extreme conditions, depending on the limiting nutrient. Sterols were the limiting nutrient at 1.0 mg/L of sterols, while it was nitrogen at 4.0 mg/L of sterols. A striking result was that the limiting nutrient varied depending of the strain at 2.5 mg/L of sterols (Fig. 3A). Sterol limitation was characterized by residual amino acids, which prevented adequate yeast multiplication and resulted in lower Vmax, in agreement with the literature (Casalta et al., 2019; Duc et al., 2017; Girardi Piva et al., 2022a). Moreover, strains could not maintain a high viability in the later part of the fermentation, as their cell membranes were not well protected from ethanol toxicity due to the lack of sterols. As a result, longer fermentation durations were observed.

As expected, all nitrogen content was consumed at 4.0 mg/L of sterols, allowing both higher biomass production and higher Vmax. In addition, yeast cell membranes were reinforced thanks to sterols, resulting in increased viability and a reduction in fermentation time, in agreement with the literature (Casalta et al., 2013; Ochando et al., 2017).

A number of strains were still limited by sterols at 2.5 mg/L of sterols, while strains L2 and L17 were able to assimilate all amino acids with either sterols. This shows for the first time that sterol requirements to assimilate all grape must nitrogen is strain dependent. Moreover, despite the positive impact of sterol concentration increase during wine fermentation for all strains, its intensity also varied according to the *S. cerevisiae* strain.

Interestingly, sterol type effect was stronger under sterol starvation than in the other conditions tested. Indeed, ergosterol allowed better viability maintenance and shorter fermentation durations, compared to phytosterols. However, almost all strains were able to complete fermentation with this latter sterol, as showed by Girardi Piva et al. (2022a) but in contrast to Luparia et al. (2004). Moreover, we could observe that the impact of sterol type was variable depending on the strain and that the difference between ergosterol and phytosterols was more significant for strains that had difficulties to cope with sterol limitation.

Regarding some key metabolites, we observed that an increase in sterol concentration resulted in a lower production of acetate for all strains. Acetate is an intermediate in the lipid synthesis

pathway (Fig. 6). Thus, more of this metabolite would be synthesized under sterol limitation, which could explain its decrease at higher sterol concentrations, as already noted by Ochando et al. (2017).

A possible explanation for the reduction of succinate synthesis under sterol deficiency would be the management of the intracellular pool of α-ketoglutarate, an intermediate of succinate synthesis and of the catabolism of amino acids. Indeed, at high sterol content, nitrogen consumption increased, resulting in a stimulation of amino acids catabolism that provoked an accumulation of α-ketoglutarate, which is a key metabolic intermediate in that cellular process. This accumulation of α-ketoglutarate would then result in a higher synthesis of succinate. So, succinate evolution appeared to be an indirect consequence of the effect of sterols addition on nitrogen consumption (Ochando et al., 2017).

A striking result was the higher amount of acetate synthetized with ergosterol, compared to phytosterols, for all sterol doses and all 10 *S. cerevisiae* strains tested. We could hypothesize that the lipid synthesis pathway was impacted by sterol type. Ergosterol being the native yeast sterol, it would better protect the membrane of yeast cells than phytosterols, resulting in a lower demand in lipid synthesis. This difference of the management of lipid production would then impact acetate content. Thus, it can be hypothesized that a lower demand in lipids would result in a lower conversion of acetate into acetyl-CoA, which would lead to acetate accumulation when ergosterol is the sterol source in the fermentation medium.

Acetate is an intermediate in the lipid synthesis pathway (Fig. 6). Thus, more of this metabolite would be synthesized under sterol limitation, which could explain its decrease at higher sterol concentrations, as already noted by Ochando et al. (2017).

In parallel, a smaller increase in glycerol was in the presence of ergosterol compared to phytosterols. A hypothesis to explain this glycerol increase would be the link between glycerol and the triglycerides pathway, of which glycerol-3-phosphate is one of the precursors (Fig. 6). Indeed, ergosterol would better reinforce yeasts membrane than phytosterols so less triglycerides would be necessary (Ochando et al., 2017). Consequently, the triacylglycerol pathway would be less activated in presence of ergosterol; so glycerol-3-phosphate flow would be mainly directed towards glycerol synthesis, resulting in a higher production of this compound with ergosterol.

4.2 Timing of sterol addition

In agreement with literature, the addition of ergosterol in case of sterol deficiency intensified the aroma profile for both *S. cerevisiae* strains (Mauricio et al., 1997; Varela et al., 2012). However, the addition during stationary phase was less efficient than at the beginning of fermentation. This could be explained by a decrease in metabolic and anabolic activities during the stationary phase, which would consequently reduce fermentative aroma synthesis, compared to initial ergosterol addition.

Another observation common to both strains was that sterol addition at the start of fermentation (versus during the stationary phase) was more efficient in terms of fermentation management and metabolites production from the central carbon metabolism: shorter fermentation time and lower production of acetate and glycerol. The increase in acetate after ergosterol addition during stationary phase was not expected. It can thus be suggested that, after ergosterol addition at 33% of fermentation progress, the pathway for de novo lipids synthesis would be less active and less acetyl-CoA (its precursor) would be synthetized. As a consequence, we might observe an accumulation of acetate (acetyl-CoA precursor). Finally, concerning succinate, it was inversely correlated with acetate, probably due to the decrease in acetyl-CoA availability (its precursor), resulting in a lower flux in the TCA cycle.

Nevertheless, despite common behaviours, important differences were noted between the two strains, revealing their different sensitivity to ergosterol addition. Indeed, the response to sterol addition at T30 for L10 under sterol limitation had some similarities to nitrogen addition in a fermentation medium lacking nitrogen during the stationary phase (Seguinot et al., 2018). This similarity probably originates from the fact that, in the present work, ergosterol addition enabled L10 to consume more amino acids and resulted in an increased biomass. However, for L6, amino acid assimilation and biomass production were not impacted by ergosterol addition at T30. These results suggest that, in the two stains, different adaptation mechanisms were activated in response to sterol limitation that induced different responses to sterol supply.

5. Conclusion

This study presents original findings regarding the response of several yeast strains to various strategies for managing sterol additions, including the quantity of sterols added, the type of sterols used and the timing of their addition. Especially, it was demonstrated, for the first time, that the disparities between the two types of sterols were mainly noticeable under sterol limitation. Indeed, ergosterol made it possible to maintain a higher viability (resulting in a shorter fermentation time) compared to phytosterols under sterol starvation; but at higher sterol concentrations, these differences between the two types of sterol were significantly reduced or nullified. Moreover, the impact of sterol type and content varied depending on the strain, which underlines *S. cerevisiae* sterol requirement diversity during wine fermentation.

A striking result was that ergosterol addition during stationary phase improved fermentation, reducing fermentation time. However, a late addition of sterols was less efficient, from an enological point of view, than an early one, as it resulted in an acetate and glycerol increase, as well as a lower production of fermentative aromas.

These findings highlight the importance of implementing a sterol management strategy during alcoholic fermentation for both the completion of the fermentation process and the production of the MCC metabolites. In case of excessive clarified grape musts, it is important to manage not only the quantity of sterols added, but also the nature and timing of this addition.

Further research should be undertaken to test the impact of the timing of sterol addition with a larger set of strains and test earlier times of sterol addition during stationary phase. Moreover, it would be interesting to better understand the molecular mechanisms associated with sterol assimilation.

CRediT authorship contribution statement

Giovana Girardi Piva: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Erick Casalta:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Jean-Luc Legras:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Isabelle Sanchez:** Data curation, Formal analysis. **Martine Pradal:** Investigation. **Faïza Macna:** Investigation. **David Ferreira:** Conceptualization, Writing – review & editing, Supervision. **Anne Ortiz-Julien:** Conceptualization, Writing – review & editing, Project administration. **Virginie Galeote:** Conceptualization, Methodology, Writing – review & editing, Project administration. **Jean-Roch Mouret:** Conceptualization, Methodology, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Datas are available from the corresponding author upon reasonable request.

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Table 1. Experimental designs of sterol dose and timing of sterol addition experiments: strains used and modalities tested.

Experiment	Strains	Modalities
Sterol dose	L2, L3, L4, L6, L8, L10, L16, L17, L18 and L21	1.0 mg/L of sterol (ergosterol or phytosterols) 2.5 mg/L of sterol (ergosterol or phytosterols) 4.0 mg/L of sterol (ergosterol or phytosterols) Control: without ergosterol addition
Timing of sterol addition	L6 and L10	T0: Ergosterol addition at the beginning of fermentation T30: ergosterol addition at 30 g/L of released CO_2

Table 2. Evaluation of the significance of effect of strain, sterol type and sterol dose on different variables representative of fermentation kinetics and central carbon metabolism. Fermentation kinetic variables: maximum fermentation rate (Vmax), time to reach 30 (tCO_2_30) and 80 g/L (tCO_2_80) of released CO_2; biological variables at tCO_2_80: viability, yeast biomass and assimilated amino acids (assimilated AA); central carbon metabolism variables at the end of fermentation: acetate, glycerol and succinate. Effects and interactions are colored according to P-value threshold. White: Not significant; Gray: P-value < 5×10^{-2}; Light blue: P-value < 1×10^{-2}; Dark blue: P-value < 1×10^{-3}.

Variables	Effects			Interactions		
	Sterol type	Sterol dose	Strain	Sterol type : sterol dose	Sterol type : Strain	Sterol dose : Strain
Vmax	7.8×10^{-6}	3.8×10^{-102}	4.2×10^{-67}	2.1×10^{-1}	2.1×10^{-3}	2.6×10^{-15}
Viability	2.4×10^{-4}	1.2×10^{-93}	2.1×10^{-52}	1.4×10^{-12}	1.5×10^{-8}	1.1×10^{-27}
Biomass	9.4×10^{-3}	7.1×10^{-101}	8.3×10^{-74}	3.7×10^{-1}	6.2×10^{-5}	1.4×10^{-4}
Assimilated AA	3.0×10^{-1}	1.5×10^{-115}	6.4×10^{-46}	4.1×10^{-1}	7.6×10^{-6}	1.1×10^{-25}
tCO_2_30	1.7×10^{-6}	2.5×10^{-66}	1.4×10^{-82}	3.6×10^{-1}	2.6×10^{-2}	2.3×10^{-9}
tCO_2_80	1.0×10^{-15}	8.5×10^{-94}	5.1×10^{-60}	1.0×10^{-5}	9.7×10^{-8}	1.1×10^{-41}
Acetate	7.1×10^{-62}	5.9×10^{-91}	4.2×10^{-93}	1.8×10^{-4}	1.5×10^{-18}	1.7×10^{-35}
Glycerol	3.4×10^{-60}	4.4×10^{-20}	1.6×10^{-116}	8.9×10^{-2}	5.6×10^{-15}	3.6×10^{-30}
Succinate	2.4×10^{-34}	1.3×10^{-57}	6.1×10^{-90}	1.3 x 10-16	3.1×10^{-7}	2.7×10^{-22}

Figure 1. PCA for variables triplicate means of 10 wine yeast strains for the evaluation of sterol dose and type. **(A)** Projection of the variables used to describe fermentation kinetics (red), biological variables (purple) and central carbon metabolites (green) on the 2 main components of PCA. PCA variables are: maximum fermentation rate (Vmax), times to achieve 30 and 80 g/L of released CO_2 and the end of fermentation (tCO_2_30, tCO_2_80 and tCO_2_End, respectively); viability, biomass and assimilated amino acids at 85 % of fermentation progress (Viability, Biomass and Assimilated_AA, respectively); acetate, glycerol, succinate at the end of fermentation. **(B)** Projection of the individuals in function of sterol dose and type tested: Ergosterol at 1.0, 2.5 and 4.0 mg/L (E1.0, E2.5 and E4.0, respectively) and phytosterols at 1.0, 2.5 and 4.0 mg/L (P1.0, P2.5 and P4.0, respectively).

Figure 2. Kinetic variables results. **(A)** Maximum fermentation rate (Vmax) and **(B)** time to release 80 g/L of CO_2 of *S. cerevisiae* strains with ergosterol (orange and red) or phytosterols (blue) at 1.0, 2.5 or 4.0 mg/L. Boxplot with means for all *10 S. cerevisiae*; Barplots with means for each strain according to sterol dose and type: Ergosterol at 1.0, 2.5 and 4.0 mg/L (E1, E2 and E4, respectively) and phytosterols at 1.0, 2.5 and 4.0 mg/L (P1, P2 and P4, respectively). Barplots with the same letters had statistically equal values for the variable tested; Barplots with different letters displayed a significant difference at a 5% level for the correspondent variable. Strains were not compared statistically.

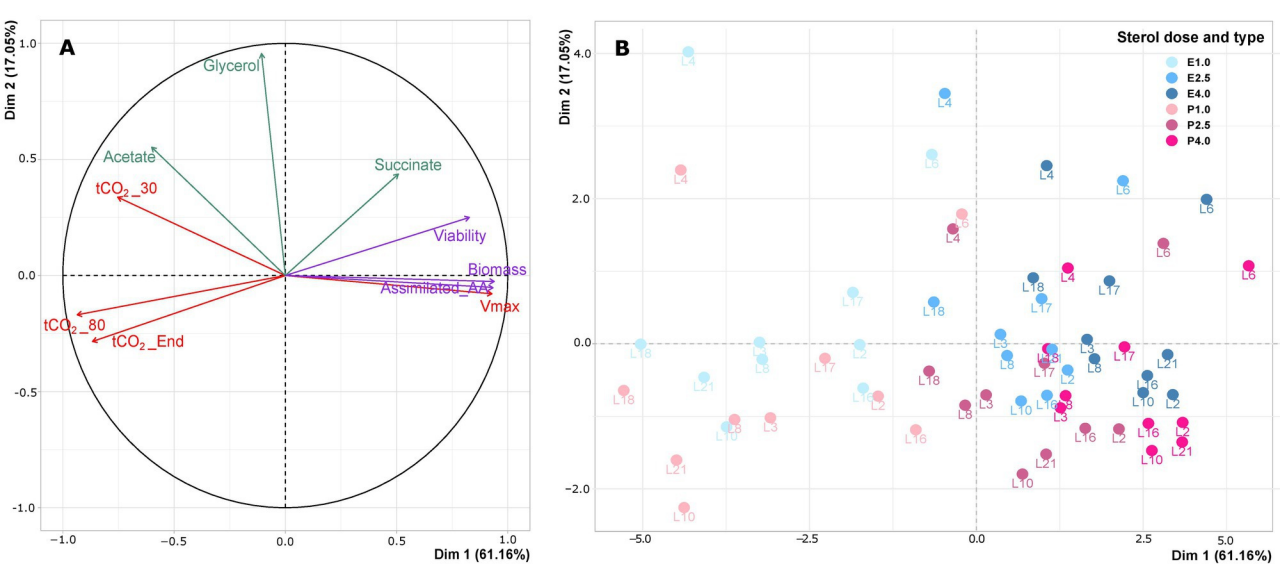
Figure 3. Biological variables results. **(A)** Assimilated amino acids, **(B)** biomass and **(C)** Viability of *S. cerevisiae* strains with ergosterol (orange and red) or phytosterols (blue) at 1.0, 2.5 or 4.0 mg/L. Boxplot with means for all *10 S. cerevisiae*; Barplots with means for each strain according to sterol dose and type: Ergosterol at 1.0, 2.5 and 4.0 mg/L (E1, E2 and E4, respectively) and phytosterols at 1.0, 2.5 and 4.0 mg/L (P1, P2 and P4, respectively). Barplots with the same letters had statistically equal values for the variable tested; Barplots with different letters displayed a significant difference at a 5% level for the correspondent variable. Strains were not compared statistically.

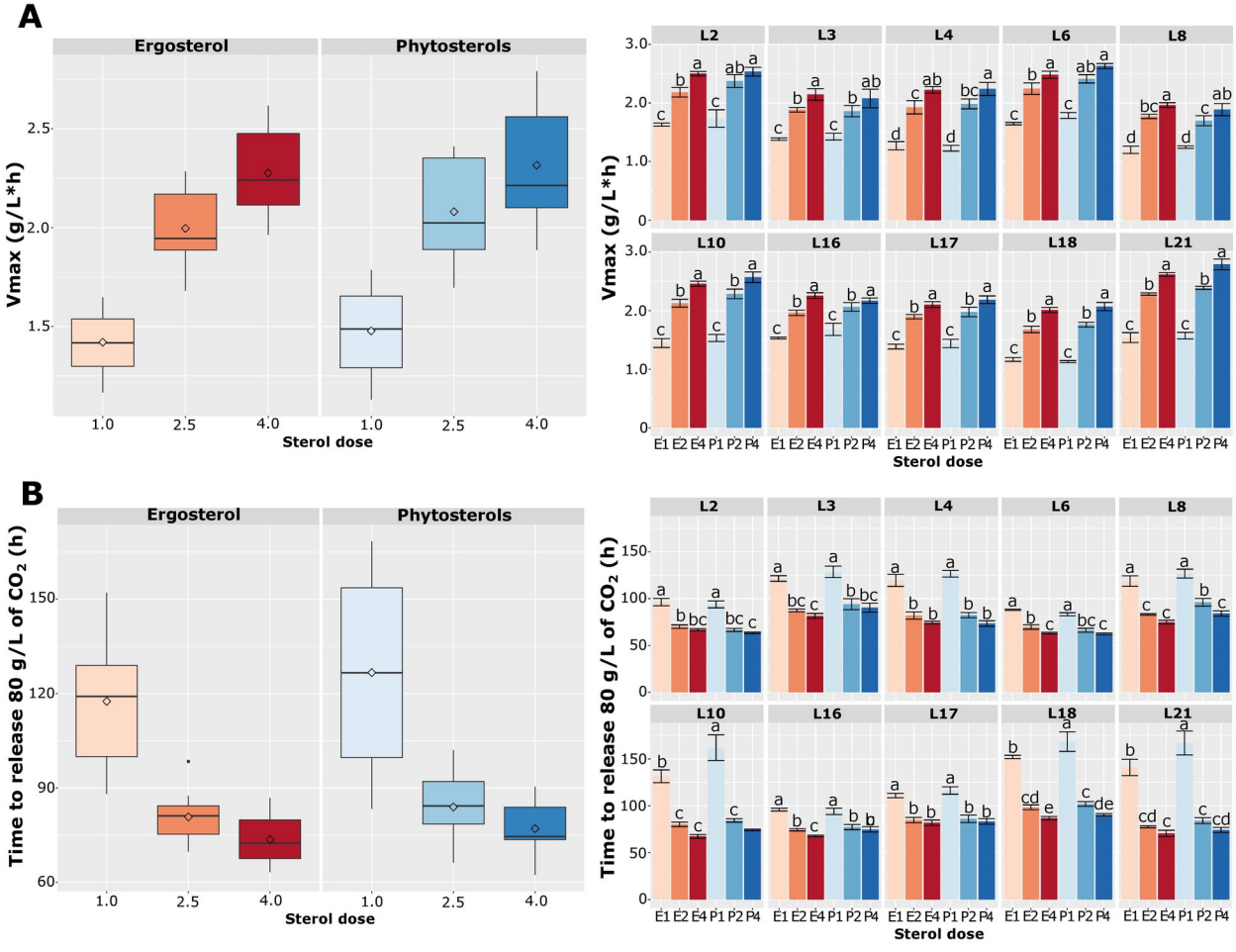
Figure 4. Barplots with the means for the impact of the timing of ergosterol addition for strains L6 and L10: control (without ergosterol addition), T0 (ergosterol addition at the start of fermentation) and T30 (ergosterol addition at 33% of fermentation progress). The variables represented are **(A)** assimilated amino acids, **(B)** biomass and **(C)** viability at 85% of fermentation progress and **(D)** acetate, **(E)** glycerol and **(F)** succinate contents at the end of fermentation. Barplots with the same letters had statistically identical values for the variable tested; Barplots with different letters had a significant difference at a 5% level for the correspondent variable. Strains L6 and L10 were not compared

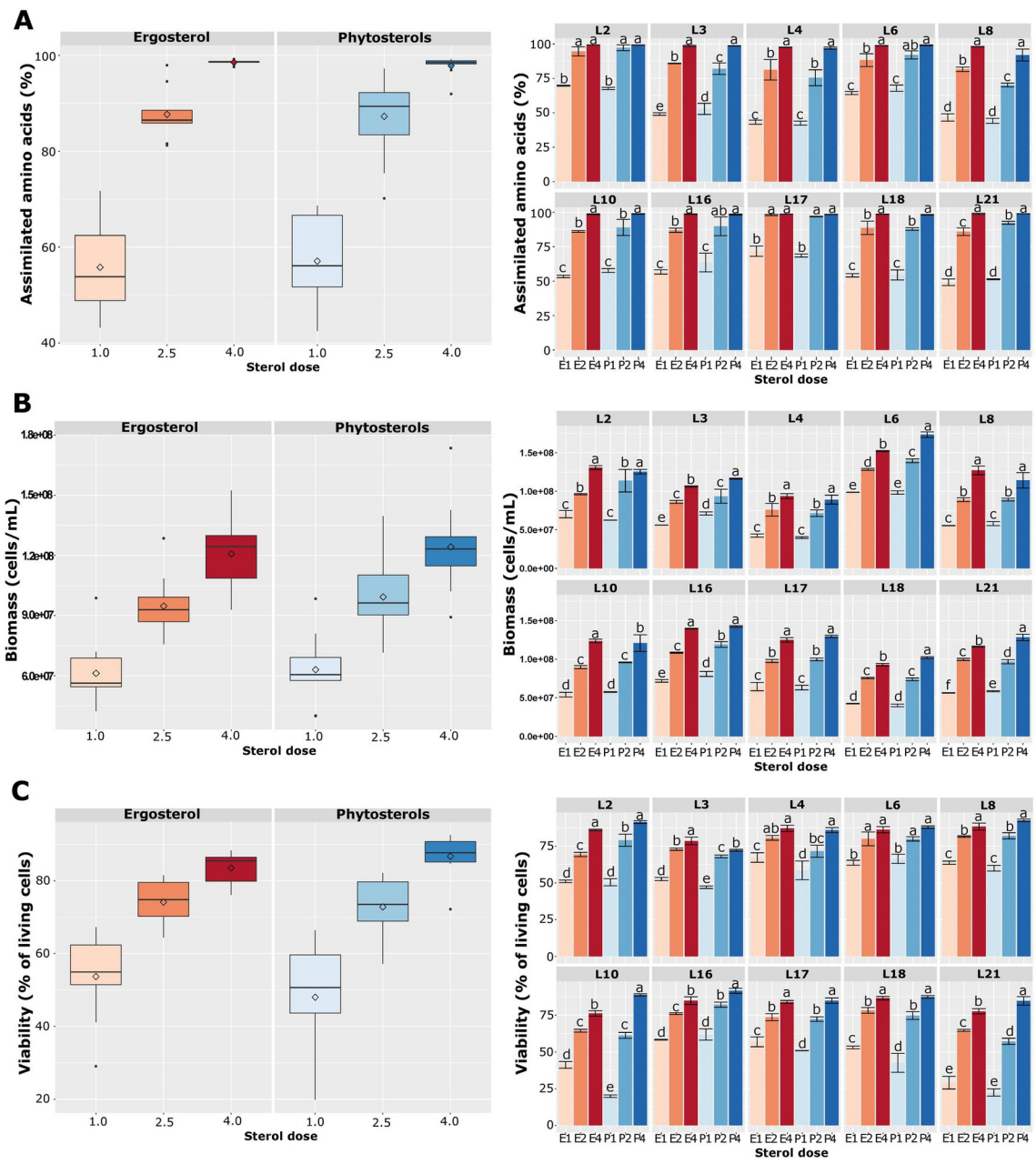
statistically. The maximum point of the bars corresponds to the mean and the vertical lines are the standard deviation for L6 and L10 strains, performed with the corresponding modality.

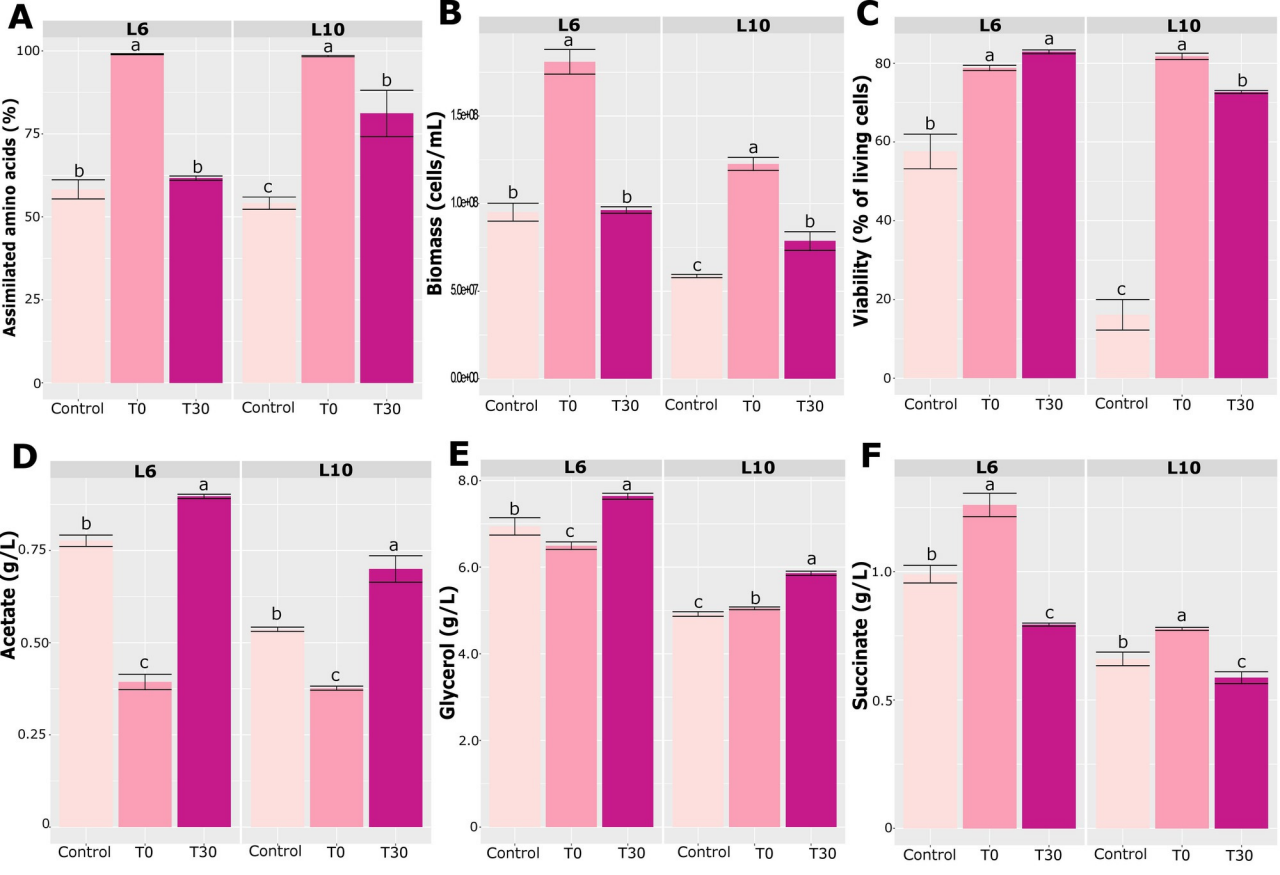
Figure 5. PCA for variables triplicate means of L6 and L10 strains for timing of ergosterol addition experiment. **(A)** Projection of the variables used to describe fermentative aromas on the 2 main components of PCA. The PCA variables are: higher alcohols, ethyl esters, acetate esters and acids. at 85 % of fermentation progress. **(B)** Projection of the individuals: control (without ergosterol addition); T0 (ergosterol addition at the start of fermentation); T30 (ergosterol addition at 33% of fermentation progress).

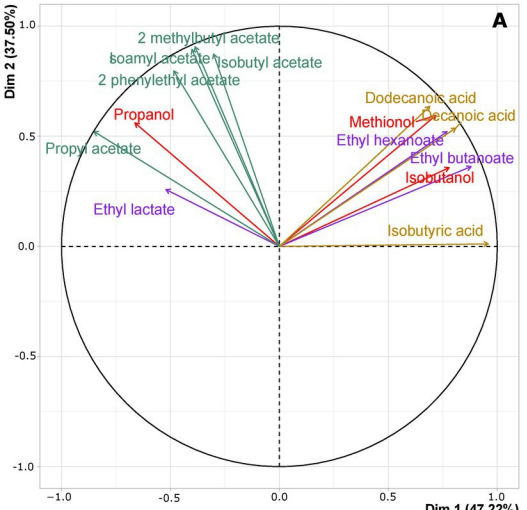
Figure 6. Biosynthesis of CCM metabolites (green), varietal aroma compounds (purple) and associated pathways (gray). Reduction reactions are in orange and pink; oxidation reactions are in blue and purple. *ALD2 to ALD6* are genes involved in acetate synthesis.

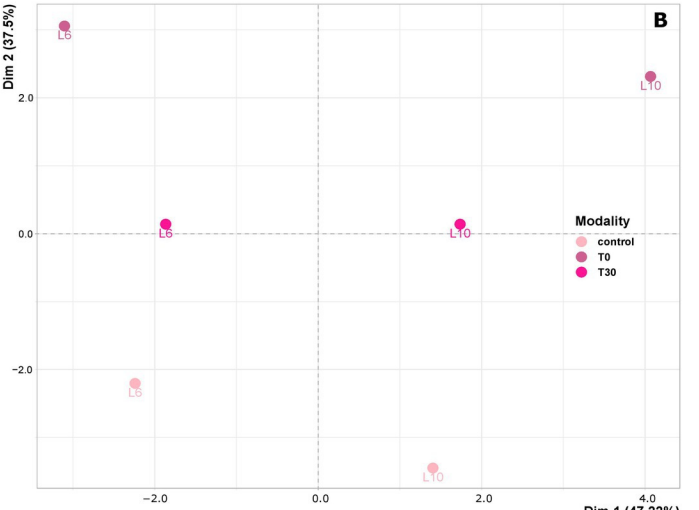

A
Glycerol
Acetate
Succinate
tCO2_30
Viability
Biomass
Assimilated_AA
Vmax
tCO2_80
tCO2_End
Dim 2 (17.05%)
Dim 1 (61.16%)
B
Sterol dose and type
E1.0
E2.5
E4.0
P1.0
P2.5
P4.0
Dim 2 (17.05%)
Dim 1 (61.16%)

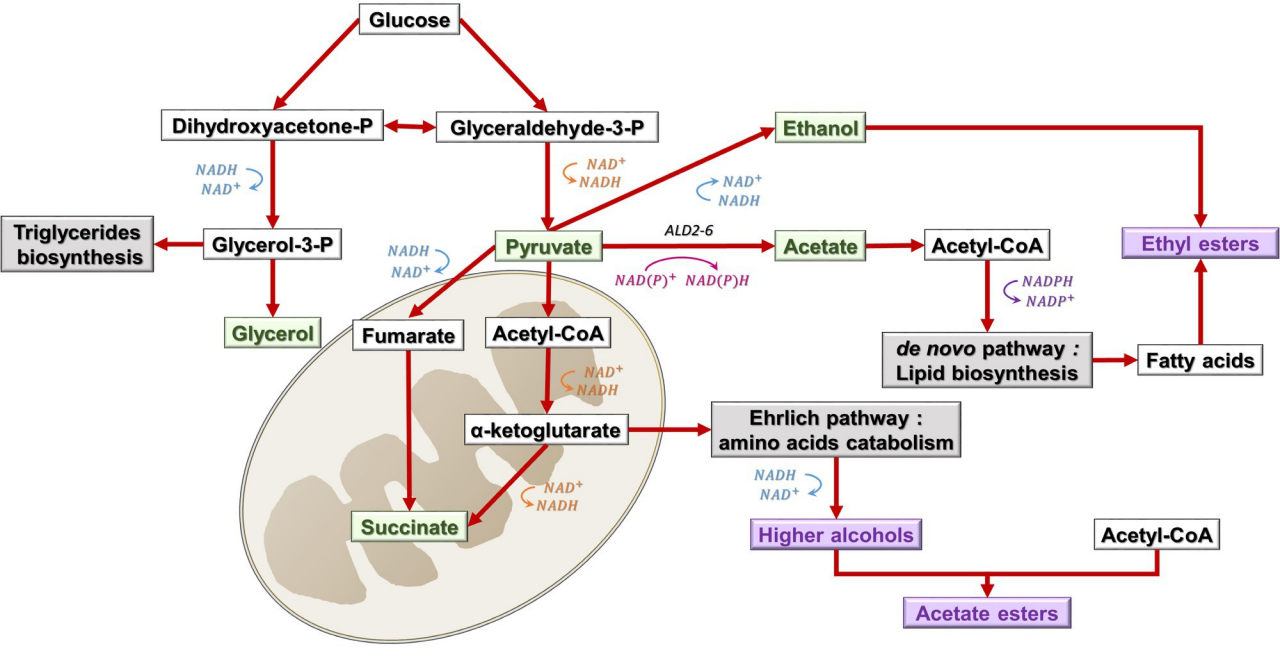

A
Ergosterol
Phytosterols
Vmax (g/L*h)
Sterol dose
L2
L3
L4
L6
L8
L10
L16
L17
L18
L21
E1 E2 E4 P1 P2 P4
B
Time to release 80 g/L of CO2 (h)


A
Ergosterol
Phytosterols
Assimilated amino acids (%)
Sterol dose
L2
L3
L4
L6
L8
L10
L16
L17
L18
L21
E1 E2 E4 P1 P2 P4
B
Biomass (cells/mL)
C
Viability (% of living cells)


A
L6
L10
Assimilated amino acids (%)
100
75
50
25
0
a
b
b
c
a
b
Control
T0
T30
Control
T0
T30
B
L6
L10
Biomass (cells/mL)
1.5e+08
1.0e+08
5.0e+07
0.0e+00
b
a
b
c
a
b
Control
T0
T30
Control
T0
T30
C
L6
L10
Viability (% of living cells)
80
60
40
20
0
b
a
a
c
a
b
Control
T0
T30
Control
T0
T30
D
L6
L10
Acetate (g/L)
0.75
0.50
0.25
0
b
c
a
b
c
a
Control
T0
T30
Control
T0
T30
E
L6
L10
Glycerol (g/L)
8.0
6.0
4.0
2.0
0
b
c
a
c
b
a
Control
T0
T30
Control
T0
T30
F
L6
L10
Succinate (g/L)
1.0
0.5
0
b
a
c
b
a
c
Control
T0
T30
Control
T0
T30


A
Dim 2 (37.50%)
Dim 1 (47.22%)
2 methylbutyl acetate
Isoamyl acetate
Isobutyl acetate
2 phenylethyl acetate
Propanol
Propyl acetate
Ethyl lactate
Dodecanoic acid
Decanoic acid
Methionol
Ethyl hexanoate
Ethyl butanoate
Isobutanol
Isobutyric acid
1.0
0.5
0.0
-0.5
-1.0
−1.0
-0.5
0.0
0.5
1.0


B
Dim 2 (37.5%)
Dim 1 (47.22%)
L6
L10
Modality
control
T0
T30
2.0
0.0
−2.0
−2.0
0.0
2.0
4.0


Glucose
Dihydroxyacetone-P
Glyceraldehyde-3-P
Ethanol
NADH
NAD+
NAD+
NADH
NAD+
NADH
Triglycerides biosynthesis
Glycerol-3-P
NADH
NAD+
Pyruvate
ALD2-6
Acetate
Acetyl-CoA
Ethyl esters
NAD(P)+
NAD(P)H
NADPH
NADP+
Glycerol
Fumarate
Acetyl-CoA
de novo pathway : Lipid biosynthesis
Fatty acids
NAD+
NADH
α-ketoglutarate
Ehrlich pathway : amino acids catabolism
NAD+
NADH
NADH
NAD+
Succinate
Higher alcohols
Acetyl-CoA
Acetate esters