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ORIGINAL RESEARCH ARTICLE

Impact of phytosterol addition on fermentation progress and volatile compounds synthesis during alcoholic fermentation in synthetic and natural grape musts

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ABSTRACT

Lipid nutrition is an important factor for yeast during alcoholic fermentation. Although recent research reports have revisited the role of sterols during alcoholic fermentation, our knowledge of lipids assimilation and volatile compound biogenesis remains partial. This study aimed to find out more about the impact of grape must phytosterol content on fermentative kinetics, nitrogen assimilation by yeast and fermentative aroma synthesis. To that end, experimental fermentations were performed in synthetic and Chardonnay musts supplemented with different phytosterol concentrations (0, 1, 3 and 5 mg/L). Sterols addition significantly increased the maximum CO_2 production rate while reducing fermentation duration. This can be explained by higher nitrogen assimilation by yeast due to sterols, which leads to higher yeast growth and better viability at the end of the fermentation process. Regarding the aromatic profile, sterol addition also significantly increased acetate esters, ethyl esters, fusel alcohols and medium-chain fatty acids production. These new advances highlight the major role of phytosterols in fermentation control and wine aroma profile.

KEYWORDS: sterol, nitrogen, *Saccharomyces cerevisiae*, fermentation, esters, wine

INTRODUCTION

In white winemaking, lipids can be the limiting nutrient for yeast growth during alcoholic fermentation. This situation occurs in case of a very high concentration of nitrogen combined with a low concentration of lipids (as in highly clarified musts) (Casalta *et al.,* 2021) and leads to sluggish and sometimes stuck fermentations.

In grape must, the main source of lipids are solid particles. Lipids are a major source of nutrients for fermenting yeasts, especially in the form of phytosterols and fatty acids (Ribéreau-Gayon *et al.,* 1975). A white grape must contains, on average, 8 % of lipids (as a percentage of dry weight) (Alexandre *et al.,* 1994). The total fatty acid mean value in different grape solids from white musts (Chardonnay, Maccabeu and Viognier) is 34 mg/g dry weight, while total phytosterols content is 8 mg/g dry weight (Casalta *et al.,* 2019). The main phytosterol in grape must is β-sitosterol (75 %), while campesterol and stigmasterol represent about 5 % of total phytosterols (Casalta *et al.,* 2019). Lipids tend to accumulate in berries as ripening progresses, indicating a link between lipids and grape maturity (Sherman *et al.*, 2023). Indeed, in this work, it is observed a strong correlation between lipids/major fatty acids and other commonly used ripeness parameters such as total soluble solids, sugars, titratable acidity and yeast available nitrogen.

Sterols are part of the eukaryotic lipidome diversity and are essential for maintaining yeast cell membrane integrity and optimal functionality. They are mainly responsible for regulating the fluidity, rigidity and permeability of cell membranes; they are surrounded by proteins and phospholipids and protected by a sphingolipids head (Henneberry and Sturley, 2005; Rosenfeld *et al.,* 2003). Under anaerobiosis, *S. cerevisiae* cells are unable to synthesise their own sterols (Van Der Rest *et al.,* 1995), although they can assimilate phytosterols from grape must. Depending on the presence or absence of oxygen, yeasts can synthesise (or assimilate) and accumulate significant amounts of sterols (reviewed in Girardi-Piva *et al.*, 2022a). These compounds are associated with yeast growth, metabolism and viability during alcoholic fermentation (Daum *et al.,* 1998; Ribéreau-Gayon *et al.,* 1975). Sterol content directly affects the maximum fermentation rate and duration when it is the limiting nutritional factor (Casalta *et al.,* 2019). Yeast sterol requirement is evaluated to be about 5 mg /L when assimilable nitrogen (N) must content is 300 mg N/L (Deytieux *et al.,* 2005). However, Ochando *et al.* (2017) reported a complete nitrogen consumption (360 mg N/L) with an initial grape must phytosterol concentration of 8 mg/L. Indeed, this requirement threshold value depends on the strain and the initial nitrogen content (Rollero *et al.,* 2015).

These recent results have re-evaluated the role of phytosterols on fermentation kinetics during white wine winemaking. They pointed out the importance of taking into consideration phytosterols in fermentation management.

Thus, the purpose of this study was to better understand the effect of must phytosterols addition on alcoholic fermentation progress and volatile compounds synthesis with different strains.

MATERIALS AND METHODS

1. Experimental design

A first set of fermentations was carried out in a synthetic medium (SM) that mimics a standard grape juice must (Bely *et al.,* 1990). Its composition has been described by Rollero *et al.* (2015). Briefly, SM contained 200 g/L of sugars (100 g of glucose and 100 g of fructose) and 400 mg N/L of assimilable nitrogen with a ratio (w/w) of 72 % from assimilable amino acids and 28 % from ammoniacal nitrogen (NH_4Cl).

A second set of fermentations was performed in a natural must (NM) of Chardonnay harvested from our experimental vineyard (Gruissan, Languedoc, France). After cold settling (2 °C during 12 h with a clarification enzyme (Rapidase Clear, DSM, Oenobrands, Montpellier, France) at 2 g/hL), grape juice was flash-pasteurised (82 °C, 30 s) and stored at 4 °C until fermentation. Before fermentation, bentonite (Electra, Martin Vialatte, Magenta, France) was added (0.6 g/L), and a second cold settling (12 h at 4 °C) was applied to obtain a low concentration of natural phytosterols (provided by solid particles). The final must turbidity was 35 NTU. The must contained 225 g/L of sugars and 452 mg /L of assimilable N (ratio of 70 % N from assimilable amino acids and 30 % of N from ammoniacal nitrogen). This condition was chosen to enhance the effect of added sterols.

Both synthetic and natural musts were used without phytosterol supplementation (negative control) and with 1, 3 and 5 mg/L of β-sitosterol (85451, Sigma Aldrich). The solution used was composed of 1 g/L of β-sitosterol in Tween 80 (P1754, Sigma Aldrich) and ethanol (0.05:0.95 v/v). Tween 80 contains oleic acid that may impact yeast metabolism. To avoid this bias, a solution of Tween 80 (5 % v/v) in ethanol was added to obtain the same concentration of Tween 80 in each modality, i.e., 0.025 % (v/v).

Fermentations were performed in triplicate in 300 mL cylindrical glass bottles fitted with fermentation locks (CO_2 bubbling outlets filled with water). Each fermenter was filled with 250 mL of must. Sampling was made by introducing a needle connected to a syringe through a sealed septum preventing oxygen ingress. Then, prior to inoculation, the medium was de-aerated by bubbling pure argon for 30 min to reach an O_2 concentration below 1 % saturation. Then, β-sitosterol and Tween 80 solutions were added as described above. Four commercial *Saccharomyces cerevisiae* yeast strains (available upon request) labelled A, B, C and D from Lallemand Œnologie (Blagnac, France) were used. Fermentation flasks were inoculated with 10 g/hL active dry yeast previously rehydrated for 30 min at 37 °C in a 50 g/L glucose solution (1 g of dry yeast diluted in 10 mL of this solution). Fermentations were carried out under isothermal conditions at 24 °C, with permanent stirring (300 rpm).

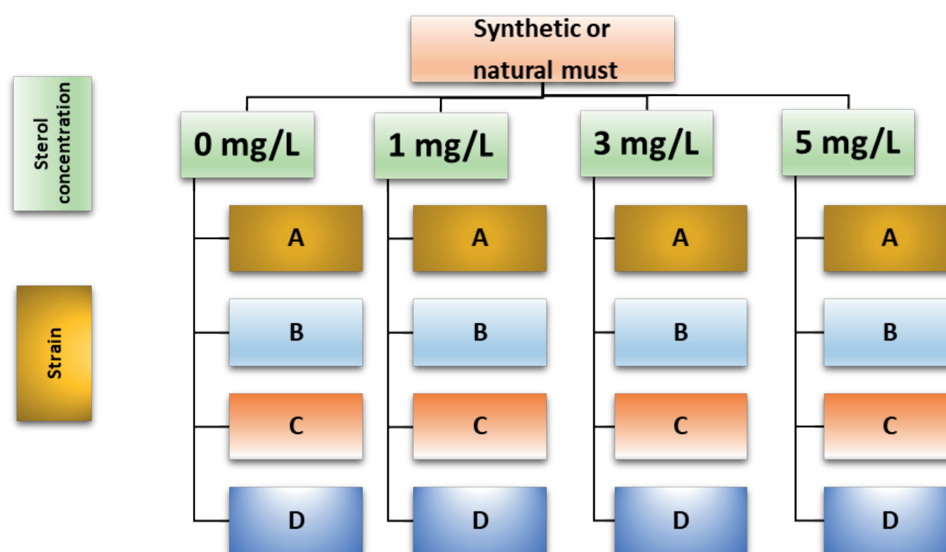

FIGURE 1. Experimental design.

The experimental design shown in Figure 1 was implemented for synthetic and natural musts.

2. Monitoring fermentation kinetics

Each fermenter was weighed every 40 min with a robotic arm (LabServices, Breda, Netherlands), capable of moving the fermenter from its location on the stirring plate to a precision balance. A custom-developed Labview application (National Instruments, Austin, Texas, USA) automatically calculated the amount of CO_2 (g/L h) released from the weight loss that is proportional to the amount of sugars consumed. The CO_2 production rate (g/L.h) was calculated by polynomial smoothing (Sablayrolles *et al.,* 1987). Data were stored on the Alfis database (Alcoholic Fermentation Information System, INRAE, UMR Mistea, UMR Sciences pour l'Œnologie, Montpellier, France).

3. Analytical methods

3.1. Metabolite concentrations

Reducing sugar, ethanol, glycerol and acetate concentrations were measured in media at the end of fermentation by HPLC (HPLC 1290 Infinity, Agilent Technologies™, Santa Clara, California, USA) using a Phenomenex Rezex ROA column (Agilent Technologies, Santa Clara, California, USA) at 60 °C. Elution was done with 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min. Analysis was carried out using the Agilent EZChrom software package. Fermentations were considered to be complete when the residual sugar concentration was below 2 g/L.

3.2. Assimilated nitrogen

Ammonium concentration (NH_4) was determined enzymatically (R-Biopharm AG™, Darmstadt, Germany). The free amino acid content of the must (Naa) was determined by cation exchange chromatography, with post-column ninhydrin derivatisation (Biochrom 30, Biochrom, Cambridge, UK), as described by Crépin *et al.* (2012). These two determinations were carried out when 80 % of total CO_2 was produced. Assimilated nitrogen (Assimilated $N_{80\%}$) was calculated as follows:

Assimilated $N_{80\%}$ = (Naa in must + NH_4 in must) – (Naa $_{80\%}$ + $NH_{4\ 80\%}$).

The percentage of assimilated nitrogen was determined as follows:

(Assimilated $N_{80\%}$x100) / (Naa in must + NH_4 in must)

3.3. Volatile compounds

The concentrations of volatile compounds were measured by GC-MS. Sampling (5 mL) was done at the end of AF and stored at – 20°C until analysis. After thawing, volatile compounds were extracted twice by liquid–liquid extraction with 1 mL of dichloromethane in the presence of deuterated standards (Rollero *et al.,* 2015). Then, the organic phase was concentrated under nitrogen flow to a final volume of 0.5 mL. Samples (final volume of 0.5 mL) were analysed with a Hewlett Packard (Agilent™ Technologies, Santa Clara, CA, USA) 6890 gas chromatograph equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu™, Columbia, USA) and coupled to a Hewlett Packard 5973 Mass Spectrometry detector (Agilent™ Technologies, Santa Clara, CA, USA) (Rollero *et al.,* 2015).

3.4. Cell counting

Must samples collected at 80 % of fermentation progress were first diluted (2500-fold) with Isoton II® (Beckman-Coulter, Margency, France). After sonication (35 s, 10W), cells were counted with a Coulter Z2 electronic counter (Coulter Multisizer3, Beckman Coulter™, Fullerton, California, USA) fitted with a 100 µm aperture probe. All measurements and analyses were carried out in duplicate.

3.5. Cell viability

Cell viability was determined by flow cytometry using a C6 Flow cytometer (Accuri Cytometers, Inc, BD Biosciences, Ann Arbor, MI USA): 5 µL of aqueous propidium iodide (PI)

solution (0.1 mg of PI/mL) (Calbiochem, San Diego, CA, USA) was added to 500 µL of the cell suspension and the samples mixed by gentle shaking. PI flow cytometry analysis was performed 15 min after staining. Fluorescence data for cells stained by PI were collected in channel FL3. Viability was determined at 80 % of fermentation progress as the percentage of intact and fragile cells among all cells (Delobel *et al.,* 2012).

3.6. Statistical analysis

A first two-way ANOVA (analysis of variance) with two factors (phytosterols addition and strain used) and their interaction was performed on data provided through the SM experiment and a second one on data obtained through the NM experiment. The aim was to know whether each factor and their interaction significantly affect the kinetics and microbiological parameters, assimilated nitrogen, glycerol, acetate and volatile compounds.

The packages tidyverse v1.3-0 (Wickham *et al.*, 2019), agricolae v1.3-3 (De Mendiburu, 2020), broom and the functions aov and lm were used.

ANOVA was evaluated using the aov function with a statistical significance level of 0.5 % after Bonferroni

TABLE 1. Two-way ANOVA of kinetics and microbiological parameters, assimilated nitrogen, glycerol, acetate and volatile compounds according to phytosterol, strain and their interaction on synthetic (SM) and natural must (NM). Effects of the factors and the interaction between factors: NS: not significant; *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

	SM			NM		
	Phytosterol	Strain	Interaction Phytosterol x strain	Phytosterol	Strain	Interaction Phytosterol x strain
Maximum CO_2 production rate	***	*	*	***	***	***
Total Fermentation duration	***	***	***	***	***	***
Yeast population at 80 % of fermentation progress	***	***	***	***	***	***
Viability at 80 % of fermentation progress	***	***	***	***	***	***
Assimilated nitrogen at 80 % of fermentation progress	***	***	NS	***	**	NS
Glycerol	***	***	NS	***	***	***
Acetate	***	***	NS	***	***	***
Isobutyl acetate	NS	*	NS	***	***	***
Isoamyl acetate	***	***	NS	***	***	***
2-Phenylethyl acetate	***	***	NS	***	***	***
Ethyl butanoate	***	**	**	***	***	***
Ethyl hexanoate	***	**	NS	***	***	**
Ethyl octanoate	***	*	NS	***	***	*
Ethyl decanoate	**	NS	NS	***	*	NS
Isobutanol	***	*	NS	***	***	***
Isoamyl alcohol	NS	*	NS	***	***	NS
2 Phenylethanol	***	***	***	***	***	**
1-Propanol	***	***	*	***	***	***
Butanoic acid	NS	*	NS	***	***	**
Hexanoic acid	NS	NS	NS	***	***	***
Octanoic acid	***	***	NS	***	***	**
Decanoic acid	***	***	NS	***	*	NS

Yeast population at 80 % of fermentation progress, viability at 80 % of fermentation progress and assimilated nitrogen at 80 % of fermentation progress were determined when 80 % of total CO_2 was produced. Assimilated nitrogen is the nitrogen consumed by yeast.

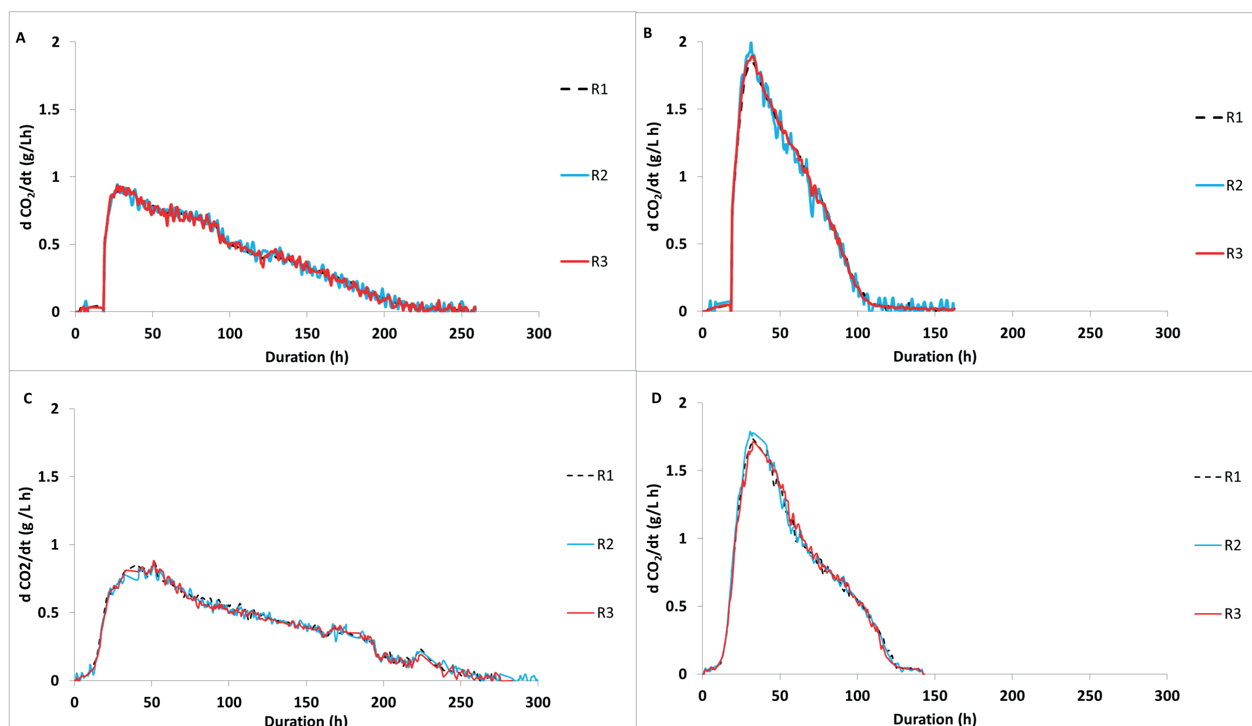

FIGURE 2. Kinetics obtained with strain A with the triplicates (R1, R2 and R3) in the synthetic must (SM) without phytosterol (A) and with 5 mg/L (B) phytosterol; in the natural must (NM) without phytosterol (C) and with 5 mg/L (D) phytosterol.

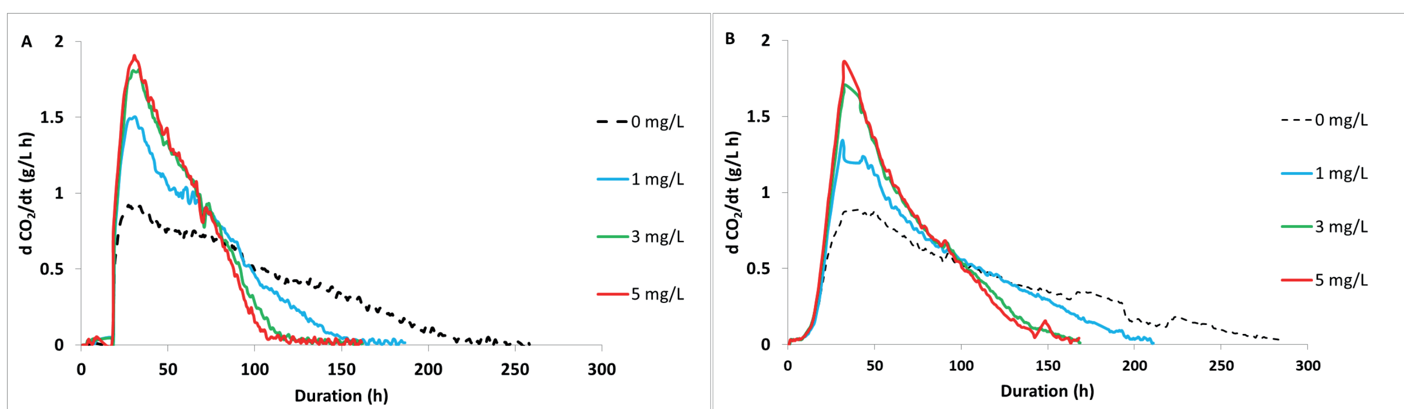

FIGURE 3. Kinetics according to sterol addition in synthetic SM (A) and natural must NM (B). Mean of the 4 strains (n = 3 triplicates × 4 strains = 12).

adjustment, following the model below (Eq. 1), where β_1 Strain is the effect of the strain, β_2 Sterol, the sterol effect, β_{12} Strain*Sterol, the interaction effect and ϵ are independent $N(0, \sigma 2)$ error terms:

$$Y = \beta_0 + \beta_1 \text{ Strain} + \beta_2 \text{ Sterol} + \beta_{12} \text{ Strain*Sterol} + \epsilon \quad \text{(Eq. 1)}$$

When the effect of the factor was significant, a Tukey test was performed with packages tidyverse v1.3-0 (Wickham *et al.*, 2019), .agricolae v1.3-3 (De Mendiburu, 2020) and Hmisc v4.5-0 (Harrell and Dupont, 2021) and the function HSD to determine the significant differences between means.

Then, a Principal Component Analysis (PCA) was carried out on volatile compounds (esters, fusel alcohols and medium-chain fatty acids).

Statistical analyses were performed with R software version 3.6.2 (R Development Core Team, 2019).

RESULTS

The study aimed to evaluate the impact of sterol content on fermentation progress and volatile compound production using different strains of *Saccharomyces cerevisiae*.

1. Sterol impact on wine fermentation

In SM, the effect of sterol addition was significant ($p < 0.001$) for all studied parameters shown in Table 1, except isobutyl acetate, isoamyl alcohol, butanoic and hexanoic acids. In NM, the effect was significant ($p < 0.001$) for all parameters.

TABLE 2. Kinetics and microbiological parameters, assimilated nitrogen, glycerol, acetate and volatile compounds obtained through the experimental design on synthetic (SM) and natural (NM) musts. Mean and standard deviation on 4 strains (n = 3 replicates × 4 strains = 12). Means significantly different ($p < 0.05$) according to sterol addition when denoted by different letters for each medium.

Must	SM				NM			
Sterol addition (mg/L)	0	1	3	5	0	1	3	5
Maximum CO_2 production rate (g/ L h)	0.98 ± 0.05d	1.56 ± 0.09 c	1.73 ± 0.07 b	1.84 ± 0.08 a	0.98 ± 0.10 d	1.55 ± 0.26 c	1.79 ± 0.25 b	1.94 ± 0.38 a
Total fermentation duration (h)	239 ± 23 a	153± 23 b	131 ± 18 c	125 ± 17 c	248 ± 32 a	173 ± 16 b	140 ± 15 c	132 ± 11 d
Yeast population at 80 % of fermentation progress (10^6 cells/mL)	42.8 ± 8.7 d	98.1 ± 18.7 c	139.3 ± 44.6 b	171.8 ± 63.7 a	42.5 ± 8.6 d	84.3 ± 5.0 c	105.6 ± 21.0 b	121.2 ± 26.5 a
Viability at 80 % of fermentation progress (%)	63.9 ± 8.6 c	81.6 ± 3.6 c	90.0 ± 4.5 a	91.7 ± 4.7 a	72.6 ± 5.6 c	85.7 ± 5.2 b	87.0 ± 6.1 ab	87.3 ± 7.8 a
Assimilated nitrogen at 80 % of fermentation progress (mg/L)	201 ± 17 d	296 ± 16 c	309 ± 17 b	329 ± 22 a	250 ± 12 c	357 ± 11 b	419 ± 11 a	427 ± 13 a
Glycerol (g/L)	6.46 ± 0.21 a	5.55 ± 0.26 b	5.55 ± 0.19 b	5.56 ± 0.14 b	7.23 ± 0.53 a	6.72 ± 0.65 c	6.72 ± 0.47 b	6.72 ±0.32 bc
Acetate (g/L)	0.80 ± 0.05 a	0.62 ± 0.11 b	0.55 ± 0.07 c	0.55 ± 0.09 c	1.11 ± 0.02 a	0.84 ± 0.06 b	0.67 ± 0.09 c	0.62 ± 0.04 d
Isobutyl acetate (mg/L)	0.10 ± 0.00	0.15 ± 0.04	0.13 ± 0.00	0.14 ± 0.01	0.21 ± 0.01 d	0.32 ± 0.03 c	0.51 ± 0.02 b	0.55 ± 0.02 a
Isoamyl acetate (mg/L)	0.68 ± 0.05 c	1.67 ± 0.25 b	2.00 ± 0.15 a	1.97 ± 0.15 a	2.65 ± 0.14 d	4.31 ± 0.40 c	6.18 ± 0.15 b	6.87 ± 0.22 a
2-Phenylethyl acetate (mg/L)	0.16 ± 0.03 b	0.47 ± 0.11 a	0.60 ± 0.05 A	0.50 ± 0.08 a	0.50 ± 0.05 d	1.16 ± 0.25 c	2.40 ± 0.20 b	3.14 ± 0.48 a
Ethyl butanoate (mg/L)	0.18 ± 0.01 b	0.26 ± 0.02 a	0.27 ± 0.01 a	0.27 ± 0.02 a	0.31 ± 0.01 c	0.42 ± 0.03 b	0.46 ± 0.01 a	0.46 ± 0.02 a
Ethyl hexanoate (mg/L)	0.29 ± 0.03 c	0.38 ± 0.03 a	0.32 ± 0.05 bc	0.35 ± 0.04 ab	0.51 ± 0.04 c	0.72 ± 0.06 b	0.78 ± 0.02 a	0.77 ± 0.05 ab
Ethyl octanoate (mg/L)	0.44 ± 0.04 b	0.55 ± 0.14 a	0.50 ± 0.04 ab	0.46 ± 0.03 ab	0.73 ± 0.06 b	1.03 ± 0.12 a	0.97 ± 0.07 a	0.99 ± 0.13 a
Ethyl decanoate (mg/L)	0.30 ± 0.04 b	0.36 ± 0.08 ab	0.40 ± 0.04 ab	0.42 ± 0.10 a	0.23 ± 0.02 b	0.29 ± 0.05 a	0.28 ± 0.03 ab	0.32 ± 0.06 a
Isobutanol (mg/L)	24.30 ± 2.57 b	24.48 ± 2.08 ab	27.32 ± 1.57 a	26.30 ± 1.20 ab	29.05 ± 1.29 d	31.84 ± 2.56 c	46.36 ± 1.65 b	47.27 ± 1.78 a
Isoamyl alcohol	76.09 ± 4.62	88.70 ± 8.01	85.80 ± 10.31	92.36 ± 14.18	100.73 ± 7.37 b	97.85 ± 8.94 b	119.74 ± 5.65 a	124.88 ± 9.70 a
2-Phenylethanol (mg/L)	11.40 ± 0.87 c	15.42 ± 0.86 b	17.34 ± 0.82 a	15.51 ± 0.72 b	13.71 ± 0.55 d	17.33 ± 2.08 c	24.14 ± 1.50 b	28.27 ± 1.80 a
1-Propanol (mg/L)	28.37 ± 4.85 b	27.76 ± 2.78 b	33.54 ± 1.95 a	33.48 ± 2.44 a	37.22 ± 1.98 c	47.48 ± 3.00 b	52.47 ± 1.11 b	62.03 ± 3.60 a
Butanoic acid (mg/L)	0.91 ± 0.11	1.47 ± 0.30	1.54 ± 0.11	1.43 ± 0.46	1.19 ± 0.32 c	1.78 ± 0.30 b	2.47 ± 0.18 a	2.24 ± 0.25 a
Hexanoic acid (mg/L)	0.78 ± 0.23	1.08 ± 0.25	0.87 ± 0.02	1.09 ± 0.13	1.40 ± 0.18 d	2.36 ± 0.12 c	2.58 ± 0.06 a	2.42 ± 0.12 b
Octanoic acid (mg/L)	1.46 ± 0.20 b	2.81 ± 0.54 a	2.59 ± 0.12 a	3.03 ± 0.23 a	2.63 ± 0.32 c	5.08 ± 0.20 b	5.67 ± 0.12 a	5.58 ± 0.56 b
Decanoic acid (mg/L)	1.83 ± 0.17 b	1.86 ± 0.36 b	2.16 ± 0.26 b	2.60 ± 0.26 a	1.49 ± 0.18 c	1.83 ± 0.13 b	1.85 ± 0.07 a	1.82 ± 0.10 ab

Yeast population at 80 % of fermentation progress, viability at 80 % of fermentation progress and assimilated nitrogen at 80 % of fermentation progress were determined when 80 % of total CO2 was produced. Assimilated nitrogen is the nitrogen consumed by yeast.

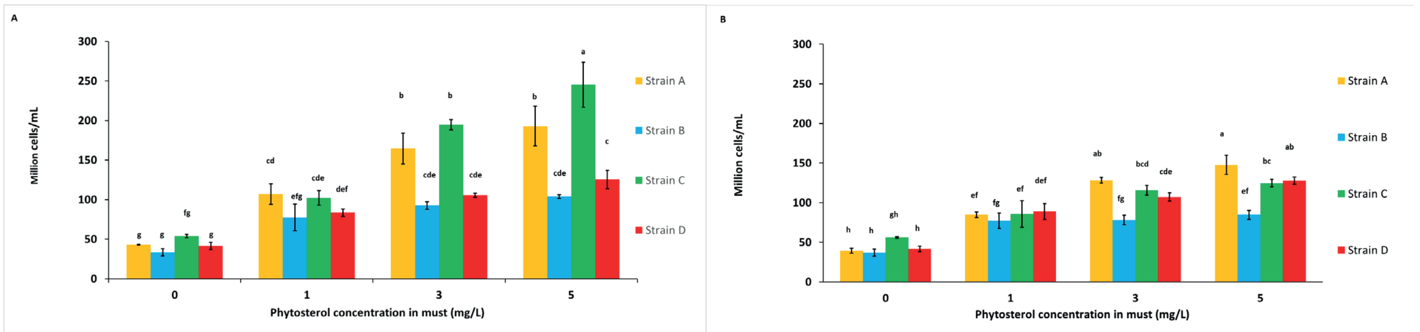

FIGURE 4. Yeast population at 80 % of fermentation progress according to sterol addition with the different strains in synthetic SM (A) and natural must NM (B). Means (n = 3) are significantly different ($p < 0.05$) when denoted by different letters.

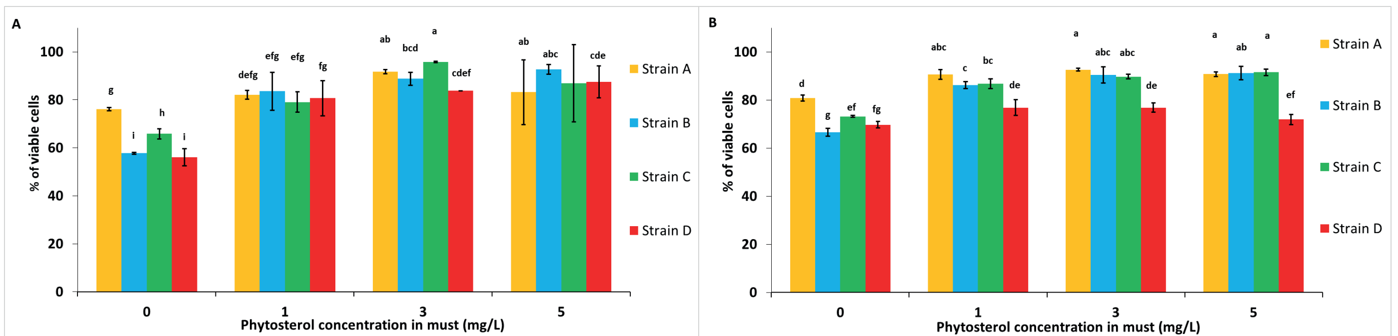

FIGURE 5. Viability at 80 % of fermentation progress according to sterol addition with the different strains in synthetic SM (A) and natural must NM (B). Means (n = 3) are significantly different ($p < 0.05$) when denoted by different letters.

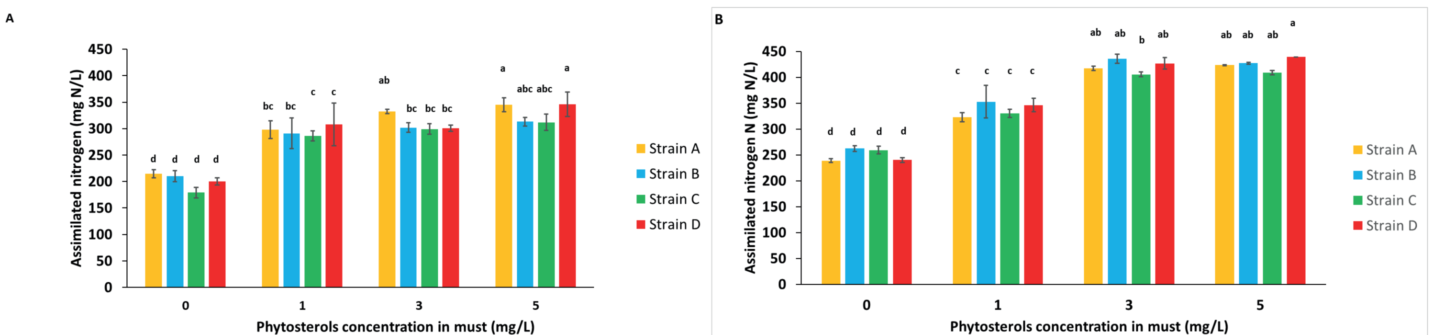

FIGURE 6. Assimilated nitrogen by yeast at 80 % of fermentation progress according to sterol addition with the different strains in synthetic SM (A) and natural must NM (B). Means (n = 3) are significantly different ($p < 0.05$) when denoted by different letters.

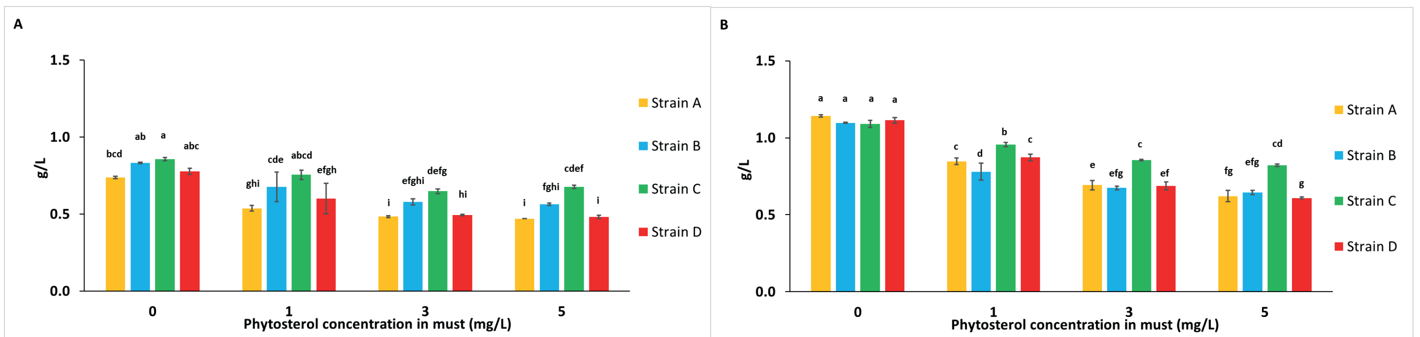

FIGURE 7. Acetate concentration at the end of the fermentation according to sterol addition with the different strains in synthetic SM (A) and natural must NM (B). Means (n = 3) are significantly different ($p < 0.05$) when denoted by different letters.

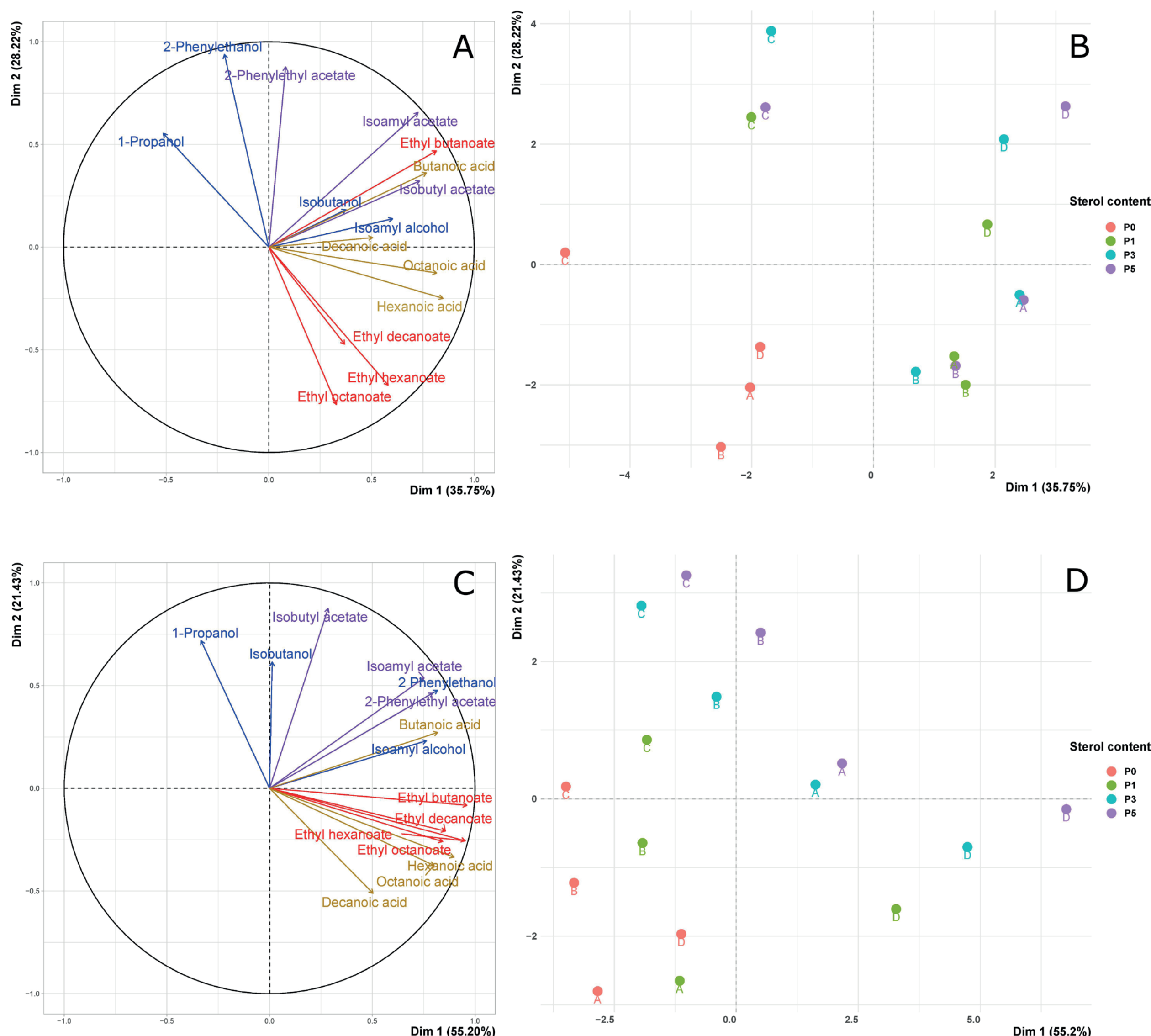

FIGURE 8. Principal Component Analysis on volatile compounds. Projection on the two main components PC1 and PC2 in synthetic (SM), variables (A) and individuals (B); in natural must (NM) variables (C) and individuals (D).

The kinetics of the three triplicates performed with strain A are shown in Figure 2. Kinetics were similar, displaying very good repeatability, as in the other strains (results not shown).

Table 2 shows the means obtained, according to the sterol content (n = 3 replicates × 4 strains = 12) and Tukey test results. There were significant differences ($p < 0.05$) between means of maximum CO_2 production rate, according to sterol addition on both media. This rate increased with sterol concentration from 0.98 (without sterol) to 1.84 g/L h (with 5 mg /L of sterol) in SM and was doubled in NM, rising from 0.98 to 1.94 g/L (n = 12) (Table 2 and Figure 3).

On average, the population at 80 % significantly increased from 43 million (without sterol) to 171 million cells/mL (with 5 mg /L of sterol) in SM and from 42 million to 121 million cells/mL in NM (Table 2 and Figure 4 which shows the individual evolution according to strain).

On average, viability also significantly increased with sterol addition from 64 % (without sterol) up to 92 % (with 5 mg /L of sterol) in SM and from 72 % to 87 % in NM (Table 2 and Figure 5 shows the individual evolution according to strain).

In addition, assimilated nitrogen means significantly increased from 200 (without phytosterol) to 328 mg N/L (with 5 mg/ L of sterol) in SM and from 250 to 427 mg N/L in NM (Table 2 and Figure 6 shows the individual evolution according to strain). In all modalities, there remained some assimilable nitrogen at the end of the fermentation. In all cases, fermentations were complete (residual sugar content under 2 g/L), except in SM without sterol addition. Moreover, in all conditions, ethanol content was not significantly impacted by sterol addition and strain (data not shown).

Glycerol concentration was significantly higher without phytosterol in both media (Table 2).

Acetate concentration, on average, decreased from 0.80 to 0.55 g/ L in SM and was divided by 2 (1.11 to 0.62 g/L) in NM when sterol content increased from 0 to 5 mg/L (Table 2

and Figure 7 that shows the individual evolution according to strain).

Isoamyl acetate and phenylethyl acetate concentration strongly increased (3-fold in SM for the 2 compounds, 2.6-fold for isoamyl acetate and 6.3-fold for the phenylethyl acetate in NM) when the sterol content varied in the same range. In parallel, the corresponding fusel alcohols, i.e. isoamyl alcohol and 2-phenylethanol, increased to a smaller extent (1.21 and 2.06-fold in SM, 1.36 and 2.06-fold, respectively). The highest increases were observed on 2-phenyethanol and propanol in NM (about 2-fold for the 2 molecules). Overall, ethyl esters and corresponding fatty acids tended to increase with sterol content but to a lesser extent than acetate esters.

Generally, the volatile compounds increase was higher in NM than in SM.

2. Strain impact

In SM, the strain effect on fermentation parameters, assimilated nitrogen, glycerol, acetate and volatile compounds were significant ($p < 0.05$), except for ethyl decanoate and hexanoic acid (Table 1). In MN, the strain effect was significant ($p < 0.05$) for all the parameters.

PCA on volatile compounds is shown in Figure 8. PC1 and PC2 explained 64 % of the variance in SM (Figure 8A) and 77 % in NM (Figure 8C). In SM, ethyl butanoate was positively correlated with butanoic acid. The other ethyl esters were also linked with their corresponding fatty acids but to a lesser extent. Generally, acetate esters were positively linked with their corresponding alcohols. In NM, ethyl esters were, in general, positively correlated with their corresponding fatty acids and acetate esters with their corresponding alcohols. On the 2 PCA, most of the variables are located on the right half of the correlation circle.

We can observe on the two media that when sterol content increases, the four strain positions shift in the same direction, i.e., upside (positive on PC2) and right side (positive on PC1) (Figures 8B and 8D). PCA allows differentiating the strains according to the volatile compounds profile. At high sterol concentrations, strain C is characterised by a high production of propanol, phenylethanol and phenylethyl acetate. By contrast, strains B and A showed an important synthesis of ethyl octanoate, ethyl hexanoate and ethyl decanoate. Strain D is characterised by a high concentration of isoamyl acetate, ethyl butanoate, butanoic acid, isobutyl acetate, isoamyl alcohol and isobutanol. Thus, generally, we observed three profiles: strain C with a profile rich in 2-phenylethanol and phenylethyl acetate, strains A and B with a profile rich in ethyl decanoate, hexanoate and octanoate and strain D with an intermediate profile.

On NM, strain C position is quite similar to that on SM. At a high sterol content, the strain D profile is rich in ethyl esters and medium-chain fatty acids. Strains A and B are intermediate between C and D (rich in acetate esters, phenylethanol and isoamyl alcohol). Strains C and D move along the periphery towards higher volatile compounds concentrations while A and, to a lesser extent, B move from the periphery to the centre of the circle. We observed three profiles: strains C and B, rich in isobutyl acetate; strain A, with low content of volatile compounds and strain D, rich in ethyl esters.

3. Impact of the interaction between sterol and strain

In SM, the effect of this interaction is significant ($p < 0.05$) for fermentation parameters, yeast population, viability, ethyl butanoate, 2-phenylethanol and 1-propanol (Table 1). In NM, the interaction is significant ($p < 0.05$) for all the parameters except assimilated nitrogen, ethyl decanoate, isoamyl alcohol and decanoic acid.

DISCUSSION

To study the effect of phytosterol on fermentation progress and volatile compounds synthesis, our experimental design was implemented on SM and NM. Negative controls were without phytosterol on SM and at low turbidity (35 NTU) on NM (Charrier *et al.*, 2013).

At first, we focused our attention on the impact of phytosterol on fermentation kinetics. Higher phytosterol concentrations reduced fermentation duration and increased maximum CO_2 production rate in synthetic and natural musts. This impact was due to the fact that phytosterols significantly i) improved yeast nitrogen assimilation allowing better cell growth and, thus, a larger population and ii) resulted in higher viability at the end of fermentation. These results are consistent with several other studies (Jahnke and Klein, 1983; Fornairon-Bonnefond *et al.*, 2002; Girardi-Piva *et al.*, 2022b).

In the next step, we compared the results obtained in natural and synthetic musts, focusing on nitrogen consumption. Without sterol addition, the quantity of assimilated nitrogen was lower in SM (200 mg N/L) than in NM (250 mg N/L). This could be due to natural sterols provided by residual solid particles present in this medium. This hypothesis is corroborated by the fact that, without added sterol, the viability in NM was higher than in SM. The effect of sterol concentration on the assimilation of nitrogen was stronger in NM (+177 mg N/L from 0 to 5 mg/L of sterol versus +128 mg N/L in SM). Nevertheless, in all the modalities, yeasts were unable to deplete nitrogen.

Our results raised an interesting question: with a must sterol concentration over 5 mg/ L, would yeasts have assimilated more nitrogen, or have they reached their nitrogen assimilation limit? In SM, the quantity of assimilated nitrogen increased by 20 mg N/L when sterol addition increased from 3 to 5 mg/L (mean on four strains). However, the limit of yeast assimilation was not reached. In NM, when sterol addition went up from 3 to 5 mg/L, the quantity of assimilated nitrogen increased slightly (from 417 to 423 mg N/L, mean on four strains). In this case, the limit of yeast assimilation seemed to have been reached. This is consistent with the result obtained by Mouret *et al.,* (2014), who reported an assimilation of 410 mg N/L in a must with an excess of sterols (15 mg /L of sterol).

After the study of the fermentation kinetics (and associated parameters), the impact of sterol content on the production of metabolites of the central carbon metabolism (CCM) was evaluated. Results obtained in the present study point out that the addition of sterols significantly reduces acetate concentration in wine, confirming previous research (Rollero *et al.,* 2015; Ochando *et al.,* 2017). According to Rollero *et al.* (2015), a plausible explanation for this metabolic variation is that the requirement for acetyl-CoA, a precursor in lipid biosynthesis, is low in the presence of exogenous lipids (phytosterols). Thus, it can be hypothesised that supplementing the culture medium with lipids decreases the intensity of the metabolic flux from acetyl-CoA to lipid synthesis resulting in the lower production of acetic acid (Ochando *et al.,* 2017), an intermediate in this metabolic pathway (Moreno-Arribas and Polo, 2009). Under our experimental conditions, where lipids are the limiting nutrient, glycerol concentration is negatively correlated with the phytosterol content. This impact on glycerol production may be explained by the modulation of the triglyceride pathway. In the case of lipid deficiency, triglyceride biosynthesis is probably activated. In this situation, yeast produces L-Glycerol 3-phosphate, the excess flow being converted to glycerol. When the initial lipid content increases, the amount of medium-chain fatty acids is higher - as shown in our results - and triglyceride biosynthesis is triggered. Thus, the accumulation of glycerol in the medium is lower. Our results are consistent with those of Ochando *et al.* (2017). Glycerol, which may contribute positively to wine quality, is produced by yeasts to maintain redox stability (NADH/NAD turnover) and as a response to cell stress (Vriesekoop *et al.,* 2009; Nevoigt and Stahl, 1997); in particular, osmotic stress (Hohmann, 1997). Glycerol formation results from L-Glycerol 3- phosphate dephosphorylation. L-Glycerol 3-phosphate is also the first step in triglyceride biosynthesis (Zheng and Zou, 2001).

Finally, the effect of sterol on fermentative aroma synthesis was evidenced. Our study shows the positive and significant impact of phytosterol addition on the synthesis of acetate esters such as isoamyl acetate and 2-phenylethylacetate. This result is consistent with the study of Varela *et al.* (2012), who reported that ergosterol addition favoured ester production both in synthetic and Chardonnay wines. We hypothesise that the impact of sterols on acetate esters is an indirect effect. At first, when lipids are the limiting nutrient, phytosterols addition allows higher assimilation of nitrogen; second, the resulting higher nitrogen consumption leads to increased acetate esters accumulation in wine (considering the existence of a proportional relationship between the initial nitrogen content and the final liquid concentrations of acetate esters, as shown by Garde- Garde-Cerdán and Ancín-Azpilicueta, 2008; Hernandez-Orte *et al.,* 2006; Rollero *et al.,* 2015; Torrea *et al.,* 2011; Ugliano *et al.,* 2010). Moreover, our results showed that supplementation with sterols also allowed an increase in higher alcohols. This impact had already been evidenced, but only in synthetic grape must, by Rollero *et al.,* (2015) and Fairbairn *et al.* (2019). Higher alcohol production is linked with yeast growth and metabolism (Valero *et al.,* 2002). Thus, lipid supplementation entails higher alcohol production by enhancing yeast sugar metabolism and growth (Varela *et al.,* 2012). Valero *et al.* (2002) showed that must initial oxygenation favoured the production of esters and higher alcohols. Oxygenation leading to ergosterol synthesis, our results showed that an addition of β-sitosterol has the same effect, and so appeared as an alternative to this process.

In our experiment, when sterol is added, acetate esters increase more than the corresponding fusel alcohols. For example, in NM, phenylethyl acetate concentration increased by 6.3-fold while phenylethanol was only multiplied by 2.06. In parallel, acetate content decreased. Thus, this difference can be explained by a higher esterification rate that could be favoured in the presence of sterol.

Finally, considering the final content of volatile compounds in the two tested musts, it appeared that acetate and ethyl esters, medium-chain fatty acids and fusel alcohol production were higher in NM when compared with SM. This result is consistent with those of Varela *et al.* (2012), who reported that ergosterol supplementation resulted in a greater accumulation of higher alcohols in Chardonnay compared to synthetic wine. In our experiment, the higher nitrogen quantity assimilated by yeast in NM could explain the higher production of volatile compounds, nitrogen being the major factor affecting the production of volatile compounds (Mouret *et al.,* 2014). This could also be linked to differences in composition between the two media. Indeed, in the natural must, the presence of natural phytosterols and medium-chain fatty acids could have favoured the activation of the ergosterol pathway.

Lastly, we compared the performances of the yeast strains. Indeed, despite common responses to sterol addition (as shown on the PCA in Figure 8), significant differences in fermentation progress and nitrogen assimilation were observed according to the strain. This phenomenon could be explained by the fact that *Saccharomyces cerevisiae* strains exhibited significantly different ergosterol requirements for growth, in accordance with Girardi-Piva *et al.,* (2022b). A possible explanation for these divergences can be attributed to differences in initial sterol reserves (Deytieux *et al.,* 2005). In addition, an interesting result is the significant effect of the interactions between sterol addition and the strain on kinetics and the microbiological parameters on the two musts and the major part of the volatile compounds on NM. This result indicates that even if strain choice is a key element to orient the wine sensory profile, nutrient management—and especially sterol content—should also be carefully adapted to better control fermentation progress and wine volatile profile.

CONCLUSION

The aim of this research was to study the effect of phytosterol addition on fermentation progress and volatile compound synthesis. This compound had a significant impact on fermentation kinetics with a decrease in fermentation duration. This effect can be explained by i) a better nitrogen assimilation in the presence of phytosterols and ii) a higher

viability at the end of the fermentation. Results confirm that the limit of nitrogen assimilation is about 400 mg/L. Another important point is confirming the interaction between phytosterol and nitrogen from a nutritional point of view: an increase in sterol content results in a higher assimilation of nitrogen by yeast. This result highlights the importance of taking into account both these factors for better control of fermentation. Another advantage of sterol supplementation is to decrease acetate concentration in wine and, thus, to limit the risk of the correlated sensory defect. Finally, phytosterol allows for greater production of acetate esters, particularly when added to a natural must containing a very low content of lipids (i.e., resulting from excessive clarification). These results evidenced the importance of taking sterol into account to modulate the fruity taste of wine. They highlight the major role of phytosterol as a key nutrient for yeast during alcoholic fermentation and their contribution to the elaboration of the wine aroma profile. Another new interesting finding is the impact of the interaction between sterol content and strain on fermentation.

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