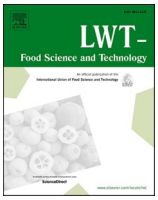
LWT - Food Science and Technology 196 (2024) 115863

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt



Lachancea thermotolerans fermentative metabolism is enhanced by chitosan under winemaking conditions

Javier Vicente [a], Paul-Petrut Manea [a], Santiago Benito [b], Domingo Marquina [a], Niina Kelanne [c], Baoru Yang [c], Antonio Santos [a,*]

[a] Department of Genetics, Physiology and Microbiology, Unit of Microbiology, Faculty of Biological Sciences, Complutense University of Madrid, 28040, Madrid, Spain
[b] Department of Chemistry and Food Technology, Polytechnic University of Madrid, 28040, Madrid, Spain
[c] Food Science, Department of Life Technology, University of Turku, FI-20014, Turku, Finland

ARTICLE INFO

Keywords:
Chitosan
Lachancea thermotolerans
Wine stability
Acidic content
Sugars reduction

ABSTRACT

Wine industry is currently exploring different alternatives to sulphur dioxide as an antimicrobial agent, and chitosan is emerging as a highly promising option due to its multiple applications. In the context of climate change, the use of *Lachancea thermotolerans* to regulate pH and acidity levels during fermentation has become a promising alternative to other physicochemical approaches. This work analysed the impact of chitosan in sequential-mixed fermentations between *L. thermotolerans* and *Saccharomyces cerevisiae*. The presence of chitosan significantly affected the overall fermentation kinetics, resulting in faster fermentations and lower residual sugars, all without an increase in ethanol production. Wines produced under these conditions exhibited increased lactic acid levels (up to 50% more) and decreased malic acid content. This differences significantly influenced final pH (around 0.2 units) and acidity content (up to 1.65 g/L more) when compared to sulphur dioxide-supplemented controls. Chitosan also notably affected to other fermentative by-products, such as glycerol, contributing to improved wine complexity and quality. The addition of chitosan in wine fermentation impacted not only the fermentative performance of key non-*Saccharomyces* yeasts but the overall quality of the final product. This study provides new insights into the different effects of chitosan during the wine fermentative process.

1. Introduction

Traditionally, wine production was described as a single species process. However, years ago, it became evident that a great diversity of non-spoilage and essential groups of microorganisms are involved in this complex process. As the significance of this microbial diversity became evident, the importance of preserving wine and must against spoilage microorganisms also became apparent. Traditionally, sulphur dioxide has been the most widely used preservative in winemaking. However, its use has been associated with the development of resistance in several microorganisms and allergies in consumers. One of the current trends in winemaking is the reduction of sulphur dioxide to reduce consumer concerns, promote the natural aspects of winemaking, and mitigate the potential adverse effects of this antimicrobial (Castro-Marín, Colangelo, Lambri, Riponi & Chinnici, 2021).

In the recent years, different alternatives have been explored to replace (completely or partially) the use of sulphur dioxide in the wine industry. Among them, the use of chitosan, a natural cationic polysaccharide polymer derived from chitin through its N-deacetylation, is the most promising one. Chitin is the second most abundant polymer on Earth, forming different biological structures, from fungal cell walls to arthropods exoskeleton. In winemaking, the OIV has allowed only fungal-derived chitosan (from *Aspergillus niger* or *Agaricus bisporus*) since 2009. The main application of chitosan is to reduce undesirable microorganisms, with its addition limited to 0.1 g/L for this purpose by the OIV. Chitosan is recommended for controlling not only yeasts, such as *Brettanomyces* spp. (Taillandier et al., 2015; Miot-Sertier et al., 2022), but also lactic and acetic acid bacteria (Bağder-Elmaci et al., 2015).

The most comprehensive exploration regarding the effect of chitosan on wine-related microorganisms up to date (Miot-Sertier et al., 2022), concluded significant findings: (1) notable differences regarding chitosan susceptibility, when *in-vitro* tested, are shown among different non-*Saccharomyces* species (without including *Brettanomyces* spp.); (2) the use of chitosan do not reduce the microbial populations present in

* Corresponding author.
E-mail address: ansantos@ucm.es (A. Santos).

https://doi.org/10.1016/j.lwt.2024.115863
Received 1 December 2023; Received in revised form 11 February 2024; Accepted 14 February 2024
Available online 14 February 2024

natural musts; (3) the fermentation carried out by *Saccharomyces cerevisiae* cannot be stopped by adding this antimicrobial; (4) microbial populations could be controlled by this compound after the alcoholic fermentation; and (5) the use of low molecular weight chitosan is more suitable for antimicrobial purposes.

Other applications of chitosan involve the use of higher concentrations, up to 1 g/L according to OIV regulations, for purposes such as clarification, prevention of protein haze, and reduction of heavy metals such as copper, iron, or cadmium (Colangelo, Torchio, De Faveri, & Lambri, 2018). Additionally, concentrations of up to 5 g/L are employed for the reduction of ochratoxin A. However, several other potential applications are currently under investigation, although they have not yet received official authorization. These potential applications include its use as an antioxidant (demonstrated to be effective in minimizing browning in white wines), as an anti-radical agent, and in reducing the content of volatile phenols (Nunes et al., 2016; Castro-Marín et al., 2021).

Despite the different uses of chitosan, little is known about its influence on the fermentation process and its impact on the resulting wines. While its effect on the population of several spoilage microorganisms through different mechanisms has been widely tested and demonstrated (Castro-Marín et al., 2021; Miot-Sertier et al., 2022), few studies have addressed the impact of this molecule on the fermentative performance of these communities and the resulting wines beyond its effect on yeast populations (Castro-Marín, Buglia, Riponi, & Chinnici, 2018; Marchante et al., 2021; Scansani, Rauhut, Brezina, Semmler, & Benito, 2020).

L. thermotolerans, among the non-*Saccharomyces* yeasts species that form must and wine natural microbial populations, is one of the most abundant (Porter, Divol & Setati, 2019). The evolving climatic conditions, particularly impacting warm viticultural regions, result in elevated sugar concentrations, leading to excessive final ethanol content and reduced acidity, thereby affecting the microbial stability of the product (Vicente, Kelanne, Rodrigo-Burgos, et al., 2023). To address these new climatological conditions, several alternatives have been considered, developed, or applied, with biological approaches generally preferred. This species has lately gained impact since it is one of the unique biological alternatives to face global warming effects in wine fermentation.

The most valuable attribute of *L. thermotolerans* is its ability to produce lactic acid and its moderate resistance to alcohol (Vilela, 2018). Lactic acid production by this yeast occurs during the initial stages of fermentation when ethanol concentration is low, allowing *L. thermotolerans* to effectively compete with *S. cerevisiae*. This characteristic renders the species a valuable strategy for acidifying wines from warm viticultural regions. It is usually employed in sequential fermentations since *L. thermotolerans* follows an aerobic fermentative metabolism through a moderate Crabtree effect remaining important residual sugar amounts when fermenting alone (Vicente et al., 2021). The resistance of selected *L. thermotolerans* strains to sulphur dioxide seems to be enough to cope with the usual concentrations employed in the wine industry (around 75 ppm). Nevertheless, its sensitivity to chitosan has been proved to be intermediate to low since all the studied strains showed sensitivity to low molecular weight chitosan, despite a significant surviving population in lees (Miot-Sertier et al., 2022).

Therefore, the primary aim of this investigation was to determine the response exhibited by different strains of *L. thermotolerans* in the vinification process to the introduction of a commercially available chitosan product. The study specifically determined how the introduction of a commercially available chitosan product impacted sequential fermentations involving both *L. thermotolerans* and *S. cerevisiae* on a laboratory scale. We described the effects on essential oenological parameters, as well as the volatile composition of the final wines, to decipher the practical implications of using this molecule.

2. Materials and methods

2.1. Yeast strains and vinification assays

Several L. *thermotolerans* strains (Excellence X'Fresh (EXC; Lamothe-Abiet, France), Levulia Alcomeno (LEV; AEB Group, Italy), EnartisFerm Qkappa (QKK; Enartis, Italy), L1, and A11-612 (Complutense Yeast Collection, Complutense University of Madrid, Madrid, Spain)) were co-fermented with *S. cerevisiae* AWRI-796 (Maurivin, Australia). All strains were preserved at −80 °C and maintained in YMA (Yeast Malt Agar) at 28 °C for routinary use.

For vinification assays, grape juice from the Vitis vinifera L. cultivar Tempranillo, grown in the Ribera del Duero Appellation of Origin (Spain) from the 2022 vintage, was employed. Grape must was obtained after destemming and crushing the grapes, followed by the removal of seeds and skins. Di-ammonium phosphate was then added to achieve a final concentration of 0.20 g/L. Initial parameters of the must were determined using Fourier-transformed infrared spectroscopy (Bacchus 3 analyser, TDI, Barcelona, Spain): fermentable sugars, 236.25 g/L; malic acid, 2.17 g/L; titratable acidity, 5.21 g/L; pH, 3.39.

The control condition consisted of grape must (devoid of seeds and skins) without any antimicrobial agent. Treated grape musts were supplemented with 0.40 g/L of a commercially available product containing chitosan from *A. niger* (Bactiless, Lallemand, Canada), or with 60 mg/L potassium metabisulphite. Fermentations were conducted in triplicate using 100 mL bottles with polypropylene caps, each filled with 100 mL of the corresponding must, and maintained at 25 °C. Bottles caps were left slightly open to allow CO_2 release while preventing microbial contamination.

Precultures were prepared in 250 mL flasks containing 100 mL of YMB and incubated for 24 h at 28 °C with orbital shaking at 150 rpm. All yeasts strains were inoculated at a final cellular concentration of 10^6 cells/mL (≈0.2 O.D. at λ_{600nm}). Subsequently, 96 h after the *L. thermotolerans* inoculation, *S. cerevisiae* was introduced.

Fermentation monitoring was performed by measuring the weight loss every 24 h. Fermentation was considered complete when the weight loss was less than 0.01% for two consecutive days. The initial weight of each fermentation was considered as 100%.

2.2. Basic oenological parameters and volatile profile

Fourier-transformed infrared spectroscopy (Bacchus 3 analyser, TDI, Barcelona, Spain) was used to analyse acetic acid, succinic acid, ethanol, residual sugars, pH, and glycerol in the resulting wines. L-malic acid, L-lactic acid, Primary Amino Nitrogen (PAN), and ammonia were determined enzymatically using the Y15 autoanalyzer (Biosystems, Barcelona, Spain).

The analysis of volatile compounds at the end of fermentation followed a previously described methodology (Vicente, Kelanne, Rodrigo-Burgos, et al., 2023). Briefly, samples were analysed in triplicate using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS). Retention indices (RIs) of the volatiles were calculated by co-injection with an alkane mixture (C7–C21, Sigma-Aldrich, St. Louis, USA). Identification of volatile compounds was performed by matching obtained mass spectra with the NIST 08 library, and RIs were compared with those of compounds reported in the literature and the NIST Webbook (https://webbook.nist.gov/chemistry/). Additionally, the identification of a selected number of volatile compounds was confirmed by comparing retention indices and mass spectra with those of authentic reference compounds.

2.3. Statistical analysis

All statistical analyses were performed using R software version 4.1.2 (R Development Core Team, Vienna, Austria, 2013). Principal

Components Analysis (PCA) was calculated using the mean values of all the oenological parameters of the fermentations using *factoextra* library (v1.0.7). Analysis of variance (ANOVA) and Tukey *post hoc* tests were applied to compare the different groups and values within trials involving the same strain combination using *agricolae* library (v1.3-7).

3. Results

In our study, the presence of different antimicrobials during grape must (without skins and seeds) fermentation impacted not only the microbial communities but also the metabolism of yeasts. Both chitosan and sulphur dioxide had a different effect in both the populations and fermentative performance of the studied yeasts. We employed five different *L. thermotolerans* wine strains in combination with a well-known fermentative *S. cerevisiae* strain. The impact of both chitosan and sulphur dioxide was observed to be similar in all the studied non-*Saccharomyces* yeasts. However, these molecules displayed different responses in yeast physiology, resulting in a clear differentiation of the resulting wines regarding their chemical composition (Fig. 1, Fig. S1).

The increased concentration of lactic acid, along with the reduction in glycerol content resulting from the presence of *L. thermotolerans*, appears to be the primary factor differentiating the use of this non-*Saccharomyces* yeast in wine fermentation (PC1 accounts for 88.15% of the variance in sample differentiation) (Fig. S1). In contrast, the impact of the different antimicrobial treatments seems to be less pronounced (PC2 accounts for 6.45% of the variance in sample differentiation), with the residual sugar content in chitosan-protected wines being lower when compared to controls (grape must without any antimicrobial treatment) or sulphur dioxide-protected ones (Fig. 1).

Despite minor differences in fermentative kinetics, ethanol production, and residual sugars, all conditions showed similar values. Although no statistically significant differences were observed in these parameters, total weight loss showed different values (Fig. 2, Fig. S2). Despite this effect, total fermentation time was not affected by the presence of the antimicrobials, reaching the end of alcoholic fermentation in 792 h (33 days). Generally, chitosan-treated fermentations resulted in greater overall weight loss compared to both sulphur and control conditions (e.g., A11–612, L1, or SC), particularly evident from 24 to 72 h (Fig. S2). Ethanol production and residual sugars (Table 1), directly linked to the released weight (as it is proportional to the released CO_2 during fermentation), were similar in all the trials. In all fermentations, the final ethanol concentration exceeded 15%, with some chitosan trials showing the lowest concentration (e.g., EXC, L1, LEV, or QKK). However, these differences were not statistically significant. Despite having the lowest ethanol concentration, these chitosan trials also exhibited the lowest levels of residual sugars.

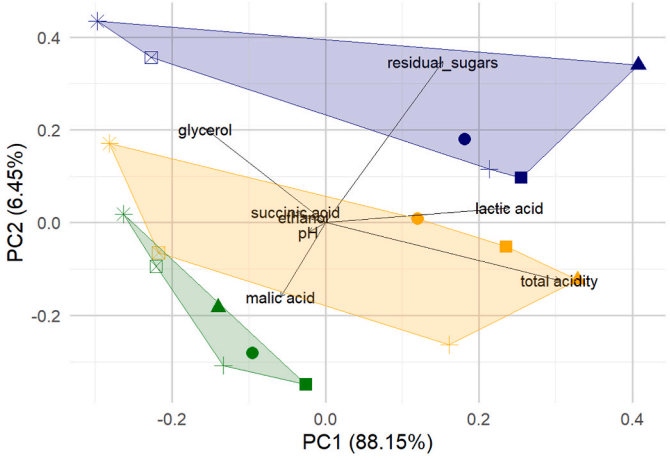


Fig. 1. Principal Component Analysis (PCA) of the resulting wines regarding their chemical composition. Different colours indicate the employed treatment: chitosan-protected (blue), sulphur-protected (green), and control fermentations (orange). Different shapes represent the used strains: A11-12 (circle), EXC (triangle), L1 (square), LEV (plus), QKK (square cross), and SC (star).

The presence of the studied antimicrobials primarily affected other metabolites produced during the alcoholic fermentation. Chitosan fermentations showed a decrease in the glycerol content of the resulting wines (e.g., A11-612), whereas sulphur dioxide-treated fermentations exhibited slightly higher values compared to the control condition (e.g., EXC).

The impact of the antimicrobials on the organic acids content of the resulting wines was not negligible. Lactic acid production by *L. thermotolerans* was significantly influenced by the presence of both compounds. Wines treated with sulphur dioxide showed significantly lower lactic acid levels, with up to an 80% reduction, while the presence of chitosan resulted in a slight increase, up to 0.23 g/L more in its concentration. Malic acid content was also affected, chitosan-treated fermentations showed lower values compared to the control condition, while sulphur-protected ones showed the highest. Despite the effect of chitosan on malic acid metabolism in *L. thermotolerans*, the overall acidic content (total acidity and pH) was not affected by the presence of chitosan in the trials involving this non-*Saccharomyces* species, as it was compensated by the high lactic acid production. *S. cerevisiae* controls in all the trials did not show changes in lactic acid concentration, whereas malic acid showed a slight decrease under the presence of chitosan, affecting the final pH and the total acidity content. The presence of *L. thermotolerans* impacted the final concentration of acetic acid in all cases compared to the *S. cerevisiae* control, despite no differences were observed regarding the different conditions in which every strain was tested.

The nitrogen metabolism appears to remain unaffected when applying any of the described treatments. Neither PAN (Primary Amino Nitrogen) nor residual ammonia levels exhibited any statistically significant differences when compared to the control condition in each trial. Nevertheless, mixed fermentations, when compared with single fermentations using *S. cerevisiae*, showed remarkable differences regarding the organic fraction in the resulting wines. The content of residual PAN was up to 53% higher in the single fermentations when sulphur was employed, and up to 40% higher in the chitosan-treated ones.

Other fermentative by-products considered the volatile fraction of the wines were not affected by any of the applied treatments. However, esters, followed by fatty acids, showed some changes. These groups exhibited significantly increased concentrations under the presence of sulphur dioxide. Despite the presence of *L. thermotolerans* reducing the concentration of esters compared to the *S. cerevisiae* control condition in each trial, the production of ethyl hexanoate and ethyl octanoate was notably increased (Table S1). QKK showed the highest esters levels in the three trials compared to the rest of mixed fermentations. On the contrary, the production of fatty acids was not clearly modified in mixed fermentations, but its production was increased when sulphur dioxide was employed. Higher alcohols were not greatly affected by the presence of the antimicrobials. Fermentations involving sulphur dioxide showed increased values in the production of this kind of compounds, whereas chitosan was related to a decrease in the production of fusel alcohols, showing significant reductions in specific trials (e.g., A11-612). As far as acetaldehyde is concerned, in some cases, chitosan-treated wines showed values like sulphur-treated ones when compared to the control condition without any antimicrobial (i.e. LEV). Other volatile compounds, such as ketones, did not display significant variations in response to the different yeasts or antimicrobial treatments (Table 1).

4. Discussion

The use of alternative antimicrobial agents, different from sulphur dioxide, is a primary focus in current wine industry research initiatives. Various factors underscore the need to develop and explore novel

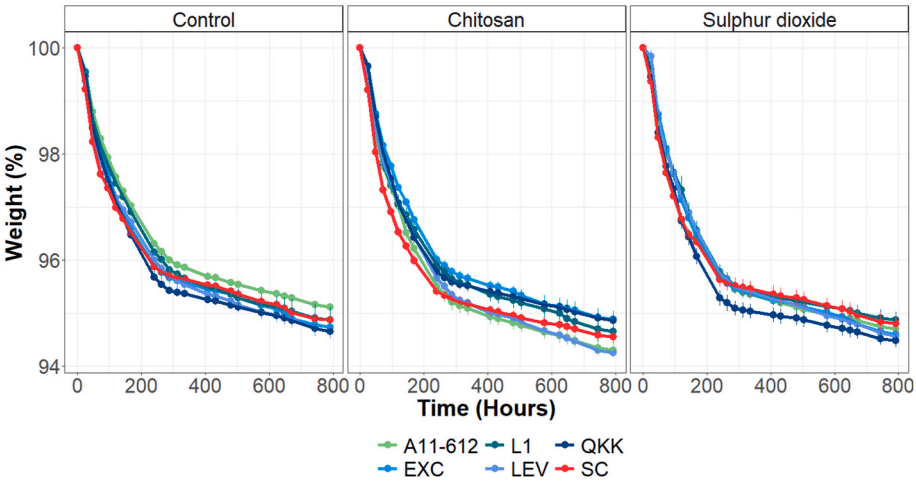


Fig. 2. Fermentative kinetics (weight loss vs. time) of the fermentations regarding each treatment. Data points represent the average value of the three biological replicates with the standard deviation bars.

antimicrobial compounds, their impact on wine yeasts, and their role in the fermentative processes throughout winemaking. Chitosan stands out as one of the most promising alternatives since it primarily derives from biological sources (mainly fungal). No adverse effects have been reported in consumers, and its potential applications are extensive (Castro-Marín et al., 2021). Another challenge that the wine industry is currently facing is climate change. Increased temperatures, together with reduced water availability, are significantly affecting vine phenology and must composition. This ultimately impacts the fermentative process carried out by yeasts and, consequently, wine quality and stability (Vicente et al., 2022).

Several studies have determined the impact of chitosan on various wine-related yeast species (Miot-Sertier et al., 2022) and its influence on specific metabolic pathways of *S. cerevisiae* (Lage, Coelho, Mira, & Mendes-Ferreira, 2023), as well as its effects on wine stability. In this study, we investigated its influence on the fermentative performance of non-*Saccharomyces* yeasts, along with its consequences on the resulting wines compared to a standard sulphur dosage under winemaking conditions.

In all fermentations, the overall fermentative performance remained largely unchanged in the presence of either chitosan or sulphur dioxide, despite minor fluctuations in different parameters. The presence of chitosan was associated with a shorter lag phase, leading to a higher fermentative rate at the beginning of the process (from 24 to 72 h); however, no significant effects were observed at the end of fermentation regarding total weight loss. In *S. cerevisiae*, the effect of a chemical-grade low molecular weight chitosan is even greater in the lag phase than the effect of sulphur dioxide, a fact that was not observed in our trials (Castro-Marín et al., 2018). In previous studies, ethanol production and residual sugars in *S. cerevisiae* (Castro-Marín et al., 2018; Marchante et al., 2021) or *Schizosaccharomyces pombe* (Scansani et al., 2020) were not significantly altered in chitosan-treated fermentations.

Chitosan has been proposed to enhance oxidative metabolism and respiration in yeasts, potentially influencing the levels of residual sugars and total weight loss, while leaving ethanol levels unaffected (López-Moya, Suárez-Fernández & López-Llorca, 2019). This alteration in general metabolism could also impact glycerol production. However, our chitosan-treated fermentations exhibited similar glycerol content to the control condition, consistent with previous findings (Castro-Marín et al., 2018). Despite these observations, chitosan has been proposed to be a glycerol production enhancer in both *S. cerevisiae* and *S. pombe*, as part of the general response to osmotic stress and to maintain redox balance (NAD+/NADH) (Marchante et al., 2021; Scansani et al., 2020). Similarly, glycerol concentration is usually increased under the presence of a sulphite salt through Neuberg fermentation (Semkiv, Ruchala, Dmytruk, & Sibirny, 2020).

The regulation of carbon metabolism in *L. thermotolerans* is still partially unknown. The existence of an alternative and highly active pathway facilitating lactic acid production, absent in other yeasts, could exert a direct influence on the overall metabolic pathways. This pathway has been identified as a crucial alternative for maintaining redox balance thought the production of reductive power (Vicente et al., 2021). Moreover, *L. thermotolerans* has the capacity to consume significant quantities of malic acid, allowing its reduction during fermentation (Vicente, Kelanne, Navascués, et al., 2023). The interaction between the intrinsic metabolic pathways of the yeast and the potential role of chitosan in enhancing oxidative metabolism could profoundly affect organic acid metabolism. However, the altered acidic equilibrium in chitosan-treated fermentations may not solely be attributed to these factors but also by the physicochemical conditions imposed by the presence of chitosan. The positively charged glucosamine groups form electrostatic interactions with anions from the dissociated organic acids (Castro-Marín et al., 2018). This dissociation is determined by the pKa of the organic acids, which typically falls within the pH range of wines (3.20–3.80); for instance, the pKa values are 3.86 for lactic acid, 3.51 for malic acid, and 3.07 for tartaric acid (Robles, Fabjanowicz, Chmiel, & Plotka-Wasylka, 2019).

In our study, both the control and chitosan-treated conditions involving *L. thermotolerans* exhibited increased lactic acid levels and reduced malic acid levels. This trend was particularly noticeable in the chitosan-treated wines compared to the control ones, although the differences were not statistically significant. In contrast, sulphur negatively impacted yeasts metabolism, resulting in lower lactic acid content and similar levels of malic acid. The *S. cerevisiae* controls did not exhibit significantly altered levels of lactic or malic acid, although a slight reduction in malic acid was observed in chitosan-treated wines, that could be explained by the physicochemical interactions. The differences in lactic and malic content across different trials could not be attributed to the development of lactic acid bacteria from the natural grape must, as these changes were only observed in trials involving non-*Saccharomyces* yeast. When working with non-*Saccharomyces*, a major concern is the risk of volatile acidity resulting from acetic acid production. However, in this study, no increases were observed, as all trials exhibited similar values well below the perception threshold.

Membrane permeability is a critical factor in yeasts when facing acidic stress induced by organic acids, such as lactic acid produced by *L. thermotolerans*. Organic acids, at a pH value below their pKa, exist in the undissociated (lipophilic) form, allowing them to permeate through the plasma membrane via simple diffusion. Within the cell, weak acids dissociate, releasing protons that cannot pass through the lipid bilayer

Table 1
Main analytical parameters of the resulting wines (mean ± SD of the three biological replicates).

Parameter	Treatment	A11-612	EXC	L1	LEV	QKK	SC
Ethanol (%, v/v)	C	15,27 ± 0,08 a	15,18 ± 0,26 a	15,37 ± 0,09 a	15,31 ± 0,1 a	15,36 ± 0,12 a	15,39 ± 0,18 ab
	CH	15,32 ± 0,08 a	15,04 ± 0,3 a	15,03 ± 0,39 a	15,22 ± 0,16 a	15,27 ± 0,11 a	15,61 ± 0,03 a
	SO_2	15,34 ± 0,04 a	15,16 ± 0,12 a	15,28 ± 0,11 a	15,31 ± 0,14 a	15,35 ± 0,05 a	15,28 ± 0,04 b
Residual sugars (g/L)	C	0,93 ± 0,04 a	0,98 ± 0,08 a	0,94 ± 0,13 a	0,85 ± 0,17 a	0,93 ± 0,29 a	0,87 ± 0,14 a
	CH	0,83 ± 0,1 a	0,8 ± 0,16 a	0,81 ± 0,05 a	0,65 ± 0,1 a	0,71 ± 0,21 a	0,88 ± 0,13 a
	SO_2	0,91 ± 0,12 a	0,92 ± 0,03 a	0,92 ± 0,12 a	0,87 ± 0,14 a	1,04 ± 0,2 a	1,05 ± 0,14 a
Glycerol (g/L)	C	6,72 ± 0,07 a	6,47 ± 0,21 a	6,46 ± 0,35 a	6,3 ± 0,2 a	7,22 ± 0,11 a	7,89 ± 0,19 a
	CH	6,4 ± 0,11 b	6,18 ± 0,2 a	6,59 ± 0,4 a	6,21 ± 0,18 a	7,48 ± 0,22 a	7,52 ± 0,08 a
	SO_2	6,74 ± 0,08 a	7,05 ± 0,17 b	6,68 ± 0,2 a	6,5 ± 0,15 a	7,1 ± 0,14 a	7,4 ± 0,32 a
pH	C	3,32 ± 0,01 a	3,29 ± 0,05 a	3,28 ± 0,03 a	3,29 ± 0,01 a	3,39 ± 0,04 a	3,36 ± 0,01 a
	CH	3,29 ± 0,01 a	3,25 ± 0,03 a	3,29 ± 0,01 a	3,29 ± 0,01 a	3,41 ± 0,01 b	3,42 ± 0,02 b
	SO_2	3,42 ± 0,01 b	3,45 ± 0,01 b	3,41 ± 0,01 b	3,42 ± 0,01 b	3,44 ± 0,01 c	3,45 ± 0,01 b
Total acidity (g/L)	C	6,52 ± 0,07 a	7,5 ± 0,88 a	6,95 ± 0,35 a	6,62 ± 0,12 a	5,25 ± 0,05 a	5,19 ± 0,17 a
	CH	6,42 ± 0,17 a	7,27 ± 0,55 a	7 ± 0,06 a	6,48 ± 0,08 a	5,01 ± 0,04 b	4,61 ± 0,06 b
	SO_2	5,67 ± 0,04 b	5,62 ± 0,12 b	6,04 ± 0,05 b	5,35 ± 0,08 b	5,23 ± 0,1 a	5,07 ± 0,31 ab
Acetic acid (g/L)	C	0,25 ± 0,03 a	0,28 ± 0,12 a	0,31 ± 0,05 a	0,28 ± 0,03 a	0,19 ± 0,03 a	0,07 ± 0,03 a
	CH	0,29 ± 0,02 a	0,28 ± 0,01 a	0,47 ± 0,22 a	0,31 ± 0,02 a	0,19 ± 0,04 a	0,07 ± 0,03 a
	SO_2	0,31 ± 0,04 a	0,32 ± 0,02 a	0,35 ± 0,02 a	0,3 ± 0,01 a	0,23 ± 0,03 a	0,09 ± 0,03 a
Lactic acid (g/L)	C	1,1 ± 0,07 a	1,7 ± 0,58 a	1,45 ± 0,24 a	1,23 ± 0,12 a	0,08 ± 0,07 a	0 ± 0,03 a
	CH	1,33 ± 0,13 a	1,93 ± 0,41 a	1,5 ± 0,33 a	1,41 ± 0,08 a	0,16 ± 0,01 a	0 ± 0,01 a
	SO_2	0,5 ± 0,05 b	0,33 ± 0,12 b	0,68 ± 0,08 b	0,35 ± 0,05 b	0,05 ± 0,06 a	0,01 ± 0,03 a
Malic acid (g/L)	C	1,1 ± 0,07 a	1 ± 0,19 a	1,06 ± 0,08 a	1,15 ± 0,04 a	1,42 ± 0,05 a	1,3 ± 0,06 a
	CH	0,96 ± 0,06 b	0,81 ± 0,09 a	0,85 ± 0,1 a	0,92 ± 0,09 b	1,1 ± 0,07 b	1,09 ± 0,02 a
	SO_2	1,38 ± 0,02 c	1,39 ± 0,08 b	1,34 ± 0,01 b	1,39 ± 0,03 c	1,44 ± 0,03 a	1,37 ± 0,08 a
Succinic acid (g/L)	C	1,04 ± 0,07 a	1,13 ± 0,26 a	0,91 ± 0,11 a	0,81 ± 0,04 a	1,18 ± 0,02 a	1,32 ± 0,11 a
	CH	0,86 ± 0,03 b	0,84 ± 0,05 a	1,01 ± 0,25 a	0,7 ± 0,02 b	1,22 ± 0,08 a	1,05 ± 0,07 a
	SO_2	0,99 ± 0,05 a	1,14 ± 0,08 a	1,02 ± 0,14 a	0,85 ± 0,03 a	1,17 ± 0,04 a	1,18 ± 0,21 a
Ammonia (mg/L)	C	7,67 ± 1,15 a	7,67 ± 2,52 a	7,67 ± 0,58 a	8,33 ± 2,08 a	6,33 ± 0,58 a	7 ± 1 a
	CH	8,33 ± 4,04 a	7,33 ± 1,15 a	9,67 ± 1,53 a	9,67 ± 5,51 a	8,33 ± 4,16 a	7 ± 1 a
	SO_2	5 ± 4,36 a	7,67 ± 3,06 a	9,67 ± 2,31 a	7,67 ± 3,51 a	4 ± 0 a	10 ± 2,65 a
PAN (mg/L)	C	22 ± 1 ab	23,67 ± 1,15 a	22 ± 1 a	25,33 ± 8,08 a	26,33 ± 4,93 a	25,67 ± 1,53 a
	CH	20,67 ± 0,58 a	23 ± 1 a	20 ± 2 a	20,33 ± 0,58 a	24 ± 1 a	28,33 ± 2,52 a
	SO_2	24 ± 1 b	25 ± 1,73 a	22,67 ± 2,08 a	23,67 ± 1,15 a	28,5 ± 2,12 a	34,67 ± 7,23 a
Esters (Area Units)	C	2,9 ± 0,11 ab	2,99 ± 0,13 a	2,66 ± 0,16 a	2,48 ± 0,07 a	3,31 ± 0,11 a	3,36 ± 0,08 a
	CH	2,71 ± 0,21 a	2,85 ± 0,19 a	2,72 ± 0,17 a	2,77 ± 0,04 b	3,19 ± 0,19 a	3,66 ± 0,11 b
	SO_2	3,12 ± 0,04 b	3,24 ± 0,25 a	3,18 ± 0,09 b	3,24 ± 0,08 c	3,57 ± 0,18 a	3,7 ± 0,1 b
Higher alcohols (Area Units)	C	6,85 ± 0,16 a	6,73 ± 0,15 ab	6,72 ± 0,47 a	6,45 ± 0,04 a	6,09 ± 0,16 a	6,61 ± 0,26 a
	CH	6,22 ± 0,19 b	6,34 ± 0,14 a	6,4 ± 0,28 a	6,52 ± 0,08 a	6,01 ± 0,22 a	6,16 ± 0,12 a
	SO_2	6,59 ± 0,17 ab	6,86 ± 0,26 b	7,12 ± 0,29 a	6,79 ± 0,41 a	6,47 ± 0,1 a	6,52 ± 0,24 a
Fatty acids (Area Units)	C	0,16 ± 0,02 a	0,16 ± 0,02 a	0,18 ± 0,12 a	0,15 ± 0,02 a	0,24 ± 0,03 a	0,24 ± 0,01 a
	CH	0,18 ± 0,03 a	0,21 ± 0,04 ab	0,14 ± 0,02 a	0,17 ± 0,01 a	0,25 ± 0,02 a	0,27 ± 0,02 ab
	SO_2	0,26 ± 0,01 b	0,26 ± 0 b	0,23 ± 0,02 a	0,24 ± 0 b	0,27 ± 0,05 a	0,29 ± 0,03 b
Aldehydes (Area Units)	C	0,65 ± 0,18 a	0,54 ± 0,06 a	0,55 ± 0,06 a	0,72 ± 0,06 a	0,64 ± 0,14 a	0,53 ± 0,14 a
	CH	0,58 ± 0,07 a	0,55 ± 0,06 a	0,68 ± 0,22 a	0,54 ± 0,12 ab	0,63 ± 0,19 a	0,55 ± 0,1 a
	SO_2	0,65 ± 0,04 a	0,66 ± 0,1 a	0,54 ± 0,11 a	0,5 ± 0,03 b	0,53 ± 0,04 a	0,57 ± 0,12 a
Ketones * (Area Units)	C	4,21 ± 0,35 a	4,21 ± 0,29 a	4,03 ± 0,5 a	4,28 ± 0,31 a	3,94 ± 0,25 a	4,12 ± 0,15 a
	CH	4,3 ± 0,24 a	4,06 ± 0,74 a	4,51 ± 0,36 a	4,16 ± 0,24 a	4,36 ± 0,14 a	4,19 ± 0,45 a
	SO_2	4,25 ± 0,05 a	4,53 ± 0,15 a	4,29 ± 0,18 a	4,36 ± 0,17 a	4,08 ± 0,38 a	4,2 ± 0,28 a

Different letters show statistical differences between treatments in each strain-combination (p-value >0.05) according to the ANOVA and Tukey *post hoc* analysis. C: Control condition; CH: chitosan treated fermentations; SO_2: sulphur dioxide treated fermentations. * Data has been multiplied by 1000.

and thus accumulate inside cells. Consequently, organic acids exert a much more inhibitory effect than pH on microorganisms. This alteration in pH homeostasis is not the sole impact of organic acids on microbial cells. Organic acids significantly affect lipid organization and the function of cellular membranes, with their effect becoming more pronounced as the hydrophobicity of organic acids increases (Mira, Teixeir & Sá-Correia, 2010). Some authors have hypothesized that the increased concentration of medium-chain fatty acids (MCFA) in fermentations carried out by *S. cerevisiae* under chitosan presence may be related to increased membrane permeability due to electrostatic interactions formed between the membrane and chitosan. This interaction may affect the fatty acids content (as MCFA), as well as the derived esters, in the final wines (Castro-Marín et al., 2018). Nevertheless, this effect has not been observed in other *S. cerevisiae* or *S. pombe* wine-related strains (Scansani et al., 2020). In our case, no effect regarding fatty acids was observed, even though we employed several strains of *L. thermotolerans* with different levels of lactic acid production. When using antimicrobials other than sulphur dioxide, some positive aspects of this molecule, such as its antioxidant capacity, are lost, especially in wines requiring long aging. Acetaldehyde, which could be considered an indicator of wine oxidation (Bueno, Culleré, Cacho, & Ferreira, 2010), did not show increased values in our wines when comparing different treatments or yeasts employed. Remarkably, some chitosan-treated fermentations exhibited values similar to sulphur-treated wines, though chitosan may exert some effect concerning premature oxidation.

L. thermotolerans and *S. cerevisiae* present a similar nitrogen metabolism under standard fermentative conditions (Roca-Mesa, Sendra, Mas, Beltran, & Torija, 2020). The presence of both species in mixed fermentations did not affect nitrogen content compared to control fermentations involving *S. cerevisiae* alone. However, under stressful conditions, *L. thermotolerans* may consume higher amounts of amino acids, as indicated by the lower residual PAN levels compared to *S. cerevisiae* controls in the chitosan- and sulphur-treated fermentations. Despite this, no significant differences were found when comparing different treatments within the same trial. This could be attributed to the nitrogen compound metabolism by *L. thermotolerans* which appear to modulate its lactic acid metabolism during the fermentative process (Battjes et al., 2023). In fermentations involving *S. cerevisiae*, chitosan treatment

typically results in a decrease in residual nitrogen levels, in both inorganic and organic sources, compared to a standard wine treated with sulphur dioxide (Marchante et al., 2021).

5. Conclusions

The results presented here indicated that chitosan may have a significant effect on yeast physiology during wine fermentation. Notwithstanding the observed subtle effects, which encompass factors such as fermentative kinetics and lactic acid production by *L. thermotolerans*, and considering the scale utilized, significant insights emerge regarding the impact of chitosan on yeast fermentative metabolism. Traditionally consider as an antimicrobial, chitosan could also serve as a fermentation adjuvant, accelerating some fermentations while leveraging its antimicrobial properties. The potential role of this molecule may vary depending on its characteristics (molecular weight, purity, or concentration) and the strain tested. Therefore, the impact of chitosan should be further investigated, not only in *S. cerevisiae* but also in other wine-related non-*Saccharomyces* species, especially when considering *L. thermotolerans* as a biological tool for pH management during wine fermentation.

CRediT authorship contribution statement

Javier Vicente: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paul-Petrut Manea:** Methodology, Investigation. **Santiago Benito:** Supervision, Investigation, Funding acquisition, Conceptualization. **Domingo Marquina:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Niina Kelanne:** Methodology, Investigation. **Baoru Yang:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Antonio Santos:** Writing – review & editing, Supervision, Project administration, Formal analysis, Conceptualization.

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

Data will be made available on request.

Acknowledgements

Funding for this research was provided by the LowpHWine Companies Consortia through the CDTI project LowpHWine (IDI-20210391) and the Spain Ministry of Science and Innovation under the VinSeg-CalClim project (PID2020-119008RB-I00/AEI/10.13039/501100011033). Javier Vicente conducted this research under a fellowship from Complutense University of Madrid (CT58/21-CT59/21).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.115863.

References

Bağder Elmacı, S., Gülgör, G., Tokatlı, M., Erten, H., İşçi, A., & Özçelik, F. (2015). Effectiveness of chitosan against wine-related microorganisms. *Antonie van Leeuwenhoek, 107*, 675–686. https://doi.org/10.1007/s10482-014-0362-6

Battjes, J., Melkonian, C., Mendoza, S. N., Haver, A., Al-Nakeeb, K., Koza, A., et al. (2023). Ethanol-lactate transition of *Lachancea thermotolerans* is linked to nitrogen metabolism. *Food Microbiology, 110*. https://doi.org/10.1016/j.fm.2022.104167

Bueno, M., Culleré, L., Cacho, J., & Ferreira, V. (2010). Chemical and sensory characterization of oxidative behavior in different wines. *Food Research International, 43*(5), 1423–1428. https://doi.org/10.1016/j.foodres.2010.04.003

Castro Marin, A., Colangelo, D., Lambri, M., Riponi, C., & Chinnici, F. (2021). Relevance and perspectives of the use of chitosan in winemaking: A review. *Critical Reviews in Food Science and Nutrition, 61*(20), 3450–3464. https://doi.org/10.1080/10408398.2020.1798871

Castro-Marín, A., Buglia, A. G., Riponi, C., & Chinnici, F. (2018). Volatile and fixed composition of sulphite-free white wines obtained after fermentation in the presence of chitosan. *LWT–Food Science and Technology, 93*, 174–180. https://doi.org/10.1016/j.lwt.2018.03.003

Colangelo, D., Torchio, F., De Faveri, D. M., & Lambri, M. (2018). The use of chitosan as alternative to bentonite for wine fining: Efects on heat-stability, proteins, organic acids, colour, and volatile compounds in an aromatic white wine. *Food Chemistry, 264*, 301–309. https://doi.org/10.1016/j.foodchem.2018.05.005

Lage, P., Coelho, B. B., Mira, N. P., & Mendes-Ferreira, A. (2023). A genome-wide phenotypic analysis of *Saccharomyces cerevisiae*'s adaptive response and tolerance to chitosan in conditions relevant for winemaking. *Fermentation-Basel, 9*(2). https://doi.org/10.3390/fermentation9020172

Lopez-Moya, F., Suarez-Fernandez, M., & Lopez-Llorca, L. V. (2019). Molecular mechanisms of chitosan interactions with fungi and plants. *International Journal of Molecular Sciences, 20*(2). https://doi.org/10.3390/ijms20020332

Marchante, L., Mena, A., Izquierdo-Canas, P. M., Garcia-Romero, E., Perez-Coello, M. S., & Diaz-Maroto, M. C. (2021). Effects of the pre-fermentative addition of chitosan on the nitrogenous fraction and the secondary fermentation products of SO(2) -free red wines. *Journal of the Science of Food and Agriculture, 101*(3), 1143–1149. https://doi.org/10.1002/jsfa.10725

Miot-Sertier, C., Paulin, M., Dutilh, L., Ballestra, P., Albertin, W., Masneuf-Pomarede, I., et al. (2022). Assessment of chitosan antimicrobial effect on wine microbes. *International Journal of Food Microbiology, 381*, Article 109907. https://doi.org/10.1016/j.ijfoodmicro.2022.109907

Mira, N. P., Teixeira, M. C., & Sá-Correia, I. (2010). Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: A genome-wide view. *OMICS: A Journal of Integrative Biology, 14*(5), 525–540. https://doi.org/10.1089/omi.2010.0072

Nunes, C., Maricato, É., Cunha, Á., Rocha, M. A. M., Santos, S., Ferreira, P., et al. (2016). Chitosan–genipin film, a sustainable methodology for wine preservation. *Green Chemistry, 18*(19), 5331–5341. https://doi.org/10.1039/C6GC01621A

Porter, T. J., Divol, B., & Setati, M. E. (2019). *Lachancea* yeast species: Origin, biochemical characteristics and oenological significance. *Food Research International, 119*, 378–389. https://doi.org/10.1016/j.foodres.2019.02.003

Robles, A., Fabjanowicz, M., Chmiel, T., & Plotka-Wasylka, J. (2019). Determination and identification of organic acids in wine samples. Problems and challenges. *TRAC Trends in Analytical Chemistry, 120*. https://doi.org/10.1016/j.trac.2019.115630

Roca-Mesa, H., Sendra, S., Mas, A., Beltran, G., & Torija, M. J. (2020). Nitrogen preferences during alcoholic fermentation of different non-*Saccharomyces* yeasts of oenological interest. *Microorganisms, 8*(2). https://doi.org/10.3390/microorganisms802157

Scansani, S., Rauhut, D., Brezina, S., Semmler, H., & Benito, S. (2020). The impact of chitosan on the chemical composition of wines fermented with *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *Foods, 9*(10). https://doi.org/10.3390/foods9101423

Semkiv, M. V., Ruchala, J., Dmytruk, K. V., & Sibirny, A. A. (2020). 100 years later, what is new in glycerol bioproduction? *Trends in Biotechnology, 38*(8), 907–916.

Taillandier, P., Joannis-Cassan, C., Jentzer, J. B., Gautier, S., Sieczkowski, N., Granes, D., et al. (2015). Effect of a fungal chitosan preparation on *Brettanomyces bruxellensis*, a wine contaminant. *Journal of Applied Microbiology, 118*(1), 123–131. https://doi.org/10.1111/jam.12682

Vicente, J., Baran, Y., Navascues, E., Santos, A., Calderon, F., Marquina, D., et al. (2022). Biological management of acidity in wine industry: A review. *International Journal of Food Microbiology, 375*. https://doi.org/10.1016/j.ijfoodmicro.2022.109726

Vicente, J., Kelanne, N., Navascués, E., Calderón, F., Santos, A., Marquina, D., et al. (2023b). Combined use of *Schizosaccharomyces pombe* and a *Lachancea thermotolerans* strain with a high malic acid consumption ability for wine production. *Fermentation-Basel, 9*(2). https://doi.org/10.3390/fermentation9020165

Vicente, J., Kelanne, N., Rodrigo-Burgos, L., Navascués, E., Calderón, F., Santos, A., et al. (2023a). Influence of different *Lachancea thermotolerans* strains in the wine profile in the era of climate challenge. *FEMS Yeast Research, 23*. https://doi.org/10.1093/femsyr/foac062

Vicente, J., Navascues, E., Calderon, F., Santos, A., Marquina, D., & Benito, S. (2021). An integrative view of the role of *Lachancea thermotolerans* in wine technology. *Foods, 10*(11). https://doi.org/10.3390/foods10112878

Vilela, A. (2018). *Lachancea thermotolerans*, the non-*Saccharomyces* yeast that reduces the volatile acidity of wines. *Fermentation, 4*(3), 56. https://doi.org/10.3390/fermentation4030056