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Ozone in gaseous and aqueous phases as a sanitizing agent for grapes used in winemaking and its impact on the implantation of *Lachancea thermotolerans*

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Ozonation is an effective and sustainable method for grape sanitation, facilitating the implementation of non-*Saccharomyces* yeasts such as *Lachancea thermotolerans*. This study evaluated gaseous (28 g h⁻¹ for 30 min) and aqueous ozone (0.5 g h⁻¹ for 30 min) treatments on *L. thermotolerans* implantation in Red Globe grapes. Fermentations with *L. thermotolerans* and *Saccharomyces cerevisiae* were conducted at different inoculation rates, assessing must acidification, sugar consumption and volatile compounds. Ozone treatments increased lactic acid production, lowered pH and enhanced *L. thermotolerans* metabolic activity. Volatile analysis revealed a higher production of 2-phenyl ethanol, a characteristic compound of *L. thermotolerans*. These findings suggest ozonation as a potential alternative to sulfitation, improving yeast implantation and modulating wine acidity and aroma.

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Sustainability spotlight

The use of ozone (O₃) to sanitize grapes is a sustainable and environmentally friendly technology. Its powerful oxidizing capacity allows the effective elimination of microorganisms without generating toxic waste, as it quickly decomposes into oxygen. This feature significantly reduces the need for chemicals and minimizes the environmental impact of the wine industry. Furthermore, the implementation of ozonation systems requires minimal infrastructure and does not require additional products for cleaning or maintenance, which simplifies its application and reduces the consumption of resources. Its use contributes directly to the United Nations Sustainable Development Goals (SDGs), in particular Goal 12 (responsible production and consumption) and Goal 13 (climate action) by reducing the use of chemicals and the environmental footprint of wine production. Moreover, the application of *Lachancea thermotolerans* in winemaking presents an innovative biotechnological strategy for the sustainable acidification of wine. This non-*Saccharomyces* yeast naturally produces significant amounts of L-lactic acid during alcoholic fermentation, which leads to a controlled reduction of pH and an increase in total acidity. Unlike traditional acidification methods, which often rely on the addition of exogenous acids or chemical agents, *L. thermotolerans* allows for a more sustainable alternative. Furthermore, bioacidification through *L. thermotolerans* aligns with sustainable production goals by decreasing chemical inputs and improving wine stability under conditions of overripe grapes and low acidity induced by climate change.

Introduction

In recent years, there has been an increasing interest in the use of non-*Saccharomyces* yeasts to improve the quality and complexity of wines.¹ In addition to this, there is a trend occurring in hot climates, where wines often have excess ethanol but lack acidity. The yeast *Lachancea thermotolerans* offers a solution to this problem, as it is capable of partially converting sugars into lactic acid during alcoholic fermentation.² The reduction in pH occurs naturally during fermentation and avoids the addition of tartaric acid or the use of resins,

which are very effective in reducing pH, but have undesirable effects on wine quality. By consuming sugars for the production of lactic acid, this yeast also contributes to a slight reduction in the alcohol content of the wine.³

Due to this natural acidification capacity, several trials have been done to determine the potential of *L. thermotolerans* in winemaking. It has been shown that both pure and mixed fermentations with *Saccharomyces cerevisiae* not only improved acidity, reducing pH by 0.3 or even more than 0.5 units from an initial pH of 3.8–4 (ref. 3 and 4) but also reducing volatile acidity and increasing the concentration of beneficial aromatic compounds, such as 2-phenyl ethanol.⁵

However, many non-*Saccharomyces* yeasts have limited fermentative capacity; in the case of *L. thermotolerans*, pure cultures have reported a moderate fermentative power with 38.8–54.73 g of residual unfermented sugar and ethanol production of 7.58–10.46% v/v.^{1,6} Furthermore, they also exhibit

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Fig. 1 Experimental procedure and sample codes for the application of different ozonation treatments and their subsequent inoculation.

Evolution of lactic acid and pH during alcoholic fermentation

To measure lactic acid concentration, daily samples were taken from each of the fermentations in order to monitor the production of this compound. The analysis was made with a Y25 multienzymatic analyzer (BioSystems, Barcelona, Spain), and the method of analysis is based on the use of the enzyme lactate dehydrogenase.

For pH, a portable pH meter METRIA model M22 (Labbox Labware S.L., Premià de Dalt, Spain) was used to make an initial measurement before inoculation and a final measurement when the fermentations were finished. This made it possible to calculate the pH variation throughout the process.

Fermentative volatile analysis

After finishing the fermentations, samples were taken from all of them to analyze the concentration of different volatile compounds present using gas chromatography coupled to a flame ionization detector (GC-FID) with Agilent Technologies 6850 equipment (J&W Scientific, Folsom, CA, USA) according to the method described by ref. 23. Before being analyzed, the samples were filtered using a 0.45 μm membrane. After this, 100 μL of internal standard (4-methyl-2-pentanol) was added to 1 mL of each filtered sample. A DB-624 column (60 m \times 250 μm \times 1.40 μm) with a 1 : 10 split was used to separate the different compounds for further analysis. The temperature program started with an initial value of 40 $^{\circ}\text{C}$ followed by an increase of 10 $^{\circ}\text{C}$ per minute until reaching 250 $^{\circ}\text{C}$ which was maintained for 5 minutes. The gas used as the mobile phase was hydrogen with a flow rate of 2.2 mL min^{-1} . The detector was programmed at a temperature of 300 $^{\circ}\text{C}$ and allowed the quantification of the following compounds: acetaldehyde, methanol, 1-propanol, diacetyl, ethyl acetate, isobutanol, acetoin, 3-methyl-1-butanol, 2-methyl-1-butanol, isobutyl acetate, ethyl butyrate, 2,3-

butanediol, 2-phenylethyl alcohol and 2-phenylethyl acetate. All these compounds were previously calibrated in the equipment.

Statistical analysis

All calculations, including means, standard deviations and other statistics, were obtained using Rstudio software (Posit, PBC, Boston, USA). Analysis of variance (ANOVA) was used to evaluate the effects of the different factors and to determine if they were significant. In cases where significant differences were observed, pairwise comparisons were performed to determine the level of significance among the different factors. Principal Component Analysis (PCA), which was applied to the analysis of volatiles, was obtained using XLSTAT software (Addinsoft, Paris, France), which provides the corresponding graph with all the information necessary for its interpretation.

Results and discussion

Plate count

A remarkable reduction of the initial yeast population was observed after applying both treatments, since the plate count of the control samples showed a population higher than 5×10^4 CFU mL^{-1} compared to 9×10^3 CFU mL^{-1} and 5×10^3 CFU mL^{-1} for O_3L and O_3G , respectively. This indicates that with the ozonation methodology applied, it was possible to reduce the yeast population of the grapes by an order of magnitude.

The reduction of the yeast population using ozonation treatments has been previously studied, obtaining decreases of around 0.5–1 log CFU mL^{-1} of the initial population in the grape.^{14,24} A similar result was observed in this study, with the decrease in the O_3G treatment being 1 log CFU mL^{-1} while in the case of O_3L it was smaller, being only 0.6 log CFU mL^{-1} . The variability present in the different methods may be due to the fact that, as previously described by other authors, the efficiency of ozone can change taking into account different factors such as the species and strain of organisms in the grape, the density of the microbiota or the methodology used for ozone application.^{13,25} A recent study on the application of gaseous ozone on this same grape variety, Red Globe, also highlights the potential of this treatment to be used as a safe and effective fungicide.²⁶

Fermentative kinetics

All fermentations were completed without difficulty in 8 days, consuming all the sugars present in the starting must. It can be observed that, especially in the case of the *L. thermotolerans* yeast, the sugar consumption of the control sample during the initial days was less pronounced than in the case of the O_3G and O_3L treated samples (Fig. 2). This could be the first indicator that the ozone treatment has facilitated the implantation of this yeast. This effect is likely related to the higher initial microbial load in the control grapes, which did not undergo any sanitizing treatment. Such microbial competition can hinder the proliferation and implantation of *L. thermotolerans*, resulting in slower sugar consumption. In contrast, *S. cerevisiae* is less affected due to its greater fermentative capacity, which allows it to grow efficiently even under these conditions.





Fig. 3 Evolution of lactic acid concentration (g L^{-1}) during fermentation. The figure shows the average obtained for the different treatments (C, O₃G and O₃L) grouped according to the concentration of the added inoculum of *L. thermotolerans* (Lt) or *S. cerevisiae* (Sc) (5, 10 and 20 g hL^{-1}). The error bars correspond to the standard deviation associated with each average.

A more detailed analysis of the final lactic acid concentration data confirms the trend previously described in relation to lactic acid evolution (Fig. 3). A significant increase in lactic acid

production is detected in all the samples treated with ozone, both O₃G and O₃L, in comparison with the control samples (Fig. 4). After calculating the percentage increase in lactic acid



Fig. 4 Final concentration of lactic acid (g L^{-1}). The figure shows the mean obtained for the different treatments (C, O₃G and O₃L) grouped according to the concentration of the added *L. thermotolerans* inoculum (5, 10 and 20 g hL^{-1}). The error bars correspond to the standard deviation associated with each average. The asterisks indicate the presence of significant differences between treatments: *, ** and *** indicate the significance at $P < 0.05$, 0.01 and 0.001 respectively.





Fig. 5 Final pH. The figure shows the mean obtained for the different treatments (C, O₃G and O₃L) grouped according to the concentration of the *L. thermotolerans* (Lt) inoculum added (5, 10 and 20 g hL⁻¹). The error bars correspond to the standard deviation associated with each mean. Asterisks indicate the presence of significant differences between treatments: *, ** and *** indicate the significance at $P < 0.05$, 0.01 and 0.001 respectively.

production with respect to the control samples, it can be seen that, for the O₃G treatment, production is 62.7%, 22.3% and 18.6% higher in the samples with an inoculum of 5, 10 and 20 g hL⁻¹, respectively. In the case of the O₃L treatment, the difference is even more noticeable, reaching an increase of 82.3%, 36.7% and 26.3% for the inoculum concentrations of 5, 10 and 20 g hL⁻¹, respectively. It should be noted that the difference between samples C with respect to O₃G and O₃L progressively decreases as the concentration of the *L. thermotolerans* inoculum increases.

The production of lactic acid indicates the presence of *L. thermotolerans* since it is a compound characteristic of its metabolism. This formation of lactic acid is produced from pyruvate in the glycolytic metabolism of sugars in which the enzyme lactate dehydrogenase is involved. The acidification occurs at the beginning of the alcoholic fermentation, at 3–5 days of fermentation.²⁷ Its production range can oscillate to around 1–16 g L⁻¹ of concentration in wine depending on the strain used.²⁸ All these data are consistent with those obtained in this study and allow correlating the concentration of lactic acid present in the samples with the degree of *L. thermotolerans* implantation: the highest production of lactic acid occurred during the first 4 days of fermentation (Fig. 3) and the final concentration reached 4.15–8.85 g L⁻¹ (Fig. 4).

As mentioned previously, significant differences were recorded between the control and treatment groups, indicating that ozonation facilitated the implantation of *L. thermotolerans*, allowing for greater production of lactic acid during

fermentation. However, it should also be considered that this difference was not similar for all the inoculum concentrations used. Bigger differences were observed in the samples with the lowest inoculum concentrations, which confirms that the treatments had a clear influence on yeast implantation. This means that even under the most unfavourable conditions (when there was a lower ratio of inoculum to the yeasts that could be present in the original grapes), it was possible to achieve lactic acid concentrations similar to those of the samples with the highest inoculum concentrations.

pH

For all samples in which *L. thermotolerans* was inoculated, there was a decrease in the pH of the wine compared to the measurements conducted initially before fermentation. These values correlate with the lactic acid concentrations observed in these fermentations (Fig. 4). Furthermore, this decrease showed significant differences when comparing the pH values of the O₃G and O₃L samples with the C samples, being higher in the treatment samples (Fig. 5). An influence of the concentration of the added inoculum is also observed: for both treatments, the difference in acidification, when compared with the C samples, is more pronounced when the concentration of the added inoculum is lower. This difference progressively reduces as the concentration of inoculum increases. For the O₃G treatment, acidification is 28.7% higher for a concentration of 5 g hL⁻¹ of inoculum, 17.9% higher for 10 g hL⁻¹ and 15% higher for 20 g



Table 2 Average concentration of volatile compounds (mg L^{-1}) present in the samples together with their standard deviation. The letters associated with each value come from the statistical analysis; different letters indicate significant differences for $P < 0.05$. The results have been divided into three different tables according to the concentration of inoculum used (5, 10 and 20 g hL^{-1}). Within each table they are in turn divided according to the yeast inoculated (Lt and Sc) and the treatment used (C, O₃G and O₃L)

	5 g hL^{-1}					
	Lt			Sc		
	C	O ₃ G	O ₃ L	C	O ₃ G	O ₃ L
Methanol	38.60 ± 4.85ab	38.71 ± 4.01ab	12.60 ± 3.26b	37.77 ± 4.48ab	34.88 ± 1.69ab	40.87 ± 22.00a
1-Propanol	99.04 ± 4.39a	82.33 ± 10.46a	80.40 ± 20.66a	79.11 ± 5.20a	95.98 ± 4.00a	89.15 ± 12.53a
Diacetyl	0.00 ± 0.00b	1.87 ± 0.44a	1.45 ± 0.08ab	0.00 ± 0.00b	0.97 ± 0.84ab	1.53 ± 1.35ab
Ethyl acetate	53.15 ± 3.18a	29.79 ± 3.93a	37.67 ± 19.91a	59.50 ± 17.88a	44.52 ± 13.70a	62.68 ± 15.91a
2-Butanol	nd	nd	nd	nd	nd	nd
Isobutanol	30.35 ± 0.64b	38.11 ± 1.75a	36.68 ± 0.54a	18.93 ± 0.46c	19.90 ± 1.17c	20.79 ± 1.63c
1-Butanol	3.20 ± 5.54a	1.52 ± 2.63a	4.79 ± 0.49a	4.20 ± 0.27a	5.24 ± 0.25a	3.96 ± 0.17a
Acetoin	42.11 ± 23.36a	27.26 ± 24.82a	17.10 ± 17.90a	15.54 ± 8.24a	10.05 ± 0.84a	12.33 ± 13.58a
3-Methyl-1-butanol	137.38 ± 22.38a	132.48 ± 29.05a	94.40 ± 8.15a	97.47 ± 3.09a	99.02 ± 3.87a	95.83 ± 9.08a
2-Methyl-1-butanol	34.35 ± 1.32bc	37.39 ± 2.78abc	29.13 ± 2.81c	39.99 ± 3.53ab	45.39 ± 3.85a	34.59 ± 3.45ac
Isobutyl acetate	nd	1.26 ± 2.19a	1.87 ± 3.23a	nd	3.75 ± 3.43a	1.91 ± 3.31a
Ethyl butyrate	nd	nd	nd	1.68 ± 0.15a	nd	nd
2,3-Butanediol	371.84 ± 16.83a	328.32 ± 32.66a	279.04 ± 45.77a	648.74 ± 114.77a	674.67 ± 60.81a	514.25 ± 44.64a
Hexanol	nd	nd	nd	nd	nd	nd
2-Phenyl ethanol	10.89 ± 0.36b	15.20 ± 6.90ab	22.69 ± 0.41a	9.46 ± 0.07b	9.82 ± 0.21b	13.42 ± 4.52b
2-Phenylethyl acetate	4.73 ± 0.01b	4.87 ± 0.19b	5.86 ± 0.03a	nd	5.03 ± 0.04b	5.01 ± 0.31b
Esters	57.87 ± 3.18a	35.92 ± 5.40a	45.40 ± 16.67a	61.18 ± 17.73a	53.30 ± 12.69a	69.60 ± 13.08a
Higher alcohols	315.21 ± 31.50a	307.01 ± 30.50ab	268.09 ± 30.36ab	249.17 ± 5.86b	275.35 ± 8.75ab	257.74 ± 5.42ab
Total volatiles	882.03 ± 78.15bc	804.95 ± 29.16c	713.29.12 ± 46.78c	1065.98 ± 55.40a	1067.89 ± 49.74a	1017.94 ± 106.05ab
	10 g hL^{-1}					
	Lt			Sc		
	C	O ₃ G	O ₃ L	C	O ₃ G	O ₃ L
Methanol	35.67 ± 1.36ab	26.35 ± 2.29bc	16.06 ± 2.30c	42.66 ± 5.35a	39.66 ± 10.07ab	49.85 ± 6.47a
1-Propanol	97.83 ± 2.26a	80.17 ± 13.29a	80.02 ± 11.52a	82.54 ± 8.36a	107.07 ± 9.11a	90.93 ± 10.87a
Diacetyl	1.42 ± 0.01a	0.51 ± 0.88ab	nd	nd	0.94 ± 0.81ab	nd
Ethyl acetate	41.33 ± 6.43a	33.24 ± 11.86a	32.55 ± 9.48a	57.91 ± 30.23a	57.31 ± 21.15a	54.54 ± 18.80a
2-Butanol	nd	nd	nd	nd	nd	nd
Isobutanol	27.11 ± 0.31b	40.27 ± 5.62a	38.47 ± 5.15a	21.07 ± 0.76b	22.91 ± 1.96b	22.37 ± 2.34b
1-Butanol	5.08 ± 0.33a	4.28 ± 0.44a	4.45 ± 0.18a	4.25 ± 0.23a	1.58 ± 0.08a	4.46 ± 0.56a
Acetoin	19.43 ± 16.98a	55.00 ± 38.43a	27.79 ± 28.82a	11.27 ± 1.85a	33.05 ± 27.41a	19.09 ± 12.37a
3-Methyl-1-butanol	123.52 ± 4.30a	109.78 ± 11.81ab	100.24 ± 2.20ab	101.93 ± 2.12ab	108.03 ± 14.57ab	92.38 ± 8.30b
2-Methyl-1-butanol	35.38 ± 4.77ab	36.96 ± 2.81ab	29.25 ± 5.22b	37.58 ± 0.76ab	34.78 ± 1.71ab	40.46 ± 1.44a
Isobutyl acetate	3.16 ± 2.76a	0.70 ± 1.21a	nd	nd	nd	nd
Ethyl butyrate	nd	0.44 ± 0.76a	nd	nd	nd	nd
2,3-Butanediol	308.96 ± 39.13a	263.93 ± 47.92a	292.25 ± 34.84a	573.78 ± 99.53a	622.62 ± 68.15a	627.40 ± 92.08a
Hexanol	1.71 ± 2.96a	nd	nd	nd	nd	nd
2-Phenyl ethanol	10.91 ± 0.11bcd	17.90 ± 6.18ab	21.21 ± 1.02a	9.94 ± 0.11d	14.43 ± 0.60cd	17.30 ± 1.23abc
2-Phenylethyl acetate	4.77 ± 0.03a	4.95 ± 0.13a	6.08 ± 0.36a	4.73 ± 0.01a	4.82 ± 0.07a	3.45 ± 3.02a
Esters	49.26 ± 5.79a	39.32 ± 12.89a	38.62 ± 9.52a	62.65 ± 30.24a	62.13 ± 21.11a	57.99 ± 15.79a
Higher alcohols	301.55 ± 12.41a	289.36 ± 30.89a	273.64 ± 15.09a	257.32 ± 10.77a	288.80 ± 20.85a	267.91 ± 5.54a
Total volatiles	778.40 ± 82.70b	723.89 ± 38.94b	752.48 ± 78.43b	1067.06 ± 36.02a	1083.02 ± 121.92a	1059.59 ± 113.66a
	20 g hL^{-1}					
	Lt			Sc		
	C	O ₃ G	O ₃ L	C	O ₃ G	O ₃ L
Methanol	38.84 ± 4.12bc	35.22 ± 3.36bc	48.26 ± 5.22b	64.44 ± 9.50a	33.02 ± 7.47bc	29.06 ± 1.19c
1-Propanol	115.00 ± 8.05a	81.13 ± 5.94c	75.14 ± 6.29c	86.39 ± 9.20bc	103.19 ± 7.18ab	104.11 ± 3.92ab
Diacetyl	nd	2.02 ± 0.26a	1.64 ± 0.30ab	1.39 ± 0.00ab	0.95 ± 0.83bc	1.47 ± 0.05ab
Ethyl acetate	40.69 ± 6.52ab	34.92 ± 11.07ab	27.39 ± 4.68b	58.04 ± 19.92a	47.72 ± 8.79ab	57.60 ± 7.26a
2-Butanol	nd	0.83 ± 1.44b	2.80 ± 0.55a	nd	nd	nd



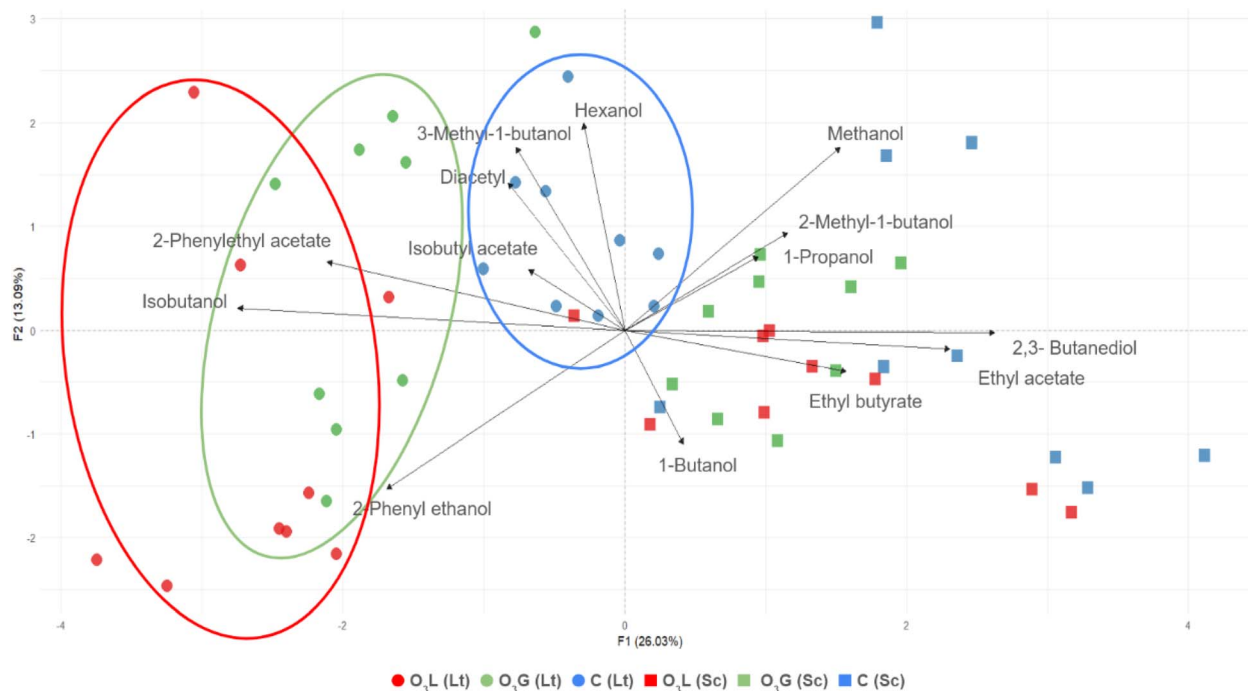


Fig. 6 Principal Component Analysis (PCA) of the values obtained for the volatile aromas present at the end of fermentation. The figure shows the samples from the different treatments (C, O₃G and O₃L) of both *S. cerevisiae* (Sc) and *L. thermotolerans* (Lt) together with the active variables that characterize each of the axes. In addition, the Lt samples have been manually grouped according to the treatment used.

concentration of the inoculum used, the greater the difference between these samples.

Based on the aroma data mentioned above, a Principal Component Analysis (PCA) was performed. Due to the lack of significant differences, the axes only explain 39.12% of the variability but still allow the samples to be grouped according to the yeast inoculated and the treatment used (Fig. 6). Of this variability, 26.03% is attributed to the first component (F1) and the remaining 13.09% to the second component (F2).

First, all the inoculations carried out with *S. cerevisiae* are in the positive values of the first component, so compounds such as 2,3-butanediol and ethyl acetate are more representative of these samples, while, in the case of *L. thermotolerans*, they would be more related to isobutanol and 2-phenylethyl acetate. Furthermore, within the *L. thermotolerans* samples, they can be grouped according to the treatment applied. The C samples are clearly separated from the O₃L samples, although in the case of O₃G, it overlaps with the other treatment and it is closer to the C samples, remaining in an intermediate position. Thus, for the C samples, being more grouped in the center of the axes, the values obtained were more similar to each other and close to the general average of all the samples. Instead, in the case of both treatments (particularly for O₃L), they are further away from the center and more dispersed from each other, which indicates greater variability with respect to the rest of the sample groups and also greater internal variability.

Although the aromatic profiles do not present significant differences, the samples can be clearly divided according to the yeast inoculated, which could be another indication of the

successful implantation of these yeasts. In addition, the possibility of grouping the *L. thermotolerans* samples according to the treatment applied shows that ozonation has had an influence on the production of certain compounds.

Conclusions

Ozone has proven to be an effective method for sanitizing grapes, both in its gaseous form and in aqueous solution, significantly reducing the population of microorganisms present on the surface of the grapes. In addition, its application has favored the implantation of the yeast *Lachancea thermotolerans*.

All the parameters analyzed indicate that ozonation has facilitated the implantation of the inoculated yeasts, allowing, in the case of *L. thermotolerans*, the wines obtained from their fermentations to show characteristics specific of this yeast, such as acidification or the presence of certain aromatic compounds such as 2-phenyl ethanol.

Another relevant aspect was the impact of inoculum concentration, as the lower the concentration of yeast inoculated, the greater the differences between the controls and the ozone treatments. This proves that both treatments allow for successful yeast implantation, even under unfavorable conditions.

These results indicate that ozonation has the potential to be considered a viable alternative to sulphitation, especially when used with non-*Saccharomyces* yeasts. However, future research should focus on comparing its effectiveness with other



sanitization methods and on evaluating its scaling under winery conditions.

This work provides new evidence that ozone treatments, both in gaseous and aqueous forms, effectively sanitize grapes while significantly facilitating the implantation and metabolic activity of *L. thermotolerans* during fermentation. The study demonstrates that the impact of ozone is particularly pronounced at lower yeast inoculum concentrations, where significant differences were observed between ozone-treated and control samples. Compared to traditional sulphitation, ozone emerges as a promising alternative sanitization method, especially in combination with non-*Saccharomyces* yeasts to enhance wine acidity and aroma profiles. By linking ozone sanitization directly with yeast ecology and inoculum-dependent implantation dynamics, these findings expand current understanding and offer valuable directions for more sustainable and targeted microbial management practices in the wine industry. Future research should further compare ozone with other sanitization approaches and assess scalability under real winery conditions.

Author contributions

Yaiza Rodríguez: writing – original draft, investigation. Juan Manuel del Fresno: writing – review & supervision. Carmen González: writing – review & editing. María Antonia Bañuelos: writing – review & editing. Antonio Morata: writing – review & editing, validation, project administration, methodology, funding acquisition, conceptualization.

Conflicts of interest

There are no conflicts to declare.

Data availability

The authors declare that most of the main data are included in the tables and figures of the article. Some of the raw data as Excel files or statistic files will be available upon request.

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