OENO One vine & wine

DOI: https://doi.org/10.20870/oeno-one.2023.57.3.7376

OPEN ACCESS

ORIGINAL RESEARCH ARTICLE

Biological acidification of "*Vino Santo di Gambellara*" by mixed fermentation of *L. thermotolerans* and *S. cerevisiae*. Role of nitrogen in the evolution of fermentation and aroma profile

Adelaide Gallo[1], Raffaele Guzzon[1*], Marco Ongaro[2], Mauro Paolini[1], Tiziana Nardin[1], Mario Malacarne[1], Tomas Roman[1], and Roberto Larcher[1]

[1] Fondazione Edmund Mach, Centro di Trasferimento Tecnologico. Via Mach 1. 38010 San Michele all'Adige, Trento, Italy

[2] Università di Trento, Dipartimento C3A. Via Mach 1. 38010 San Michele all'Adige, Trento, Italy

*correspondence:
raffaele.guzzon@fmach.it

Associate editor:
Patricia Taillandier

Received:
2 February 2023

Accepted:
17 July 2023

Published:
29 August 2023

This article is published under the **Creative Commons licence** (CC BY 4.0).

Use of all or part of the content of this article must mention the authors, the year of publication, the title, the name of the journal, the volume, the pages and the DOI in compliance with the information given above.

ABSTRACT

In this work, *Lachancea thermotolerans* was exploited as a biological acidifier of Vino Santo di Gambellara, a traditional Italian wine made from long-dried grapes. The drying of grapes before winemaking is a traditional technique widely applied in Italy in the production of sweet wines, but in recent years, global warming reduces dramatically the acidity of grapes, causing microbial instability during drying and winemaking. The ability of *L. thermotolerans* to convert sugars in lactic acid was already applied in the acidification of red wines, but the peculiar features of dried grape (e.g., osmotic stress, lack of nutrients, presence of mould-derived toxic compounds during drying) impose specific tests. *L. thermotolerans* was employed in sequential fermentation in combination with a strain of *Saccharomyces cerevisiae*. Considering the lack of information about the nutritional requirement of the non-*Saccharomyces* yeast, three protocols of nitrogen supplementation (mineral, organic and organic at high dosage) were tested. Alcoholic fermentation experiments were followed by plate counts onto differential media to discriminate between the two yeast species and by chemical analysis. Moreover, a GC-MS-MS approach carried out a complete characterisation of the volatile profile of wines. Results evidenced a long permanence of *L. thermotolerans* during alcoholic fermentation, which remained over the 7 log units until the 14th day of fermentation. The nitrogen supplementation protocol influenced cell growth and fermentative activity. Inorganic nitrogen supplementations allowed the accomplishment of alcoholic fermentation and the maintenance of pH below 3.35, with respect to the control wine (made only by *S. cerevisiae*), which was over pH 3.50. *L. thermotolerans* also influenced the wine's volatile aroma profile. Statistical differences were found in the main families of the yeast-derived aroma: acetate, esters, lactates, fatty acids and C_6 compounds.

KEYWORDS: *L. thermotolerans*, straw wine, Vino Santo, yeast assimilable nitrogen (YAN), lactic acid, mixed fermentation, wine acidification.

INTRODUCTION

The progressive southernisation of the climate is a fact in many European wine regions. The responses to this phenomenon have been various, the evolution of agronomic practices, the use of different cultivars of *Vitis vinifera* or winemaking strategies aimed at minimising the impact of climate change on wine composition (Ollat *et al*., 2016). However, there are wines more sensitive to sudden climatic changes because of having an ancient tradition, refractory to changes due to the artisanal productive process or their peculiar organoleptic profile.

The production of Vino Santo, sweet wines obtained from post-harvest dried grapes and subject to a prolonged oxidative ageing in little casks called “Caratelli”, is traditional in some Italian regions: Tuscany (Domizio and Lencioni, 2011), Emilia Romagna (Laureati *et al.,* 2020), Veneto (Torriani *et al.,* 2011) and Trento province (Guzzon *et al.,* 2014). Although peculiarities related to the different regional traditions, there are some common traits in the production and in the organoleptic profile of the different Vino Santo. Grapes of traditional Italian white varieties, the most employed are Trebbiano, Garganega, Malvasia di Candia or Nosiola, are chosen among sound bunches and dried in the attic of the winery, in a ventilated room called “Vinsantaia” (Laureati *et al.,* 2020). To favour the water evaporation and avoid the growth of grey mould (*Botrytis cinerea*), grapes are conserved in small, perforated boxes, racks or hanging from vertical nets for 3–4 months, reaching a sugars concentration of 35–40 % g/g (Domizio and Lencioni, 2011). Traditionally spontaneous alcoholic fermentation takes place after grape pressing by a consortium of *Saccharomyces* and non-*Saccharomyces* yeasts (Stefanini *et al.,* 2016) that colonises the Caratelli. Alcoholic fermentation stops naturally for the simultaneous presence of several limiting factors, such as volatile acidity, osmotic stress, high ethanol content and lack of nutrients (Guaragnella & Bettiga, 2021), and the Vino Santo ages some years (4–10 years) in barrels under oxidative conditions because the “Caratelli” are not fully filled. This sweet wine typically has an alcohol degree of up to 14 % (v/v) and a residual sugar content between 50 and 100 g/L (Laureati *et al.,* 2020).

The organoleptic equilibrium and the durability of “Vino Santo” are based on maintaining an adequate acidity that regulates microbial evolution during grape drying and through the winemaking process. The acidity of Vino Santo cannot be represented only by acetic acid, accumulated by yeast due to osmotic stress (Erasmus *et al.,* 2004; Bely *et al.,* 2003) because the wine would be unbalanced. To obtain a high-quality Vino Santo, it is necessary to maintain a composite acidic profile based on the contribution of tartaric, lactic and succinic acids. The control of acetic acid accumulation can be done in many ways. Apart from careful hygiene and the use of osmotolerant yeast strains, the nitrogen supplementation of grapes must play a key role. Bely *et al*. (2008) demonstrated that the production of volatile acidity is inversely correlated with cell concentration during fermentation. High nitrogen availability in the first stage of winemaking is essential because it stimulates cell growth, improves the redox-equilibrating process, and consequently reduces volatile acidity formation.

Despite Vino Santo’s capability to age for a very long time (Domizio and Lencioni, 2011), there are numerous microorganisms able to spoil Vino Santo. Moulds, already during grape dying (Guzzon *et al.,* 2018; Lorenzini *et al.,* 2013), acetic or lactic bacteria and some kinds of non-*Saccharomyces* yeasts can create organoleptic faults, interfere with fermentation and accumulate toxic molecules. The artisanal production of the Vino Santo, as well as a peculiar organoleptic profile, discourage relevant changes in the production process. Therefore, it is necessary to resolve the main critical issues of this wine-producing process in a discreet and sustainable way; in this sense, a relevant contribution can be given using non-*Saccharomyces* yeasts (Mateo and Maicas, 2016).

Lachancea thermotolerans is a yeast widespread in the oenological environment, surviving up to 13 % (v/v) of ethanol (Morata *et al.,* 2018). As with other non-*Saccharomyces* yeasts, it is common to combine this yeast with a co-starter, generally a *Saccharomyces cerevisiae* strain. The timing and ratio of *S. cerevisiae* inoculum regulate the activity of *L. thermotolerans*, thanks to different phenomena of antagonism: cell–cell contact mechanisms, secretion of antimicrobial peptides and mere competition for nutritional sources (Nissen and Arneborg, 2003; Kemsawasd *et al.,* 2015). There are two features of *L. thermotolerans* that arouse interest in the production of straw wines. The main is the production of L(+)-lactic acid, already during alcoholic fermentation (Banilas *et al.,* 2016; Hranilovic *et al.,* 2018; Morata *et al.,* 2018). With oxygen deficiency, typical of alcoholic fermentation, *L. thermotolerans* activates genes involved in the pentose phosphate pathway and the citric acid cycle. However, in opposition to what occurs in *S. cerevisiae*, the hyperactivation of genes encoding for enzymes of the lactate dehydrogenase class (LDH) was observed which allows the production of lactic acid from glucose and fructose. It can be assumed that the high expression of these genes is linked to the lack of production of alcohol dehydrogenase enzymes (only two genes involved are expressed, compared to seven of the other yeasts); therefore, the production of lactic acid is an alternative way for the reduction of acetaldehyde to ethanol to maintain intracellular redox balance, in the absence of oxygen (Gatto *et al*., 2020; Vicente *et al*., 2021). The highest accumulation of lactic acid so far reported reached 16 g/L, the maximum observed among non-genetically modified yeasts (Banilas *et al.,* 2016). The acidification induced by *L. thermotolerans* positively affects the microbiological stability of the wine and the organoleptic balance, avoiding alterations or exogenous acidifications. Another characteristic of grapes subjected to withering is the excessive sugar content, which leads to high concentrations of ethanol and the massive production of fermentation bioproducts, such as acetic acid, which is a symptom

of the osmotic stress of common winemaking yeasts. Several studies (Comitini *et al.,* 2011; Gobbi *et al.,* 2013; Morata *et al.,* 2019; Sgouros *et al.,* 2020) have reported significantly lower ethanol contents in co-fermentations with *L. thermotolerans* and *S. cerevisiae* with reductions ranging from 0.2 % to 0.9 % (v/v). Other interesting aspects are the increase in the concentration of glycerol, the decrease in the acetic acid content and a peculiar modulation of the volatile compounds in the wine.

In this work, we evaluated for the first time the use of this yeast in the production of Vino Santo di Gambellara, a traditional sweet wine produced in the Veneto region. The evolution of sequential fermentation made by combining *L. thermotolerans* and *S. cerevisiae* was followed by microbiological and chemical determinations, also evaluating the aromatic profile of obtained wines by a comprehensive GC-MS-MS approach. Among the different technological variables, particular attention was paid to the nutrition protocol furnished to the yeasts, considering the need to increase the knowledge regarding the nutritional requirements of non-*Saccharomyces* yeasts employed in oenology.

MATERIALS AND METHODS

1. Winemaking protocol and nitrogen supplementation schema

According to the traditional winemaking of Vino Santo, grapes of *cv.* Garganega were hand-harvested in small boxes of about 25 kg, then further transferred on grids in a ventilated attic to allow a natural drying of bunches. During the period of grape drying, the temperature remained in the interval between 5 and 15 °C, and the relative humidity of the atmosphere was 80±30 %. After drying, grapes were transferred to the experimental winery of the Edmund Mach Foundation (Italy) and further crushed using a vertical pneumatic press (Alfa 40, Polisnelli Enologia, Italy). Grape must was statically decanted at 10 °C for 48 h, adding pectolytic enzyme (10 mg L^{-1}, Rapidase Clear Extreme, Oenobrands, France) and potassium metabisulfite salt (30 mg L^{-1}, Lafood, Italy). After decanting, the grape must was subdivided into aliquots to perform the experimental plan detailed in Table 1. For alcoholic fermentation starters, two Active Dry Yeast strains were employed at an inoculum dose of 250 mg L^{-1}: *S. cerevisiae var. bayanus* (PDM, Oenobrands) and *L. thermotolerans* (Laktia™, Lallemand Inc, Canada). Active dry yeast was inoculated in grape must after rehydration according to the OIV standards (OIV, 2023a). Nitrogen supplementation of grape must was performed by two different products, a source of organic nitrogen (Natuferm Bright, Oenobrands) and diammonium phosphate salt (Oenobrands). Table 1 lists the amount of nitrogen furnished at each trial and the timing of supplementation; each trial was performed in triplicate. Alcoholic fermentation occurred in stainless steel containers of 10 L of volume at a temperature of 21±2 °C. After fermentation, wines were decanted and stored at 4±2 °C in stainless steel kegs with minimal ullage filled by argon until analysis.

2. Microbiological analysis and monitoring of alcoholic fermentation

Microbiological analyses were performed following the OIV methods (OIV, 2023b). Total yeasts were enumerated onto Wallerstein Laboratory agar medium (WL Agar, Oxoid, UK), while non-*Saccharomyces* yeasts, including *L. thermotolerans,* were differentiated using Agar Lysine Medium (Oxoid). Both samples were incubated aerobically at 25±2 °C for 3 days. The amount of live and dead cells in active dry yeast samples was determined by flow cytometry analysis using a Cube6 apparatus (Sysmex, Germany) coupled with a live & dead staining of samples, according to Guzzon and Larcher (2015). Flow cytometry analyses revealed that the *S. cerevisiae* var. *bayanus* employed in this work has a concentration of live cells of $9.8 \pm 1.1 \times 10^9$ cell/g, while the strain of *L. thermotolerans* showed a concentration of live cells of $2.5 \pm 2.1 \times 10^{10}$ cell/g. Dead cells in both cases resulted below the 10^9 cell/g. Considering the ratio of dilution due to the inoculum of yeast in wine, the nominal concentration of *S. cerevisiae* at the beginning of fermentation is about 2.3×10^6 cell/mL and 6.3 × 106 cell/mL for *L. thermotolerans.*

TABLE 1. Experimental plan performed in the work to evaluate the potential of *L. thermotolerans* in the acidification of Vino Santo during alcoholic fermentation, in function of different nitrogen supplementation. Each trial was performed in 3 replicates in a volume of about 80 L. Organic nitrogen: Natuferm Bright, Oenobrands; DAP: diammonium phosphate salt.

Experimental trial	*S. cerevisiae*	*L. thermotolerans*	Nitrogen supplementation (Each dose 300 mg L-1)				
			Day 1	Day 5	Day 7	Day 10	Day14
SC	Inoculum at Day 0	NO	Organic N	Organic N	-	-	DAP
LTNM	Inoculum at Day 5	Inoculum Day 0	DAP	DAP	-	-	DAP
LTNO	Inoculum at Day 5	Inoculum Day 0	Organic N	Organic N	-	-	DAP
LTNO+	Inoculum at Day 5	Inoculum Day 0	Organic N ×3	Organic N ×3	Organic N	Organic N	DAP

The evolution of alcoholic fermentation was monitored by measuring the weight loss due to CO_2 evolution and the °Brix with an optical refractometer (ATC, Polsinelli Enologia, Italy). pH and titratable acidity were measured by a pH meter and an automatic titrator (Crison Instruments, Spain). The fermentation rate (V) was calculated as the first derivative of the weight loss expressed as g L^{-1} day^{-1}. At the end of alcoholic fermentation, reducing sugars, ethanol and organic acids were determined by enzymatic assay (Miura One, Exacta-Optech, Italy).

3. GC-MS/MS analysis of wines

The analysis of volatile compounds in wine was performed according to the method reported by Paolini *et al.* (2018). A total of 50 mL of wine was diluted to 100 mL with H_2O milliQ after adding 100 µL of internal standard (n-heptanol), and volatile compounds were extracted by solid-phase extraction (SPE) using ENV+ cartridges. The GC-MS/MS Analysis was performed on an Agilent (California, USA) Intuvo 9000 coupled with an Agilent 7000 Series Triple Quadrupole mass spectrometer working in electron impact (EI) mode at 70 eV. Chromatographic separation was achieved by injecting 2 µL in split mode (1:5) into a DB-Wax Ultra Inert capillary column (20 m, 0.18 mm di × 0.18 µm film thickness) and using helium (He) as carrier gas (0.8 mL min^{-1}). The oven temperature was programmed starting at 40 °C for 2 minutes, increased to 55 °C at 10 °C min^{-1}, then increased to 165 °C at 20 °C min^{-1}, and finally increased to 240 °C at 40 °C min^{-1}, maintaining this temperature for 5 minutes. The mass spectrum was acquired in MRM (multiple reaction monitoring) mode, setting the instrument in a dynamic system. The injector, transfer line and source temperatures were 260 °C, 250 °C and 230 °C, respectively.

4. Amino acid analysis by HPLC

"The quantification of amino acids was performed with the method reported by Gallo *et al*. (2023) by creating o-phthalaldehyde (OPA) adducts. The measurements were carried out using an HPLC 1260 Infinity system from Agilent Technologies (Santa Clara, USA), which had a fluorescence detector (Ex = 336 nm, Em = 445 nm). Separation was accomplished using a Chromolith Performance RP-18e column (100 × 4.6 mm; Merck, Darmstadt, Germany) with a Guard Cartridge Chromolith RP-18e (10 × 4.6 mm; Merck, Darmstadt, Germany) at a temperature of 40 °C. The mobile phase consisted of sodium acetate 0.05 M (pH 6.9; eluent A) and methanol (eluent B). The flow rate was set at 2 mL/min. The analytical gradient used was as follows: 100 % A for 1 minute, 80 % A and 20 % B over 10 minutes, 60 % A and 40 % B over 5 minutes, and 100 % B over 4 minutes, held for 5 minutes. This was followed by a gradient of 90 % A, 10 % B in 0.5 minutes, held for 2 minutes, and 100 % A for 3 minutes. Prior to separation, a 10 µL sample was automatically derivatised with a mixture of OPA (4.5 g/L; Sigma-Aldrich, St. Louis, USA) in sodium tetraborate 0.1 M, adjusted to pH 10.5, methanol (10 %; Sigma-Aldrich, St. Louis, USA), and 2-mercaptoethanol (2 %; Sigma-Aldrich, St. Louis, USA). Agilent OpenLab CDS 3.1 software was used for data acquisition and processing."

5. Statistical treatment of data

The wine data were subjected to a one-way analysis of variance (ANOVA) followed by Tukey's HSD test post-hoc (HSD) in R (R Foundation for Statistical Computing, Austria) version 1.4.1103 in RStudio (RStudio, Massachusetts, USA).

RESULTS

1. Grapes drying and grape must feature.

Table 2 reports the evolution of the main parameters of grapes during withering from November to April. In addition to the acidity (as g L^{-1} of tartaric acid) and sugars concentration (as °Brix), the weight of a berry sample (n = 30) was measured, considering it a good index of the evolution of water loss from bunches. During drying, the sugar concentration inside berries increased by about 42 % in the function of grape drying time and relative humidity, while the temperature of the environment, in the interval of the test, appeared less relevant in driving the evaporation process. The rate of sugar concentration and weight loss followed a nonlinear trend, with two peaks, the first one observed between December and January and the second one before the crushing, from March and April. (Table 2). The total acidity of grapes showed a peculiar trend, with a decrease in the first month and a stabilisation in the final part of the drying process. At the end of withering, grapes were crushed, and grape must was characterised for the main oenological quality control parameters. The total soluble solids level in the grape must was 39.0° Brix, with a pH of 3.35 and titratable acidity equal to 6.01 g L^{-1} (expressed as tartaric acid), yeast-assailable nitrogen was below 40 mg/L.

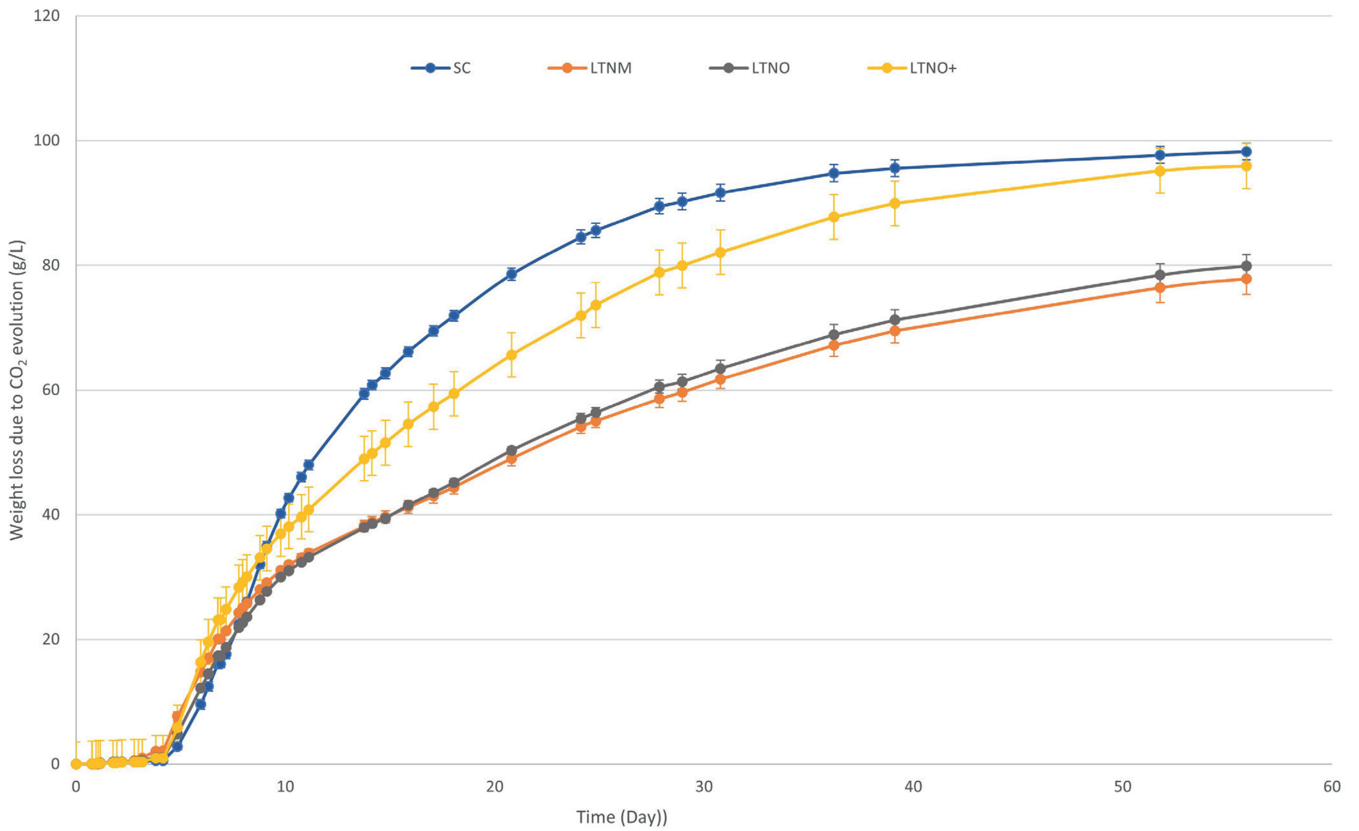
TABLE 2. Evolution of main characters of grapes *cv.* Garganega during natural dying and relative humidity of the environment (Data obtained by analysing a sample of 30 berries randomly sampled).

Sampling data	Sugars content	Total acidity	Weight of 30 berries	Mean berry weight	Relative humidity
Day/month	°Brix	g L^{-1} of tartaric acid	g		%
28/11	20.5	6.5	46.1	1.5	87.0
19/12	21.0	6.1	49.0	1.6	83.5
7/01	24.0	6.8	36.8	1.2	80.0
27/01	23.5	7.1	32.1	1.4	76.5
18/02	25.0	7.6	33.8	1.1	67.5
06/03	26.2	8.0	35.5	1.2	63.0
20/03	27.1	7.8	35.0	1.2	57.5
09/04	34.0	8.0	31.2	0.9	48.0

2. Evolution of alcoholic fermentation and microbial populations

Considering the importance of nitrogen supplementation in the experimental plan, the characterisation of the two nitrogen preparations employed was performed. For diammonium phosphate salt, we estimated that the addiction of 300 mg/L corresponds to about 60 mg N/L; regarding organic

A



B

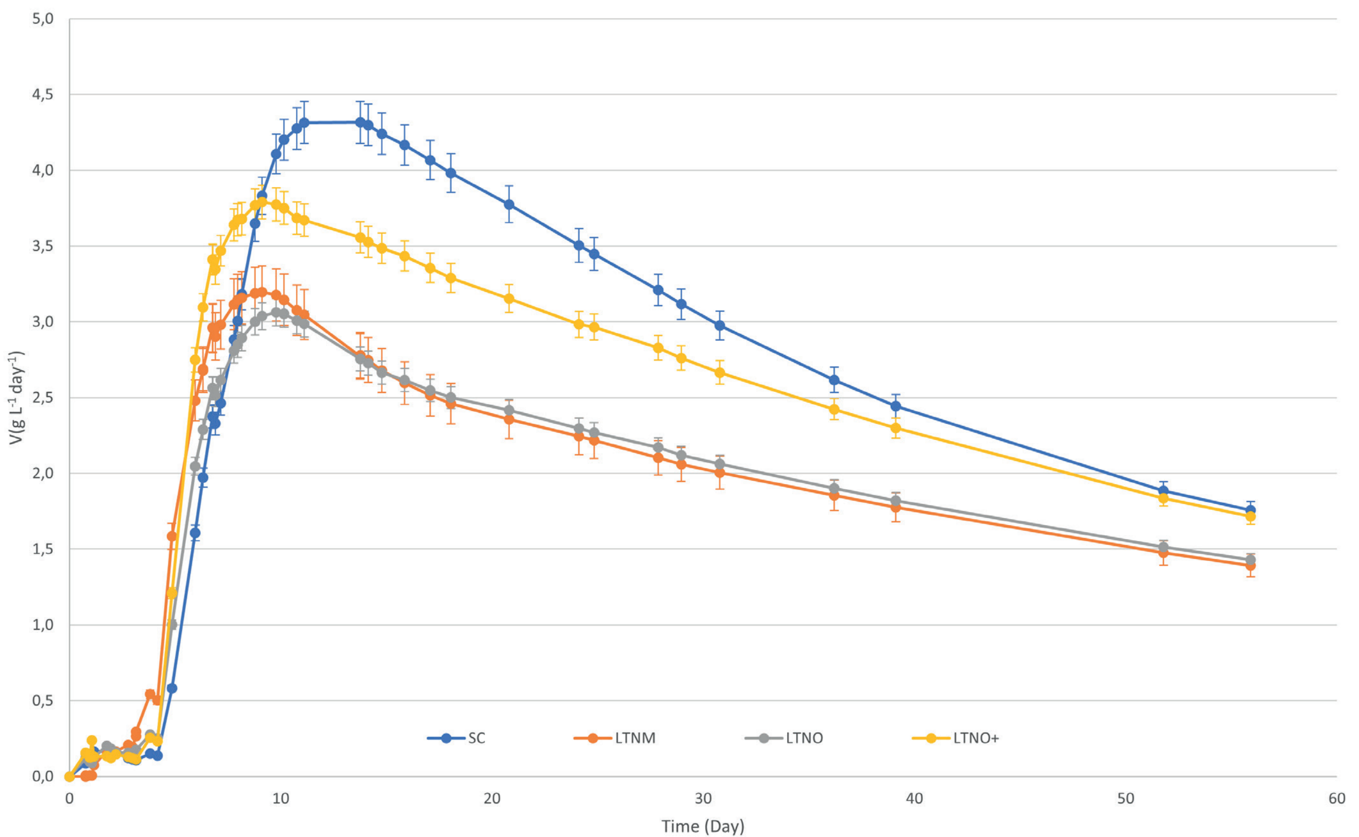


FIGURE 1. Evolution of alcoholic fermentation (A) and V (B) of grape must obtained from dried grapes of Garganega suitable for production of Vino Santo di Gambellara. Mean data ± SD (n=3).

supplementation (Natuferm Bright), Table S1 shows the amino acid composition of preparation. Considering the observed abundance of each amino acid and the dose of addiction, it is possible to estimate that each dose of Natuferm Bright furnishes at wine 9.2 mg/N/kg. Nevertheless, it is known that organic formulates deriving from yeast autolysates contain further compounds influencing the nitrogen metabolism of yeasts (e.g., di- and tri-peptides, oligopeptides, vitamins) and a YAN comparison would be underestimated in terms of nutrient supplementation.

Diammonium phosphate salt in Figure 1A shows the trend of alcoholic fermentations as weight loss (g $L^{-1}day^{-1}$). In the SC experiment, the lag phase lasted 4 days; after that, the maximum fermentation rate (V_{max}) of 4.30±0.22 g $L^{-1}day^{-1}$ was reached 13 days before *S. cerevisiae* inoculum. Experiments conducted by sequential inoculum of *L. thermotolerans* and *S. cerevisiae* (LTNM, LTNO and LTNO+, Figure 1) showed a reduction of the lag phase. The promptest activation of alcoholic fermentation was observed in the experiment LTNM (lag phase duration 3.2 days), while LTNO and LTNO+ required 3.8 days to start sugar consumption. The readiness in the activation of alcoholic fermentation did not correspond to a higher sugar degradation rate which, independently from the nutritional protocol applied, remained lower than that observed in the SC trial for both experiments conducted with the sequential inoculation of the two yeasts. The LTNM trial reached a maximum fermentation rate of 3.16±0.18 g $L^{-1}day^{-1}$ after 8 days, the LTNO experiment showed a V_{max} of 3.06±11 g $L^{-1}day^{-1}$ after 9.8 days, and LTNO+ V_{max} was 3.77±0.26 g $L^{-1}day^{-1}$, measured at the 9th day of fermentation. The inoculation of *S. cerevisiae* performed 5 days after the beginning of the test did not alter the behaviour of the LTNM and LTNO experiments, which maintained a relevant delay with respect to the test performed only by *S. cerevisiae* in terms of sugar degradation rate (SC, Figure 1). In the LTNO+ experiment, the evolution of alcoholic fermentation resulted comparable to that of the SC test, especially in the last days of winemaking. Another difference observed among the four experiments concerns the amount of CO_2 produced, which corresponds to the level of sugar consumption. SC and LTNO+ experiments produced 98.30 and 95.92 g L^{-1} of CO_2, respectively, after 55 days of fermentation, while the LTNM and LTNO CO_2 production was in the range between 77.87 and 79.90 g L^{-1} at the same time (Figure 1A).

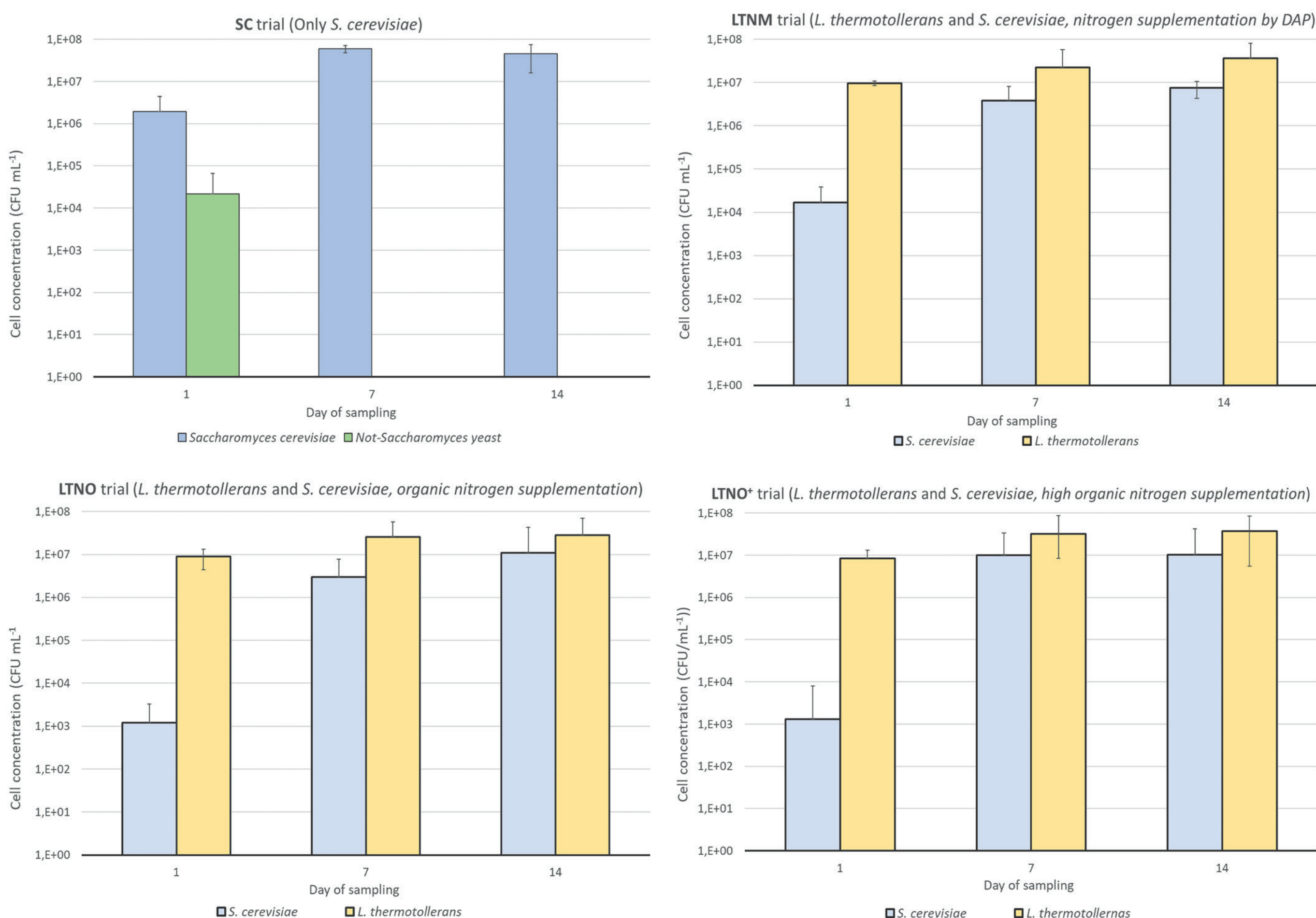


FIGURE 2. Estimation of populations of *S. cerevisiae* and *L. thermotolerans* during alcoholic fermentation of grape must obtained from dried grapes of Garganega suitable for production of Vino Santo di Gambellara. Mean data ± SD (n=3), different letters in the different trials (SC, LTNM, LTNO and LTNO+) in the same day of sampling and yeast group indicate data that are significantly different. ANOVA and Tukey's HSD test α = 5 % ANOVA and Tukey tests (p = 0.05).

In wine, the yeast population was monitored in the first two weeks of alcoholic fermentation, obtaining an estimation of the populations of *L. thermotolerans* (when present) and *S. cerevisiae* by the difference between plate count onto a non-selective (WL agar) and selective (Lysine agar) medium. (Figure 2). The different types of inoculum and nutritional protocols influenced the development of the yeasts. In the SC experiment, the population of *S. cerevisiae* reached the maximum density ($6.0 \pm 0.4 \times 10^7$ CFU mL^{-1}) on the 7th day of fermentation; the population of non-*Saccharomyces* yeast was in the order of 4 log units and disappeared (Detection limit 50 CFU/mL) after 24 hours of fermentation. In the LTNM experiment (Figure 2), the population of *L. thermotolerans* was over the 7 log units for the entire duration of observations, while *S. cerevisiae* increased after the 6 log units only after the inoculum was performed on day 5. This trend is amplified in the experiments conducted by organic nitrogen supplementation, with a spread among non-*Saccharomyces* and *Saccharomyces* yeast in the order of 5 log units after 24 hours and 1 log units after 7 days of alcoholic fermentation. However, the maximum density of *S. cerevisiae* was reached in the LTNO+ experiments on the 14th day of fermentation ($1.1 \pm 0.2 \times 10^7$ CFU mL^{-1}).

3. Grape must acidification and chemical features of wines

The evolution of pH was monitored daily during the first 25 days after grape crushing, corresponding to the hypothesised period of activity of *L. thermotolerans.* As reported in Figure 3, the experiments performed only with *S. cerevisiae* (SC) started from a pH of 3.36±0.02 and increased up to 3.51±0.01 after 25 days, while in the experiments performed by sequential inoculum of *L. thermotolerans* and *S. cerevisiae,* the wine pH remained below 3.50. More in detail, the LTNM experiment showed after 25 days of fermentation, a pH value of 3.30±0.02, less than that of the grape must. In the LTNO and LTNO+ experiments, the acidification was less efficient, reaching a pH value of 3.43±0.01 and 3.46±0.02, respectively. In both cases, it is interesting to observe that the acidification trend is not linear; in the first days of alcoholic fermentation, a sudden decrease in pH was observed, probably due to the lactic acid production due to *L. thermotolerans*, followed by an increase attributable at the precipitation of tartaric acid salts during alcoholic fermentation. This hypothesis is supported by the acidic profile of the resulting wines, which revealed an accumulation of lactic acid only in the test performed in the presence of LT (Table 3). Table 3 lists the chemical features of the wines. The addition of *L. thermotolerans* significantly ($p \leq 0.05$) reduced the sugar consumption and ethanol accumulation, considering that wines were sampled at the same time; also, the volatile acidity was lowered in the trials performed by sequential inoculum of the two yeasts. Titratable acidity increased due to the accumulation of lactic acid, whose content in wines made by non-*Saccharomyces* yeast was statistically different ($p \leq 0.05$) with respect to SC trials.

A total of thirty-three free volatile compounds were identified and quantified in wines (Table 3). These were represented mainly by yeast-derived metabolites. Acetates, Ethyl Esters, Alcohols and Fatty acids underwent statistically significant differences among treatments. The profile of volatile compounds was significantly altered by the presence of *L. thermotolerans* in all main aroma groups.

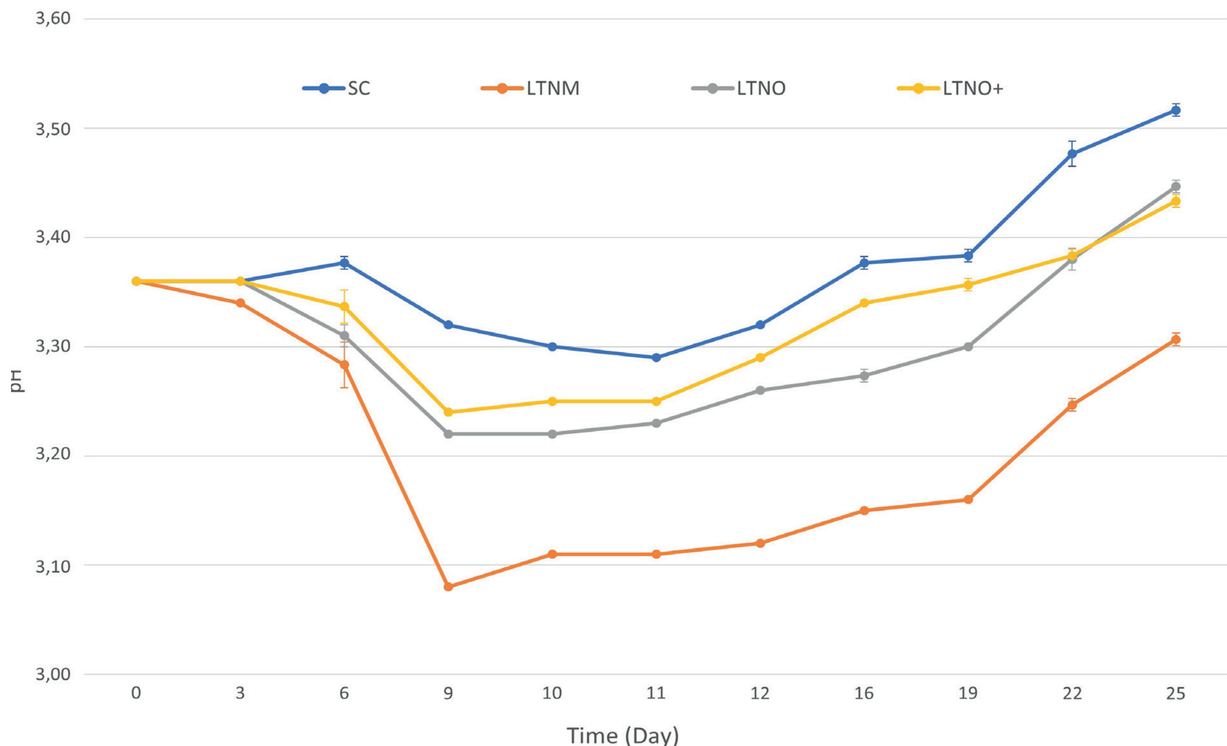


FIGURE 3. Evolution of pH during alcoholic fermentation of grape must obtained from dried grapes of Garganega suitable for production of Vino Santo di Gambellara. Mean data ± SD (n = 3).

TABLE 3. Main chemical parameters of Vino Santo obtained by different oenological protocols that consider the use of *L. thermotolerans* as natural acidifiers (Mean ± SD, n=3). Different superscript letters indicate data statistically different (ANOVA and Tukey's HSD test α = 5 %).

	SC	LTNM	LTNO	LTNO+
EtOH (% vol)	15.85±0.15^{a}	12.54±0.39^{b}	13.15±1.08^{b}	14.18±0.99^{ab}
pH	3.51±0.02^{a}	3.30±0.01^{b}	3.43±0.04^{a}	3.46±0.05^{a}
Titratable acidity (g L^{-1} of tartaric acid)	7.00±0.00^{c}	8.87±0.15^{a}	8.07±0.25^{b}	7.83±0.32^{b}
Volatile acidity (g L^{-1} of acetic acid)	1.80±0.03^{a}	1.65±0.01^{b}	1.68±0.09^{ab}	1.75±0.05^{ab}
Reducing sugars (g L^{-1})	150.23±3.65^{b}	195.53±5.73^{a}	184.03±21.65^{ab}	165.93±20.35^{ab}
Lactic acid (g L^{-1})	0.01±0.00^{c}	2.51±0.24^{a}	1.70±0.20^{b}	1.57±0.15^{b}
Malic acid (g L^{-1})	0.61±0.01^{a}	0.54±0.03^{b}	0.56±0.02^{ab}	0.53±0.04^{b}

The family of acetates resulted in significantly differences both among yeast and nutrition treatments. The lowest concentration of acetates (sum of isobutyl acetate, isopentyl acetate, 2-phenylethyl acetate, ethyl phenyl acetate) was detected in SC wines (0.225 mg L^{-1}). On the other hand, LTNO wines were in all the cases characterised by higher values of acetates. On the other hand, calculating the sum of ethyl esters of medium-chain fatty acids (ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate), emerged higher concentrations of these compounds in SC wines compared to LT treatments. The concentrations of the ester precursors were mainly differentiated between yeast species rather than nutrient treatments. However, when considering ethyl lactate, the situation is overturned; in fact, LTNM, LTNO and LTNO+ were about 10 to 30 times higher than the control. Regarding ethyl esters of short-chain fatty acids, ethyl butyrate in LTNO was higher than in the controls. Medium-chain fatty acid concentrations resulted in a significant difference in yeast strain management, following the trend found in their derivative ethyl esters, as seen previously: higher values in SC wines. Likewise, the same tendency was detected in the quantification of short-chain fatty acids. Another strain-derived difference was noticeable in C6 compounds, such as 1-hexanol, higher in SC wines (0.218 mg L^{-1}). Regarding alcohols, 2-phenylethanol was found to be higher in LT-fermented wines than in the controls; among the LT fermentations, LTNM significantly decreased compared to LTNO. The family of terpenes were not present in relevant amounts in all wines, according to the features of native grapes.

DISCUSSION

The main objective of this work is to improve the acidic profile of Vino Santo, without relevant changes in the productive process or in the features of obtained wines to ensure, at the same time, wine quality, microbiological control and recognisability of this ancient oenological production. Previous experiences in the biological acidification of different wines, such as Merlot (Hranilovic *et al*., 2021), Emir (Balikci *et al*., 2016) and, more generally, wines made from warm regions (Morata *et al*., 2019), suggested that *L. thermotolerans* could be a promising solution in the fermentation of grape must made from dried bunches. At the same time, the peculiar features of Vino Santo require an in-depth study of the interaction among different yeast species, *S. cerevisiae* and *L. thermotolerans*, and the nutritional requirement of the last one in terms of nitrogen sources.

In our experiments, the early inoculum of *L. thermotolerans* ensured the prompt activation of alcoholic fermentation, while the pure culture of *S. cerevisiae* was slower (SC, Figure 1A). This behaviour is not surprising because the sensitivity of *S. cerevisiae* to the osmotic stress typical of high-sugar grape must made from dried grapes is already known. On the contrary, *L. thermotolerans*, as reviewed by Vicente *et al.* (2022), can better adapt to high osmotic pressure environments (García *et al.,* 2021), such as over-ripe grapes; then, *S. cerevisiae* ensures a prompt activation of alcoholic fermentation useful to protect grape must from the early proliferation of spoilage microorganisms (Morata *et al.,* 2021). However, in the continuation of the fermentation, the poor fermentative capacity of the non-*Saccharomyces* yeast emerges, resulting in a low fermentation rate, both in terms of sugar consumption and in the V_{max} value, which resulted in a mean of 23 % lower than that of the SC trial. This difference is more relevant if we consider the addition of *S. cerevisiae* on the 5th day, resulting that the V_{max} measured after 10 days is due to the contribution of both yeasts. The lowering of fermentative activity observed in the LT tests confirms the presence of negative interactions between the two species of yeast utilised in this work, as already observed by some authors (Ciani *et al.,* 2006; Hranilovic *et al.,* 2018a; Hranilovic *et al.,* 2021). A different hypothesis regarding the nature of these interactions was advanced (Renault *et al.,* 2013; Kemsawasd *et al.,* 2015; Ciani *et al.,* 2016). In this work, we observe the role of nitrogen supplementation in reducing these detrimental effects, suggesting the key role of the competition among yeasts for nutritional sources. The non-mixed nitrogen supplementation protocols employed in this study (only inorganic or organic nitrogen) did not ensure the optimal evolution of sugar consumption, according to the observation of Roca-Mesa *et al.* (2020). On the contrary, in the thesis LTNO+, the high organic nitrogen supplementation reduces the gap with the SC trial regarding fermentation behaviour and V_{max} (Figure 1).

TABLE 4. Volatile profile of Vino Santo obtained by different oenological protocols that consider the use of *L. thermotolerans* as natural acidifiers. (Mean ± SD, n = 3). Different superscript letters indicate data statistically different (ANOVA and Tukey's HSD test α = 5 %).

	SC	LTNM	LTNO	LTNO+
		µg/L		
Isobutyl acetate	20±1^{b}	30±1^{a}	31±2^{a}	30±1^{a}
Isopentyl acetate	169±16^{c}	229±20^{ab}	253±8^{a}	212±11^{b}
2-phenylethyl acetate	34±4^{b}	39±1^{ab}	44±0^{a}	36±2^{b}
Ethyl phenyl acetate	3±0^{b}	3±0^{ab}	4±0^{a}	3±1^{ab}
Acetate (sum)	225±21^{c}	302±19^{b}	332±8^{a}	281±12^{ab}
Ethyl butyrate	49±49^{b}	56±6^{ab}	65±4^{a}	55±3^{ab}
Ethyl-2-methylbuthyrate	5±0^{a}	5±0^{a}	5±1^{a}	5±0^{a}
Ethyl isovalerate	0±0^{b}	1±0^{a}	1±0^{a}	1±0^{a}
Ethyl pentanoate	0±0^{a}	1±0^{a}	1±0^{a}	0±0^{a}
Ethyl hexanoate	72±5^{a}	37±10^{b}	37±3^{b}	37±4^{b}
Ethyl octanoate	71±17^{a}	22±9^{b}	18±2^{b}	24±1^{b}
Ethyl decanoate	49±8^{a}	21±1^{b}	16±5^{b}	21±3^{b}
Ethyl dodecanoate	5±1^{a}	3±0^{b}	2±0^{b}	3±0^{b}
Ethyl lactate	2239±83^{d}	58043±7215^{a}	33939±4527^{b}	26101±2424^{c}
Esters + lactate (sum)	2494±643^{c}	58192±7190^{a}	34089±4516^{b}	26250±2417^{b}
Butanoic acid	146±6^{a}	122±5^{b}	137±3^{a}	137±6^{a}
Isobutytric acid	268±27^{a}	156±9^{b}	161±9^{b}	131±7^{b}
Valeric acid	8±0^{a}	4±0^{b}	4±1^{b}	5±1^{b}
Isovaleric acid	312±13^{a}	213±12^{c}	245±5^{b}	237±15^{bc}
Hexanoic acid	267±3^{a}	102±19^{b}	99±11^{b}	104±14^{b}
Octanoic acid	143±4^{a}	33±12^{b}	31±6^{b}	38±6^{b}
Nonanoic acid	3±0^{a}	3±0^{a}	1±0^{c}	2±0^{b}
Decanoic acid	98±9^{a}	29±8^{b}	22±3^{b}	26±1^{b}
Fatty acids (sum)	1245±28^{a}	662±43^{b}	702±28^{b}	680±48^{b}
1-hexanol	218±7^{a}	142±10^{b}	134±11^{b}	147±12^{b}
Trans-3-hexen-1-ol	7±0^{a}	8±1^{a}	8±0^{a}	8±0^{a}
Cis-3-hexen-1-ol	2±0^{a}	2±0^{a}	1±0^{ab}	1±0^{ab}
Terpinen-4-ol	7±0^{a}	7±0^{a}	8±0^{a}	7±0^{a}
3-methylthio-1-propanol	92±4^{b}	224±48^{ab}	279±95^{a}	226±54^{ab}
2-phenylethanol	27,897±2364^{c}	33,557±886^{b}	40,070±1717^{a}	35,835±1300^{ab}
Benzaldehyde	142±3^{a}	78±5^{b}	103±15^{b}	145±17^{a}
Benzothiazole	1±0^{a}	1±0^{a}	1±0^{a}	1±0^{a}
Diethyl-succinate	653±32^{b}	782±96^{ab}	890±66^{a}	937±46^{a}
Beta-damascone	97±9^{a}	76±11^{a}	83±19^{a}	77±13^{a}
Guaiacol	2±0^{a}	2±0^{a}	2±0^{a}	2±0^{a}
Benzyl alcohol	43±7^{a}	65±2^{b}	59±9^{ab}	37±4^{a}
Geranic acid	22±2^{c}	29±1^{b}	33±1^{a}	29±1^{b}
Zingerone	9±1^{a}	1±0^{b}	2±0^{b}	2±0^{b}

The microbiological analysis performed on the active dry yeast samples and the wines during alcoholic fermentation (Figure 2) ensures additional information about the interaction between yeasts during mixed fermentation. The *L. thermotolerans* strain showed a slightly higher concentration in terms of viable cells per gram compared to the *S. cerevisiae* strain, which justified the high cell concentration measured already after 24 hours from the inoculum. In SC trials, the population of *S. cerevisiae* after 24 hours was in the order of 6 log units in accordance with the inoculum ratio, reaching after 5 days 7 log units and remaining constant for the entire duration of sugars degradation.

The presence of a high concentration of *Saccharomyces* counteracted the growth of non-*Saccharomyces* yeasts made from the raw material. In all the experiments, *L. thermotolerans* showed good tolerance to the wine limiting factors, remaining up to 7 log units for the entire duration of alcoholic fermentation. *S. cerevisiae* suffered, however, from the competition of the other yeast. Two days after the inoculum of *S. cerevisiae* (Day 7, Figure 2), it did not reach 7 log units, remaining significantly less concentrated ($p \leq 0.05$) than that observed in the SC test. In synthesis, the presence of *L. thermotolerans* allowed a faster activation of sugar degradation but, at the same time, created an environment less favourable for the activity of *S. cerevisiae*; the chemical analysis performed on the resulting wines can aid in explaining how these differences impact the composition of wines and their organoleptic profile.

Regarding the composition of wines (Table 4), the early inoculation with *L. thermotolerans* ensured the main objective of this work was achieved. Compared to the SC test, wines produced by the two yeasts showed a higher content of lactic acid that resulted in a pH below 3.50. This pH value is generally recognised as critical for wine microbiological stability (Bartowsky, 2009; Rankine, 2006). This result is even more interesting if we consider that the malolactic fermentation did not occur, as demonstrated by the malic acid residue. The sequential fermentation of *L. thermotolerans* and *S. cerevisiae* positively impacted the other parameters: the wines had a more moderate alcohol content and a lower volatile acidity, according to that observed by Vilela (2018).

The volatile profile of wines (Table 4) was influenced both by the yeast specie and the nutrition protocol in LT wines. As previously reported (Vaquero *et al.,* 2021; Hranilovic *et al.,* 2021), *L. thermotolerans* produced a higher concentration of acetate esters compared to *S. cerevisiae*. Acetate esters are derived from higher alcohols, which can be formed by yeast catabolism through the Ehrlich pathway from amino acids (Labuschagne and Divol, 2021). Among the LT wines, LTNO showed a greater value of the total acetates than LTNM, perhaps due to the presence of amino acids in the organic nutrient composition (Mendes-Ferreira *et al.,* 2011). However, no statistically significant differences were noticed between LTNO+ and the other LT treatments. The level of ethyl esters of medium chain fatty acids was yeast-dependent, with higher values in SC wines, and related to the availability of their respective precursors, hexanoic, octanoic and decanoic acid (Dennis *et al.,* 2012), which followed the same trend.

FIGURE 4. Principal component analysis (PCA) of compositive profile of Vino Santo di Gambellara obtained by different yeast inoculum and nitrogen supplementation protocol. Projection in the plane of first two factors of variables (Compositive data) and cases (Experimental trials).

Those observations agreed with some previous studies (Vaquero *et al.,* 2021; Sgouros *et al.,* 2020; Hranilovic *et al.,* 2021). However, only ethyl hexanoate and ethyl octanoate were above the ODT. Moreover, branched-chain fatty acids (isobutyric acid, valeric acid and isovaleric acid) resulted higher in SC wines, with some differences in concentration also among the nutrient treatments; LTNM always reported lower values, results in contrast with previous works (Whitener *et al*., 2017; Sgouros *et al.,* 2020). The correspondent ethyl esters were influenced by the yeast strain, in agreement with previous studies (Mallouchos *et al.,* 2003; Sgouros *et al.,* 2020; Hranilovic *et al.,* 2021). SC wines had, in general, either equal or significantly lower concentrations with respect to LT wines. Whereas, as expected, ethyl lactate was the ester predominant in wines fermented by *L. thermotolerans*, probably due to the high availability of lactic acid as its precursor. Ethyl lactate is formed because of the esterification of D-lactic or L-lactic acid, rarely produced by *S. cerevisiae* (Ishida *et al.,* 2006). Ethyl lactate gives the wine buttery and fruity notes. In this case, the concentrations, even if important, did not reach the perception threshold (146 mg L^{-1}; Moyano *et al.,* 2012). This result is in accordance with previous studies (Whitener *et al.,* 2017; Vaquero *et al*., 2021; Sgouros *et al*., 2020; Hranilovic *et al*., 2021).

Besides the univariate analysis, the chemical dataset was also subjected to multivariate analysis with the principal component analysis (PCA; Figure 4). Wines are clearly separated in the space depending both on yeast and nutrient treatments. The first two principal components accounted for 70.3 % of the total variation in the samples. Along the first principal component, which accounts for 57.6 % of the variance explained, we observe a clear distinction between wines fermented with different yeasts. In fact, wines fermented by *L. thermotolerans* occupy the left side, in correspondence with higher values of lactic acid, titratable acidity (TA), and some acetates. While the SC samples are in the far-right, this is driven mainly by higher values of fatty acids, 1-hexenol and ethanol, as a confirmation of results seen in Table 3. The major distinction between nutrient treatments is observable along PC2 (12.72 % of the variance explained). LTNM is in the left-highest quadrant, correlated to the highest accumulation of lactic acid, residual sugars, ethyl lactate and titratable acidity. Finally, the two experiments made by organic nutrition of yeasts (LTNO and LTNO+) are placed in the left lower quadrant, corresponding to greater values of some acetates and esters.

In conclusion, *L. thermotolerans* showed promising potentiality in restoring an adequate acidic profile in wines made from dried grapes, also giving peculiar character due to the synthesis of specific volatile metabolites. These results are in accordance with previous studies on different wines (Whitener *et al*., 2017, Vaquero *et al*., 2021; Roca-Mesa *et al*., 2020; Sgouros *et al*., 2020; Hranilovic *et al*., 2021); however, further investigations into the nutrition management of *L. thermotolerans* and volatile metabolites would be interesting as a confirmation of the present work.

ACKNOWLEDGEMENTS

We thank Dr Paola Vagnoli and the staff of Lallemand Italy for their support in this work and for the supply of the commercial strain of *L. thermotolerans*.

REFERENCES

Balikci, E.K., Tanguler, H., Jolly, N.P., & Erten, H. (2016) Influence of *Lachancea thermotolerans* on cv. Emir wine fermentation. *YEAST* 33, 312-321. https://doi.org/10.1002/yea.3166

Banilas, G., Sgouros, G., & Nisiotou, A. (2016). Development of microsatellite markers for *Lachancea thermotolerans* typing and population structure of wine-associated isolates. *Microbiological Research*, 193, 1-10. https://doi.org/10.1002/yea.3166

Bartowsky, E.J. (2009) Bacterial spoilage of wine and approaches to minimize it. *Letters in Applied Microbiology.* 48, 149-156. https://doi.org/10.1111/j.1472-765X.2008.02505.x

Bely, M., Rinaldi, A., & Dubourdieu, D. (2003). Influence of assimilable nitrogen on volatile acidity production by *Saccharomyces cerevisiae* during high sugar fermentation. *Journal of Bioscience and Bioengineering,* 9, 507-512. https://doi.org/10.1016/S1389-1723(04)70141-3

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., & Dubourdieu, D. (2008). Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. *International Journal of Food Microbiology*. 20, 312-20. https://doi.org/10.1016/j.ijfoodmicro.2007.12.023

Ciani, M., Beco, L., & Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multi starter wine yeast fermentations. *International Journal of Food Microbiology*, 108(2), 239-245. https://doi.org/10.1016/j.ijfoodmicro.2005.11.012

Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., & Romano, P. (2016). Yeast interactions in inoculated wine fermentation. *Frontiers in Microbiology*, 7, 1-7. https://doi.org/10.3389/fmicb.2016.00555

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., & Ciani, M. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multi starter fermentations with *Saccharomyces* cerevisiae. *Food Microbiology*, 28(5), 873–882. https://doi.org/10.1016/j.fm.2010.12.001

Dennis, E. G., Keyzers, R. A., Kalua, C. M., Maffei, S. M., Nicholson, E. L., & Boss, P. K. (2012). Grape contribution to wine aroma: Production of hexyl acetate, octyl acetate, and benzyl acetate during yeast fermentation is dependent upon precursors in the must. *Journal of Agricultural and Food Chemistry, 60*(10), 2638-2646. https://doi.org/10.1021/jf2042517

Domizio, P. & Lencioni, L. (2011). Vino Santo. *Advances in Food and Nutrition Research*, Vol 63, 41-100. https://doi.org/10.1016/B978-0-12-384927-4.00003-8

Erasmus, D. J., Cliff, M., & Van Vuuren, H. J. (2004). Impact of yeast strain on the production of acetic acid, glycerol, and the sensory attributes of icewine. *American Journal of Enology and Viticulture, 55*(4), 371-378. https://doi.org/10.5344/ajev.2004.55.4.371

Gallo, G., Larcher, R., Cappello, G., Paolini, M., Moser., R., Schneider, R., Carrau., F., Nardin., T., & Roman, T. (2023). Insights into the grape must composition effect on *Hanseniaspora vineae* performance and metabolic aroma compounds in Chardonnay base wine for sparkling wine production. *Journal of Food Composition and Analysis*, 105514. In press. https://doi.org/10.1016/j.jfca.2023.105514

García, M., Crespo, J., Cabellos, J. M., & Arroyo, T. (2021). Growth of non-*Saccharomyces* native strains under different fermentative stress conditions. *Fermentation*, 7(3). https://doi.org/10.3390/fermentation7030124

Gatto, V., Binati, R., L., Lemos Junior, W.J.F., Basile, A., Treu, L., de Almeida, O.G.G., Innocente, G., Campanaro, S., & Torriani, S. (2020). New insights into the variability of lactic acid production in *Lachancea thermotolerans* at the phenotypic and genomic level. *Microbiological Research,* 238, 126525. https://doi.org/10.1016/j.micres.2020.126525

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., & Ciani, M. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. *Food Microbiology*, 33(2), 271–281. https://doi.org/10.1016/j.fm.2012.10.004

Guaragnella, N., & Bettiga, M. (2021). Acetic acid stress in budding yeast: From molecular mechanisms to applications. *Yeast*, 38, 391–400. https://doi.org/10.1002/yea.3651

Guzzon, R., & Larcher, R. (2015). The application of flow cytometry in microbiological monitoring during winemaking: two case studies. *Annals of Microbiology* 65, 1865–1878. https://doi.org/10.1007/s13213-014-1025-6

Guzzon, R., Franciosi, E., & Larcher, R. (2014). A new resource from traditional wines: characterisation of the microbiota of Vino Santo grapes as a biocontrol agent against *Botrytis cinerea*. *European Food Research and Technology,* 239(1), 117-126. https://doi.org/10.1007/s00217-014-2195-y

Guzzon, R., Franciosi, E., Moser, S., Carafa, I., & Larcher, R. (2018). Application of ozone during grape drying for the production of straw wine. Effects on the microbiota and compositive profile of grapes. *Journal of Applied Microbiology,* 125, 513-527. https://doi.org/10.1111/jam.13774

Hranilovic, A., Albertin, W., Capone, D. L., Gallo, A., Grbin, P. R., Danner, L., Bastian, S. E. P., Masneuf-Pomarede, I., Coulon, J., Bely, M., & Jiranek, V. (2021). Impact of *Lachancea thermotolerans* on chemical composition and sensory profiles of Merlot wines. *Food chemistry 349*, 129015. https://doi.org/10.1016/j.foodchem.2021.129015

Hranilovic, A., Li, S., Boss, P. K., Bindon, K., Ristic, R., Grbin, P. R., Van der Westhuizen, T., & Jiranek, V. (2018a). Chemical and sensory profiling of Shiraz wines co-fermented with commercial non-*Saccharomyces* inocula. *Australian Journal of Grape and Wine Research*, 24(2), 166–180. https://doi.org/10.1111/ajgw.12320

Hranilovic, Ana, Gambetta, J. M., Schmidtke, L., Boss, P. K., Grbin, P. R., Masneuf-Pomarede, I., Bely, M., Albertin, W., & Jiranek, V. (2018b). Oenological traits of *Lachancea thermotolerans* show signs of domestication and allopatric differentiation. *Scientific Reports*, 8(1), 1–13. https://doi.org/10.1038/s41598-018-33105-7

Ishida, N., Suzuki, T., Tokuhiro, K., Nagamori, E., Onishi, T., Saitoh, S., ... & Takahashi, H. (2006). D-Lactic acid production by metabolically engineered Saccharomyces cerevisiae. Journal of Bioscience and Bioengineering, 101(2), 172-177. https://doi.org/10.1263/jbb.101.172

Kemsawasd, V., Viana, T., Ardö, Y., & Arneborg, N. (2015). Influence of nitrogen sources on growth and fermentation performance of different wine yeast species during alcoholic fermentation. *Applied Microbial and Cell Physiology*. https://doi.org/10.1007/s00253-015-6835-3

Labuschagne, P. W. J., & Divol, B. (2021). Thiamine: a key nutrient for yeasts during wine alcoholic fermentation. *Applied Microbiology and Biotechnology, 105*, 953-973. https://doi.org/10.1007/s00253-020-11080-2

Laureati, M., Cattaneo, C., Tateo, F., & Bononi, M. (2020). Identification of the volatile compounds and sensory attributes of long-term aging Vino Santo wine from Malvasia di Candia aromatic grapes. *Foods*, 9(12), 1736. https://doi.org/10.3390/foods9121736

Lorenzini, M., Azzolini, M., Tosi, E., & Zapparoli., G. (2013). Postharvest grape infection of *Botrytis cinerea* and its interactions with other moulds under withering conditions to produce noble-rotten grapes. *Journal of Applied Microbiology*, 114(3), 762-70. https://doi.org/10.1111/jam.12075

Mallouchos, A., Komaitis, M., Koutinas, A., & Kanellaki, M. (2003). Wine fermentations by immobilized and free cells at different temperatures. Effect of immobilization and temperature on volatile by-products. *Food Chemistry, 80*(1), 109-113. https://doi.org/10.1016/S0308-8146(02)00247-9

Mateo, J., & Maicas, S. (2016). Application of Non-*Saccharomyces* Yeasts to Wine-Making Process. *Fermentation, 2*(4), 14. MDPI AG. Retrieved from http://dx.doi.org/10.3390/fermentation2030014

Mendes-Ferreira, A., Barbosa, C., Lage, P., & Mendes-Faia, A. (2011). The impact of nitrogen on yeast fermentation and wine quality. *Ciencia E Tecnica Vitivinicola, 26*, 17-32. https://api.semanticscholar.org/CorpusID:59154344

Morata, A., Bañuelos, M. aA, Vaquero, C., Loira, I., Cuerda, R., Palomero, F., González, C., Suárez-Lepe, J. A., Wang, J., Han, S., & Bi, Y. (2019). *Lachancea thermotolerans* as a tool to improve pH in red wines from warm regions. *European Food Research and Technology*, 245(4), 885–894. https://doi.org/10.1007/s00217-019-03229-9

Morata, A., Loira, I., González, C., & Escott, C. (2021). Non-*Saccharomyces* as bio tools to control the production of off flavours in wines. *Molecules*, 26(15). https://doi.org/10.3390/molecules26154571

Morata, A., Loira, I., Tesfaye, W., Bañuelos, M. A., González, C., & Suárez Lepe, J. A. (2018). *Lachancea thermotolerans* applications in wine technology. *Fermentation*, 4(3). https://doi.org/10.3390/fermentation4030053

Moyano, L., Zea, L., Moreno, J. A., & Medina, M. (2010). Evaluation of the active odorants in Amontillado sherry wines during the aging process. *Journal of agricultural and food chemistry, 58*(11), 6900-6904. https://doi.org/10.1021/jf100410n

Nissen, P., & Arneborg, N. (2003). Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. *Archives of Microbiology*, 180(4), 257–263. https://doi.org/10.1007/s00203-003-0585-9

OIV (2023a). *COEI-2-CONBAC Bacteriological control. Paragraph 1 "Preliminary rehydration of yeasts"*. International Organization of Vine and Wine (OIV), Dijon, F.

OIV (2023b). *OIV-MA-AS4-01 Microbiological analysis of wines and musts. Detection, differentiation and counting of micro-organisms*. International Organization of Vine and Wine (OIV), Dijon, F.

Ollat, N., Touzard, J.M., & van Leeuwen, C. (2016). Climate change impacts and adaptations: new challenges for the wine industry. *Journal of Wine Economics,* 11(1), 139-149. PMID: 18262301. https://doi.org/10.1017/jwe.2016.3

Paolini, M., Tonidandel, L., Moser, S., & Larcher, R. (2018). Development of a fast gas chromatography–tandem mass spectrometry method for volatile aromatic compound analysis in oenological products. *Journal of Mass Spectrometry*, 53, 801–810. https://doi.org/10.1002/jms.4259

Rankine, B. (2006). *Making good wine: a manual of winemaking practice for Australia and New Zealand*. Sun Books, South Melbourne (Macmillan Australia).

Renault, P. E., Albertin, W., & Bely, M. (2013). An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Applied Microbiology and Biotechnology*, 97(9), 4105–4119. https://doi.org/10.1007/s00253-012-4660-5

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), 157. MDPI AG. http://dx.doi.org/10.3390/microorganisms8020157

Sgouros, G., Mallouchos, A., Filippousi, M. E., Banilas, G., & Nisiotou, A. (2020). Molecular characterization and enological potential of a high lactic acid producing *Lachancea thermotolerans* Vinoeyard strain. *Foods*, 9(5), 1–16. https://doi.org/10.3390/foods9050595

Stefanini, I., Albanese, D., Cavazza, A., Franciosi, E., De Filippo, C., Donati, C., & Cavalieri, D. (2016). Dynamic changes in microbiota during spontaneous "Vino Santo Trentino' fermentation. *Microbial Biotechnology*, 9(2), 195-208. https://doi.org/10.1111/1751-7915.12337

Torriani, S., Lorenzini, M., Salvetti, E., & Fells, G. E. (2011). *Zygosaccharomyces gambellarensis sp* nov., an ascosporogenous yeast isolated from an Italian 'passito' style wine. *International Journal of Systematic and Evolutionary Microbiology*. 61, 3084-3088. https://doi.org/10.1099/ijs.0.031146-0

Vaquero, C., Izquierdo-Cañas, P. M., Mena-Morales, A., Marchante-Cuevas, L., Heras, J. M., & Morata, A. (2021). Use of *Lachancea thermotolerans* for Biological vs. Chemical Acidification at Pilot-Scale in White Wines from Warm Areas. *Fermentation, 7*(3), 193. MDPI AG. http://dx.doi.org/10.3390/fermentation7030193

Vicente, J., Baran, Y., Navascués, E., Santos, A., Calderón, F., Marquina, D., ... & Benito, S. (2022). Biological management of acidity in wine industry: A review. *International Journal of Food Microbiology, 375*, 109726. https://doi.org/10.1016/j.ijfoodmicro.2022.109726

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, 278. https://doi.org/10.3390/foods10112878

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

Whitener, M. B., Stanstrup, J., Carlin, S., Divol, B., Du Toit, M., & Vrhovsek, U. (2017). Effect of non-*Saccharomyces* yeasts on the volatile chemical profile of Shiraz wine. *Australian journal of grape and wine research, 23*(2), 179-192. https://doi.org/10.1111/ajgw.12269